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► To cite this version:

Sara Montanari. Identification and mapping of genomic regions controlling fire blight and psylla resistance and hybrid necrosis in pear. Agricultural sciences. Université d'Angers, 2015. English. NNT : 2015ANGE0063 . tel-02170683

HAL Id: tel-02170683

<https://theses.hal.science/tel-02170683>

Submitted on 2 Jul 2019

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Thèse de Doctorat

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*Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université d'Angers
sous le label de L'Université Nantes Angers Le Mans*

École doctorale : *VENAM*

Discipline : *Biologie des organismes; Biotechnologies animales, végétales et microbiennes*

Spécialité : *Biologie cellulaire et moléculaire végétale*

Unité de recherche : *Institut de Recherches en Horticulture et Semences (France)*

Fondazione Edmund Mach (Italy)

Plant & Food Research (New Zealand)

Soutenue le 03 Juillet 2015

Thèse N° : 1493

Identification and mapping of genomic regions controlling fire blight and psylla resistance and hybrid necrosis in pear

JURY

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Titre en français

IDENTIFICATION ET CARTOGRAPHIE DE REGIONS DU GENOME CONTROLANT LA RESISTANCE AU FEU BACTERIEN ET AU PSYLLE ET LA NECROSE HYBRIDE CHEZ LE POIRIER

Résumé

Le feu bactérien et le psylle causent d'importantes pertes économiques dans les zones de production du poirier dans le monde entier. Le développement de nouvelles variétés de poirier résistantes à ces bio-agresseurs constitue un enjeu majeur dans le cadre d'un programme de lutte intégrée. L'objectif de ce projet de thèse est l'étude du déterminisme génétique de la résistance vis-à-vis de ces deux bio-agresseurs. La thèse a été réalisée dans le cadre d'une collaboration internationale entre Fondazione Edmund Mach (Italie), Institut de Recherches en Horticulture et Semences (France) et Plant & Food Research (Nouvelle-Zélande). Une descendance interspécifique de poirier PEAR3 x 'Moonglow' a été développée avec pour objectif de cumuler les résistances au feu bactérien et au psylle provenant de variétés asiatiques et européennes de *Pyrus*. Deux cartes génétiques ont été élaborées pour PEAR3 et 'Moonglow' sur la base de marqueurs SNP (Single Nucleotide Polymorphism) et SSR (microsatellite), et la cartographie de QTLs (Quantitative Trait Loci) a permis de démontrer le déterminisme polygénique de la résistance à ces bio-agresseurs. Une sélection assistée par marqueurs (MAS) peut donc être engagée pour ces deux caractères. Des incompatibilités génétiques ont aussi été observées dans une partie de la descendance, ce qui a permis de cartographier pour la première fois chez le poirier les zones du génome liées au phénomène de "nécrose hybride". Le développement de marqueurs liés aux gènes létaux devrait permettre aux sélectionneurs d'éviter les combinaisons incompatibles en croisement qui peuvent impacter certains caractères agronomiques co-ségrégant avec ces gènes létaux.

Mots clés: *Pyrus x bretschneideri*; *Pyrus communis*; cartographie génétique; détection de QTL; *Cacopsylla pyri*; *Erwinia amylovora*; gènes létaux; incompatibilités génétiques.

Abstract

The goal of this PhD project was to study the genetic architecture of pear resistance to two of its most significant diseases and pests, fire blight and psylla, which cause severe yield losses in all the main pear production regions worldwide. The development of new pear varieties with resistance against these two biotic stresses is of major interest for Integrated Pest Management. This project was designed in a joint collaboration among Fondazione Edmund Mach (Italy), Institut de Recherches en Horticulture et Semences (France) and Plant & Food Research (New-Zealand). The interspecific pear F1 progeny PEAR3 x ‘Moonglow’ was developed with the purpose of cumulating resistances to fire blight and psylla deriving from Asian and European pear cultivars. Single nucleotide polymorphism (SNP) and simple sequence repeat (SSR)-based genetic maps were built for PEAR3 and ‘Moonglow’. Quantitative Trait Loci (QTLs) were detected for the resistances, demonstrating their polygenic nature. Marker-assisted selection (MAS) can now be applied for these two traits. Furthermore, the segregating population exhibited genetic incompatibilities, and the genomic regions associated with hybrid necrosis were mapped for the first time in pear. Development of molecular markers linked to the lethal genes should allow breeders to avoid crosses leading to incompatible combinations that could affect the expression of important agronomic traits co-segregating with these genes.

Key Words: *Pyrus x bretschneideri*; *Pyrus communis*; genetic mapping; QTL detection; *Cacopsylla pyri*; *Erwinia amylovora*; lethal genes; genetic incompatibility.

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Résumé substantiel en Français

Introduction

Le poirier (*Pyrus* spp.) est un des arbres fruitiers les plus importants dans les régions tempérées et il est aujourd'hui cultivé dans plus de 50 pays dans le monde entier (Song et al. 2014). Il y a vingt-deux espèces largement connues de poirier, qui sont habituellement divisés en deux grands groupes: les poiriers occidentaux ou européens (avec *Pyrus communis* L. comme espèce principale) et les poiriers orientaux ou asiatiques (notamment *P. x bretschneideri* Rehd., *P. pyrifolia* (Burm.) Nakai, aussi appelé 'nashi' en Europe, *P. ussuriensis* Maxim. et *P. sinkiangensis*) (Wu et al. 2013). En Europe, Afrique, Océanie et les Amériques, l'espèce de poirier principalement cultivée et commercialisée est *P. communis*, qui a été diversifiée en milliers de variétés (Hedrick et al. 1921); cependant, seulement quelques cultivars sont effectivement utilisés pour la production de fruits. 'Williams Bon Chrétien' (WBC), encore appelé 'Bartlett', est certainement le cultivar de poirier européen le plus cultivé dans le monde entier (McGregor, 1976). *P. communis* n'est pas communément cultivé en Chine, mais plutôt les espèces asiatiques, surtout *P. x bretschneideri*, suivi par *P. pyrifolia* et *P. ussuriensis* (<http://www.fao.org/docrep/004/ab985e/ab985e06.htm>).

Le poirier appartient à la famille des *Rosaceae*, tribu des *Pyreae*, qui comprend également le pommier (*Malus* spp.) (Potter et al. 2007). Au cours de ces dernières années les connaissances sur la génomique du poirier ont bien progressé, notamment avec le séquençage des génomes du poirier chinois (*P. x bretschneideri* cv 'Dangshansuli', également connu sous le nom 'Suli', (Wu et al. 2013)) et européen (*P. communis* WBC, (Chagné et al. 2014)). Le poirier est fortement hétérozygote, en raison de son système d'auto-incompatibilité gamétophytique et de sa compatibilité interspécifique (Crane and Lewis 1942; Zheng et al. 2014). Les espèces de la tribu des *Pyreae* se caractérisent par un fruit distinctif, fruit à pépins, et un nombre haploïde (x) de chromosomes de 17 (Velasco et al. 2010).

Une des préoccupations principales dans l'agriculture a toujours été la lutte contre les maladies et les ravageurs, qui causent des pertes de rendement, des dégâts sur les cultures et réduisent la

qualité des aliments. Dans un contexte de changements climatiques majeurs et d'une croissance démographique rapide de la population humaine, la protection des cultures est encore plus importante. L'application de composés chimiques, même si elle est bien souvent l'unique stratégie de lutte efficace pour contrôler les maladies et les ravageurs, est très nocive pour l'environnement et la santé humaine et augmente considérablement les coûts de production. En outre, il existe des agents pathogènes et des parasites qui ne peuvent pas être contrôlés complètement avec des pesticides (par exemple *Erwinia amylovora* (Norelli et al. 2003)). De plus, dans de nombreux cas l'émergence de souches résistantes aux produits phytosanitaires les plus utilisés a limité l'éventail des principes actifs applicables (par exemple pour le psylle du poirier, *Cacopsylla* spp. (Harries and Burts 1965; Buès et al. 2003; Civolani et al. 2007)). Par conséquent, durant les dernières décennies la recherche a mis l'accent sur des stratégies de lutte alternatives aux produits chimiques, tels que les méthodes biologiques et les pratiques agronomiques, afin de réduire les applications de pesticides sans compromettre la production. Le concept de Lutte Intégrée, né dans les années 70, est basé sur l'intégration de stratégies de contrôle des bio-agresseurs différentes (les bio-agresseurs sont les insectes, les agents pathogènes et les adventices), prenant en compte "l'intérêt et l'impact sur les producteurs, la société et l'environnement" (Kogan 1998). Dans ce cadre, une importance particulière est accordée à la sélection de variétés résistantes, qui est confortée par les incroyables progrès récemment accomplis dans le domaine de la génomique végétale.

Les espèces de poirier sont généralement attaqués par plusieurs insectes et agents pathogènes. Le "pear decline" (*Candidatus Phytoplasma pyri*), le feu bactérien (*Erwinia amylovora*), la tavelure (*Venturia pirina* et *V. nashicola*), le psylle du poirier (surtout *Cacopsylla pyri* et *C. pyricola*) et les pucerons (*Dysaphis pyri*, *Myzus persicae*, *Aphis gossypii* et *A. fabae*, principalement) comptent parmi les bio-agresseurs les plus importants.

Les résistances des plantes aux bio-agresseurs sont héritées soit de manière qualitative (présence de gènes majeurs encore appelés "gènes *R*"), soit de manière quantitative, comme le sont par ailleurs la plupart des autres caractères agronomiques importants (Collard et al. 2005; Würschum 2012). Un locus de caractère quantitatif, ou QTL (Quantitative Trait Locus), correspond à une région du génome qui contient un (éventuellement plusieurs) gène(s) contrôlant une part de la

variation phénotypique d'un caractère quantitatif (Collard et al. 2005). La détection de QTLs débute avec la construction de cartes génétiques basées sur des marqueurs moléculaires, suivie de l'identification de liaison entre des données génotypiques et phénotypiques au sein d'une population en ségrégation (en général une descendance issue d'un croisement contrôlé). L'objectif d'une étude de cartographie de QTLs est de mieux connaître le déterminisme génétique (ou "architecture génétique") d'un caractère d'intérêt: il s'agit en particulier d'identifier le nombre de gènes impliqués dans la variation du caractère, leur contribution relative à cette variation, leurs éventuelles interactions, leur localisation précise sur le génome et in fine leur fonction. Ces informations sont importantes pour mieux comprendre comment le caractère (et en particulier sa variation) est génétiquement contrôlé. À travers l'identification des marqueurs génétiques étroitement liés aux QTLs, il est aussi possible d'utiliser ces informations pour mettre en œuvre une sélection assistée par marqueurs (SAM). Avec cette technique de sélection, les plantules peuvent être testées avec des marqueurs moléculaires, et celles portant des caractéristiques indésirables éliminées; de cette façon, les plantes n'attendent pas d'être arrivées à maturité pour être évaluées, ce qui fait gagner de temps et de l'argent aux sélectionneurs. En outre, les variétés utilisées comme parents de population de sélection peuvent être sélectionnées sur la base de leurs génotypes et sur la connaissance de l'hérédité de caractères importants (Myles 2013; van Nocker and Gardiner 2014). La résistance aux bio-agresseurs est l'un des objectifs de sélection qui a trouvé le plus grand intérêt pour l'application des stratégies de SAM, particulièrement parce que l'évaluation phénotypique est généralement coûteuse et longue, et les résistances sont parfois fortement influencées par l'environnement (Muranty et al. 2014). Un certain nombre de sources de des résistances aux insectes et maladies ont un déterminisme polygénique, bien que de nombreux gènes majeurs soient aussi été détectés. Les marqueurs moléculaires liés aux résistances qualitatives et quantitatives peuvent être combinés avec des marqueurs liés à d'autres caractères polygéniques importants, dans une approche de SAM, et ce afin d'accélérer la sélection du poirier.

Objectif de la thèse

L'objectif de ce projet de thèse était l'étude du déterminisme génétique de la résistance du poirier vis-à-vis de deux de ses plus importants agents pathogènes et ravageurs, le feu bactérien et le psylle. En effet, ces derniers entraînent des pertes économiques élevées dans toutes les principales régions de production de poirier à l'échelle mondiale. Le développement de nouvelles variétés de poirier ayant des résistances durables vis-à-vis de ces deux bio-agresseurs constitue un enjeu majeur dans le cadre d'un programme de lutte intégrée. Cette thèse a été réalisée dans le cadre d'une collaboration internationale entre la Fondazione Edmund Mach (FEM, Italie), l'Institut de Recherches en Horticulture et Semences (INRA/ACO/UA, France) et le Plant & Food Research (PFR, Nouvelle-Zélande). La résistance au feu bactérien est un des critères de sélection majeur dans les programmes d'amélioration génétique du poirier à PFR et à l'INRA depuis plus de 20 ans; plus récemment, la résistance à *C. pyri* a également été intégrée comme caractère cible pour la création variétale.

Une approche de cartographie de QTLs dans la descendance interspécifique de poirier PEAR3 (*P. x bretschneideri* X *P. communis*) x 'Moonglow' (*P. communis*) a été utilisée pour étudier l'architecture génétique de ces deux résistances polygéniques. PEAR3 est un hybride interspécifique dérivé de la variété de poirier chinois 'Xue Hua Li' (*P. x bretschneideri*). Ce cultivar, comme plusieurs espèces asiatiques, est une bonne source de résistance aux ravageurs et aux maladies, y compris *C. pyri*. La variété européenne (*P. communis*) 'Max Red Bartlett' (MRB) était considérée comme le parent mâle de PEAR3, mais cette généalogie s'est avérée fautive. MRB est une variété à peau rouge générée par une mutation de la variété WBC. MRB et WBC sont censés être génétiquement identiques, sauf pour le gène contrôlant la couleur rouge de la peau du fruit, qui a été cartographié sur le groupe de liaison (GL) 4 (Dondini et al. 2008). Le génotypage de WBC à l'aide de marqueurs microsatellites (SSRs) effectué dans le cadre de cette thèse afin d'étudier l'hérédité des allèles de résistance au feu bactérien et des allèles d'incompatibilité causant le phénomène de "nécrose hybride" pour d'une partie de la descendance PEAR3 x 'Moonglow', a révélé que WBC/MRB, n'est pas apparenté à PEAR3. Des tests supplémentaires devront, donc, être effectués dans la collection de poiriers de PFR, où PEAR3 a été créé, afin d'identifier le parent mâle de PEAR3. La variété 'Moonglow' dérive d'un

croisement entre l'hybride Michigan-US 437 et la variété 'Roi Charles de Wurtemberg' (RCW). Ces deux génotypes de poirier européens sont résistants au feu bactérien, tout comme 'Moonglow' qui a été montré très peu sensible à cette maladie (Quamme 1977; Paulin 1990). Les parents de RCW ne sont pas connus de manière faïble, mais RCW pourrait être un semis de la variété 'Beurré Clairjeau', qui serait elle-même un semis de la variété 'Duchesse d'Angoulême' (<http://www.ars.usda.gov/SP2-UserFiles/Place/20721500/catalogs/pyrcult.html>). Michigan-US 437 est une sélection provenant du croisement entre WBC et 'Barseck', ce dernier dérivant à son tour d'un croisement entre WBC et 'Seckel'. WBC est la plus cultivée des variétés de poirier européen dans le monde entier, grâce à ses fruits de bonne qualité. 'Seckel' est un cultivar bien connu pour sa résistance au feu bactérien. Il a été utilisé dans plusieurs croisements de nombreux programmes d'amélioration génétique de poirier (Van Der Zwet et al. 1974; Quamme 1977). La première étape de cette thèse était de construire la carte génétique des parents PEAR3 et 'Moonglow', grâce au génotypage et à l'analyse de leur descendance. Une puce Illumina avec 9000 marqueurs de polymorphisme nucléotidique (SNPs) de pommier et de poirier, "*apple and pear Illumina Infinium® II 9K SNPs array*", a été utilisée pour le génotypage des 220 descendants disponibles, permettant la construction d'une carte génétique haute densité. De plus, des marqueurs SSR développés chez le pommier et le poirier ont été rajoutés à la carte génétique. Par la suite, la population PEAR3 x 'Moonglow' a été évaluée phénotypiquement pour les résistances au psylle et feu bactérien. Des QTLs de résistance à ces deux caractères ont ainsi pu être détectés. Un autre objectif de cette thèse était d'étudier les bases génétiques qui ont causé la mortalité ("nécrose hybride") de plus de 50% des semis du croisement PEAR3 x 'Moonglow'. Des analyses génétiques ont permis d'identifier les régions chromosomiques impliquées dans les incompatibilités causant cette mortalité.

Construction de la carte génétique haute densité de la population interspécifique de poirier PEAR3 x 'Moonglow'

Les SNPs sont des variations nucléotidiques de l'ADN qui sont abondantes dans les génomes de plantes et sont utiles pour déterminer les polymorphismes au sein des individus ou des populations, mais aussi pour identifier et localiser les loci contrôlant la variation phénotypique.

Même si les SNPs ont été considérés comme les outils les plus efficaces pour les études de déterminisme génétique depuis plusieurs années, chez le poirier peu de SNPs étaient disponibles avant le début de cette thèse, et du coup, aucune des cartes génétiques développées pour *Pyrus* ne contenait de marqueurs SNP. La première carte de poirier avait été construite à l'aide de marqueurs ADN de type RAPD (Random Amplified Polymorphic DNA) dans un croisement entre *P. pyrifolia* 'Kinchaku' et 'Kosui' (Iketani et al. 2001). Yamamoto et al. (2002; 2004) ont mis au point la deuxième génération de cartes génétiques du poirier grâce à des marqueurs de type AFLP (Amplified Fragment Length Polymorphism) et des SSRs de pommier et poirier, en utilisant un croisement interspécifique entre 'Bartlett' (*P. communis*) et 'Hosui' (*P. pyrifolia*). La réalisation de la carte génétique de 'Bartlett' x 'Hosui' avec des SSRs développés chez le poirier et le pommier a permis de montrer la synténie entre les génomes de poirier et de pommier, et de numéroter les GLs du poirier selon la numérotation déjà établie chez le pommier. Des marqueurs SSR et AFLP de pommier et poirier avaient également été utilisés pour générer des cartes génétiques de deux cultivars de poirier européens, 'Passe Crassane' et 'Harrow Sweet' (Dondini et al. 2004). Des SSRs développés chez le poirier et le pommier ont aussi servis à Celton et al. (2009) pour générer une carte intégrée des cultivars *P. communis* 'Bartlett' et 'La France', et de deux porte-greffes de pommier, *Malus x domestica* 'Malling 9' et *M. robusta* 'Robusta 5'. Finalement, Lu et al. (2010) ont génotypé la population interspécifique 'Mishirazi' (*P. pyrifolia* X *P. communis*) x 'Jinhua' (*P. x bretschneideri*) avec des SSRs de pommier et ont également construit une carte génétique de poirier.

La révolution technologique initiée par la nouvelle génération de séquençage (NGS) a facilité l'identification de variations de séquence de l'ADN à l'échelle du génome entier, par le re-séquençage de multiples accessions d'une même espèce et l'alignement de ces séquences sur un génome de référence, et ce dans le but de détecter *in silico* des sites nucléotidiques polymorphes (Bentley 2006; Li et al. 2009; Hyten et al. 2010; Stothard et al. 2011; Chagné et al. 2012; Hand et al. 2012; Verde et al. 2012; Xu et al. 2012). Une fois que les polymorphismes SNP ont été détectés par NGS, le défi suivant est le criblage de grandes populations génétiques avec de multiples marqueurs simultanément. Si le re-séquençage peut être utilisé pour la découverte des SNPs et le génotypage de l'ensemble des polymorphismes d'une espèce (Elshire et al. 2011), les

puces SNP à haut débit, tels que la technique Infinium® II (Illumina Inc.), sont des outils efficaces pour le génotypage simultané de milliers de SNPs sur de grandes populations d'individus. Au moment où ce projet de thèse a été initié, quelques puces SNP avaient été développées pour une gamme d'espèces d'arbres fruitiers. Chez les *Rosaceae*, une puce SNP de pommier avait été développée par le consortium international RosBREED (*International RosBREED SNP Consortium* ; IRSC) (www.rosbreed.org) (Chagné et al. 2012). Cette puce Infinium® II 8K IRSC de pommier contient 7867 SNPs, dont 5554 sont polymorphes et couvrent le génome à haute densité. Le *International Peach SNP Consortium* (IPSC) avait mis au point une puce SNP 9K pour le pêcher qui inclut 8144 SNPs, dont 84.3 % sont polymorphes pour 709 accessions de pêcher, comprenant des cultivars de pêcher, des espèces sauvages de *Prunus* et des hybrides interspécifiques (Verde et al. 2012). Le projet international RosBREED a aussi dirigé le développement d'une puce SNP 6K pour la cerise, avec 1825 SNPs polymorphes chez la cerise et 2058 chez la griotte (Peace et al. 2012).

Nous avons utilisé des technologies NGS pour re-séquencer trois cultivars de poirier européen (*P. communis*) et détecter les SNPs dans le génome de poirier. Nous avons choisi 1096 SNPs de poirier, qui ont été combinés avec un ensemble des 7692 SNPs de pommier sur la puce Infinium® II 8K IRSC de pommier (Chagné et al. 2012). C'était la première fois que des SNPs de poirier étaient inclus dans une puce de génotypage. Ces nouveaux SNPs de poirier ont été choisis pour leur positionnement sur la séquence de gènes candidats, afin d'assurer leur utilité dans des études d'association marqueur-phénotype et pour les programmes d'amélioration génétique futurs. L'incorporation des SNPs de poirier et de pommier dans une seule puce a permis l'étude de la transférabilité des SNPs non seulement au sein du genre *Pyrus*, mais aussi entre les genres *Malus* et *Pyrus*. C'est la première fois qu'une puce de SNPs inter-générique est développée et évaluée. Nous avons ensuite évalué cette puce Infinium® II 9K SNP de pommier et poirier pour le génotypage grande échelle dans le poirier, et pour la construction de la carte génétique de cinq descendance de poiriers d'origine européennes et asiatiques, y compris la descendance PEAR3 x 'Moonglow'. L'évaluation de la puce de SNPs de pommier et poirier a été combinée à d'autres descendance pour maximiser le nombre de SNPs polymorphes chez le poirier européen, asiatique et les hybrides. Cette évaluation a été réalisée en collaboration avec deux autres

étudiantes en thèse. Les résultats présentés ci-dessous et résumant les performances des SNPs de poirier combinent les 5 descendance utilisées.

Le regroupement de 873 descendants génotypés a assuré une analyse précise des SNPs par le logiciel GenomeStudio. Une grande proportion (83.8 %) des 1096 SNPs de poirier étaient polymorphes dans au moins une population, et 857 de ces marqueurs polymorphes (93.4 %) ont été utilisés pour la construction des cartes génétiques. Ces cartes sont les premières cartes génétiques à haute densité sur la base de marqueurs SNPs chez le poirier. De plus, nous avons constaté que 1482 SNPs provenant de pommier (19.3 % du nombre total des SNPs de pommier présents sur la puce) étaient polymorphes chez le poirier, dont 1031 d'entre eux ont été placés sur les cartes génétiques. Les SNPs de pommier ont permis d'améliorer considérablement la densité en marqueurs des cartes génétiques de poirier. Ces résultats sont les premiers à démontrer la transférabilité des SNPs entre différents genres, et entre *Malus* et *Pyrus* en particulier. La plupart des nombreuses études sur la transférabilité de marqueurs génétiques ont mis l'accent sur les SSRs, y compris entre le pommier et poirier (Yamamoto et al. 2004; Pierantoni et al. 2004; Yamamoto et al. 2007; Lu et al. 2010). Les tentatives précédentes de transférer des SNPs entre genres ont impliqué seulement quelques accessions d'espèces non ciblées, y compris l'étude de Micheletti et al. (2011), qui a estimé le taux de transférabilité de l'état hétérozygote de *M. x domestica* vers *P. communis* et *P. pyrifolia* utilisant 237 SNPs de pommier. Dans la présente étude, nous avons observé que 7562 SNPs de pommier (98.3 %) était monomorphes ou polymorphes chez le poirier dans au moins une population, alors que seulement 130 n'ont pas hybridé parmi toutes les populations étudiées. Le pourcentage élevé d'hybridation de l'ADN génomique de poirier sur les SNPs de pommier (et vice versa) obtenus dans cette étude n'est pas surprenant, étant donné que *Malus* et *Pyrus* sont des genres phylogénétiquement strictement proches et sont supposés partager des séquences très similaires. De plus, les SNPs inclus dans la puce ont été sélectionnés pour être situés dans les séquences codantes des gènes, avec pour conséquence que les séquences flanquantes sont susceptibles d'être mieux conservées entre espèces.

Au total, 337 marqueurs SNP de poirier se sont révélés polymorphes dans la population PEAR3 x 'Moonglow' et 330 ont été utilisés avec succès pour construire les cartes génétiques des parents;

279 marqueurs SNPs dérivés de pommier étaient polymorphes et 255 ont également pu être cartographiés. Le nombre de SNPs de poirier polymorphes chez le cultivar européen ('Moonglow') était significativement plus élevé que chez l'hybride (PEAR3), ce qui s'explique assez logiquement par le fait que les SNPs proviennent de séquençage d'accessions de *P. communis*.

Un aspect supplémentaire et intéressant de cette étude de génotypage SNP est l'identification d'allèles nuls. Ces allèles nuls peuvent s'expliquer par des délétions dans la séquence flanquante d'un site polymorphe, par des polymorphismes secondaires dans cette même séquence, ou par des situations tri-alléliques du polymorphisme primaire (Carlson et al. 2006; Ollitrault et al. 2012). Comme la technologie de génotypage SNPs Infinium® II de Illumina ne permet pas de génotyper plus de trois allèles, dans notre étude les SNPs à allèles nuls pouvaient être classés seulement dans les deux premières catégories. Les allèles nuls sont une source importante de polymorphisme, mais, ils sont difficiles à détecter et analyser à l'aide du logiciel GenomeStudio de lecture et analyse des puces SNP. Un nombre plus élevé de SNPs avec allèles nuls a été détecté dans les populations interspécifiques par rapport à la population de *P. communis* pure. Ceci s'explique par une augmentation de la fréquence des allèles nuls avec la distance génétique entre les échantillons génotypés et le lot de variétés re-séquencées pour la découverte des SNPs (Ollitrault et al. 2012). En effet, des polymorphismes additionnels dans les séquences flanquantes des SNPs de la puce Infinium® sont plus fréquemment observés entre les différentes espèces (Asiatiques versus Européennes) ou genre (*Malus* versus *Pyrus*) qu'à l'intérieure de l'espèce européenne. Par ailleurs, nous avons constaté que la fréquence d'allèles nuls était similaire entre les SNPs de pommier et poirier. En définitive, puisque les allèles nuls hétérozygotes sont utiles pour la cartographie génétique, nous les avons utilisés pour augmenter la densité de la carte dans les populations interspécifiques. Dans le croisement PEAR3 x 'Moonglow', un total de 102 SNPs avec allèles nuls ont été ainsi inclus dans la carte génétique.

Nous avons donc démontré dans cette première partie l'utilité de la puce SNP Infinium® II 9K de pommier et poirier pour le génotypage à haut débit de populations de *P. communis*, et d'hybrides entre *P. communis*, *P. pyrifolia* et *P. x bretschneideri*. De plus, nous avons prouvé que les SNPs de la puce sont transférables, non seulement dans l'ensemble de ces espèces de *Pyrus*, mais aussi

entre les deux genres *Malus* et *Pyrus*. Cette étude a fait l'objet d'une première publication dans PLOS ONE (Montanari et al. 2013). Les cartes génétiques des cinq populations de poirier construites ont ensuite servi à ancrer l'assemblage de la séquence du génome de 'Bartlett', publiée en 2014 par Chagné et al.

La descendance PEAR3 x 'Moonglow' a également été génotypée avec des marqueurs SSRs de pommier et de poirier. Au total, 54 SSRs ont été choisis sur la carte intégrée de 'Bartlett' construite par Celton et al. (2009) et un autre SSR, Md-Exp 7, développé par Costa et al. (2008). De ces 55 marqueurs SSRs, 38 ont été cartographiés, 25 loci sur la carte de PEAR3 et 31 sur la carte de 'Moonglow'. Cette information a été suffisante pour confirmer l'identité des GLs et les orienter par rapport aux cartes publiées chez le pommier et le poirier.

Les cartes génétiques de PEAR3 et 'Moonglow' ont encore été améliorées au cours de la thèse par l'ajout d'autres SSRs et de nouveaux marqueurs développés par l'analyse des courbes de fusion à haute résolution (HRM). Au total, la carte de PEAR3 comprend 256 marqueurs couvrant 988 cM et celle de 'Moonglow' 515 marqueurs couvrant 1067 cM.

La construction de cartes génétiques à haute densité grâce aux SNPs chez le poirier constitue une étape importante vers l'identification de régions chromosomiques associées à la variation de plusieurs caractères, tels que la résistance aux maladies et ravageurs, la qualité des fruits et l'amélioration des conditions de culture dans les vergers de poirier.

Étude du déterminisme génétique de la résistance au psylle du poirier dans le croisement interspécifique PEAR3 x 'Moonglow'

Les psylles (*Hemiptera*, *Psyllidae*) sont un des plus grands ravageurs du poirier. Les espèces de psylles identifiées comme causant le plus de dégâts sont *Cacopsylla pyri* (Linnaeus), endémique en Europe, *C. pyricola* (Förster) en Europe et Amérique du Nord, et *C. bidens* (Sulc) en Europe et Moyen-Orient (Shaltiel-Harpaz et al. 2014). Le cycle de vie du psylle du poirier commence avec les œufs, pondus individuellement ou par groupe sur la plante hôte, qui éclosent ensuite en larves en passant par cinq stades larvaires (L1 à L5). Après la dernière mue, les larves se développent en adultes mâles ou femelles, qui sont capables de se reproduire sexuellement en quelques jours (Hodkinson 2009). Les psylles adultes et jeunes se nourrissent de la plante en

insérant leurs stylets dans le phloème. Cependant, le principal dégât sur l'hôte est causé par la production de miellat généré par les larves qui se nourrissent activement. Ce miellat est à son tour un substrat idéal pour le développement des champignons de la fumagine. Le miellat excrété, qui bloque la photosynthèse, provoque des nécroses sur les feuilles des plantes infestées (Salvianti et al. 2008) et le brûnissement des fruits, réduisant leur valeur économique (Pasqualini et al. 2006). Pendant l'été, le psylle peut donner naissance à plusieurs générations qui se chevauchent (Schaub et al. 2005), conduisant à de très fortes densités d'insectes qui peuvent provoquer la chute des feuilles et des fruits et réduire le calibre des poires (Shaltiel-Harpaz et al. 2014), induisant ainsi des pertes de rendement élevées. De plus, le psylle du poirier est le principal vecteur du phytoplasme (*Candidatus Phytoplasma pyri*) responsable de la maladie du "pear decline" (Salvianti et al. 2008). La lutte contre le psylle du poirier dans les vergers est basée principalement sur l'utilisation d'insecticides (par exemple l'amtiazine, l'abamectine, les organophosphorés, les pyréthrinoides) (Civolani 2012). Toutefois, l'insecte a développé une résistance à un grand nombre d'antiparasitaires chimiques (Harries and Burts 1965; Buès et al. 2003; Civolani et al. 2007), tandis que les stratégies de lutte biologique basées sur l'utilisation des ennemis naturels ne sont pas suffisantes pour empêcher les dégâts (Berrada et al. 1995). Par conséquent, le développement de nouveaux cultivars de poirier avec une résistance durable apparaît comme une stratégie efficace pour contrôler le psylle.

Les trois typologies de résistance des plantes aux insectes sont l'antixénose, l'antibiose et la tolérance (Hesler and Tharp 2005; Bell 2013a). L'antixénose empêche les insectes de coloniser l'hôte ou de s'alimenter durablement et l'antibiose affecte la biologie du parasite, alors que la tolérance est la capacité de la plante à croître malgré l'infestation (Hesler and Tharp 2005). L'antixénose envers le psylle du poirier est caractérisé par la dissuasion de ponte des œufs et l'inhibition de l'alimentation, tandis que l'antibiose est exprimée par la mortalité larvaire et le retard du développement (Bell and Stuart 1990). Il est possible que ces types de résistance ne partagent pas un déterminisme moléculaire et biologique commun, parce que certains génotypes de poirier ne montrent que l'un ou l'autre (Pasqualini et al. 2006). Des résistances de type antixénose et antibiose envers *C. pyri* en Europe et *C. pyricola* en Amérique du Nord ont été caractérisées, et des cultivars avec différents niveaux de résistance ont été identifiés parmi les

poiriers asiatiques et européens et les hybrides interspécifiques (Bell and Stuart 1990; Bell 1992; Robert et al. 2004; Robert and Rimbault 2004; Bell 2013a). L'étude de tous les types de résistance au psylle du poirier est fondamentale pour déterminer si un cultivar sera utile pour les programmes d'amélioration génétique.

La résistance du poirier au psylle est considérée comme un caractère polygénique (Pasqualini et al. 2006; Lespinasse et al. 2008), mais à ce jour un seul QTL a été détecté sur le GL17 chez la descendance interspécifique 'Angélys' (*P. communis*) x NY10355 (*P. ussuriensis* X *P. communis*) (Bouvier et al. 2011a). À notre connaissance, il n'y a qu'une autre étude axée sur la cartographie de locus de résistance aux ravageurs de poirier. En effet, Evans et al. (2008) ont cartographié un gène majeur de résistance à *D. pyri* sur GL17 de *P. nivalis*. En revanche, dans le génome de pommier (*M. x domestica*) plusieurs loci liés à la résistance aux insectes, en particulier aux pucerons, ont été cartographiés (Roche et al. 1997; Cevik and King 2002; Stoeckli et al. 2008b; Bus et al. 2008; Stoeckli et al. 2008a; Bus et al. 2010).

Nous avons étudié une nouvelle source de résistance au psylle du poirier, dérivé de l'espèce asiatique *P. x bretschneideri*, dans le pedigree de PEAR3. PEAR3 a été précédemment évalué comme moyennement résistant aux psylles (données non publiées), tandis que 'Moonglow' a été montré comme étant modérément à fortement sensible (Bell 1984; Berrada et al. 1995). Dans un verger de poirier monovariétal l'insecte est plus proche d'une situation de "non choix" (Pasqualini et al. 2006), nous nous sommes donc concentrés sur la résistance de type antibiose, principalement exprimée sous forme de développement larvaire réduit. Les cartes génétiques parentales élaborées lors de l'étape précédente de la thèse ont été utilisées pour détecter des QTL de résistance au *C. pyri*.

Recueillir des données phénotypiques quantitatives et reproductibles avec un effet de l'environnement le plus faible possible sur un grand nombre de plantes est crucial pour la cartographie de QTL. Plusieurs protocoles ont été développés précédemment pour le phénotypage de l'antibiose au psylle (Berrada et al. 1995; Pasqualini et al. 2006; Bell 2013a; Bell 2013b), mais aucun d'entre eux ne convenait pour l'évaluation d'une grande population. Les défis en termes de logistique et de reproductibilité ont été exacerbés par la nécessité d'une stricte synchronisation phénologique entre la plante et le parasite et la création d'un environnement avec

des conditions de croissance optimales pour chacun d'eux. Nous avons élaboré un nouveau protocole de phénotypage pour étudier la résistance de type antibiose et pour recueillir des données quantitatives sur des centaines de plantes. Afin de réaliser un test de non choix et garantir la ponte sur tous les génotypes, les 3-4 feuilles du haut de chaque pousse ont été recouvertes avec des sachets en organza et deux adultes (un mâle et une femelle) de *C. pyri* ont été introduits dans chaque sachet. Après huit jours, les sachets ont été retirés, en veillant de ne pas laisser des adultes vivants dans la serre, et les œufs ont été dénombrés à l'aide de loupes binoculaires, avec six classes de dénombrement. Dès lors, les plantes ont été observées et ce afin de déterminer le moment où les œufs seraient éclos, sans aucun adulte émergent, et par conséquent le début du dénombrement des larves (trois à quatre semaines après l'infestation). L'évaluation du nombre des larves était la partie la plus cruciale de l'expérience. En effet, afin de réduire le plus possible la variabilité des caractères phénotypiques, le juste équilibre entre le temps (l'évaluation devait être terminée en quelques jours) et la subjectivité inévitable du notateur (plus il y a de notateurs, plus il y a de variabilité) devait être atteint. Les larves jeunes (stades L1, L2 et L3) et âgées (stades L4 et L5) vivantes ont été dénombrées à l'aide de loupes binoculaires. Néanmoins, dans la pratique l'antixénose et l'antibiose ne pouvaient pas être complètement dissociées, nous avons donc aussi mesuré une variabilité significative du nombre d'œufs parmi les génotypes. De plus, comme l'antixénose intervient plus tôt dans le processus parasitaire que l'antibiose, elle peut entraver la détection correcte de l'antibiose en masquant sa variation génétique, surtout pour les génotypes présentant une forte antixénose. Une telle dépendance chronologique crée un biais dans l'exactitude de l'évaluation de l'antibiose. Ce biais ne peut pas être corrigé simplement par des approches statistiques. Ainsi, l'antixénose peut générer une pseudo-antibiose. Ici, la présence constante d'un faible nombre d'œufs entre les répétitions de plusieurs génotypes a généré une héritabilité modérée mais significative pour ce caractère, ce qui démontre qu'il y avait une contribution importante de l'antixénose à la résistance au psylle dans notre expérience. Systématiquement, nous avons pu détecter des QTLs pour le nombre d'œufs. Néanmoins, nous avons considéré que l'antibiose était présente et correctement cartographiée dans notre expérience, puisque le nombre d'œufs était assez élevé pour un grand nombre des génotypes. Ainsi, le nouveau protocole de phénotypage mis au point a permis un contrôle

incomplet, mais acceptable du mécanisme d'antixénose et une étude correcte de l'antibiose. De plus, ce protocole s'est avéré répétable sur plusieurs années.

Un QTL stable à effet majeur a été détecté sur le GL8 de PEAR3 ($R^2 = 17.2 - 39.1 \%$). De plus, des QTLs ont été cartographiés sur le GL5 ($R^2 = 10.8 \%$) de PEAR3 et le GL15 de 'Moonglow' ($R^2 = 13.7 \%$). En outre, une interaction significative (épistasie) a été détectée entre les QTLs sur les GLs 8 et 5. Les résultats de la cartographie QTL pour le nombre d'œufs et de larves de différents stades montrent que le QTL situé sur le GL8 de PEAR3 est responsable d'une forte antibiose, mais aussi d'antixénose. Étant donné que l'intervalle de confiance de ce QTL est assez large, deux loci différents, mais étroitement liés, un lié à l'antibiose et l'autre à l'antixénose, pourraient être situés dans la même région chromosomique. La taille de la population (~100 descendants) n'est pas assez élevée pour faire la distinction entre les deux hypothèses: avec des tailles de populations inférieures à 500, les QTLs étroitement liés (environ 20 cM ou moins) ne peuvent pas être détectés (Collard et al. 2005). En revanche, le QTL sur le GL15 du parent sensible 'Moonglow', même si sa présence doit être confirmée par des tests supplémentaires, pourrait être spécifiquement lié au mécanisme d'antibiose (c'est-à-dire un retard de développement larvaire). L'observation de plusieurs lignées transgressives parmi la descendance laissait supposer la présence de facteurs de résistance provenant des deux parents. Par conséquent, des sources inconnues de résistance au psylle du poirier pourraient être présentes parmi les cultivars de *P. communis* dans le pedigree de 'Moonglow'. Des expériences de phénotypage et génotypage additionnelles seront nécessaires pour réduire l'intervalle de confiance du QTL sur le GL8 et pour confirmer l'importance des QTLs mineurs sur les GLs 5 et 15, et ce dans le but d'identifier des marqueurs moléculaires utilisables en SAM. En outre, re-tester la même population avec plus de précision sur la ponte (avec un dénombrement réel et non par classe) pourrait être utile pour vérifier l'hypothèse de la présence de deux loci distincts sur le GL8, un pour l'antibiose et un pour l'antixénose.

Par le biais de marqueurs SSRs communs, la carte génétique de PEAR3 pourrait être alignée sur les cartes de poiriers et de pommiers générées pour la détection de QTLs et gènes majeurs de résistance aux bio-agresseurs. Le QTL majeur que nous avons détecté sur le GL8 co-localise avec deux gènes de résistance au puceron lanigère du pommier cartographiés par Bus et al. (2008;

2010). Ce n'est pas la première fois que des loci associés à la résistance aux psylles et aux pucerons se trouvent dans des régions chromosomiques orthologues entre différentes espèces (et genres). D'autres gènes de résistance aux pucerons ont été cartographiés sur le GL17 chez le pommier (Bus et al. 2008; Stoeckli et al. 2008a; Bus et al. 2010) dans la même région que le QTL de résistance au psylle détecté par Bouvier et al. (2011) chez l'hybride 'NY10355' (*P. ussuriensis* X *P. communis*). Les pucerons et les psylles sont tous les deux des insectes de type piqueur-suceur, et notamment suceurs du phloème, donc trouver des régions orthologues liées à l'antibiose vis-à-vis de ces insectes pourraient indiquer certains mécanismes moléculaires communs impliqués dans la résistance. Civolani et al. (2013) ont mené des expériences sur le comportement exploratoire de *C. pyri*, et ont émis l'hypothèse que des facteurs de résistance à effet majeur seraient présents dans le phloème des accessions de poirier résistantes.

En conclusion, les résultats de notre expérience confirment le déterminisme polygénique de la résistance au psylle du poirier. La principale source de résistance dans la population PEAR3 x 'Moonglow' était *P. x bretschnideri*, source différente de celle étudiée par Bouvier et al. (2011), *P. ussuriensis*. Les deux principaux QTLs détectés dans ces deux études sont situés sur des GLs différents, sur le GL8 dans notre cas et sur le GL17 dans la cadre de l'étude de Bouvier et al. (2011). Le cumul de ces deux loci pourrait être une stratégie efficace pour le développement de variétés de poirier résistantes aux psylles. De plus, les cultivars de poirier cumulant des QTLs responsables de l'antixénose et de l'antibiose pourraient présenter une résistance plus durable, plus difficile à contourner par de nouvelles races de psylle. Enfin, il serait intéressant d'étudier la localisation éventuelle sur les GLs 8 et 17 de pommier et de poirier de gènes responsables de la production de facteurs de résistance dans le phloème, agissant en réponse à l'infestation de pucerons et de psylles. Pour ce faire, des descendants très résistants et très sensibles des deux populations de cartographie de résistance au psylle et au puceron pourraient être choisis, avec les parents, pour des études d'electropénétrographie (EPG). De plus, le séquençage ARN (*RNA-Seq*) de ces mêmes génotypes sélectionnés pourrait être utile pour trouver les gènes impliqués dans la résistance aux psylles chez PEAR3 et NY10355.

Cette étude a fait l'objet d'une publication récemment acceptée dans le journal *Trees Genetics and Genomes*.

Cartographie de QTLs de résistance au feu bactérien

Le feu bactérien est une maladie dévastatrice des espèces de *Rosaceae* (Vanneste 2000) et la plus importante économiquement pour les producteurs et vendeurs de poire. Elle est causée par la bactérie gram-négative *Erwinia amylovora* (Burrill) Winslow et al. e (Vanneste 2000), qui sévit dans plusieurs pays du monde (Bonn and Van Der Zwet 2000). *E. amylovora* est considérée comme un organisme de quarantaine par l'Organisation Européenne et méditerranéenne pour la Protection des Plantes (OEPP) (EPPO 1977), par la Commission Phytosanitaire pour l'Asie et le Pacifique (APPPC) et par d'autres organisations régionales de la protection des végétaux (Bokszczanin et al. 2009; <http://www.cabi.org/isc/datasheet/21908>), pour lesquelles la présence de bactéries sur le matériel végétal et les fruits peut contraindre le commerce. L'agent pathogène pénètre dans la plante par des ouvertures naturelles dans les fleurs ou des plaies et ensuite il se développe et est véhiculé de façon systémique dans les vaisseaux de la plante, provoquant la nécrose rapide de tous les tissus infectés et la production de gouttelettes d'exsudats (Malnoy et al. 2012). Les dégâts directs sont liés à une réduction très importante de la production (EPPO 1977), mais une conséquence plus grave encore est que la plante, après avoir été infectée, doit être détruite, puisque tous les organes infectés sont des sources potentielles de dissémination de la bactérie (Thomson 2000). Le contrôle de cet agent pathogène est difficile et aucune stratégie n'est totalement efficace (Paulin 1990): l'application de composés chimiques, principalement des antibiotiques et le cuivre, ainsi que les stratégies de lutte biologique doivent être combinées avec l'éradication des plantes infectées (EPPO 1977; Norelli et al. 2003). Mais l'utilisation d'antibiotiques n'est pas autorisée dans tous les pays. Le développement de cultivars avec une résistance durable a une importance primordiale dans le cadre d'une lutte intégrée efficace contre le feu bactérien (Lepinasse and Aldwinckle 2000), et un certain nombre de programmes d'amélioration génétique de poiriers et de pommiers dans le monde ont mis l'accent sur cet objectif depuis le début des années 80.

Malgré le fait que les espèces de poirier asiatique d'importance économique, tels que *P. ussuriensis*, *P. pyrifolia* Nakai, *P. calleryana* et *P. betulaefolia*, aient tendance à être plus résistantes à *E. amylovora* que le poirier européen (*P. communis*) et d'autres espèces asiatiques (Van Der Zwet et al. 1974; Bell and Ranney 2005) et soient par conséquent utilisés plus fréquemment pour le développement de cultivars résistants au feu bactérien, des accessions résistantes peuvent être trouvées chez toutes les espèces (Paulin 1990; Lespinasse and Aldwinckle 2000). Deux études phénotypique (par exemple Durel et al. 2004) et génotypique (par exemple Dondini et al. 2004) suggèrent que ce caractère est polygénique chez toutes les espèces, et des QTLs de résistance ont été détectés chez *P. ussuriensis* et *P. communis*. À ce jour, trois de ces QTL ont été cartographiés chez le cultivar de poirier européen résistant 'Harrow Sweet' (Dondini et al. 2004; Le Roux et al. 2012), un chez l'accession résistante n°18 de l'espèce asiatique *P. ussuriensis*, et un autre dans le cultivar sensible de *P. communis* 'Doyenné du Comice' (Bokszczanin et al. 2009) sur les GLs 2, 4 et 9 respectivement.

Deux sous-ensembles de la descendance PEAR3 x 'Moonglow' ont été évalués pour la résistance au feu bactérien en France et en Nouvelle-Zélande, à l'aide de deux souches locales d'*E. amylovora* (CFBP 1430 et Ea9148, respectivement). 'Moonglow' est une variété bien connue pour sa faible sensibilité (Quamme 1977; Paulin 1990), tandis que PEAR3 est très sensible au feu bactérien (données non publiées). Les cartes génétiques des parents construites pour cette population ont été utilisées pour détecter des QTLs de résistance au feu bactérien. Les évaluations phénotypiques ont été réalisées grâce à la méthode d'inoculation, largement utilisée, de découpage de la feuille avec des ciseaux préalablement trempés dans la solution bactérienne (Maas Geesteranus and Heyting 1981). Nous avons effectué ensuite plusieurs notations de la maladie, chaque semaine jusqu'à 28 jours après l'inoculation (jai). Ceci nous a permis de calculer le taux de développement de la maladie avec l'aire sous la courbe de la progression de la maladie (AUDPC) (Shaner and Finney 1977). Deux variables ont donc été étudiées : la sévérité mesurée à 28 jai et l'AUDPC.

En France et en Nouvelle-Zélande, un QTL stable à effet majeur a été localisé en haut du GL2 de 'Moonglow' ($R^2 = 12.9 - 34.4 \%$). Trois autres QTLs ont été cartographiés sur les GLs 7, 12 et 15 de PEAR3 en Nouvelle Zélande, dont deux présentaient des interactions épistatiques (entre les

QTLs des GLs 7 et 12). Ces QTLs peuvent être spécifiques de la souche Ea9148, puisqu'ils n'ont pas été détectés dans le sous-ensemble de descendants phénotypés en France avec la souche CFBP 1430. À l'inverse, un QTL à effet mineur a été détecté sur le GL9 de PEAR3 en France et en Nouvelle-Zélande (putatif); cependant, en Nouvelle Zélande le LOD score était inférieur au seuil, et la localisation sur le GL différente du QTL cartographié en France. On ne peut pas, donc, clairement affirmer si ce QTL sur le GL9 est spécifique à la souche CFBP 1430 ou à large spectre. Les variations de conditions environnementales entre les tests réalisés en France et en Nouvelle Zélande pourraient également influencer sur l'identification des QTLs sur les GLs 7, 9, 12 et 15. La détection de plusieurs QTLs chez PEAR3, le parent très sensible, ainsi que la présence de certaines lignées transgressives, confirment le déterminisme polygénique de la résistance au feu bactérien dans cette population, conformément à ce qui a été précédemment décrit dans d'autres descendance de poirier (Dondini et al. 2004; Le Roux et al. 2012) et de pommier (Calenge et al. 2005; Durel et al. 2009).

Les parents de 'Moonglow', Michigan-US 437 et 'RCW', sont tous les deux résistants au feu bactérien (Paulin 1990; Durel et al. 2004). Nous avons effectué une analyse génétique avec des SSRs positionnés dans l'intervalle de confiance du QTL du GL2 de 'Moonglow', et nous avons démontré que les allèles favorables ont été hérités de RCW. Nous ne connaissons pas l'identité du parent mâle de PEAR3, mais il a été possible de vérifier que les allèles favorables aux QTLs des GLs 9 et 15 de PEAR3 avaient été hérités par *P. x bretschneideri* 'Xue Hua Li'. PEAR3 et 'Xue Hua Li' sont deux génotypes très sensibles au feu bactérien. Cependant, les espèces asiatiques de poirier ont fréquemment été rapportées comme sources de résistance au feu bactérien (Paulin 1990; Bell and Ranney 2005; Peil et al. 2009), il n'est donc pas surprenant que 'Xue Hua Li' ait transmis des allèles conférant une certaine résistance (faible) à sa descendance. Il n'est pas rare que les parents sensibles transmettent des allèles de résistance à leur descendance, ceci a été décrit lors d'études de différentes interactions plante-pathogène (Foulongne et al. 2003; Perchepped et al. 2005; Perchepped et al. 2006).

Des SSRs communs entre le QTL localisé sur le GL2 de 'Moonglow' et celui de 'Harrow Sweet', détecté par Dondini et al. (2004) et dont la position a été précisée par Le Roux et al. (2012), nous ont permis d'observer leur co-localisation. Cependant les deux allèles favorables correspondants

pourraient ne pas être identiques, ne pas provenir du même ancêtre. Le Roux et al. (2012) ont identifié les allèles favorables de résistance de ‘Harrow Sweet’ et ont retracé leurs origines comme provenant de ‘Early Sweet’. Toutefois, un autre grand-parent de ‘Harrow Sweet’, ‘Old Home’, est aussi très résistant au feu bactérien (Van Der Zwet et al. 1974; Quamme 1977), plus résistant que ‘Early Sweet’, mais sa résistance au feu bactérien n’a jamais été cartographiée. L’alignement de la carte génétique SNP du GL2 de ‘Old Home’ avec le GL2 de ‘Moonglow’ a mis en évidence la colinéarité entre les deux régions homologues qui sous-tendent le pic du QTL de résistance au feu bactérien de ‘Moonglow’. De plus, les marqueurs SNPs dans cette région présentent le même haplotype pour les deux cultivars. Par conséquent, il est fortement probable que ‘Old Home’ porte le même allèle de résistance au feu bactérien que ‘Moonglow’ au niveau de ce QTL. Nous pourrions donc conclure que ce QTL majeur de résistance au feu bactérien du poirier détecté dans le cadre de notre étude sur le LG2 est stable, non seulement dans des environnements différents, mais également dans fonds génétiques différents. Nous sommes donc en mesure de proposer des marqueurs SNPs et SSRs pour la SAM sur la résistance au feu bactérien. Toutefois, avant que les sélectionneurs puissent utiliser ces marqueurs pour la SAM, ils devront être validés dans des fonds génétiques plus diversifiés. Pour cela, nous proposons d’étudier des populations de poirier issus de croisement avec le cultivar ‘Old Home’. Ceci pourrait permettre de confirmer l’hypothèse que ce cultivar porte le même QTL que ‘Moonglow’ sur le GL2. Avec les nouvelles technologies de test ADN disponibles à ce jour, les marqueurs SNPs pourraient être plus appropriés pour la SAM que les SSRs. En effet, les marqueurs basés sur les SNPs sont maintenant utilisés en routine pour la SAM dans le programme d’amélioration génétique de pommier en Nouvelle Zélande, car la technique HRM est simple (Chagné 2015) et se prête bien à l’automatisation. De plus, maintenant il est possible de créer à bas prix de mini-puce SNPs pour le criblage de populations avec des marqueurs associés à plusieurs caractères différents simultanément (Peace et al. 2012; Gasic and Peace 2013; Ru et al. 2015).

En ce qui concerne les autres QTLs à effet mineur détectés pour PEAR3, nous n’avons trouvé aucune homologie avec d’autres populations de poirier utilisées pour l’identification des loci de résistance au feu bactérien (Dondini et al. 2004; Bokszczanin et al. 2009; Le Roux et al. 2012). Cependant, les génomes de poirier et de pommier étant fortement synténiques (Yamamoto et al.

2004; Pierantoni et al. 2004; Celton et al. 2009a), nous avons également comparé les localisations de nos QTLs nouvellement cartographiés avec celles qui ont été cartographiées dans des régions orthologues du pommier. Un QTL pour la résistance au feu bactérien sur le GL9 a été cartographié chez *M. x domestica* ‘Nova-Easygro’ (Le Roux et al. 2010), dans une région synténique à celle du QTL détecté sur le GL9 de PEAR3. En outre, des QTLs ont été cartographiés sur les GLs 7, 12 et 15 de plusieurs accessions de pommier, et en particulier: sur le GL7 de ‘Fiesta’ (*M. x domestica*) croisé avec ‘Prima’ et ‘Discovery’ et sur le GL12 de ‘Discovery’ dans le même croisement (Calenge et al. 2005; Khan et al. 2007); dans la même population ‘Fiesta’ x ‘Discovery’ sur le GL7, en utilisant une autre souche d’*E. amylovora* (Khan et al. 2006); sur le GL7 de ‘Robusta 5’ dans un croisement avec ‘Ottawa 3’ phénotypé avec les souches Ea273 et Ea2002a (Gardiner et al. 2012); sur les GLs 12 et 15 de ‘Evereste’ (*M. floribunda* X *M. x domestica*) dans un croisement avec ‘MM106’ et sur le GL12 de *M. floribunda* clone 821, en croisement avec ‘Golden Delicious’ (Durel et al. 2009); sur le GL15 dans la population F1 ‘Co-op 16’ x ‘Co-op 17’ (*M. x domestica*) (Khan et al. 2012b). Les QTLs détectés sur les GLs 7 et 12 du pommier ont été localisés dans la partie inférieure de ces deux GLs, de même que les QTLs de résistance au feu bactérien du poirier détectés chez PEAR3.

En résumé, la détection d’un QTL majeur de résistance au feu bactérien sur le GL2 du parent européen de la population PEAR3 x ‘Moonglow’ est d’une importance remarquable. Nous avons démontré que ce QTL est à spectre large et stable dans plusieurs environnements (après avoir testé la descendance en France et en Nouvelle Zélande et utilisé deux souches différentes d’*E. amylovora*) et cultivars (‘Old Home’, qui semble n’avoir aucun lien avec ‘Moonglow’, porterait le même QTL). Nous avons également proposé des marqueurs SSRs et SNPs pour la SAM pour la résistance au feu bactérien chez le poirier, après une validation de ces marqueurs dans un panel de fonds génétiques.

Vu que le QTL du GL2 de ‘Moonglow’ est un QTL à effet majeur, des gènes majeurs pourraient être localisés dans cette région. Chez le pommier, un gène de type CC-NBS-LRR (Coiled-Coil-Nucleotide-Binding site-Leucine-Rich Repeat) *FB_MR5* a été identifié comme étant le gène responsable de la résistance au feu bactérien sur le GL3 de *M. x robusta* 5 (Broggini et al. 2014). Il est à noter que le chromosome 2 de *P. x bretschneideri* est riche en clusters de paralogues des

gènes de résistance (*R*) (Wu et al. 2013), et il est possible que celui de *P. communis* le soit aussi. La récente publication de la séquence du génome de *P. communis* (Chagné et al. 2014) facilitera la réalisation d'études de cartographie fine, nécessaires pour réduire l'intervalle de confiance du QTL et identifier les gènes candidats pour la résistance au feu bactérien.

Nous avons de plus détecté quatre QTLs à effet mineur sur la carte génétique de PEAR3, dont les allèles favorables de deux de ces QTLs ont été hérités de 'Xue Hua Li', ce qui montre que ce cultivar asiatique, même en étant sensible, pourrait servir comme source de résistance au feu bactérien.

Cette étude fait l'objet d'une publication encore en cours de rédaction à soumettre dans *Molecular Breeding*.

Cartographie des zones génomiques liées aux incompatibilités génétiques entraînant le phénomène de la nécrose hybride dans la descendance PEAR3 x 'Moonglow'

Le phénomène de la "nécrose hybride" est défini comme la viabilité réduite d'un hybride en raison d'incompatibilités génétiques. Bien que les interactions entre les gènes peuvent avoir un effet positif sur l'hybride, résultant en de meilleures performances que ses parents (la "vigueur hybride"), elles peuvent aussi être préjudiciables et causer la stérilité, le manque de vigueur ou la létalité (Bomblies et al. 2007). Les incompatibilités génétiques peuvent survenir à différents stades de la reproduction. Elles sont généralement divisées en incompatibilité pré-zygotique et post-zygotique, en agissant, respectivement, avant et après la fécondation. Le phénomène de la nécrose hybride, qui est aussi appelé "manque de vigueur de l'hybride" ou "non-viabilité", est un type de barrière post-zygotique de flux de gènes qui est associée à un phénotype typique des plantules, caractérisé par la mort cellulaire, la nécrose, le flétrissement, le jaunissement, la chlorose, le nanisme, une baisse de la croissance et dans certains cas la létalité (Bomblies and Weigel 2007; Bomblies 2009). La nécrose hybride a été observée dans plusieurs taxons végétaux, chez les espèces sauvages et cultivées, aussi bien dans des populations de lignées et des populations allogames, mais son phénotype semble être caractéristique au sein d'une gamme d'hôtes, ce qui suggère un mécanisme commun sous-jacent (Bomblies and Weigel 2007;

Bomblies 2009). Selon le modèle de Bateson-Dobzhansky-Muller (BDM), la génétique de la nécrose hybride est simple et implique des interactions épistatiques entre au moins deux loci (Orr 1996). Le modèle BDM postule que des substitutions indépendantes se produisant dans deux lignées divergentes, non délétère dans leur contexte génomique natif, pourraient devenir délétères quand elles sont combinées chez l'hybride. La plupart des cas de nécrose hybride rapportés dans la littérature est expliquée par l'épistasie entre deux gènes (Bomblies and Weigel 2007).

Bien que la non-viabilité de l'hybride soit connue depuis longtemps parmi les sélectionneurs et les scientifiques de la spéciation, et qu'il y ait des exemples dans la littérature depuis le début du XXe siècle (Hollingshead 1930), seulement récemment des efforts ont été faits afin d'en expliquer les bases moléculaires. Le phénotype associé à la nécrose hybride ressemble à l'ensemble des symptômes survenant après l'attaque d'agents pathogènes, et les recherches sur *Arabidopsis* spp. (Bomblies et al. 2007; Alcázar et al. 2009; Tahir et al. 2013) et la tomate (Krüger et al. 2002) ont montré qu'elle était liée à des réactions d'auto-immunité impliquant des gènes *R*. Au cours de cette réaction d'hypersensibilité (HR), la plante subit des stress oxydatifs, suivies d'une mort cellulaire programmée (Greenberg et al. 2003; Takken et al. 2006). La progression de l'agent pathogène, qui nécessite que les tissus soient vivants (Dangl et al. 1996), est ainsi stoppée. Dans le cas de la non-viabilité de l'hybride, le système immunitaire de la plante est activé en l'absence de l'attaque d'un agent pathogène, en raison de l'incompatibilité génétique, ce qui provoque une nécrose des tissus semblable à celle observée au cours de la HR. Une hypothèse est que différentes protéines *R* (au moins deux), codées par des gènes *R* ayant évolué indépendamment, causent l'"auto-nécrose" lorsqu'elles interagissent chez l'hybride (Bomblies et al. 2007). Sinon, un locus coderait pour une protéine hôte qui régulerait l'activation de la protéine *R* codée par le second locus, comme expliqué par le "modèle de garde" (Jones and Dangl 2006; Bomblies 2009). La plupart des gènes *R* montrés comme étant impliqués dans la nécrose hybride appartiennent à la classe NB-LRR. Les gènes *R*, et en particulier les domaines LRR, sont connus pour être très polymorphes, même au sein de la même espèce, évoluant à un rythme très rapide sous la pression de sélection naturelle pour la résistance (Bergelson et al. 2001), ce qui est cohérent avec l'hypothèse de leur implication dans les incompatibilités génétiques BDM.

Il existe plusieurs exemples dans la littérature d'événements de nécrose hybride survenant dans des populations en ségrégation générées pour augmenter la résistance aux agents pathogènes dans une gamme d'espèces (Bomblies and Weigel 2007; Bomblies et al. 2007), y compris le blé (Morrison 1957), le riz (Ichitani et al. 2012) et les pommes de terre diploïdes (Valkonen and Watanabe 1999). De plus, des ratios de ségrégation distordus en faveur ou contre la résistance à la tavelure ont été signalés également chez le pommier (Tartarini 1996; Conner et al. 1997; Gao and Van de Weg 2006) et le poirier (Iketani et al. 2001; Bus et al. 2013), et de fortes mortalités ont été observées chez des hybrides inter-génériques pommier-poirier (Shimura et al. 1980; Inoue et al. 2003).

Le croisement entre l'hybride interspécifique de première génération PEAR3 et le poirier européen 'Moonglow' a généré une large proportion de plantules non viables (plus de 50%), qui présentaient le phénotype typique de la nécrose hybride (Bomblies and Weigel 2007; Bomblies 2009). En particulier, nous avons observé deux types de létalité (que nous avons appelé 'Type 1' et 'Type 2'), exprimés à deux moments différents; nous avons aussi qualifié les plantules qui ont poussés normalement de 'Type 3'. Un mois après la germination, la non-viabilité des plantules de 'Type 1' était déjà visible, par rapport à leurs tailles beaucoup plus petites au regard des autres plantules et à la présence des nécroses importantes. À ce moment-là, les plantules de 'Type 2' sont aussi grandes que celles de 'Type 3', montrant seulement de petites nécroses et un enroulement des feuilles; cependant, leur surface foliaire est déjà significativement plus petite que celle des plantules de 'Type 3'. La non-viabilité de 'Type 2' devient apparente 50 jours après la germination, et encore plus 85 jours après la germination, quand les plantules sont irréversiblement bloquées ou mortes. Par conséquent, ce deuxième type de nécrose hybride agit plus lentement que celui de 'Type 1', atteignant son expression complète seulement trois mois après la germination. Des régions de génome très distordues ont été détectées dans les cartes génétiques des parents, suggérant la présence d'incompatibilités pré-zygotiques (non caractérisées) et post-zygotiques affectant le développement des descendants. Les gènes létaux impliqués dans la nécrose hybride des semis de 'Type 1' et 'Type 2' pourraient être localisés dans certaines de ces régions. En combinant des marqueurs génétiques précédemment cartographiés et nouvellement développés, nous avons identifié trois régions chromosomiques

associées à ces deux types de létalité, qui sont génétiquement indépendants. L'analyse de la ségrégation des phénotypes a montré que des incompatibilités de type BDM impliquant l'épistasie entre différents loci sont à la base de la nécrose hybride chez cette population de poirier, résultat qui est cohérent avec les études déjà publiées sur les autres plantes (Song et al. 2009; Alcázar et al. 2009; Mizuno et al. 2010). La létalité de 'Type 1' résulte d'une interaction épistatique négative entre deux loci, l'un cartographié sur le GL5 de PEAR3 et l'autre sur le GL1 de 'Moonglow'. En revanche, la nécrose hybride de 'Type 2' semble due à un locus situé sur le GL2 de PEAR3, qui soit agit seul, ou, plus probablement, interagit avec gène non localisé hérité de 'Moonglow'. L'hypothèse des deux loci interagissant est plus probable que celle d'un locus unique, étant donné que les incompatibilités post-zygotiques décrites sont habituellement causées par des interactions épistatiques entre au moins deux gènes (Orr 1996; Bomblies and Weigel 2007). Le rétrocroisement de la descendance F1 viable (qui porte l'allèle de létalité seulement au locus inconnu et pas au locus GL2) avec PEAR3 pourrait valider cette hypothèse.

Comme une réponse auto-immune est susceptible de se produire dans des combinaisons incompatibles montrant le phénotype de nécrose hybride (Krüger et al. 2002; Bomblies et al. 2007; Tahir et al. 2013), nous avons mis en lien nos résultats avec les résistances cartographiées chez le poirier. Le marqueur SSR CHVf1 est associé avec le gène létal sur le GL1 de 'Moonglow' et impliqué dans l'incompatibilité de 'Type 1'. Chez le pommier, ce marqueur est étroitement lié aux deux principaux gènes conférant la résistance à la tavelure (*Venturia inaequalis*), *Rvi6* et *Rvi17* (Bus et al. 2011), anciennement nommé *Vf* (Vinatzer et al. 2004) et *Val* (Dunemann and Egerer 2010), respectivement. Les génomes de pommier et poirier étant très synteniques (Yamamoto et al. 2004; Pierantoni et al. 2004; Celton et al. 2009a), il est possible qu'un locus orthologue du gène *Rvi6* de pommier soit impliqué dans la létalité de 'Type 1' au sein de la population PEAR3 x 'Moonglow'. Chez le poirier, le gène de résistance à la tavelure asiatique (*V. nashicola*) *Vnk*, rebaptisé *Rvn1*, a également été localisé sur le GL1, en amont de la région orthologue du pommier porteuse du gène *Rvi6* (Iketani et al. 2001; Terakami et al. 2006; Bouvier et al. 2011b). Le gène *Rvi6* a été fréquemment associé à des événements de distorsion de ségrégation et de nécrose hybride chez le pommier (Alston 1976; Gao and Van de Weg 2006). Puisque cette résistance provient de *M. floribunda*, largement utilisé par les sélectionneurs de

pommier pour effectuer des croisements interspécifiques afin d'obtenir des cultivars combinant des facteurs de résistance à la tavelure (Crosby et al. 1992), les incompatibilités entre les espèces pourraient bien être à l'origine de la nécrose hybride chez le pommier, comme montré ici chez le poirier. Il est à noter que pour une des deux cartes génétiques parentales construites pour une population interspécifique différente du poirier le GL1 manque complètement (Won et al. 2014). Cela pourrait avoir été causé par de fortes distorsions de ségrégation sur les marqueurs qui avaient été choisis d'après leur cartographie préalable sur le GL1 du poirier et du pommier.

En ce qui concerne le deuxième locus impliqué dans la nécrose de l'hybride de 'Type 1', nous avons cartographié un QTL de résistance au psylle du poirier sur ce même GL. De plus, un QTL spécifique d'une souche de *V. pirina* a été détecté chez un hybride interspécifique de poirier (Won et al. 2014). En effet, le GL5 est un des chromosomes de *P. x bretschneideri* avec le plus grand nombre des clusters de paralogues des gènes *R* (Wu et al. 2013). En outre, Calenge et al. (2004) ont cartographié un QTL pour la résistance à la tavelure sur le GL5 chez le pommier. Toutefois, il est également possible qu'une interaction de type modèle de garde provoque la non viabilité, similaire à celle rapportée chez la tomate par Krüger et al. (2002). Il est à noter que Yamamoto et al. (2007) ont montré une forte distorsion de ségrégation sur les GLs 2 et 5 du poirier européen 'La France' dans un croisement avec une accession de *P. pyrifolia* (poirier asiatique): des gènes létaux causant l'incompatibilité entre les espèces pourraient être à l'origine de cette distorsion de ségrégation, comme dans notre population, même si nous avons observé la distorsion chez le cultivar asiatique (*P. x bretschneideri*), plutôt que chez le cultivar européen. L'espèce *P. x bretschneideri* est considéré comme un hybride interspécifique entre *P. ussuriensis* et *P. betulaefolia*, mais *P. pyrifolia* pourrait également y être impliqué (Bell 1991).

Dans le génome de *P. x bretschneideri*, le GL2 (où est localisé le locus causant la létalité de 'Type 2'), comme le GL5, sont riches en clusters de paralogues de gènes *R* (Wu et al. 2013), et plusieurs QTLs et gènes majeurs de résistance aux ravageurs et aux maladies chez le poirier ont été cartographiés à cet endroit du génome (Dondini et al. 2004; Bouvier et al. 2011b; Le Roux et al. 2012; cette thèse). Il est donc possible que des gènes *R* puissent être aussi associés à la létalité de 'Type 2', comme postulé pour le 'Type 1'. Des travaux supplémentaires seront nécessaires pour tester ces hypothèses.

En plus, des segments génomiques identifiés sur les GLs 2 et 5 de PEAR3 et sur le GL1 de ‘Moonglow’, des régions distordues ont aussi été détectées sur le GL10 des deux parents et sur les GLs 9 et 16 de ‘Moonglow’. Cependant, ces régions ne semblent pas être impliquées dans les létalités de ‘Type 1’ et ‘Type 2’, puisque les génotypes des marqueurs cartographiés dans ces régions étaient répartis de façon équilibrée entre les plantules nécrosées et non nécrosées. Nos données ne permettent pas de déterminer si ces régions participent à une incompatibilité pré-zygotique, ou plutôt à des anomalies dans le processus de germination. Parmi les GLs montrant de la distorsion de ségrégation, le GL10 est particulièrement intéressant, non seulement parce qu’il est distordu chez les deux parents, mais aussi à cause de l’homologie entre les GLs 10 et 5 des génomes du poirier (Wu et al. 2013) et du pommier (Velasco et al. 2010). Des distorsions de ségrégation de marqueurs cartographiés sur GL10 ont été précédemment décrites chez plusieurs populations de pommier (Conner et al. 1997; Maliepaard et al. 1998; Liebhard et al. 2003; Kenis and Keulemans 2005).

En résumé, il s’agit ici de la première description de la nécrose de l’hybride chez le poirier. Nous avons montré que, bien que l’hybridation interspécifique dans ce genre soit possible, il existerait des barrières génétiques qui pourraient causer la perte d’au moins une partie de la descendance. Notre détection de régions chromosomiques impliquées dans des incompatibilités post-zygotiques chez les hybrides de poirier est d’une importance majeure, car elle contribue aux études tant sur la spéciation et l’évolution, que pour la sélection. Tout d’abord, les incompatibilités entre les deux espèces auraient pu survenir quand elles ont divergé au cours du processus d’évolution, et leur identification pourrait aider à la découverte des événements sélectifs qui ont conduit à la différenciation des espèces. En particulier les incompatibilités BDM impliquant des mutations d’allèles qui n’abaissent pas la *fitness* dans les lignées divergentes, peuvent être rapidement accumulées (Rieseberg et al. 2003), et leur identification pourrait aider à localiser les forces de la spéciation dans le temps (Orr 1995). Deuxièmement, les sélectionneurs cumulant des résistances pour améliorer la durabilité devraient noter qu’ils pourraient perdre la combinaison de résistance désirée, à cause d’incompatibilités biaisant la ségrégation de la descendance. En outre, les gènes associés aux autres caractères désirés pourraient co-ségrégier avec les gènes létaux et être perdus dans la population de sélection. Par conséquent, notre

identification de marqueurs moléculaires liés aux gènes létaux sera utile pour les sélectionneurs de poirier, qui seront désormais davantage capables de croiser des parents en évitant les combinaisons incompatibles affectant potentiellement l'expression des caractères d'intérêt. La récente publication des séquences de génomes des poiriers chinois (Wu et al. 2013) et européen (Chagné et al. 2014) offre l'opportunité de développer de nouveaux marqueurs afin de réduire l'intervalle des trois régions liée à la nécrose de l'hybride et d'identifier les gènes létaux candidats.

Cette étude fait l'objet d'une publication encore en cours de rédaction à soumettre dans *Theoretical and Applied Genetics*.

Conclusion

Ces dernières années, les connaissances sur la génomique du poirier ont progressé considérablement, et ce projet de thèse a permis d'y contribuer de manière significative.

Bien que nous ayons développé et cartographié de nombreux marqueurs SNP de poirier, le nombre de SNP découvert chez cette espèce reste limité par rapport à d'autres espèces de *Rosaceae* plus étudiées. Malgré la possibilité d'utiliser des marqueurs SNP de pommier pour le génotypage du poirier, ce problème devra être traité dans un avenir très proche si nous voulons accélérer l'identification d'associations loci-caractère et mettre en œuvre la SAM chez le poirier. Les génomes des poiriers chinois (Wu et al. 2013) et européen (Chagné et al. 2014) peuvent servir de référence pour le re-séquençage d'accessions de *Pyrus* et la détection de SNPs. Ces nouveaux SNPs pourraient être inclus dans des puces avec un plus grand nombre de SNPs par rapport à la puce 9K utilisée dans cette thèse, comme celles développées plus récemment sur le pommier (20K, (Bianco et al. 2014) et la fraise cultivée (90K, (Bassil et al. 2015). Une puce SNP très haute densité permettra la construction de cartes génétiques avec une encore meilleure résolution que celles de PEAR3 et 'Moonglow'. Toutefois, l'élaboration de puces avec un nombre plus élevé de SNPs augmente aussi les frais de génotypage. Une technique plus prometteuse est celle offerte par le génotypage-par-séquençage (GBS – Genotyping-by-Sequencing) (Elshire et al. 2011). La technique GBS est basée sur la réduction de la complexité du génome avec des enzymes de restriction et le séquençage haut débit des fragments génomiques clivés. Cette

méthode, qui est techniquement simple et hautement multiplexable, s'applique également aux génomes plus grands et plus complexes. Il y a déjà plusieurs exemples de l'application du GBS pour la construction des cartes génétiques saturées chez des plantes cultivées (par exemple, Ward et al. 2013; Gardner et al. 2014; Bastiaanse et al. 2015).

Les QTLs de résistance au feu bactérien et au psylle détectés lors cette thèse s'ajoutent aux QTLs et gènes majeurs de résistance aux maladies et ravageurs déjà cartographiés chez le poirier. Bien que la gamme de bio-agresseurs étudiées jusqu'à maintenant en poirier couvre presque complètement tous les bio-agresseurs d'importance économique, ce n'est certainement pas exhaustif en terme de loci de résistance existants chez *Pyrus*. On peut supposer que de nombreux autres loci de résistance pourront être détectés dans les années à venir, grâce aux progrès rapides des technologies de biologie moléculaire.

Le projet RosBREED (<http://www.rosbreed.org/portfolioimpactstatements>), une collaboration internationale vise à la SAM des principales cultures de *Rosaceae*, a été lancé en 2010 et pendant quatre ans a eu comme objectif l'amélioration génétique de pommier, pêcher, cerisier et fraisier, mais pas du poirier. La suite de ce projet, RosBREED 2, inclus un nombre plus élevé d'espèces, dont le poirier. RosBREED 2 ciblera principalement la qualité des fruits et la résistance au bio-agresseurs, et donc les résultats obtenus au cours de cette thèse concernant la résistance du poirier au psylle et au feu bactérien, et la nécrose de l'hybride pourront être d'une grande utilité dans le cadre de ce projet. En effet, la mise en œuvre de la SAM pour la résistance au psylle et au feu bactérien du poirier, exploitant les résultats que j'ai acquis au cours de ma thèse, est l'un des l'objectifs de RosBREED 2.

Enfin, l'étude de la nécrose hybride d'un point de vue moléculaire contribuera à accroître la compréhension des forces évolutives représentées par des ravageurs et pathogènes sur les génomes de plantes. Les incompatibilités entre les allèles mutés dans des fonds génétiques différents pourraient pu avoir un rôle important dans le processus de spéciation (Bomblies and Weigel 2007). Chez le poirier, par exemple, plusieurs espèces orientales sont “non hôte” pour les organismes qui sont pathogènes des espèces occidentales, et vice-versa. Il est possible que l'évolution des deux groupes d'espèces de *Pyrus* dans différents environnements, où ils ont été soumis à des pressions sélectives de pathogènes différents, ait pu causer la divergence des gènes

R au départ communs, qui, par effet de pléiotropie, aurait causé des incompatibilités entre les deux groupes.

Toutes les données génotypiques et phénotypiques du poirier sont recueillies dans une base de données commune à toutes les espèces de *Rosaceae*, le *Genome Database for Rosaceae* (GDR, <http://www.rosaceae.org/>). Cette base de données fournit un accès centralisé aux données de génétique, de génomique et de sélection, ainsi que des outils d'analyse pour aider la recherche fondamentale, translationnelle et appliquée sur les *Rosaceae*.

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List of abbreviations

ABA: Absciscic Acid

ADT: Assay Design Tool

AFLP: Amplified Fragment Length Polymorphism

ANOVA: Analysis Of Variance

APPPC: Asian and Pacific Plant Protection Commission

AUDPC: Area Under Disease Progress Curve

Avr (gene): Avirulence (gene)

BDM: Bateson-Dobzhansky-Muller

CC: Coiled-Coil

CMV: Cucumber Mosaic Virus

DH: Double Haploid

DNA: Deoxyribonucleic Acid

dNTP: deoxy-Nucleoside Triphosphate

dpi: days post inoculation

dsRNA: double stranded RNA

eLRR: Extracellular LRR

ELS: Endocytosis Cell Signaling

EPG: Electrical Penetration Graph

EPPO: European and Mediterranean Plant Protection Organization

EST: Expressed Sequence Tag

ET: Ethylene

ETI: Effector-Triggered Immunity

FEM: Fondazione Edmund Mach

GBS: Genotyping-by-sequencing

GEBV: Genomic Estimated Breeding Value

GS: Genomic Selection

GS: Genomic Selection

GWAS: Genome-Wide Association Study

GWAS: Genome-Wide Association Study

GWD: Genome-Wide Duplication

GxE: Genotype x Environment interaction

HK: Haley-Knott

HR: Hypersensitive Response

HRM: High Resolution Melting

IM: Interval Mapping

INRA: Institut National de Recherche Agronomique

IPSC: International Peach SNP Consortium

IRSC: International RosBREED SNP Consortium

JA: Jasmonic Acid

JA-Ile: JA-Isoleucine

KIN: Kinase

LBJ: 'Louise Bon Jersey'

LD: Linkage Disequilibrium

LG: Linkage Group

LOD: Logarithm of Odds

LOD: Logarithm of Odds

LRR: Leucine-Rich Repeat

LZ: Leucine-zipper

MAB: Marker-Assisted Breeding	PTI: Pathogen-Triggered Immunity
MAF: Minor Allele Frequency	QTL: Quantitative Trait Locus
MAMP: Microbe-Associated Molecular Pattern	<i>R</i> (gene): Resistance (gene)
MARS: Marker-Assisted Recurrent Selection	RAPD: Random Amplified Polymorphic DNA
MAS: Marker Assisted Selection	RCW: ‘Roi Charles de Württemberg’
MAS: Marker-Assisted Selection	RGC6/7: 6 th /7 th Rosaceae Genomics Conference
MeJA: Methyl JA	RH: Relative Humidity
miRNA: micro RNA	RIC: Recombinant Inbred Line
MRB: ‘Max Red Bartlett’	RLK: Receptor-Like Kinase
mRNA: messenger RNA	RLP: Receptor-Like Protein
MT: Millions of Tons	rMQM: restricted Multiple QTL Mapping
NBS: Nucleotide-Binding Site	RNA: Ribonucleic acid
NGS: Next Generation Sequencing	RNA-Seq: RNA-Sequencing
NLS: Nuclear Localization signal	ROI: Reactive Oxygen Intermediate
OH: ‘Old Home’	<i>S</i> (locus): Self-incompatibility (locus)
PAMP: Pathogen-Associated Molecular Pattern	SA: Salicylic Acid
PCR: Polymerase Chain Reaction	SAR: Systemic Acquired Resistance
PEST: Pro-Glu-Ser-Thr (Proline-Glutamic Acid-Serine-Threonine)	SCRI: Specialty Crop Research Initiative
PFR: Plant & Food Research	SIGA: Italian Society of Agricultural Genetics
PI: Proteinase Inhibitor	siRNA: small interfering RNA
PIP: Potential Intron Polymorphism	SNP: Single Nucleotide Polymorphism
PPO: Polyphenol Oxidase	SSR: Simple Sequence Repeat
<i>PR</i> (gene): Pathogenesis Related (gene)	ssRNA: single stranded RNA
PRR: Pattern Recognition Receptor	TGS: Transcriptional Gene Silencing
PTGS: Post-Transcriptional Gene Silencing	TIR: Toll-Interleukin-1-Receptor
	TM: Transmembrane

TMV: Tobacco Mosaic Virus

VOC: Volatile Organic Compound

TTSS: Type III Secretion System

WBC: ‘Williams Bon Chrétien’

USA: United States of America

WRKY: 60 amino acid domain

USDA: US Department of Agriculture

CHAPTER 1. General Introduction

1.1 The pear crop

1.1.1 Origin and diversity of the genus *Pyrus* and its origin

Pear (*Pyrus* spp.) is one of the most important tree fruit crops in the temperate regions and is nowadays grown in more than 50 countries over the world (Song et al. 2014). The first written mentions of pear can be found in Plutarch's "Greek Questions" and in Homer's "Odyssey" (where he called the pear fruit as one of the "gifts of gods"), although the domestication of this crop is assumed to have started long before ancient Greece (Hedrick et al. 1921). Pear originated presumably during the Tertiary Era, in western and southwestern China, from which it then spread to the whole temperate Asia, to Europe and to Northern Africa, encountering different natural selection forces which led to speciation (Bell 1991; Wu et al. 2013). Moreover, several pear species are suspected to be arisen from natural hybridization events (Bell 1991). In particular, Vavilov (1951) identified three centers of diversity: one in China, where *Pyrus pyrifolia* and *P. ussuriensis* arose, one in Central Asia, where *P. communis* and its hybrids occurred, and one in the Caucasus region/Asia Minor, where the domesticated forms of *P. communis* originated (Bell 1991; Jackson 2003). There are twenty-two widely recognized species of pear, which are usually divided into two major groups: the Occidental or European pears (with *P. communis* as the most important species) and the Oriental or Asian pears (including *P. x bretschneideri* Rehd., *P. pyrifolia* (Burm.) Nakai, *P. ussuriensis* Maxim. and *P. sinkiangensis*) (Wu et al. 2013).

In Europe, Africa, Oceania and in the Americas, the pear species mainly grown and commercialized is *P. communis*, which is diversified in thousands of varieties (Hedrick et al. 1921); however, only few cultivars are actually used for fruit production, and these include 'Conference', 'Abbé Fétel' and 'Williams Bon Chrétien' (syn. 'Bartlett') in Europe, 'Williams Bon Chrétien' and 'Packman's Triumph' in the Southern Hemisphere (South America, Oceania and South Africa), and 'Williams Bon Chrétien' and 'Anjou' in the USA (data from 2012, the

World Apple and Pear Association, <http://www.wapa-association.org/asp/index.asp>). ‘Williams Bon Chrétien’ (WBC) is certainly the most cultivated pear cultivar over the world (McGregor 1976). European pear is not commonly grown in China, where the Asian species can be found instead, mostly *P. x bretschneideri*, followed by *P. pyrifolia* and *P. ussuriensis* (<http://www.fao.org/docrep/004/ab985e/ab985e06.htm>). *P. x bretschneideri* (Chinese white pear) is a natural hybrid involving *P. ussuriensis*, *P. pyrifolia* (once called *P. serotina*) and *P. betulaefolia*, although its exact pedigree is not known (Teng et al. 2002).

1.1.2 Production and economy

The fruits of pear are produced mainly for the fresh consumption market, and secondly for canning (Jackson 2003). In the last 20 years the worldwide pear production has constantly increased, equaling 25.2 millions of tons (MT) in 2013, corresponding to a value of more than 10,000 million \$ (<http://faostat3.fao.org/browse/Q/QC/E>). Asia, and in particularly China, has long been the world most important center of pear fruits production, having taken over Italy by 1980 (Jackson 2003); moreover, in the last 20 years its production has increased consistently, passing from 4.9 MT (47% of the world production) in 1993, to 10.4 MT (64% of the world production) in 2000, to 19.5 MT (77% of the world production) in 2013. Italy was the second country, after China mainland, for the pear production until 2012, when it was surpassed by the United States of America (USA). Nowadays, China is also the main exporter of pear fruits, while the most important importer country in the world is Russia (data from 2012, the World Apple and Pear Association, <http://www.wapa-association.org/asp/index.asp>). The countries with the highest yields of pear fruits in the last two decades were Switzerland, Slovenia and New Zealand (in 2013 they delivered 559,000 hg/Ha, 516,000 hg/Ha and 453,000 hg/Ha of pears, respectively).

The market demand of pear fruits lasts all year round, thanks to the different harvesting season of the commercial cultivars, the import-export trade and the use of long-term storage (Jackson 2003).

1.1.3 Botany of pear and development stages

Pear is a medium sized, upright growing tree; its size is heavily dependent on rootstock and training system. The root system of a mature pear tree has a main rootstock stem from which a network of horizontal scaffold roots and of vertical “sinkers” departs (Jackson 2003). The development of a pear tree root systems in the orchard is dependent upon the soil composition (which affects air, water and nutrients contents), temperature, competition with other trees and with shoots and fruits of the same tree (Jackson 2003). Pear has simple, alternate, deciduous leaves, with an elliptic/ovate shape with acute tips and finely serrate or entire margins. Pear trees bring two types of buds: the “leaf” or “wood buds”, responsible only for the vegetative growth, and the “mixed buds”, which then develop into a new shoot and a flower (Fideghelli 2007). Hermaphrodite flowers have five petals, usually white in color, and are borne in corymbs in groups of 5-7 on short spurs or lateral branchlets; the ovary is 5-celled, the styles usually free (Hedrick et al. 1921; Fideghelli 2007). The flower is protogynous (i.e. anthers release pollen after the stigma has stopped being receptive) (McGregor 1976). Fruit is a pyriform (European) or round (Asian) pome, and the fleshy edible portion is derived from the hypanthium tissue (Hedrick et al. 1921). The flesh may contain stone cells (termed brachysclereids), which give gritty texture characteristic of many Asian pears (from which the name “sand pear” of *P. pyrifolia*) and some cultivars of European pear (Fideghelli 2007). The color of the fruit skin is very variable, depending on the ground color and the intensity of red surface coloration. As the fruit matures, the green color of the skin may fade into a cream, pale yellow or greenish-yellow; in some cultivars the green do not disappear. The blush developing on the ground color can be more or less intense, or completely absent, according to the anthocyanin production (Jackson 2003; Fideghelli 2007). The variability of pear botanic characteristics is very large throughout the species and cultivars.

Pear seedlings juvenile phase, defined as the initial period after seed germination while the vegetative development takes place and there is no production of flowers, lasts several years (Zimmerman 1972). The duration of this phase is different from cultivar to cultivar. There are some agronomic practices which can reduce the length of the juvenile period, e.g. particular pruning system or the use of dwarfing rootstocks.

Pear trees go through seasonal dormancy, which allows the plant to survive regularly recurring periods of drought or low temperature (Jackson 2003). Endo-dormancy is induced by a certain number of days at low temperatures, and is broken after a certain amount of chilling hours (or better, chilling units) and accumulated high temperatures (growing-degree-hours). The length of the period which induces dormancy and the chilling units and growing-degree-hours needed to break it are typical of each cultivar, and are an indication of the optimal regions of cultivation. When the dormancy is broken, the buds open and start to develop into shoots and corymbs. Pear flowering time varies from cultivar to cultivar and climate to climate; however, in general, it flowers during the spring season (March – April in Italy), slightly earlier than apple. The flowering on a tree usually lasts about a week (McGregor 1976). Petals loss is followed by fruit set, growth and ripening. Pear fruit maturation time is largely variable (90-200 days), depending on cultivars and climate conditions; therefore, the harvesting season ranges between the beginning of summer and mid-autumn. Typically only 5-10% of the flowers give harvestable fruits, while the rest fail to set and are shed (Jackson 2003).

1.1.4 Cultivation

Pear crop can be grown in a wide range of different climatic conditions, thanks to its high variability, and nowadays it is cultivated in the whole temperate-zone and also in some countries in the subtropical and tropical area (Jackson 2003).

Pear, like most tree fruits, is usually grafted or budded onto compatible rootstocks. This practice, which has been used for thousands of years (Jackson 2003), enables the propagation of clones and the combination of beneficial characteristics from the rootstock (like control of vigor and tolerance to biotic and abiotic stresses related to soil) and the scion (mainly fruit quality). Common rootstocks for pears are ‘Old Home’, ‘Old Home’ x ‘Farmingdale’ selections (which turned out to be ‘Old Home’ x ‘WBC’ (Postman et al. 2013)), quince (*Cydonia oblonga*) (Webster 1998), and occasionally *P. calleryana*, *P. ussuriensis* and *P. betulaefolia*.

In the orchard, pear trees are spaced at 4-2.5 m x 2-0.30 m (2000-13,000 trees/Ha) depending on the vigor of the rootstock-scion combination and the training system used. There are several

different training system for fruit trees, but the most common are palmette, slender spindle and Y-shaped (Musacchi 2007).

In areas where the spring-summer season is warm and dry, irrigation is necessary in the pear orchards. In the last years, the most used irrigation system was the drip, which has high efficiency and requires the application of a reduced amount of water in comparison with spraying, with benefits for the fruit production, the soil characteristics and the production costs (Musacchi 2007).

Pears are insect-pollinated, predominantly by honey bees (*Apis mellifera* L.) (McGregor 1976). Most pear cultivars are completely or almost completely self-incompatible (Crane and Lewis 1942), i.e. the male and female gametes of the same genotype are not compatible and the pollen tube growth in the style or ovary is inhibited (Brewbaker 1957). Hence, these genotypes are not able to set seed when self-pollinated, and in the orchard the introduction of compatible cultivars for cross-pollination is required. Self-incompatibility in plants has evolved to prevent successive self-fertilizations and deleterious inbreeding (Jackson 2003). This trait is controlled by a single multi-allelic (*S*) locus: if the *S* allele of the pollen matches one of the two *S* alleles of the pistil, incompatibility takes place and the pollen growth is blocked (Ishimizu et al. 1998; Sanzol and Herrero 2002; Okada et al. 2008a). Also cross-incompatibility has been reported in pear, particularly between cultivars which turned out to be genetically related (Sanzol and Herrero 2002). In both Japanese and European pears, some cultivars have been screened to identify their *S* locus genotype and grouped based on their cross-incompatibility (Ishimizu et al. 1999; Sanzol and Herrero 2002; Okada et al. 2008b; Quinet et al. 2014). Pear can also develop parthenocarpic (seedless) fruits in absence of fertilization (Jackson 2003). Fruits are usually harvested before complete ripeness and then subjected to particular conditions for long-term storage.

1.2 Diseases, pests and crop protection

One of the main concerns in agriculture has always been the control of diseases and pests, which cause yield losses, damage the cultivated plants and reduce the quality and healthiness of food. In a context of major climatic changes and quick human population growth, crop protection is even more important. Tilman et al. (2011) forecasted a 100-110% increase in the global agricultural production demand between 2005 and 2050; the improvement of the efficiency and sustainability of crop management practices will be crucial to meet this demand without leading our planet to collapse. Chemical compounds application, although often the most effective strategy to control diseases and pest, is very harmful to the environment and humans, and increases substantially the production costs; moreover, there are pathogens and pests which cannot be completely controlled with pesticides (e.g. *Erwinia amylovora*, (Norelli et al. 2003)), and in many cases the arisen of resistant strains to the most sprayed compounds has limited the range of applicable active ingredients (e.g. for pear psylla (Harries and Burts 1965; Buès et al. 2003; Civolani et al. 2007)). Therefore, in the last decades research has been focusing on crop protection strategies alternative to the chemical ones, such as biological methods and agronomical practices, in order to reduce the pesticide applications without compromising the production. The concept of “Integrated Pest Management”, born in the early 70s, is based on the integration of different pest control strategies (where pest means non-arthropod animals, pathogens and weeds), taking into account “the interest of and impacts on producers, society and the environment” (Kogan 1998). In this scenario, particular importance goes to the breeding for resistant varieties, which has been enhanced by the incredible progresses recently achieved in the plant genomics area.

1.2.1 Pathogens and pests

The term “pathogen” includes all the microorganisms, such as viruses, bacteria, fungi, protozoa, and nematodes, which cause diseases, while with “pest”, in its stricter meaning, is usually meant any animal which is harmful to plants (while sometimes, in a wider meaning, “pest” also includes pathogens). Pathogens of plants and their control are the object of Plant Pathology, while insects, which are the most common pests for crops, are studied in the discipline of Entomology.

Organisms are called parasites when they live on or in some other organism (in this context a plant) and they use its resources to feed; a parasite become a pathogen when it interferes with the plant metabolism, thereby causing disease (Agrios 2005).

Pathogens can be generally classified in: i) biotrophic, which colonize living cells and alter their metabolism to favor their own growth and reproduction; ii) necrotrophic, which live most of the time and thrive well on dead organic matter (these can be broad or narrow-spectrum); iii) and hemibiotrophic, which act like the biotrophic pathogens at first stages of the infection, but later kill the host cells and continue their life cycle on dead tissues (Hammond-Kosack and Jones 1997).

The infection and development of the disease is dependent on the interaction among three components: the pathogen, which has to be virulent and sufficiently abundant; the plant, which has to be susceptible to the pathogen; and the environment, which includes all the external conditions affecting somehow the instauration of the plant-pathogen complex. When one of these conditions is not optimal for the disease, than its severity is reduced, or even nullified. Plant pathologists have long referred to this tri-components interaction as the “disease triangle” (Stevens 1960) (Figure 1.1).

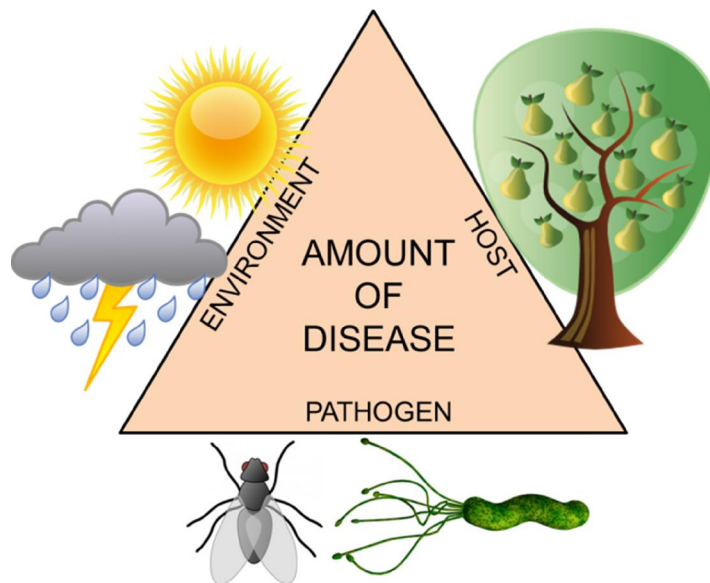


Figure 1.1: The disease triangle

Insects damage directly the host plant mainly by chewing or sap-feeding on above-ground and below-ground plant parts (Dangl and Jones 2001). Furthermore, insects function often as vectors of plant pathogens.

1.2.2 Physiology and genetics of plant resistance to disease

The ability of a particular pathogen species or variety to infect only a specific range of related plants (hosts) is due to their genetic makeup. Most plant species are immune to almost all potential pathogen species: this phenomenon is called non-host resistance. Conversely, host resistance is exhibited by particular genotypes of plants to pathogens that are usually able to infect them (Agrios 2005).

Van der Plank (1963) proposed to divide the host resistance in vertical (or oligogenic or qualitative), controlled by one or few major genes and completely effective against only one or few strains of pathogen species, and horizontal (or polygenic or quantitative), controlled by many different genes and only partial, but usually effective against all strains of a pathogen species. This classification has been lately criticized, since these two types of resistance sometimes overlap, and some of the genetic mechanisms at the base of quantitative and qualitative resistances are hypothesized to coincide (Poland et al. 2009). Currently, plant pathologists recognize two related categories of resistance mechanisms: basal defense and resistance (*R*) genes-mediated immunity (Jones and Dangl 2006).

Plant resistance results from a combination of constitutive and induced defense mechanisms (Niks and Marcel 2009). The first are represented by preformed physical and chemical barriers, such as secondary cell wall (Miedes et al. 2014) or the constitutive expression of defense-related genes (Vergne et al. 2010), which limit the growth of the pathogen. The second is induced resistance, which is the result of three subsequent events: the recognition of the pathogen by the plant, the signal transduction, which, then, leads to the induction of defense responses. Recognition can be operated by receptors located on the plant cell surface (pattern recognition receptors - PRRs), which detect pathogen or microbe-associated molecular patterns (PAMP or MAMP) and induce the basal defense, or by receptors in the cytoplasm of the host cell, which interact with pathogen effectors and activate the effector-triggered immunity (ETI, in

contraposition with the pathogen-triggered immunity (PTI) induced by PAMP and MAMP) (Deslandes and Rivas 2011; Stael et al. 2014). In the case of PTI and basal defense, recognition is relatively non-specific: the plant detects broadly conserved pathogen features, like flagellin (the main constituent of bacterial flagella) and chitin (the main component of fungal cell walls) (Jones and Dangl 2006; Newman et al. 2013). On the contrary, ETI is dependent upon a more specific-recognition pattern, which causes an oxidative burst, disruption of cell membranes and alkalinization of the cytoplasm, release of toxic compounds (e.g. Reactive Oxygen Intermediates (ROIs) and phytoalexins), and finally cell death, which consequently inhibits the pathogen growth: this phenomenon is known as hypersensitive response (HR) (Morel and Dangl 1997; Stael et al. 2014).

ETI is often based on gene(s)-for-gene(s) interactions, where avirulence (*Avr*) genes in the pathogen are recognized by the corresponding *R* genes in the plant, which then activates the defense mechanism in the plant (Flor 1946). Often, both the avirulence in the pathogen and the resistance in the plant are dominant (*Avr* and *R*); in this case, resistance is exhibited only in the presence of the dominant alleles at both loci (Flor 1971). Other than a simple direct gene-for-gene interaction, plant defense can be activated by a more complex, indirect mechanism which is termed the “guard hypothesis”. This mechanism was first described for the resistance of tomato to *Pseudomonas syringae* pv. *tomato*, which requires the action of both the Pto protein kinase and the LZ-NBS-LRR (Leucine Zipper-Nucleotide Binding Site-Leucine Rich Repeat) protein Prf to activate defense upon recognition of the AvrPto elicitor (Salmeron et al. 1996). In this model, the pathogen effector entering a resistant host cell interacts with a target (the “guardee”), and by altering this guardee protein, it activates the corresponding *R* protein (the “guard”), which then triggers the disease resistance (Dangl and Jones 2001; Jones and Dangl 2006; Gassmann and Bhattacharjee 2012).

Whereas HR is effective against biotrophic pathogens, it is beneficial for necrotrophs, which thrive on dead host tissue. Plant basal defense, on the contrary, acts against both biotrophs and necrotrophs. Resistance against necrotrophic pathogens is usually quantitative (Poland et al. 2009).

There is a continuous co-evolution process between plants and their pathogens, an evolutionary arms race resulting in an oscillation between susceptibility and resistance over time. Jones and

Dangl (2006) illustrated this phenomenon as a four phased “zigzag” model (valid for biotrophic pathogens): i) first, the plant basal defense, based on the broad-spectrum recognition of pathogens by plant transmembrane receptors, activates PTI and halts the pathogens spread inside the cell; ii) some pathogens manage to elude the basal defense, secreting effector proteins inside the host cell and causing disease; iii) in response, susceptible plants develop mechanisms which enable them to recognize the pathogen effectors and trigger ETI, usually generating the HR; iv) finally, the pathogen evolves new, or additional, genes encoding for virulence effectors able to overcome the plant *R* genes.

Whereas qualitative resistance is determined by single (or few) major *R* genes, quantitative resistance generally consist in the joint effect of several genes, each contributing partially to the global resistance. Quantitative (or partial) resistance is thus generally seen as (and shown to be) the combined effect of several QTLs (Quantitative Trait Loci). A QTL is defined as the genomic region which includes the gene partially contributing to the overall quantitative resistance. As a QTL is generally statistically detected with an imprecise localization on the genome due to its partial contribution to the overall phenotypic variation, the genomic region can be somewhat large and thus includes several linked genes. Co-localizations of QTLs and *R* genes for different diseases and pests’ resistance have often been reported. Some chromosomic regions in the plant genomes can be particularly rich in resistance loci; however, it is also possible that some genes have a pleiotropic effect, resulting in a resistance to multiple-diseases (Poland et al. 2009). Indeed, *R* proteins recognizing more than one *Avr* effector from the pathogen have been identified (Dangl and Jones 2001). For example, the gene *RPM1* in *Arabidopsis thaliana* has a dual specificity for *Avr* genes in *P. syringae* (Bisgrove et al. 1994; Grant et al. 1995), and the *Mi* gene in tomato confers resistance to the nematode *Meloidogyne incognita*, the aphid *Macrosiphum euphorbiae* (Rossi et al. 1998) and the whitefly *Bemisia tabaci* (Nombela et al. 2003).

Sometimes an “apparent resistance” of plants to pathogens can also be observed. This is the case of susceptible plants that do not get infected by their pathogens (disease escape) because of the non-optimal environment conditions, the absence of synchronization between plant and pathogen or the low density of one or the other (which then do not allow the spread of the disease in the

field), or of plants that are able to produce a good crop (although not excellent) even if they are infected (the so-called tolerance) (Agrios 2005).

1.2.3 Pathogen avirulence effectors and plant resistance proteins

Pathogen effectors are extremely diverse. They are responsible for the instauration of the disease in susceptible hosts (virulence), but they are also specifically recognized by R proteins in case of a resistant genotype (avirulence). Bacteria have several genes associated with pathogenicity, like those encoding for toxins or exopolysaccharides and those composing the different secretion systems. The Type III Secretion System (TTSS) of an individual phytopathogenic bacterium secretes 20–30 proteins, which have molecular or enzymatic activities on host targets involved in PTI or ETI responses (Jones and Dangl 2006). For example, *P. syringae* effectors AvrPtoB and AvrPto suppress multiple PRR kinases, perhaps by acting as kinase inhibitors or inducing their degradation (Xiang et al. 2008; Gimenez-Ibanez et al. 2009). Bacteria that lack the TTSS are non-pathogenic (Dodds and Rathjen 2010). Very little is known about eukaryotic effectors with respect to bacteria. Plant pathogenic fungi have genes involved in the recognition and adhesion to the host plant, the production of enzymes that degrade the cuticle and the cell wall, the release of phytotoxic molecules. For example, pathogens like the fungus *Cladosporium fulvum* and the oomycete *Phytophthora infestans* secrete effectors targeting apoplastic hydrolytic enzymes (produced by the plant in its immunity response), such as chitinases and proteases (Rovenich et al. 2014). Fungal pathogens are also able to overcome plant secondary metabolites, finally leading to the suppression of defense mechanisms, like PRR-mediated immunity, salicylic acid biosynthesis (see below) or host cell death (Rovenich et al. 2014). Additionally, fungi like, for example, *Botrytis cinerea*, were reported to be able to deliver small RNAs into the host cell, which suppress plant immunity by silencing the resistant genes (Weiberg et al. 2013; Weiberg et al. 2014). Biotrophic and hemibiotrophic fungi traffic disease effectors inside the plant cell from the haustoria, specialized feeding structures that invaginate the host cell and make near-direct contact with the host plasma membrane (Birch et al. 2006; Dodds et al. 2009). In general, eukaryotic pathogens secrete a wide diversity of effectors, which moreover have extremely versatile functions, involved in any step of the immunity and in any part of the plant cell (Figure

1.2) (Rovenich et al. 2014); this may be a consequence of their high specialized nutrient acquisition strategies (Dodds and Rathjen 2010). Viruses have a limited number of genes that are involved in all the steps of pathogenicity (Agrios 2005); typically, they encode specific suppressors which interfere with single or multiple steps of the small RNAs pathway activated by the plant defense system (see below), finally preventing the degradation of their genomes and/or abrogation of viral gene expression (Muthamilarasan and Prasad 2013). Also nematodes and some insects are able to secrete effector proteins (Martin et al. 2003), apparently through their saliva.

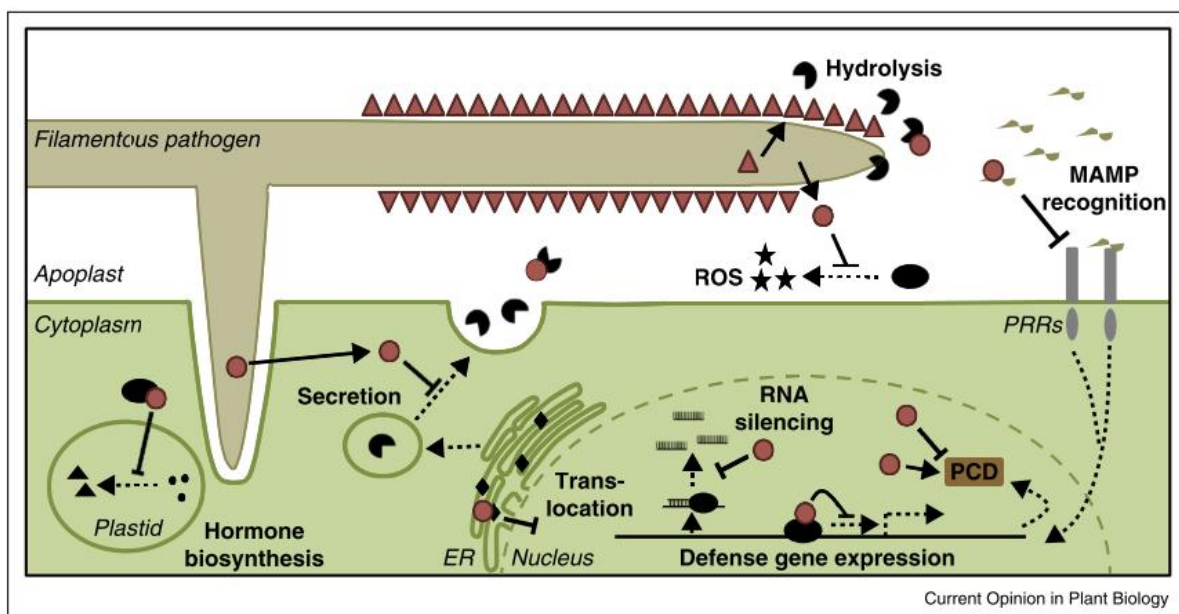


Figure 1.2: Schematic of the defense mechanism occurring inside the plant cell and on its surface in response to a pathogen attack

Pathogens secrete effectors (red symbols) to deregulate plant immunity. Whereas one group of effectors (red circles) interacts with host targets that act in immunity (black shapes), another group of effectors (red triangles) acts in self-defense to protect the pathogen from host-derived antimicrobials (Rovenich et al. 2014). Pathogen effectors can interact with all the steps of the plant immunity system to cause disease

Several *R* genes, acting against bacteria, viruses, fungi, oomycetes and even nematodes and insects, outside or inside the plant cell, have been characterized (Dangl and Jones 2001).

Table 1.1: Eight classes of plants resistance (R) proteins, as identified by Gururani et al. (2012).

The domains of the proteins and their location in the plant cell are reported. For each class, examples of R proteins described in the literature, with the corresponding reference, are also shown.

	R proteins class	Domains	Location in the plant cell	Examples	References
1	TIR-NBS-LRR	A leucine-rich repeat (LRR) domain, a putative nucleotide binding site (NBS) domain and an N-terminal similar to the Toll-Interleukin-1-receptor (TIR)	cytoplasm	Arabidopsis RPS4, RPS6 (against <i>Pseudomonas syringae</i>) and RPP5 (against <i>Peronospora parasitica</i>); tobacco N (against Tobacco Mosaic Virus); flax L6 (against <i>Melampsora lini</i>)	Whitham et al. 1994; Lawrence et al. 1995; Parker et al. 1997; Kim et al. 2009
2	LZ/CC-NBS-LRR	A leucine-rich repeat (LRR) domain, a putative nucleotide binding site (NBS) domain and an N-terminal putative leucine-zipper (LZ) or coiled-coil (CC) domain	cytoplasm	Arabidopsis RPS2, RPM1, RPS5 (against <i>Pseudomonas syringae</i>)	Kim et al. 2009
3	Receptor-like proteins (RLPs)	An extracellular LRR (eLRR) domain, a single transmembrane (TM) domain and a small cytoplasmic tail	extracellular space/cytoplasm	tomato Cf proteins (against <i>C. fulvum</i>)	De Wit and Joosten 1999; Luderer et al. 2002
4	Receptor-like kinases (RLKs)	A cytoplasmic serine/threonine kinase (KIN) domain in addition to an extracellular LRR (eLRR) domain and a TM domain	extracellular space/cytoplasm	Rice Xa21 protein (against <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>); the Arabidopsis FLS2 protein	Song et al. 1995; Gómez-Gómez and Bolter 2000; Chinchilla et al. 2006
5*	CC	Putatively anchored N-terminally in the plasma membrane and containing a coiled-coil (CC) domain	cytoplasm	Arabidopsis RPW8 (against <i>Erysiphe cichoracearum</i>)	Xiao et al. 2001
6	LRR-LZ/PEST-ECS	An extracellular LRR (eLRR) domain, a single transmembrane (TM) domain, leucine-zipper (LZ) domain or a PEST (Pro-Glu-Ser-Thr) domain for protein degradation and short proteins motifs (ECS) that might target the protein for receptor mediated endocytosis	extracellular space/cytoplasm	Tomato Ve1 (LZ) and Ve2 (PEST) (against <i>Verticillium albo-atrum</i>)	Kawchuk et al. 2001
7	TIR-NBS-LRR-NLS-WRKY	TIR-NBS-LRR proteins with a putative nuclear localization signal (NLS) and a WRKY domain (a 60 amino acids conserved sequence characteristic of transcription factors identified only in plants and involved in many biological processes) at the C-terminal	cytoplasm	Arabidopsis RRS1-R (against <i>Ralstonia solanacearum</i>)	Deslandes et al. 2002
8	Protein encoded by enzymatic R genes	not containing either LRR or NBS	cytoplasm	Maize HM1 (against <i>Cochliobolus carbonum</i>); tomato Pto (against <i>P. syringae</i>); barley Rpg1 (against <i>Puccinia graminis</i> f. sp. <i>tritici</i>)	Johal and Briggs 1992; Ronald et al 1992; Martin et al 1993; Brueggeman et al 2002; Kim et al 2002

Gururani et al. (2012) identified eight classes of plant R proteins, based on their structural motifs (Table 1.1); however, this classification is continuously improving when new R proteins are discovered.

The LRRs domain has an important role in recognition specificity and is present in the majority of R proteins. Transmembrane R proteins, like RLPs and RLKs, whose LRR domains are located in the extracellular space, detect surface components from the pathogen and act by preventing the host cell colonization; on the other hand, NBS-LRR proteins residing inside the cytoplasm recognize elicitors secreted by the pathogen into the cell and trigger the biochemical and metabolic processes which lead to HR (Dangl and Jones 2001).

Signal transduction leading to the activation of the plant defense system is regulated by specific molecules, mainly salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Jones and Dangl 2006), which interact extensively (Glazebrook 2005). These molecules are differentially involved in the plants' defense against biotrophic and necrotrophic pathogens, with SA-mediated pathways activated upon infection from the first ones and JA from the second ones. Commonly, when a biotroph (or a hemibiotroph) attacks a host plant, the activation of NBS-LRR proteins leads to HR and the subsequent activation of the SA-dependent signaling pathway. SA is involved in the long lasting and broad-spectrum Systemic Acquired Resistance (SAR): SA production promotes the expression of pathogenesis-related (*PR*) genes and enables the development of the resistance in the cells surrounding the infection site and in distal parts of the plant (Ward et al. 1991; Kunkel and Brooks 2002). Conversely, intracellular R proteins are not effective against necrotrophs, and SA is not involved in plants' resistance to this type of pathogens, while in this case JA and ET-dependent responses are usually activated (McDowell and Dangl 2000). Increase in JA synthesis, following a necrotrophic pathogen attack or in response to wounding and insect feeding, triggers the expression of specific defense effector genes; some of these JA-regulated genes are also dependent on ET production (Glazebrook 2005). However, exceptions to these general rules have been reported (Robert-Seilanianantz et al. 2011). The situation is complicated by the cross-talks occurring at multiple points among SA, JA and ET-signaling pathways: usually, SA and JA have a mutual repression effect, while JA and ET interact positively with each other, although with some exceptions; SA an ET interaction is rare and contradictory (Kunkel and Brooks 2002). Moreover, several findings have suggested the involvement of a multitude of

signaling molecules, other than SA, in the mediation of SAR (including JA), which were dependent on the environmental conditions (Muthamilarasan and Prasad 2013). Additionally, other phytohormones, such as auxins, abscisic acid (ABA) and cytokinins have been recently demonstrated to function as modulators of SA and JA signaling pathways (Robert-Seilaniantz et al. 2011; Denancé et al. 2013).

Another mechanism involved in the plant immunity is RNA interference, usually adopted against viruses. Following a viral infection, plants can degrade the RNA of the virus by gene silencing. There are two distinct gene silencing phenomena: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), which both use small regulating RNAs to specifically target and inactivate invading nucleic acids (Muthamilarasan and Prasad 2013). PTGS is initiated by synthesis of double-stranded RNA (dsRNA) from the viral genome; the dsRNA is then diced by an endoribonuclease (RNase) enzyme, generating a pool of small interfering RNAs (siRNA) of ~21–24 nt. Viral mRNA strands are then produced complementary to their bound siRNAs, and the duplex siRNA-mRNA is cleaved in two parts. In TGS, firstly single-stranded transcripts (ssRNAs) from the viral genome are generated, and then they are converted into dsRNA and subsequently diced to 24-nt siRNAs. These siRNAs act as a guiding strand for heterochromatin formation and methylation (Muthamilarasan and Prasad 2013). Furthermore, Li et al. (2012) suggested a role of micro RNAs (miRNAs) and siRNAs in regulating plant LRR genes expression. By studying the TMV-tobacco pathosystem, they identified miRNAs targeting the tobacco TIR-NB- LRR gene *N* and causing the attenuation of the resistance. They proposed that this mechanism has the function of limiting the potential fitness costs associated with the evolution of multicopy *R* genes, forcing then the plants to diversify their *R* genes set. They also hypothesized that the suppression of miRNAs and siRNAs from some viral and bacterial effectors, released by these pathogens to enhance their virulence, might instead favorite the expression of those *R* genes otherwise blocked by the small RNAs, in a complex co-evolutionary model between plants and pathogens. This hypothesis was strengthened by the parallel work of Shivaprasad et al. (2012) in tomato.

The mechanisms underlying quantitative resistance are not perfectly clear yet, and several hypothesis have been outlined (Poland et al. 2009). Genes acting at different levels of the plant immune system can be responsible for a quantitative, instead of qualitative, resistance to diseases

and insects: i) genes linked to morphological and developmental characteristics of the plant, such as stomata density, plant height and leaf area (Albar et al. 1998; Zhu et al. 1999; Melotto et al. 2006); ii) multiallelic genes involved in basal defense (Dunning et al. 2007); iii) genes for the synthesis of antimicrobial (i.e. phytoalexins) and other detoxifying compounds, deployed by the plants against toxins producing pathogens (typically necrotrophs) (Denby et al. 2004; Kliebenstein et al. 2005); iv) genes responsible for the SA, JA and ET-dependent signaling pathways (Zheng et al. 2006); v) small effect *R* genes (Parlevliet and Zadoks 1977). It has been demonstrated, that sometimes when a pathogen overcomes a strong effect *R* gene, the plant keeps a “residual resistance”, i.e. its resistance is reduced, but not completely nullified (Brodny et al. 1986; Li et al. 1999). Although quantitative resistance was presumed to be broad-spectrum (Van der Plank 1963), several exceptions to this assumption have been reported (e.g. Perchevied et al. 2005; Whitaker et al. 2007). These examples support the hypothesis of an involvement of high numbers of small effect *R* genes in the quantitative resistance. Nonetheless, it is probable that all the hypotheses mentioned above are true.

1.2.4 Plant responses to insect herbivory

Like for pathogens, plant resistance to insects can be constitutively present or induced upon herbivore attack. Constitutive defense is based on preformed physical barriers, such as trichomes, hairs, and waxes, and on the accumulation and storage of compounds during the normal growth and development of the plant, which are released against the insect in case of an attack. On the contrary, in the induced defense resistance compounds are produced by the plant only in response to insect wounding (Gatehouse 2002). Furthermore, plant resistance to insects can be divided into direct and indirect (Figure 1.3). Direct defense mechanisms include the production of secondary metabolites, such as: i) proteinase inhibitors (PI), which inhibit insect digestive enzymes; ii) polyphenol oxidases (PPO), anti-feedant enzymes that decrease the nutritive value of the wounded plant; iii) toxic compounds (e.g., alkaloids, terpenoids, phenolics), which are poisonous for herbivores (Dangl and Jones 2001; Kessler and Baldwin 2002; Wang and Wu 2013). Direct wounding responses can also act as physical barriers, like the lignification or the production of resin (Gatehouse 2002). Indirect resistance is based on the volatile organic compounds (VOCs)

release, which can have a repellent or toxic effect on the insect, inhibit oviposition, attract predators and parasitoids of the pest and also serve as airborne phytohormones inducing defense responses in the non-attacked tissues of the same plant or of neighboring plants (Kessler and Baldwin 2002; Baldwin et al. 2006; Heil and Silva Bueno 2007; Staudt et al. 2010; Broekgaarden et al. 2011).

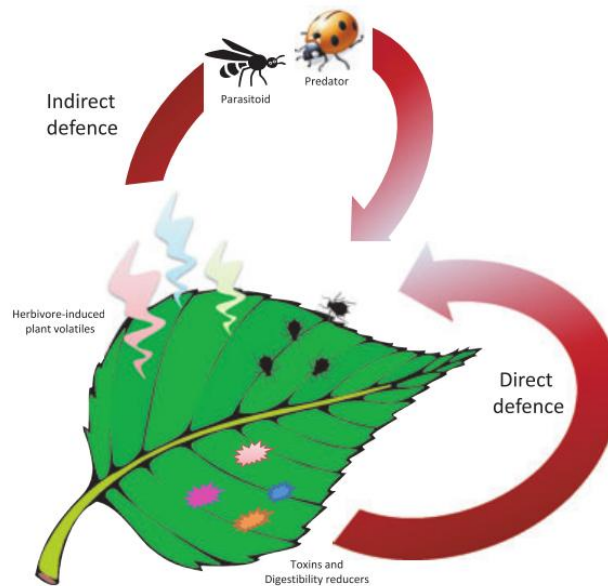


Figure 1.3: Direct and indirect defences induced in plants by insects herbivory.

Direct defense mechanisms include the production of proteinase inhibitors, polyphenol oxidases, anti-feedant enzymes and toxic compounds. Indirect resistance is based on the volatile organic compounds release, which can attract predators and parasitoids of the pest (Broekgaarden et al. 2011).

Plants have the ability to distinguish between herbivory wounding and mechanical damage, such as hail and wind. Plants are also able to recognize compounds in insect oral secretions and in the oviposition fluids (Fürstenberg-Hägg et al. 2013). Most of wounding-induced direct (such as the production of defensive proteins like PI and PPO) and indirect (VOCs release) defenses are elicited by the JA signaling pathway (Howe and Jander 2008; Wang and Wu 2013). JA is synthesized from linolenic acid in chloroplasts and peroxisomes via the octadecanoid pathway (Gatehouse 2002; Wang and Wu 2013). JA turnover is extremely complex (Figure 1.4). The most important JA metabolites in the plant defense mechanisms appear to be JA-Ile, generated from conjugation of JA with the amino acid isoleucine, and the methyl JA (MeJA) (Wang and Wu

2013). Interestingly, defense is often induced also in undamaged distal leaves, far from the wounding site, indicating a systemic signaling pathway, which allows the plant to cope with highly mobile herbivores. This process remains unclear, although the most plausible hypothesis seems that transmissible electric signals activate systemic responses (Fürstenberg-Hägg et al. 2013; Wang and Wu 2013). The signal transduction induced by wounding and leading to plant resistance to herbivores is complex and very diverse across the range of plant-insect interaction systems, involving several genes. Phytohormones, such as ABA, auxin, ET and SA, negatively or positively modulate the defense pathway by interacting with the JA-mediate signaling.

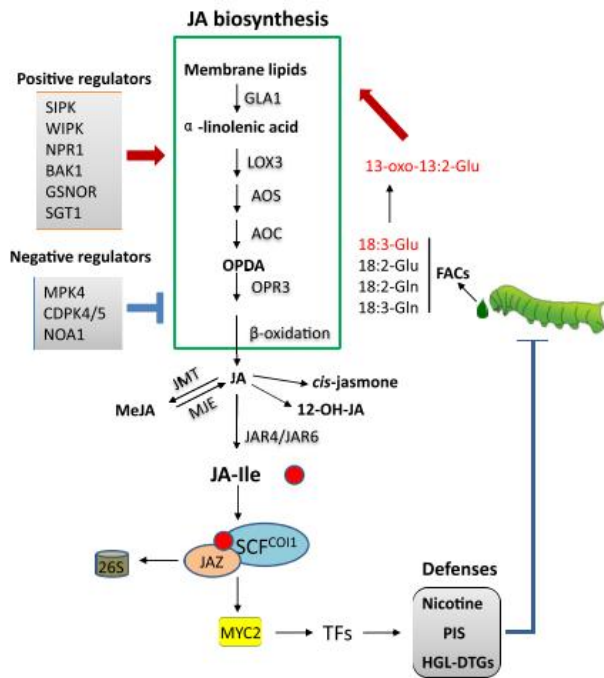


Figure 1.4: Model summarizing herbivory-induced jasmonic acid (JA) signaling and its regulation

During insect feeding, factors in the oral secretion are introduced into the host leaf tissue and thereafter activate JA biosynthesis from α -linolenic acid. JA is further converted to JA-Ile, MeJA and other compounds. JA-Ile finally induces the production of defensive compounds, such as proteinase inhibitors (PIs) (Wang and Wu 2013)

Sap-feeding insects, such as aphids and other Hemiptera, release elicitors inside the host cell, by inserting their stylets into the vascular system, and induce plant responses that are similar to those described for a pathogen attack (Walling 2000). Indeed, both PTI and ETI-like reactions have been reported in plant-aphid interactions. Interestingly, the aphids endosymbionts, bacteria

located inside the hemocoel of the insect, which contribute to its production of amino acid, have been found to release elicitors responsible for PTI in the attacked plants, like those activated in *Arabidopsis thaliana* by the saliva of *Myzus persica* (Figure 1.5) (Jaouannet et al. 2014).

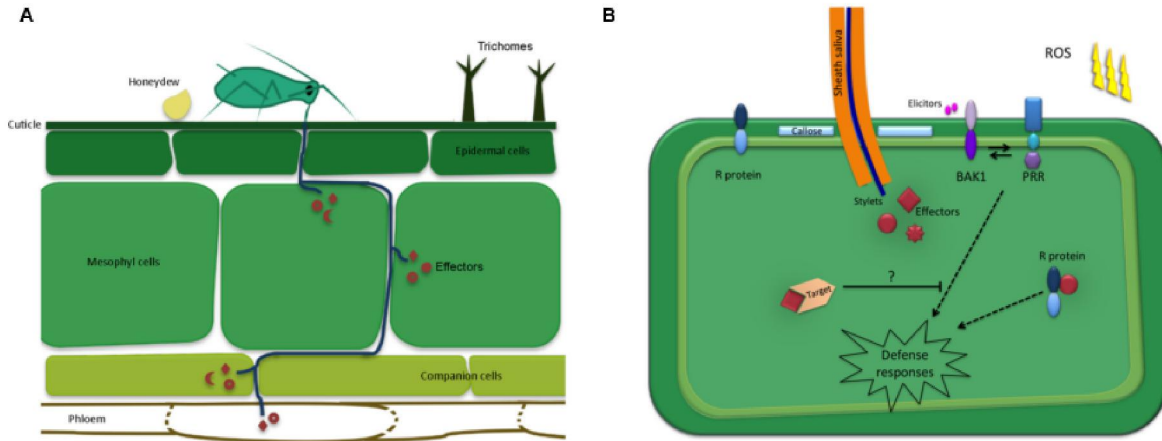


Figure 1.5: Schematic presentation of the aphid-plant interaction

(A) Aphid stylets penetrate the leaf surface after having encountered preformed defenses such as trichomes and waxes. The aphid stylets follow a mainly extracellular pathway while probing and locating the phloem. Most cells along the stylets pathway are punctured, including the phloem cells. Saliva, containing effectors is secreted into the different cell types as well as in the apoplast. (B) Upon probing, aphids secrete effectors inside the host cell cytoplasm, which interact with targets to modulate host cell processes. In resistant plants, these effectors may be recognized by resistance (R) proteins leading to effector-triggered immunity. In addition, the plant may perceive aphid elicitors by means of pattern recognition receptors (PRRs). This perception induces defense responses, including callose formation and the production of reactive oxygen species (ROS) (Jaouannet et al. 2014)

To date, there is a number of examples of plant-insect gene-for-gene interactions leading to ETI, and both arthropod effectors and the corresponding *R* genes have been identified (Stuart 2015). By transient expression in tobacco and *Arabidopsis*, Elzinga et al. (2014) studied the effect of a number of salivary proteins from *M. persicae* (the green peach aphid), and found some that increase and some that decrease the aphid reproduction. The first insect *Avr* gene cloned was the *vH13* in the Hessian fly (*Mayetiola destructor*), a gall midge pest of wheat (Aggarwal et al. 2014). This *Avr* gene does not have similarities with any other gene in the GeneBank. The corresponding *R* gene in wheat is *H13*, which belongs to a rich cluster of NB-LRR genes. Via transcriptome sequencing of the salivary gland of the potato aphid (*M. euphorbiae*) and subsequent transient expression in model plants, Atamian et al. (2013) identified two candidate

effectors, Me10 and Me23, which increase the aphid fecundity. An interesting example is also that observed in tomato plants, where the *Mi-1.2* gene confers resistance to the aphid *M. euphorbiae*, the whitefly *B. tabaci* and the root-knot nematodes *M. incognita*. This gene encodes an NBS-LRR protein, but the mechanisms of recognition and subsequent defense activation are unknown, although they are supposed to differ for each pest (Rossi et al. 1998; Nombela et al. 2003).

Much more is expected to be discovered in the future about the defense mechanisms of the plants against herbivores and the pests virulence effectors, a topic which has revealed to be extremely wide and diverse.

1.2.5 Diseases and pest in pear

Pyrus spp. is generally affected by several pests and pathogens. Here I described the most economically important ones.

Pear decline. This disease is caused by a phytoplasma (*Candidatus Phytoplasma pyri*) and is transmitted naturally by pear psylla (*Cacopsylla pyri* or *C. pyricola*), or artificially by budding or grafting. Symptoms expression and the economic impact of the disease strongly depend on the rootstock (Seemüller et al. 2011). Symptoms can appear quickly (quick decline), with the tree suddenly wilting and dying within a few days or weeks, or slowly, extending through a few years (slow decline), during which the general growth of the tree is arrested (Jackson 2003). A mild form of slow decline can be observed on more tolerant cultivars, and is usually associated with reddening of the leaves, leaf-curling and premature defoliation. The disease can be extremely catastrophic. Oriental species such as *P. pyrifolia* and *P. ussuriensis* are highly susceptible, however pear decline has also been observed in the more resistant or tolerant cultivars of *P. communis* and *P. betulaefolia*. The phytoplasma can overwinter inside psylla adults and in the roots of the infected pear trees. The most effective control of pear decline is obtained by growing disease-free pear varieties on resistant rootstocks (Carraro et al. 2001). Injection of a tetracycline solution in the trunk of infected trees soon after fruit harvest results in a temporary remission of

symptoms. Antibiotic treatments must be repeated annually, however, or the disease will reappear (Agrios 2005).

Fire blight. Fire blight is the most important bacterial disease of Rosaceae, especially the *Pyraea*, widespread in almost all apple and pear growing areas in the world, where it causes serious economic losses. The unique causative agent of fire blight is the gram-negative bacterium *Erwinia amylovora* (Burrill) Winslow et al., which belongs to the family Enterobacteriaceae (Vanneste 2000). After its first known observation in eastern New York in the late 18th century, fire blight has been reported from more than 40 countries around the world, in Europe, the Mediterranean area, the Americas and New Zealand (Peil et al. 2009). Long-distance dissemination was caused by human transportations of infected plant material. *E. amylovora* is considered a quarantine pest by many Regional Plant Protection Organizations (Bokszczanin et al 2009, <http://www.cabi.org/isc/datasheet/21908>).

E. amylovora sources of primary inoculum consist mainly in the previous year's cankers on branches, where the bacterium overwinters. On cankers, viable bacteria are contained in the ooze, a hygroscopic polysaccharide matrix, from which, at spring, they are transmitted to flowers by flies and ants or rain (Vanneste 2000). Primary inoculum can originate from trees in the orchard or from other host plants close to it (e.g. *Crataegus*, *Cotoneaster*, *Pyracantha*, wild *Malus*, *Photinia*), since strains of *E. amylovora* are not strictly species-specific (Momol and Aldwinckle 2000). The use of infected material for the propagation (bud woods, nursery stocks) and contaminated tools for pruning is also a way for spreading *E. amylovora* (Vanneste 2000). Secondary dissemination of the inoculum from infected flowers to other flowers or foliage occurs via insects and rain. Bacteria enter the flower through natural openings or injuries and they begin to multiply in the intercellular spaces, quickly spreading throughout the corymb; ooze droplets come out of pedicels. Shoots are inoculated via wounds on leaves and stems, caused either by natural (e.g. hail, strong wind) or artificial (pruning) events, or via natural openings, like hydathodes, stomata and lenticels. From the inoculation point, bacteria move systemically inside the plant through xylem vessels and even phloem and cortical parenchyma (Vanneste 2000). A schema of the fire blight disease cycle in apple and pear is shown in Figure 1.6.

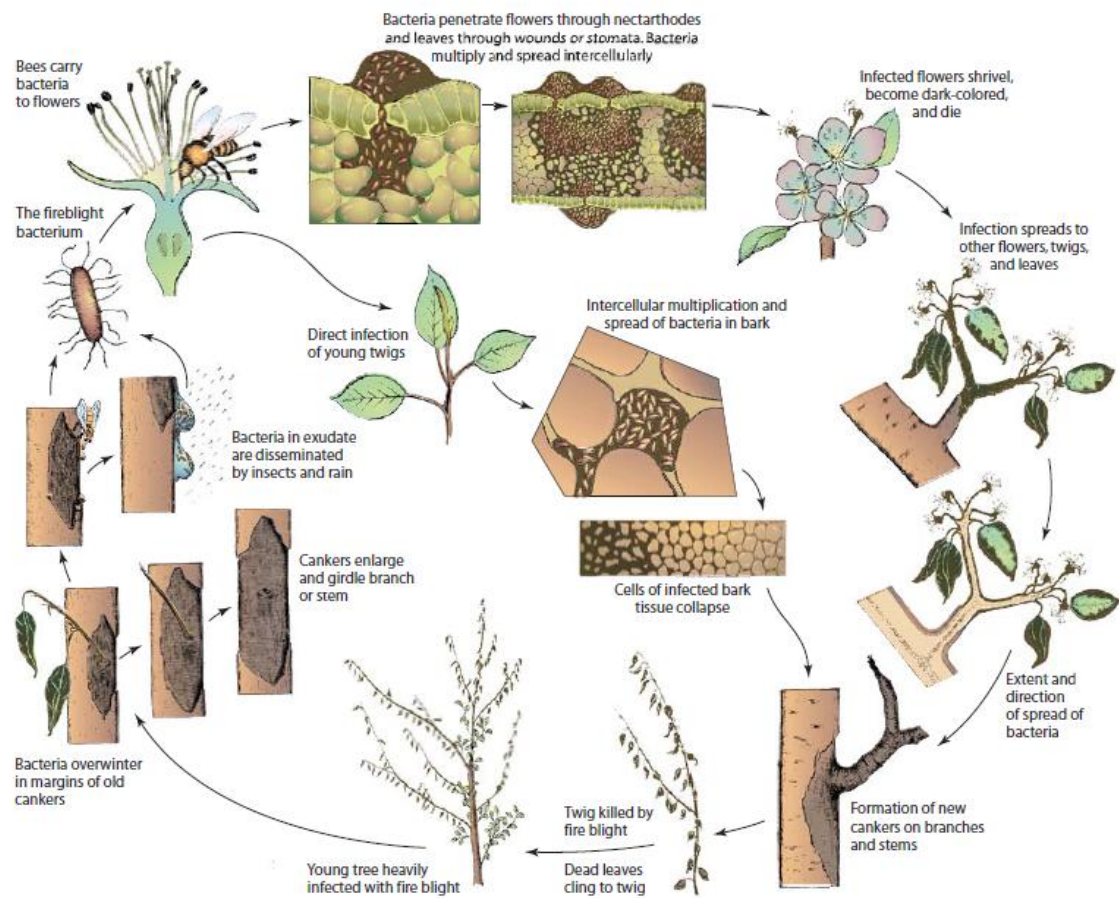


Figure 1.6: Disease cycle of fire blight in pear and apple (Agrios 2005)

Symptoms of fire blight are due to the presence of *E. amylovora* in the intercellular space of the cortical parenchyma, where it multiplies and absorbs water, causing an increased physical pressure which made the tissue collapse and the bacteria move, either inside the plant, invading other tissues, or outside, in the form of a sticky exudate (Vanneste 2000). Infected flowers become water soaked, then dry, turn brownish black, and fall or remain attached to the plant. Infected young succulent shoots and twigs wither, rapidly necrotize and in most of the cases the tip hooks (symptom known as “Shepherd’s crook”) and the leaves turn black and cling to the twig. Infected leaves develop brown-black blotches along the midrib and main veins or along the margins and the petiole. When the disease spreads to larger twigs and branches, it causes cankers and then may continue into the scaffold limbs and the trunk. The bark of cankers appears water

soaked at first, later becoming darker, sunken, and dry. Cankers cause quick death of branches or the whole tree by girdling. Infected fruits also become water soaked, turn brown to black, shrivel, and may cling to the tree for several months after infection, taking on a mummified appearance. In warm, wet conditions, drops of bacterial ooze may exude from infected shoots, petioles, cankered bark and infected fruits and blossoms (EPPO 1977; Vanneste 2000; Agrios 2005).

E. amylovora is quite a homogeneous species, although a rather important genetic diversity has been discovered among different strains in the last decades (Momol and Aldwinckle 2000; Malnoy et al. 2012). After the publication of the genome sequence of the *E. amylovora* strain CFBP 1430 (Smits et al. 2010), other strains genomes have been completely or partially sequenced, and from their comparison two main groups of diversity could be identified (Malnoy et al. 2012): the *Maloideae* group, which is very monomorphic, and the *Rubus* group, which includes isolates more genetically diverse. Nevertheless, even within the same group, there could be a differential reaction basing on the strain-cultivar combination (Momol and Aldwinckle 2000).

The coexistence of three factors is required for the exhibition of pathogenesis in *E. amylovora*: *hrp* genes, *dsp* genes and the exopolysaccharide amylovoran. Exopolysaccharides associated with *E. amylovora* virulence are amylovoran and the homopolymer levan, which are contained in the bacteria exudate (Geider 2000; Vrancken et al. 2013). Biosynthesis of amylovoran is dependent on 12 structural genes, located in the *ams* region of the chromosome, and 2 genes adjacent to the *ams* cluster, involved in precursor formation. Levan is synthesized via the secreted enzyme levansucrase, encoded by the *lsc* gene (Geider 2000). Lack of levan synthesis can result in a slow development of symptoms in the host plant. Both elicitation of HR in a non-host/resistant plant and pathogenicity in a susceptible one are controlled by the *hrp* genes, which in the *E. amylovora* chromosome are clustered within the so-called “Hrp pathogenicity island” (Kim and Beer 2000; Vrancken et al. 2013). Expression of the *hrp* genes is dependent upon particular environmental conditions (carbon and nitrogen sources, pH, temperature and osmolarity) which are met only inside the plant. Proteins encoded by *hrp* genes can be classified in three categories: i) regulatory proteins, which control the expression of the other *hrp* genes; ii) secretory proteins, the structural components of a transmembrane secretion apparatus; and iii) secreted proteins, including the effector protein harpin. The Hrp secretion apparatus in *E. amylovora* is a TTSS, a secretion

pathway which is cosmopolitan among important animal and plant pathogens. Harpins elicit HR and induce SA-dependent SAR in non-host plants. Other genes involved in *E. amylovora* pathogenesis (but not in the non-host defense responses) are the *dsp* genes, named after their disease-specific function from their first discoverers (Barry et al. 1990). The *dsp* region is located next to one end of the *hrp* gene cluster and includes two genes, *dspA/E* and *dspB/F* (Bogdanove 2000; Malnoy et al. 2012). Expression of *dspA/E* is under the control of a *hrp* gene and is dependent on the same environmental conditions (specifically found inside the plants) described above for *hrp* genes; DspA/E protein is secreted via the TTSS. The disease factor DspA/E, other than being fundamental for pathogenicity, is an effector protein interacting with the intracellular domains of host plant receptor kinases and peroxidase (Vrancken et al. 2013). DspB/F protein functions as a chaperone to DspA/E.

Fire blight disease management in the orchard is difficult. Chemical control is based mainly on copper compounds and antibiotics (i.e. streptomycin, oxytetracycline and kasugamycin). However, no completely effective systemic chemical bactericide exists that is also environmentally safe and non-phytotoxic. In addition, antibiotics can easily cause the development of resistance mutants, not only by *E. amylovora*, but also by other microorganisms present in the environment, including human and animal pathogens; their use has hence been prohibited in many countries (mostly European) (Psallidas et al. 2000). Therefore, pesticides application should be combined with other measures, like proper agronomic practices, employment of biological agents, use of resistant rootstocks and scions, as part of an integrated program (Norelli et al. 2003). The most effective biological strategy to control fire blight is based on bacterial species used as biocontrol agents; the main ones are *Pseudomonas fluorescens*, *Pantoea vagans*, *P. agglomerans*, *Bacillus subtilis* and *Aureobasidium pullulans* (Malnoy et al. 2012). These bacteria produce antibiotics and/or compete for nutrients with *E. amylovora*, suppressing its colonization and growth on the plant (Johnson et al. 2000; Peil et al. 2009). In an integrated fire blight management strategy, great importance goes to the prompt and complete removal of all visibly infected limbs and, when necessary, entire trees. When pruning off an infected shoot, the cut has to be made at least 20-30 cm below any visible symptoms, and pruning tools must be sterilized between each cut. All cut plant material should be removed from the orchard and destroyed, since it may continue to provide sources of inoculum (Steiner and

Vanneste 2000). An important component of the integrated management of fire blight is the development of resistant cultivars, both for scions and for rootstocks (Lespinasse and Aldwinckle 2000; Peil et al. 2009). Major pear breeding programs aimed at fire blight resistance are based in the USA, New Zealand, Canada, Turkey, Poland, Germany, Italy, Switzerland, France, and Czech Republic (Peil et al. 2009).

Pear scab. Two fungus species inciting scab on pear have been described: *Venturia nashicola* and *Venturia pirina* Aderh., which are specific to Asian pears and European pears, respectively (Ishii and Yanase 2000). Symptoms of pear scab are characteristic black scab lesions, consisting of sporulating mycelia growing under the epidermis, which appear on the lower surface of sepals or young leaves and on fruits. High infection can cause premature defoliation. Infected fruits sometimes crack, become misshapen and frequently drop prematurely. The economic damage of pear scab is mainly linked to reduction of the quality of fruits, which are often not marketable.

Control of pear scab mainly relies on chemicals application. Fungicides are first sprayed in spring, when the bud dormancy is broken and the weather conditions are favorable for infection; sprays are usually repeated several more times during the growing season. So far, no effective practical biological control of scab has been developed. The research on pear scab resistance is very active, and breeding strategies often exploit the non-host resistance of Asian pear species to European scab, and vice versa (Bus et al. 2013).

Pear psylla. Pear psylla is one of the most serious insect pests of pears. Several species exist, but the three major ones are *C. pyri*, *C. pyricola* and *C. bidens*, which are widespread mostly in Europe, North America and the Middle East. Pear psylla adults overwinter in sheltered places in the bark or under the ground. Eggs are tiny, elongated, and yellow and are barely visible without a hand lens. As buds open, females lay eggs, singly or in clusters, mainly along midribs and petioles of developing leaves, stems and leaves of blossoms. Nymphs pass through five instars, the youngest ones almost completely encased in honeydew. First and second instar nymphs are flat and oval, have red eyes, small antennae and no wings. The wing pads become visible in the third stage and develop gradually during the fourth instar, while the antennae elongate. The fifth instar has prominent wing pads. Nymphs get larger at every stage. After the last molt, nymphs develop into male or female adults, which are able to reproduce sexually within a few days

(Hodkinson 2009). The psylla has three or four generations per year, depending on the length of the growing season.

Honeydew, produced by feeding psylla nymphs, blocks photosynthesis, causing necrosis on the leaves (Salvianti et al. 2008), and drops onto fruit. Black sooty mould grows on the honeydew and the fruit skin russets, which downgrades fruit for fresh-market use (Pasqualini et al. 2006). High infestation on trees causes leaves to become yellow and sometimes fall; growth and productivity of the tree can be severely reduced for one or more seasons. Moreover, losses can occur from pear decline disease, which is transmitted by psylla (see above).

For an effective management of pear psylla in the orchard, it is essential to keep populations low through summer, because control is difficult when generations overlap and all life stages are present, since not all stages are susceptible to chemical treatments. Orchards should be sprayed also after harvest, to prevent migration of adults in fall and then reduce the size of the overwintering population. However, pear psylla has developed resistance to a great number of insecticides (Harries and Burts 1965; Buès et al. 2003; Civolani et al. 2007), and these also destroy many of the naturally occurring predators and parasites of pear psylla (e.g. green lacewings, brown lacewings, and minute pirate bugs).

Aphids. Several aphid species occasionally attack pears; the most common are the pear bedstraw aphid (*Dysaphis pyri*), the green peach aphid (*M. persicae*), the cotton aphid (*Aphis gossypii*, also known as melon aphid), and the bean aphid (*A. fabae*, also known as dock aphid). These aphids overwinter as adults on various weeds and field crops in or outside the orchards. Usually after pear bloom, when trees are growing rapidly, these aphids appear on foliage and shoots, establishing colonies, and several generations may occur in cool spring weather. Aphid feeding on pear foliage cause leaves to become yellow and curl, forming a refuge for the colonies and sheltering the aphids from any subsequent chemical treatments; more importantly, aphids produce honeydew, which falls on the fruit, causing the same damage as describe earlier for pear psylla. Aphids are infrequently encountered in pear orchards and seldom require special treatments.

1.3 Genetics and breeding approaches in crop plants

1.3.1 Genetic mapping and QTL analysis

Most important agronomic traits, such as yield, quality and many resistances to abiotic and biotic stresses, are quantitatively inherited (Collard et al. 2005; Würschum 2012); as indicated above, a QTL corresponds to a genomic region which control part of the phenotypic variation of a quantitative trait (Collard et al. 2005).

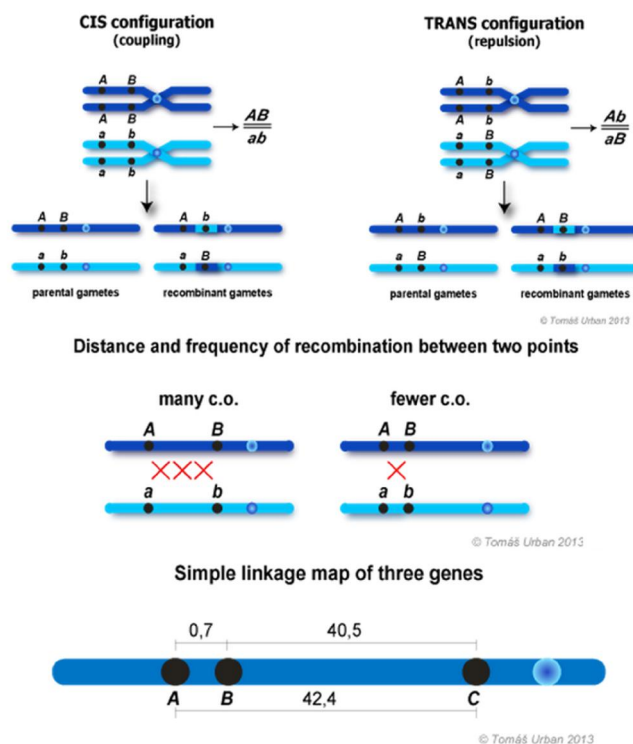


Figure 1.7: Linkage map construction

The genetic distance between two loci (A and B) is inferred from their recombination frequency. The closer the two loci are on the chromosome, the less likely is a crossing-over to occur between them, the lower is the recombination frequency (http://web2.mendelu.cz/af_291_projekty2/vseo/stranka.php?kod=284)

QTL detection starts with the construction of linkage maps based on molecular markers. Markers that are polymorphic in the segregating population under study are screened across the entire progeny and the parents. Linkage between markers is usually calculated using the logarithm of the likelihood ratio of linkage versus no linkage, and is called a logarithm of odds (LOD) value or

LOD score. For example, a LOD score of 3 indicates that linkage is 1000 times more likely than no linkage. Based on the LOD score chosen as a threshold for the linkage calculation, markers are grouped into 'linkage groups' (LG), which represent chromosomal segments or entire chromosomes. Subsequently, the genetic distance between markers belonging to the same LG is calculated. Each individual of the progeny is a mixture of parental and recombinant genotypes, and the genetic distance between markers can be inferred by calculating the frequency of recombination (Figure 1.7). Markers with a recombination frequency of 50% are described as 'unlinked' and assumed to be located at long distance on the same chromosome or on different chromosomes. Mapping functions (e.g. the Kosambi and the Haldane mapping functions) are used to convert recombination frequencies into map units called centiMorgans (cM) ($1 \text{ cM} \cong 1\%$ recombination). There are several software packages available (e.g. JoinMap (Van Ooijen 2006)) for linkage analysis.

QTL mapping analysis is based on the statistical association between genotypic and phenotypic data of a segregating population: basically, the mapping population is divided into genotypic groups according to the alleles of a particular marker locus, and the phenotypic means for each group are compared. If the marker and the QTL are closely linked, they have a higher chance to be inherited together in the progeny (the chance of a crossing over occurring between them is lower), and the phenotypic means are significantly different between the genotypic groups; conversely, markers and QTLs which are unlinked segregate independently, and the phenotypic means between the genotypic groups will not be significantly different (Figure 1.8) (Collard et al. 2005). There are three methods for the detection of QTLs: single-marker analysis, simple interval mapping and composite interval mapping. The first method involves studying single genetic markers one-at-a-time, and is commonly performed via non-parametric (e.g., Kruskal-Wallis) tests, analysis of variance or linear regression between the phenotypes and each marker: the most likely position of the QTL corresponds to the marker with the higher coefficient of determination (R^2). This method is extremely simple and computationally fast; however, if the genetic map has less than 1 marker every 10 cM, the effect of QTLs are underestimated, their genetic locations inaccurate, and the number of progeny required for detecting QTLs is larger than necessary (Lander and Botstein 1989; Collard et al. 2005). Simple interval mapping (IM) is more powerful

for QTL detection, since it analyses the intervals between adjacent pairs of linked markers along chromosomes simultaneously (Lander and Botstein 1989), and thus allows to accurately localize the QTL even between two markers which are quite distant, as long as the population size is sufficiently large. However, the most precise and effective method for QTL detection, especially when linked QTLs are involved, is the composite IM, which combines multiple linear regression with simple IM. In this method, the phenotype is explained by a single putative QTL in a given interval and at the same time by a number of markers that serve as cofactors, to eliminate the major part of the variation induced by the corresponding QTLs located in other regions of the genome, thus reducing the background noise (Jansen 1993; Jansen and Stam 1994). The results of the test statistics for simple and composite interval mapping are typically presented using a LOD: the position on the linkage maps where the highest LOD value is obtained is the most likely position for a QTL (maximum likelihood method) Figure 1.9. Permutation tests are usually performed for the determination of the empirical significance threshold values above which the LOD score of a QTL should fall in order for it to be considered “true”. In a permutation test, the phenotypic values of the population are “shuffled” whilst the marker genotypic values remain fixed; subsequently, QTL analysis is performed to assess the level of false positive marker-trait associations (Churchill and Doerge 1994). This process is repeated at least 1000 times and the significance threshold can then be determined based on the level of false positive marker-trait associations. Several software (e.g. MapQTL (Van Ooijen 2004)) have been developed for QTL mapping analysis.

Each QTL explains a certain amount of total phenotypic variation (estimated with the R^2): the higher the R^2 , the higher the effect of the QTL. In general, QTLs are considered having a major effect when their R^2 are higher than 10% and/or they are stable across different environments or experimental conditions. The total genetic variation of a quantitative trait is determined by additive and putatively dominance effects of each QTL, and by putative epistatic effects between different QTLs (Collard et al. 2005).

There are several factors affecting the power and accuracy of a QTL mapping study: mainly the effect (R^2) of the QTLs, the size of the mapping population, genotyping and/or phenotyping errors and missing data, the density of the genetic map and the presence of closely-linked (20 cM or less) QTLs, especially when acting in repulsion. Furthermore, environmental factors may have a profound influence on the expression of quantitative traits. In order to account for the effect of external factors on the phenotype, a number of replicates for each genotype of the mapping population should be tested, which in turn allows to compute the heritability of the quantitative trait of interest. Moreover, to confirm the QTL effects and positions, the experiment should be replicated with at least another study in a different season and/or site (Lander and Kruglyak 1995). When considering different sites, the putative QTL x E (environment) interactions can be evaluated.

1.3.2 Traditional breeding and marker assisted selection

Tree fruit crops cultivars with new fruit qualities and improved agronomic features are commonly developed by skillfully designing and making bi-parental crosses, and then selecting for the offspring with the desired performance (Ru et al. 2015). For several years this has been achieved by traditional breeding, where selection is based on phenotype. The traditional breeding technique is extremely laborious, time consuming and expensive, especially for woody perennial crops, which have a long juvenility phase and hence breeding cycles that can take even more than 10 years (van Nocker and Gardiner 2014). Moreover, the expression of complex traits is also affected by the environment and by genotype-environment interaction (GxE) (Mitchell-Olds 2013), and then elite cultivars selected in a particular location might not have the same performance in a different cultivation site.

Consequently, marker assisted selection (MAS) (also called marker assisted breeding – MAB), which, exploiting linkage disequilibrium (LD) between markers and trait loci, results in fewer breeding cycles, has a great potential for tree fruit crops. With this breeding technique, seedlings are screened with molecular markers when they are small, and those bringing undesirable characteristics are early culled; this way, they do not have to reach maturity for their evaluation, and time and money are saved. Moreover, the cultivars to be used as parents for improved

breeding populations can be selected based on their genotypes and on knowledge of the inheritance pattern of important traits (Myles 2013; van Nocker and Gardiner 2014).

Hospital (2009) distinguished five MAS breeding strategies for plants: i) marker-assisted introgression or marker-assisted backcrossing, ii) simple population screening, iii) gene pyramiding schemes, (iv) marker-based (or assisted) recurrent selection, and (v) selection based on an index combining molecular and phenotypic scores.

The most used MAS methods is marker-assisted introgression or backcrossing (i) (Figure 1.10), which starts by crossing the cultivated species to improve (recurrent parent) with a wild relative species (donor parent) carrying the specific trait (and allele) to introgress in the cultivated species. Half of their offspring (F1) will also have this trait/allele (foreground selection), and they are backcrossed with the recurrent parent (either the same genotype, or another genotype of the cultivated species if self-incompatibility exists), resulting in a heterozygous progeny (BC1) composed by different individuals with a variable proportion of their ancestry derived from the wild and cultivated species. The BC1 is then repeatedly backcrossed to the recurrent parent for a number of generations, in order to obtain individuals containing practically all of the recurrent parent genome except for the target trait/allele inherited by the donor parent (background selection) (Myles 2013). Homozygous F2 lines can be obtained by selfing the final BC generation plant. Using traditional breeding, this procedure typically takes 6–8 backcrosses to fully recover the recurrent parent genome, which with tree fruit crops translates to several decades. Moreover, in the case of generally self-incompatible species, such as pear and apple, selfing is often not possible. The theoretical proportion of the recurrent parent genome after n generations of backcrossing is given by $(2^{n+1} - 1)/2^{n+1}$ (where n is the number of backcrosses; assuming an infinite population size). However, this formula calculates the average percentage of the recurrent parent genome for the entire BC1 population, while some individuals will possess a higher proportion of the recurrent parent ancestry than others (Collard et al. 2005). MAS with markers tightly linked to the target trait and markers evenly spaced in the other chromosomes can extremely simplify and accelerate this process. First, the foreground selection step can be performed by genotyping with markers associated to the trait of interest, early discarding the half of the F1 which does not carry it. Then, each BC generation can be screened with both the trait-

linked and unlinked molecular markers, to identify those individuals that carry the target allele and have the minimum amount of wild ancestry (Ribaut et al. 2002).

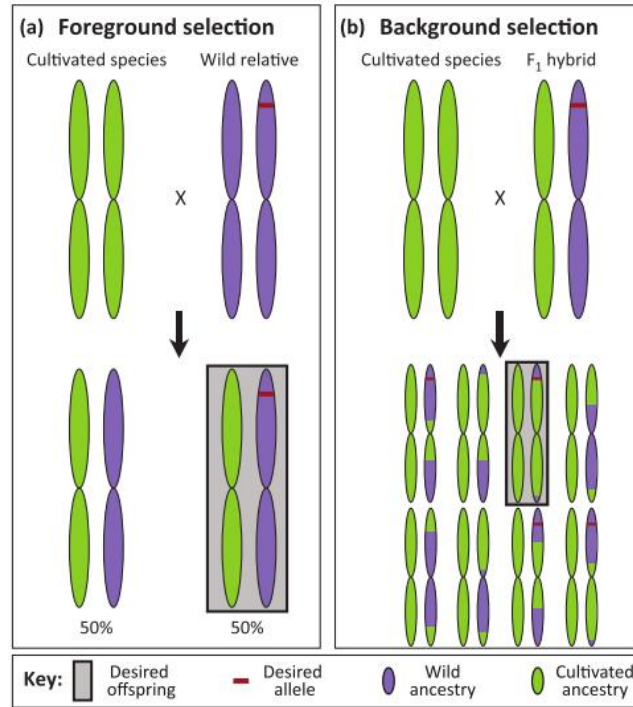


Figure 1.10: Schematic of marker-assisted introgression or backcrossing

(a) The recurrent parent is crossed to a wild relative species, the donor parent, with a desired trait. The half of the offspring (F₁) carrying the desired trait is selected (foreground selection). (b) These F₁ hybrid is backcrossed with the recurrent parent, and again the progeny (BC₁) carrying the desired trait is selected (background selection) and repeatedly backcrossed to the recurrent parent (Myles 2013)

MAS via population screening (ii) is simply the selection of the genotypes of any type of population (F₂, RIL, DH...) based on the marker data.

In gene pyramiding schemes (iii) two parental lines, each carrying one (or more) allele(s) of interest, are crossed, and the offspring population is screened with markers linked to those alleles of interest, in order to identify the individuals carrying all of them; this process can be repeated more times with additional parental lines if more alleles are to be accumulated in one genotype.

With marker-assisted recurrent selection (MARS) (iv), selection is based solely on markers data from several genomic regions (up to 20 or even more) for complex traits within a single population, while phenotypic data are not available.

Finally an extension of the selection based on an index combining molecular and phenotypic scores (v, as proposed by Hospital (2009)) is the genomic selection (GS), which is revolutionizing the genetic improvement of animals and plants species (Calus 2010; Kumar et al. 2012b), particularly since the implementation of the Single Nucleotide Polymorphism (SNP) markers-based genotyping technologies. More in particular, GS is making use of all available markers covering a plant genome to compute genomic estimated breeding values (GEBVs) (Calus 2010), which can further be used for ranking and selecting individuals. GS can be particularly convenient for breeding for quantitative traits determined by several low effect genes, since it relies on high-density genotyping, so that all the genes affecting the target trait are expected to have a tight correlation with at least one or possibly multiple markers (Meuwissen et al. 2001; Meuwissen 2007). At first, the genotypic and phenotypic data of a ‘training population’ must be collected, in order to estimate the effects of the genetic markers and build a prediction model. Subsequently, this prediction model is applied in a ‘selection population’ for which only the genotypic data are available, and the GEBVs are computed/predicted (Meuwissen et al. 2001). The GEBVs can be directly used to rank individuals for the selection of elite accessions to be used as parents of next-generation breeding populations, or to be further tested as potential commercial cultivars (Kumar et al. 2012a).

In summary, we can distinguish between two types of MAS: post-QTL MAS, which depends on the previous detection of genes and QTLs and of the linked molecular markers, and GS, which applies molecular markers densely spread all over the genome, without necessarily knowing the location of specific traits-associated loci.

With the rapid advancement of the DNA testing technologies in the last decade, post-QTL MAS and GS have become even more affordable. Nonetheless, the effectiveness of MAS over traditional breeding needs to be considered on a case by case basis, being dependent on the heritability and the genetic architecture of the trait (in particular for GS), the phenotyping method, the field/glasshouse and labor costs and the cost and accessibility of resources (including DNA testing platforms and services). With high-heritability traits, post-QTL MAS tends to perform like phenotypic selection, while with low-heritability traits QTL effects are poorly estimated, reducing MAS efficiency. In this case, GS can be much more powerful, especially if the prediction model is strongly established with high replicated phenotypic data. Furthermore,

post-QTL MAS efficiency increases with QTLs accounting for larger percentages of phenotypic variation (Muranty et al. 2014). Another important factor to take into account when evaluating the advantages of MAS over traditional breeding is the initial cost of molecular markers development and the establishment and routine implementation of a MAS program (Ru et al. 2015).

1.3.3 Linkage drag and lethal genes

One of the advantages of MAS is the possibility of avoiding the transfer of undesirable or deleterious genes. One of the drawback of marker-assisted backcrossing, in particular when the donor parent is a wild species, is the ‘linkage drag’ (Collard et al. 2005), which refers to the reduction in fitness in a cultivar due to deleterious genes introduced along with the beneficial one during backcrossing. This occurs when the undesirable genes lie close to the genes or QTL we are trying to introgress. Overcoming linkage drag requires searching for recombinants (possibly rare) between the target QTL and the undesirable gene. Of course, this is achievable only if markers associated to the deleterious genes have also been developed.

An extreme case of linkage drag is when the trait of interest is linked to lethal genes. In this context, an interesting phenomenon, which has a great relevance in plant breeding, is hybrid necrosis. Hybrid necrosis is a type of post-zygotic genetic incompatibility that is associated with a typical phenotype, common to several plant taxa, characterized by cell death, tissue necrosis, wilting, yellowing, chlorosis, dwarfism and reduced growth rate, and in some case lethality (Bomblies and Weigel 2007; Bomblies 2009). It usually results from deleterious epistatic interactions between two (or even more) loci, inherited from the different parents, which are expressed in the hybrid. The most exemplifying model for hybrid necrosis is the Bateson–Dobzhansky–Muller (BDM) model (Orr 1996), which posits that independent substitutions occurring in two diverging lineages, not detrimental in their native genomic context, might be deleterious when combined in the hybrid (Figure 1.11). This can occur in the F1 generation when both loci are heterozygous (that is, the alleles are dominant), or in the F2 or backcross generations if one or both loci must be homozygous (that is, the alleles are recessive) (Bomblies and Weigel 2007).

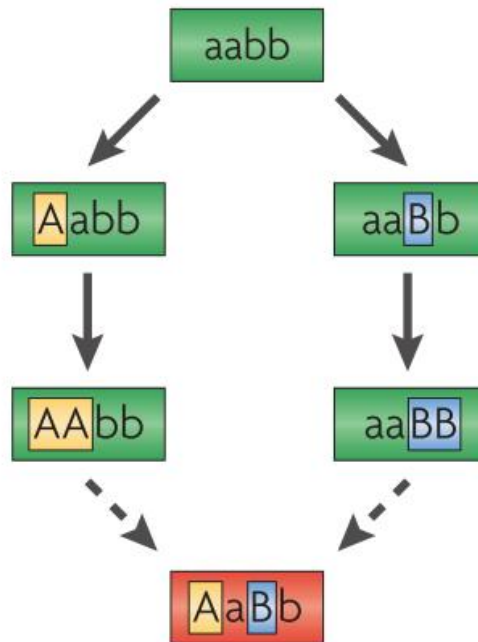


Figure 1.11: The two-locus Bateson–Dobzhansky–Muller (BDM) model for the genetic incompatibility

Two lineages, diverged from a common ancestral population, evolve independent substitutions (shown as capitalized alleles in the figure) at different loci, which are not detrimental (represented by a green box) in their native genomic contexts. The two mutated alleles can cause genetic incompatibilities (represented by a red box) when combined in a hybrid, if they interact negatively (Bomblies and Weigel 2007)

With the development of molecular markers associated with genes involved in the hybrid necrosis, it will be possible to screen the parent pool of a breeding line in order to identify the incompatible alleles and then avoid the deleterious combinations, particularly if the lethal genes turn out to be linked to the target trait(s). Special attention must be taken when breeding for disease and pest resistances. Several studies, on both model plants (e.g. *Arabidopsis* (Bomblies et al. 2007; Alcázar et al. 2009; Tahir et al. 2013) and tomato (Krüger et al. 2002)) and crops (such as wheat (Mishra et al. 2005; Mizuno et al. 2010) and apple (Alston 1976; Gao and Van de Weg 2006; Fernández-Fernández et al. 2013)) have reported a relation between resistances and hybrid necrosis, either showing linkage between *R* and lethal genes, or demonstrating the functional involvement of NBS-LRR genes in the phenomenon. Therefore, breeders pyramiding several

resistance genes to enhance durability should note that they may end up with the loss of the wanted resistance because of incompatibilities skewing the progeny segregation.

1.3.4 Breeding for durable resistant varieties

Resistance to biotic stresses is one of the breeding targets which has found the largest interest for the application of MAS breeding strategies, particularly because the phenotypic evaluation is usually expensive and time consuming, and resistances are often strongly affected by the environment (Muranty et al. 2014). Many pest and disease resistances have a polygenic determinism, which makes them good subject traits for the application of GS (that might accelerate breeding) and gene pyramiding (to obtain more durable resistant cultivars).

Sources of resistance to several pests and pathogens of most important crops can be found in some commercial varieties, in older varieties earlier abandoned or amongst wild relatives. Once resistant varieties are identified, they are crossed with highly productive and superior quality cultivars, in an effort to confer to them the resistance. In case of a mono or oligogenic resistance, the introgression of one or few major genes can make a plant completely resistant to a pathogen, which is not the case for resistances associated with several minor genes. Furthermore, mono/oligogenic resistances are easier to manipulate in a breeding program. However, they break down easily when new pathogen races evolve and bypass or overcome the (few) *R* gene(s). A well-known example has been thoroughly described for the interaction between *Rvi6(Vf)*-carrying resistant apple cultivars and the fungus *Venturia inaequalis* (Parisi and Lespinasse 1996). On the contrary, quantitative (polygenic) resistances were generally thought to confer a more durable protection, because multiple genes with a small effect are probably less easy to overcome than a single (or few) *R* gene(s). Moreover, resistance is not completely lost if one of these genes is overcome by the pathogen, and the selection pressure exerted on pathogen populations by quantitative resistance genes, each with a minor effect, is certainly lower and more diversified than that of major *R* genes (Poland et al. 2009; Mundt 2014). Nevertheless, pathogens can also adapt to quantitative resistance, causing its “erosion”, as was documented for *Venturia inaequalis* in apple (Caffier et al. 2014), or a complete breakdown, as demonstrated for the Potato Virus Y in resistant pepper genotypes (Montarry et al. 2012). It has also been observed that pathogens

adapted to quantitatively resistant cultivars become more aggressive (on susceptible cultivars) than pathogens which overcome qualitative resistance. This could be explained by the fact that an increase in the pathogen aggressiveness enables it to infect hosts carrying quantitative resistance, but not those carrying qualitative resistance, which is an “all-or-nothing” response (Gandon and Michalakis 2000).

Reaching durable resistance is of utmost importance in perennial species, such as pear or apple, whose selected cultivars are planted for dozens of years in the same orchards, thus exerting a continuous selection pressure on the pathogen populations (Caffier et al. 2014). Strategies to avoid the rapid and sudden breakdown of resistance are gene rotation, the use of multilines and gene pyramiding (Djian-Caporalino et al. 2014). Gene rotation, based on the prompt replacement of the cultivar when the *R* gene is overcome, is more theoretical, since several issues make its practical application difficult (Mundt 2014). Multilines are mixtures of cultivars with the same agronomic features but different *R* genes. There are both negative and positive examples of application of this approach for the increase of durability. An issue to multilines, although still theoretical, is the putative emergence of pathogen “super-races”, i.e. complex and polymorphic strains which are virulent to a wider range of plant genotypes (Mundt 2002). Gene pyramiding (Pedersen and Leath 1988) is probably the most successful approach to increase resistance durability, although it is still not clear whether it is the number of pyramided genes or the particular gene combinations that confers a more durable resistance (Mundt 2014). The combination of qualitative and quantitative resistances appears particularly promising to improve resistance durability (Palloix et al. 2009; Brun et al. 2010).

1.4 Genomics of pear

1.4.1 SNP markers and genetic maps

Several linkage maps have been reported for pear. However, most of them were based on Random Amplified Polymorphic DNA (RAPD) markers (Iketani et al. 2001), which are not reproducible and generally not transferable across populations, and Amplified Fragment Length Polymorphism (AFLP) markers (Yamamoto et al. 2002b; Yamamoto et al. 2004; Yamamoto et al. 2007; Yamamoto et al. 2009), which are not easily transferable as well. Consequently, despite that they could be used for QTL mapping studies, their application in MAS is not straightforward (Collard et al. 2005). A number of studies have developed Expressed Sequence Tag (EST)-based (Nishitani et al. 2009; Zhang et al. 2014) and genomic Simple Sequence Repeat (SSR) markers (Yamamoto et al. 2002a; Yamamoto et al. 2002c; Sawamura et al. 2004) from pear, which, along with apple SSR markers, were used to build low to medium density genetic maps for *P. communis* ‘Bartlett’ and ‘La France’, *P. pyrifolia* ‘Hosui’ (Nishitani et al. 2009; Celton et al. 2009a) and ‘Mishirazi’ (*P. pyrifolia* x *P. communis*) x ‘Jinhua’ (*P. x bretschneideri*) (Lu et al. 2010). Moreover, Yamamoto et al. (2007; 2009) constructed high-density genetic maps, mainly based on AFLP markers, but also included some apple and pear SSRs. More recently, new SSR markers have been developed from pear and were used to build a genetic map with a high resolution (Fan et al. 2013; Chen et al. 2014).

Although microsatellites are robust, reliable and transferable across populations (and related species), SNPs are considered to be the most efficient tools for comprehensive genetic studies (Yamamoto and Chevreau 2009). SNPs are the most abundant DNA sequence variations found in genomes, including coding regions, of most organisms. Moreover, with the evolution of next-generation sequencing (NGS) technologies, the detection of SNPs in a determined species, through the re-sequencing of multiple accessions and the alignment of these sequences to a reference genome, has become extremely cost-effective (Bentley 2006). Several high-throughput platforms for the whole-genome genotyping of a variable number of samples with one to up to one million SNPs in parallel are available, including array-based technologies from Illumina (GoldenGate[®] and Infinium[®]) (Steemers and Gunderson 2007; Hyten et al. 2008) or Affymetrix

(Close et al. 2004). Genetic maps with a high density of SNP (and SSR) markers are necessary for fine dissection of functional genetic variation.

In *Pyrus*, the number of available SNPs was marginal before the beginning of this project. We developed an Infinium[®] II array with more than 1000 SNPs from European pear, which also included about 8000 apple SNPs, and we used it to build the first high-density SNP-based genetic maps for pear (Montanari et al. 2013). In parallel, Terakami et al. (2013) used Potential Intron Polymorphism (PIP) markers designed from apple ESTs to identify intron regions and to detect SNPs in pear; about a hundred of these markers were then mapped on the genetic linkage maps of ‘Bartlett’ and ‘Housui’.

1.4.2 The sequence of the Chinese and European pear genomes

Pear belongs to the angiosperm family of Rosaceae, tribe *Pyreae*, which also includes apple (*Malus* spp.) (Potter et al. 2007). During the last few years the knowledge on the genomics of this crop has advanced well, culminating in the sequencing of the Chinese (*P. x bretschneideri* Rehd. cv. ‘Dangshansuli’ (also known as ‘Suli’), (Wu et al. 2013)) and of the European (*P. communis* WBC, (Chagné et al. 2014)) pear genomes. Pear is highly heterozygous, due to self-incompatibility and general interspecies compatibility (Crane and Lewis 1942; Zheng et al. 2014). The haploid genome size has been estimated by flow cytometry to approximately 496-536 Mb for *P. communis* (Arumuganathan and Earle 1991) and 527 Mb for *P. x bretschneideri* (Wu et al. 2013), and the two draft sequences covered more than 90% of both genomes (Wu et al. 2013; Chagné et al. 2014).

Species of the *Pyreae* are characterized by a distinctive fruit, the pome, and a haploid (x) chromosome number of 17, while most of the other members of Rosaceae have 7, 8 or 9 chromosomes pairs. The most supported hypothesis for the origin of *Pyreae* is based on an autopolyploidization event of *Gillenia* or another similar taxon (x = 9) followed by a genome-wide duplication (GWD) (x = 18) and, in a parsimony model, a chromosome rearrangement which caused the loss of one pair of homologous chromosomes (x = 17) (Evans and Campbell 2002; Velasco et al. 2010) (Figure 1.12). The occurrence of two GWD events has been postulated in the *Pyreae*: the most recent one, which led to the 18 chromosomes, supposedly occurred 30-45

million years ago (Velasco et al. 2010), while the ancient one must have resulted from an acknowledged paleohexaploidization event that took place ~140 million years ago and which is shared by most eudicots (Fawcett et al. 2009). The extremely high synteny among the European pear, Chinese pear and apple genomes suggests that the *Pyraea* genome reorganization occurred before the divergence of the two genera (Velasco et al. 2010). Both in pear and apple, large orthologous segments have been identified between chromosomes 3 and 11, 5 and 10, 9 and 17, and 13 and 16, and shorter orthologous segments between chromosomes 1 and 7, 2 and 7, 2 and 15, 4 and 12, 12 and 14, 6 and 14, and 8 and 15 (Velasco et al. 2010; Wu et al. 2013).

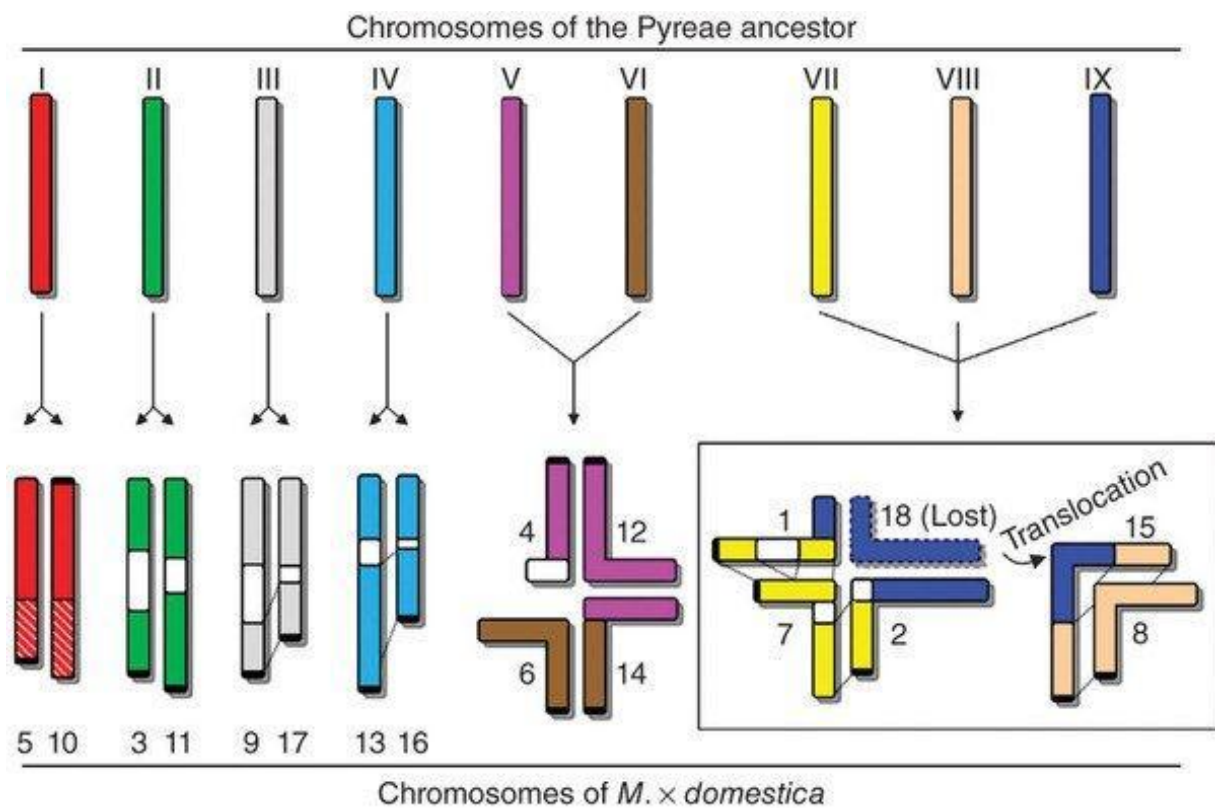


Figure 1.12: Genome wide duplication (GWD) and chromosomes rearrangements in *Pyraea*

The 17-chromosome karyotype of *Pyraea* evolved from a 9-chromosome ancestor. A GWD event followed by a parsimony model of chromosome rearrangements is postulated. Shared colors indicate homology (or partial homology in the case of white-hatched portions) and white fragments indicate lack of a duplicated counterpart (Velasco et al. 2010).

One of the characteristic features of the *Pyraea* tribe is the pome fruit, which is not found in any other species (Potter et al. 2007) and that has probably evolved after the more recent GWD event.

Chagné et al. (2014) identified almost 1500 protein clusters specific of both pear species and apple, which may then include products of genes determining the pome fruit character.

A total of 469 NBS-LRR genes were identified by Zhong et al. (2015) in the Chinese pear genome (*P. x bretschneideri*), less than in *M. x domestica*, but more than in the other Rosaceae species *Fragaria vesca*, *Prunus persica* and *P. mume*. Different numbers of *R* genes in these related species might be attributed to gene duplication events, deletions, pseudogenization (the mutation of a gene causing its loss of biological function) and functional diversification (Demuth and Hahn 2009). *R* genes are often grouped in clusters in the genomes, and molecular studies have demonstrated that this clustering usually results from tandem duplications of paralog sequences (Meyers et al. 2005). In the Chinese pear genome, *R* paralog genes clusters were found to be more abundant on chromosomes 2, 5, and 11 (Wu et al. 2013). Whereas *R* genes are duplicated in response to pathogens or natural selective pressures, and thus different Rosaceae species, which have evolved in different ecological environments, might have species-specific *R* genes, similar NBS-LRR genes are still shared in *Pyrus*, *Malus* and *Prunus*, which support their monophyletic origin (Zhong et al. 2015).

1.4.3 High genome synteny between *Pyrus* and *Malus*

High level of genome co-linearity between apple and pear was frequently reported. SSR markers were demonstrated to be transferable across the two genera (Pierantoni et al. 2004; Yamamoto et al. 2007; Celton et al. 2009a). The sequencing of the apple, Chinese pear and European pear genomes emphasized the high syntenic relationship among these species (Velasco et al. 2010; Wu et al. 2013; Chagné et al. 2014).

The extensive knowledge about the apple genome has been (and will be) employed to increase the understanding of structural and functional genomics of the less studied pear. QTLs and major genes for many important agronomic and quality traits detected in apple, along with their underlying candidate genes, can be used to discover gene-trait associations in pear as well.

1.5 The thesis - General objectives and background

When this project started, in 2011, a considerable number of microsatellite markers was available for linkage analysis in pear (including apple SSRs, which were demonstrated to be transferable to pear), but very few SNP markers had been developed. Concerning breeding for resistance to the main pests and pathogens of pear, a few major genes and QTLs had been mapped by 2011. Progress in breeding for pear psylla (*C. pyri* and *C. pyricola*) had been very limited, despite a number of highly resistant accessions had been identified; the first QTL for this trait was mapped in that year, by Bouvier et al. (2011a). On the contrary, the study of fire blight resistance was a little more advanced. This disease is a main concern for apple and pear growers worldwide, and the development of cultivars resistant to fire blight has been the objective of many pear breeding programs for many years. Several sources of resistance had been identified within European and Asian *Pyrus* species, and by 2011 a total of six QTLs had been detected in two separate studies (Dondini et al. 2004; Bokszczanin et al. 2009). Moreover, breeding efforts were addressed to pear scab (*V. pirina* and *V. nashicola*) host and non-host resistance, with a few major genes and QTLs being mapped (Terakami et al. 2006; Pierantoni et al. 2007; Cho et al. 2009; Bouvier et al. 2011b). Nonetheless, pear breeding was based on traditional techniques, and the application of MAS for pest and disease resistances in this crop had not even been postulated.

The objective of this project was to study the genetic determinism of pear resistance to two of the most significant diseases and pests of this crop, fire blight and psylla, which cause high yield losses in all the main pear production regions internationally. The development of new pear varieties with resistance to these two biotic stresses is of major interest for Integrated Pest Management. This project was designed in a joint collaboration among Fondazione Edmund Mach (FEM), the INRA of Angers and the Plant & Food Research (PFR). Resistance to fire blight has been one of the objectives of both the PFR and INRA pear breeding programs for more than 20 years; in addition, resistance to *C. pyri* had more recently become a goal. The

interspecific pear segregating population PEAR3¹ x ‘Moonglow’ was developed at PFR with the purpose of accumulating resistances to fire blight and psylla deriving from Asian and European pear cultivars. Both these resistances being postulated as polygenic, QTL mapping was evaluated as the best approach for their genetic characterization in this population.

The first step of this project was to build the genetic map of PEAR3 x ‘Moonglow’ population. An Illumina Infinium[®] II array including 1000 pear and 8000 apple SNP markers was developed and 220 progeny were genotyped with this tool, enabling the construction of high-density genetic maps. These maps were then used to anchor the scaffolds of the ‘Bartlett’ genome sequence, which was published in 2014 by Chagné et al.

In 2012 tests were performed in order to set up a novel phenotyping protocol for the antibiosis resistance to pear psylla, and PEAR3 x ‘Moonglow’ progeny was evaluated in 2013 and 2014 at the INRA site of Angers. At the same time, a first trial for the fire blight resistance phenotyping was carried out in 2012 both at INRA and PFR, with the aim of standardizing the protocol between the two sites and identifying the most suitable *E. amylovora* isolates to use for the inoculations; subsequently, PEAR3 x ‘Moonglow’ population was tested for fire blight resistance in Angers in 2013 and in New Zealand in 2013 and 2014. The phenotypic data collected for both psylla and fire blight resistance and the high-density genetic map previously developed were then used to detect QTLs for these two traits.

During the growing of PEAR3 x ‘Moonglow’ seedlings, an extremely high rate of lethality (more than 50%) was observed. When the genetic maps of this population were constructed, distorted chromosomal regions were identified, and it was thus postulated the hypothesis of the involvement of genetic incompatibilities in this extended mortality. Molecular-based experiments were then initiated in order to genetically characterize this phenomenon.

¹ The complete name of this hybrid was erroneously disclosed in the paper “Identification of *Pyrus* Single Nucleotide Polymorphisms (SNPs) and evaluation for genetic mapping in European pear and interspecific *Pyrus* hybrids” (see Chapter 2). However, for variety protection reasons, in this thesis it has been substituted with the PEAR3 term, according to the Plant & Food Research request.

CHAPTER 2. The Genetic Map

QTL mapping analyses for characters of any kind need two datasets for the segregating population under study: the genotypic data for the parents and the progeny, along with linkage maps built from these data, and the quantitative phenotypic data. In this project, the genotyping and genetic map construction for PEAR3 x ‘Moonglow’ population was carried out as a first step. The F1 seeds obtained from the cross between PEAR3 and ‘Moonglow’ at PFR were split in two subsets, one of which remained at PFR and was grown at the Motueka site (New Zealand), while the other was reared at the INRA of Angers (France). A total of 220 seedlings (111 from the New Zealand subsets and 109 for the French one), among those that were successfully growing, were selected for the QTL mapping studies of this project.

In the last decade, SNPs have become the markers of choice for the genetic mapping of plant species, being abundant across the genomes and enabling high-multiplexing genotyping and, consequently, the construction of high-density genetic maps. SNP markers have been chosen also in this project for the genome scanning of PEAR3 x ‘Moonglow’ population. NGS technologies from Illumina Inc. (San Diego, USA) were used for the re-sequencing of three *P. communis* accessions and SNPs discovery, and subsequently for the development of a SNP array for high-throughput genotyping. A total of 1096 pear SNPs were included in this array and combined with 7692 previously developed apple SNPs, making the apple and pear Infinium® II 9K SNP array the first cross-genera SNP chip. In order to assess the polymorphism of the apple and pear SNPs across different pear species, this array was evaluated in one European (*P. communis*) and four interspecific (*P. x bretschneideri*, *P. communis* and *P. pyrifolia*) pear populations, including PEAR3 x ‘Moonglow’.

The two subsets of the PEAR3 x ‘Moonglow’ progeny were genotyped with the apple and pear Infinium® II 9K SNP array separately, using the parents and a reference genotype as controls. The French subset was scanned at FEM (Italy), and the New Zealand one at the AgResearch Limited, Invermay (New Zealand). The French subset was also genotyped with SSR markers evenly distributed across the 17 LGs, which was sufficient for assigning the number and

orientation to the LGs of all the five populations in the study, by comparison with the consensus map of ‘Bartlett’ and ‘La France’ built by Celton et al. (2009).

The results of the pear SNP markers development and the genotyping and genetic map construction of the five segregating pear populations with the apple and pear Infinium[®] II 9K SNP array were carried out in collaboration with two other PhD students and were published on *PLOS ONE* in 2013. I also presented this work with an oral communication at the 6th International Rosaceae Genomics Conference (RGC6) which was held in Italy in 2012.

The list of SSRs tested in PEAR3 x ‘Moonglow’ population during this study is reported in the Annex 1. The genetic maps of PEAR3 and ‘Moonglow’ developed in this work are reported in Annex 2.

This article has been published as follow:

Montanari et al., 2013. PLOS ONE 8(10): e77022. doi:10.1371/journal.pone.0077022

Identification of *Pyrus* Single Nucleotide Polymorphisms (SNPs) and evaluation for genetic mapping in European pear and interspecific *Pyrus* hybrids

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Abstract

We have used new generation sequencing (NGS) technologies to identify single nucleotide polymorphism (SNP) markers from three European pear (*Pyrus communis* L.) cultivars and

subsequently developed a subset of 1096 pear SNPs into high throughput markers by combining them with the set of 7692 apple SNPs on the IRSC apple Infinium® II 8K array. We then evaluated this apple and pear Infinium® II 9K SNP array for large-scale genotyping in pear across several species, using both pear and apple SNPs. The segregating populations employed for array validation included a segregating population of European pear ('Old Home' x 'Louise Bon Jersey') and four interspecific breeding families derived from Asian (*P. pyrifolia* Nakai and *P. x bretschneideri* Rehd.) and European pear pedigrees. In total, we mapped 857 polymorphic pear markers to construct the first SNP-based genetic maps for pear, comprising 78% of the total pear SNPs included in the array. In addition, 1031 SNP markers derived from apple (13% of the total apple SNPs included in the array) were polymorphic and were mapped in one or more of the pear populations. These results are the first to demonstrate SNP transferability across the genera *Malus* and *Pyrus*. Our construction of high density SNP-based and gene-based genetic maps in pear represents an important step towards the identification of chromosomal regions associated with a range of horticultural characters, such as pest and disease resistance, orchard yield and fruit quality.

Keywords: transferability, orthologous markers, *Pyrus*, Rosaceae, SNP array

Introduction

One of the biggest challenges for plant biologists has long been to associate genetic variations with phenotypic traits. The recent technological revolution initiated by new generation sequencing (NGS) has enabled the sequencing of the entire genome of complex organisms, including the higher plants grape (Velasco et al. 2007; Jaillon et al. 2007), maize (Schnable et al. 2009), peach (Verde et al. 2013), apple (Velasco et al. 2010), potato (Xu et al. 2011), tomato (Sato et al. 2012) and most recently, Chinese pear (Wu et al. 2013). NGS also enables the inventory of entire sets of DNA variations in genomes, through the re-sequencing of multiple accessions of the same species and alignment of these sequences to the reference genome, for the purpose of *in silico* detection of DNA polymorphisms (Bentley 2006; Li et al. 2009; Hyten et al. 2010; Stothard et al. 2011; Chagné et al. 2012; Hand et al. 2012; Verde et al. 2012; Xu et al. 2012).

Single nucleotide polymorphisms (SNPs) are single base variations in DNA sequences that are abundant in plant genomes and are useful for identifying differences within individuals or populations as well as identifying genetic loci associated with phenotypic variation. Within coding regions, SNPs may be defined as non-synonymous or synonymous (resulting in an amino acid change or not) and are also found in gene-regulating regions (e.g. in promoters, untranslated mRNA regions and introns). Once polymorphisms have been detected by NGS, the next challenge is to screen large genetic populations with multiple markers simultaneously. While re-sequencing can be used for both SNP discovery and genotyping of the entire set of polymorphisms of a species (Elshire et al. 2011), high throughput SNP arrays, such as the Infinium[®] II assay (Illumina Inc.), are effective technologies for genotyping of large populations. High throughput SNP arrays have been recently developed for a range of fruit tree species. In Rosaceae, an apple SNP array was developed by the International RosBREED SNP consortium (IRSC) (www.rosbreed.org) (Chagné et al. 2012). This 8K SNP array v1 contains 7867 SNPs, of which 5554 proved to be genome-wide polymorphic SNPs in apple. The International Peach SNP Consortium (IPSC) developed a 9K SNP array for peach that includes 8144 SNPs, 84.3% of which exhibit polymorphism when screened over 709 accessions of peach (comprising peach cultivars, wild related *Prunus* species and interspecific hybrids) (Verde et al. 2012). IRSC also led the development of a 6K SNP array for cherry, with 1825 verified polymorphic SNPs in sweet cherry and 2058 in sour cherry (Peace et al. 2012). In *Citrus*, 54 accessions and 52 interspecific hybrids between pummelo and Clementine were genotyped using a 1457 GoldenGate[®] SNPs assay developed from clementine BAC-end sequencing. Out of 622 SNPs showing consistent results, 80.5% were demonstrated to be transferable to the whole *Citrus* gene pool (Ollitrault et al. 2012).

The genus *Pyrus* includes both European (*Pyrus communis*) and Asian pears (*P. pyrifolia* or Japanese pear, and *P. x bretschneideri*, commonly known as Chinese pear). To date, only a few genetic maps have been developed for *Pyrus* and none of these contains SNP markers. The first map was constructed using random amplified polymorphic DNA (RAPD) markers in a *P. pyrifolia* cross between ‘Kinchaku’ and ‘Kosui’ (Iketani et al. 2001). Yamamoto et al. Yamamoto et al. (2002b; 2004) developed the second generation of pear maps based on amplified fragment length polymorphism (AFLPs) and transferrable apple and pear simple sequence repeat (SSRs),

using an interspecific cross between ‘Bartlett’ (*P. communis*) and ‘Hosui’ (*P. pyrifolia*). As the ‘Bartlett’ x ‘Hosui’ map contained SSRs derived from both pear and apple, this study enabled the assessment of genome synteny between pear and apple and suggested that these species have co-linear genomes. Apple and pear markers had also been used earlier to generate maps for the two European pear cultivars ‘Passe Crassane’ and ‘Harrow Sweet’ (Dondini et al. 2004). SSR markers developed from both apple and pear were also used by Celton et al. (2009) to build an integrated map of the *P. communis* cultivars ‘Bartlett’ and ‘La France’, along with two apple rootstocks. Lu et al. (2010) screened the interspecific pear population ‘Mishirazi’ (*P. pyrifolia* X *P. communis*) x ‘Jinhua’ (*P. x bretschneideri*) with apple SSRs and were able to construct a genetic map. However, the number of markers used in all these studies was limited to few hundreds. Recently, NGS was used to develop a genetic map of ‘Bayuehong’ (*P. x bretschneideri* X *P. communis*) x ‘Dangshansuli’ (*P. x bretschneideri*) to anchor the Chinese pear genome; however, these SNPs were not evaluated for the screening of large segregating populations (Wu et al. 2013).

In this study, we used NGS to detect SNPs in the pear genome, to enable the design of a medium throughput SNP assay. These new pear SNPs were evaluated for genetic map construction using five segregating populations of European and Asian pear origin. Our incorporation of the new pear SNPs into the IRSC apple Infinium® II 8 K array (Chagné et al. 2012), enabled the study of SNP transferability not only within the genus *Pyrus*, but also between the genera *Malus* and *Pyrus*.

Materials and Methods

NGS Sequencing of Pear Cultivars

A SNP detection panel consisting of three European pear (*P. communis*) cultivars was chosen for low coverage whole-genome sequencing. The individuals were ‘Bartlett’ (a.k.a. ‘Williams Bon Chrétien’), ‘Old Home’ (OH) and ‘Louise Bon Jersey’ (LBJ). These accessions were chosen as ‘Bartlett’ is a founder of most breeding programs worldwide, and OH and LBJ are the parents of a segregating population developed at Plant & Food Research (PFR). Each accession was

sequenced using one lane of Illumina GA II with 75 cycles per read and small insert paired-end sequencing, as described in (Chagné et al. 2012).

Two pear unnormalized cDNA libraries were prepared by vertis Biotechnologie AG for the European pear cultivar ‘Max Red Bartlett’ following VERTIS customized protocol (<http://www.vertis-biotech.com/>). One run of 454 sequencing on a Roche/454 GS FLX Sequencer was performed.

Bioinformatics Detection and Selection of SNPs for Array

A *de novo* assembly was performed for the ‘Bartlett’ sequencing data using AbySS 1.2.1 (k=43). Contigs of 600 bp or larger were used as a reference genome set. The sequencing data from OH and LBJ were mapped to the reference genome set of ‘Bartlett’ using *Soap2.20* (-p 8 -M 4 -v 5 -c 52 -s 12 -n 5 -r 2 -m 50 -x 600). *Soap* output files were split into a single file per contig and each contig file sorted by location of the mapped reads. *SoapSNP* was used for SNP detection and filtering with the same parameters as described in (Chagné et al. 2012). The detected SNPs were then subjected to filtering, where calls were discarded when the quality score was less than 20; fewer than two reads per genotype were present; overall coverage depth was greater than the average coverage plus three standard deviations; the site was at least 25 bases away from another SNP call; and the SNPs were not located within regions associated with a set of candidate genes. The candidate gene set used for filtering consisted of 2559 transcription factor sequences from *Malus x domestica* (Velasco et al. 2010). Locations within pear were defined by mapping these sequences to the reference genome set of ‘Bartlett’ using *gmap* with command line options -K 3000–L 50000.

454 cDNA reads were assembled using CAP3 (Huang and Madan 1999). Contigs were aligned to the reference *M. x domestica* genome and only unique alignments were considered to avoid paralogy issues. SNPs were predicted using a customized bioinformatics pipeline and selected to be well spread over the 17 apple chromosomes.

The Illumina Infinium[®] assay design tool (ADT) was used on the detected SNPs with a threshold of 0.7. These pear SNPs were synthesized as probes and located on the same array as the IRSC apple Infinium[®] II 8 K array (Chagné et al. 2012).

Plant Material for SNP Array Evaluation

Five pear segregating populations were screened using the apple and pear Infinium® II 9K SNP array. No permission was required to collect plant material and pear is not an endangered or protected species. These were one *P. communis* intraspecific family and four interspecific (*P. x bretschneideri*, *P. communis* and *P. pyrifolia*) pear populations: OH x LBJ, of 297 F1 individuals and both parents; P128R068T003 x ‘Moonglow’ (T003 x M), of 220 F1 individuals and both parents; P019R045T042 x P037R048T081 (T042 x T081), of 142 F1 individuals and both parents; P202R137T052 x P128R068T003 (T052 x T003), of 91 F1 individuals and T003 parent only; and P202R137T052 x P266R225T064 (T052 x T065), of 123 F1 individuals and T064 parent only, since parent T052 has been lost. Figure 2.1 shows the relationships among the interspecific populations. The interspecific hybrid populations were developed as part of the PFR pear breeding program (Brewer et al. 2005). Half the P128R068T003 x ‘Moonglow’ population was grown at INRA, Angers (France) and genotyped at the Fondazione Edmund Mach (FEM, Italy), and the other half was grown at PFR, Motueka and genotyped at AgResearch Limited, Invermay in New Zealand, together with the other four populations. DNA extraction of OH x LBJ, T042 x T081 and T052 x T003 populations was performed using a CTAB extraction method (Doyle and Doyle 1987), followed by purification with NucleoSpin® columns (Macherey-Nagel GmbH & Co. KG). DNA from the T003 x M and T052 x T064 populations was extracted using the QIAGEN DNeasy Plant Kit (QIAGEN GmbH, Hilden, Germany). DNA quantifications were carried out using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc.).

SNP Genotyping and Data Analysis

Genomic DNA was amplified and hybridized to the apple and pear Infinium® II 9K SNP array following the Infinium® HD Assay Ultra protocol (Illumina Inc., San Diego, USA) and scanned with the Illumina HiScan. Data were analyzed using Illumina’s GenomeStudio v 1.0 software Genotyping Module, setting a *GenCall* Threshold of 0.15. The software automatically determines the cluster positions of the AA/AB/BB genotypes for each SNP and displays them in normalized graphs (Figure 2.2). A systematic method was used to evaluate the SNP array data employing

quality metrics from GenomeStudio (Illumina): *GenTrain* score ≥ 0.50 , minor allelic frequency (MAF) ≥ 0.15 and call rate $> 80\%$. A Chi-square test at a significance of 0.01 was performed to determine distortion of markers from the expected segregation. SNPs that were highly distorted or which had the genotype of one or both parents missing were manually edited in GenomeStudio. The SNPs for which 25% or 50% of the individuals were not called in clusters were manually edited, since this kind of segregation may have been due to SNPs with null alleles.

Simple Sequence Repeat Genotyping

The T003 x M population was genotyped with apple and pear microsatellite markers as well as SNPs. Fifty-four SSRs were selected based on the ‘Bartlett’ consensus map developed by Celton et al. (2009) and one SSR, Md-Exp 7, from the work of Costa et al. (2008). They were first screened for polymorphism over DNA extracted from both parents and five individuals of the progeny, and then screened over the subset of the T003 x M population raised at INRA (Annex 1). PCR amplifications were performed in a final volume of 12.5 uL containing 10 ng of genomic DNA, 1x buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.4 uM of each forward and reverse primer and 0.75 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems® by Life Technologies™). All SSR amplifications were performed in a Biometra T gradient Thermocycler (Biometra GmbH, Göttingen, Germany) or in a Bio-Rad C-1000 thermocycler (Bio-Rad Laboratories, Hercules, CA) at FEM (Italy) and INRA, Angers (France) under the following conditions: an initial denaturation at 95°C for 5 min, followed by 36 cycles of 95°C for 30 sec, TA (an optimal annealing temperature for each primer was used) for 30 sec, 72°C for 1 min, finishing with a final extension at 72°C for 7 min. Fragment analysis was performed with an ABI PRISM_3730 capillary sequencer (Applied Biosystems® by Life Technologies™) in a final mix of 0.5 uL of PCR product, 9.97 uL formamide and 0.03 uL of 500-LIZ dye, denaturated for 3 min at 95°C. Fragment sizing was performed with GeneMapper software v. 4.0 (Applied Biosystems® by Life Technologies™).

Linkage Mapping Analysis

The genetic maps of both parents of all five populations were constructed using JoinMap v3.0 and v4.0 software (Van Ooijen 2006), based on the SNP data for each individual population, except for the T003 x M population, where both the SNP and SSR data were used. Linkage groups were determined with a LOD score of 5 and higher for grouping and the Kosambi function was used for map calculation. The maps were drawn and aligned using MapChart v2.2 (Voorrips 2002).

Pear SNP Alignment to the Apple Genome Sequence

The pear SNPs included in the array were aligned to the apple genome assembly (Velasco et al. 2010) using BLASTN analysis of the SNP flanking sequence against the ‘Golden Delicious’ (GD) genome assembly. A BLASTN cutoff of an alignment length >100 nucleotides and an e-value < e-30 were used.

Results

SNP Detection and Selection for 1 K Pear Array

In total, 34,082,435, 35,687,533 and 25,167,853 paired-end reads were generated for ‘Bartlett’, OH and LBJ, respectively. The *de novo* assembly genome set of ‘Bartlett’ consisted of 78,748 contigs of 600 bp or greater in length containing a total of 79,067,993 bases, with a maximum contig length of 15,094 bases, N50 of 1004 bases, N90 of 658 bases, and an average contig length of 1004 bases.

A total of 73,214 SNPs were predicted by *SoapSNP* when reads of OH and LBJ were aligned to the genome of ‘Bartlett’ using the *Soap* aligner, corresponding to one SNP per 1079 bases. In total, 1456 SNPs passed the filtering criteria and were then subjected to the Illumina ADT. This yielded 1107 SNPs, of which 1064 were included in the final SNP array. A total of 144,816 high quality 454 sequence reads were generated. Total sequence output was 32,418,987 bases, with an average read length of 224 bases. Quality filtered sequences were *de novo* assembled using

CAP3. The average depth of assembly for all samples was ~2.5. A total of 1751 cDNA SNPs were predicted using a customized bioinformatics pipeline and 69 experimentally validated by M. Troggio (unpublished data) that passed the Illumina ADT design, were selected for inclusion in the SNP array.

In total, 1133 pear SNPs were incorporated in the final array, making a grand total of 9000 attempted apple and pear SNPs.

SNP Chip Evaluation

Of the 1133 attempted pear SNPs, 1096 (96.7%) were successful bead types on the IRSC Infinium[®] II (Illumina Inc.) array. When the 1096 pear and 7692 apple bead types were evaluated using five segregating populations, twelve and three individuals from the T003 x M and T052 x T003 populations, respectively, did not hybridize well to the BeadChip and were excluded from the clustering, which resulted in 873 F1 individuals that were used for evaluating the SNP array. All the 1096 pear SNPs hybridized well, resulting to be either polymorphic or monomorphic in at least one population. Of the apple SNPs, 7562 out of the total 7692 bead typed (98.3%) were either polymorphic or monomorphic in at least one population, while only 130 showed low quality hybridization. All 1096 pear SNPs hybridized pear DNA and were either monomorphic or polymorphic.

In total, 1528 unique pear and apple-derived SNPs (872 pear SNPs and 656 apple SNPs) were polymorphic in at least one segregating population, with 713, 508, 437, 442 and 711 polymorphic SNPs for the OH x LBJ, T003 x M, T042 x T081, T052 x T003 and T052 x T064 populations, respectively (Table 2.1). For the newly developed pear SNPs, the polymorphism rate was variable and depended on the informative parent. *P. communis* parents had higher polymorphism rate (from 25.9% to 35.1%, for ‘Moonglow’, OH and LBJ) than Asian x European hybrid parents (from 2.9% to 21.4%, for T003 and T064, respectively). The number of polymorphic apple SNPs per pear population ranged from 115 to 381 out of 7692 bead types (1.5 to 5.0% polymorphic SNPs per population). When the transfer rate of the new pear SNPs was evaluated in the apple ‘Royal Gala’ x ‘Granny Smith’ segregating population, it was similar to the apple SNP to pear transfer rate, with 13 (1.2%) polymorphic pear SNPs.

Identification and Genotyping of SNPs with Null Alleles

The analysis of SNP polymorphism in segregating populations highlighted the presence of SNP markers with potential null alleles. By default, the standard SNP calling algorithms of GenomeStudio clustered heterozygous A0 and B0 genotypes together with homozygous AA and BB genotypes, and called homozygous null genotypes (00) as missing genotypic calls. However, some SNPs containing null alleles do not follow the expected Mendelian segregation based on the parental genotypes. Therefore, manual editing of clusters for all the SNPs with strong deviation from Mendelian ratio or around 25% or 50% of no calls was performed and the SNPs which displayed a clear clustering and for which genotypes could be unequivocally determined as containing potential null alleles, were selected for further linkage analysis (Figure 2.3). The following null allele segregation types were observed in the segregating populations: 00xA0, A0xAA, A0xA0, A0xB0, ABxA0, A0xBB and ABx00. The number of polymorphic null allele SNPs varied throughout the five populations: 115 in OH x LBJ, 108 in T003 x M, 112 in T042 x T081, 702 in T052 x T003, and 436 in T052 x T064 (Table 2.2). The percentage of polymorphic null allele markers from attempted bead types seemed to be similar for pear and apple SNPs: 2% and 1.2% in OH x LBJ, 2.9% and 1% in T003 x M, 2.4% and 1.1% in T042 x T081, 9.9% and 8.1% in T052 x T003, and 4.9% and 5% in T052 x T064. Of the total of 1132 unique pear and apple SNPs exhibiting null alleles, 255 were polymorphic markers without a null allele in at least one other segregating population. When the polymorphic null allele markers were mapped, the null allele markers were used to increase the density of the maps for the interspecific crosses, but were not required for the already dense OH x LBJ map (Table 2.3).

The total number of unique polymorphic markers, including both apple and pear-derived SNPs and SNPs with null alleles, was 2400 for all five populations. For the pear SNPs, 918 (83.8%) were polymorphic in at least one segregating population, and 623 (56.8%) were polymorphic in OH x LBJ, 384 (35%) in T052 x T064, 337 (30.7%) in T042 x T081, 337 (30.7%) in T003 x M, and 295 (26.9%) in T052 x T003.

Genetic Map Construction

Parental genetic maps were constructed for five segregating populations using the 2400 unique polymorphic SNPs. All maps contained 17 linkage groups except T003, T042 and T081. For the OH x LBJ population, the parental maps spanned 825 and 974 cM and consisted of 356 and 393 SNP markers for OH and LBJ, respectively. For the T003 x M population, the parental maps spanned 980 and 1016 cM and consisted of 182 and 434 SNP markers for T003 and M, respectively. For the T042 x T081 population, the parental maps spanned 923 and 1133 cM and consisted of 250 and 312 SNP markers for T042 and T081, respectively. For the T052 x T003 population, the parental maps spanned 1018 and 1101 cM and consisted of 370 and 255 SNP markers for T052 and T003, respectively. For T052 x T064 the parental maps spanned 1485 and 1580 cM and consisted of 628 and 682 SNP markers for T052 and T064, respectively. In total, 1888 unique SNPs were mapped, including null allele markers.

The markers in common among the five segregating populations enabled the alignment of parental genetic maps as shown in Figure 2.4 for four maps of LG9. However, the bridges among the 10 parental maps were insufficient for the construction of a unique integrated map. The common polymorphic markers (with and without null alleles) between pairs of parents of the segregating populations are shown in Table 2.3. For example, there are 105 common polymorphic markers (without null alleles) between the European pears ‘Moonglow’ and ‘Old Home’. In comparison, only 52 markers (without null alleles) are in common between ‘Moonglow’ and the interspecific parent T081. The parent T003 from the T003 x M cross has 20 null allele markers in common with the same parent from the T052 x T003 cross and only 5 with T081.

SSR Mapping

Of the 54 SSR markers derived from the published ‘Bartlett’ consensus map (Celton et al. 2009a) that were screened over the T003 x M population, 38 were mapped, 25 loci to T003 and 30 to ‘Moonglow’ (Annex 1). This information on linkage group assignment, taken together with data on SNP markers in common, was sufficient to enable the application of the ‘Bartlett’ LG nomenclature across all the pear genetic maps in this study.

Pear SNP Alignment to the Apple Genome Sequence

A total of 1009 pear SNPs (92%) were successfully anchored to the GD genome using bioinformatics analysis. Using the OH x LBJ consensus map as an example, 433 (42.9%) of the pear SNPs were anchored to apple and enabled the comparison of this genetic map with the GD genome assembly. On average, 20 markers per LG were in common between the OH x LBJ map and the GD genome (Figure 2.5), with LG2 having the most markers in common (32 markers) and LG17 the least (9 markers).

Discussion

SNPs are considered to be the most efficient tools for comprehensive genetic studies (Yamamoto and Chevreau 2009). In *Pyrus*, the number of available SNPs was marginal. We developed more than 1000 SNPs from the re-sequencing of *P. communis* cultivars and for the first time we included them in an array, making them easily available for further studies. These SNPs were selected based on their location within candidate genes, to ensure their usefulness for marker-trait association and for future breeding programs.

We used the apple and pear Infinium® II 9K SNP array for the genotyping of five segregating pear populations, for a grand total of 873 individuals. The clustering of the SNPs using the GenomeStudio software depends on the minor allele frequency of the SNPs: the lower the minor allele frequency, the more samples are required to achieve accurate representation of all clusters. Illumina recommends a population of 100 or more. In our case, all the populations had largely more than 100 individuals (except for T052 x T003, with 91 progenies), and this large dataset of 873 individuals ensured an accurate clustering of array SNPs. Moreover, the threshold of 15% for the MAF is relatively high, in comparison with other studies using the same technique (Antanaviciute et al. 2012).

High Polymorphism Rate for the Newly Developed Pear SNPs

A large proportion (83.8%) of the 1096 pear SNPs used to construct the first pear genotyping array were polymorphic in at least one segregating population, and 857 of these unique

polymorphic pear markers (93.4%) were demonstrated to be useful for construction of genetic maps, using five populations of a range of genetic backgrounds across *P. communis*, *P. pyrifolia* and *P. x bretschneideri*. These maps are the first dense SNP-based genetic maps for pear of any species. The previously developed maps in *Pyrus*, including those of Yamamoto et al. (2002b; 2004) and Celton et al. (2009), as well as an earlier map using pear SNPs constructed in ‘Bartlett’ and ‘Hosui’ (Terakami et al. 2013), are not sufficiently dense to be useful for QTL analysis. Although Wu et al. (2013) reported the development of 2005 SNPs in the course of anchoring the *P. x bretschneideri* genome sequence, these SNPs are not available as a genotyping array, as they were obtained using genotyping by sequencing. In addition to the new *P. communis* pear SNPs developed in this study, we found that 1482 SNP markers derived from apple (19.3% of the total apple SNPs on the IRSC array) were polymorphic in pear, and 1031 of them were positioned on the pear genetic maps. The apple SNPs considerably improved the density of all maps, in some cases, e.g. T052 x T003 and T052 x T064, even doubling the number of mapped markers. In fact, because of the lower polymorphism of pear SNPs in the interspecific hybrid parents compared with the *P. communis* parents, the apple SNPs were necessary to saturate these maps.

The higher number of polymorphic pear markers identified in the European pear cross OH x LBJ compared with the four populations with an Asian pear background is because sequence data from OH and LBJ were used to design the pear SNPs, which also validates the bioinformatic SNP detection method used. In the T003 x M population, the number of polymorphic pear SNPs in the European parent (‘Moonglow’) was significantly higher than in the hybrid (T003), again because the SNPs were derived from sequencing of *P. communis* accessions. However, the number of pear SNPs that were polymorphic in the interspecific parents was more variable, and reflects both the number of SNPs that are conserved between European and Asian pear and those that were introgressed from the European parent into the interspecific hybrid parents. The transferability of SNPs between species of the same genus has been reported previously in a few studies. These include the plant genera *Vitis* (Vezzulli et al. 2008), *Citrus* (Ollitrault et al. 2012) and *Eucalyptus* (Grattapaglia et al. 2011), as well as the mammalian genus *Bubalus* (Matukumalli et al. 2009). It is noteworthy that the transferability of SNPs between species was as high in these studies as observed in this study in *Pyrus*.

*SNP Transferability between Genera *Pyrus* and *Malus**

The distinguishing feature of the apple and pear Infinium® II 9K SNP array is its combination of SNPs from both *Malus* and *Pyrus*, making it the first cross-genera SNP array created. It therefore enables, for one of the first time, the assessment of SNP marker transferability between genera. Most of the numerous studies on genetic marker transferability in recent years have focused on SSR markers, including those concerning apple and pear (Yamamoto et al. 2004; Pierantoni et al. 2004; Yamamoto et al. 2007; Lu et al. 2010). Previous attempts to transfer SNPs between genera involved a few accessions only of the non-targeted species, including the study of Micheletti et al. (2011), who estimated the rate of transferability of the heterozygous state from *M. x domestica* to *P. communis* and *P. pyrifolia* using 237 apple SNPs. In the present study, we observed that 7562 apple SNPs (98.3%) were either monomorphic or polymorphic in at least one pear population, while only 130 did not hybridize well in all of them. The high percentage of hybridization of pear genomic DNA to apple SNPs and vice versa obtained in the present study are not surprising, given that *Malus* and *Pyrus* are closely related genera and might be expected to share high sequence similarity. Furthermore, both the pear and apple SNPs included in the array were selected to be located in coding genes, with the consequence that the flanking sequences are more likely to be conserved between species. Although many of the apple SNPs were monomorphic (but still hybridized to pear DNA) and were not useful for genetic mapping in the five pear populations, we were able to map 99 apple markers in the OH x LBJ population, 255 in T003 x M, 199 in T042 x T081, 365 in T052 x T003, and 631 in T052 x T064.

SNPs with Null Alleles

The existence of null or unexpected alleles has been already demonstrated in several other SNP genotyping studies. Such alleles can be explained as deletions spanning a polymorphic site, secondary polymorphisms, or tri-allelic sites at the primary polymorphism (Carlson et al. 2006; Ollitrault et al. 2012). Since the SNP genotyping technology we used was the Infinium® II from Illumina, any putative third allele of polymorphic SNPs was not detectable and, therefore, in our study the SNPs with null alleles can fall only into the first two categories. Null alleles are an important source of polymorphisms; however, they are challenging to detect and analyze using

SNP array software. In the present study, a higher number of SNPs with null alleles was detected in the interspecific populations than in the *P. communis* population. This was expected, as the frequency of null alleles increases with genetic distance between the samples genotyped and the discovery panel (Ollitrault et al. 2012), because additional SNPs in the flanking sequence used for the Infinium[®] array design are more likely to occur between different species (Asian versus European pear) or genus (*Malus* versus *Pyrus*). We found that the within-species frequency of null alleles was similar in apple and pear SNPs. As heterozygous null alleles are useful for genetic mapping, we used them to increase map density in interspecific populations. It must be noted, however, that null alleles are a potential source of increased false positives in marker-trait association studies (Rice and Holmans 2003; Sawcer et al. 2004).

Pear and Apple Genome Synteny

In total, 92% of the pear SNPs included in the Infinium[®] II array were successfully anchored to the ‘Golden Delicious’ genome (Velasco et al. 2010), and the alignment of the physical map with the OH x LBJ genetic map resulted in an average of 20 orthologous markers per LG. Nevertheless, the apple SNPs were not always located at the same position on the pear genetic map as in the apple genome, which, however, can also be explained by the finding that approximately 15% of the SNPs included in the 9 K array have been assigned erroneous positions on the ‘Golden Delicious’ reference sequence (Antanaviciute et al. 2012). However, the number of orthologous markers between apple and pear identified in the present work (433 pear SNPs and 99 apple SNPs for OH x LBJ) is almost double the total found in previous studies (227). These studies included those by Pierantoni et al. (2004), who demonstrated good genome colinearity between one apple and two pear genetic maps, using 41 and 31 mapped apple SSRs, respectively; Yamamoto et al. (2007), who mapped apple and pear markers in European pear cultivars, and found that the position of 66 apple SSRs showed colinearity with the apple reference map; and (Celton et al. 2009a), who aligned the genetic maps of two apple and pear cultivars constructed using apple and pear SSRs, and identified 90 colinear markers (53 pear and 37 apple SSRs) in common between the apple and pear genomes.

Conclusions

We have thoroughly validated the apple and pear Infinium® II 9K SNP array, and demonstrated its usefulness for high throughput genotyping in breeding populations of *P. communis*, as well as those of a mixed genetic background that includes *P. communis*, *P. pyrifolia* and *P. x bretschneideri*. Furthermore, we attested that the arrayed SNPs are transferable not only across these species, but also between the two closely related genera *Malus* and *Pyrus*.

The construction of high density gene-based genetic maps using our SNP array represents an important step for the discovery of chromosomal regions associated with commercially important horticultural traits, such as pest and disease resistance, orchard productivity and fruit quality (Yamamoto and Chevreau 2009) in pears derived from *P. communis*, *P. pyrifolia* and *P. x bretschneideri*. The OH x LBJ population was a repeat of a cross (Jacob 1998) used to develop an understanding of genetic determinants of vigor control and precocity in pear rootstocks. The 400 seedlings planted in Motueka (New Zealand) are grafted with ‘Doyenne du Comice’ (*P. communis*) scions for the purpose of a QTL analysis of rootstock induced dwarfing in pear. The T003 x M population was developed to study the genetic basis of resistance to pear scab (*Venturia pirina*), fire blight (*Erwinia amylovora*), pear psylla (*Cacopsylla pyri*) and pear sawfly (*Caliroa cerasi*). T003 (as most Asian pears in general) is not host to *V. pirina* (Brewer and Alspach 2009; Bus et al. 2013) and a good source of resistance to *C. pyri* and *C. cerasi* (Brewer et al. 2002), while ‘Moonglow’ derives from fire blight-resistant cultivars ‘Roi Charles de Wurtemberg’ and ‘Seckel’. The T042 x T081 population was created to develop an understanding of the genetic control of scab resistance in pear. We are using the T052 x T003 and T052 x T064 populations to investigate the genetic basis of a storage-related disorder “friction discoloration”, using genetic mapping in combination with metabolomics phenotyping to identify QTLs controlling the disorder. Such examples of applications of the apple and pear Infinium® II 9K SNP array demonstrate that it will produce a range of outcomes that can be applied to pear breeding programs worldwide.

Genomic Resources

The pear SNPs detected by sequencing, the pear SNPs chosen for the apple and pear Infinium® II 9K SNP array, and the GenomeStudio cluster file developed are deposited in the Genome Database for Rosaceae (www.rosaceae.org). SNPs are available in dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) under accessions ss527787751 to ss527789916.

Acknowledgments

We thank Dianne Hyndman and Rosemary Rickman (AgResearch Invermay, New Zealand) and Elisa Banchi (IASMA, Italy) for providing the Illumina genotyping service. DC thanks the Rosaceae genomics community for kindly enabling the inclusion of pear SNPs in the apple and pear Infinium® II 9K SNP array. We also thank the INRA Experimental Unit (UE Horti, Angers, France) for care of the T003 x M population and the INRA GENTYANE platform (UMR1095, Clermont-Ferrand, France) for SSR genotyping of this progeny.

Author Contributions

Conceived and designed the experiments: DC. Performed the experiments: SM MS MK YKK. Analyzed the data: SM MS MK YKK MT PF RS RNC. Contributed reagents/materials/analysis tools: MM RV KHW CED LP RS CW VB LB SEG DC. Wrote the paper: SM MS MK DC.

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Figures

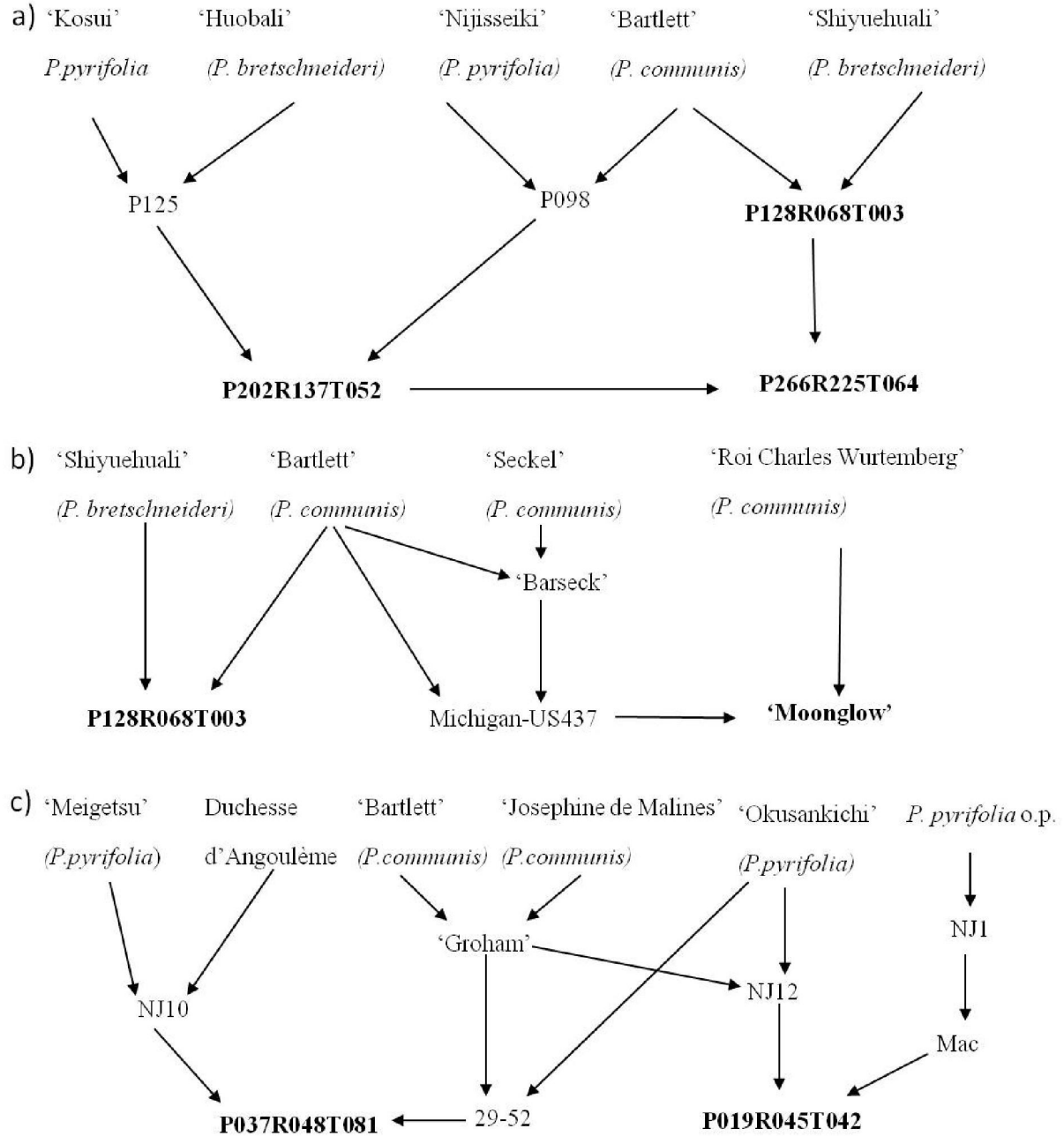


Figure 2.1: Pedigree diagrams for segregating populations used for SNP evaluation

A) P128R068T003 x 'Moonglow'; B) P037R048T081 x P019R045T042, and C) P202R137T052 x P128R068T003 and P202R137T052 x P266R225T064

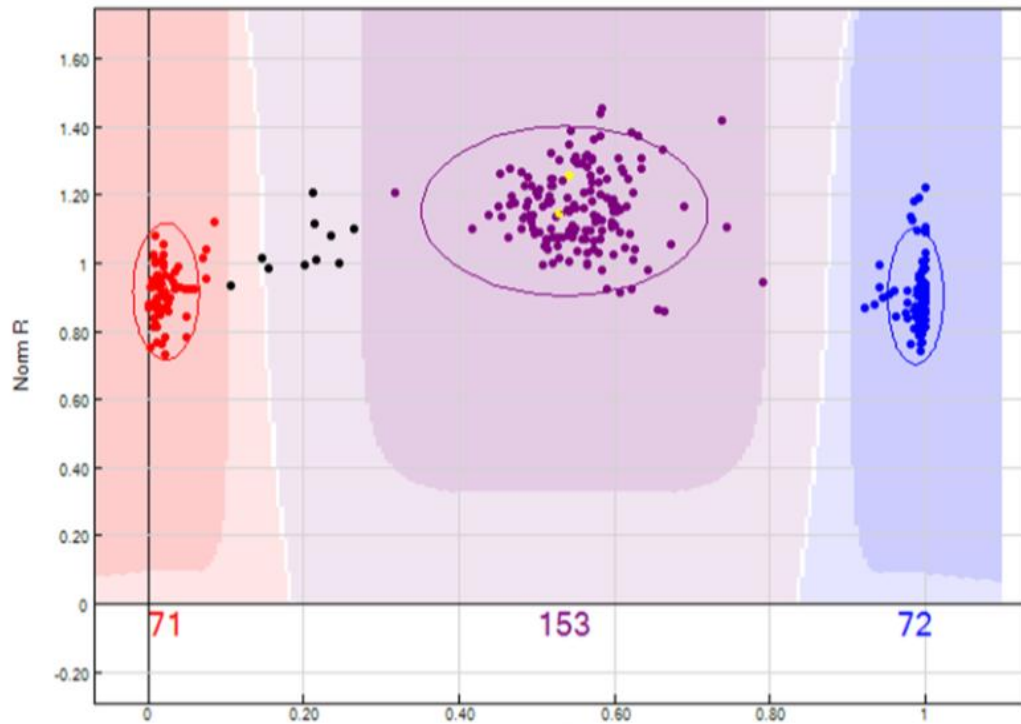


Figure 2.2: A typical example of an ABxAB SNP (ss527787957), as represented in GenomeStudio

Parents 'Old Home' and 'Louise Bon Jersey' are indicated in yellow; the red cluster is identified as AA, the blue as BB and the purple as AB genotype. The total number of the individuals analyzed here is 297 and the segregation ratio is 1:2:1

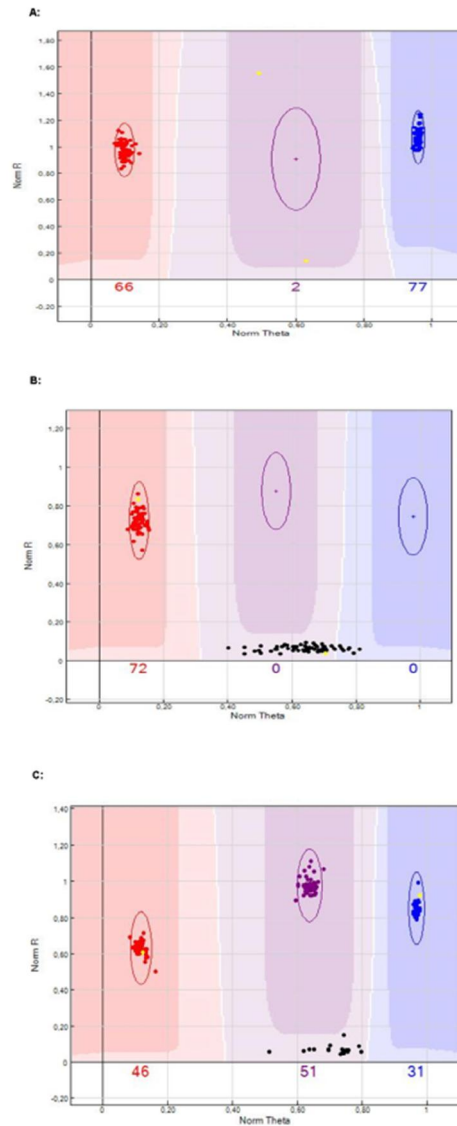


Figure 2.3: Typical examples of SNPs with null allele as represented in GenomeStudio

A) A 00xAB SNP (ss527789894), as represented in GenomeStudio. Parents P128R068T003 and 'Moonglow' are indicated in yellow; the red and blue clusters are identified as A0 and B0 genotypes, respectively. The total number of the individuals analyzed is 143 and the segregation ratio is 1:1. B) A 00xA0 SNP (ss475879014), as represented in GenomeStudio. Parents P128R068T003 and 'Moonglow' are indicated in yellow; the red cluster is identified as heterozygous genotypes (A0), while genotypes with missing call (in black) are identified as homozygous for the null allele (00). The total number of the individuals analyzed is 143 and the segregation ratio is 1:1. C) A A0xB0 SNP (ss475882353), as represented in GenomeStudio. Parents P128R068T003 and 'Moonglow' are indicated in yellow; the red, blue and purple clusters are identified as A0, B0 and AB genotypes, respectively, while genotypes with missing call (in black) are identified as homozygous for the null allele (00). The total number of the individuals analyzed is 143 and the segregation ratio is 1:1:1

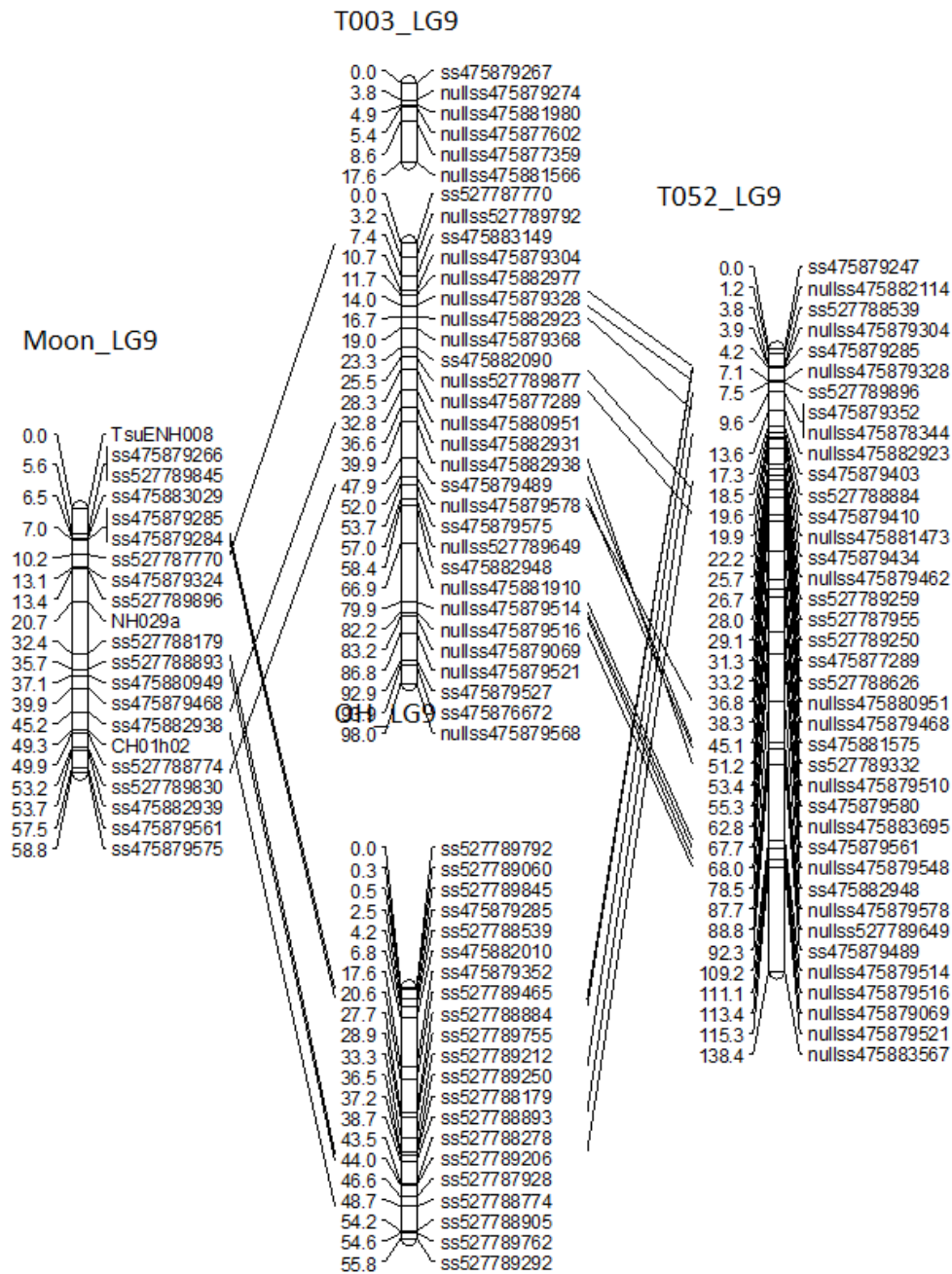


Figure 2.4: Alignment of LG9 from four parental maps P128R068T003, ‘Moonglow’, P202R137T052 and ‘Old Home’

The lines between the maps each show markers in common with two other parents

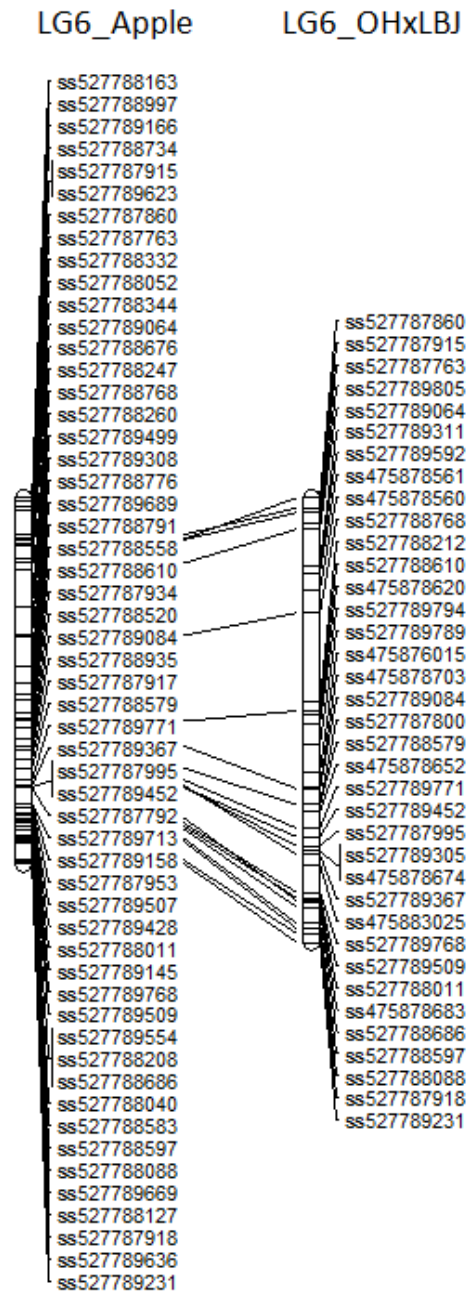


Figure 2.5: Alignment of OH x LBJ LG6 with chromosome 6 of the 'Golden Delicious' genome

Lines show the markers in common

Tables

Table 2.1: Number of polymorphic and mapped apple and pear markers for each segregating population.

			Polymorphic markers			Mapped markers		
	Population	Marker segregation	Pear SNPs	Apple SNPs	Total	Pear SNPs	Apple SNPs	Total
<i>Pyrus</i>	OHxLBJ (n=297)	ABxAA/BB	213	50	263	194	41	235
		ABxAB	128	9	137	123	9	132
		BB/AAxAB	257	56	313	229	49	278
		total	598	115	713	546	99	645
	T003xM (n=220)	ABxAA/BB	21	113	134	16	105	121
		ABxAB	11	4	15	11	3	14
		BB/AAxAB	273	86	359	271	77	348
		total	305	203	508	298	185	483
	T042xT081	ABxAA/BB	146	47	193	140	42	182
		ABxAB	23	3	26	23	3	26
		BB/AAxAB	142	76	218	139	75	214
		total	311	126	437	302	120	422
	T052xT003 (n=91)	ABxAA/BB	179	83	262	131	66	197
		ABxAB	28	67	95	15	43	58
		BB/AAxAB	12	73	85	11	52	63
		total	219	223	442	157	161	318
	T052xT064	ABxAA/BB	96	113	209	82	89	171
		ABxAB	137	130	267	132	111	243
		BB/AAxAB	97	138	235	89	121	210
		total	330	381	711	303	321	624
	Unique		872	656	1528	829	569	1398
<i>Malus</i>	RGxGS (n=186)	ABxAA/BB	3	1020	1023			
		ABxAB	3	587	590			
		BB/AAxAB	7	1203	1210			
		total	13	2810	2823			

Table 2.2: Number of polymorphic and mapped null allele markers for each segregating population.

			Null-allele markers			Null-allele markers		
	Population	Marker segregation	Pear SNPs	Apple SNPs	Total	Pear SNPs	Apple SNPs	Total
<i>Pyrus</i>	OHxLBJ*	00xA0/00xB0/BBxB0	1	45	47	1	39	40
		A0xA0/B0xB0	17	46	63	9	28	37
		ABx00	0	0	0	0	0	0
		A0x B0	4	0	7	3	0	3
		A0xAB/ABxB0/ABxA0	0	2	2	0	1	1
		total	22	93	115	13	68	81
	T003xM (n=220)	00xA0/00xB0/BBxB0	3	57	60	3	51	54
		A0xA0/B0xB0	0	6	6	0	6	6
		ABx00	11	5	16	11	5	16
		A0xB0	0	2	2	0	2	2
		A0xAB/ABxB0/ABxA0	9	2	11	9	2	11
		A0xBB/B0xAA	9	4	13	9	4	13
		total	32	76	108	32	70	102
	T042xT081	00xA0/00xB0/BBxB0	3	63	66	3	57	60
		A0xA0/B0xB0	9	20	29	9	20	29
		ABx00	1	0	1	0	0	0
		A0xAB/ABxB0/ABxA0	2	1	3	1	1	2
		A0xBB/BBxA0	11	2	13	10	1	11
		total	26	86	112	23	79	102
	T052xT003	00xA0/00xB0/BBxB0	30	193	223	24	123	147
		A0xA0/B0xB0	40	421	461	10	76	86
		A0x B0	5	7	12	3	2	5
		A0x	1	5	6	2	3	5
		total	76	626	702	39	204	243
	T052xT064	00xA0/00xB0/BBxB0	32	213	245	18	134	152
		A0xA0/B0xB0	12	156	168	13	169	182
		A0xAB	4	1	5	2	1	3
		A0xB0	6	12	18	3	6	9
		total	54	382	436	36	310	346
	Unique		163	969	1132	117	557	674

*null allele not used for mapping

Table 2.3: Common mapped polymorphic SNP markers in each parent of the different segregating populations.

Diagonal in bold, total number of mapped markers in a specified parent (including null alleles); above the diagonal, null alleles; below the diagonal, polymorphic markers without null alleles.

		OHxLBJ		T003xM		T042xT081		T052xT003		T052xT064	
		OH	LBJ	T003	M	T042	T081	T052	T003	T052	T064
OHxLBJ	OH	356*	NA	NA	NA	NA	NA	NA	NA	NA	NA
	LBJ	104	393*	NA	NA	NA	NA	NA	NA	NA	NA
T003xM	T003	8	11	182	18	6	20	4	84	17	25
	M	105	130	13	434	76	52	52	12	51	48
T042xT081	T042	56	80	2	6	250	19	34	4	29	27
	T081	63	70	5	6	19	312	34	18	44	35
T052xT003	T052	32	50	8	10	4	2	370	58	40	50
	T003	10	12	20	14	6	3	6	255	27	43
T052xT064	T052	31	48	6	6	4	6	164	27	628	125
	T064	37	52	11	14	7	7	90	52	215	682

* no null alleles mapped

CHAPTER 3. QTL Mapping for Pear Psylla Resistance

Pear psylla is a highly damaging pest of pear in Europe, North America and the Middle East. Pear psyllids are still not present in New Zealand. Consequently, only the French subset of the PEAR3 x ‘Moonglow’ progeny could be assessed for the resistance to this insect. *C. pyri* was chosen for the phenotyping of this population, being the most common species of psylla in Europe. Since in a mono-varietal pear orchard the insect cannot choose the genotype to attack (i.e. it is in a no-choice situation), the object of this study was the evaluation of pear antibiosis resistance. Before the assessment of the entire French subset of the population, a phenotyping protocol well suited for the collection of quantitative data on the antibiosis resistance had to be developed. Working with a team of plant geneticists and entomologists at the INRA of Angers, I set up the novel protocol reported in this study, which was then applied to PEAR3 x ‘Moonglow’ population for two consecutive years, enabling the identification of a stable QTL involved in the antibiosis resistance.

This work was reported in a paper recently accepted with minor revisions in the peer-reviewed journal *Tree Genetics and Genomes*. Moreover, I presented the results of the first year of QTL mapping with a poster (reported in the Annex 3) at the 57th Italian Society of Agricultural Genetics (SIGA) Annual Congress, which was held in Italy in 2013, and the final results with an oral communication at the 7th International Rosaceae Genomics Conference (RGC7), held in the USA in 2014, for which I got the award for best PhD student oral presentation.

The LOD score curves for all the QTLs detected in this study are reported in the Annex 4.

This article has been recently accepted with minor revisions for publication in *Tree Genetics and Genomes*.

Genetic mapping of *Cacopsylla pyri* resistance in an interspecific pear (*Pyrus* spp.) population

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Abstract

Cacopsylla pyri (pear psylla) is one of the most serious pests of pear (*Pyrus* spp.) in Europe. It can cause high yield losses, and its control has become difficult since it has developed resistance to a wide range of pesticides. Pear breeders are developing new cultivars resistant to pear psyllids, and Asian species, such as *Pyrus ussuriensis* and *P. x bretschneideri*, are good sources of resistance. Antixenosis and antibiosis resistance to psylla were both identified in pear; they may differ in the biological mechanism and probably have different genetic backgrounds. We crossed interspecific *P. x bretschneideri* x *P. communis* hybrid PEAR3, resistant to pear psylla, with the susceptible European pear cultivar 'Moonglow' to obtain an F1 population for the genetic mapping of the resistance. Quantitative trait locus (QTL) analysis was carried out for antibiosis by measuring the number of surviving nymphs and the nymphal development, using a novel phenotyping protocol and a saturated genetic map made

of single nucleotide polymorphism (SNP) and microsatellite (SSR) markers. A stable QTL was detected on linkage group (LG) 8 of PEAR3 ($R^2 = 17.2 - 39.1\%$). In addition, QTLs were detected on LG5 ($R^2 = 10.8\%$) of PEAR3 and on LG15 of 'Moonglow' ($R^2 = 13.7\%$).

Keywords: *Pyrus x bretschneideri*; *Pyrus communis*; psylla; antixenosis; antibiosis; QTL mapping

Introduction

Pear psyllids (Hemiptera, Psyllidae) are one of the most serious pests of pear (*Pyrus* spp.). The most damaging psylla species identified are *Cacopsylla pyri* (Linnaeus), endemic to Europe, *C. pyricola* (Förster) to Europe and North America, and *C. bidens* (Šulc) to Europe and the Middle East (Shaltiel-Harpaz et al. 2014). The pear psylla life cycle begins with the eggs, laid singly or in clusters on the host plant, which hatch into nymphs that go through five instars (Figure 3.1a). First and second instar nymphs (L1 and L2) are 0.4 and 0.5 mm long, respectively, with small antennae and no wings. The wing pads become visible in L3 and develop gradually during L4 and L5, while the antennae elongate. L3 nymphs are about 0.8 mm long, L4 1.3 mm and L5 1.8 mm. After the last molt, nymphs develop into male or female adults (Figure 3.1b), which are able to reproduce sexually within a few days (Hodkinson 2009). The development rate of all immature psylla stages is highly affected by temperature: the egg stage duration ranges between 6 and 28 days, with a direct linear correlation with temperature, while the young nymphs (L1 to L3) and old nymphs (L4-L5) stages last 10-19 days and 12-18 days, respectively, with an asymptotic relationship with temperature (Kapatos and Stratopoulou 1999). Both young and adult psylla feed on the plant by inserting their stylets into the phloem. However, the main damage on the host is caused by the production of honeydew by actively feeding nymphs, which in turn is a favorite substrate for sooty mould fungi. The excreted honeydew, which blocks photosynthesis, causes necrosis on the leaves of infested plants (Salvianti et al. 2008) and russets the fruits, reducing their market value (Pasqualini et al. 2006). During summer, psylla can give rise to several overlapping generations (Schaub et al. 2005), leading to high pest densities that can induce leaf and fruit drop, and reduce fruit size (Shaltiel-Harpaz et al. 2014), hence causing high yield losses. Moreover, pear psylla is the major vector of the phytoplasma (*Candidatus Phytoplasma pyri*) responsible for pear decline disease (Salvianti et al. 2008).

Control of pear psylla in orchards is based mainly on the use of insecticides (for example amitraz, abamectin, organophosphates, pyrethroids) (Civolani 2012). However, the insect has developed resistance to a great number of them (Harries and Burts 1965; Buès et al. 2003; Civolani et al. 2007), while biological control strategies based on the use of natural enemies are not sufficient to prevent the damage (Berrada et al. 1995). Therefore, development of new pear cultivars with durable resistance is an effective and sustainable strategy for psylla control.

The three types of plant resistance to insects are antixenosis, antibiosis and tolerance (Hesler and Tharp 2005; Bell 2013a). Antixenosis prevents insects from colonizing the host or sustained feeding, and antibiosis affects the pest biology, while tolerance is the ability of the plant to grow despite infestation (Hesler and Tharp 2005). Antixenosis to pear psylla is characterized by ovipositional deterrence and feeding inhibition, whereas antibiosis is expressed by nymphal mortality and delayed development (Bell and Stuart 1990). These types of resistance may not share a common molecular and biological determinism, because some pear genotypes only show one or the other (Pasqualini et al. 2006). Antixenosis and antibiosis resistances to *C. pyri* in Europe and *C. pyricola* in North America have been characterized, and cultivars with different levels of resistance were identified among European and Asian pears and interspecific hybrids (Bell and Stuart 1990; Bell 1992; Robert et al. 2004; Robert and Raimbault 2004; Bell 2013a). Most of the *P. communis* resistant varieties originate from Eastern Europe and were found, or supposed, to be triploid, which means they might not be pure *P. communis* (Bell 2013a). Asian pear cultivars have long been used as sources of resistance to psylla (Westigard et al. 1970); Harris and Lamb (1973) showed that *P. ussuriensis* resistance, based on counts of nymphs on the seedlings, was heritable and dominant when crossed with *P. communis*. Also, Pasqualini et al. (2006) showed that the Asian species *P. ussuriensis* and *P. pyrifolia* were able to transmit psylla resistance to their progeny, although some variability was observed, depending on the parent combinations. They evaluated the resistance in terms of settling of adults, ovipositional antixenosis and nymphal antibiosis, and concluded that the last one was the most important type of resistance in the observed crosses. On the other hand, resistant cultivars of East European origin did not appear able to transmit high degrees of resistance to nymphal feeding to their progeny, except for ‘Erabasma’. Moreover, in crosses involving European pear cultivars, susceptibility was dominant (Bell 2013b). It is important to underline that the studies of Harris and Lamb (1973)

and of Bell (2013b), other than using different sources of resistance, evaluated two distinct modes of resistance. Therefore, it is clear that the investigation of all types of resistance to pear psylla is fundamental to determine whether a cultivar will be useful for breeding programs.

Pear psylla resistance is considered to be a polygenic trait (Pasqualini et al. 2006; Lespinasse et al. 2008) and to date only one quantitative trait locus (QTL) has been detected on pear linkage group (LG) 17 using the interspecific population ‘Angélys’ (*P. communis*) x NY10355 (*P. ussuriensis* X *P. communis*) (Bouvier et al. 2011). To our knowledge, only one other study focused on mapping pest resistance loci in pear: Evans et al. (2008) mapped a major gene for resistance to *Dysaphis pyri* to LG17 of the snow pear (*P. nivalis*). In contrast, in the apple (*Malus x domestica*) genome several loci linked to insect resistance, especially to aphids, have been mapped: a resistance gene and a QTL for the leaf-curling aphid (*Dysaphis devecta* (Walker)) on LG7 (Roche et al. 1997; Cevik and King 2002; Stoeckli et al. 2008b); a QTL for the rosy apple aphid (*Dysaphis plantaginea* (Passerini)) resistance on LG17 (Stoeckli et al. 2008b); a QTL for antibiosis resistance to the green apple aphid (*Aphis pomi* De Geer) on LG11 (Stoeckli et al. 2008a); four major genes conferring resistance to woolly apple aphid (*Eriosoma lanigerum* (Hausmann)) on LGs 7, 8 and 17 (Bus et al. 2008; 2010); and a QTL associated to the carpophagous codling moth (*Cydia pomonella* L.) susceptibility on LG10 (Stoeckli et al. 2009).

We investigated a new source of resistance to pear psylla derived from the Asian species *P. x bretschneideri*. Interspecific hybrid PEAR3 (*P. x bretschneideri* ‘Xue Hua Li’² x *P. communis* ‘Max Red Bartlett’) was crossed with the European cultivar ‘Moonglow’ to develop a segregating population for QTL mapping. PEAR3 was previously proven to be moderately resistant to psylla (unpublished data), while ‘Moonglow’ was reported as moderately to highly susceptible (Bell 1984; Berrada et al. 1995). In a mono-varietal pear orchard the insect is closer to a no-choice situation (Pasqualini et al. 2006), therefore we focused on antibiosis resistance, predominantly expressed as a reduced development rate of the insects. A novel phenotyping protocol was developed to screen large numbers of plants simultaneously, and its repeatability was tested over two years. (Montanari et al. 2013) scanned 220 progeny of the PEAR3 x ‘Moonglow’ segregating population with single

² The name ‘Xue Hua Li’ for this cultivar substitutes the name ‘Shiyuehuali’, which was used in (Montanari et al. 2013). *P. x bretschneideri* ‘Xue Hua Li’ is also known as Snowflake pear (Wang 2002).

nucleotide polymorphism (SNP) and simple sequence repeats (SSR) markers, and constructed two high density parental genetic maps. PEAR3 map consisted of 208 markers spanning 979.8 cM (with a density of one marker every 4.7 cM and a LG average length of 57.6 cM), and ‘Moonglow’ map consisted of 464 markers spanning 1016.6 cM (with a density of one marker every 2.2 cM and a LG average length of 59.8 cM). These parental maps were used to detect QTLs for *C. pyri* resistance.

Materials and Methods

Plant Material

An F1 population derived from PEAR3 x ‘Moonglow’ was screened for pear psylla resistance in 2013 and 2014 at the INRA site of Angers (France), testing respectively 96 and 98 progeny, along with the two parents and five controls: *P. communis* cultivars ‘Angélys’, ‘Harrow Sweet’, Michigan-US 437 and ‘Williams Bon Chrétien’, and the interspecific *P. ussuriensis* x *P. communis* hybrid NY10355. All genotypes were grafted on ‘Kirchensaller’ rootstocks and randomized in the greenhouse with an average of 5 and 7 replicates in 2013 and 2014, respectively. Potted plants were placed on benches and the climatic conditions in the greenhouse were controlled in order to keep an average temperature of 22/18°C day/night and 55% of relative humidity (RH). Pots were fertilized with a nutrient solution (N17 – P10 – K30) one to seven times per week, depending on growth condition of the plants. Irrigation was applied manually when needed.

At the infestation dates, the shoots were at least 15 cm tall and actively growing.

Infestation and assessments

C. pyri was reared on ‘Williams Bon Chrétien’ in insect-proof cages placed in a climatic chamber (16/8 h day/night photoperiod, 100 $\mu\text{M}/\text{m}^2/\text{s}$ minimum photosynthetic photon flux density, 22/18°C temperature and 70-90% of RH) (Figure 3.2a). Adults were collected for infestation one week after the last molt, when males and females were visually recognizable and put in separate tubes (Figure 3.2b). In order to perform a no-choice test and guarantee oviposition on all genotypes, the upper 3-4 leaves of each shoot were covered with light net bags, and one male and one female were introduced in each bag (Figure 3.2c). After eight

days the bags were removed, making sure not to leave any live adults in the greenhouse, and the number of eggs were counted with the aid of binocular magnifiers, using six classes (0 = no eggs, 1 = 1-10 eggs, 2 = 11-30 eggs, 3 = 31-50 eggs, 4 = 51-100 eggs and 5 = more than 100 eggs). From this moment on, plants were monitored constantly to determine when all the eggs had hatched, but no new adults had appeared, which was when the nymph assessment was performed (three to four weeks after infestation). Nymph assessment was the most crucial part of the experiment: in order to introduce as little variability as possible to the phenotypic traits, the right balance between time (the assessment had to be completed within very few days) and the unavoidable subjectivity of the scorer (more observers, more variability) had to be achieved. The number of living young (L1, L2 and L3 instars) and old (L4 and L5 instars) nymphs were counted (Figure 3.1a) with the use of a stereomicroscope.

Statistical analysis and QTL mapping

R studio (<http://www.rstudio.com>) was used for statistical analyses. Shoots that stopped growing were excluded from the analyses.

Raw data (eggs, total nymphs, young nymphs and old nymphs) were tested for normality using the Lilliefors and Shapiro-Francia tests (Thode 2002), in which the null hypothesis is that the data were normally distributed.

In both years, the nymph counting on all plants took three days, during which the insects continued to develop, and involved ten scorers. Therefore, the significance of the “scoring date” and “scorer” effects, considered as fixed effects, on the number of nymphs at different stages was tested, using ANOVA. The model of the analysis of variance was considered reliable when the residual errors were normal, which condition was verified with “residual versus fitted” and “normal quantile-quantile” plots. For each year, averages were adjusted according to the significant ($p < 0.05$) effects and the distributions of the adjusted means were again tested for normality with the Lilliefors and Shapiro-Francia tests. The egg phenotypic data were treated both as a factor affecting the number of nymphs and a trait for QTL mapping. In the first case, the ordered “factor eggs” (obtained from the variable “eggs” by applying the function *factor* in R with the argument *ordered=TRUE*) was added to the ANOVA model for the means adjustment.

For each year of phenotyping, the correlations between the adjusted means of the different traits were tested, and in particular: “eggs versus total nymphs”, “young versus total nymphs”,

“old versus total nymphs” and “young versus old nymphs”. The correlation coefficients were calculated using the Pearson formula when the traits were normally distributed, or Spearman formula (for ranked data) when at least one of the two traits was not normal. The statistical significance of the correlations was also evaluated.

The traits considered for QTL mapping were: i) class of eggs (“eggs”); ii) number of total nymphs (“total nymphs”) and iii) the ratio of the number of old / number of total nymphs (“old/total nymphs”). The broad sense heritability (H^2) of genotypic means within each

progeny for all these traits was estimated using the formula $H^2 = \sigma_g^2 \div \left(\sigma_g^2 + \sigma_e^2/n \right)$, where

n is the mean number of replicates per genotype, σ_g^2 is the genetic variance (i.e. inter-genotype variance) and σ_e^2 is the residual error variance (Calenge et al. 2005; Durel et al. 2009).

QTL mapping was performed with the MapQTL 5.0 software (Van Ooijen 2004), using Interval Mapping (IM) (Lander and Botstein 1989) and, when multiple QTLs were detected, restricted Multiple QTL Mapping (rMQM) (Jansen 1993; Jansen and Stam 1994), selecting the closest marker to the QTL peak as cofactor. QTLs were also detected using the Haley-Knott (HK) regression method (Haley and Knott 1992), with the package ‘qtl’ of R (Rqtl) (<http://www.rqtl.org>). The significant LOD threshold ($\rho = 0.05$) for each trait was determined after genome-wide permutation tests (Churchill and Doerge 1994) using 1000 permutations. The genetic maps of the parents PEAR3 and ‘Moonglow’ used for QTL mapping were the ones published by Montanari et al. (2013), with minor modifications: i) eight new markers were added to LG5 of PEAR3 (ss475882774, ss475883501, ss475878404, ss475879604) and LG15 of ‘Moonglow’ (ss475881341, ss475881255, NB129a, ss527789616) and ii) the SNPs heterozygous with the same alleles in both parents were removed prior to analyses.

Possible epistatic interactions between detected QTLs were tested using ANOVA with the formula $Y_k = \mu + M1 + M2 + (M1 * M2) + \varepsilon_k$, where Y_k is the phenotypic value of the genotype k , μ is the phenotypic mean of the population, $M1$ and $M2$ are the proper effects of markers $M1$ (the closest to the peak of QTL1) and $M2$ (the closest to the peak of QTL2), $(M1 * M2)$ is the interaction effect between the markers $M1$ and $M2$, and ε_k is the residual effect. The normality of the residual errors was verified as explained before. The percentage of the phenotypic variation explained by all the significant ($\rho < 0.05$) QTLs and epistatic

interactions (R^2 or coefficient of multiple determination) was estimated using the formula $globalR^2 = 1 - \left(SS_{res} / SS_{tot} \right)$, where SS_{res} is the residual sum of squares and SS_{tot} is the total sum of squares.

Comparison between the two years

The data collected in 2013 and 2014 were compared in two ways: first, they were pooled together and the significance of the effect “year” was tested; then, the adjusted means from 2013 and 2014 were compared in R and the correlation coefficients were estimated. As for the comparisons between different traits, the Pearson formula was used when the two compared sets of data followed a normal distribution, otherwise the Spearman rank correlation coefficient was calculated. In order to verify if our interpretation of the correlation coefficient was correct, we also tested the statistical significance of the correlations.

Results

Egg and nymph assessments

In total, 405 and 504 trees from 96 and 98 replicated seedlings were screened for host resistance to pear psylla in 2013 and 2014, respectively. When the infestations were carried out, plant shoots were actively growing and psylla females were ready for oviposition. By the time the bags were removed, eight days later, the plants were just a little withered, and the oviposition was well advanced. The nymph assessment started 22 and 26 days after infestation in 2013 and 2014, respectively. When looking at the response of the controls, ‘Williams Bon Chrétien’ and NY10355 were always highly susceptible and highly resistant, respectively (Figure 3.3). For the total number of nymphs in 2013, the parent ‘Moonglow’ was comparable to ‘Williams Bon Chrétien’, and PEAR3 to NY10355, and both parents were not significantly different from each other (according to Tukey test); no significant difference was observed between any of the controls for “old/total nymphs”. Also in 2014 the PEAR3 response was similar to that of NY10355, while the total number of nymphs for this parent, although not significantly different from ‘Williams Bon Chrétien’, was lower than Michigan-US 437. This year, like in 2013, the total number of nymphs and the old/total nymphs ratio in

‘Moonglow’ were not significantly different from that of PEAR3 (Figure 3.3). It is worth mentioning that while the number of replicates for ‘Moonglow’ in 2013 and 2014 was comparable (four and three), in 2013 PEAR3 had only one replicate, and six in 2014.

The number of eggs was expected to be consistent amongst all plants, because the insects were in a no-choice situation. However, only few eggs were counted on the antixenotic and antibiotic NY10355 (Salvianti et al. 2008) (Figure 3.3), which demonstrates that antixenosis was possible. Several transgressive seedlings were observed amongst the progeny when looking at the arithmetic means for each genotype. This was consistent with the polygenic nature of the trait, and it was probably emphasized by the level of resistance of the two parents, which was not as different as expected.

Phenotypic distribution, environmental effects and heritability

The results of the Lilliefors and Shapiro-Francia tests performed on the raw data were consistent, with only the latter reported here. The tests indicated that none of the measured variables had a normal distribution ($p < 0.05$) (Table 3.1) and all were biased towards resistance, except for “eggs” (Online resources 3.1). Nevertheless, the residual errors turned out to be normally distributed, and no transformation or non-parametric analysis was needed. Consequently, ANOVA was used to evaluate the significance of the effects “factor eggs” (i.e. the number of laid eggs considered as an ordered factor affecting the number of nymphs), “year”, “scoring date” and “scorer” on the phenotypic traits. A higher infestation was observed in the second year (2014), since significantly higher numbers of eggs and total nymphs were scored. Moreover, nymphal development was faster in 2014 than in 2013, as revealed by the quicker evolution of the numbers of young and old nymphs over the three days of assessment. Indeed, in 2014 the decrease of young nymphs and the increase of the old ones from the first to the third day of assessment were significantly greater than in 2013 ($\Delta(\text{young}) = -6.62$ and $\Delta(\text{old}) = +2.12$ in 2013, and -14.58 and $+25.5$ in 2014), with the number of old nymphs largely surpassing the number of young in 2014 (Figure 3.4). The genotype significantly affected all the traits in both years. The environmental effects “factor eggs”, “scoring date” and “scorer” were also significant in both years, and the phenotypic means were adjusted according to them. The distribution of the adjusted means was normal for the traits “total nymphs” and “young nymphs” in 2013, and for “eggs”, “young nymphs” (although only according to Lilliefors test, and not to Shapiro-Francia) and “old/total

nymphs” in 2014, while it was not normal for the other traits (Table 3.2, Online resources 3.2). The number of eggs and the total number of nymphs were significantly ($\rho < 0.05$) positively correlated and had Spearman coefficients $r_s = 0.747$ and 0.672 in 2013 and 2014, respectively (Figure 3.5). The total number of nymphs was very highly correlated with the number of young nymphs in 2013 ($r_s = 0.958$) and to the number of old nymphs in 2014 ($r_s = 0.946$) (Figure 3.5).

The estimated broad sense heritability (H^2) was generally high for each trait (Table 3.3), with the highest values observed for “total nymphs” (0.63 in 2013 and 0.68 in 2014). The heritability for “eggs” was lower than the other traits in 2013 (0.45), but in the same range as for “total nymphs” in 2014 (0.58), thus indicating that antixenosis was significantly contributing to psylla resistance in this experiment. In contrast, the ratio “old/total nymphs” had a high heritability in 2013 (0.65), but it was lower in 2014 (0.45). The H^2 was lower when considering the “factor eggs” in ANOVA (data not presented).

Phenotypic correlation between years

A significant ($\rho < 0.05$) linear correlation was observed for the trait “total nymphs” between 2013 and 2014, with a Spearman coefficient of $r_s = 0.474$ (Figure 3.6a). Conversely, the traits “eggs” and “old/total nymphs” showed very weak or no correlation (Figure 3.6b). For the trait “eggs” the Spearman coefficient was $r_s = 0.249$.

QTL detection

QTLs were detected for all measured traits except the old/total nymphs in 2013 by IM or rMQM using MapQTL, and by HK regression using Rqtl, with the significance of genome-wide LOD thresholds ranging between 3.1 and 3.3 after permutation tests (Table 3.4). The phenotypic variation explained by each QTL (R^2) is reported in Table 3.4, as well as the global R^2 estimated for each trait taking into account possible epistatic interactions between QTLs ($globalR^2$), when several QTLs were present. The detected QTLs were the same whether or not the “factor eggs” was added into the model. However, the LOD scores were more significant without the “factor eggs”. Therefore, the results obtained with the “factor eggs” were not reported.

QTL detection in 2013. A QTL on LG8 of PEAR3 was detected with both methods for “eggs” and “total nymphs”, while for “old/total nymphs” no QTL was found. The marker closest to the QTL peak was SSR CH05a02, with its 130 bp allele associated with resistance. This QTL on LG8 explained between 17.2% (calculated with HK for the “eggs”) and 39.1% (calculated with rMQM for “total nymphs”) of the phenotypic variation. When using MapQTL, two other putative QTLs, just a little below the threshold, were mapped to LG5 of PEAR3 for “eggs” ($R^2 = 9.9\%$), associated with SNP ss475875754, and to LG11 of PEAR3 for “total nymphs” ($R^2 = 8.4\%$), associated with SNP ss475877524. However, neither of these QTLs was detected when using the HK method. The residual errors calculated post-QTL analysis were normally distributed for the trait “total nymphs”, but not for “eggs”.

QTL detection in 2014. The QTL on LG8 of PEAR3 was confirmed in 2014 for “eggs” (just below the threshold) and “total nymphs” using both methods. Its peak was located on the upper part of the LG, at the same position as in 2013 or above it. By looking at marker CH05a02, the favorable allele was 130 bp, as in 2013. Moreover, a QTL just below the threshold was found on the same location also for “old/total nymphs”. The R^2 of the LG8 QTL ranged between 10.9% and 29.5%, again with the highest value for the “total number of nymphs”. The QTL on LG5 of PEAR3 for “eggs” (marker ss475875754) was not detected in 2014, but a QTL in a very close position (marker ss475878404) was found for the trait “total nymphs”, although not confirmed when using the regression method. For “total nymphs” another putative QTL was found on LG11 of PEAR3 using MapQTL, close to the one detected in 2013; however, its peak was below the threshold. Furthermore, a QTL was mapped to LG15 of ‘Moonglow’ for the “old/total nymphs” ratio, significant only with the HK method. LG5, LG11 and LG15 QTLs had usually smaller effects than the one on LG8, with R^2 values ranging between 7.7% and 13.7%. Both in 2014 and 2013, the resistance was associated to allele “G” of SNP ss475875754 and to allele “0” (null allele) of ss475878404 on LG5, and to allele “G” of ss475877524 and to allele “0” (null allele) of ss475882338 on LG11. On LG15, the QTL peak was close to SNP ss475883269 and “T” was the favorable allele. The global R^2 was estimated for the total number of nymphs ($globalR^2 = 50.5\%$), and a significant interaction effect (epistasis) was detected between the LG8 and LG5 QTLs. The residual errors were normally distributed for all the traits except for “eggs”, like in 2013. The positions of all the QTLs detected in 2013 and 2014 are shown on the genetic map (Figure 3.7).

When R^2 was compared with the estimated broad sense heritability (Table 3.3), it was noticed that it was always lower than H^2 .

Discussion

A robust, repeatable and high throughput phenotyping protocol for psylla resistance genetic analysis

Collecting quantitative and reproducible phenotypic data with minimal environmental effect over large numbers of segregating seedlings is crucial for QTL mapping. Multiple protocols have been developed previously for phenotyping antibiosis to psylla (Berrada et al. 1995; Pasqualini et al. 2006; Bell 2013a; 2013b), but none of them was suitable for the purpose of assessing resistance in a large segregating population. The logistical and reproducible challenges were exacerbated by the necessity of a strict phenological synchronization between the plant and the pest, and the creation of an environment with optimal growing conditions for both of them. The phenotyping protocol we developed employed about ten people for only five days each year to study the antibiosis resistance of pear to psylla and to collect quantitative data from hundreds of plants. The egg and nymph assessments were carried out over a short time frame in order to minimize non-genetic factors, such as the influence of temperature and relative humidity on insect development, and allowed the detection of robust QTLs. Furthermore, this protocol has proved to be repeatable across years.

Sensitivity of the assessment period

When considering both tests performed in 2013 and 2014, it was interesting to notice that the date of the nymph assessment (“scoring date”) was a very sensitive parameter. Firstly, considering each year separately, this effect turned out to be significant (Figure 3.4), despite the scoring had been performed on three consecutive days with a complete randomization of the assessed genotypes and a stable involvement. Consequently, the number of young and old nymphs was quickly evolving in a short period of time, which aspect was fixed year-per-year by adjusting the phenotypic means of the lines according to the “scoring date” effect. Secondly, when comparing 2013 and 2014 tests, the nymph assessment was performed with a small discrepancy regarding the number of days after infestation (22 and 26 days,

respectively). Moreover, nymph development was faster in 2014 than in 2013 (Figure 3.4), even though the temperature and the relative humidity measured inside the greenhouse were comparable. The later assessment and quicker nymphal development in 2014 with respect to 2013 explain the stronger correlation between “young nymphs” and “total nymphs” in 2013, and between “old nymphs” and “total nymphs” in 2014 (Figure 3.5). As “total nymphs” = “young nymphs” + “old nymphs”, a later and faster (respectively earlier and slower) assessment gave higher emphasis to old relative to nymphs in agreement with the nymph developmental process.

Discrimination between antibiosis and antixenosis

In this experiment, we initially wanted to evaluate the antibiosis resistance to pear psylla, putting insects in a no-choice situation in order to reduce as much as possible the variability in the oviposition rate among the different genotypes. The reason for that was to mimic a mono-varietal pear orchard where the insect has no choice for the variety on which to lay its eggs. Nevertheless, in practice ovipositional antixenosis and antibiosis resistance could not be completely separated, and we also measured significant variability among the genotypes for the “eggs” trait. An extreme situation was observed for NY10355, where the number of eggs laid was particularly small, thus indicating that this genotype exhibits a strong ovipositional antixenosis resistance. Basically, there is a (chronological) dependency between the final number of total nymphs and the initial number of eggs. A small number of laid eggs prevents one from observing a large number of nymphs, whereas a large number of laid eggs allows the observation of small, medium, or large number of nymphs according to the subsequent antibiosis resistance of the genotypes. This was clearly shown in the “triangle” relationship between “total nymphs” and “eggs” (Figure 3.5), with less variation in “total nymphs” for small values of “eggs”, and larger variation for high values of “eggs”. We tried to correct for the number of nymphs according to the number of eggs in order to focus on antibiosis, but we came across the imprecision of our initial egg assessment protocol, as an ordinal scale with large intervals and only six classes was used. Moreover, as antixenosis applies earlier in the parasitic process than antibiosis, it can hamper the correct detection of antibiosis resistance by hiding its genetic variation, especially for those genotypes with strong ovipositional antixenosis resistance. Such a chronological dependency creates a bias in the accuracy of the antibiosis assessment, which cannot be simply corrected by statistical approaches. Thus,

ovipositional antixenosis can generate seeming antibiosis. Here, the consistency of low numbers of eggs across the replicates of several genotypes generated a moderate but significant heritability for this trait, demonstrating that there was an important contribution of antixenosis to psylla resistance in our experiment. Consistently, we were able to detect QTLs for the “eggs” trait. For the number of nymphs, the detected QTLs were the same whether or not the “factor eggs” was added into the ANOVA model, indicating that antibiosis was also most probably contributing to psylla resistance. The lower LOD scores significance for the QTLs detected with “factor eggs” could be interpreted as a signature of the antixenosis impact on the antibiosis assessment. We nevertheless considered that antibiosis resistance was present and correctly mapped in the present experiment, since the number of laid eggs was rather high for many of the genotypes, with a majority of 4 or 5 scoring at the egg assessment. Thus, the new phenotyping protocol allowed an incomplete, but acceptable control of the antixenosis mechanism of resistance and a correct examination of the antibiosis.

New QTLs for pear resistance to psylla

A large effect and stable QTL inherited from the resistant parent PEAR3 was detected on LG8 for all the traits (Figure 3.7). The position of this QTL was confirmed after two years of experiments and by using two QTL mapping methods, IM and HK regression. QTL detection by regression is more robust for non-normally distributed data (Feenstra et al. 2006), which was the case for the trait “eggs” (non-normal distribution of the residual errors after the QTL analysis). In 2014, when the infestation was higher, a QTL was also detected on LG15 of ‘Moonglow’ for the ratio “old/total nymphs”. No QTL was found for this trait in 2013, probably because of the very low numbers of old nymphs scored. Furthermore, two small effect QTLs were detected on LG5 (for “eggs” in 2013 and for “total nymphs” in 2014) and on LG11 of PEAR3 (for “total nymphs” both in 2013 and 2014). However, their LOD scores were low and neither of them was confirmed using HK regression, indicating that they could be spurious QTLs (Table 3.4).

The number of total nymphs was the measure less prone to error. In fact, the distinction between young and old nymphs can be difficult, especially between the L3 and L4 instars (Figure 3.1a); hence, some nymphs could have been allocated to the wrong class. This may explain why we found the strongest QTLs for the trait “total nymphs”, with the highest LOD score and R^2 (Table 3.4). For the trait “eggs” we also found a QTL on LG8 of PEAR3 in 2013

and in 2014 (just below the threshold), whose position was consistent with the QTLs found for the other traits (Figure 3.7). The broad sense heritability was usually high for all the traits (Table 3.3), indicating that the phenotypic variance was mostly attributable to differences in genotypes and less to the environment, and that the results of our QTL mapping were reliable. However, the R^2 explained by the QTLs were always lower than the H^2 , which indicates that we were not able to detect all the loci linked to psylla resistance. The reasons were imputable to the type and size of the mapping population we used. Being an interspecific F1 population, all individuals were supposedly highly heterozygous, hence the progeny was highly variable with possible complex genetic architecture of the studied traits involving gene interactions, which are more difficult to map. Moreover, our population consisted of just fewer than 100 genotypes, which is sufficient to detect only the largest effect QTLs. The strong QTL we discovered on LG8 of PEAR3 also has epistatic relationships with other loci. Therefore, a larger number of genotypes would be necessary for the detection of further smaller effect QTLs in this family, if present (Collard et al. 2005). Since the parental genetic maps, especially the one of PEAR3, were not saturated, it is also possible that some QTLs are located in genomic regions not covered by markers, hence could not be detected in this experiment.

From these results, we can assume that a locus responsible for a strong antibiosis resistance, but also for ovipositional antixenosis, was located on LG8 of PEAR3. Since the confidence intervals of the QTL detected for the different traits were quite large (one-LOD support interval ranging from 6 to 25 cM, but usually higher than 18 cM) (Figure 3.7), two different, but closely linked loci, one for antibiosis and one for antixenosis, could be located in the same interval. Here again, the population size (~100 progeny) was not large enough to discriminate between both hypotheses: closely linked QTLs (approximately 20 cM or less) are not distinguishable with populations size lower than 500 (Collard et al. 2005). On the other hand, the QTL for “old/total nymphs” on LG15 of the “susceptible” parent ‘Moonglow’, even if its presence should be confirmed with other tests, may be more strictly linked to the antibiosis mechanism (i.e. delayed nymphal development). The observation of several transgressive lines amongst the progenies had already predicted the possible presence of resistance factors in both parents. Therefore, unknown sources of psylla resistance may be present among the *P. communis* cultivars in the ‘Moonglow’ pedigree (Montanari et al. 2013): ‘Seckel’ and ‘Bartlett’ are known to be susceptible to psylla species (Butt et al. 1988; Bell and Stuart

1990), Michigan-US 437 was tested in our experiments and resulted to be susceptible (Figure 3.3), while there is no information available about ‘Roi Charles de Wurtemberg’, which could thus be more resistant. Concerning the LG8 QTL, while we do not have any information about the genotypes at this locus for the PEAR3 parents, with psylla resistance mostly found in Asian pear species (Westigard et al. 1970; Bell and Stuart 1990; Bell 2013a) it is most likely to have been inherited from ‘Xue Hua Li’. Previously, the cultivar ‘Xue Hua Li’ was reported as having good field resistance to psylla (Stanica 2002).

Co-localization with genes and QTLs for aphid resistance

The genetic map of PEAR3 could be compared with other pear and apple maps generated for the detection of QTLs and major loci for pest and disease resistance through common microsatellite markers. Two major genes for woolly apple aphid resistance were mapped to the upper part of LG8 in apple (Bus et al. 2008; 2010), the same region where we detected the major QTL for psylla resistance. Moreover, the putative QTL for the resistance of apple to *A. pomi* found on LG11 by Stoeckli et al. (2008a) co-localizes with the small effect QTL we detected on this LG for “total nymphs”. This is not the first time that loci associated to psylla and aphid resistance are found in chromosomal regions orthologous between species: aphid resistance genes were mapped to LG17 in both pear (Evans et al. 2008) and apple (Stoeckli et al. 2008b; Bus et al. 2008; 2010), where Bouvier et al. (2011) also detected a QTL for pear psylla resistance in the *P. ussuriensis* X *P. communis* hybrid NY10355. Aphids and psylla are both phloem feeders, therefore finding orthologous regions linked to antibiosis resistance to these insects may indicate some common molecular resistance mechanisms. Civolani et al. (2013) conducted experiments on the probing behavior of *C. pyri*, and they introduced the hypothesis that strong resistance factors are present in the phloem of resistant pear accessions.

Conclusion

The results of our experiment confirmed pear psylla resistance to be a polygenic trait. Although the parents PEAR3 and ‘Moonglow’ turned out to have a much more similar response to psylla infestation than we expected, we were able to detect a stable QTL on LG8 of PEAR3. Until now, only Bouvier et al. (2011) had published results from a QTL mapping study for pear psylla resistance, but they used a different source of resistance (*P. ussuriensis*),

and the major QTL they found was located on LG17. Pyramiding these two sources of resistance (*P. x bretschneideri* and *P. ussuriensis*) could be an effective breeding strategy for the development of pear cultivar highly resistant to psylla.

Other experiments will be necessary to reduce the confidence interval of the QTL on LG8 and to confirm the significance of the minor QTLs on LGs 5, 11 and 15, with the final purpose of identifying markers useful for marker assisted selection (MAS). Moreover, scoring the same population more accurately for oviposition could be useful to verify the hypothesis of the presence of two distinct loci on LG8, one for antibiosis and one for antixenosis. Pear cultivars bringing both the QTLs responsible for antixenosis and antibiosis would have a more durable resistance, more difficult to be overcome by newly evolved psylla races. Indeed, the experiment carried out by Puterka (1997) with different *C. pyricola* biotypes (originating from different regions in the USA) on susceptible and resistant pear varieties, suggested the ability of this pest to adapt to the host resistance.

Finally, it would be interesting to study the possible localization on apple and pear LG8 and LG17 of genes responsible for the production of phloem resistance factors, which act in response to psylla and aphid infestation.

The recent publication of *P. x bretschneideri* (Wu et al. 2013) and *P. communis* (Chagné et al. 2014) genome sequences will facilitate studies on the molecular determinism of agronomic traits of interest in pear, such as pest and disease resistance. The development of new markers and the functional analysis of genes in the genomic regions linked to psylla resistance will lead to a better understanding of this important, but complex trait.

Acknowledgements

SM was funded by the Fondazione Edmund Mach (FEM) PhD School. We greatly thank Déborah Renault, Jasmine McCarthy, Ferreol Braud, Sophie Aligon, Medhi Al-Rifai, Clement Joffrion, Sylvain Hanteville, Valérie Le Mignon, Arnaud Guyader and Christine Boursier (UMR1345 IRHS, Angers, France) for their precious help in the scoring on *C. pyri* eggs and nymphs. We also thank Lester Brewer at PFR for generating the PEAR3 x 'Moonglow' cross, and the INEM team of IRHS, especially Michel Boucourt, and the INRA Experimental Unit (UE Horti, Angers, France), especially Christian Cattaneo, for taking care of the seedling population.

Data archiving statement

QTL data are reported in Table 3.4 and will be made publicly available through the Genome Database for Rosaceae (www.rosaceae.org).

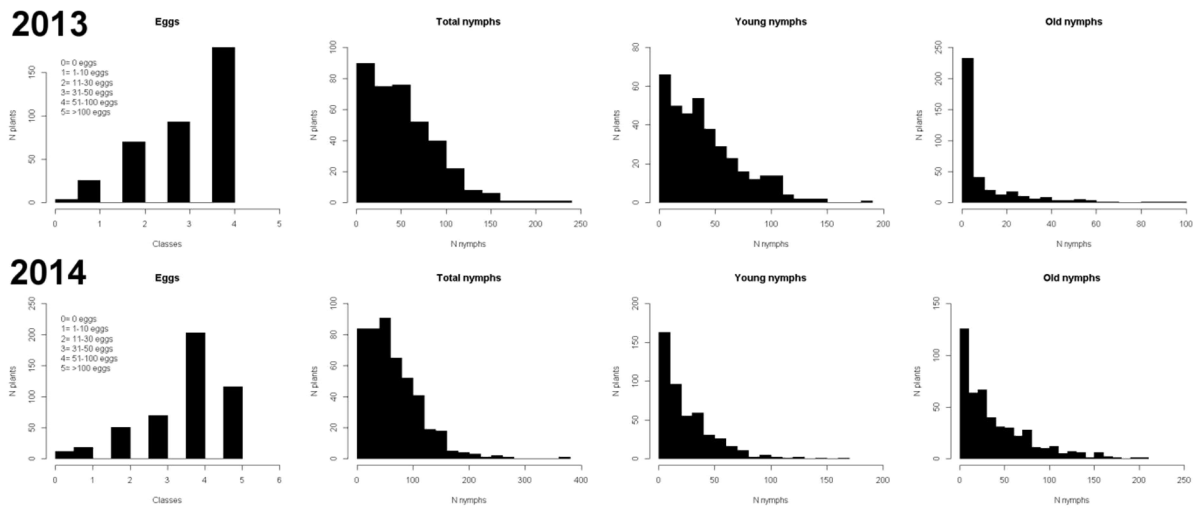
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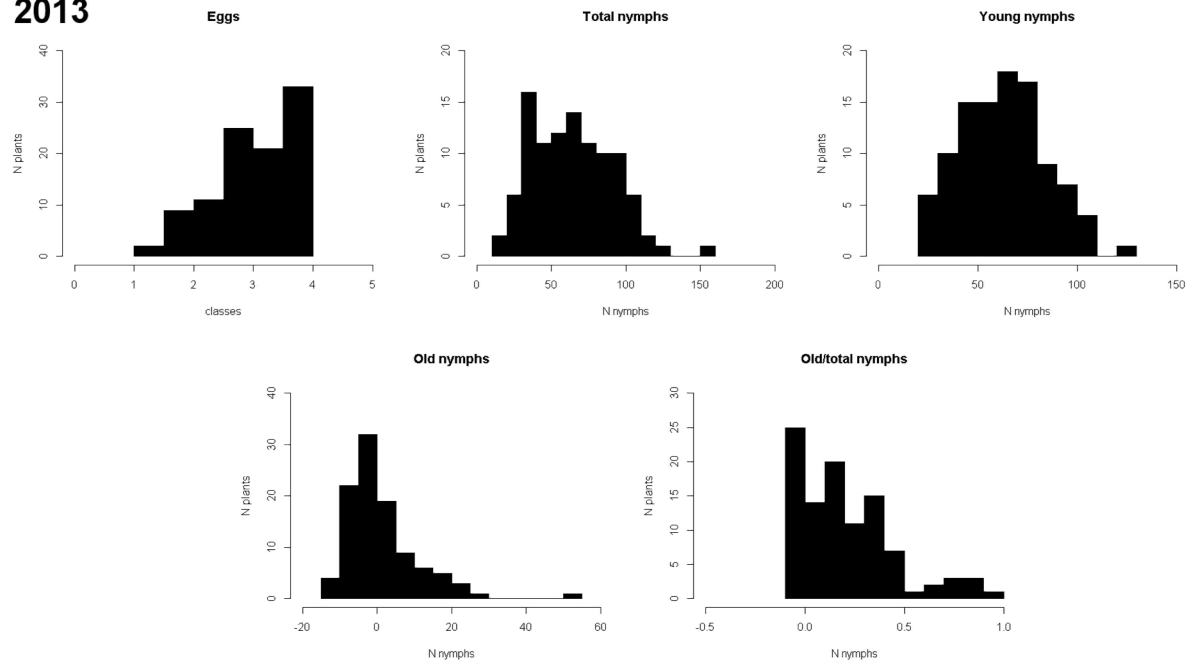
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Electronic supplementary material

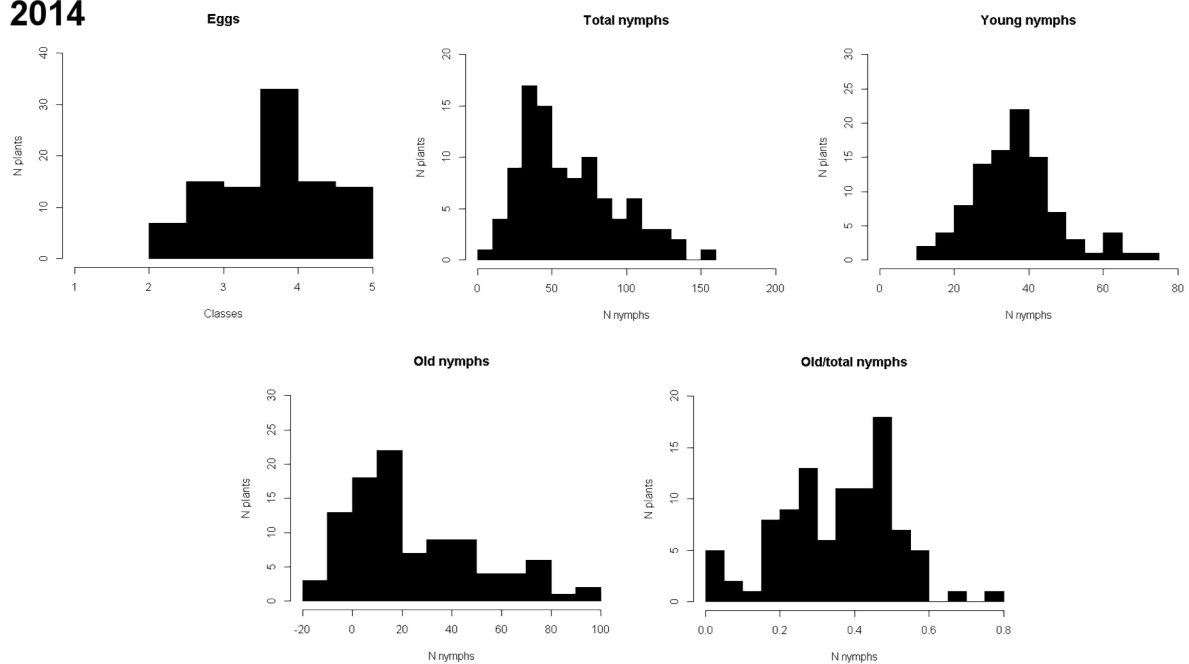


Online resources 3.1: Psylla resistance phenotypic data distributions in a segregating interspecific pear population in 2013 and 2014

2013



2014



Online resources 3.2: Distribution of the phenotypic means of psylla resistance adjusted for environmental factors in a pear segregating population in 2013 and 2014

Figures

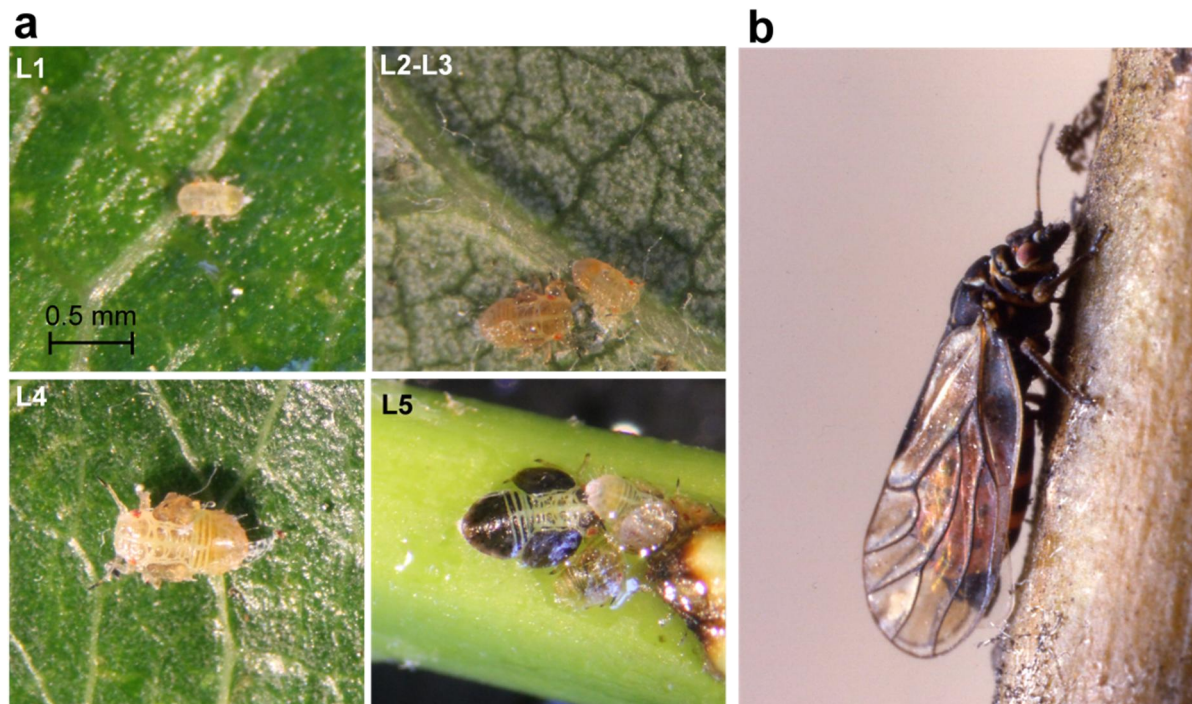


Figure 3.1: Development stages of psylla

(a) Nymphs go through five instar. For the assessment in an interspecific pear population the number of young (L1, L2 and L3 instars) and old (L4 and L5 instars) nymphs on each shoot was counted using a stereomicroscope. (b) Adult of psylla

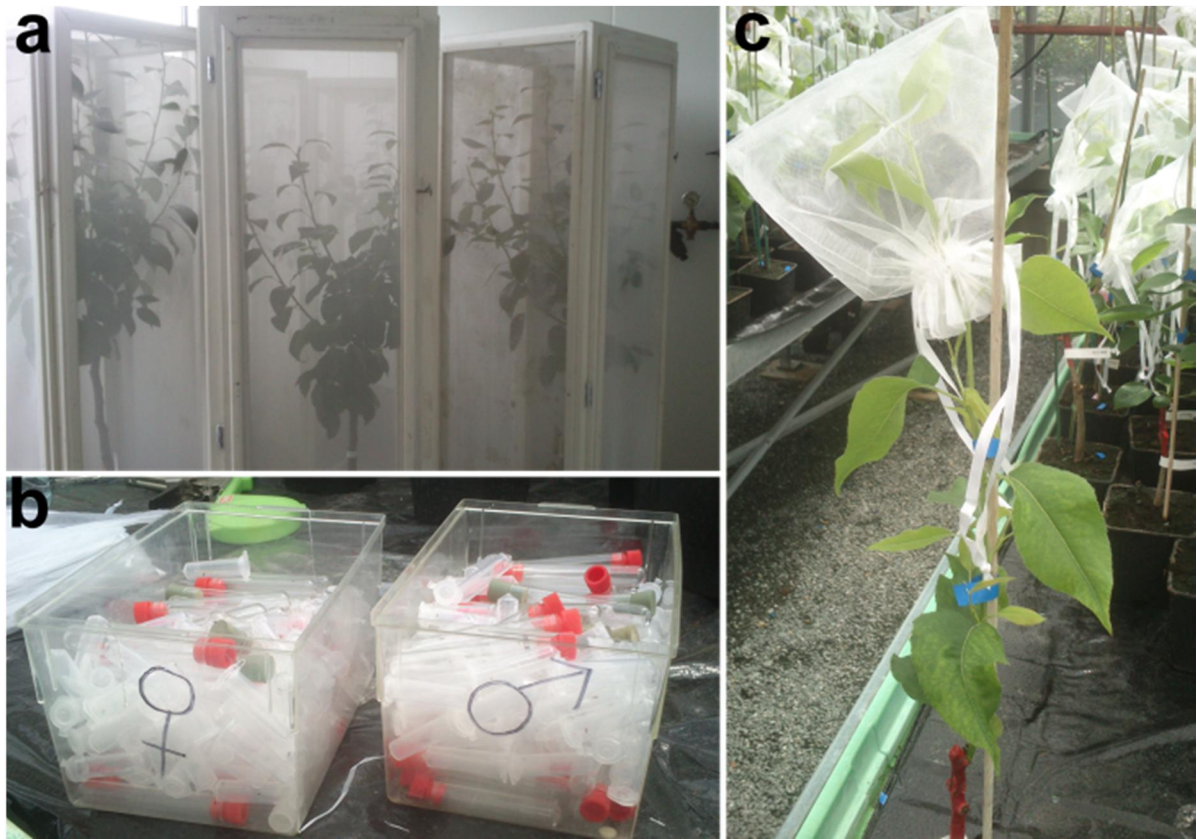


Figure 3.2: Method for psylla infestation in an interspecific pear population

(a) *Cacopsylla pyri* was reared on ‘Williams Bon Chrétien’ trees placed in insect-proof cages; (b) on the infestation date male and female adults were captured into separate tubes; (c) the main shoot for each genotype grown in the greenhouse was covered with a light net bag, and one male and one female were introduced inside each bag

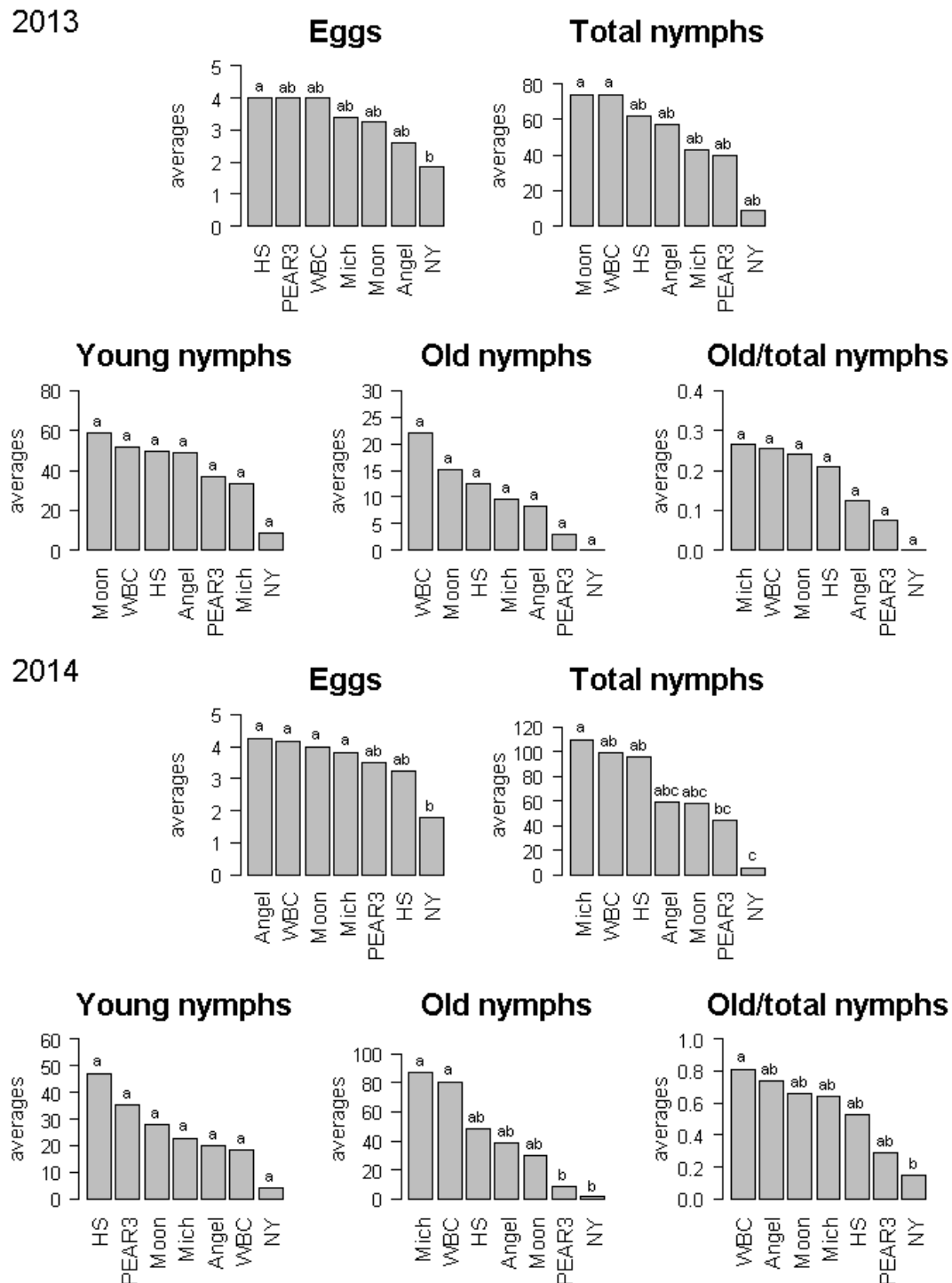


Figure 3.3: Comparison between the different pear accessions used as controls in a psylla resistance phenotyping in 2013 and 2014

Significantly different genotypes, according to Tukey test, are identified by different letters. PEAR3 and 'Moonglow' (Moon) are the parents of the interspecific pear population tested, 'Williams Bon Chrétien' (WBC), 'Harrow Sweet' (HS), 'Angélus' (Angel) and Michigan-US 437 (Mich) the susceptible controls, and NY10355 (NY) the resistant control

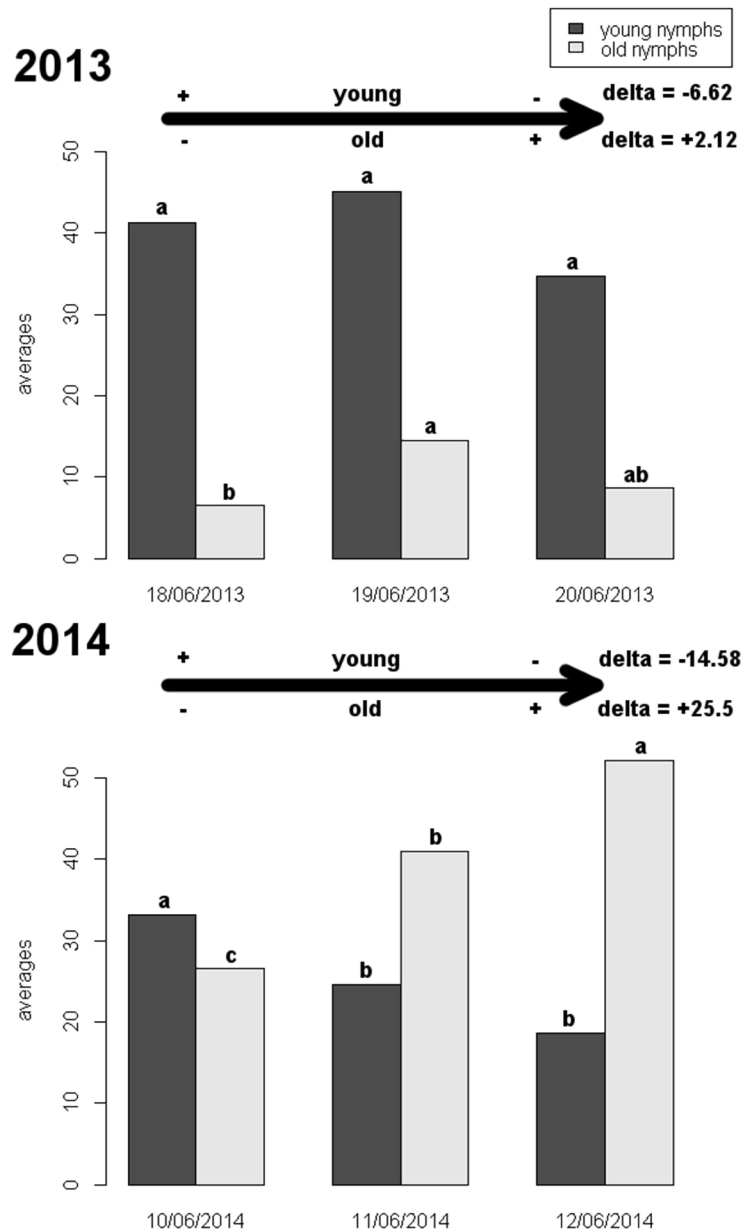
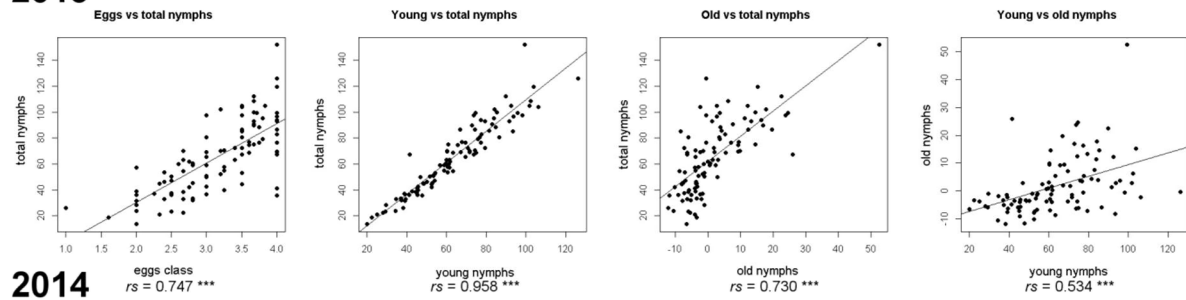


Figure 3.4: Effect of the three nymph scoring dates on the number of young and old nymphs counted in an interspecific pear population in 2013 and 2014

The different letters represent significance difference (according to Tukey test) within each category: young nymphs in 2013, old nymphs in 2013, young nymphs in 2014 and old nymphs in 2014. Young nymphs are represented by the dark grey bars and old nymphs by the light grey bars. For each year, the difference between the third and the first day of assessments for the numbers of young and of old nymphs (delta) was calculated

2013



2014

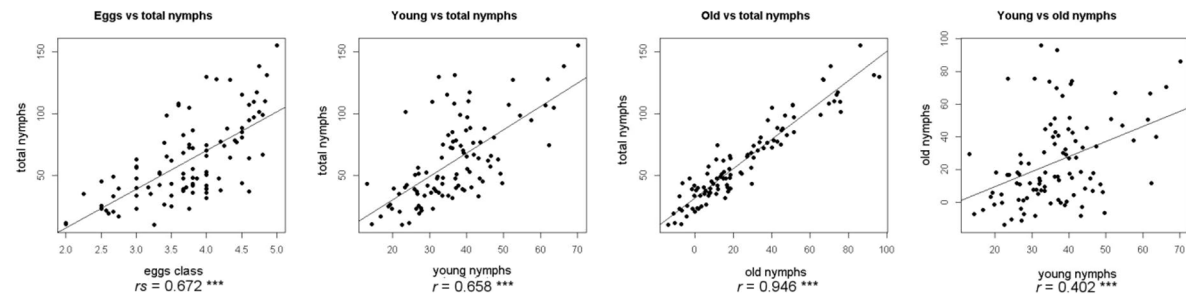


Figure 3.5: Correlation between different psylla resistance phenotypic traits measured in a pear segregating population in 2013 and 2014

For each comparison, correlation coefficients and their significance level ($*** = \rho < 0.001$; $** = \rho < 0.01$; $* = \rho < 0.05$; ns = not significative) are shown. The Pearson formula (r) was used when both traits were normally distributed, otherwise the Spearman formula (r_s) was used

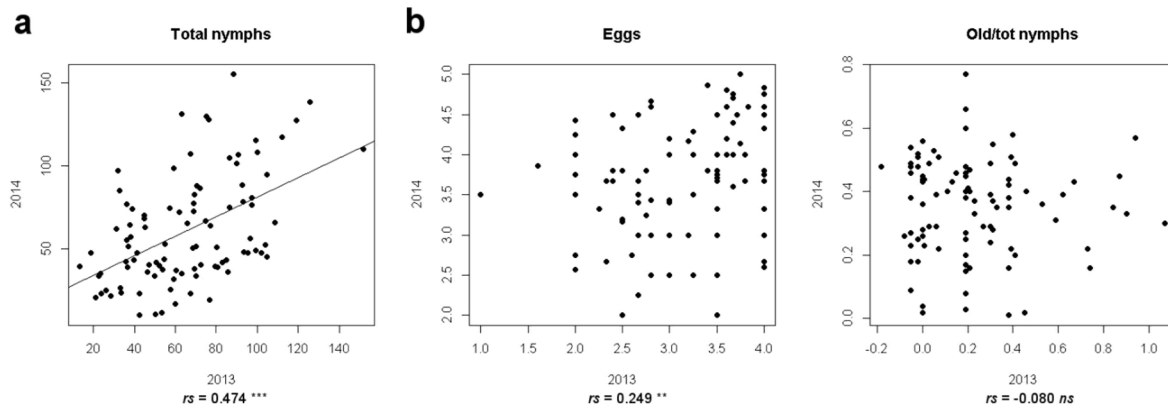


Figure 3.6: Phenotypic variability between years for psylla resistance in a pear segregating population

For each trait, the means adjusted for the environmental factors were used. Correlation coefficients, calculated using Spearman formula (r_s), and their significance level (***) = $\rho < 0.001$; ** = $\rho < 0.01$; * = $\rho < 0.05$; *ns* = not significant) are also shown. (a) For the total number of nymphs (“Total nymphs”) a linear correlation was observed between 2013 and 2014. (b) The number of eggs (“Eggs”) and the ratio of old/total nymphs (“Old/tot nymphs”) showed weak or absence of correlation between 2013 and 2014

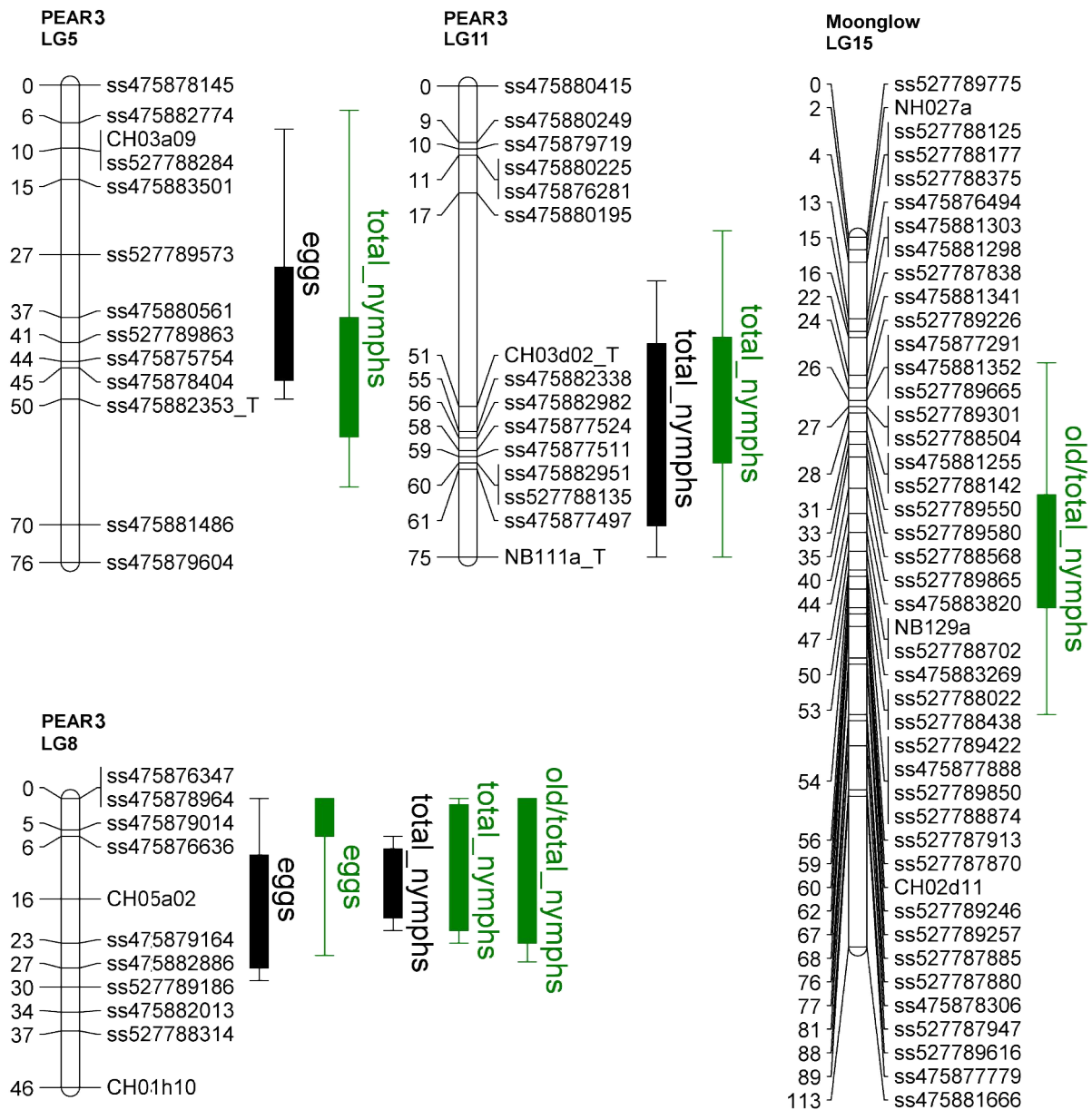


Figure 3.7: Quantitative trait loci detected for three psylla resistance traits in an interspecific pear population in 2013 (black bars) and 2014 (green bars)

Tables

Table 3.1: Shapiro-Francia normality test on the phenotypic data for psylla resistance in a segregating interspecific pear population.

The statistical values and the p-values are reported for each measured variable (number of eggs, total nymphs, young nymphs and old nymphs). For $p < 0.05$, the data distribution is not normal.

Raw data	Shapiro-Francia test	
	statistic value	p-value
2013		
eggs	0.871	< 2.2e-16
total nymphs	0.869	< 2.2e-16
young nymphs	0.847	< 2.2e-16
old nymphs	0.602	< 2.2e-16
2014		
eggs	0.846	< 2.2e-16
total nymphs	0.902	8.14e-15
young nymphs	0.848	< 2.2e-16
old nymphs	0.861	< 2.2e-16

Table 3.2: Lilliefors and Shapiro-Francia normality test for phenotypic means of psylla resistance adjusted for environmental factors in a pear segregating population.

The statistical values and the p-values are reported for each measured variable (number of eggs, total nymphs, young nymphs, old nymphs and old/total nymphs ratio). For $p < 0.05$, the data distribution is not normal.

Adjusted means	Lilliefors test		Shapiro-Francia test	
	statistic value	p-value	statistic value	p-value
2013				
eggs ^a	0.153	4.482e-06	0.939	3.152e-04
total nymphs	0.075	0.173	0.978	0.079
young nymphs	0.063	0.418	0.989	0.475
old nymphs	0.152	4.285e-06	0.837	4.016e-08
old/total nymphs	0.121	8.190e-01	0.893	2.747e-06
2014				
eggs ^a	0.085	0.075	0.975	0.060
total nymphs	0.134	1.840e-04	0.947	0.001
young nymphs	0.084	0.087	0.970	0.024
old nymphs	0.166	5.303e-07	0.930	1.430e-04
old/total nymphs	0.080	0.123	0.983	0.213

a = arithmetic means

Table 3.3 Broad sense heritability estimation (H^2) and phenotypic variation explained by all the significant QTLs (R^2) for pear psylla resistance in a segregating interspecific pear population

	2013		2014	
Trait	H^2	R^2 (%)	H^2	R^2 (%)
eggs	0.45	22	0.58	13
total nymphs	0.63	39	0.68	51
old/total	0.65	no QTL	0.45	24

Table 3.4: QTL mapping for psylla resistance in a segregating interspecific pear population

QTLs were detected using Interval Mapping or restricted Multiple QTL Mapping with MapQTL 5.0 and Haley-Knott regression with Rqtl. Putative QTLs that were slightly below the threshold are in italic. For each trait and method, the LOD threshold, the linkage groups (LGs) and the parental map on which the QTLs were detected, the marker closest to the peak and the LOD score and R^2 are shown. For the trait "total nymphs" the $globalR^2$ was also calculated

	Interval Mapping or restricted Multiple QTL mapping (MapQTL 5.0)							Haley-Knott regression (Rqtl)					
Trait	LOD Threshold	LG	parental map	Marker closest to peak	LOD	R ²	globalR ²	LOD Threshold	LG	parental map	Marker closest to peak	LOD	R ²
2013													
eggs	3.2	8	PEAR3	CH05a02	5.10	22.2	44.9	3.1	8	PEAR3	CH05a02	3.89	17.2
		5	PEAR3	ss475875754	2.57	9.9							
total nymphs	3.3	8	PEAR3	CH05a02	9.90	39.1	56.0	3.2	8	PEAR3	CH05a02	7.65	30.7
		11	PEAR3	ss475877524	2.56	8.4							
old/total nymphs	3.5	no QTL detected						3.2	no QTL detected				
2014													
eggs	3.1	8	PEAR3	ss475878964	3.08	12.5	NA	3.2	8	PEAR3	ss475878964	2.46	10.9
total nymphs	3.1	8	PEAR3	CH05a02	7.54	29.5	50.5	3.2	8	PEAR3	CH05a02	6.24	25.4
		5	PEAR3	ss475878404	3.19	10.8							
		11	PEAR3	ss475882338	2.56	7.7	65.7						
old/total nymphs	3.1	8	PEAR3	ss475876636	2.90	11.3	24.4	3.1	15	Moonglow	ss475883269	3.15	13.7
		15	Moonglow	ss475883269	2.56	10.1			8	PEAR3	ss475876636	2.98	13.1

NA = not applicable

CHAPTER 4. Mapping QTLs for Fire Blight Resistance

Fire blight is probably the most serious disease for pear. It is present worldwide, including France and New Zealand; hence the two subsets of PEAR3 x ‘Moonglow’ progeny could be screened for the resistance to fire blight. *E. amylovora* is considered a quarantine pest both in France and New Zealand, as in many other countries around the world, and thus two local isolates were employed for the experiments at the two locations. At the INRA of Angers the highly aggressive strain CFBP 1430 from the French Collection of Phytopathogenic Bacteria (Paulin and Samson 1973), isolated from *Crataegus* spp., has been used for years for several fire blight experiments. On the contrary, such a reference strain does not exist at PFR in New Zealand; therefore, here a number of *E. amylovora* isolates have been tested with the attempt to identify the most aggressive one on PEAR3 x ‘Moonglow’ population. In particular, I compared six different isolates: Ea236, Ea241, Ea9910, Ea9148, Ea4450 and Ea233. The test was performed by inoculating with these six isolates the parents and some offspring of PEAR3 x ‘Moonglow’ cross, as well as the European cultivars ‘Doyenne du Comice’, ‘Magness’, ‘Williams Bon Chrétien’, ‘Packman’s Triumph’ and the Chinese cultivar ‘Xue Hua Li’, as controls. Inoculations were performed in the glasshouse, and the severity of the infections was assessed 4 weeks later. This test was sufficient to allow the selection of the best *E. amylovora* isolate to employ in the phenotyping experiments of PEAR3 x ‘Moonglow’ population. The average severities for all the genotypes tested were higher for the three isolates Ea9148, Ea4450 and Ea233. However, the severity observed on the susceptible parent, PEAR3, inoculated with Ea4450 was extremely low (less than 20%), hence this isolate was excluded. Between Ea9148 and Ea233, the first one was chosen because it was isolated from the Japanese pear *P. pyrifolia*, while the other was isolated from *M. x domestica*.

Once the *E. amylovora* isolates were selected, the French and New Zealand subsets of the population were phenotyped. The experiment in New Zealand was repeated across two subsequent years, and although in the first one we experienced some technical difficulties, the

results from the two years were consistent. A major QTL, stable across the two environments, was detected, along with other small effect QTLs, putatively strain-specific. SSRs scanning of the progenitors of PEAR3 and ‘Moonglow’ was also performed, and the origin of the QTLs resistant allele determined. Furthermore, similarities with QTLs for fire blight resistance previously detected in other pear segregating populations allowed the identification of candidate markers for MAS.

This work is the object of an article still under editing, which will be submitted to *Molecular Breeding*. Together with the study on pear psylla resistance, I presented the early results of the QTL mapping for fire blight resistance with a poster (reported in the Annex 3) at the 57th Italian Society of Agricultural Genetics (SIGA) Annual Congress, which was held in Italy in 2013, and the final results with an oral communication at the 7th International Rosaceae Genomics Conference (RGC7), held in the USA in 2014.

The LOD score curves for all the QTLs detected in this study are reported in the Annex 5.

This article is planned to be submitted for publication in *Molecular Breeding*.

A QTL detected in an interspecific pear population confers stable fire blight resistance across different environments and genetic backgrounds

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Abstract

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is one of the most serious diseases of pear. To effectively control fire blight, the development of pear cultivars with a durable resistance is extremely important and is a key objective of most pear breeding programs throughout the world. We phenotyped seedlings from the interspecific pear population PEAR3 (*P. x bretschneideri* X *P. communis*) x 'Moonglow' (*P. communis*) for fire blight resistance at two different geographic locations, in France and New Zealand respectively, employing two local *E. amylovora* isolates. Using a genetic map constructed with single nucleotide polymorphism (SNP) and microsatellite (SSR) markers previously developed for this segregating population, we detected a major quantitative trait locus (QTL) on linkage group (LG)

2 of ‘Moonglow’ ($R^2 = 12.9 - 34.4\%$), which was stable in both environments. We demonstrated that this QTL co-localizes with another major QTL for fire blight resistance previously detected in ‘Harrow Sweet’, and that the two corresponding favorable alleles could be not identical by descent. We also identified some small effect ($R^2 = 8.1 - 14.8\%$) QTLs derived from the susceptible parent PEAR3. In the discussion of our results, we draw conclusions regarding the large effect QTL on LG2 and we propose SNP and SSR markers as candidates for marker assisted breeding (MAB) for fire blight resistance.

Key words: *Erwinia amylovora*; *Pyrus communis*; *Pyrus x bretschneideri*; marker assisted breeding

Introduction

Fire blight is a devastating disease of *Rosaceae* species (Vanneste 2000) and the most economically significant disease for pear (*Pyrus communis* L.) growers and traders. It is caused by the gram-negative bacterium *Erwinia amylovora* (Burrill) Winslow et al. (Vanneste 2000), which is widespread in several countries all over the world (Bonn and Van Der Zwet 2000). *E. amylovora* is considered a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO) (EPPO 1977), by the Asia and Pacific Plant Protection Commission (APPPC) and by other Regional Plant Protection Organizations (Bokszczanin et al. 2009, <http://www.cabi.org/isc/datasheet/21908>), hence presence of bacteria on fruit and plant material can constrain trade. The organism enters the plant through natural openings in flowers or through wounds and then develops systemically into the plant vessels, causing the rapid necrosis of all infected tissues and the production of exudation droplets (Malnoy et al. 2012) (Figure 4.1). Its direct damage is linked to an extreme reduction of yield (EPPO 1977), but more importantly the plant, once infected, has to be completely destroyed, since all organs are potential sources for dissemination (Thomson 2000). Control of this pathogen is difficult and no strategy is completely effective by itself (Paulin 1990): application of chemical compounds, mainly antibiotics and copper, as well as biological control strategies must be combined with the eradication of infected plants (EPPO 1977; Norelli et al. 2003). The development of cultivars with durable resistance is of extreme importance for an effective integrated management of fire blight disease (Lespinnasse

and Aldwinckle 2000), and a number of pear and apple breeding programs around the world have focused on this objective since the early 80s.

While the Asian pear species of economic importance, such as *P. ussuriensis*, *P. pyrifolia* Nakai, *P. calleryana* and *P. betulaeifolia*, tend to be more resistant to *E. amylovora* than *P. communis* and other Asian species (Van Der Zwet et al. 1974; Bell and Ranney 2005), and hence are used more frequently for the development of fire blight resistant cultivars, resistant accessions can be found in all species (Paulin 1990; Lespinasse and Aldwinckle 2000). Both phenotypic (e.g. Durel et al. 2004) and genotypic (e.g. Dondini et al. 2004) studies suggest that the trait is polygenic in all species, as quantitative trait loci (QTLs) for the control of resistance have been detected in both *P. ussuriensis* and *P. communis*. To date, three such QTLs have been mapped to linkage groups (LGs) 2, 4 and 9 respectively of the fire blight resistant European pear ‘Harrow Sweet’ (Dondini et al. 2004; Le Roux et al. 2012), one QTL was identified in resistant accession 18 of the Asian species *P. ussuriensis* and another in the susceptible *P. communis* ‘Doyenne du Comice’ (Bokszczanin et al. 2009).

The development of fire blight resistant pear cultivars with high fruit quality characteristics are key objectives of the Plant & Food Research (PFR, New Zealand) (White and Brewer 2002) and INRA (Angers, France) (Durel et al. 2004) Pear Breeding Programs. An interspecific pear breeding population PEAR3 (*P. x bretschneideri* X *P. communis*) x ‘Moonglow’ (*P. communis*) was employed for our study. ‘Moonglow’ is a well-known cultivar with low susceptibility to fire blight (Quamme 1977; Paulin 1990), while PEAR3 is highly susceptible (unpublished data). Subsets of the progeny were evaluated for fire blight resistance both in France and New Zealand, using two local *E. amylovora* isolates. Dense parental genetic maps constructed in this interspecific pear population by Montanari et al. (2013) were subsequently employed to detect QTLs for control of resistance to fire blight.

Materials and Methods

Plant materials

A subset of the F1 population derived from the cross PEAR3 x ‘Moonglow’ was grown at the INRA site at Angers, France, and another subset at PFR in Havelock North, New Zealand. Three

phenotyping experiments for fire blight resistance were performed: one in 2013 on 85 individuals from the French progeny subset and two on the New Zealand one, using 90 individuals in 2013 and 105 in 2014, with 86 progeny used in both experiments. Both parents (PEAR3 and ‘Moonglow’) and control cultivars were included in these experiments: in France, *P. communis* ‘Williams Bon Chrétien’ and ‘Angélys’ were used as susceptible controls and Michigan-US 437, ‘Harrow Sweet’ and the interspecific hybrid NY10355 (*P. ussuriensis* X *P. communis*) as resistant controls, while in New Zealand the susceptible *P. communis* ‘Williams Bon Chrétien’ and *P. x bretschneideri* ‘Xue Hua Li’, and the resistant *P. communis* cultivar ‘Magness’ were employed. All plants were grafted on *P. communis* ‘Kirchensaller’ rootstock in France, on *P. calleryana* or *P. betulaefolia* rootstocks in New Zealand in 2013, and on *P. betulaefolia* in 2014, and were grown on PB5 planter bags filled with standard “apple cutting” potting mix. An average of eight, three and four replicates per genotype were evaluated in France, in New Zealand in 2013 and in New Zealand in 2014, respectively, randomized in the greenhouse, with one or two shoots per replicate inoculated. Potted plants were placed on benches and the climatic conditions in the greenhouse were controlled in order to keep optimal growing conditions for both plants and bacteria. In France, average temperature was 20°C day/18°C night (16/8 hours) and relative humidity (RH) 85%, while in New Zealand temperature was 22-24°C day/18-20°C night and RH 90%. In France, water was applied automatically by drip-irrigation two times per day, one of which with the addition of fertilizers (N 15 – P 10 – K 30); in New Zealand plants were watered once every 2-3 days in early stages of development and daily when larger.

Inoculation and disease assessments

Different local *E. amylovora* isolates were used for the inoculations in France and in New Zealand respectively. CFBP 1430, isolated from *Crataegus* spp., is the reference strain at Angers (Smits et al. 2010) and was used for our phenotyping experiment there. In New Zealand, the Ea9148 strain, isolated from *P. pyrifolia*, was chosen for both phenotyping experiments.

E. amylovora was grown for 28 h on King’s medium B at 26°C and on the day of the inoculation the bacterial cells were harvested from the plates and re-suspended in sterile water. The inoculum concentration was adjusted to 10⁷ colony forming units (cfu)/mL for the CFBP 1430 isolate and

to 10^9 cfu/mL for the Ea9148 isolate. Actively growing shoots were inoculated by bisection of the two youngest unfolded leaves with scissors previously dipped into the inoculum solution (Figure 4.2). Multiple inoculations were performed on a weekly basis in order to ensure an average length of the shoots of 30 cm.

At 7, 14, 21 and 28 days post inoculation (dpi) the length of the necrosis developing on the stem below the inoculated leaves was measured for each shoot. The severity of infection (percentage of necrosis length over total shoot length) was calculated at each time point and the Area Under Disease Progress Curve (AUDPC or ADPC) was computed as in Shaner and Finney (1977).

Statistical analysis and QTL mapping

All statistical analyses were performed with R studio (<http://www.rstudio.com>); shoots that had stopped growing after inoculation were removed prior to analyses for a more correct evaluation of resistance.

Each phenotyping experiment was analyzed separately: raw data were checked for normality using the Shapiro-Francia test (Thode 2002), and the significance of the “inoculation date” and “rootstock” (when more than one was used) effects on severity and AUDPC were tested with ANOVA (Analysis of Variance). Residual error distributions were checked graphically for normality (with “residual versus fitted” and “normal quantile-quantile” plots), in order to ascertain the validity of the ANOVA model. Averages were adjusted according to the significant ($\rho < 0.05$) effects and the distributions of the adjusted means were again tested for normality with the Shapiro-Francia test. The correlation between severity at 28 dpi and AUDPC was evaluated for each experiment and the Spearman rank correlation coefficient calculated.

QTL mapping was carried out on severity at 28 dpi and AUDPC adjusted means from the three experiments separately. The broad sense heritability (H^2) of genotypic means within each progeny was estimated for both traits as explained in Calenge et al. (2005). Interval mapping (IM) (Lander and Botstein 1989) and, when multiple QTLs were detected, restricted Multiple QTL Mapping (rMQM) (Jansen 1993; Jansen and Stam 1994), were conducted with MapQTL 5.0 software (Van Ooijen 2004). The genome-wide significance LOD thresholds ($\rho = 0.05$) were determined by permutation tests (Churchill and Doerge 1994) with 1000 permutations. The

genetic maps of the parents PEAR3 and ‘Moonglow’ used for QTL mapping were published by Montanari et al. (2013), with minor modifications as explained in Chapter 3. Possible epistatic interactions between the QTLs detected were tested and the percentage of the phenotypic variation explained by all the significant ($\rho < 0.05$) QTLs and epistatic interactions (*globalR*²) was estimated as described by Montanari et al. (unpublished).

SSR-based analysis of the pedigree

Apple and pear Simple Sequence Repeat markers (SSRs) mapping within the confidence interval of the detected major QTL and of two small effect QTLs were selected for the analysis of pedigree (Gianfranceschi et al. 1998; Liebhard et al. 2002; Yamamoto et al. 2002; Sawamura et al. 2004; Silfverberg-Dilworth et al. 2006; Nishitani et al. 2009) (Table 4.1). CH02f06, CH05c07, NB106a, NB129a, NB130b, NH212a, TsuENH017 and TsuENH062 were used to scan PEAR3, ‘Moonglow’, their progenitors (*P. communis* Michigan-US 437, ‘Roi Charles de Würtemberg’, ‘Williams Bon Chrétien’ and *P. x bretschneideri* ‘Xue Hua Li’), as well as 16 and 109 individuals from the French and New Zealand progeny subsets, respectively, and ‘Harrow Sweet’. All forward primers were tailed with an M13 sequence to allow binding with a fluorescent dye, as in Oetting et al. (1995). PCR mixtures consisted of 20 ng of genomic DNA, 1x Platinum[®] PCR buffer (Invitrogen, Carlsbad, CA), 1.5 mM of MgCl₂, 0.2 mM of dNTPs, 0.013 and 0.2 μ M of each forward and reverse primer, respectively, 0.5 unit of Platinum[®] Taq DNA Polymerase (Invitrogen) and 0.3 μ l of fluorescent dye (6-FAM, PET, NED or HEX), in a final volume of 15.5 μ l. Amplifications were performed in an Applied Biosystems[®] GeneAmp[®] PCR System 9800 (Applied Biosystems[®] by Life Technologies[™]) at PFR, as described by Bus et al. (2005), with the following modifications: the number of touchdown cycles was 5, with a decrease of 1°C/cycle (63°C to 58°C or 61°C to 56°C, depending on the marker, Table 4.1) and the main amplification reactions consisted of 35 cycles. Fragments were analyzed as outlined in Montanari et al. (2013). These 8 SSR markers were incorporated in the PEAR3 and ‘Moonglow’ genetic maps using JoinMap v4.0 software (Van Ooijen 2006) and maps were drawn using MapChart 2.2 (Voorrips 2002).

Results

Disease assessment and comparison between French and New Zealand experiments

Temperature and humidity in the greenhouses were regulated automatically, in order to assure optimal growing conditions for both the plants and the bacterium. Furthermore, the inoculation protocols were standardized, as far as possible, between France and New Zealand, with plants of the same age and size; however, the *E. amylovora* isolates employed, as well as their inoculum concentrations (10^7 and 10^9 cfu/mL, respectively) differed. The severity of the disease (also called PLL – i.e. percentage lesion length) was calculated at 7, 14, 21 and 28 dpi in order to estimate the AUDPC.

The year did not have a significant effect (according to the Kruskal Wallis test) on the phenotypic data collected in New Zealand, however, the adjusted means for severity at 28 dpi and AUDPC were significantly ($\rho < 0.05$) not correlated (Spearman's correlation) between the two years. As the results of the QTL analysis from 2013 were consistent with those from 2014, but less powerful, we present the data from the second year of experiments only, for simplicity.

A non-parametric Kruskal-Wallis test revealed a significant difference between the two parents for both fire blight severity at 28 dpi and AUDPC in both environments (Figure 4.3). PEAR3's vulnerability to infection was similar to that of the most susceptible controls 'Williams Bon Chrétien' and 'Xue Hua Li', and higher than that of 'Angély's' (especially for the AUDPC trait). 'Moonglow' resistance was comparable to that of Michigan-US 437 in France and 'Magness' in New Zealand. The ranking of the averages showed the existence of some transgressive lines in the segregating population, either more susceptible than PEAR3, or more resistant than 'Moonglow', consistent with the hypothesis of a polygenic control of fire blight resistance.

When PEAR3, 'Moonglow' and 'Williams Bon Chrétien' were phenotyped in France and New Zealand using different *E. amylovora* isolates, disease incidence differed between the two sites, with respect both to severity at 28 dpi and AUDPC values (Table 4.2). The 2014 New Zealand experiment exhibited significantly less severe disease by both measures (Chi-squared test $\rho = 0.02$ and $\rho < 2.2e^{-16}$, respectively for severity and AUDPC) than the experiment in France in 2013.

Statistical analysis on the phenotypic data

The pear scion growth was uneven in all the experiments, both in parental material and segregating populations. For this reasons we performed multiple inoculations, with shoots inoculated within a range of 20-40 cm long (but mainly 25-35 cm), to ensure that plants were at a similar development stage. Four and six inoculations were carried out in France in 2013 and in New Zealand in 2014, respectively, with the effect of “inoculation date” being significant for both severity at 28 dpi and AUDPC, according to Kruskal-Wallis test. The data from the last inoculation were excluded from the New Zealand 2014 experimental analysis, because they increased the heterogeneity of the whole data set, since a very small number of plants was inoculated at this date and just a few genotypes were represented. Even though the raw data distribution showed deviation from normality according to the Shapiro-Francia test (Table 4.3, Supplemental Figure 4.1), the residual error distribution was always normal, and hence the analysis of variance was reliable and the means were adjusted according to “inoculation date”. The adjusted means distribution was normal only for AUDPC in France according to the Shapiro-Francia test (Table 4.4), and it appeared more skewed towards resistance in New Zealand than in France, reflecting the more severe disease symptoms observed in France than in New Zealand (Supplemental Figure 4.2). The adjusted means for severity at 28 dpi and AUDPC were highly correlated both in France (Spearman coefficient $r_s = 0.98$) and in New Zealand in 2014 ($r_s = 0.98$) (Supplemental Figure 4.3).

QTL detection, heritability and phenotypic variation

Following the permutation tests, the significance threshold for QTL detection was established between LOD = 3.2 and 3.3, depending on the trait and the experiment (Table 4.5, column 2). A QTL was detected for all traits and experiments on LG2 of ‘Moonglow’, the resistant parent (Table 4.5, Figure 4.4). The highest LOD score for this QTL was observed in France (LOD = 7.87 and 8.50, respectively for severity and AUDPC), where it explained more than 30% of the phenotypic variation. In New Zealand in 2014, the LOD scores for this QTL were 6.55 for severity and 4.47 for AUDPC, and the amount of phenotypic variance explained (R^2) were 17% and 13%, respectively. The closest SNP markers to the QTL peak were ss527789563, with

resistance associated with the “C” allele, or ss527789655, with resistance associated with the “G” allele (Table 4.5, Supplemental Figure 4.4). These two markers are 2 cM apart. The percentage of French and New Zealand progeny carrying the favorable allele for these two markers was higher in the French subset (58% and 61%) than in the New Zealand one (43% and 45%). Smaller effect QTLs were detected, with some differences among the two experiments (Table 4.5). In France, a QTL was mapped to LG9 of PEAR3 for both severity and AUDPC, with LOD score of 4.07 and 4.02 and explaining 14.8% and 13.9% of the phenotypic variation, respectively for the two traits. The QTL peak co-located with marker ss475879846, with resistance associated with the “C” allele. Although a peak in the LOD curve for severity was observed on the same LG in New Zealand in 2014, it was below the threshold and did not co-locate with the QTL found in France (they were 13 cM apart) (Supplemental Figure 4.4). In the New Zealand 2014 experiment, three additional QTLs, that were not found in France, were detected for both traits, and these were mapped to LGs 7, 12 and 15 of the susceptible parent PEAR3 (Figure 4.4), with R^2 ranging between 8% and 12% (Table 4.5). The $globalR^2$ calculated for both traits in France and in New Zealand in 2014 was higher than 30% (Table 4.5). Epistasis was significant only between the QTLs detected in New Zealand on LGs 7 and 12 of PEAR3. The residual error calculated following the QTL analysis for both traits, severity and AUDPC, were normally distributed both in France and in New Zealand in 2014.

Heritability was always very high, over 0.80 in France and 0.60 in New Zealand in 2014 (Table 4.6).

Origin of the favorable QTL alleles for resistance

Eight microsatellite markers polymorphic in the PEAR3 x ‘Moonglow’ population were screened over DNA from 125 progeny and mapped to LG2, LG9 and LG15 (four, three and one markers, respectively) (Table 4.1, Figure 4.4). The five SSR markers located in the genomic regions where QTLs were detected were then used to identify the source of the alleles associated with resistance in the pedigrees of the parents (Table 4.7, Figure 4.5). The size of the alleles was adjusted by subtracting 18 bp of the M13 tail. On LG2 of ‘Moonglow’, the resistance was associated with the 176 bp and 179 bp alleles of CH02f06 and TsuENH017, respectively, which

were inherited from ‘Roi Charles de Würtemberg’. ‘Harrow Sweet’ and ‘Williams Bon Chrétien’ profiles for TsuENH017 were 169-189 bp and 189-195 bp, respectively, like those reported by Le Roux et al. (2012). For the small effect QTL on LG9 of PEAR3, the favorable alleles at CH05c07 and NB130b were 141 bp and 90 bp, respectively, both inherited from ‘Xue Hua Li’. ‘Harrow Sweet’ did not carry any of these favorable marker alleles for either LG2 or LG9. For the QTL on LG15 of PEAR3, the allele in coupling phase with the resistance at NB129a (131 bp) derived from ‘Xue Hua Li’.

Discussion

Phenotyping of two subsets from the interspecific pear breeding family PEAR3 x ‘Moonglow’ for fire blight resistance in France and in New Zealand enabled us to identify a major QTL on LG2 of the European parent, ‘Moonglow’. This QTL is stable across the two environments, and SSR analysis of the pedigree of the parents indicated that the favorable allele is inherited from the European pear cultivar ‘Roi Charles de Würtemberg’. Four small effect and possibly strain-specific QTLs were detected in the susceptible parent PEAR3, two of which were inherited from *P. x bretschneideri* ‘Xue Hua Li’. SSR markers in common enabled us to compare the location of the ‘Moonglow’ LG2 QTL with the ‘Harrow Sweet’ LG2 QTL detected by Dondini et al. (2004), and whose position was refined later by Le Roux et al. (2012). Because the pear and apple genomes are highly syntenic (Yamamoto et al. 2004; Pierantoni et al. 2004; Celton et al. 2009), we also compared the locations of our newly mapped QTLs with those mapped to orthologous regions in apple.

Optimization of the protocol for inoculation and disease assessment

Because of quarantine restrictions both in France and New Zealand, artificial *E. amylovora* inoculations had to be performed inside the greenhouse, which is, however, the common practice for the evaluation of fire blight resistance in breeding populations (Peil et al. 2009). Nevertheless, greenhouse assays are more efficient than field assays relying on natural occurring inoculations; moreover, Quamme et al. (1976) demonstrated that there is a high correlation between tests

performed in controlled conditions, with the employment of several replicates, and the resistance observed in the orchards (Peil et al. 2009).

Although a range of phenotyping techniques for assessing fire blight resistance are available to breeders (Peil et al. 2009), inoculation by the cut-leaf method (Maas Geesteranus and Heyting 1981) is widely applied in both apple and pear. Durel et al. (2004) performed the inoculation on 20-30 cm tall pear seedlings, while Bokszczanin et al. (2009) used plants of 50 cm. In our study we inoculated shoots longer than 25 cm, since smaller pear plants frequently stopped growing after the inoculation, compromising the disease development. In order to ensure as much homogeneity as possible among the replicates, a balance between the uneven growth of the shoots and the number of inoculation dates had to be found; therefore, in our experiments inoculations were performed on plants in the 25-35 cm length range, with only few replicates inoculated at smaller (but no less than 20 cm) and greater (no more than 40 cm) length. Assessment of the disease incidence usually involves measuring the necrosis length at multiple (2-4) time points during symptoms development (e.g. Calenge et al. 2005; Durel et al. 2009; Bokszczanin et al. 2009; Khan et al. 2013), or a single assessment of severity at 21 (Durel et al. 2004; Fahrentrapp et al. 2013) or 28 dpi (Peil et al. 2011; Le Roux et al. 2012; Vogt et al. 2013; Emeriewen et al. 2014). Because of the variability in the responses of replicates to fire blight, a single assessment may result in an inaccurate evaluation of the resistance (Taylor et al.. 2002). Performing multiple weekly disease assessments of 4-8 replicates up to 28 dpi enabled us to calculate the disease development rate based on the AUDPC method (Shaner and Finney 1977) and to compare the QTL map with that based on the severity at 28 dpi measure. The correlation between the two methods was very high in both the France 2013 and New Zealand 2014 experiments (Supplemental Figure 4.3), with the difference at higher disease levels being explained by variations in disease progress in the middle stages of symptom development, which are neglected in the single final severity observations. This in turn may explain the tendency of QTL intervals based on AUDPC to be longer than those based on severity (Figure 4.4). Nevertheless, the consistency of the results of the QTL analysis between the two measures of phenotype (Table 4.5) indicates that they both gave a reliable evaluation of the resistance.

Evaluation of the differences between fire blight phenotyping in France and New Zealand

We performed phenotyping in two different environments (in France and in New Zealand), using in each case local isolates of *E. amylovora* (CFBP 1430 and Ea9148, respectively) to inoculate different subsets of the PEAR3 x ‘Moonglow’ progeny, for reasons related to biosecurity legislation in the two countries. A higher proportion of resistant genotypes was observed in New Zealand, as shown by the distributions of the adjusted means for both severity at 28 dpi and AUDPC (Supplemental Figure 4.2). At first, we evaluated the possibility that there was a difference in the genetic profiles of the progeny subsets due to the random partitioning of the seeds, with more resistant genotypes occurring in New Zealand than in France. Conversely, there were more genotypes carrying the allele in coupling phase with resistance at the LG2 QTL in France, where the disease was more severe, than in New Zealand. Furthermore, this trend was supported by the comparison of PEAR3, ‘Moonglow’ and ‘Williams Bon Chrétien’ responses between the two experiments, which demonstrated a significantly greater severity of the disease in France, as well as a faster development (indicated by AUDPC) (Table 4.2). There are two possible reasons for the phenotyping differences observed in France and New Zealand. Firstly, differences in the environmental conditions for plant growth, and secondly, different aggressiveness of the two isolates employed. In spite of the 100-fold higher inoculum concentration used and the maintenance of both temperature and humidity at higher values in New Zealand, reflecting the optimal conditions for field infection, disease severity was lower in New Zealand than in France, which strongly suggests that the difference between the two sites is mainly due to differences in pathogenicity of the isolates used. While the respective *E. amylovora* isolates were selected for high aggressiveness in their country of origin, CFBP 1430 appeared to be much more aggressive than Ea9148, in line with earlier findings on variability in pathogenicity among *E. amylovora* isolates (Taylor et al. 2002; Vrancken et al. 2013). CFBP 1430 has been employed for fire blight resistance screening in Angers for many years because of its highly aggressive nature. It is noteworthy that, although the lower pathogenicity of the New Zealand Ea9148 isolate might be the basis of the smaller effect of the LG2 QTLs (Table 4.5), it did enable the detection of additional QTLs not exhibited following inoculation with CFBP 1430.

Large and small effect QTLs were detected

A stable large effect QTL was mapped to the top of ‘Moonglow’ LG2, with a LOD peak close to SNP markers ss527789563 (15cM) and ss527789655 (17cM) (Table 4.5, Supplemental Figure 4.4). In France, this QTL contributed most of the observed phenotypic variation for both measures of phenotype, since its R^2 was only 12% lower than the $globalR^2$ calculated with the additive effect of the smaller QTL on PEAR3 LG9. As discussed above, in New Zealand, the ‘Moonglow’ LG2 QTL exhibited a lower R^2 than in France; however, it is probable that the effect of this QTL was mitigated by the presence of the other three QTLs mapped to LGs 7, 12 and 15 of PEAR3, two of which also had epistatic interactions (LG7 and LG12 QTLs). These QTLs may be strain-specific to isolate Ea9148, since they were not observed in the population subset phenotyped in France with isolate CFBP 1430. A putative QTL on LG9 was detected in New Zealand as well, however the LOD score is below the threshold (LOD = 2.56, threshold = 3.3) and it is in a different location than the one found in France (Supplemental Figure 4.4), then it is not clear whether it is strain-specific or broad-spectrum. It is also possible that the different environmental conditions experienced by the plants in France and in New Zealand might have affected QTLs identification on LGs 7, 9, 12 and 15.

The detection of multiple QTLs in PEAR3, the highly susceptible parent, as well as the presence of some transgressive lines, is an indication of the polygenic nature of fire blight resistance in this population, consistent with what has been previously reported in other pear (Dondini et al. 2004; Le Roux et al. 2012) and apple (Calenge et al. 2005; Durel et al. 2009) families. The broad-sense heritability was estimated to be very high in both experiments, supporting the reliability of the QTLs detected. As the $globalR^2$ was lower than the H^2 (Table 4.6), there might be other loci linked to fire blight resistance that were not identified. This may be due to the small size of the progeny subsets in the two separated experiments (85 in France and 105 in New Zealand in 2014), which allowed only the identification of higher effect QTLs, or to the possible presence of additional QTLs in regions not covered by markers, since neither parental genetic map was saturated. With regards to this last point, it is important to note that the interspecific population under study turned out to be subject to the pre- and post-zygotic incompatibilities described in Chapter 5, which might be the cause of some of the gaps in the parental genetic maps. Hence, the

effect of putative resistant loci derived from either parent and mapping to one of these regions might not be observed in the progeny, because of linkage to a lethal locus.

Favorable allele for resistance were inherited from a highly resistant European cultivar and a susceptible Asian cultivar

The parents of ‘Moonglow’, Michigan-US 437 and ‘Roi Charles de Wurtemberg’, are both resistant to fire blight (Paulin 1990; Durel et al. 2004). However, the SSR scan we performed with markers mapped within the ‘Moonglow’ LG2 QTL interval demonstrated that the favorable alleles were inherited from ‘Roi Charles de Wurtemberg’ (Table 4.7, Figure 4.5).

Although the identity of the pollen parent of PEAR3 is unknown (Montanari et al., unpublished), it was possible to ascertain that the favorable allele at the PEAR3 LG9 and LG15 QTLs were inherited from *P. x bretschneideri* ‘Xue Hua Li’. Both PEAR3 and ‘Xue Hua Li’ are extremely susceptible to fire blight (Figure 4.3). However, Asian species of pear have frequently been reported as sources of fire blight resistance (Paulin 1990; Bell and Ranney 2005; Peil et al. 2009), and it is not surprising that ‘Xue Hua Li’ carried alleles conferring resistance and passed them to its offspring. Indeed, this phenomenon has been observed in many other host-pathogen interactions: for example, in peach-*Sphaerotheca pannosa* var. *persicae* (powdery mildew) (Foulongne et al. 2003); in melon-*Fusarium oxysporum* f. sp. *melonis* (Perchepped et al. 2005); in *Arabidopsis-Pseudomonas syringae* pv. *tomato* (Perchepped et al. 2006).

The QTLs on LG2 of ‘Moonglow’ and ‘Harrow Sweet’ co-localize, however they are different

The first fire blight resistance QTLs in pear were mapped by Dondini et al. (2004) to LGs 2a, 2b, 4 and 9 of the resistant European cultivar ‘Harrow Sweet’. Later, Le Roux et al. (2012) reported the combination of LGs 2a and 2b in this cultivar and the accurate location of the major QTL on this LG, as well as confirming the QTL on LG4. The alignment of our ‘Moonglow’ LG2 map with that of ‘Harrow Sweet’ using two SSR markers in common (CH02f06 and TsuENH017), indicates that both QTLs co-locate immediately downstream to TsuENH017. Le Roux et al. (2012) identified the favorable alleles for the resistance derived from ‘Harrow Sweet’, using TsuENH017 and another SSR marker, and traced back their origin to ‘Early Sweet’. In our study

of the ‘Moonglow’ QTL, the 179 bp allele of TsuENH017 was in coupling phase with the resistance, while in ‘Harrow Sweet’ the favorable allele was 169 bp (Table 4.7, Figure 4.5). This means that, although the QTLs on LG2 co-localize between the two cultivars, the resistance is associated with different alleles of TsuENH017. Interestingly, Le Roux et al. (2012) reported the SSR TsuENH017 profile for ‘Old Home’ as 179-189 bp, which is the same as we found for ‘Moonglow’. ‘Old Home’ is another ‘Harrow Sweet’ grandparent and it is highly resistant to fire blight (Van Der Zwet et al. 1974; Quamme 1977), even more so than ‘Early Sweet’, however its fire blight resistance has never been mapped. We suggest that part of the ‘Old Home’ resistance is linked to the 179 bp allele of marker TsuENH017 on LG2, as we found in ‘Moonglow’. Alignment of the SNP-based genetic map of LG2 of ‘Old Home’ (Montanari et al. 2013) with the ‘Moonglow’ LG2 (Figure 4.6) highlights the co-linearity between the two homologous regions underlying the fire blight resistance QTL peak of ‘Moonglow’ (except for the inversion of two neighboring markers, which could be due to genotyping errors or missing data). Moreover, the SNP-markers in this region have the same haplotype for the two cultivars (Figure 4.6). Consequently, it is highly probable that ‘Old Home’ carries the same fire blight resistance QTL as ‘Moonglow’. We can therefore conclude that this major LG2 QTL for fire blight resistance in pear is stable not only in different environments, as demonstrated by our analysis in France and New Zealand, but also in different genetic backgrounds.

Candidate molecular markers for Marker Assisted Breeding

The SSR marker TsuENH017, located at the upper border of the LG2 QTL interval, is a good candidate for marker assisted breeding (MAB) for fire blight resistance in pear. However, before pear breeders could use it for MAB, studies in different genetic backgrounds need to be performed in order to validate the marker. Mapping in pear segregating populations involving ‘Old Home’ could help to confirm the hypothesis that this cultivar carries the same QTL as ‘Moonglow’ on LG2 and to validate the use of TsuENH017 for MAB. Le Roux et al. (2012) also mapped the SSRs TsuENH001 and NH033b within the LG2 QTL interval on ‘Harrow Sweet’; however, in the PEAR3 x ‘Moonglow’ population TsuENH001 had too complex a profile, and NH033b was monomorphic, and were thus not suitable for linkage analysis. However, in a

separate study (reported in Chapter 5) Montanari et al. mapped other SSR markers within the ‘Moonglow’ fire blight resistance QTL, Hi02a07 (11cM) and CN493139 (19 cM), and we verified that the alleles which at these two loci were in coupling phase with fire blight resistance were inherited from the ‘Moonglow’ pollen parent, ‘Roi Charles de Wurtemberg’. Thus, Hi02a07 or CN493139 could also be used to screen breeding populations derived from ‘Harrow Sweet’ or ‘Old Home’, and may turn out to be more suitable for MAB for fire blight resistance than TsuENH017, since they mapped closer to the QTL peak. In order to unambiguously identify the correct allele in coupling phase with the resistance, SSR markers, when used for analysis in different laboratories, need standardization prior application (Patocchi et al. 2009), as well as validation (Troggio et al. 2012).

Although microsatellite markers are normally more informative than SNP markers, SNP-based markers are now used routinely for marker assisted selection in the New Zealand apple breeding program, as the High-Resolution Melting Technique is simple (Chagné 2015) and amenable to automation. The SNPs mapped to the ‘Moonglow’ fire blight resistance QTL on LG2 would be well suited for such application in pear. Moreover, now it is possible to create affordable SNP mini-arrays to screen breeding populations with markers associated with different traits simultaneously (Peace and Bassil 2012; Gasic and Peace 2013; Ru et al. 2015).

Comparison of the small effect QTLs with other pear and apple fire blight resistance QTLs

The alignment of the LG9 genetic maps of PEAR3 and ‘Harrow Sweet’ from Dondini et al. (2004) was more difficult, because there was only one marker in common. However, we are confident that the two QTLs are located in two different regions of LG9. In fact, in the European pear cultivar the QTL was detected in relation to SSR CH05a03, while in our family it was close to SSRs CH05c07 and NB130b (Figure 4.4), which were mapped by Celton et al. (2009) about 20 cM above CH05a03. Since our LG9 QTL originated from *P. x bretschneideri* ‘Xue Hua Li’ (Figure 4.5), it is not surprising that it would be different from the one mapped to the *P. communis* cultivar. A QTL for fire blight resistance in LG9 was also mapped in apple, in ‘Nova Easygro’ (*Malus x domestica*), below CH05c07 (Le Roux et al. 2010) in a region syntenic to the PEAR3 LG9 QTL.

Concerning the other small effect QTLs mapped to PEAR3 in New Zealand (Table 4.5), no homology could be found with other pear populations used to identify fire blight resistance loci (Dondini et al. 2004; Bokszezanin et al. 2009; Le Roux et al. 2012). However, QTLs have been mapped to LGs 7, 12 and 15 in several apple accessions, and in particular: on ‘Fiesta’ (*M. x domestica*) LG7 in a cross with both ‘Prima’ and ‘Discovery’ and on ‘Discovery’ LG12 in the same cross (Calenge et al. 2005; Khan et al. 2007); on the same ‘Fiesta’ x ‘Discovery’ population on LG7, using another *E. amylovora* strain (Khan et al. 2006); on LG7 of ‘Robusta 5’ in a cross with ‘Ottawa 3’ inoculated with Ea273 and Ea2002a isolates (Gardiner et al. 2012); on LGs 12 and 15 of ‘Evereste’ (*M. x domestica* X *M. floribunda*) in a cross with ‘MM106’, and on LG12 of the *M. floribunda* clone 821, in a cross with ‘Golden Delicious’ (Durel et al. 2009); on LG15 in the *M. x domestica* F1 population ‘Co-op 16’ x ‘Co-op 17’ (Khan et al. 2013). All QTLs detected on LG7 and LG12 in apple were mapped to the bottom part of these two LGs, like those that we found in PEAR3 (Figure 4.4).

Although none of the QTLs we detected on PEAR3 was previously reported in other pear accession, QTL mapping studies for fire blight resistance in pear are not as numerous as in apple, and since European pear, Asian pears and apple genomes have all macrosyntenic relationships (Chagné et al. 2014), it is possible that these QTLs will be detected in other pear populations in the future.

Conclusion

Our detection of a major QTL for fire blight resistance in LG2 of the European parent of the PEAR3 x ‘Moonglow’ population is of remarkable importance. We have demonstrated this QTL to be broad-spectrum and stable through environments (having tested the progeny in France and in New Zealand, using two different *E. amylovora* isolates) and cultivars (‘Old Home’, which appears to have no relationship to ‘Moonglow’, carries the same QTL). We also propose SSR and SNP markers suitable for MAB for fire blight resistance in pear, after proper validation in a range of genetic backgrounds.

As the QTL on LG2 of ‘Moonglow’ had a high effect on the phenotypic variance, major genes might be located in this region. A parallel in apple would be *FB_MR5* CC-NBS-LRR, which has

been confirmed as the gene responsible for fire blight resistance on LG3 of *Malus x robusta* 5 (Broggini et al. 2014). It is worth mentioning that *P. x bretschneideri* chromosome 2 is rich in resistance (*R*) genes paralogs clusters (Wu et al. 2013), and it is possible that *P. communis* might be too. The recent publication of the *P. communis* genome sequence (Chagné et al. 2014) will facilitate the realization of fine-mapping studies, necessary to reduce the QTL confidence interval and identify candidate genes for fire blight resistance.

Apart from the ‘Moonglow’ LG2 QTL, we detected four minor effect QTLs on the genetic map of PEAR3, two of which were inherited from ‘Xue Hua Li’, demonstrating that this Asian cultivar could be used as a source of resistance to fire blight even though susceptible.

Furthermore, as the sequences of *M. x domestica* ‘Golden Delicious’ (Velasco et al. 2010), *P. x bretschneideri* ‘Dangshansuli’ (Wu et al. 2013) and *P. communis* ‘Bartlett’ (Chagné et al. 2014) genomes are now available, comparative studies focusing on the regions linked to fire blight resistance should be performed, to provide additional useful information about this extremely important trait.

Acknowledgements

We greatly thank Jasmine McCarthy (UMR1345 IRHS, Angers, France) for helping with the phenotyping experiments, Philippe Guérif (IRHS) and Gagandeep Singla (PFR) for taking care of the seedling population and helping with the disease assessment, and Roland Chartier (IRHS) for preparing the inoculum solutions in France. We also thank Lester Brewer at PFR (Motueka, New Zealand) for generating the PEAR3 x ‘Moonglow’ cross, and the INEM team of IRHS, especially Michel Boucourt, and the INRA Experimental Unit (UE Horti, Angers, France), for taking care of the plants.

Compliance with ethical standards

The experiments conducted in this research comply with the current laws of the countries in which they were performed.

Funding

SM was funded by the Fondazione Edmund Mach (FEM) PhD School. Part of this work was funded by the New Zealand Ministry of Business Innovation and Employment (MBIE) and Plant & Food Research's Pipfruit Core Funding.

Conflict of Interest

The authors declare that they have no conflict of interest.

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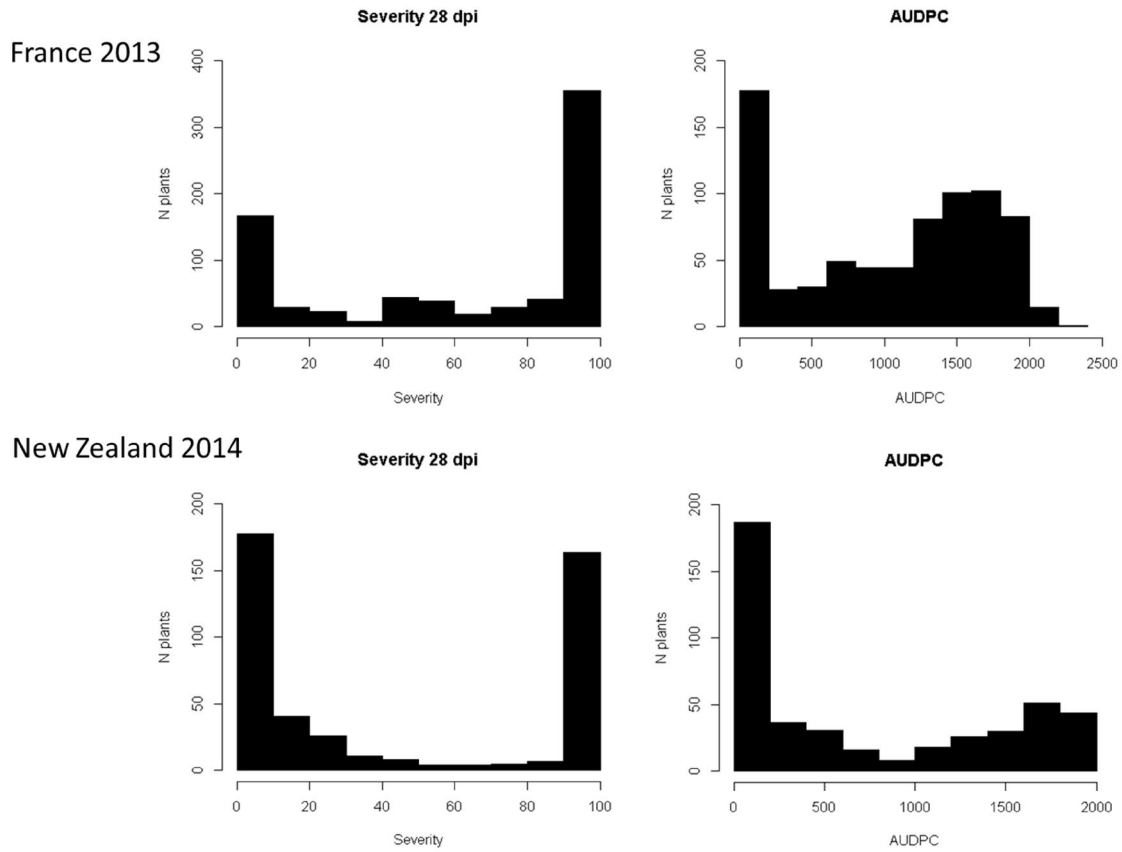
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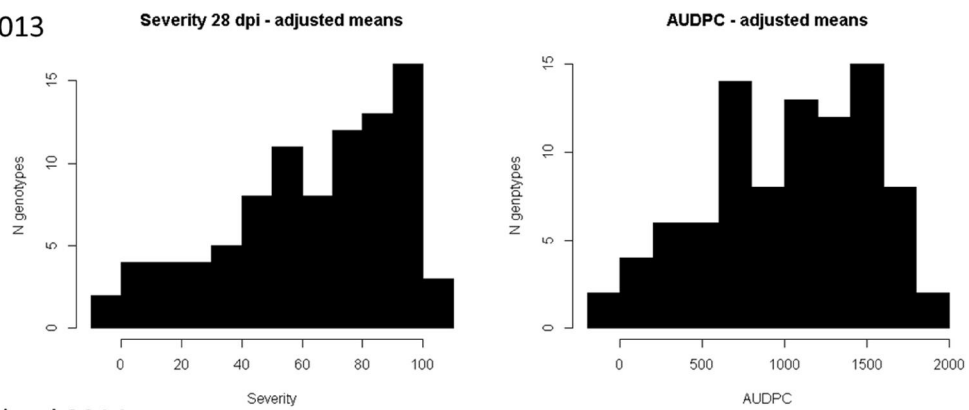
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Supplemental material

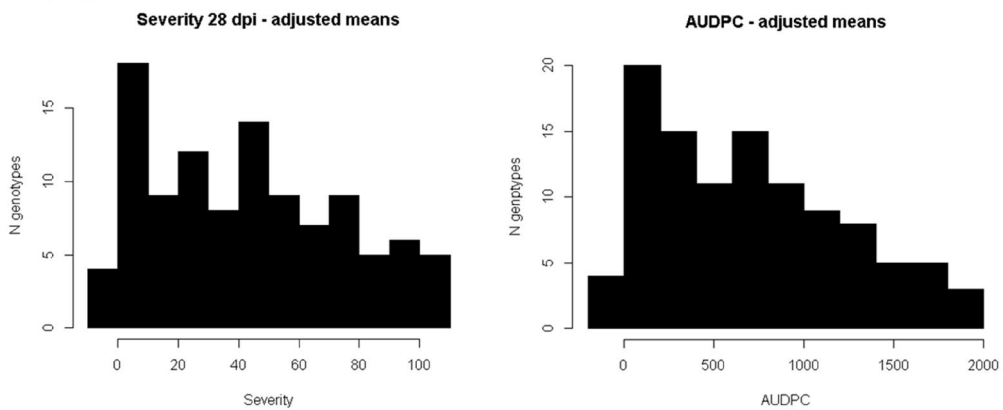


Supplemental Figure 4.1: Fire blight resistance phenotypic data distributions in a pear interspecific population in France in 2013 and in New Zealand in 2014

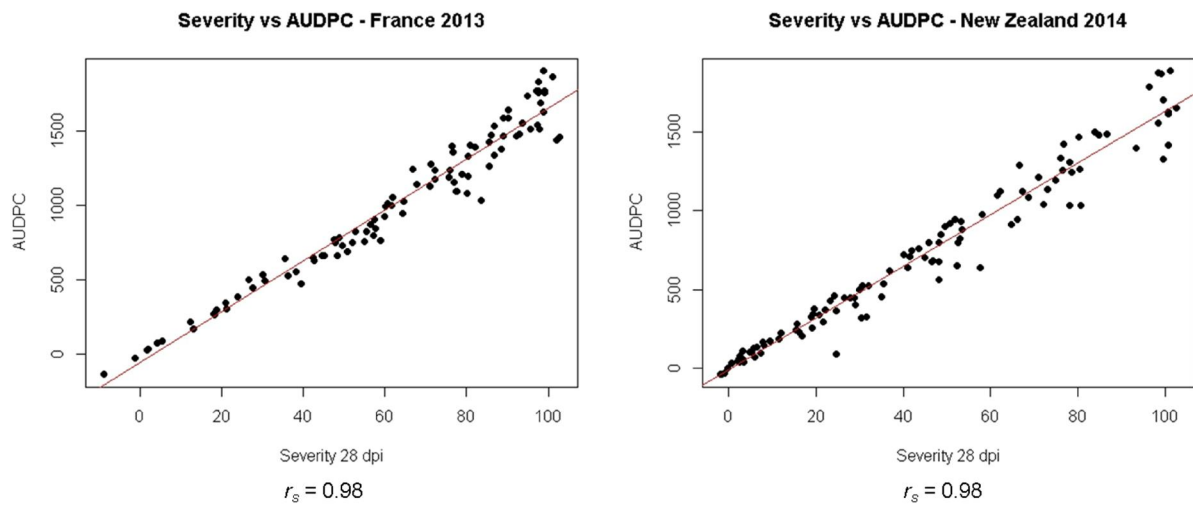
France 2013



New Zealand 2014

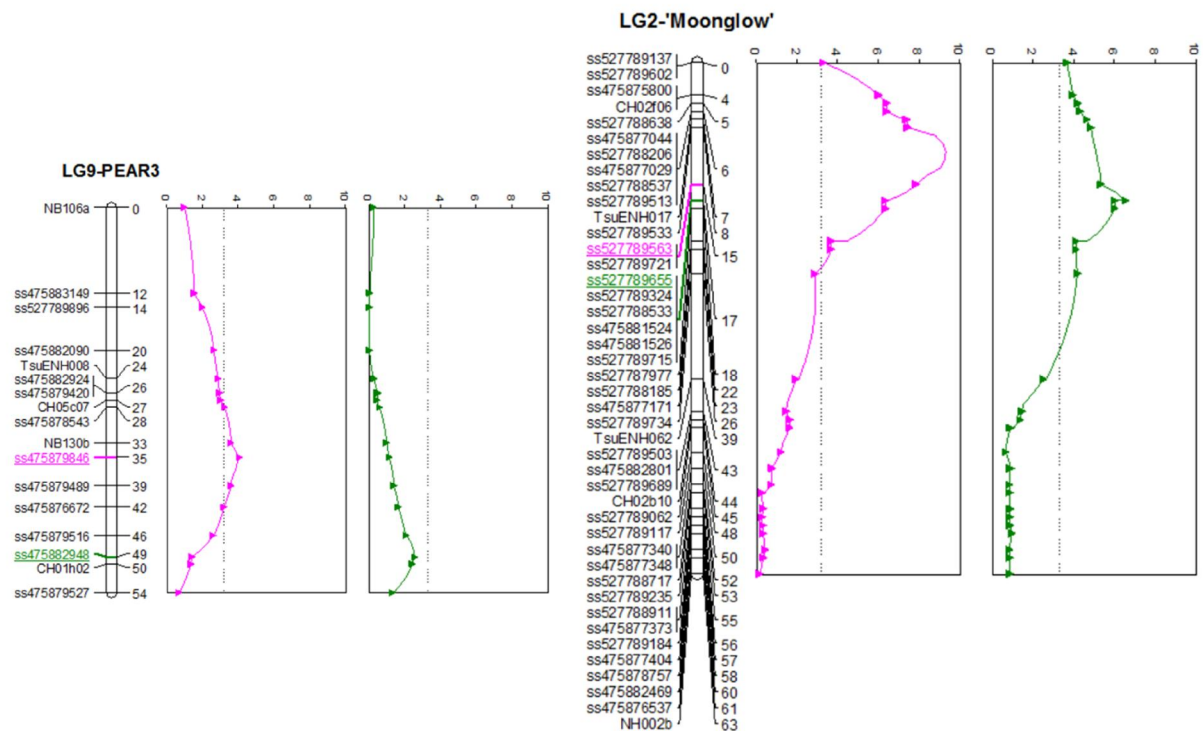


Supplemental Figure 4.2: Distribution of the phenotypic means of fire blight resistance adjusted for the inoculation date in a pear segregating population in France in 2013 and in New Zealand in 2014



Supplemental Figure 4.3: Correlation between fire blight Severity at 28 dpi and AUDPC (area under disease progress curve) measured in a pear segregating population (a) in France in 2013 and (b) in New Zealand in 2014

The Spearman coefficient (r_s) is shown for comparisons at both sites



Supplemental Figure 4.4: LOD curves on linkage group (LG)9 of PEAR3 and LG2 of 'Moonglow' for fire blight resistance

The LOD for the Severity at 28 dpi and marker at the peak in France (pink) and in New Zealand in 2014 (green), calculated with Multiple QTL Mapping analysis, are reported

Figures



Figure 4.1: Symptoms of fire blight disease on pear seedlings

Leaves and stems of the infected plants rapidly become necrotic and exudation droplets are secreted from the vessels

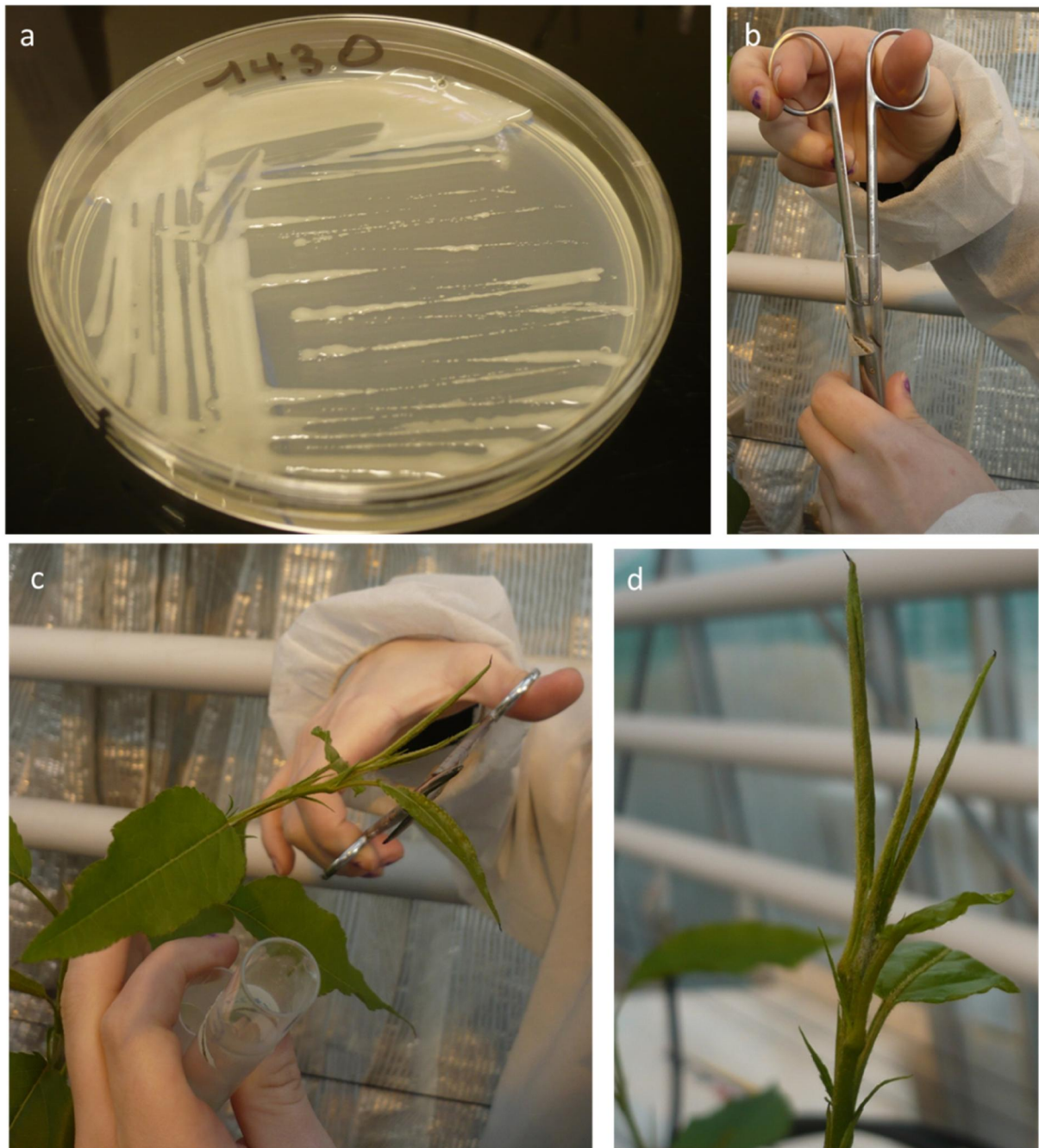


Figure 4.2: Method for fire blight inoculation adopted for the phenotypic evaluation of an interspecific pear population

(a) *Erwinia amylovora* was grown on King's medium B; (b) scissors were dipped into the inoculum solution and (c, d) used to bisect the two youngest unfolded leaves of actively growing shoots

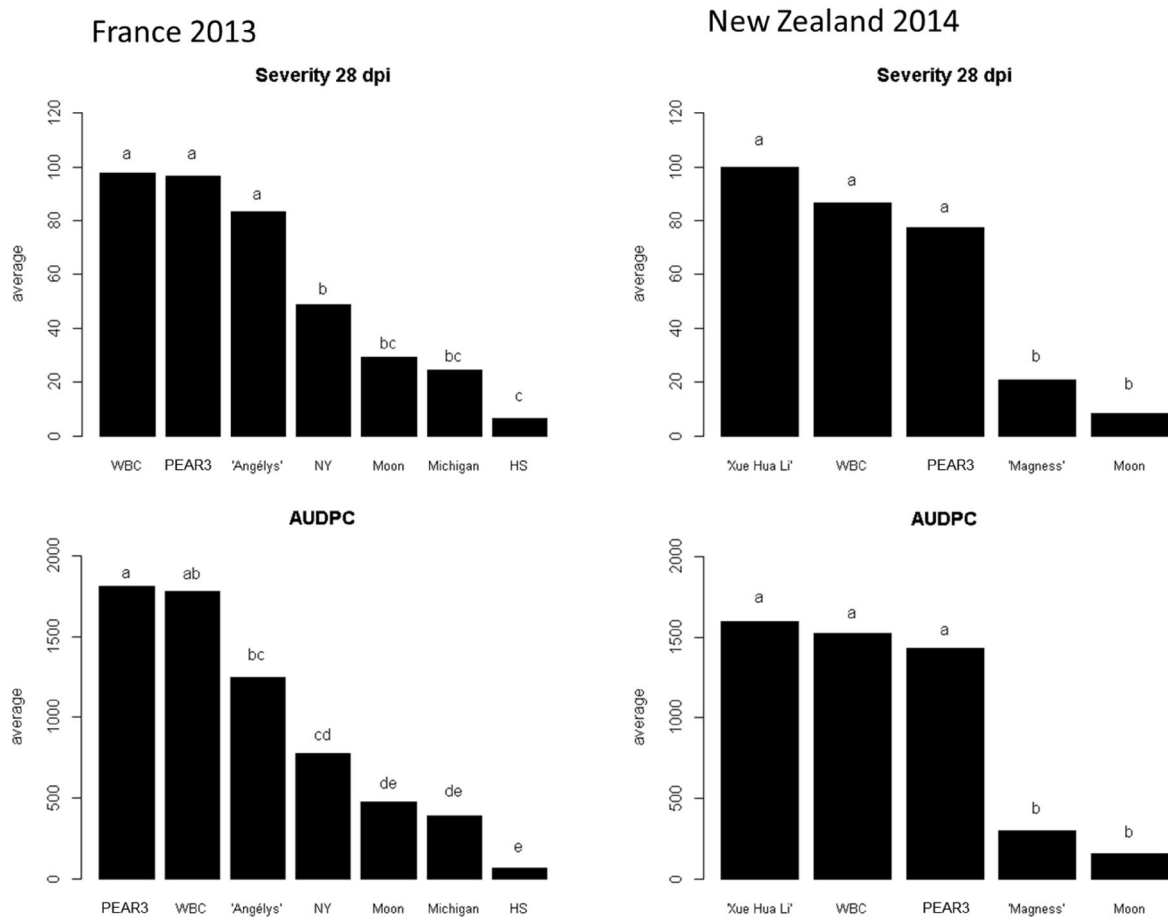


Figure 4.3: Comparison of disease incidence among the different pear accessions used as controls during fire blight resistance phenotyping in France in 2013 and in New Zealand in 2014

Significantly different genotypes, according to the Kruskal-Wallis test, are identified by different letters. Plots were constructed for both phenotypes, severity at 28 dpi and the area under disease progress curve (AUDPC). PEAR3 and 'Moonglow' (Moon) are the parents of the interspecific pear population tested, 'Williams Bon Chrétien' (WBC), 'Angélys' and 'Xue Hua Li' are the fire blight susceptible controls and 'Harrow Sweet' (HS), Michigan-US 437 (Michigan), NY10355 (NY) and 'Magness' are the resistant controls

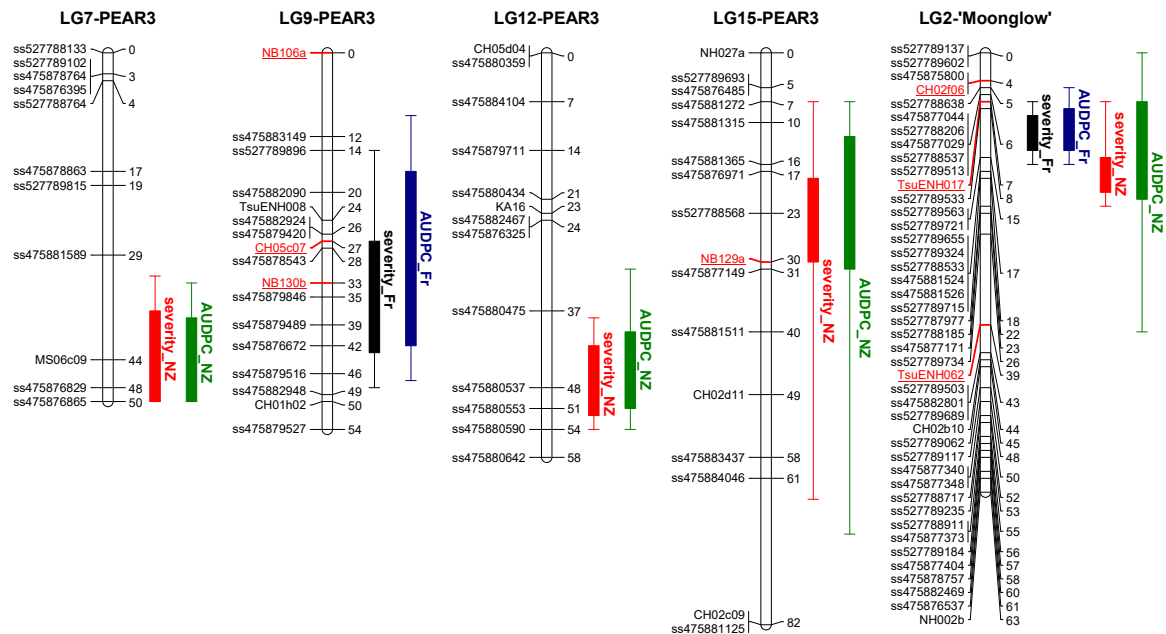


Figure 4.4: Quantitative trait loci (QTLs) detected for fire blight resistance in an interspecific pear population

QTLs for Severity at 28 dpi and AUDPC (area under disease progress curve) in France in 2013 (black and blue bars) and Severity at 28 dpi and AUDPC in New Zealand in 2014 (red and green bars) are reported. For each QTL, the one and two-LOD support interval are shown

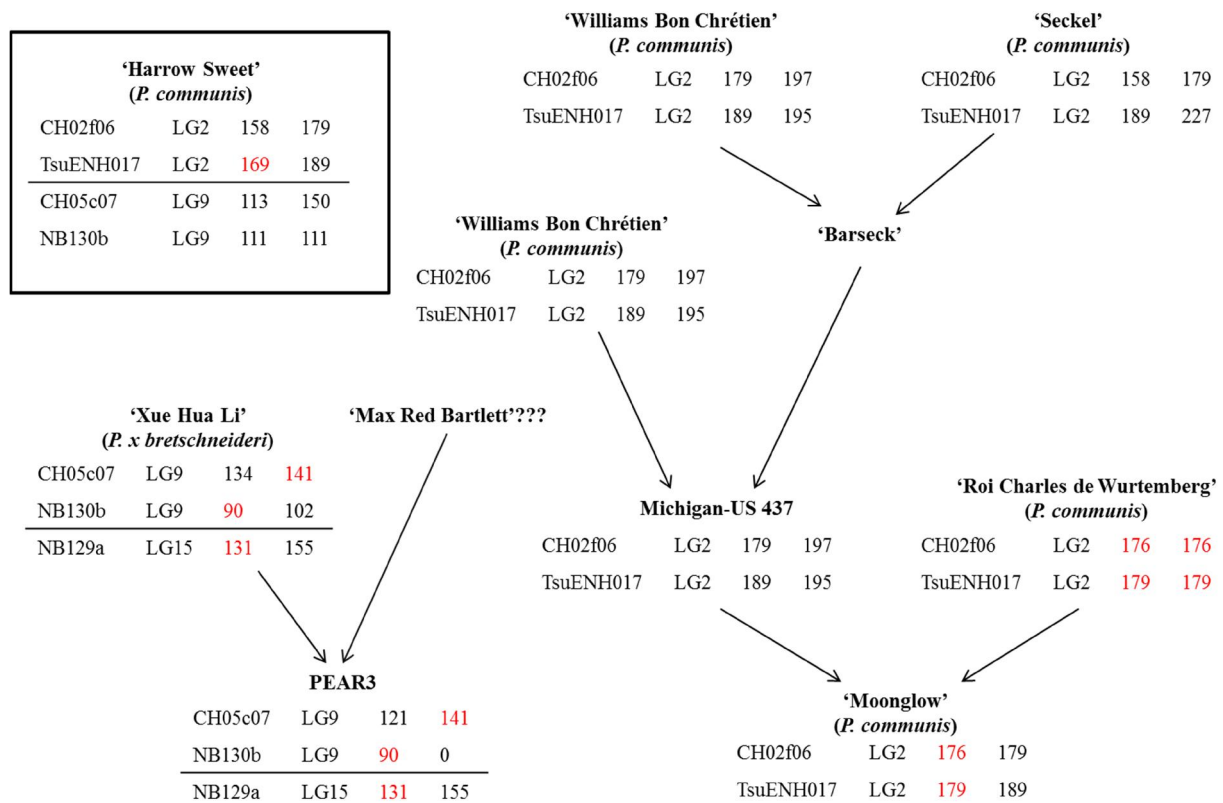


Figure 4.5: Inheritance of the alleles in coupling phase with fire blight resistance in the PEAR3 x 'Moonglow' pedigree

Progenitors of PEAR3 and 'Moonglow', and 'Harrow Sweet', were scanned with Simple Sequence Repeat (SSR) markers mapped within the linkage group (LG)2, LG9 and LG15 quantitative trait loci (QTLs) controlling fire blight resistance. For each marker, the favorable allele (in bp) is highlighted in red

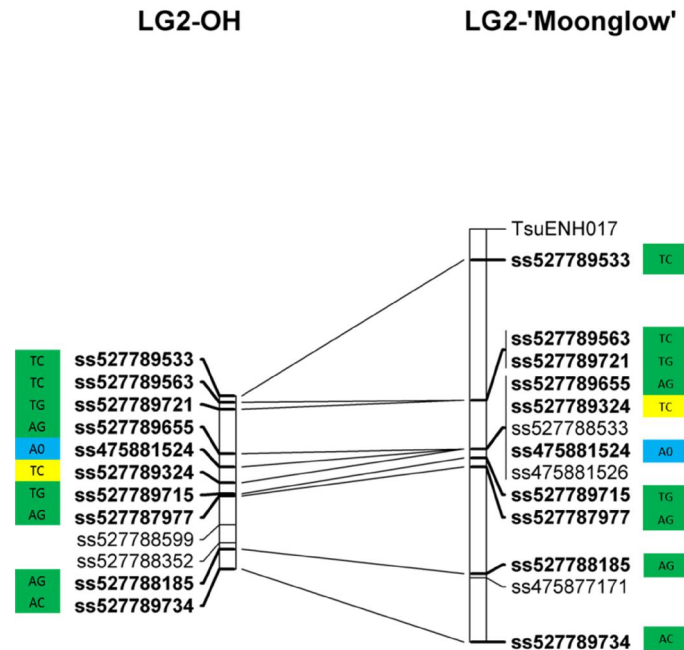


Figure 4.6: Comparison of ‘Old Home’ (Montanari et al. 2013) and ‘Moonglow’ genotypes within the region spanned by the quantitative trait locus (QTL) for fire blight resistance detected on linkage group (LG)2 of ‘Moonglow’

The common SNP markers between the two homologous regions are in bold and are connected with a line. On the side of both regions, the SNP genotype for the markers in common is reported: green color indicates co-linearity between the two regions; inverted markers are highlighted with different colors

Tables

Table 4.1: Microsatellite (SSR) markers selected for pedigree studies on linkage group (LG) 2, 9 and 15 Quantitative Trait Loci (QTLs) for fire blight resistance in a pear interspecific segregating population.

For each marker, the primers sequence, the allelic composition and the LG of PEAR3 and/or 'Moonglow' are shown, as well as the amplicon size range and the annealing temperature. The reference of each marker is also reported.

SSR locus	Location on other maps	Primer sequence	Segregation type in PEAR3x'Moonglow'	Location on PEAR3x'Moonglow' map	Amplicon size range (bp)	Annealing temperature (°C)	Reference
CH01f03b	LG9	for: GAGAAGCAAATGCAAAACCC rev: CTCCCCGGCTCCTATTCTAC	monomorphic	--	--	touchdown 63-58	Liebhard et al. 2002
CH02f06	LG2	for: CCCTCTTCAGACCTGCATATG rev: ACTGTTTCCAAGCGATCAGG	a0xbc	LG2 PEAR3 and 'Moonglow'	165-215	touchdown 63-58	Gianfranceschi et al. 1998
CH03h03	LG2	for: TAAGAAATCGGATCCAAAACAAC rev: GTTTCCTCAAAGATTGCTCCTG	complex	--	--	touchdown 63-58	Liebhard et al. 2002
CH05c07	LG9	for: TGATGCATTAGGGCTTGACTT rev GGGATGCATTGCTAAATAGGAT	abxcd	LG9 PEAR3 and 'Moonglow'	130-170	touchdown 63-58	Liebhard et al. 2002
Hi07d12	LG2	for: GGAATGAGGGAGAAGGAAGTG rev: GTTTCCTCTTCACGTGGGATGTACC	no amplification	--	--	touchdown 63-58	Silfverberg-Dilworth et al. 2006
NB106a	LG9	for: GTACGTCGACATGAGAGAG rev: TCTCTTGTTCCTTCCTGCAC	a0xbb	LG9 PEAR3	110-145	touchdown 61-56	Yamamoto et al. 2002b
NB129a	LG2/ LG15	for: TAACCACTGAAGAGAGAGAGAG rev: CCCTTATGTATTTTCCTGTG	abxcd	LG15 PEAR3 and 'Moonglow'	135-200	touchdown 61-56	Published Only in Database (2007)
NB130b	LG9	for: GTACGTCGACATGAGAGAGAGA rev: TGCACAGGAAATATCATCTCTT	a0xbb	LG9 PEAR3	105-130	touchdown 61-56	Published Only in Database (2007)
NB134a	LG9	for: TTTGGTTAGACATTTGGCGGAG rev: ATTTGGGCTGTATGTTTTGGCT	complex	--	--	touchdown 63-58	Published Only in Database (2007)
NH010a	LG2	for: GGTGGAGCAGGAGGGAAGAG rev: TATAGCCGGGTTTGGGTTGT	complex	--	--	touchdown 63-58	Published Only in Database (2007)
NH046a	LG2	for: TTGATTCTAAAACTCGTCTCCT rev: CATGTTATTTGTGCACTTCT	complex	--	--	touchdown 61-56	Published Only in Database (2007)
NH212a	LG2	for: TCCGAAAGCCAAATATTGAAAG rev: TTGGCAGGAGGCGTGGGTAG	abx00	LG2 PEAR3	175-190	touchdown 63-58	Sawamura et al. 2004
TsuENH001	LG2	for: AAAGACGGCATTGACTGGATAGA rev: GATGCAAAGACTTTCGCCTATCT	complex	--	--	touchdown 63-58	Nishitani et al. 2009
TsuENH017	LG2	for: ACTTCAAGTAGCCAATATCAG	abxcd	LG2 PEAR3 and	185-245	touchdown	Nishitani et al. 2009

		rev: GGCACTCTGTTTCTTATCAAC		'Moonglow'		63-58	
TsuENH062	LG2	for: ACTCAGATCGTACGCAGAACAAA rev: CGATAAAGATCGATAATCCTCATGC	aaxab	LG2 'Moonglow'	205-220	touchdown 63-58	Nishitani et al. 2009

Table 4.2: Comparison of fire blight phenotyping in France and in New Zealand.

PEAR3, 'Moonglow' and 'Williams Bon Chrétien' accessions were screened in both locations, using isolates CFBP 1430 in France and Ea9148 in New Zealand. The adjusted means for the phenotypes, severity at 28 dpi and area under disease progress curve (AUDPC), are reported for each pear accession and the result of the Chi-squared test between sites is also shown. At $p < 0.05$, the disease incidence is significantly different between sites.

	Severity		AUDPC	
	France	New	France	New
PEAR3	97.60	76.76	1831.55	1425.42
'Moonglow'	27.60	52.96	444.59	822.18
'Williams Bon Chretien'	95.01	84.75	1735.97	1484.19
Chi-squared	7.74		77.79	
p-value	0.02		<2.2e-16	

Table 4.3: Shapiro-Francia normality test on the phenotypic data for fire blight resistance in a pear interspecific segregating population.

For both France 2013 and New Zealand 2014 experiments, the statistical values and the p-values are reported for each measured variable (severity at 28 dpi and AUDPC – area under disease progress curve). At $\rho < 0.05$, the data distribution is not normal.

Raw Data	Shapiro-Francia test	
	statistical	p-value
France 2013		
Severity at 28 dpi	0.779	< 2.2e-16
AUDPC	0.899	< 2.2e-16
New Zealand 2014		
Severity at 28 dpi	0.740	< 2.2e-16
AUDPC	0.817	< 2.2e-16

Table 4.4: Shapiro-Francia normality test for phenotypic means of fire blight resistance adjusted for the inoculation date in a pear segregating population.

For both France 2013 and New Zealand 2014 experiments, the statistical values and the p-values are reported for each measured variable (severity at 28 dpi and AUDPC – area under disease progress curve). At $\rho < 0.05$, the data distribution is not normal.

Adjusted means	Shapiro-Francia test	
	statistical	p-value
France 2013		
Severity at 28 dpi	0.944	0.001214
AUDPC	0.976	0.09222
New Zealand 2014		
Severity at 28 dpi	0.949	0.0008825
AUDPC	0.952	0.001206

Table 4.5: Quantitative trait locus (QTL) mapping results for fire blight resistance in a segregating interspecific pear population.

For each trait and experiment, the LOD threshold, the linkage groups (LGs) and the parental map on which the QTLs were detected, the marker closest to the peak, the LOD score and R^2 and the favorable allele are shown. The $globalR^2$ was also calculated.

Trait	LOD Threshold	LG	parental map	Marker closest to peak	LOD	R ²	Favourable allele	globalR ²
France 2013								
Severity 28 dpi	3.2	2	'Moonglow'	ss527789563	7.87	31.9	C	44.2
		9	PEAR3	ss475879846	4.07	14.8	C	
AUDPC	3.2	2	'Moonglow'	ss527789563	8.50	34.4	C	46.2
		9	PEAR3	ss475879846	4.02	13.9	C	
New Zealand 2014								
Severity 28 dpi	3.3	2	'Moonglow'	ss527789655	6.55	16.6	G	51.5
		12	PEAR3	ss475880537	4.33	10.4	T	
		7	PEAR3	ss475876829	4.31	12.3	A	
		15	PEAR3	ss527788568	3.57	8.1	null	
AUDPC	3.3	2	'Moonglow'	ss527789655	4.47	12.9	G	32.1
		7	PEAR3	ss475876829	3.71	10.5	A	
		12	PEAR3	ss475880537	3.71	10.9	T	
		15	PEAR3	ss527788568	3.55	9.9	null	

Table 4.6: Broad sense heritability estimation (H^2) and phenotypic variation explained by all the significant QTLs (R^2) for fire blight resistance in a pear interspecific segregating population.

	France 2013		New Zealand 2014	
Trait	H^2	R^2	H^2	R^2
Severity at 28 dpi	0.86	0.44	0.61	0.52
AUDPC	0.87	0.46	0.64	0.32

Table 4.7: Simple Sequence Repeats (SSRs) profile for PEAR3, ‘Moonglow’, their progenitors and ‘Harrow Sweet’.

For each marker, the allele (in bp) in coupling with the resistance is highlighted.

Marker	CH02f06	TsuENH017	CH05c07	NB130b	NB129a
LG	LG2-'Moonglow'	LG2-'Moonglow'	LG9-PEAR3	LG9-PEAR3	LG15-PEAR3
Position (cM)	4.1	6.9	26.5	33.1	30.1
PEAR3	154-0	193-202	121- 141	90 -0	131 -155
'Moonglow'	176 -179	179 -189	117-150	102-102	118-139
Michigan-US 437	179-197	189-195	117-150	102-102	139-180
'Roi Charles de Wurtemberg'	176-176	179-179	117-117	102-102	118-155
'Seckel'	158-179	189-227	117-150	102-111	135-180
'Williams Bon Chretien'	179-197	189-195	113-150	102-102	139-180
'Xue Hua Li'	154-154/154-0	195-202	134- 141	90 -102	131 -155
'Harrow Sweet'	158-179	169-189	113-150	111-111	139-155

CHAPTER 5. Genetic Mapping of Loci Associated with Hybrid Necrosis

In both the French and the New Zealand progeny subsets, a high proportion (more than 50%) of the seedlings died a few months after germination, part within one month, and part two months later. Moreover, already in the first weeks after germination chlorosis and necrosis could be observed in those plantlets, as well as leaf cupping and dwarfism. When building the genetic maps of PEAR3 and ‘Moonglow’, a considerable number of distorted markers was detected. These markers were not all discarded and a few distorted regions, where these distorted markers clustered, were identified across the LGs. It was then that the existence of genetic incompatibilities causing the lethality of such a high proportion of seedlings was hypothesized, and the phenotype observed associated with the phenomenon of “hybrid necrosis”.

Thanks to the prompted observation of those particular symptoms in the stunted seedlings, leaf samples could be collected from many of them. The screening of the DNA extracted from both necrotic and non-necrotic seedlings with previously mapped and new genetic markers enabled us to identify three chromosomic regions linked to the phenomenon of hybrid necrosis in PEAR3 x ‘Moonglow’ population. Furthermore, an *ad hoc* protocol for the measurement of traits associated with hybrid necrosis was developed, and a new set of seedlings from the cross between PEAR3 and ‘Moonglow’ was phenotyped, confirming the results obtained with the molecular analysis. This new protocol could now be adopted for a more systematic observation of the hybrid necrosis in PEAR3 x ‘Moonglow’ population, as well as in other inter-specific pear families, which are likely to exhibit the same type of lethality.

This work is the object of an article still under editing, which will be submitted to *Theoretical and Applied Genetics*. Moreover, I presented these results, together with the study on pear psylla and fire blight resistance, with an oral communication at the 7th International Rosaceae Genomics Conference (RGC7), held in the USA in 2014.

The lists of the SSRs and of the newly developed HRM markers used in this study are reported in the Annex 6, Annex 7 and Annex 8.

The final genetic maps of PEAR3 and ‘Moonglow’, reporting the location of all the SNP, SSR and HRM markers used in this project, of all the QTLs detected and of the regions involved in the hybrid necrosis are in Annex 9.

This article is planned to be submitted for publication in *Theoretical and Applied Genetics*.

Genome mapping of post-zygotic hybrid necrosis in an interspecific pear population

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Abstract

Key message: We identified three chromosome regions associated with two distinct phenotypes for post-zygotic hybrid necrosis in an interspecific pear population, providing useful information for speciation studies and pear breeding.

Abstract: Deleterious epistatic interactions in plant inter- and intraspecific hybrids can cause a phenomenon known as hybrid necrosis, characterized by a typical seedling phenotype whose main distinguishing features are dwarfism, tissue necrosis and in some cases lethality. Identification of the chromosome regions associated with this type of incompatibility is important not only to increase our understanding of the evolutionary diversification which led to speciation, but also for breeding purposes. Development of molecular markers linked to the lethal genes will allow breeders to avoid incompatible inbred combinations that could affect the expression of

important agronomic traits co-segregating with these genes. Although hybrid necrosis has been reported in several plant taxa, including Rosaceae species, this phenomenon has not been described previously in pear. In the interspecific pear population resulting from a cross between PEAR3 (*Pyrus x bretschneideri* X *P. communis*) and ‘Moonglow’ (*P. communis*) we observed two types of hybrid necrosis, expressed at different stages of plant development. Using a combination of previously mapped and newly developed genetic markers, we identified three chromosome regions associated with these two types of lethality, which were genetically independent. One type resulted from a negative epistatic interaction between a locus on linkage group (LG) 5 of PEAR3 and a locus on LG1 of ‘Moonglow’, while the second type was due to a gene that maps to LG2 of PEAR3 and which either acts alone, or more probably, interacts with another gene of unknown location inherited from ‘Moonglow’.

Keywords: *Pyrus x bretschneideri*; *Pyrus communis*; lethal genes; genetic incompatibility; segregation distortion; *R* genes

Introduction

Hybrid necrosis is defined as the reduced viability of a hybrid due to genetic incompatibilities. Although interactions between genes may have a positive effect on the hybrid, resulting in it having better performance than its parents (hybrid vigor), they may also be detrimental and cause sterility, weakness or lethality (Bomblies et al. 2007). Genetic incompatibilities can occur at different stages of the reproduction process, and they are generally divided into pre-zygotic and post-zygotic, acting, respectively, before and after fertilization. Hybrid necrosis, which is also termed hybrid weakness or inviability, is a class of post-zygotic gene-flow barrier that is associated with a typical seedling phenotype, characterized by cell death, tissue necrosis, wilting, yellowing, chlorosis, dwarfism and reduced growth rate, and in some case lethality (Bomblies and Weigel 2007; Bomblies 2009). Hybrid necrosis has been observed in several plant taxa, in wild and cultivated species, both in inbred populations and outcrosses, however its phenotype appears to be characteristic across a range of hosts, suggesting a common underlying mechanism (Bomblies and Weigel 2007; Bomblies 2009). According to the Bateson–Dobzhansky–Muller (BDM) model, the genetics of hybrid necrosis is simple and involves epistasis between at least

two loci (Orr 1996). The BDM model posits that independent substitutions occurring in two diverging lineages, not detrimental in their native genomic context, might be deleterious when combined in the hybrid. Most of the cases of hybrid necrosis reported in the literature are explained by two-gene epistasis (Bomblies and Weigel 2007). However, there are some examples of three-locus interactions (Alcázar et al. 2009), as well as lethality controlled by a single locus (Hollingshead 1930; Heuer and Miézan 2003; Mishra et al. 2005).

Although hybrid inviability has long been known among plant breeders and speciation scientists, with examples in the literature since the early 20th century (Hollingshead 1930), only recently have efforts been made to explain its molecular basis. The hybrid necrosis phenotype resembles the set of symptoms resulting from pathogen attack, and research on *Arabidopsis* spp. (Bomblies et al. 2007; Alcázar et al. 2009; Tahir et al. 2013) and tomato (Krüger et al. 2002) demonstrated that it was linked to autoimmunity reactions involving resistance (*R*) genes. During this hypersensitive response (HR), the plant undergoes oxidative stresses, followed by programmed cell death (Greenberg et al. 2003; Takken et al. 2006), in order to halt the spread of the pathogen, which requires living tissues (Dangl et al. 1996). In the case of hybrid inviability, the plant immune system is improperly activated in the absence of a pathogen attack because of the genetic incompatibility, which causes tissue necrosis similar to that observed during HR. One hypothesis is that different (at least two) *R* proteins, encoded by independently evolved *R* genes, cause autonecrosis when they interact in the hybrid (Bomblies et al. 2007). Alternatively, one locus encodes a host protein, which regulates the activation of the *R* protein encoded by the second locus, as explained by the “guard hypothesis” (Jones and Dangl 2006; Bomblies 2009). Most of the *R* genes demonstrated to be involved in hybrid necrosis belonged to the Nucleotide Binding-Leucine Rich Repeats (NB-LRR) class, the most common category of plant disease resistance genes (Rieseberg and Blackman 2010). For example, Bomblies et al. (2007) detected two unlinked regions (*DMI* and *DM2*) that were responsible for the hybrid necrosis in an *A. thaliana* segregating population, and identified *DMI* as an NB-LRR gene. Moreover, they proved that genetic interaction between those two loci was required for increased resistance to *Hyaloperonospora parasitica*. When Alcázar et al. (2009) investigated the cause of dwarfism in hybrids of two *A. thaliana* Recombinant Inbred Lines (RILs), they found that TIR (Toll-Interleukin-1 Receptor)-NB-LRR genes were the likely determinants of one of the interacting loci

responsible for the phenomenon. This gene cluster mapped to the same position as the *DM2* locus detected by Bomblies et al. (2007). The work of Krüger et al. (2002) in tomato (*Solanum lycopersicum* L.) was the first example of a “guard-guardee” interaction causing genetic incompatibility. In *S. lycopersicum* lines introgressed with the *Cf-2* gene from a wild relative of tomato, *S. pimpinellifolium* Jusl., both autonecrosis and resistance to the fungus *Cladosporium fulvum* were observed. The two phenomena were dependent on the interaction between the *Cf-2* gene from *S. pimpinellifolium*, encoding for an LRR-containing receptor-like protein (the “guard”), and the *RCR3* locus from *S. lycopersicum*, encoding for a cysteine endoprotease (the “guardee”). However, when the *RCR3* locus was introduced from *S. pimpinellifolium*, no autonecrosis was observed, and the resistance was maintained. This demonstrated that the two loci were incompatible with each other only when they had evolved in different genomic contexts.

R genes, and especially LRR domains, are known to be highly polymorphic, even within the same species, evolving at very fast rates under the pressure of natural selection for resistance (Bergelson et al. 2001) and consistent with the hypothesis of their implication in BDM-like genetic incompatibilities. Breeding for disease resistant cultivars might be expected to generate hybrid necrosis as a by-product (Bomblies and Weigel 2007), and indeed there are several examples in the literature of hybrid necrosis events occurring in segregating populations developed to increase the resistance to pathogens in a range of species (Bomblies and Weigel 2007; Bomblies et al. 2007): in wheat breeding lines resistant to species of *Puccinia* rust (Morrison 1957); in rice subspecific hybrids which showed resistance to *Xanthomonas oryzae* (Ichitani et al. 2012); and in diploid potatoes (*Solanum* spp.) resistant to the cucumber mosaic virus (CMV) (Valkonen and Watanabe 1999).

Only a few examples of hybrid inviability have been reported for Rosaceae species. Loci linked to chlorotic or albino leaf, dwarfism and lethality have been detected in strawberry and apple. Sargent et al. (2004) mapped a recessive locus for the pale green leaf trait (*pg*) in the interspecific *Fragaria vesca* X *F. nubicola* F2 progeny, and this was suggested to be orthologous to the *vir* gene mapped in *Malus* spp. by Fernández-Fernández et al. (2013), that was associated with the virescent phenotype in progeny from several East Malling rootstocks crosses. In apple, a gene for compact habit was shown to be linked to the *Vf* gene for scab resistance (Decourtye 1967), now

called *Rvi6* (Bus et al. 2011), which maps to linkage group (LG) 1. A few years later, Alston (1976) demonstrated that the pale green lethal trait in apple, which characterizes seedlings deficient in chlorophyll that die a few weeks after germination, was controlled by the recessive gene *l*, linked to *Rvi6*. In addition, two different sub-lethal recessive genes (*sl1* and *sl2*), detected by Gao and van de Weg (2006) in apple, were linked to the *Rvi6* gene. These genes control lethality at two different stages of apple seedling development, one before and one after germination, however they both interacted with another locus, *sl3*, whose map position was not identified. Distorted segregation ratios in favor or against scab resistance have been reported also in other publications, both in apple (Tartarini 1996; Conner et al. 1997) and pear (Iketani et al. 2001; Bus et al. 2013). Moreover, hybrid lethality has been described in intergeneric hybrids between apple and pear (Shimura et al. 1980; Inoue et al. 2003).

A pear interspecific segregating population was developed from a cross between PEAR3 (*Pyrus x bretschneideri* X *P. communis*) and ‘Moonglow’ (*P. communis*) at Plant & Food Research (PFR), for the purpose of detecting chromosome regions linked to resistances against fire blight (*Erwinia amylovora*), pear scab (*Venturia pirina*) and pear psylla (*Cacopsylla pyri*) (Montanari et al. 2013). A subset of the seeds originating from this cross was planted and grown at PFR, Motueka (New Zealand), and another subset at INRA, Angers (France). In both environments, stunted seedlings and lethality were observed and postulated to be due to hybrid necrosis. We describe the initial identification and subsequent validation of genomic regions linked to hybrid necrosis, utilizing genetic mapping in populations consisting of both necrotic and non-necrotic plants.

Materials and Methods

Plant material and growth conditions

Fruit was harvested from the PEAR3 x ‘Moonglow’ cross in Motueka during the summers (late February) of 2010 and 2014. Seeds were extracted, washed, treated with a 10% solution of Sodium hypochlorite (42 g/l) and dried, then stored in a refrigerator at 3-5°C until sowing. In winter 2010 (July), 760 seeds were planted in Motueka, with a further 728 sown in winter 2011 in Angers (February) and another 240 in winter 2014 in Motueka (July). In 2010 in Motueka

seeds were spread evenly through damp sphagnum moss for vernalization, in order to break the dormancy, and then stored in a refrigerator at 3-5°C until germination; then all seeds were planted. In Angers seeds were also subjected to vernalization treatments, by stratification in a moist sand and vermiculite substrate at 3-5°C for three months, after which the seeds were planted in a mixture of peat and sphagnum soil. In 2014 in Motueka, seeds were dipped in 5% Thiram 40F (400 g/l Thiram as a suspension concentrate) before sowing, to prevent fungal development, and then placed on filter paper into petri dishes (Figure 5.1) and 3 ml of 5% Thiram 40F added to each plate. Petri dishes were sealed with parafilm to prevent desiccation and stored at 3-5°C for 53 days, and then at 20°C for three days. On the second day at 20°C they were again treated with Thiram 40F as above. Petri dishes were then moved back to 3-5°C until seed germination. Seeds were planted into pots containing Daltons strawberry potting mix seven days after germination, and moved to the greenhouse. The first batch of seeds was planted 67 days after extraction from the fruit, and sowing continued on a weekly basis for another 75 days. During storage in the refrigerator, some seeds were treated a third time with Thiram 40F because of mould development, while others were moistened with 2 ml of distilled water because they were becoming dry. Seeds that had not germinated after 127 days were returned to 20°C for three days.

Phenotypic assessment, types of hybrid necrosis, and test of Mendelian ratios

The number of seedlings that stopped growing, were necrotic or dead, or were growing normally were assessed at 30, 50 and 85 days after planting. A classification of the seedlings including two types of hybrid necrosis was performed according to the morphological appearance of the seedling and a chronological criterion. In 2014, the dry weight was measured for all seeds individually, as well as the weight and the radicle length of the germinated seed at the planting date. The plant height was measured at 30, 50 and 85 days after planting, and the plant condition (chlorosis, presence of necrosis, cupped leaves) was noted. At the first assessment, the leaf area was calculated (via graphical tools from taken pictures), and at the final assessment the number of buds was noted. The segregation ratios for the seedling types were computed and confronted to

various Mendelian segregation ratios corresponding to various genetic models using chi-square (X^2) tests.

DNA extraction and design of High Resolution Melting markers for hybrid necrosis

Both in 2010 in Motueka and in 2011 in Angers, leaves developed from some of these seedlings were collected for DNA extraction before they died. Genomic DNA was extracted using the QIAGEN DNeasy Plant Kit (QIAGEN GmbH, Hilden, Germany) or the NucleoSpin® 96 Plant II (Macherey-Nagel GmbH & Co. KG). DNA quantifications were carried out using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc.).

Regions with distorted segregation ratios were identified in PEAR3 and ‘Moonglow’ genetic maps, as an initial indication of hybrid necrosis. Two to four Single Nucleotide Polymorphism (SNP) markers were randomly selected within each of these regions, on LGs 1, 2, 5, 10 and 16 of the PEAR3 x ‘Moonglow’ genetic map constructed with the apple and pear Infinium® II 9K SNPs array (Montanari et al. 2013). In addition, putative candidate lethal genes (NB-LRR genes) were identified from the orthologous regions of the apple genome on LGs 1, 5 and 10 (Velasco et al. 2010). High Resolution Melting (HRM) markers were developed both from these SNPs and from candidate gene sequence. PCR primers were designed flanking SNPs using Primer3 software (Rozen and Skaletsky 1999, <http://primer3.ut.ee/>) with the following criteria: i) PCR product size between 50 and 200 base pairs (bp); ii) primer size between 18 and 25 bases; iii) optimal melting temperature (T_m) of 59°C; iv) GC content of each primer between 40% and 55%; v) maximum alignment score and global alignment score for self-complementarity and complementarity between primer pairs set to 4 and 1, respectively. The quality of the primers was controlled by BLASTn queries against the ‘Bartlett’ v1.0 genome (Chagné et al. 2014). PCR reactions and HRM analysis were performed on DNA from necrotic and non-necrotic individuals using a LightCycler® 480 instrument (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) as described by Guitton et al. (2012).

Linkage map analysis

The new HRM markers were added to the SNPs and Simple Sequence Repeats (SSRs) dataset described in Montanari et al. (2013) and updated parental genetic maps were constructed for the target LGs using JoinMap v4.0 software (Van Ooijen 2006) following the double pseudo testcross mapping strategy (Grattapaglia and Sederoff 1994). The linkage groups were determined with a minimum LOD score of 4 for grouping and the Kosambi function was used for map calculation. Maps were drawn using MapChart 2.2 (Voorrips 2002).

SSR analysis of regions associated with hybrid necrosis

Microsatellite markers were selected from published apple and pear SSRs (Gianfranceschi et al. 1998; Liebhard et al. 2002; Yamamoto et al. 2002a; Silfverberg-Dilworth et al. 2006; Nishitani et al. 2009) within the regions associated with hybrid necrosis (detected by the HRM markers analysis), as well as SSR markers CH03a09 and CHVf1, previously mapped to LG5 of PEAR3 and LG1 of ‘Moonglow’, respectively (Montanari et al. 2013). These were used to genotype both the necrotic and non-necrotic individuals employing PCR reactions consisting of 20 ng of genomic DNA, 1x QIAGEN Multiplex PCR Master Mix and 0.2 µM of each forward and reverse primer, in a final volume of 12.5 µl. Three to four SSRs with fluorescent-labelled primers were multiplexed and amplified using an Applied Biosystems® GeneAmp® PCR System 9700 (Applied Biosystems® by Life Technologies™) at Fondazione Edmund Mach (Italy). Multiplex PCR were performed as described by Teixeira and Bernasconi (2007), with some modifications: the initial denaturation step was followed by 5 touchdown cycles with a decrease of 1°C/cycle, and the main amplification reactions consisted of 35 cycles. Fragments were analyzed as outlined by Montanari et al. (2013). All the SSR markers, as well as one necrotic phenotype, were incorporated in the PEAR3 and ‘Moonglow’ genetic maps.

In order to identify the origin of the incompatible alleles, accessions from the PEAR3 and ‘Moonglow’ pedigrees, including *P. communis* Michigan-US 437, ‘Roi Charles de Wurtemberg’, ‘Williams Bon Chrétien’ and ‘Seckel’ and *P. x bretschneideri* ‘Xue Hua Li’, were screened with CHVf1, CH03a09, Hi04d02, CH05f06, CH02f06, Hi08g12, CN493139, CN444636 and Hi24f04 markers, in order to identify the origin of the incompatible alleles.

Results

Phenotypic evaluation of hybrid necrosis

The seeds from the PEAR3 x ‘Moonglow’ cross had high rates of germination across years and locations. In total, 704 seeds out of 775, 657 out of 728 and 227 out of 240 germinated in Motueka in 2010, in Angers in 2011 and in Motueka in 2014, respectively, for an overall germination rate greater than 90%. The alternation of cold and warm temperature treatments on seeds improved germination in 2014. Three distinct phenotypic classes were identified in the segregating population over both sites and years. The ‘Type 1’ seedlings ceased growing very soon after germination, and chlorosis and necrotic lesions were apparent on their leaves (Figure 5.2a). These seedlings died within one month after germination, or remained less than 50 mm in height with small leaves. The ‘Type 2’ seedlings initially developed normally, however, the leaves began to cup downwards and became chlorotic and necrotic (Figure 5.2b), with these characteristics increasingly exacerbated at 50 and 85 days after planting. Within three months after germination, the plant development stopped and the seedlings did not grow higher than 150 mm, progressively degenerating with time. The ‘Type 3’ seedlings grew normally (Figure 5.2c). In 2014 at Motueka, 30 days after germination the ‘Type 1’ seedlings were significantly smaller (according to the non-parametric Kruskal-Wallis test) than the other two phenotypic classes, while there was no difference between the height of ‘Type 2’ and ‘Type 3’ seedlings. In contrast, the plant heights at 50 and 85 days were significantly different between each of the phenotypic classes, with the highest values for ‘Type 3’ individuals and the lowest for ‘Type 1’ (Figure 5.3a). Moreover, the leaf area (measured at 30 days) (Figure 5.3b) and the bud number (measured at 85 days) (Figure 5.3c) were significantly different among the three classes. No significant difference was observed for the seed weight, both dry and at planting, nor for the radicle length. The presence of necrotic lesions in both ‘Type 1’ and ‘Type 2’ phenotypes indicated that the lethality observed in these seedlings was due to hybrid necrosis. We hypothesized that ‘Type 1’ and ‘Type 2’ lethality had independent biological and genetic causes. The incompatibility causing the ‘Type 1’ plants to become stunted and die acted soon after germination, within a month, while the ‘Type 2’ dwarfism was activated later, 50 to 90 days after plant germination. Figure 5.4

presents a model for pre- and post-zygotic hybrid lethality, showing at which stages the ‘Type 1’ and ‘Type 2’ phenomena are expressed.

Genetic model for ‘Type 1’ and ‘Type 2’ hybrid necrosis

The observed segregation ratios for ‘Type 1’:‘Type 2’:‘Type 3’ phenotypes in the PEAR3 x ‘Moonglow’ population were 153:271:280 (22%-38%-40%), 101:260:296 (15%-40%-45%) and 44:79:104 (19%-35%-46%) in Motueka 2010, Angers 2011 and Motueka 2014, respectively (Table 5.1). A chi-square (X^2) test was performed in order to increase our understanding of the genetic basis of the observed segregation ratios. At the risk of $\alpha < 0.05$, the observed segregation ratio is significantly different from the Mendelian ratio tested. The progeny segregation for ‘Type 1’:‘Type 2’+‘Type 3’ in the Motueka 2014 experiment is consistent with either a 1:3 or a 3:13 ratio. On the other hand, the segregations observed in Motueka 2010 and in Angers 2011 experiments did not fit any of the Mendelian ratios tested, even though they were close to the 1:3 and 3:13 ratios. The pooled data was not significantly different from the 3:13 ratio, as shown by the X^2 test performed on the sum of the three experiments pooled for each class (Pooled X^2). However, the three experiments were significantly heterogeneous (X^2 heterogeneity test calculated as the difference between the Total X^2 – i.e. the sum of the three X^2 calculated for each experiment - and the Pooled X^2). As the observations were much more accurate in 2014 than in 2010, it is possible that the small discrepancy of 2010 data from either 1:3 or 3:13 ratios is due to erroneous phenotypic assessment. It is also probable that environmental conditions affected the phenotypes, with a higher number of ‘Type 1’-like seedlings in Motueka than in Angers (within the same set of seeds collected in 2010) and in 2010 than in 2014 (within the same location Motueka) (Table 5.1). Consequently, both the 1:3 and 3:13 ratio were taken into account. The observed ‘Type 2’:‘Type 3’ ratio fitted well the 1:1 Mendelian ratio and the three experiments were rather homogeneous for this data, with a pooled dataset generating a ratio just not significant (p-value = 0.051).

Detection of candidate genomic regions linked to hybrid necrosis and refinement of the intervals

The parental genetic maps of PEAR3 x ‘Moonglow’ based on SNP and SSR markers (Montanari et al. 2013) constructed using ‘Type 3’ seedlings were employed to identify regions that were potentially involved in control of ‘Type 1’ and ‘Type 2’ hybrid necrosis. These maps were searched for regions where the markers showed distorted segregation ratios by plotting the Minor Allele Frequency (MAF) value for each marker used for map construction against its position on the LG (Annex 2); MAF values around 0.5 were observed in regions with no segregation distortions and MAF lower than 0.35 indicated severe segregation distortion. Using this method, segregation distortion was detected on seven LGs: 2, 5 and 10 of PEAR3 and 1, 9, 10 and 16 of ‘Moonglow’.

When DNA extracted from 55 ‘Type 1’ and 93 ‘Type 2’ necrotic seedlings, plus 105 non-necrotic seedlings (‘Type 3’) that had been used for the genetic map construction, was screened with newly developed molecular markers designed for these seven candidate regions for hybrid necrosis, twelve out of 23 HRM markers designed from SNPs with distorted segregation frequency were polymorphic and were distributed over all the distorted regions, with 10 of them mapping close to the SNP marker from which they had been developed (Annex 6, Figure 5.5). New HRM markers were also developed from putative candidate lethal genes (NB-LRR genes) annotated in the orthologous regions of the apple genome (Velasco et al. 2010), and a total of 31 primer pairs were designed for these *R* genes, resulting in 15 polymorphic markers, with 10 mapping to the locations predicted from the whole genome sequence (Annex 7, Figure 5.5).

An examination of the genotypic ratios for the newly designed HRM markers in ‘Type 1’ versus either ‘Type 2’ or ‘Type 3’ seedlings indicated that most of them were in equilibrium among the phenotypes. In contrast, the results for markers mapping to LG5 of PEAR3 and LG1 of ‘Moonglow’ were strongly skewed between ‘Type 1’ versus ‘Type 2’ + ‘Type 3’ phenotypes. The most extreme situation was observed for markers LETss527789863 from LG5 of PEAR3 and MDP0000160413_LG1b from LG1 of ‘Moonglow’ were 79.6% of the ‘Type 1’ seedlings carried both the *b* allele of the first marker and the *n* allele of second marker (Table 5.2), while only 4.8% of the ‘Type 2’ + ‘Type 3’ seedlings carried these alleles in combination. Conversely, all the other genotypic combinations of these LG5 and LG1 markers were almost balanced in

both ‘Type 2’ and ‘Type 3’ seedlings and were much less frequently observed in ‘Type 1’ seedlings. This demonstrated a linkage between the ‘Type 1’ phenotype and a combination of loci mapping to PEAR3 LG5 and ‘Moonglow’ LG1.

An examination of ‘Type 2’ versus ‘Type 3’ seedlings for all remaining markers indicated that LETss527788384 from LG2 of the interspecific parent PEAR3 was linked to the ‘Type 2’ phenotype (Table 5.2). Based on this observation, a linkage analysis was performed by considering the ‘Type 2’ hybrid necrosis as a phenotypic marker segregating (consistent with the 1:1 segregation ratio observed for ‘Type 2’:‘Type 3’). The corresponding locus, named *let2* (as the “lethal gene causing Type 2 hybrid necrosis”), was mapped to LG2 of PEAR3 8 cM upstream from the previously considered marker LETss527788384 (Figure 5.5).

Alignment of the regions exhibiting segregation distortion detected on LGs 1, 2 and 5 with homologous regions in other segregating pear populations (Montanari et al. 2013) enabled us to identify SNPs with a strong or completely (i.e. with an entire genotypic class missing) distorted segregation, which were filtered out during the initial SNP array analysis because of the very low MAF. Eight, seven and one of the strongly distorted SNPs were mapped to LGs 2 and 5 of PEAR3 and to LG1 of ‘Moonglow’, respectively (Table 5.3, Figure 5.5). Moreover, the five SNPs with completely distorted segregations mapped to LG2 in the other pear maps and were heterozygous in PEAR3 (Table 5.3). One of those SNPs, ss527787834 (segregating *abxaa* and with *ab* genotype missing amongst ‘Type 3’), could be located with certainty between ss527788206 and ss527789268, in the region linked to ‘Type 2’ lethality (Figure 5.5).

Pedigree analysis of the incompatible alleles

Among the eighteen microsatellite markers selected within the regions linked to hybrid necrosis on LGs 2 and 5, seven and four, respectively, were polymorphic. Five and two SSR markers mapped to the LGs 2 and 5 of PEAR3, respectively, while Ch05e06, CN581493 and Hi02a07 were homozygous in PEAR3 and mapped only to ‘Moonglow’, and CN445599 did not map (Annex 8, Figure 5.5). In order to reduce the interval of the regions linked to the hybrid necrosis, these seven SSRs, plus CH03a09 and CHVf1, mapping respectively to LG5 of PEAR3 and LG1 of ‘Moonglow’, were used for genotyping 49, 76 and 74 of the ‘Type 1’, ‘Type 2’ and ‘Type 3’

seedlings, as well as progenitors of PEAR3 and ‘Moonglow’ with the purpose of identifying the origin of the incompatible alleles.

For LG5 and LG1 markers, the frequency of the allele linked to ‘Type 1’ necrotic phenotype was examined (Table 5.4), as well as the genotype of the grandparents (Table 5.5). On LG5, an allele of HRM marker LETss527789863 derived from PEAR3 (denoted as ‘b’) had the highest frequency (90.7%) in ‘Type 1’ seedlings. Three SSRs were mapped to LG5 close to this locus: CH03a09, Hi04d02 and CH05f06. PEAR3 carries a 115 bp allele of CH03a09 associated with ‘Type 1’ incompatibility and inherited from ‘Xue Hua Li’ (homozygous for the 115 bp allele). For marker CH05f06, PEAR3 carries a 181 bp allele associated with incompatibility and also inherited from ‘Xue Hua Li’. It was not possible to ascertain the origin of the allele associated with incompatibility at Hi04d02, since PEAR3 exhibits both alleles carried by ‘Xue Hua Li’ (Table 5.5, Figure 5.6). A null allele of marker CHVf1 on LG1 inherited from ‘Moonglow’ had the highest frequency (86.5%) in ‘Type 1’ seedlings. The parents of ‘Moonglow’, Michigan-US 437 and ‘Roi Charles de Wurtemberg’, showed only one peak at 127 bp for CHVf1, while they both potentially bring a null allele as well (Table 5.5, Figure 5.6).

For LG2 of PEAR3, alleles denoted as ‘e’, ‘m’, and ‘a’ for the markers CN493139, LETss527788384 and CN444636, respectively, showed the highest frequencies (87.8%, 87.6% and 87.7%, respectively) in ‘Type 2’ seedlings (Table 5.4). The ‘Type 2’ incompatibility was associated with the 148 bp and 243 bp alleles of SSR markers CN493139 and CN444636, respectively, however ‘Xue Hua Li’ did not carry any of these alleles (Table 5.5, Figure 5.6). Also, ‘Max Red Bartlett’, which is red skinned sport of ‘Williams Bon Chrétien’, could not be confirmed as the PEAR3 male parent by the SSRs analysis.

Following the rearrangement of the markers on LG2 of PEAR3 with respect to the original map of Montanari et al. (2013), after the addition of the new HRM, SSR and SNP markers, the peak of distortion could be identified within the region linked to lethality, as for PEAR3 LG5 and ‘Moonglow’ LG1 (Figure 5.5).

Discussion

The cross between first generation interspecific accession PEAR3 (*P. x bretschneideri* X *P. communis*) and European pear ‘Moonglow’ (*P. communis*) generated a large proportion of non-viable seedlings, which exhibited a typical hybrid necrosis phenotype (Bomblies and Weigel 2007; Bomblies 2009). The molecular analysis we performed enabled us to identify three chromosome regions associated with this phenomenon. Segregation analysis of phenotypes showed that BDM-like incompatibilities involving epistasis among different loci was the basis of hybrid necrosis in this pear population, a finding that is consistent with reports for other plant species (Song et al. 2009; Alcázar et al. 2009; Mizuno et al. 2010). Since an autoimmune response is likely to occur in incompatible combinations showing the hybrid necrosis phenotype (Krüger et al. 2002; Bomblies et al. 2007; Tahir et al. 2013), we discuss our findings in relation to previously mapped resistances in pear. Furthermore, we identified SSR markers linked to the lethal genes, which were used to perform a pedigree analysis that outlined the existence of post-zygotic gene-flow barriers between the two different *Pyrus* species.

Two independent post-zygotic incompatibilities

Non-viable seedlings of the PEAR3 x ‘Moonglow’ population, which never reached complete development and necrotized or died instead (Figure 5.2a and b), could be divided into two classes, ‘Type 1’ and ‘Type 2’, based on the timing of the occurrence of the lethal phenotype (Figure 5.4). One month after germination, the inviability of ‘Type 1’ seedlings was already visible, with regard to their significant smaller dimensions with respect to the other seedlings (Figure 5.3a and b), and for the presence of extensive necrosis (Figure 5.2a). At this time, ‘Type 2’ plants were as tall as the normal growing ones (which were termed ‘Type 3’), and showed only little necrosis and leaves cupping; however, their leaf area was already significantly lower compared with that of ‘Type 3’ (Figure 5.3b). ‘Type 2’ incompatibility became more apparent at 50 days after germination, and even more so at 85 days (Figure 5.3a and c), when their growth was irreversibly blocked or they had died. In summary, this ‘Type 2’ hybrid necrosis acts more slowly than the ‘Type 1’ class, reaching its complete expression only three months after germination. The existence of a number of highly distorted regions in the parental genetic maps

(Annex 2) suggested the presence of pre-zygotic (not characterized) and post-zygotic lethal loci affecting the offspring development. Lethal genes involved in ‘Type 1’ and ‘Type 2’ hybrid necrosis are likely to be located in some of these regions.

The different timing of the expression of ‘Type 1’ and ‘Type 2’ lethality indicated that they were caused by two independent post-zygotic incompatibilities. This hypothesis was supported by the molecular analysis, which clearly showed these two phenotypes to be due to different and unlinked loci (Table 5.2).

Negative epistatic interactions cause ‘Type 1’ and ‘Type 2’ lethality

The different experiments (Motueka 2010, Angers 2011 and Motueka 2014) were heterogeneous for the segregation ratios for three phenotypes, which might be attributed to the different environmental conditions, including the treatments to which seeds were subjected. However, in all the experiments the timing of the expression of ‘Type 1’ and ‘Type 2’ lethality was the same. The observed segregation ratio for ‘Type 1’:‘Type 2’+‘Type 3’ was close to either a 1:3 or a 3:13 Mendelian ratio (Table 5.1), the 1:3 ratio indicating a recessive genetic control or the action of two loci with dominant epistasis, while the 3:13 ratio indicates a two locus control with dominant suppression epistasis (Table 5.6). The molecular analysis we performed on both necrotic and non-necrotic seedlings showed ‘Type 1’ lethality to be linked to two loci, one mapping to LG5 of PEAR3 and one to LG1 of ‘Moonglow’. The genotypic combinations at these two loci for ‘Type 1’ versus ‘Type 2’ + ‘Type 3’ was close to a ratio of 1:3 (Table 5.1), as per the model of epistatic interaction between two loci with no dominance, consistent with the BDM-model of hybrid incompatibility (Orr 1996).

The 1:1 ratio of the ‘Type 2’ phenotype with normally growing ‘Type 3’ seedlings (Table 5.1) indicated a single locus or a two locus control (Table 5.6). Only markers mapping to LG2 of PEAR3 were found to be associated with ‘Type 2’ lethality (Table 5.2). However, Figure 5.7 illustrates that it is also possible that the LG2 locus interacts with another, yet unmapped, locus that would be homozygous for the viable allele in PEAR3 (“aa”) and homozygous for the lethal allele in ‘Moonglow’ (“ll”). In this case, all progeny would have genotype “al” and contribute the lethal allele, but the ‘Type 2’ inviability would only be expressed in the simultaneous presence of

the lethal allele of the gene on LG2. Since no segregation distortion was visible for this second locus in the F1 progeny, its chromosomal location could not be identified. This two-locus hypothesis is more probable than the single locus one, because post-zygotic incompatibilities have usually been demonstrated to be caused by epistatic interactions between at least two genes (Orr 1996; Bomblies and Weigel 2007). Backcrossing the viable F1 progeny (which carries the lethal allele only at the unknown locus and not at LG2 locus) with PEAR3 would validate this hypothesis (Figure 5.7).

Resistance genes might be involved in ‘Type 1’ and ‘Type 2’ inviability

The frequency of ‘Type 1’ seedlings carrying the incompatible allele inherited from LG1 of ‘Moonglow’ is higher for SSR CHVf1 than the markers flanking it (Table 5.4), indicating that the lethal gene is closely linked to this SSR and located between markers MDP0000160413_LG1b and MDP0000251943_LG1b, which spanned a region of 8 cM (Figure 5.5). In apple, SSR CHVf1 is tightly linked to two major genes conferring scab (*Venturia inaequalis*) resistance, *Rvi6* and *Rvi17* (Bus et al. 2011), historically known as *Vf* (Vinatzer et al. 2004) and *Val* (Dunemann and Egerer 2010), respectively. As the apple and pear genomes are highly syntenic (Yamamoto et al. 2004; Pierantoni et al. 2004; Celton et al. 2009), it is possible that a locus orthologous to the apple *Rvi6* gene is involved in ‘Type 1’ lethality in the PEAR3 x ‘Moonglow’ population. In pear the scab (*V. nashicola*) resistance gene *Vnk*, later re-named *Rvn1*, has also been mapped to LG1, upstream of the orthologous apple region carrying the *Rvi6* gene (Iketani et al. 2001; Terakami et al. 2006; Bouvier et al. 2011). *Rvi6* has been frequently associated with segregation distortion and hybrid necrosis events in apple (Alston 1976), and two sub-lethal genes, *s11* and *s12*, were mapped very close to it (Gao and Van de Weg 2006). As this resistance originated from *M. floribunda*, widely used by apple breeders in interspecific crosses in order to obtain high value cultivars with pyramided scab resistance (Crosby et al. 1992), inter-species incompatibilities may well be at the basis of the hybrid necrosis in apple, as reported here for pear. It is of interest that one of two parental genetic maps constructed in a different pear interspecific population, used by Won et al. (2014) to detect QTL for resistance to *V. pirina*, completely lacked LG1. This might

have been caused by high segregation distortions for the markers that had been predicted from prior knowledge in pear and apple to map to the LG.

In PEAR3 LG5 locus, interacting with ‘Moonglow’ LG1 locus, the marker with highest frequency in ‘Type 1’ seedlings was the HRM marker LETss527789863 (Table 5.4). Because the segregation distortion increased while moving down the LG from this point, we concluded that the lethal gene on PEAR3 LG5 might be located between this marker and SSR Hi04d02, within a region of 22 cM (Figure 5.5). It is possible that the lethal gene mapping to LG5 of PEAR3 might encode for an R protein that interacts with the one encoded by the locus on LG1, in line with previous results in Bomblies et al. (2007); Alcázar et al. (2009). Indeed, LG5 is one of the chromosomes in the *P. x bretschneideri* genome with the highest number of R gene paralogs clusters (Wu et al. 2013). Furthermore, Calenge et al. (2004) mapped a QTL for scab resistance to LG5 in apple. However, it is also possible that the lethal gene mapping to LG5 of PEAR3 might encode for another endogenous protein, which would have a “guard-guardee” type of interaction with the LG1 R protein, similar to that reported in tomato by Krüger et al. (2002).

For the second class of hybrid necrosis, ‘Type 2’, the highest frequencies were detected for incompatible alleles of markers CN493139, LETss527788384 and CN444636, mapping to LG2 of PEAR3 (Table 5.4), hence we postulate that the lethal locus might be located close to these markers, within a region of 13 cM (Figure 5.5). Moreover, we mapped the *let2* locus, which controls the ‘Type 2’ phenotype, 5 cM downstream of CN493139 (Figure 5.5). In the *P. x bretschneideri* genome, LG2, like LG5, is rich in R gene paralogs clusters, (Wu et al. 2013), and several QTLs and major genes for resistances to pests and diseases in pear have been mapped to this LG (Dondini et al. 2004; Bouvier et al. 2011; Le Roux et al. 2012). An example of an interaction between a resistance gene on LG2 and another linkage group causing segregation distortion is found in *Malus x domestica*, where the interaction between apple scab resistance loci *Rvi2* on LG2 and *Rvi6* on LG1 (formerly *Vh2* and *Vf*), first reported in (Bus et al. 2005), has been observed frequently since then as an outcome of pyramiding these resistances in breeding programs (unpublished). Hence, it is possible that R genes might also be associated with ‘Type 2’ lethality in pear, as postulated for ‘Type 1’. Much further work is needed to test all these hypotheses.

*Incompatible alleles were inherited from different *Pyrus* spp.*

On LG5 of PEAR3, where a locus causing ‘Type 1’ inviability was mapped, two SSR markers, CH03a09 and CH05f06, provided sufficient information to conclude that the incompatible allele originated from the Asian pear ‘Xue Hua Li’ (Table 5.5, Figure 5.6). However, it was not possible to determine the origin of the incompatibility for the interacting locus, mapped to LG1 of ‘Moonglow’, as either parent of ‘Moonglow’ (European pears Michigan-US 437 and ‘Roi Charles de Wurtemberg’) could have potentially contributed the CHVf1 null allele. Nevertheless, we can still conclude that ‘Type 1’ hybrid necrosis resulted from the interaction between an Asian pear allele from a locus on LG5 and a European pear allele from a locus on LG1. Consequently, ‘Type 1’ hybrid necrosis is a typical result of inter-species gene-flow barriers, and the mutation which caused the evolution of the incompatible alleles might date back to the time when *P. x bretschneideri* and *P. communis* diverged.

In contrast, the ‘Type 2’ lethal allele at the locus mapped to LG2 was not derived from ‘Xue Hua Li’, and might be inherited from the unknown male parent of PEAR3 (Table 5.5, Figure 5.6). We propose that this LG2 allele has to interact with one from another gene inherited from ‘Moonglow’, whose position is unknown, in order to produce incompatibility (Figure 5.7).

It is noteworthy that Yamamoto et al. (2007) reported severe segregation distortion in both LGs 2 and 5 in the European pear ‘La France’ in a cross with a *P. pyrifolia* (Japanese pear) accession: lethal genes causing inter-species incompatibility might be at the basis of this segregation distortion, as in our population, although we observed the segregation distortion in the Asian cultivar (*P. x bretschneideri*), rather than in the European one. *P. x bretschneideri* is thought to be an interspecific hybrid of *P. ussuriensis* x *P. betulaeifolia*, however it may involve *P. pyrifolia* as well (Bell 1991).

Additional lethal loci might be involved in other types of incompatibility in the PEAR3 x ‘Moonglow’ population

Apart from the genomic segments identified on LGs 2 and 5 of PEAR3 and LG1 of ‘Moonglow’, distorted regions were detected on LG10 of both parents and on LGs 9 and 16 of ‘Moonglow’ (Annex 2). However, these were not involved in either ‘Type 1’ or ‘Type 2’ lethality, since the

genotypes for markers mapped to these regions were in equilibrium for both necrotic and non-necrotic seedlings. The high germination rates observed in the three experiments indicates absence of incompatibility at this stage of plant development. However, seeds were subjected to special treatments to promote germination in our study, while under natural conditions a higher number might fail to germinate. Our data did not enable us to determine whether those regions were involved in pre-zygotic incompatibility, or in aberrations of the germination process. Among the LGs exhibiting distortion, LG10 is of particular interest, not only because it is distorted in both parents, but also because of the homology demonstrated between LGs 10 and 5 in both pear (Wu et al. 2013) and apple (Velasco et al. 2010) genomes. Distorted segregations of markers mapping to LG10 have been previously reported in several apple populations (Conner et al. 1997; Maliepaard et al. 1998; Liebhard et al. 2003; Kenis and Keulemans 2005).

In summary, this is the first reported description of hybrid necrosis in pear. We have shown that, although interspecific hybridization within the *Pyrus* genus is possible, there are genetic barriers which might cause the loss of at least a proportion of the hybrid offspring.

Our detection of chromosome regions involved in post-zygotic incompatibilities in pear hybrids is of considerable value, contributing both to studies on speciation and evolution, and to breeding. Firstly, incompatibilities between two species might have arisen when they diverged in the evolutionary process, and their identification could assist in discovery of the selective events that drove the species differentiation. In particular, BDM-incompatibilities, which involve alleles mutations that do not lower fitness within the diverging lineages, can accumulate rapidly (Rieseberg et al. 2003), and their identification might help to locate the speciation forces in the timeline (Orr 1995). Secondly, breeders pyramiding resistances to enhance durability should note that they may end up with the loss of the desired resistance combination, because of incompatibilities skewing the progeny segregation. In addition, genes associated with other desired traits could co-segregate with lethal genes and be lost to the breeding population. Consequently, our identification of molecular markers linked to lethal genes will be useful for pear breeders, who will now be able to select parents that avoid incompatible combinations that potentially affect expression of the traits of interest.

The recent publication of the Chinese (Wu et al. 2013) and European (Chagné et al. 2014) pear genome sequences offers the opportunity to develop new markers that can be used to further

reduce the interval of the three regions linked to hybrid necrosis and identify candidate lethal genes.

Author contributions

SM, DC and LB designed the experiments. SM performed the marker development, the genotyping and the genetic mapping and wrote the manuscript. LB performed the phenotyping experiments in NZ and PG in Fr. DC co-wrote the manuscript together with SM. LB and VGMB developed the PEAR3 x ‘Moonglow’ population. DC, LP, SEG and CED oversaw the genotyping and mapping part of the work. CED, RV, SEG, and DC were the co-PIs on the SM PhD project that led to this manuscript; they conceived the study and participated in its design and coordination, together with VGMB and LP. All authors read and approved the final manuscript.

Acknowledgements

SM was funded by the Fondazione Edmund Mach (FEM) PhD School. We greatly thank Robert Lamberts at PFR (Motueka, New Zealand) for taking the pictures used in this article and calculating the leaf area, Chris Morgan at PFR (Motueka, New Zealand) for helping designing the germination protocol in 2014 and for his assistance with the pollination and the seed and plant measurements. We are also grateful to the technicians of the INRA greenhouse facilities (INEM), especially Nicolas Dousset and Michel Boucourt, for taking care of the seedling growth in 2010.

Compliance with ethical standards

The experiments conducted in this research comply with the current laws of the countries in which they were performed.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

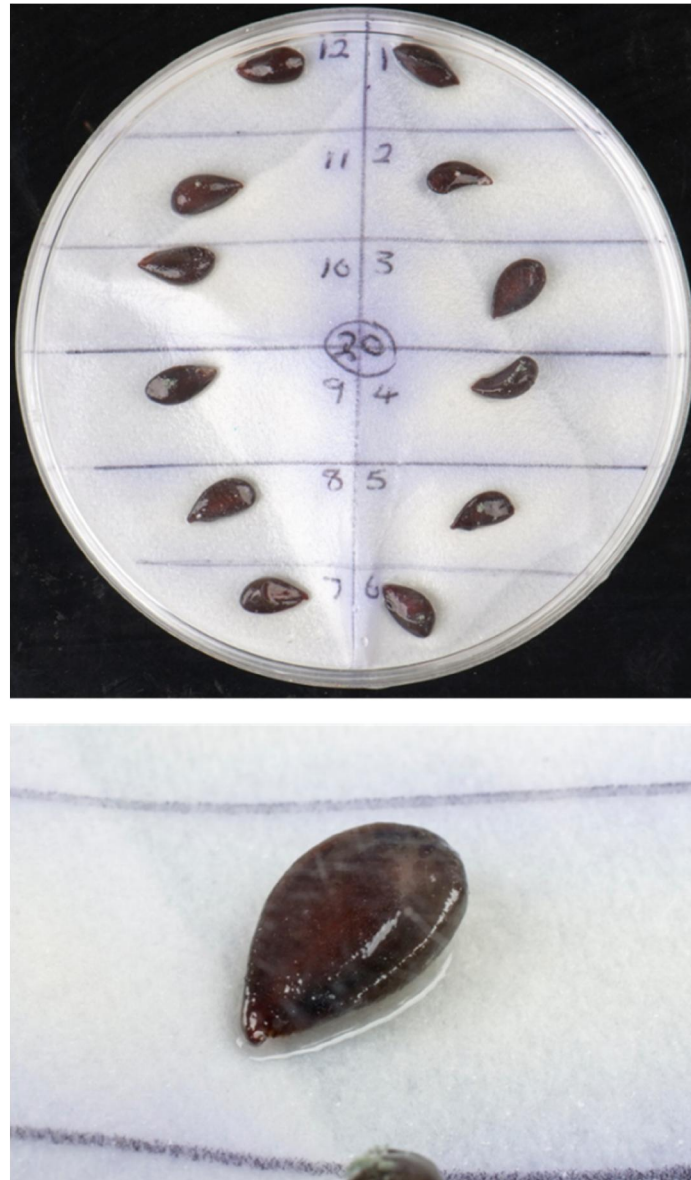


Figure 5.1: Seeds from the PEAR3 x ‘Moonglow’ cross subjected to special treatment to enhance germination in Motueka in 2014

Before sowing, the seeds were dipped into a fungicide solution and then left on moist filter paper in petri dishes until germination

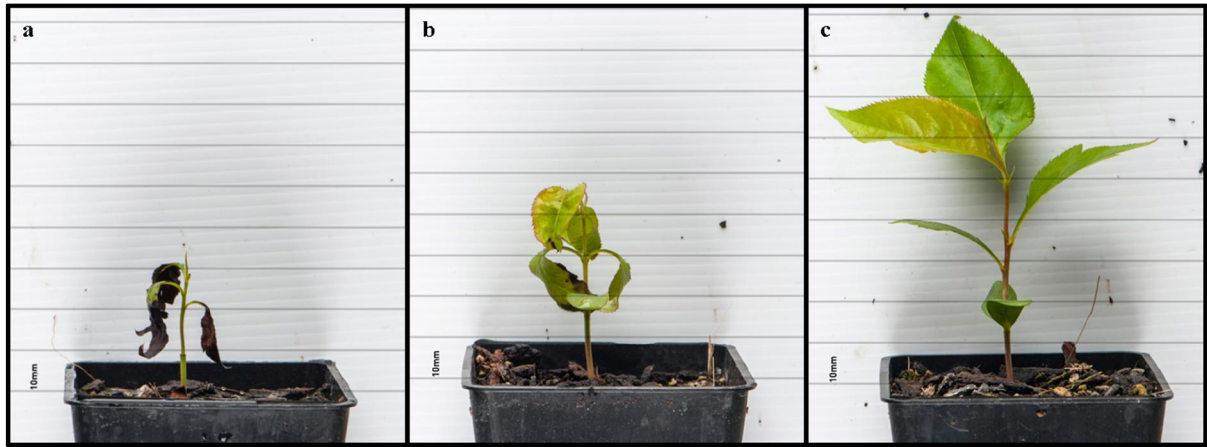


Figure 5.2: Hybrid necrosis phenotypes in the interspecific PEAR3 x ‘Moonglow’ population

Three distinct phenotypes were observed in the seedlings. Pictures were taken 30 days after germination. (a) ‘Type 1’ seedlings had stopped growing and chlorosis and necrotic lesions were apparent on their leaves. (b) ‘Type 2’ seedlings grew normally initially, however their leaves began to cup downwards and to become chlorotic and necrotic. (c) ‘Type 3’ seedlings grew normally

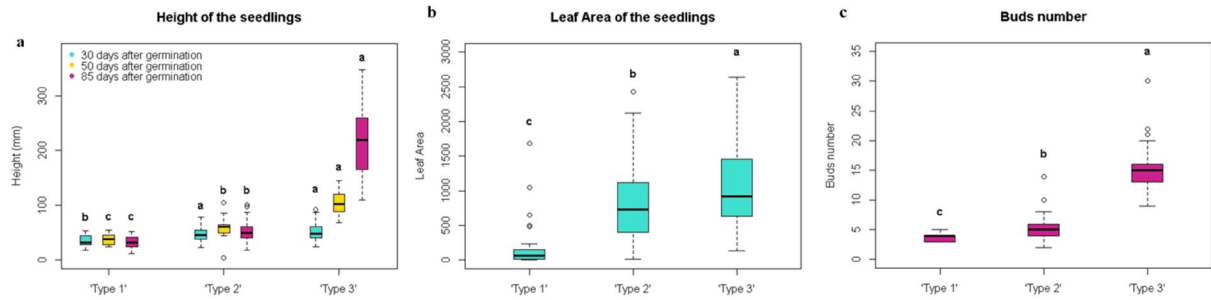


Figure 5.3: Differences in plant development among ‘Type 1’, ‘Type 2’ and ‘Type 3’ seedlings in the PEAR3 x ‘Moonglow’ progeny sowed in Motueka in 2014

The letters on top of each box (a, b and c) represent significant differences (according to the multiple comparison with Kruskal-Wallis test). (a) Height of the seedlings measured at 30 (in light blue), 50 (in yellow) and 85 (in purple) days after germination. Significant differences among the three types are shown for each assessment. (b) Leaf area measured at 30 days after germination. (c) Average number of buds counted at 85 days after germination

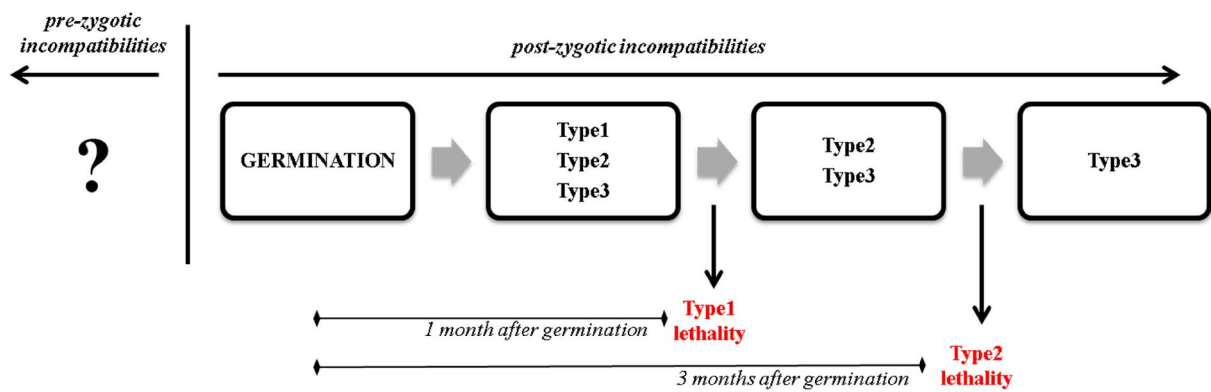


Figure 5.4: Timing for the expression of the genetic incompatibilities and lethality that occur in the PEAR3 x 'Moonglow' population

A timeline is drawn to show when 'Type 1' and 'Type 2' seedlings die or irreversibly stop growing and necrotize

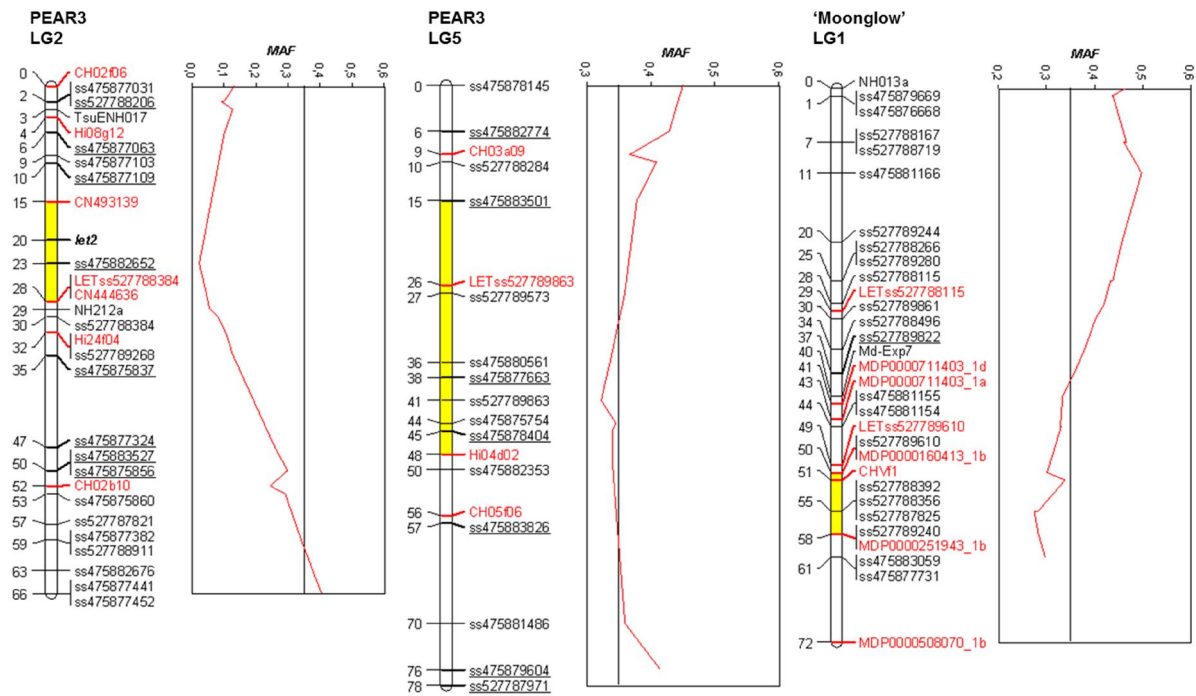


Figure 5.5: Genetic map of linkage groups (LGs) 2 and 5 of PEAR3 and LG1 of 'Moonglow' indicating regions of segregation distortion

High Resolution Melting (HRM) and Simple Sequence Repeats (SSRs) markers developed for 'Type 1' and 'Type 2' screening are highlighted in red. Newly added SNPs compared to the map of Montanari et al. (2013) are underlined. The regions involved in hybrid necrosis are marked in yellow. The locus *let2* linked to 'Type 2' phenotype is in bold and italic. The Minor Allele Frequency (MAF) is presented as a measure of segregation distortion of the markers evaluated on non-necrotic progeny

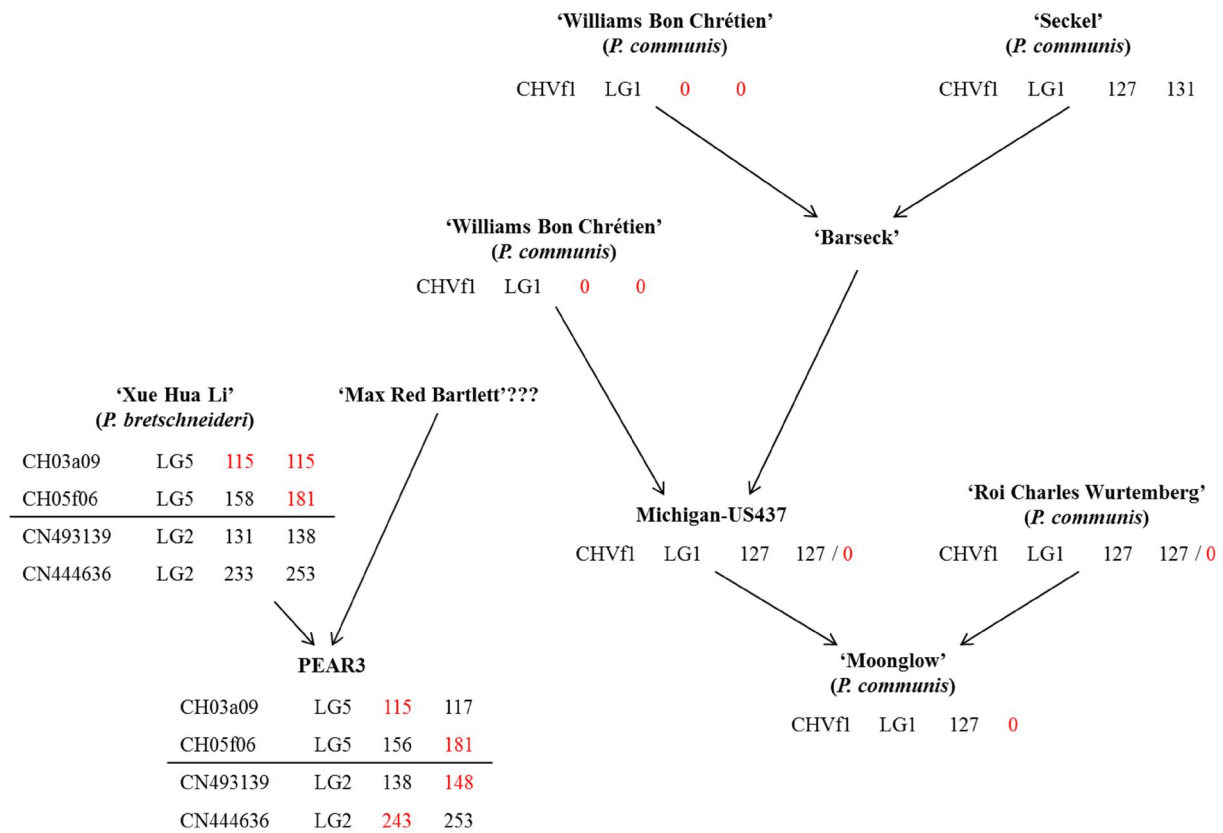


Figure 5.6: Inheritance of the lethal alleles in the PEAR3 x 'Moonglow' pedigree

Progenitors of PEAR3 and 'Moonglow' were scanned with Simple Sequence Repeats (SSR) markers mapped within the regions involved in hybrid necrosis. For each marker, the incompatible allele (in bp) is highlighted in red

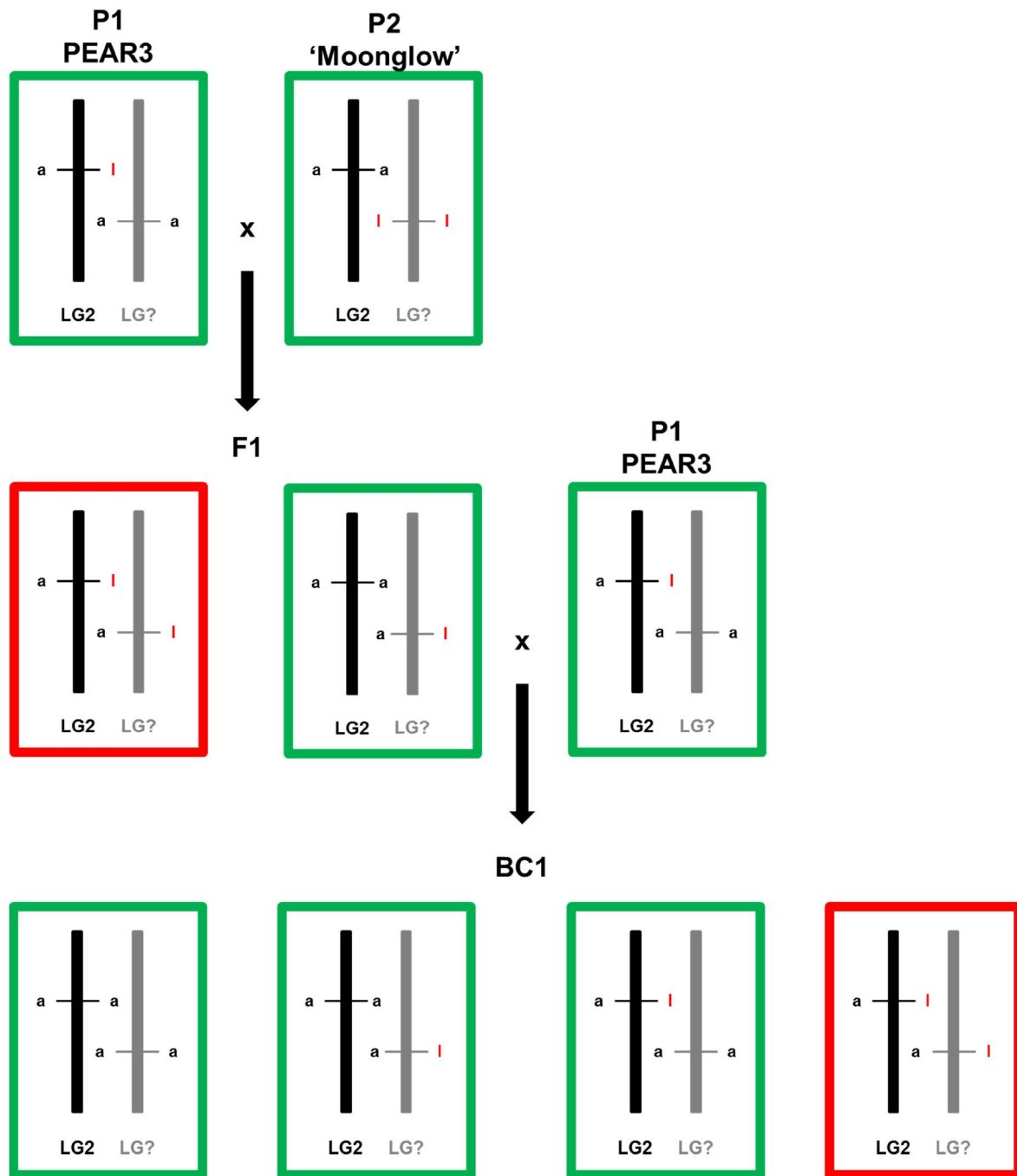


Figure 5.7: Putative genetic model for the two-locus interaction causing 'Type 2' lethality in the PEAR3 x 'Moonglow' population

The lethal alleles are marked in red. When the lethal alleles at both loci (i.e. on LG2 and an unknown LG) co-exist, that individual dies (red boxes); if none or just one lethal allele is present, that individual grows normally (green boxes). Segregation distortion can be observed in the F1 for the locus on LG2, and in the BC1 for the other locus

Table 5.1: Observed phenotypic segregation ratios for hybrid necrosis in the PEAR3 x ‘Moonglow’ population and Chi-square (X^2) test.

For each experiment (Motueka 2010, Angers 2011, and Motueka 2014), the number of seedlings was counted for each class (‘Type 1’, ‘Type 2’ and ‘Type 3’). The X^2 test was performed for ‘Type 1’:‘Type 2’+‘Type 3’ = 1:3 or 3:13 and for ‘Type 2’:‘Type 3’ = 1:1 for all three experiments individually. The Total X^2 , the Pooled X^2 and the heterogeneity were calculated. The degrees of freedom (df) and the p-values are shown. At $p < 0.05$ the observed segregation ratios are significantly different from the expected ratios.

Location and year of experiment	Number of seedlings					1:3 segregation			3:13 segregation		
	‘Type 1’		‘Type 2’+‘Type 3’		Total	X^2	df	ρ	X^2	df	ρ
Motueka 2010	153	21.7%	551	78.3%	704	4.01	1	0.045	4.11	1	0.043
Angers 2011	101	15.4%	556	84.6%	657	32.48	1	0.000	4.92	1	0.027
Motueka 2014	44	19.4%	183	80.6%	227	3.82	1	0.051	0.06	1	0.806
Total						40.30	3	0.000	9.09	3	0.028
Pooled						32.92	1	0.000	0.00	1	0.987
Heterogeneity						7.39	2	0.025	9.09	2	0.011
Location and year of experiment	Number of seedlings					1:1 segregation					
	‘Type 2’		‘Type 3’		Total	X^2	df	ρ			
Motueka 2010	271	49.2%	280	50.8%	551	0.15	1	0.699			
Angers 2011	260	46.8%	296	53.2%	556	2.33	1	0.127			
Motueka 2014	79	43.2%	104	56.8%	183	3.42	1	0.064			
Total						5.89	3	0.117			
Pooled						3.80	1	0.051			
Heterogeneity						2.09	2	0.352			

Table 5.2: Segregation ratios for the High Resolution Melting (HRM) markers mapped to the regions involved in hybrid necrosis in the PEAR3 x ‘Moonglow’ population.

The segregation ratios of the combined genotypic classes for the markers on PEAR3 linkage group (LG)5 and on ‘Moonglow’ LG1 are compared between ‘Type 1’ and ‘Type 2’+‘Type 3’ progeny. The segregation ratios of the genotypic classes for the marker on PEAR3 LG2 are compared between ‘Type 2’ and ‘Type 3’ progeny. The incompatible genotypes are underlined.

PEAR3 LG5 + ‘Moonglow’ LG1 LETss527789863 (<abxcd>) + MDP0000160413 LG1b (<nnxnp>)		
Genotype	‘Type 1’	‘Type 2’+‘Type 3’
acnn	3.7%	11.5%
adnn	1.9%	15.1%
<u>bcnn</u>	<u>38.9%</u>	<u>1.2%</u>
<u>bdnn</u>	<u>40.7%</u>	<u>3.6%</u>
acnp	3.7%	12.1%
adnp	1.9%	15.7%
bcnp	7.4%	18.1%
bdnp	1.9%	22.9%
PEAR3 LG2 LETss527788384 (<lmxll>)		
Genotype	‘Type 2’	‘Type 3’
ll	12.4%	92.3%
<u>lm</u>	<u>87.6%</u>	<u>7.7%</u>

Table 5.3: Single nucleotide polymorphism (SNP) markers with highly distorted segregations that were incorporated in the published PEAR3 x ‘Moonglow’ parental genetic maps (Montanari et al. 2013).

For each SNP, the segregation and location on the map are shown, as well as the Chi-square (X^2) test value and significance level (*=0.1, **=0.05, ***=0.01, ****=0.005, *****=0.001, *****=0.0005, *****=0.0001), and the Minor Allele Frequency (MAF). Completely distorted SNPs could not be mapped, however their imputed locations on the map are shown (based on their location in other pear genetic maps).

Newly mapped SNPs	Segregation type in PEAR3x‘Moonglow’	Location on PEAR3x‘Moonglow’ map	X^2	Significance of X^2	MAF
ss527788206	<a0xb0>	LG2 of PEAR3	145.41	*****	0.09
ss475877063	<a0xaa>	LG2 of PEAR3	131.52	*****	0.10
ss475877109	<a0xa0>	LG2 of PEAR3	101.72	*****	--
ss475882652	<a0x00>	LG2 of PEAR3	198.46	*****	0.02
ss475875837	<abx00>	LG2 of PEAR3	120.30	*****	0.13
ss475877324	<a0x00>	LG2 of PEAR3	50.07	*****	0.26
ss475883527	<abxab>	LG2 of PEAR3	57.12	*****	--
ss475875856	<abxaa>	LG2 of PEAR3	35.52	*****	0.30
ss475882774	<a0xaa>	LG5 of PEAR3	4.43	**	0.43
ss475883501	<a0x00>	LG5 of PEAR3	12.94	*****	0.38
ss475877663	<a0xa0>	LG5 of PEAR3	92.24	*****	--
ss475878404	<a0x00>	LG5 of PEAR3	22.48	*****	0.34
ss475883826	<a0xa0>	LG5 of PEAR3	72.48	*****	--
ss475879604	<abxaa>	LG5 of PEAR3	6.11	**	0.42
ss527787971	<abxab>	LG5 of PEAR3	47.15	*****	--
ss527789822	<aaxab>	LG1 of ‘Moonglow’	17.63	*****	0.36
Newly mapped SNPs	Segregation type in PEAR3x‘Moonglow’	Imputed location on PEAR3x‘Moonglow’ map	Distortion		
ss475876968	<abxaa>	LG2 of PEAR3	completely distorted		
ss527787834	<abxaa>	LG2 of PEAR3	completely distorted		
ss527788214	<abxaa>	LG2 of PEAR3	completely distorted		
ss475876969	<abxaa>	LG2 of PEAR3	completely distorted		
ss475877229	<abxaa>	LG2 of PEAR3	completely distorted		

Table 5.4: Proximity of the lethal genes to markers located within the regions linked to hybrid necrosis in PEAR3 x ‘Moonglow’ population.

For the combined loci from linkage group LG5 of PEAR3 and LG1 of ‘Moonglow’, the percentage of ‘Type 1’ contributing the incompatible alleles over the total ‘Type 1’ genotyped was calculated. For LG2 of PEAR3 the percentage of ‘Type 2’ contributing the incompatible alleles over the total ‘Type 2’ genotyped was calculated. The higher the percentage, the closer the marker is to the lethal gene. For each marker the location on the genetic map, the allelic composition, linkage phase (with respect to the parent where the marker was mapped) and the incompatible allele are shown. The closest marker to the lethal gene is indicated in bold.

PEAR3 LG5								
Marker	CH03a09	LETss527789863	Hi04d02	CH05f06				
Position (cM)	9.2	26.2	48.3	56.2				
Segregation	<lmxll>	<abxcd>	<abxcd>	<abxcd>				
Phase	repulsion	coupling	repulsion	coupling				
Incompatible allele	l (115bp)	b	a (164bp)	b (181bp)				
% of ‘Type 1’	48.8	90.7	83.7	81.3				
‘Moonglow’ LG1								
Marker	LETss527788115	MDP0000711403_LG1d	MDP0000711403_LG1a	LETss527789610	MDP0000160413_LG1b	CHVf1	MDP0000251943_LG1b	MDP00000508070_LG1b
Position (cM)	28.9	40.8	42.5	49.1	50.3	51.0	58.4	71.9
Segregation	<nnxnp>	<nnxnp>	<nnxnp>	<abxcd>	<nnxnp>	<nnxnp>	<abxcd>	<nnxnp>
Phase	repulsion	coupling	repulsion	coupling	repulsion	coupling	coupling	coupling
Incompatible allele	n	p	n	d	n	p (0)	d	p
% of ‘Type 1’	76.9	81.8	83.6	85.2	85.2	86.5	77.8	47.2
PEAR3 LG2								
Marker	CH02f06	Hi08g12	CN493139	LETss527788384	CN444636	Hi24f04		
Position (cM)	0.0	4.5	14.6	27.7	28.2	31.7		
Segregation	<abxcd>	<efxeg>	<efxeg>	<lmxll>	<abxcd>	<lmxll>		
Phase	coupling	coupling	repulsion	coupling	repulsion	coupling		
Incompatible allele	b (0)	f (196bp)	e (148bp)	m	a (243bp)	m (139bp)		
% of ‘Type 2’	70.8	84.7	87.8	87.6	87.7	83.6		

Table 5.5: Simple sequence repeats (SSRs) profile for PEAR3, ‘Moonglow’ and their progenitors.

For each marker, the incompatible allele (in bp) is in bold and underlined.

Marker	CHV1	CH03a09	Hi04d02	CH05f06	CH02f06	Hi08g12	CN493139	CN444636	Hi24f04
Linkage Group	LG1	LG5	LG5	LG5	LG2	LG2	LG2	LG2	LG2
PEAR3	129-129	<u>115</u> -117	<u>164</u> -173	156- <u>181</u>	150- <u>0</u>	179- <u>196</u>	138- <u>148</u>	<u>243</u> -253	129- <u>139</u>
‘Moonglow’	127- <u>0</u>	115-115	158-197	173-179	174-177	179-205	135-148	237-245	144-144
Michigan-US 437	127-127/ 127- <u>0</u>	112-115	158-197	173-173	177-193	194-205	135-16	228-245	144-147
‘Roi Charles de Württemberg’	127-127/ 127- <u>0</u>	112-115	158-173	179-179	174-174	179-179	148-150	232-237	144-144
‘Williams Bon Chrétien’	<u>0-0</u>	<u>115-115</u>	158-197	173-173	177-194	???	135-16	228-245	144-147
‘Seckel’	127-131	112-115	158-173	159-173	154-177	194-205	135-152	239-245	137-144
‘Xue Hua Li’	129-135	<u>115-115</u>	<u>164</u> -173	158-181	150-150/ 150- <u>0</u>	179- <u>196</u>	131-138	233-253	129-142

Table 5.6: Possible segregation types in PEAR3 x 'Moonglow' population explaining 'Type 1' and 'Type 2' lethality.

'Type 1' seedlings had a 1:3 or a 3:13 segregation ratio with 'Type 2'+ 'Type 3'; 'Type 2' seedlings had a 1:1 segregation ratio with 'Type 3'. For these three segregation ratios, the number of loci possibly involved, the dominance of the lethal alleles and the segregation type in PEAR3 x 'Moonglow' are shown. The segregation types which resulted to cause 'Type 1' and 'Type 2' lethality are highlighted.

<i>If 'Type 1': 'Type 2'+ 'Type 3' = 1:3</i>			
number of loci involved	lethal allele 1st locus (l)	lethal allele 2nd locus (L)	Segregation type in PEAR3 x 'Moonglow'
one	recessive	--	al x al
two	recessive	recessive	al-LL x al-LL
two	recessive	recessive	al-LL x ll-AL
two	recessive	dominant	al-AA x al-LL
two	recessive	dominant	al-AL x al-LL
two	recessive	dominant	al-AL x ll-AA
two	recessive	dominant	al-LL x al-LL
two	dominant	dominant	aa-AL x al-AA
two	no	no	al-AB x cd-CL
<i>If 'Type 1': 'Type 2'+ 'Type 3' = 3:13</i>			
number of loci involved	lethal allele 1st locus (l)	lethal allele 2nd locus (L)	Segregation type in PEAR3 x 'Moonglow'
two	recessive	dominant	al-AL x al-AL

<i>If 'Type 2': 'Type 3' = 1:1</i>			
number of loci involved	lethal allele 1st locus (l)	lethal allele 2nd locus (L)	Segregation type in PEAR3 x 'Moonglow'
one	no		al ₁ x l ₂ l ₂ *
two	recessive	dominant	al-LL x ll-AA
two	dominant	dominant	aa-AL x ll-AA

CHAPTER 6. General Discussion and Conclusion

The goal of this thesis was to study the genetic determinism of pear resistance to fire blight and psylla in an interspecific population, PEAR3 (*P. x bretschneideri* X *P. communis*) x ‘Moonglow’ (*P. communis*), and to detect the chromosomic regions linked to hybrid necrosis. During this work, I developed molecular tools useful for genomics studies of pear and for the implementation of MAS.

PEAR3 x ‘Moonglow’ population: its pedigree and the sources of resistances

The PEAR3 x ‘Moonglow’ population was developed to unravel the genetic basis of resistance to several pests and diseases. In this thesis, resistances to fire blight (*E. amylovora*) and pear psylla (*C. pyri*) were studied.

PEAR3 is an interspecific hybrid derived from the Chinese pear ‘Xue Hua Li’ (*P. x bretschneideri*). This cultivar, as many Asian species, is a good source of resistance to pests and diseases, including *C. pyri*. The variety *P. communis* ‘Max Red Bartlett’ (MRB) was thought to be the pollen parent of PEAR3, but this genealogy turned out to be wrong. MRB is a red-skinned sport variety of WBC, generated from a bud mutation. MRB and WBC are supposed to be genetically (as they are phenotypically) identical, except for the gene involved in the reddening of the fruit skin, mapped to LG4 (Dondini et al. 2008). The SSR scanning of WBC performed in this thesis project to study the inheritance of fire blight resistance alleles and incompatible alleles causing hybrid necrosis, revealed that WBC, and thus MRB, is not related to PEAR3. DNA tests with SSR markers should be carried out in the parent pool of PFR, in order to identify the real male parent of PEAR3. ‘Moonglow’ derives from a cross between Michigan-US 437 and ‘Roi Charles de Wurtemberg’ (RCW). Both these European pear genotypes are resistant to fire blight, and ‘Moonglow’ was previously reported to have low susceptibility to this disease as well (Quamme 1977; Paulin 1990). The progenitors of RCW are not known, although it is presumed to be an open seedling of ‘Beurré Clairjeau’, which in turn is presumed to be an open seedling of

construction of high density genetic maps, and then the more accurate detection of a greater number of QTLs and major genes.

SNPs have become the markers of choice for genetic mapping and association studies, since they are abundant across genomes and genetically more stable than SSRs. Furthermore, sophisticated and high-throughput SNP detection systems and SNP-based genotyping assays have been developed, making the construction of high-density genetic maps feasible and affordable (Troggio et al. 2007; Antanaviciute et al. 2012; Bianco et al. 2014). I took advantage of this advancements and I used the Illumina Infinium® II apple and pear 9K SNP array, containing more than 1000 newly developed European pear SNPs and about 8000 apple SNPs, to genotype a total of 220 progeny of the PEAR3 x ‘Moonglow’ population and build one of the first five SNP-based high-density genetic maps for pear (Montanari et al. 2013). I also used a set of apple and pear microsatellite markers for the genetic maps construction of PEAR3 x ‘Moonglow’, with the purpose of assigning the number and orientation to the LGs (Montanari et al. 2013); during the subsequent years of the thesis, these maps were further improved by the addition of other SSR and newly developed HRM markers (Table 6.1 and Annex 9, final maps).

Table 6.1: Number of markers and genetic length (in cM) of PEAR3 and ‘Moonglow’ maps developed during this project.

The number of single nucleotide polymorphism (SNP), microsatellite (SSR) and high resolution melting (HRM) marker are shown, as well as the total, and the density of the maps is calculated. Numbers refer to the final genetic maps of PEAR3 and ‘Moonglow’, including all the markers used in this project.

Parental map	LGs (N°)	SNP markers (N°)	SSR markers (N°)	HRM markers (N°)	Total markers (N°)	cM	Density (markers/cM)
PEAR3	17	206	37	11	256 *	988.340	0.3
‘Moonglow’	17	452	43	20	515	1067.321	0.5

* one double-locus SSR and one phenotypic marker

The high degree of polymorphism shown by the numerous apple and pear genetic markers in the interspecific PEAR3 x ‘Moonglow’ progeny indicates their transferability to other breeding populations. In particular, the SNP markers validated in this PhD project and the Illumina Infinium® II apple and pear 9K SNPs array are valuable tools for the construction of high-density genetic maps in other pear experimental families, which will increase the power of the QTLs and genes discovery for this crop. Although SSRs have been progressively replaced by SNPs in any

recent genotyping assay, they remain useful for pedigree assessments and for multiple comparisons among genetic maps generated in different populations and germplasm accessions, thanks to their multi-allelic nature (in comparison with the bi-allelism of SNPs) and their large employment in the last decades. Having used both SNP and SSR markers for the genetic map construction of PEAR3 x 'Moonglow' population, overall comparison of the relative genomic locations of already and here-newly identified genes/QTLs are possible.

The array I used in this thesis had a 9K content of SNP markers, comparable to other SNP arrays developed in the same years for other Rosaceae crops (9K for peach (Verde et al. 2012), 6K for cherry (Peace et al. 2012)). However, arrays targeting a much greater number of SNPs have been released more recently, such as the 20K and 90K SNPs arrays, respectively for apple (Bianco et al. 2014) and the cultivated strawberry (Bassil et al. 2015). Such high-density SNPs arrays will enable the construction of genetic maps with an incredibly high resolution, thereby enhancing the identification of marker-trait associations, and will be very useful for Pedigree-Based Analysis and GS. The number of SNPs discovered in *Pyrus* remains lower if compared to other more studied Rosaceae species, and despite the possibility of using apple markers for genotyping in pear, this issue might be addressed in the very next future if we want to accelerate the identification of gene-trait associations and implement MAS in this crop. The Chinese (Wu et al. 2013) and the European (Chagné et al. 2014) pear genomes can be used as reference for re-sequencing in *Pyrus* germplasm and the detection of SNPs, as has been performed for apple (Chagné et al. 2012) and peach (Verde et al. 2012). Newly developed SNP markers might be included in the 20K array of apple, or an array with a much wider specificity may even be generated for all the most important Rosaceae crops, exploiting the genomic relatedness within this family. However, the development of arrays with higher numbers of SNPs also increases the expenses of screening. A more promising technique is offered by genotyping-by-sequencing (GBS) (Elshire et al. 2011). GBS is based on the reduction of genome complexity with restriction enzymes and the subsequent high-throughput, next-generation sequencing of the cleaved genomic fragments. This method, which is technically simple and highly multiplexing, is applicable also to the most large and complex genomes. Several examples of the application of GBS in horticultural crops can already be found. Highly saturated parental genetic maps (with more than 9 and 6 markers per cM) were produced via GBS for a red raspberry (*Rubus idaeus*) segregating

population (Ward et al. 2013). GBS data were used to create a saturated genetic map of an apple segregating population and to identify a QTL underlying the skin color (Gardner et al. 2014). The genetic map of another apple population was built by Bastiaanse et al. (2015) applying GBS, with the purpose of identifying scab resistance genes in the cultivar ‘Geneva’. In the last couple of years, application of GBS in fruit tree crops was also reported in several international conferences. In kiwifruit (*Actinidia chinensis*) GBS data were employed to construct a genetic map for the identification of genetic markers for resistance to *P. syringae* pv. *actinidiae*, and for GS and genome-wide association studies in this crop (Deng et al. 2014; van Nocker and Gardiner 2014). GBS was applied for high-resolution genetic mapping also in *Vitis vinifera*, and the data were then used to identify single-marker associations for several qualitative and quantitative traits (Barba et al. 2014). In pear, the segregating population ‘Old Home’ x ‘Louise Bon Jersey’ was genotyped employing GBS, and, using both the ‘Bartlett’ v1.0 and the *P. x bretschneideri* genome assemblies as reference, 28,902 SNPs on 3150 scaffolds and 23,408 SNPs on 756 scaffolds were generated, respectively. A total of 14,433 SNPs were mapped to the consensus of ‘Old Home’ x ‘Louise Bon Jersey’ (Knäbel, unpublished). However, improvements still need to be made in the sequencing technologies, to address missing data issues in GBS analysis (Myles 2013). Nonetheless, this technique was proved to be valuable for accurate GS models in wheat (*Triticum aestivum* L.) (Poland et al. 2012).

State of the art of MAS for pest and disease resistance in pear

Pear crop cultivation, despite the large variability of the *Pyrus* species, is based on a relatively low number of cultivars (see General Introduction, “The pear crop”, “Origin and diversity of the genus *Pyrus* and its origin”), which have been selected to answer the consumers demand of particular fruit quality characteristics. However, the fast rate of evolution of pests and pathogens, facilitated by continuous monoculture, has always boosted the research of new resistances. Although in the last decade traditional breeding has started to be replaced with MAS in many crops, MAS for disease and pest resistances has not yet been implemented in pear breeding programs. One of the reasons is certainly the limited number and lack of confirmation of resistance loci discovered to date in pear. Indeed, in the more studied crop apple, where a larger

number of resistant loci have been mapped and associated markers identified, successful cases of MAS application have been reported (Tartarini et al. 2000; Kellerhals et al. 2011; Bassett et al. 2013; Jänsch et al. 2015). However, MAS for disease resistance in pear will be addressed in the USDA-SCRI RosBREED 2 project (<http://www.rosbreed.org/node/376>), an international collaboration which brings together scientists working on genomics, genetics, and breeding of rosaceous crops, with the aim of applying modern DNA tests and marker-assisted breeding to deliver new cultivars with superior horticultural quality and improved disease resistances.

The success of MAS depends on several factors. One of the most important is the validity of the target genetic marker (Ru et al. 2015). Once a QTL position has been confirmed in different mapping populations and markers associated with the trait of interest have been identified, they need to be validated in different genetic and environmental backgrounds, and in particular in the germplasm in which they have to be deployed (Dwivedi et al. 2007; Collard and Mackill 2008). Moreover, the recombination frequency between the markers and the trait loci should be as lowest as possible (Lande and Thompson 1990), and hence either they must be closely linked, or multiple flanking markers should be used (Dwivedi et al. 2007). A remarkable example of development of a set of reliable markers for MAS has been recently reported in apple (Jänsch et al. 2015). Eight loci, robustly associated with resistance to three diseases (apple scab, powdery mildew and fire blight), were the focus of this work. First, they identified SNP markers closely linked to these eight loci and tested them on the parents and the recombinant individuals of the mapping populations, and then validated the specificity of the alleles associated with the resistances in founder clones of the majority of modern apple cultivars. Such a pipeline could be applied also for the major QTLs for psylla and fire blight resistance detected in this PhD project, with the advantage that SNP markers associated with these two loci are already known.

The last frontier of marker-assisted breeding is GS. The potential of this approach in the breeding of important crops, especially for quantitatively inherited traits, has been reported (Kumar et al. 2012a; Poland et al. 2012; Zhao et al. 2012; Kumar et al. 2012b). MAS for the major QTLs for psylla and fire blight resistance here detected might be combined with GS for other important horticultural characters, such as fruit quality, tree physiology and fruit storability, leading to the identification of elite pear cultivars with several desirable traits. GS in pear will be enhanced by

the recent development of a whole-genome sequence for the Chinese (Wu et al. 2013) and European (Chagné et al. 2014) pears.

QTLs for fire blight and psylla resistance: future developments for the application of MAS in pear

The high density parental genetic maps of PEAR3 and ‘Moonglow’ were used during this project for QTL mapping analysis for fire blight and psylla resistance. Phenotyping for fire blight resistance was performed at two sites, Angers (France) and Havelock North (New Zealand). Phenotyping for *C. pyri* resistance was performed only at INRA of Angers, however replicated in two years, using a novel protocol developed during this project for the study of the antibiosis resistance of pear to this insect.

The major fire blight resistance QTL detected on LG2 of ‘Moonglow’ turned out to be stable across different environments and conserved with other pear populations (Le Roux et al. 2012); candidate markers for MAS, either SSRs or SNPs, have been identified and now need to be validated. In order to be reliably used for MAS, molecular markers must be tightly linked to the QTL for the target trait. Usually, fine-mapping of the confidence interval of a detected QTL is necessary for the identification of more closely linked markers. Fine-mapping is performed by using larger population sizes and a greater number of markers (Sen and Churchill 2001). Subsequently, markers should be validated by testing their effectiveness in determining the target phenotype in independent populations and different genetic backgrounds (Collard and Mackill 2008). With this purpose, I suggested the study of breeding populations directly derived from ‘Old Home’, one of the most important pear rootstocks resistant to fire blight, widely used in several pear breeding programs. Moreover, larger pear germplasm collections (such as that maintained at the National Clonal Germplasm Repository in Corvallis, Oregon, USA, http://www.ars.usda.gov/main/site_main.htm?modecode=20-72-15-00) should be screened with these markers and phenotyped for fire blight resistance, in order to test for phenotype-marker association and putatively confirm their tight association with this trait and the stability of the QTL in a larger germplasm. Resistant accessions should also be phenotypically evaluated under

inoculation with different *E. amylovora* isolates (or with a mixture of strains), in order to more reliably represent a fire blight infection event in the orchard.

In the last years, significant progress has been made in understanding how plant genomes function, for example with the development and improvement of technologies for expression analysis which are now capable of monitoring the entire transcriptome of a species. Whole-genome transcription profiling studies have thus been performed in apple with different types of microarray (Celton et al. 2014; Jensen et al. 2014; Segonne et al. 2014). The RNA-sequencing (RNA-Seq) technique (Wang et al. 2009) can also be used to analyze previously unidentified genes, making it possible to perform whole-genome expression studies in biological organisms whether their genome has been sequenced or not. The declining cost of sequencing has led to an increased use of RNA-Seq in the past few years (Van Verk et al. 2013). A perspective of my PhD project could thus be the use of RNA-Seq to explore the molecular mechanisms underlying the fire blight and psylla resistance QTLs detected here. Despite the accurate localization of the major QTLs is not yet reached, it would be possible to analyze bulks of progenies alternatively carrying the favorable and unfavorable alleles of a given QTL, in order to look for differentially expressed genes thanks to RNA-Seq performed within a time frame, including, for example, inoculation and the first steps of infestation. Analyzing the differential expression patterns of both progeny bulks should make it possible to identify co-regulated genes, and thus corresponding pathways putatively responsible for the observed resistance/susceptibility status of the progenies. Moreover, putative genomic co-localizations of some differentially expressed genes with the QTL region may end up with the identification of functional + positional candidate genes, thus helping in the putative characterization of the causal gene underlying the QTL. Another, more basic, application of ESTs is the development of gene-targeted SSR and SNP markers from their sequence (Dwivedi et al. 2007). In pear, a number of EST-bases SSRs (Nishitani et al. 2009; Zhang et al. 2014) and SNPs (Terakami et al. 2013) have been developed, but much less than in other important Rosaceae species (<http://www.rosaceae.org/node/32>).

Furthermore, the positional cloning of candidate genes underlying a QTL, with the identification of the polymorphic, functional motif causally associated with the phenotypic trait variation, enables the designing of functional markers, which are strictly linked to the trait of interest

(Andersen and Lübberstedt 2003). Functional markers will not suffer recombination with the trait in segregating breeding populations, hence providing the upper bound in MAS efficiency (Muranty et al. 2014). For example, rice genotypes with high yielding and two or three-gene pyramided resistances against bacterial leaf blight have been developed by functional markers-assisted selection (Perumalsamy et al. 2010); in wheat, a number of functional markers for disease resistances and other important agronomical traits have been developed (Liu et al. 2012). With the sequence of the two pear genomes available, fine-mapping and positional cloning of the region underlying a QTL detected in *Pyrus* will be more straightforward. In case of the LG2 QTL for fire blight resistance in pear, which was mapped in two European cultivars, the draft genome sequence of ‘Bartlett’ would result more advantageous. However, because of the low anchoring of this genome (~30% of the assembly scaffolds are anchored to a genetic map), the list of initial candidate genes for positional cloning might not be exhaustive; in this case, the RNA-Seq technique might turn out useful. A project aimed at improving the sequencing and anchoring of the ‘Bartlett’ genome has been initiated, and the candidate genes approach might be suitable by then.

The fire blight resistance QTLs on LG2 detected in PEAR3 x ‘Moonglow’ (this project) and in ‘Passe Crassane’ x ‘Harrow Sweet’ (Le Roux et al. 2012) populations had a strong effect on the phenotypic variation. This might indicate a more oligogenic, instead of polygenic, determinism of this resistance in pear. Interestingly, a gene-for-gene resistance to *E. amylovora* has already been found in a wild apple (Fahrenttrapp et al. 2013; Vogt et al. 2013), and the CC-NBS-LRR gene (*FB_MR5*), derived from *M. x robusta* 5, was successfully inserted in the *M. x domestica* cultivar ‘Gala’, conferring it the resistance to fire blight (Broggini et al. 2014). Likewise, if a single gene would be responsible for a large part of the resistance of ‘Moonglow’ to fire blight, this might be cloned and used to create genetically engineered pear cultivars with both high fruit quality characteristics and fire blight resistance in a much shorter time than via MAS. However, besides the limitation of many governmental laws on the release of genetically modified organisms, such a resistance will likely not be durable, as the *FB_MR5*-based resistance in apple was already broken twice by adapted *E. amylovora* strains (Norelli and Aldwinckle 1986; Peil et al. 2011; Vogt et al. 2013). Nevertheless, the phenotypic variation explained by ‘Moonglow’ LG2 QTL (~30%) is not as high as that reported by Peil et al. (2007) for *FB_MR5* QTL on *M. x*

robusta 5 (~80%) and therefore a gene-for-gene interaction is less likely, making ‘Moonglow’ resistance less strong, but putatively more durable. Consequently, with the objective of developing high valuable pear cultivars with a strong and durable resistance to fire blight, gene pyramiding strategies should be applied. Again, besides the limitations on the release of genetically modified organisms, a first combination to be proposed could be the *FB_MR5* gene transfer into ‘Moonglow’ using transgenesis. If the apple *FB_MR5* gene is functional in pear, it would generate a putatively more durable construct with the Moonglow quantitative resistance, strengthening the qualitative *FB_MR5* resistance, as shown for other pathosystems (Palloix et al. 2009; Brun et al. 2010). If other pear resistance sources are preferred, new screening of a wider genetic variability in pear would be necessary, in order to discover other strong effect and stable loci related to fire blight resistance. Intriguingly, the allele associated with resistance in ‘Moonglow’ was inherited from RCW, while no resistance factor derived from ‘Seckel’ (progenitor of Michigan-US 437, the female parent of ‘Moonglow’) was detected. ‘Seckel’ is a well-known variety for being highly resistant to fire blight, which source has been employed in several pear breeding programs. It is likely, then, that the alleles associated with resistance in ‘Seckel’ have been lost along the breeding line which led to the development of ‘Moonglow’. The genetic determinism of fire blight resistance in this cultivar should be unraveled, and MAS must be applied to select varieties carrying the pyramided alleles from both ‘Seckel’ and RCW. Furthermore, ‘Moonglow’ genetic map is not saturated and resistance loci might be located in regions not covered by markers. The resolution of ‘Moonglow’ genetic map should be increased, either with a few markers (possibly EST-based) targeted to the gaps, or with a second step of whole-genome genotyping, using, for example, GBS or the Illumina Infinium® II 20K SNPs array (Bianco et al. 2014) (as the transferability of *Malus* SNPs to *Pyrus* (and vice versa) was demonstrated (Montanari et al. 2013)).

Unlike fire blight resistance, for which pear cultivars with a high breeding value are known and have been used to make several crosses and to perform QTL mapping studies, the research on the genetic characterization of pear psylla resistance is relatively in its infancy. Moreover, resistance to pear psylla, like for other insects, is based on different biological mechanisms (antixenosis and antibiosis), and then it presumably has a more complex genetic determinism than fire blight. Nonetheless, the co-localization of both the QTLs on LG8 of PEAR3 (this project) and on LG17

of NY10355 (Bouvier et al. 2011a) with major *R* genes for aphids in apple (Stoeckli et al. 2008b; Bus et al. 2008; 2010) indicates a possible common mechanism underlying the resistance of these crops to phloem-feeders. Indeed, electrical penetration graph (EPG) studies, showed that resistance factors to this type of insects might be located in the phloem of the resistant accession NY10353 (Civolani et al. 2013b). The phenotypic characterization of an increased number of offspring from the PEAR3 x ‘Moonglow’ and genotyping with a high-density SNPs array or GBS would also improve the accuracy of the QTLs detected for pear psylla resistance. A selection of extremely resistant and extremely susceptible progeny, along with the parents, could be used for EPG studies in *C. pyri*, in order to confirm the hypothesis drawn by Civolani et al. (2013b). Furthermore, Salvianti et al. (2008) performed gene expression analysis in the susceptible pear cultivar ‘Bartlett’ and in the resistant selection NY10355 upon infestation with *C. pyri* and identified some candidate genes for the resistance to pear psylla. Although I searched for the chromosome location of those candidate genes via BLASTN against the ‘Bartlett’ sequence, I could not find any match with scaffolds anchored to LG8 or LG17, where the two major QTLs have been mapped. However, some candidate genes aligned to scaffolds not anchored to the ‘Bartlett’ genome, and they might then be located in one of these two LGs. Moreover, the type of gene expression study carried out by Salvianti et al. (2008) allowed only the identification of candidate genes homologous to already known genes for the defense against pathogen and/or insect attack in *Pyrus* spp. or other plants. Studies on the basis of plant resistance to insects have revealed the existence of a wide diversity of defense mechanisms and molecules, and many pathways are still extremely unclear (see General Introduction, “Diseases, pests and crop protection”, “Plant responses to insect herbivory”). Therefore, genes still unknown might be involved in PEAR3 and NY10355 resistance to pear psylla. Transcriptomic approaches (e.g., RNA-Seq) might again turn out useful for the identification of these genes.

What is missing in my QTL mapping analysis for the pear psylla resistance is a genetic markers-screening of PEAR3 progenitors, for the identification of the origin of the resistant alleles. However, as the identity of the pollen parent of PEAR3 remains unknown, this analysis of the pedigree might in fact have turned out challenging, therefore further clues about PEAR3 pedigree are required.

It is particularly noteworthy that the RosBREED 2 project has set among its objectives the implementation of MAS for fire blight and psylla resistance in pear, developing the results I obtained during my PhD.

Genomic organization of QTLs and major genes already detected for resistance to diseases and pests in pear

The QTLs for fire blight and pear psylla resistance detected in this PhD project add to the number of QTLs and major genes for pests and disease resistance already mapped in pear (Table 6.2). In total, 31 loci associated with resistance to pests and diseases, of which four major genes and 27 QTLs, have been identified in pear. In particular, of these 31 loci, 25 are linked to resistance to diseases (13 to fire blight, 11 to pear scab and one to brown spot) and 6 to insects (5 to *C. pyri* and one to *D. pyri*). Furthermore, two genes associated with susceptibility to black spot disease (incited by *A. alternata*) and one QTL for susceptibility to brown spot (*Stemphylium vesicarium*) were mapped. Some of the reported QTLs are putatively isolate-specific, and in particular the four QTLs for fire blight resistance detected in PEAR3 in this project, and 5 of the 6 QTLs for scab resistance detected by Won et al. (2014) on PEAR1 and PEAR2. Five out of the total 34 loci were mapped in Asian pear species (*P. pyrifolia* and *P. ussuriensis*), 14 in European species (*P. communis* and *P. nivalis*) and 15 in interspecific hybrids (from crosses among *P. pyrifolia*, *P. ussuriensis*, *P. x bretschneideri* and *P. communis*). The fire blight resistance alleles at the QTLs detected in this PhD project on LGs 9 and 15 of the hybrid PEAR3 resulted to be inherited from the Chinese pear cultivar ‘Xue Hua Li’. Therefore, both Occidental and Oriental pears can be used as sources of resistance to different pathogens and pest. It is likely that many of the resistances observed in interspecific population in pear are of the “non-host” type (like it has been demonstrated for pear scab). Interspecific hybrids with non-host resistance, which is generally considered as highly effective and durable, to the major pests and pathogens of pear would have a great potential. PEAR3 is resistant to *C. pyri*, and apparently also to *V. pirina* (unpublished data), but not to fire blight, although the cross of this hybrid with ‘Moonglow’ might have generated individuals carrying all these three resistances.

Table 6.2: Quantitative trait loci (QTLs) and major genes for the resistance to pests and diseases detected since now in pear.

Name	Type of locus	Resistance to	Pear cultivar	Linkage group	R^2	Reference
<i>Ani</i>	Gene for susceptibility	Black spot (<i>Alternaria alternata</i>)	<i>P. pyrifolia</i> ‘Osa Nijisseiki’	11	--	Terakami et al. 2007
<i>Ana</i>	Gene for susceptibility	Black spot (<i>Alternaria alternata</i>)	<i>P. pyrifolia</i> ‘Nansui’	11	--	Terakami et al. 2007
--	QTL	Brown spot (<i>Stemphylium vesicarium</i>)	<i>P. communis</i> ‘Max Red Bartlett’	2	--	De Franceschi et al. 2013; Dondini 2013
--	QTL for susceptibility	Brown spot (<i>Stemphylium vesicarium</i>)	<i>P. communis</i> ‘Abbé Fétel’	15	--	De Franceschi et al. 2013; Dondini 2013
--	QTL	<i>C. pyri</i>	<i>P. x bretschneideri</i> x <i>P. communis</i> PEAR3	5	11%	This thesis
--	QTL	<i>C. pyri</i>	<i>P. x bretschneideri</i> x <i>P. communis</i> PEAR3	8	17-39%	This thesis
--	QTL	<i>C. pyri</i>	<i>P. communis</i> ‘Moonglow’	15	14%	This thesis
--	QTL	<i>C. pyri</i>	<i>P. ussuriensis</i> x <i>P. communis</i> NY10355	17	15%	Bouvier et al. 2011a
--	QTL	<i>C. pyri</i>	<i>P. ussuriensis</i> x <i>P. communis</i> NY10353	17	--	Civolani et al. 2013a
<i>Dp-1</i>	Major gene	<i>Dysaphis pyri</i>	<i>P. nivalis</i> EM	17	--	Evans et al. 2008
--	QTL	Fire blight	<i>P. communis</i> ‘Harrow Sweet’	2	29%**	Dondini et al. 2004; Le Roux et al. 2012
--	QTL	Fire blight	<i>P. communis</i> ‘Moonglow’	2	17-32%**	This thesis
--	QTL	Fire blight	<i>P. communis</i> ‘Doyenne du Comice’	3	--	Bokszczanin et al. 2011
--	QTL	Fire blight	<i>P. communis</i> ‘Doyenne du Comice’	4	--	Bokszczanin et al. 2009; Bokszczanin et al. 2011
--	QTL	Fire blight	<i>P. communis</i> ‘Harrow Sweet’	4	12%**	Dondini et al. 2004; Le Roux et al. 2012
--	QTL *	Fire blight	<i>P. x bretschneideri</i> x <i>P. communis</i> PEAR3	7	12%**	This thesis
--	QTL	Fire blight	<i>P. ussuriensis</i> accession 18	9	62%**	Bokszczanin et al. 2011
--	QTL	Fire blight	<i>P. communis</i> ‘Harrow Sweet’	9	8%**	Dondini et al. 2004
--	QTL *	Fire blight	<i>P. x bretschneideri</i> x <i>P. communis</i> PEAR3	9	15%**	This thesis
--	QTL	Fire blight	<i>P. ussuriensis</i> accession 18	11	--	Bokszczanin et al. 2009

--	QTL	Fire blight	<i>P. communis</i> 'Doyenne du Comice'	11	--	Bokszczanin et al. 2011
--	QTL *	Fire blight	<i>P. x bretschneideri</i> x <i>P. communis</i> PEAR3	12	10%**	This thesis
--	QTL *	Fire blight	<i>P. x bretschneideri</i> x <i>P. communis</i> PEAR3	15	8%**	This thesis
<i>Rvn1 (Vnk)</i>	Major gene	Scab (<i>V. nashicola</i>)	<i>P. pyrifolia</i> 'Kinchaku'	1	--	Terakami et al. 2006
<i>Rvn2</i>	Major gene	Scab (<i>V. nashicola</i>)	<i>P. pyrifolia</i> x <i>P. ussuriensis</i> x <i>P. communis</i> PS2-93-3-98	2	--	Cho et al. 2009; Bouvier et al. 2011b
<i>Rvp1</i>	Major gene	Scab (<i>V. pirina</i>)	<i>P. communis</i> 'Navara'	2	--	Bouvier et al. 2011a; Bouvier et al. 2011b
--	QTL *	Scab (<i>V. pirina</i>)	<i>P. communis</i> x <i>P. pyrifolia</i> PEAR2	2	--	Won et al. 2014
--	QTL	Scab (<i>V. pirina</i>)	<i>P. communis</i> 'Abbé Fétel'	3	87%	Pierantoni et al. 2007
--	QTL *	Scab (<i>V. pirina</i>)	<i>P. communis</i> x <i>P. pyrifolia</i> PEAR2	5	--	Won et al. 2014
--	QTL	Scab (<i>V. pirina</i>)	<i>P. communis</i> 'Abbe Fétel'	7	86%	Pierantoni et al. 2007
--	QTL *	Scab (<i>V. pirina</i>)	<i>P. pyrifolia</i> x <i>P. communis</i> PEAR1	7	--	Won et al. 2014
--	QTL *	Scab (<i>V. pirina</i>)	<i>P. communis</i> x <i>P. pyrifolia</i> PEAR2	7	--	Won et al. 2014
--	QTL *	Scab (<i>V. pirina</i>)	<i>P. pyrifolia</i> x <i>P. communis</i> PEAR1	10	--	Won et al. 2014
--	QTL	Scab (<i>V. pirina</i>)	<i>P. pyrifolia</i> x <i>P. communis</i> PEAR1	17	--	Won et al. 2014

* isolate(s)-specific?

** for severity

Interspecific hybridization, which is at the bases of one of the lines of the PFR pear breeding program (White and Brewer 2002), is a very powerful tool for the development of varieties with enhanced agronomic and fruit quality characteristics. However, breeders exploiting non-host resistances should be aware that they tend to be lost after a few segregating generations.

In the absence of a general integrated genetic map for either species of pear (like that built in apple by Khan et al. (2012)), I used the SSR-based consensus map of ‘Bartlett’ and ‘La France’ built by Celton et al. (2009) to show the location of all the resistance loci listed above, using common SSR and SNP markers. However, some of the genetic maps used to detect these resistance loci did not have any common marker with the map of Celton et al. (2009); therefore, the inference of the loci position might be slightly shifted and the confidence intervals larger than the original (Figure 6.2). Co-localization of loci for the resistance to at least two different biotic stresses were mapped to LGs 2, 3, 5, 7, 11, 15 and 17. QTLs for fire blight resistance detected on ‘Harrow Sweet’ (Dondini et al. 2004; Le Roux et al. 2012) and ‘Moonglow’ (this thesis) co-localize with one QTL mapped to PEAR2, specific for the P35.2 isolate of *V. pirina* (Won et al. 2014); a second QTL for another isolate of *V. pirina* (P34.1), detected on the same hybrid, co-localizes with the major gene *Rvp1* (Bouvier et al. 2011b); a third locus, the *Rvn2* resistance gene to *V. nashicola* (Cho et al. 2009), is located at the bottom part of LG2; finally, a QTL for the resistance to brown spot was mapped to LG2 in MRB, but its position is not yet published (De Franceschi et al. 2013; Dondini 2013). A QTL for resistance to *V. pirina* was mapped to LG3 of ‘Abbe Fétel’ (Pierantoni et al. 2007); on the same LG of ‘Doyenne du Comice’, Bokszczanin et al. (2011) identified a QTL for fire blight resistance, but they did not report its exact position. QTLs for resistance to *C. pyri* in PEAR3 (this thesis) and to P34.1 isolate of *V. pirina* in PEAR2 (Won et al. 2014) co-locate on LG5. QTLs for resistance to *V. pirina* on ‘Abbé Fétel’ (Pierantoni et al. 2007) and PEAR1 (Won et al. 2014) on LG7 co-locate with a QTL for fire blight resistance in PEAR3 (this thesis); another QTL for *V. pirina* was mapped up-stream to these loci in PEAR2 (Won et al. 2014). QTLs for the resistance to *C. pyri* in PEAR3 (this thesis) and fire blight in *P. ussuriensis* (Bokszczanin et al. 2009) co-locate with the genes for susceptibility to the black spot disease (Terakami et al. 2007) on LG11; also, Bokszczanin et al. (2011) identified a QTL for fire blight resistance on LG11 of ‘Doyenne du Comice’, without reporting its position. QTLs for the resistance to *C. pyri* in ‘Moonglow’ and fire blight in PEAR3 (this thesis) co-locate on LG15, and on the same LG a QTL for the susceptibility to brown spot was identified in ‘Abbé Fétel’.

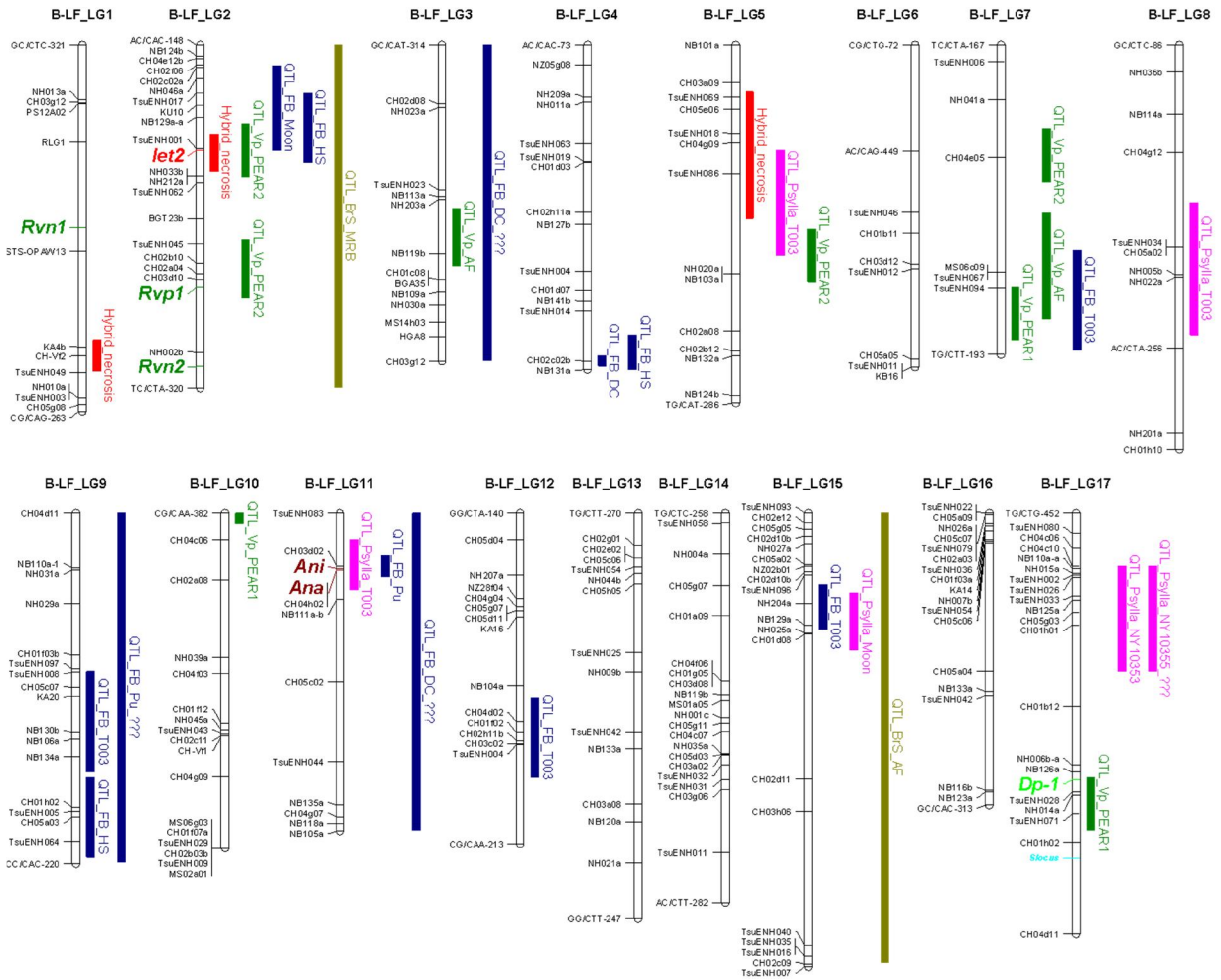


Figure 6.2: All the resistance loci detected since now in pear (and reported in Table 6.2) are projected on the simple sequence repeats (SSRs)-based consensus map of ‘Bartlett’ and ‘La France’ built by (Celton et al. 2009a)

Loci for the resistance to black spot are maroon, to brown spot olive green, to psylla pink, to *Dysaphis pyri* light green, to fire blight blue and to scab dark green

Finally, a QTL for the resistance to *V. pirina*, mapped to LG17 of PEAR1 (Won et al. 2014), co-locates with a major gene conferring resistance to *D. pyri* (Evans et al. 2008); moreover, in the top-medium part of LG17, (Civolani et al. 2013a) mapped a QTL for *C. pyri* resistance, probably co-locating with the one found by (Bouvier et al. 2011a).

LG2 carries the greatest number of resistant loci mapped since now in pear. Interestingly, also in apple the highest number of *R* genes and *R* gene clusters are located on LG2 (Perazzolli et al.

2014), with many of them involved in the resistance to *V. inaequalis* (Bus et al. 2011). Intriguingly, similarity between apple and pear resistances can be highlighted also for LG11. This LG is second to LG2 for the number of NBS genes in apple, and a rich cluster is located in its upper part (Perazzolli et al. 2014), where four (and maybe also a fifth) disease-related loci have been mapped in pear. Furthermore, LGs 7 and 15 carry a considerably high number of *R* gene analogs in apple (Perazzolli et al. 2014). These findings are consistent with the known frequent clusterisation of *R* genes in the plant genomes (Michelmore and Meyers 1998; Meyers et al. 2005). Moreover, often these clusters are composed by *R* genes of the same clade (the so-called homogeneous clusters) are usually generated by tandem duplications of the same gene (Leister 2004). In apple, for example, 71% of the *R* gene analog clusters are homogeneous (Perazzolli et al. 2014). Conversely, heterogeneous clusters originates from duplication and translocation of entire chromosomal segments (segmental duplication), or from single gene transpositions. In pear the study of *R* genes families is much less advanced than in apple. Nevertheless, Wu et al. (2013) reported that 30% of the *R* paralog genes they identified in the Chinese pear genome were clustered, and that LGs 2, 5 and 11 were particularly rich in these clusters.

Co-locating major genes and QTLs for the resistance to different biotic agents might reveal the presence of distinct *R* genes that are tightly linked, or of unique *R* genes (possibly with different resistant alleles, as for the *Vat* gene on melon, (Dogimont et al. 2008)) effective against multiple diseases and pests, either because involved in broad-spectrum mechanisms of resistance (such as those related to the plant basal defense) or because able to specifically-recognize different pathogenic effectors (like what was elucidated for the *Mi* gene in tomato (Rossi et al. 1998; Nombela et al. 2003). Poland et al. (2009) reported the existence of several examples of co-locating QTLs for resistance to different pathogens in plant crops, such as in maize (Wisser et al. 2006) and rice (Wisser et al. 2005); the identification of loci with pleiotropic effects and mechanisms that provide quantitative resistance to multiple diseases would be strategic for the development of unique cultivars. Such loci have been identified, for example, in wheat (Krattinger et al. 2009), rice (Manosalva et al. 2009) and maize (Wisser et al. 2011). Similar types of studies, for example based on positional cloning, could also be performed in pear, starting from the QTLs detected on LG2.

Resistant loci for single disease or pest were detected in LGs 1, 4, 8, 9, 10 and 12 in pear. In apple, LGs 8 and 10 were reported to carry several *R* gene analogs (Perazzolli et al. 2014). No resistance locus was ever mapped to LGs 6, 13, 14 and 16 in pear. Interestingly, these four LGs turned out to carry the lowest number of the total *R* gene analogs identified by Perazzolli et al. (2014) in the apple genome.

Although the range of pests and diseases listed above almost completely covers all the economically important biotic stresses of pear, it is certainly not exhaustive in terms of existing resistance loci in this species. Indeed, whereas a consistent number of pear accessions showing resistance to a biotic stress were identified, only very few of them have been investigated through genetic mapping studies. Moreover, alleles for resistance can be found in susceptible pear varieties as well, where their low effect on the phenotypic variation is not sufficient to confer acceptable resistance to the individuals; such small effect loci might still be exploited by GS. Consequently, several other resistant loci might be expected to be detected in the following years, being the research on pear enhanced also by the quick advancements in the molecular biology technologies. The identification of resistance loci can be achieved via QTL mapping studies, like those performed in this PhD, or, preferably, with association mapping studies (Ingvarsson and Street 2011). Although QTL mapping has allowed to genetically characterize many quantitative traits in the last decades, it has a number of drawbacks which can be overpassed with association studies, which are based on populations with a much wider genetic variation than the bi-parental crosses used for QTL mapping (Ingvarsson and Street 2011). The approach of association mapping is to study statistical associations (LD) between genetic markers and phenotypic traits in large populations where the relationships between individuals are not necessarily known. In a diverse germplasm, such as a collection of old varieties or a natural population, the number of recombination events occurred is much higher than in a F1 or F2 population, usually employed for QTL mapping, thus allowing to detect more accurate loci encompassing much smaller genomic regions. The decrease of the costs for high-throughput genotyping techniques will help implementing the adoption of genome-wide association (GWAS) analysis, which may be then preferred over QTL mapping studies. However, in order to identify loci with small effects and account for epistasis and GxE interactions, the population needs to have a very large size, and preferably be replicated over different environments. A drawback of GWAS is the lack of

statistical power when trying to identify rare alleles (e.g. with a minor QTL allele frequency < 5%), for which related molecular markers are usually excluded from the genotypic dataset; this issue can be addressed by strongly increasing the number of both markers and individuals, and taking into account also markers with low MAF.

The availability of the pear genome sequences allows the analysis of resistance gene analogs families, like that performed in *M. x domestica* by Perazzolli et al. (2014), which will increase our understanding on the *R* gene clusters organization along the chromosomes.

The co-linearity of the apple and pear genomes is striking, and is again supported by the similarities here reported in terms of the *R* genes distribution along the chromosomes of these two species. The much deeper knowledge on resistance loci in apple could be exploited to gain new insight in pear. However, we should keep in mind that, besides the similarities, apple and pear have also many differences. Indeed, the work of Zhong et al. (2015) demonstrated the existence of specific *R* genes in the different Rosaceae species.

Implication of hybrid necrosis-related markers in pear breeding

For the enhancement of the pear breeding strategies great importance goes to the identification of genetic markers associated with lethal genes. In this project, three regions linked to the early lethality of a large proportion of the PEAR3 x ‘Moonglow’ progeny have been identified, providing new insight in the genetic determinism of the hybrid necrosis, a phenomenon of great relevance for both speciation studies and breeding. The location of those regions is also projected on the ‘Bartlett’ and ‘La France’ consensus map (Figure 6.2), where the loci for pests and disease resistance detected in general in pear is reported (see above). The reduction of the interval of these regions will be necessary for the detection of candidate lethal genes, which are presumed to be directly, or indirectly (according to the guard-guardee hypothesis), associated with disease and pest resistances, and the identification of markers associated with them. Screening the parent pool of a pear breeding line with these markers will enable the identification of cross-incompatible accessions, whose inbreeding will results in skewed segregation for traits (likely resistances) closely linked to the lethal genes.

Specific NBS-LRR genes are duplicated in response to the high selective pressure exerted by pests and pathogens. In *Pyrus* spp., like in other Rosaceae species, several events of gene duplication have been demonstrated to have occurred in the past (Zhong et al. 2015). This generated (and still does) a high polymorphism in the *R* gene classes, especially in the NBS-LRR ones, even within the same species (Bergelson et al. 2001), which fits with the hypothesis of their implication in BDM-like incompatibilities (Bomblies and Weigel 2007). The study of hybrid necrosis in plants from a genetic prospective will help to increase the understanding of the evolutionary force represented by pests and pathogens on plant genomes. The incompatibilities among alleles mutated within different genetic background might have had an important role in the speciation process (Bomblies and Weigel 2007). In *Pyrus*, for example, many Oriental species are non-host to organisms which are pathogenic to Occidental species, and vice versa. It is possible that the evolution of the two groups of *Pyrus* species in different environments, where they have been subjected to selective pressures from different pathogens, might have caused the divergence of initially common *R* genes, which, as a pleiotropic effect, caused incompatibilities between the two groups.

Conclusion

In conclusion, in the last few years the knowledge about pear genomics has made considerable advances, and this project has significantly contributed to these. An international collaboration aimed at the MAS in the main Rosaceae crops, the RosBREED project (<http://www.rosbreed.org-/portfolio-impact-statements>), was started in 2010 and for four years focused on the breeding of apple, peach, cherry and strawberry, but not pear. The follow-up of this project, RosBREED 2, includes a higher number of species, and pear has gained its place in the list. RosBREED 2 will target mainly fruit quality and disease resistances, and thus the discoveries I made during this project concerning fire blight and psylla resistance and the hybrid necrosis will have a great relevance for this project.

Other than what I presented here, the work carried out by FEM, INRA of Angers and PFR for the breeding of pear has provided new insights on pear scab resistance, vigor control and precocity in pear rootstocks and storage-related disorders.

MAS, or, preferentially, GS, in pear is today finally conceivable.

All the genotypic and phenotypic data on pear are collected in a common web-based database for all the Rosaceae species, the Genome Database for Rosaceae (GDR, <http://www.rosaceae.org/>). This database provides centralized access to genomics, genetics and breeding data and analysis tools to facilitate basic, translational and applied research on Rosaceae.

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Annex 1: List of Simple Sequence Repeats (SSR) markers tested in PEAR3 x ‘Moonglow’ population during the construction of the parental genetic maps.

The name of the marker, the primer sequences, the segregation type in PEAR3 x ‘Moonglow’ population and the location on the parental maps, the location on other pear maps (‘Bartlett’ x ‘La France’ (Celton et al. 2009a) and ‘Passe Crassane’ x ‘Harrow Sweet’ (Costa et al. 2008)), the amplicon size and the annealing temperature used in this project are shown.

	SSR locus	primers sequence	segregation type in PEAR3x‘Moonglow’	Location in PEAR3x‘Moonglow’	Location in ‘Bartlett’x‘La France’	Amplicon size range (bp)	Annealing temperature (°C)
1	BGA-35	for: AGAGGGAGAAAGGCGATT rev: GCTTCATCACCCTCTGCT	no amplification	--	LG3	--	60
2	CH01b12	for: CGCATGCTGACATGTTGAAT rev: CGGTGAGCCCTCTTATGTGA	complex	--	LG17	128-139	59
3	CH01g05	for: CATCAGTCTCTTGCACTGGAAA rev: GACAGAGTAAGCTAGGGCTAGGG	aaxab	LG14	LG14	140-151	60
4	CH01h02	for: AGAGCTTCGAGCTTCGTTTG rev: ATCTTTTGGTGCTCCACAC	locus 1: monomorphic locus 2: abxcd	LG9	LG9/ LG17	205-248	62
5	CH01h10	for: TGCAAAGATAGGTAGATATATGCCA rev: AGGAGGGATTGTTTGTGCAC	abxcd	LG8	LG8	97-123	60
6	CH02a03	for: AGAAGTTTTACGGGTGCC rev: TGGAGACATGCAGAATGGAG	monomorphic	--	LG16	115-165	61
7	CH02b10	for: CAAGGAAATCATCAAAGATTCAAG rev: CAAGTGGCTTCGGATAGTTG	abxcd (n)	LG2	LG2	128-135	57
8	CH02b12	for: GGCAGGCTTTACGATTATGC rev: CCCACTAAAAGTTCACAGGC	aaxab	LG5	LG5	102-136	60
9	CH02c02a	for: CTTCAAGTTCAGCATCAAGACAA rev: TAGGGCACACTTGCTGGTC	complex	--	LG2	137-166	59
10	CH02c02b	for: TGCATGCATGGAAACGAC rev: TGGAAAAAGTCACACTGCTCC	aaxab	LG4	LG4	109-123	57
11	CH02c09	for: TTATGTACCAACTTTGCTAACCTC rev: AGAAGCAGCAGAGGAGGATG	abxaa	LG15	LG15	235-255	60
12	CH02d08	for: TCCAAAATGGCGTACCTCTC rev: GCAGACACTCACTCACTATCTCTC	abxcd (n)	LG3	LG3	208-223	54
13	CH02d11	for: AGCGTCCAGAGCAACAGC rev: AACAAAAGCAGATCCGTTGC	abxcd	LG15	LG15	113-139	60
14	CH02g01	for: GATGACGTCGGCAGGTAAAG	abxaa	LG13	LG13	183-191	60

		rev: CAACCAACAGCTCTGCAATC					
15	CH03a09	for: GCCAGGTGTGACTCCTTCTC rev: CTGCAGCTGCTGAAACTGG	abxaa	LG5	LG5	119-122	62
16	CH03c02	for: TCACTATTTACGGGATCAAGCA rev: GTGCAGAGTCTTTGACAAGGC	aaxab (n)	LG12	LG12	105-139	59
17	CH03d02	for: AAACCTTTCACCTTCACCCACG rev: ACTACATTTTTAGATTTGTGCGTC	efxeg	LG11	LG11	180-236	60
18	CH03d12	for: GCCCAGAAGCAATAAGTAAACC rev: ATTGCTCCATGCATAAAGGG	monomorphic	--	LG6	100-150	60
19	CH03g06	for: ATCCACAGCTTCTGTTTTTG rev: TCACAGAGAATCACAAGGTGGA	abxcd	LG14	LG14	136-157	54
20	CH04c06	for: GCTGCTGCTGCTTCTAGGTT rev: GCTTGGAAGGTCACTTGC	locus 1: abxaa locus 2: abxaa	LG10/ LG17	LG10/ LG17	166-211	60
21	CH04c10	for: GGGTTAGGTTGTCTTCTCCT rev: GCTTCTCGGGTGAGTTTTTC	abxcd	LG17	LG17	113-145	56
22	CH04d11	for: ATTAGGCAATACACAGCAC rev: GCTGCTTTGCTTCTCACTCC	no amplification	--	LG9/ LG17	--	52
23	CH04e05	for: AGGCTAACAGAAATGTGGTTTG rev: ATGGCTCCTATTGCCATCAT	aaxab	LG7	LG7	173-204	58
24	CH05a02	for: GTTGCAAGAGTTGCATGTTAGC rev: TTTTGACCCATAAAACCCAC	abxcd	LG8	LG8/ LG15	110-130	60
25	CH05a03	for: CGGCTGAGCATGGTTACTTC rev: TGATCGTTGTGAAAGCTCCA	no amplification	--	LG9	--	59
26	CH05a04	for: GAAGCGAATTTTGCACGAAT rev: GCTTTTGTTCATTGAATCCCC	aaxab	LG16	LG16	154-175	57
27	CH05a09	for: TGATTTAGACGTCCACTTCACCT rev: TGATTGGATCATGGTGACTAGG	no amplification	--	LG16	--	59
28	CH05d04	for: ACTTGTGAGCCGTGAGAGGT rev: TCCGAAGGTATGCTTCGATT	abxaa (n)	LG12	LG12	190-197	59
29	CH05g08	for: CCAAGACCAAGGCAACATTT rev: CCCTTCACCTCATTCTCACC	no amplification	--	LG1	--	51
30	Ch-Vf1	for: ATCACCACCAGCAGCAAAAG rev: CATACAAATCAAAGCACAAACC	aaxab (n)	LG1	LG10	131-134	58
31	KA16	for: GCCAGCGAACTCAAATCT rev: AACGAGAACGACGAGCG	abxcd	LG12	LG12	133-151	56
32	KA4b	for: AAAGGTCTCTCTACTGTCT rev: CCTCAGCCCAACTCAAAGCC	monomorphic	--	LG1	137-141	56
33	KB16	for: GATTTTGTCCGCAGGT rev: AAAGAACAGCAAGAACCA	aaxab (n)	not mapped	LG6	149-155	55

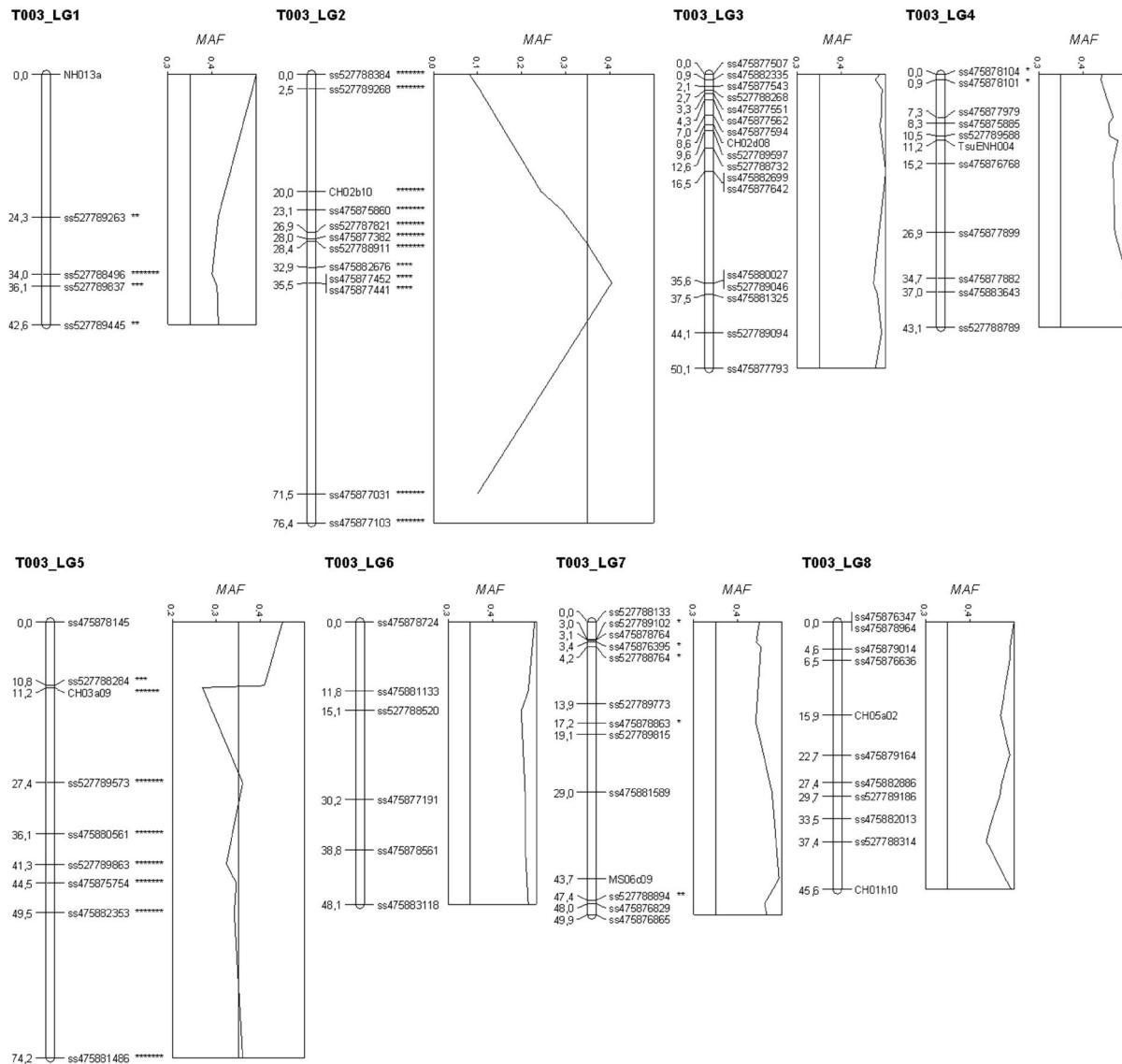
34	MS02a01	for: CTCCTACATTGACATTGCAT rev: TAGACATTTGATGAGACTG	abxcd (n)	LG10	LG10	133-149	52
35	MS06c09	for: ACTATTGGAGTAAGTCGA rev: AATATAAGAGCCAGAGGC	abxcd	LG7	LG7	107-150	52
36	MS14h03	for: CGCTCACCTCGTAGACGT rev: ATGCAATGGCTAAGCATA	monomorphic	--	LG3	120-126	54
37	NB105a	for: AAACAACCGACTGAGCAACATC rev: AAAATCTTAGCCCCAAAATCTCC	abxcd	LG11	LG11	138-153	56
38	NB111a	for: CCAAGCTGTGATTATAGGAAG rev: AGGCTGAAAAGATTGTAAGGT	efxeg (n)	LG11	LG11	149-162	57
39	NH002b	for: GGAGTCAGCGGCAAAAAAAG rev: CCCACTCCCTCCTCTTATTGT	aaxab (n)	LG2	LG2	168-185	58
40	NH004a	for: AGGATGGGACGAGTTTAGAG rev: CCACATCTCTCAACCTACCA	aaxab	LG14	LG14	74-93	59
41	NH013a	for: GGTTTGAAGAGGAATGAGGAG rev: CATTGACTTTAGGGCACATTTC	abxcd	LG1	LG1	161-225	57
42	NH014a	for: CAAACCTAACCTAAATACC rev: TGTTTCATATATTCATCACTC	no amplification	--	LG17	--	50
43	NH021a	for: ATCTCAATTTTCTCGGTAAACCA rev: CTGATATCTCTCTGCATCCCT	abxcd	LG13	LG13	138-168	58
44	NH027a	for: TAATGTGTTGGGGAGAGAGAG rev: GCTCTTGTTCCCTTGCTCTTAA	abxcd (n)	LG15	LG15	133-158	56
45	NH029a	for: GAAGAAAACCAGAGCAGGGCA rev: CCTCCCGTCTCCACCATATTAG	aaxab	LG9	LG9	88-101	62
46	NH033b	for: GTCTGAAACAAAAAGCATCGCAA rev: CTGCCTCGTCTTCCTCTATCTCC	monomorphic	--	LG2	173-205	60
47	NH041a	for: TGAGGAGTTTGACAGCATCG rev: GGCGCATTTTTATTTTGACG	monomorphic	--	LG7	126-127	55
48	NH045a	for: ATCGAGAGACGAGGGTAGCA rev: TCTCTTGGCGTCTTCCTCTC	abxaa	LG10	LG10	181-222	61
49	NZ05g08	for: CGGCCATCGATTATCTTACTCTT rev: GGATCAATGCACTGAAATAAACG	no amplification	--	LG4	--	54
50	TsuENH004	for: CGCATTAAAGTCTGGCTTTCTTC rev: GAATTGGCAGAGAGATTGAGTGG	abxcd (n)	LG4	LG4/ LG12	151-166	59
51	TsuENH008	for: CTGAGGTCTCATTCCGGTGATTCT rev: GTTCTTCCTTCTCTGCTTTCTTCTCACG	abxcd (n)	LG9	LG9	147-165	63
52	TsuENH046	for: GGTCAACCCACTTAAAAACCA rev: GTTCTTGTTGCCCTGAAGTAATTGAGATGG	monomorphic	--	LG6	147-156	60
53	TsuENH058	for: AGAAGAAGGATAAGAAGAAGGATGG rev: GTTCTTGTAACGAAAAGGAAACAGGACTTG	abxaa	LG14	LG14	293-300	61

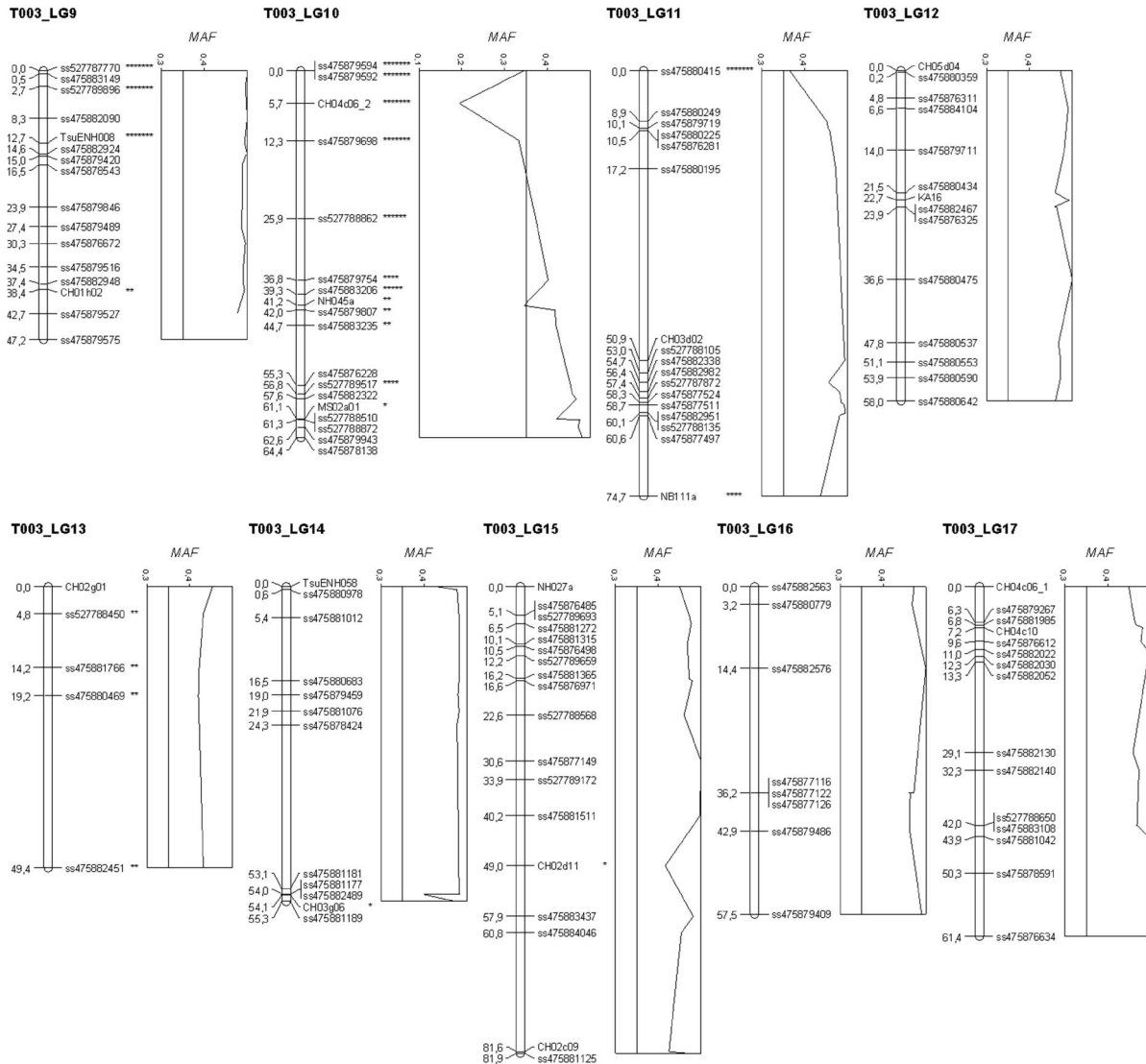
54	TsuENH086	for: CTCTGTTCTGCTTCGATTCTGCT rev: GTTCTTGTCACGTCACCATTTTCAGT	aaxab	LG5	LG5	163-183	61
55	Md-Exp 7	for: FCATAGAAGGTGGCATGAGCA rev: TTTCTCCTCACACCCAAACC	aaxab	LG1	LG1*	203-208	60

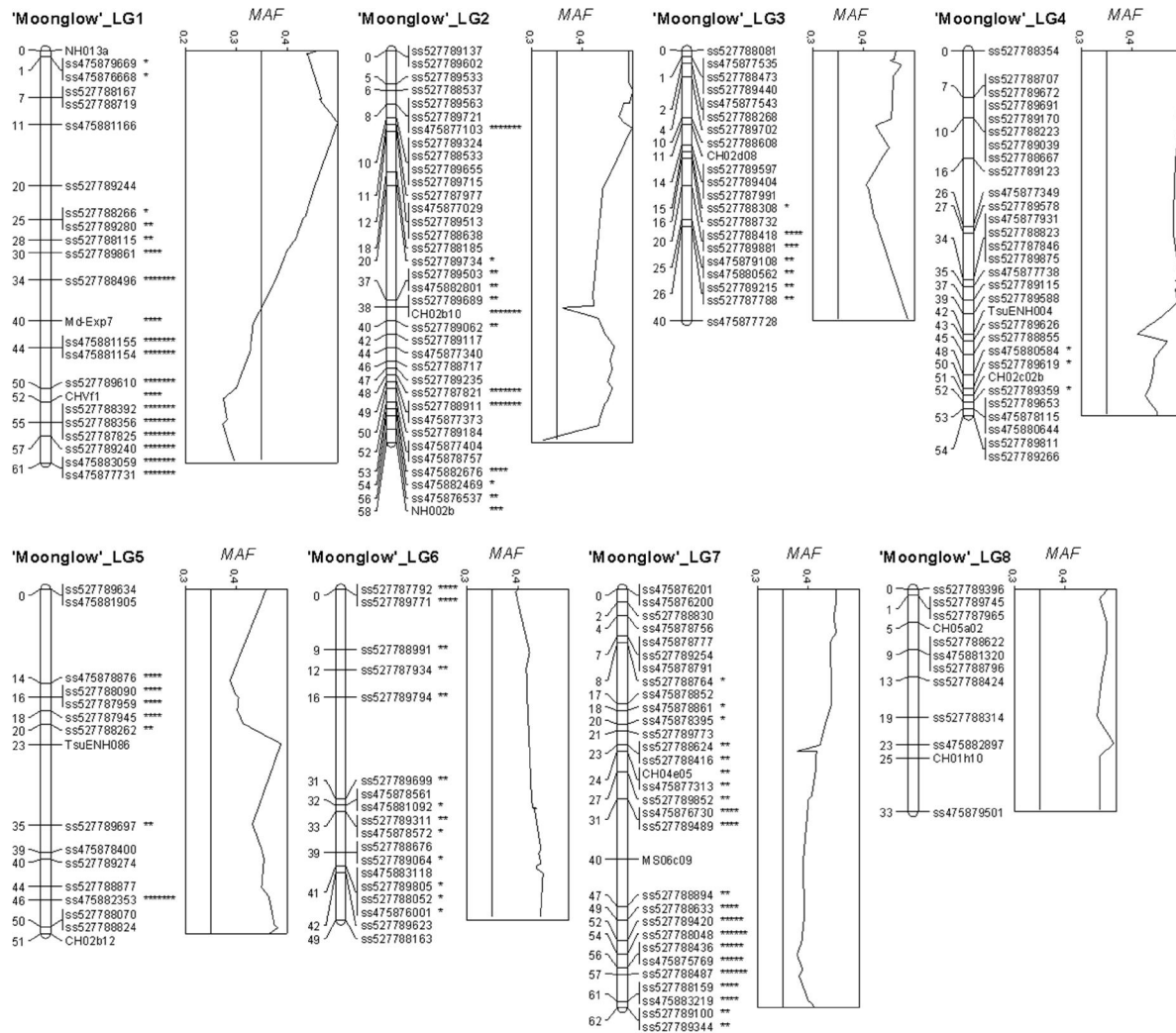
* location on 'Passe Crassane'x'Harrow Sweet' map

Annex 2: Genetic map of PEAR3 and ‘Moonglow’ based on Single Nucleotide polymorphism (SNP) and Simple Sequence Repeat (SSR) markers.

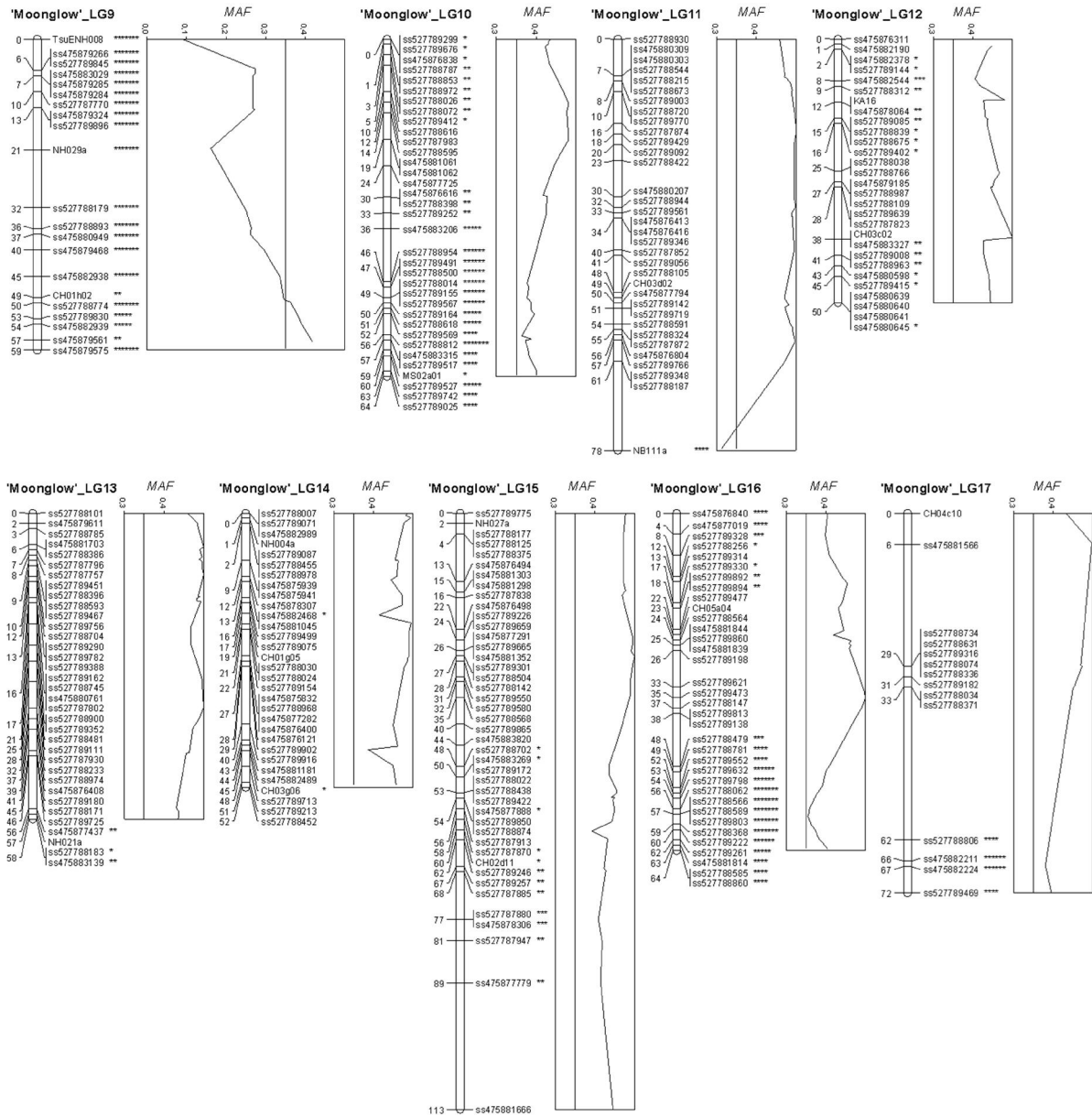
These maps were developed during the first step of this PhD project, and were published in PLOS ONE (Montanari et al. 2013). The segregation distortion along each linkage group (LG) is shown with * (representing the significance of the distortion according to Chi-squared test: * $p=0.1$, ** $p=0.05$, *** $p=0.01$, **** $p=0.005$, ***** $p=0.001$, ***** $p=0.0005$, ***** $p=0.0001$) and with the minor allele frequency (MAF) values for each marker plotted against their position in the map.





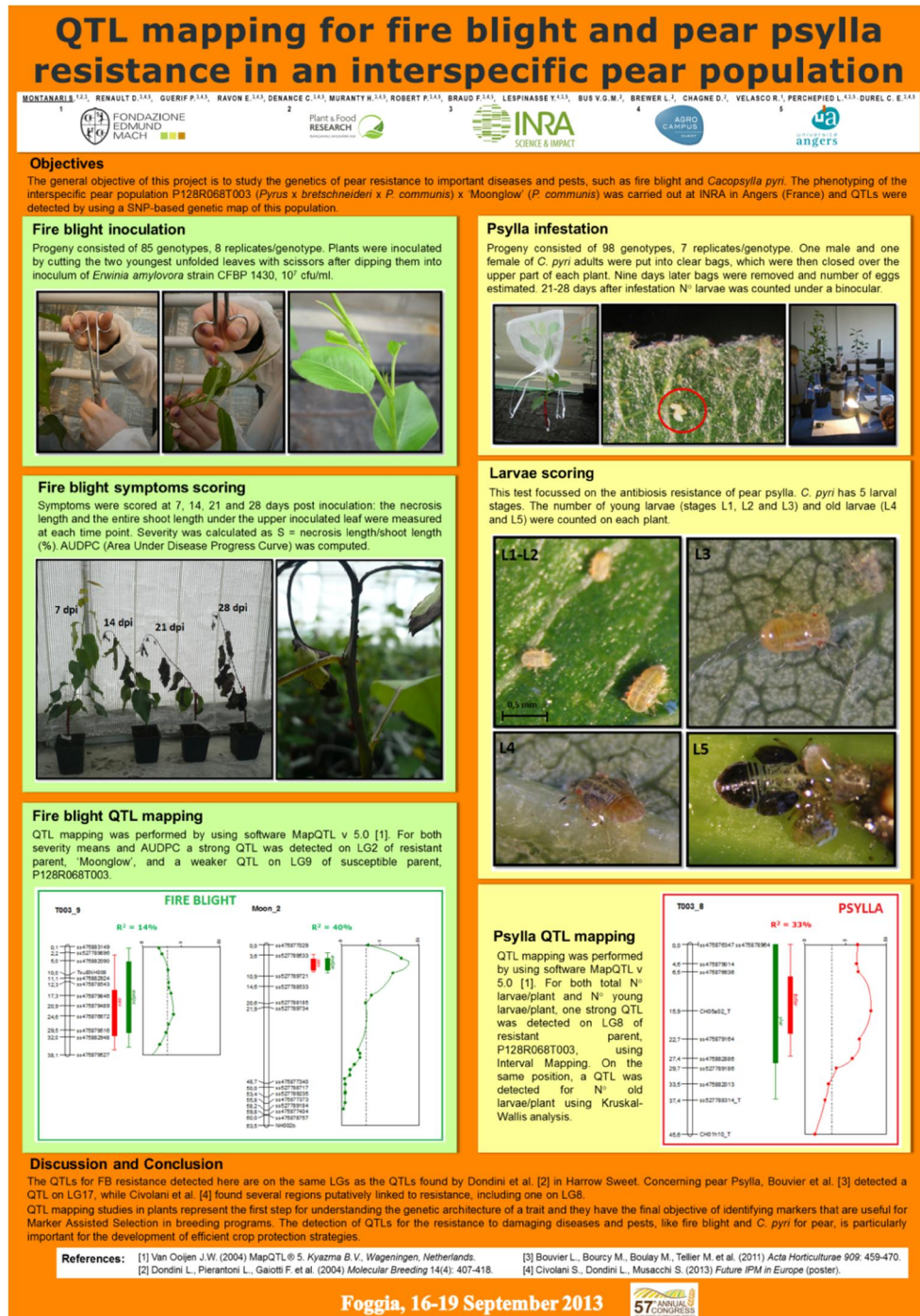


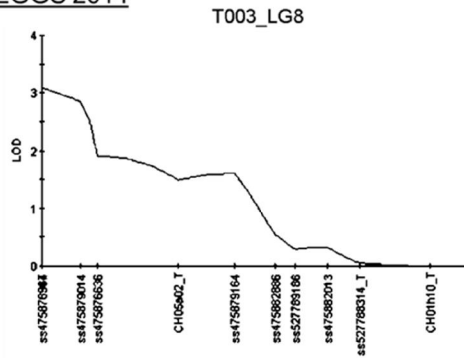
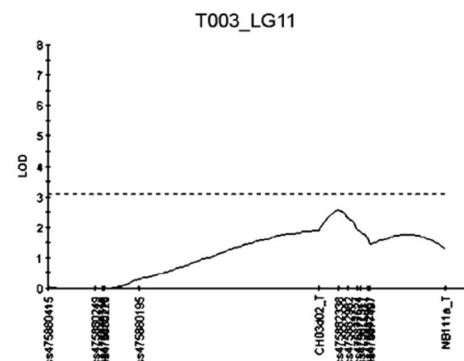
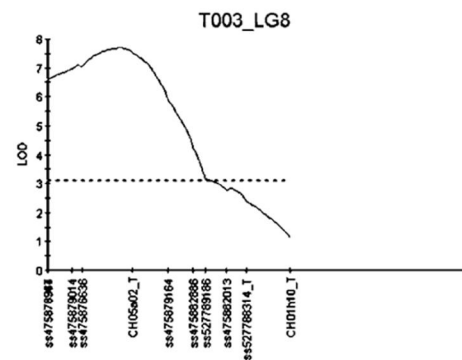
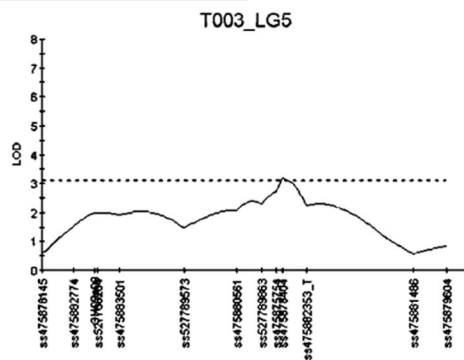
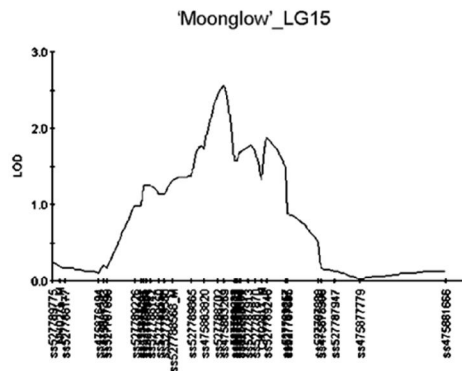
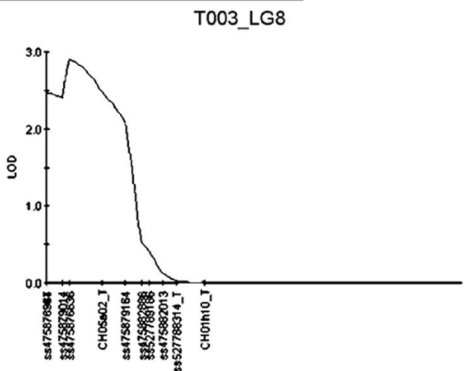
Identification and Mapping of Genomic Regions Controlling Fire Blight and Psylla Resistance and Hybrid Necrosis in Pear



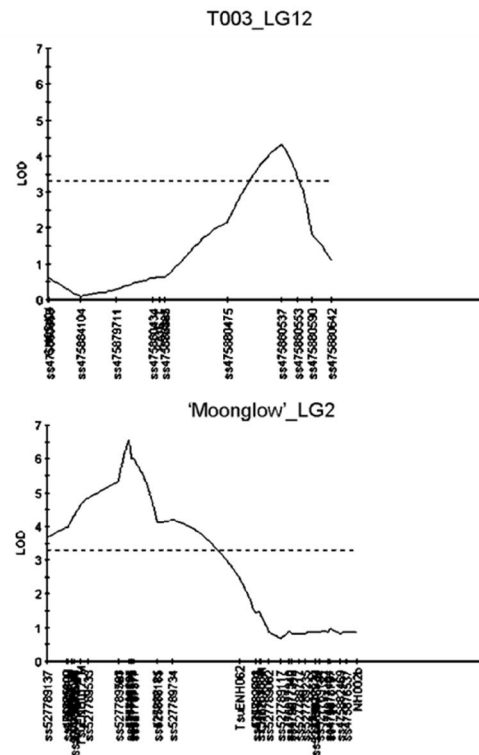
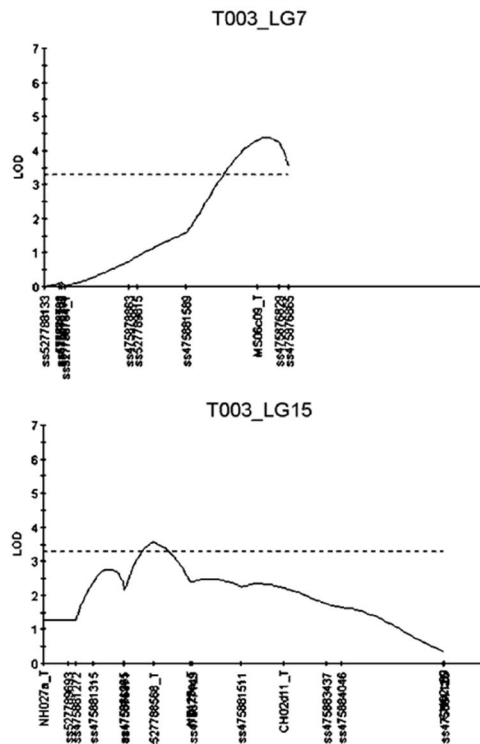
Annex 3: Poster presented at the 57th Italian Society of Agricultural Genetics (SIGA) Annual Congress (2013).

The results of the first year of phenotyping and QTL mapping for pear psylla and fire blight resistance in Angers (France) were presented.

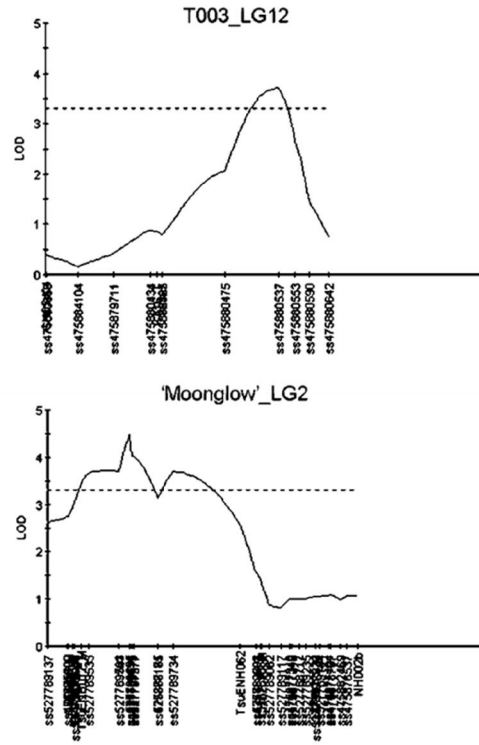
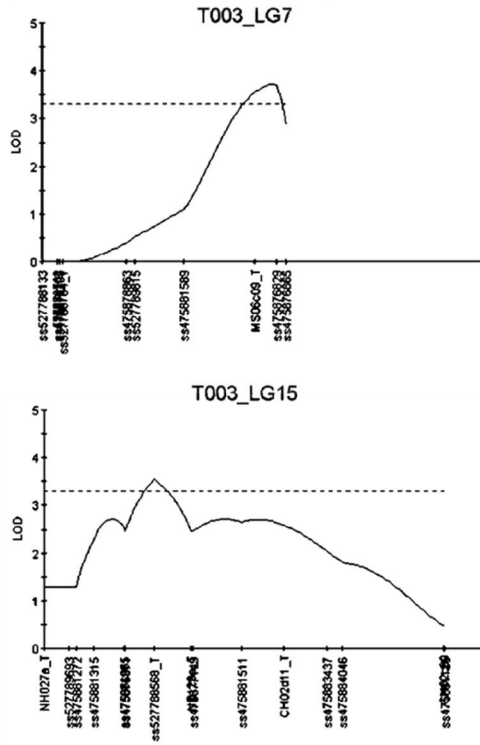


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AUDPC New Zealand 2014



Annex 6: High Resolution Melting (HRM) markers designed from the flanking regions of Single Nucleotide Polymorphisms (SNPs) showing distorted segregations in the PEAR3 x 'Moonglow' non-necrotic progeny.

Linkage groups (LGs) with severe segregation distortion were: 2, 5 and 10 of PEAR3, and 1, 9, 10 and 16 of 'Moonglow'. Polymorphic HRM markers were evaluated on 'Type 1', 'Type 2' and 'Type 3' individuals. For each marker, the allelic composition and the linkage group of PEAR3 and/or 'Moonglow' are shown.

HRM marker name	SNP accession	SNP location on PEAR3x'Moonglow' map	HRM primers sequence	HRM marker segregation in PEAR3x'Moonglow'	HRM marker location on PEAR3x'Moonglow' map
LETss527788384	ss527788384	LG2 on PEAR3	for: CATAGCATTCTTGCGTTCA rev: ACCCCCTGCCATATCATCTT	b0xaa	LG2 on PEAR3
LETss475875860	ss475875860	LG2 on PEAR3	for: TTTCTTTTGGCTCTCCCTGA rev: CGTCACTATCATCCTCCTCCA	monomorphic	--
LETss527787821	ss527787821	LG2 on PEAR3	for: CAACCATGAAGAGCTGAGGAG rev: GCAAAGTAATCAAACAGCCAAA	monomorphic	--
LETss475882676	ss475882676	LG2 on PEAR3	for: CATTTCCTCATAGCCTCCAAA rev: TGGGGTTGAAGAAGGTAGCA	efxeg	LG7 on 'Moonglow'
LETss527789863	ss527789863	LG5 on PEAR3	for: GGTGGGTTTCAGGTAAGAGG rev: CACAGCATCCCAAGAGACAA	abxcd	LG5 on PEAR3 and 'Moonglow'
LETss475882353	ss475882353	LG5 on PEAR3	for: CTCCATAGGCTGTAGCAGAAAA rev: TGTGAAGGGAGATGTGGAAA	complex	--
LETss475879594	ss475879594	LG10 on PEAR3	for: GTTCGTTTCAGGCACCATTTT rev: CCATCGTTGTCATCTCTCCA	monomorphic	--
LETss527788862	ss527788862	LG10 on PEAR3	for: ACAAACCCCAAAAGAAGTCA rev: TCCTACTGTTTCAGGCATGTT	abxcd	not mapped
LETss475879807	ss475879807	LG10 on PEAR3	for: GGGGTACAATGCCAATTCA rev: CCAAATCAACCAGCAAATACA	complex	--
LETss527788115	ss527788115	LG1 on 'Moonglow'	for: AGCAACCAAGTAGCCTTTCCA rev: TGGTGAGCACATACCGTGA	ccxab	LG1 on 'Moonglow'
LETss527789610	ss527789610	LG1 on 'Moonglow'	for: TGTCTCCTTCGACCATCTCC rev: AACATCCCATAAGTCCCAAGAA	abxcd	LG1 on PEAR3 and 'Moonglow'
LETss527789240	ss527789240	LG1 on 'Moonglow'	for: CTCTTGAGCAGGCTTAGTTGG rev: GAAAGGGGTTGCCATAACTC	monomorphic	--
LETss527789845	ss527789845	LG9 on 'Moonglow'	for: GCCGAGAAGAAGATCAAGGA rev: GATAGCGAAAACTCCGAAAAA	monomorphic	--
LETss527789896	ss527789896	LG9 on 'Moonglow'	for: TTCCAAGTGTCTTTGCTCCA rev: CCATCATTGTACTGGTCTTCTCC	no amplification	--
LETss527788179	ss527788179	LG9 on 'Moonglow'	for: TTGAGCCAATGCTTTCTCTATG rev: TCATCACCGTCCATCTTTATGT	complex	--

LETss527789830	ss527789830	LG9 on 'Moonglow'	for: AAAGTGGTCGTTGCTCTTGG rev: ACAGCAGAACCTGGAACAGAA	aaxab	LG9 on 'Moonglow'
LETss527789491	ss527789491	LG10 on 'Moonglow'	for: TCTGGCTTCAGATCCCTTCA rev: GGTTGCAGTATGTTTGTTTCTTCC	aaxab	LG10 on 'Moonglow'
LETss527789569	ss527789569	LG10 on 'Moonglow'	for: TTTCAGAGGAGGCTGTAGGAA rev: GTTGTCTACCTTAAACCCTTGGA	aaxab	LG10 on 'Moonglow'
LETss527789742	ss527789742	LG10 on 'Moonglow'	for: TGGTTCAGCAACTCCACAAG rev: AATGCAGGGTTTCAAGTGTGT	efxeg	LG10 on PEAR3 and 'Moonglow'
LETss527788479	ss527788479	LG16 on 'Moonglow'	for: GCTTACAAGGTTTTATGGTCCTTT rev: CAAAAGCAGAGTCAGGAGACATT	monomorphic	--
LETss527789632	ss527789632	LG16 on 'Moonglow'	for: TGGCGTGTTCAGAGTTTTGT rev: CAGCATGTTTCGGATTGATAGA	no amplification	--
LETss527788589	ss527788589	LG16 on 'Moonglow'	for: TGTGCAGAGAAGGCAGAGTT rev: GCTTTCCAGTAACCCGACAC	ccxab	LG16 on 'Moonglow'
LETss527788585	ss527788585	LG16 on 'Moonglow'	for: TCTTAGGCTTTGGTGCCAGT rev: GGGGACGAAGTGTATGGAGA	abxab	LG16 on PEAR3 and 'Moonglow'

Annex 7: High Resolution Melting (HRM) markers designed from NB-LRR genes annotated to linkage groups (LGs) 1, 5 and 10 of the apple genome

For each predicted gene, its physical position in the ‘Golden Delicious’ v1.0 and ‘Bartlett’ v1.0 genomes is shown. Polymorphic HRM markers were evaluated on ‘Type 1’, ‘Type 2’ and ‘Type 3’ individuals. For each marker, the allelic composition and the linkage group of PEAR3 and/or ‘Moonglow’ are shown

HRM marker name	Gene prediction on apple	LG on apple	Physical position on apple LG (bp)	Pear scaffold	Region on pear scaffold (bp)	HRM primers sequence	Segregation in PEAR3x'Moonglow'	Location on PEAR3x'Moonglow' map
MDP0000711403_LG1a	MDP0000711403	1	26198130-26199950	00175	744 – 830	for: ATCCTCGCCGCTATTGAAGA rev: TTCTTCTCCCGCCTCAGTTT	aaxab	LG1 on 'Moonglow'
MDP0000711403_LG1b	MDP0000711403	1	26198130-26199950	00175	580 – 763	for: GCGAACCATCAAGACAAGGG rev: TCTCAATAGCGGCGAGGAT	bad amplification	--
MDP0000711403_LG1c	MDP0000711403	1	26198130-26199950	00175	440 – 599	for: GAAATGGCTATCGGAGTGACC rev: CCCTTGTCTTGATGGTTCGC	monomorphic	--
MDP0000711403_LG1d	MDP0000711403	1	26198130-26199950	00175	325 – 460	for: TATGCGGCTCCAGGTAAACT rev: GGTAAGTCCGATAGCCATTTC	00xa0	LG1 on 'Moonglow'
MDP0000160413_LG1a	MDP0000160413	1	29897351-29901059	20661	1132 – 1307	for: CCGGATGAAACAAAGCGACT rev: TGCAGTGATTCCAGCCAATG	00xa0	not mapped
MDP0000160413_LG1b	MDP0000160413	1	29897351-29901059	20661	1994 – 2045	for: ACTACAGAGCCTCGATCAGTC rev: AGTCCGCATAATTCGTTGC	aaxab	LG1 on 'Moonglow'
MDP0000160413_LG1c	MDP0000160413	1	29897351-29901059	20661	1046 – 1151	for: TGCCTATTGTTCCACCTCA rev: AGTCGCTTTGTTTCATCCGG	monomorphic	--
MDP0000508070_LG1a	MDP0000508070	1	30595368-30603170	00269	231572 – 231654	for: GCTCCTTAGAAAAGCGGTGG rev: GGCGTCGAGTTATTGGCTTT	monomorphic	--
MDP0000508070_LG1b	MDP0000508070	1	30595368-30603170	00269	230070 – 230216	for: ACTCATCACCACAGAAGCGA rev: GCACTGTCAGTCACCATGTC	00xab	LG1 on 'Moonglow'
MDP0000508070_LG1c	MDP0000508070	1	30595368-30603170	00269	231353 – 231521	for: CTTTGGCATCTTTCCCGAGG rev: CCAAACCTGTGACACTTGCA	bad amplification	--
MDP0000251943_LG1a	MDP0000251943	1	34313547-34316841	00634	83949 – 84064	for: GGCTCGGTATCCTCCAGTAC rev: AGGCCTCCAACATCTTCCTC	abxaa	LG1 on PEAR3
MDP0000251943_LG1b	MDP0000251943	1	34313547-34316841	00634	84777 – 84918	for: CATGCTTGCCGAGTTTCACT rev: CTCTCAGGGAATGTGCCTCA	abxcd	LG1 on PEAR3 and 'Moonglow'
MDP0000251943_LG1c	MDP0000251943	1	34313547-34316841	00634	85425 – 85480	for: TATCATCAACTGGGTCCGCA rev: CGTTTTGGTTACTGGGGCAA	monomorphic	--
MDP0000820483_LG5a	MDP0000820483	5	14650316-14654059	00046	67830 – 68025	for: ACTGTGGGGTTACATCAGGG rev: CTGCGCTTCCTCAATCTGTC	no amplification	--
MDP0000820483_LG5b	MDP0000820483	5	14650316-14654059	00046	65822 – 65961	for: CTACCCTCAGCTGAACCCAA rev: GCCTCTTCCCTCCTTCAAGT	monomorphic	--

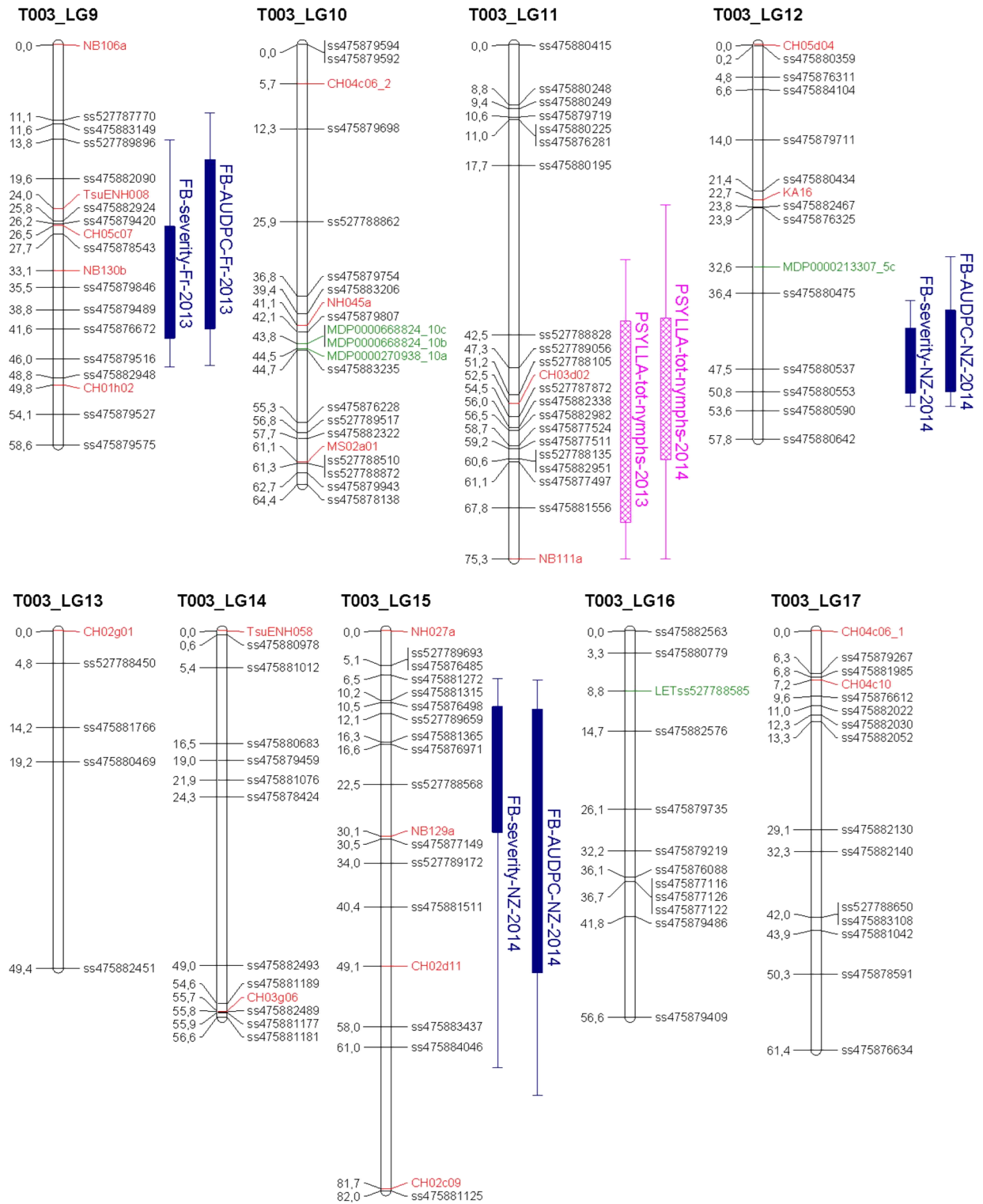
MDP0000820483_LG5c	MDP0000820483	5	14650316-14654059	00046	69024 – 69181	for: ACAACCCCAGCAACATTTC rev: CAGGATATTTGGCGCTGCT	monomorphic	--
MDP0000213307_LG5a	MDP0000213307	5	18371531-18373694	03936	5310 – 5464	for: GTCTAGGACTTGCGCCAATG rev: GCTATGACCAAGACAGCAGC	aaxab	LG12 on 'Moonglow'
MDP0000213307_LG5b	MDP0000213307	5	18371531-18373694	03936	4966 – 5035	for: TTGGGCGAATCTGATGTTGC rev: AGAAGACTCGATGGCACTGT	monomorphic	--
MDP0000213307_LG5c	MDP0000213307	5	18371531-18373694	03936	5623 – 5763	for: TGGGAATCAACCTGCACAAG rev: ACAACTCCAAACCTCCCGA	abxcc	LG12 on PEAR3
MDP0000303781_LG5a	MDP0000303781	5	21965062-21988213	00305	170258 – 170370	for: CGAAACACACACCAACCACT rev: TGCATCACAAACAGGCTTTC	abxcd	not mapped
MDP0000303781_LG5b	MDP0000303781	5	21965062-21988213	00305	171834 – 172020	for: GGAAATGCAGGGTTGACAGG rev: GGCGGAAGTTTACCAGGTTC	monomorphic	--
MDP0000303781_LG5c	MDP0000303781	5	21965062-21988213	00305	171298 – 171347	for: CCGGTGTTTGAGATTGGACC rev: CAGCGCTTGAAAAGTTTGCC	abxaa	not mapped
MDP0000431101_LG5a	MDP0000431101	5	24619224-24623097	00861	105504 – 105663	for: TGCATTGGGATTGCATGTGG rev: GGGTCATGTTGGGAGGGATA	complex	--
MDP0000431101_LG5b	MDP0000431101	5	24619224-24623097	00861	105162 – 105310	for: TGGCTGTTGTGTTGAAGGAA rev: CACATTCACGCATTACACG	1st locus aaxab 2nd monomorphic	LG5 on 'Moonglow'
MDP0000668824_LG10a	MDP0000668824	10	18606299-18610295	01100	102777 - 102847	for: CATGGGATCTTGGCAGCAAA rev: GATGATTCTCGTGGTGGTGC	no amplification	--
MDP0000668824_LG10b	MDP0000668824	10	18606299-18610295	10250	1703 - 1797	for: ACGATGATTCTGGTGGTGGT rev: GTGGACAGGGGACATTGAGA	abxcd	LG10 on PEAR3 and 'Moonglow'
MDP0000668824_LG10c	MDP0000668824	10	18606299-18610295	10250	1593 - 1722	for: GGAGTTGGCCTTGACATTCG rev: ACCACCACCAGAATCATCGT	abxcd	LG10 on PEAR3 and 'Moonglow'
MDP0000270938_LG10a	MDP0000270938	10	18781942-18786363	00278	61754 - 61834	for: GAATCGCACTTTCACAGCCA rev: GTTCTAGGCCGGGTAGTTT	efxeg	LG10 on PEAR3 and 'Moonglow'
MDP0000270938_LG10b	MDP0000270938	10	18781942-18786363	00278	60808 – 60867	for: ACCAGGTTAAAGGAGTCGGG rev: GCTGATTTGCGGGAGAGAAC	monomorphic	--
MDP0000270938_LG10c	MDP0000270938	10	18781942-18786363	00278	62338 – 62518	for: TTCAAGGTCAGCGGAAGAGT rev: TTCCAGACAGCTTGGGAGAG	no amplification	--
MDP0000270938_LG10d	MDP0000270938	10	18781942-18786363	00278	64583 – 64639	for: CAGATGCCAACCACAAACA rev: AGCAAACCTTCGTGGTTCCG	monomorphic	--

Annex 8: Microsatellite (SSR) markers selected to reduce the interval of the regions linked to hybrid necrosis in the PEAR3 x 'Moonglow' population

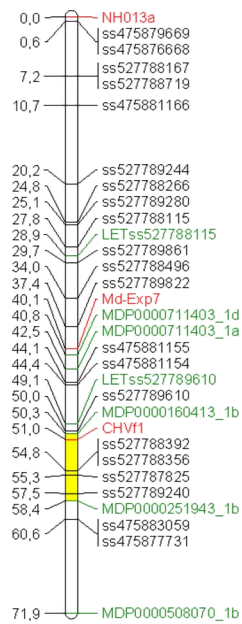
Polymorphic SSR markers were evaluated on 'Type 1', 'Type 2' and 'Type 3' individuals. For each marker, the allelic composition and the linkage group of PEAR3 and/or 'Moonglow' are shown, as well as the amplicon size range and the annealing temperature

SSR locus	Location on other maps	Primers sequence	Segregation type in PEAR3x'Moonglow'	Location on PEAR3x'Moonglow' map	Amplicon size range (bp)	Annealing temperature (°C)
AJ251116	LG2	for: GATCAGAAAATTGCTAGGAAAAGG rev: AGAGAACGGTGAGCTCCTGA	monomorphic	--	--	--
AT000400	LG2	for: CTCCTTTGCTCCCTCTCTT rev: AGGATGTCAGGGTTGTACGG	no amplification	--	--	--
AU223670	LG5	for: GGACTCAATGCCTTTTCTGG rev: AGGATGGCAGCAATCTTGAA	monomorphic	--	--	--
CH02f06	LG2	for: CCCTCTTCAGACCTGCATATG rev: ACTGTTTCCAAGCGATCAGG	a0xbc	LG2 on PEAR3 and 'Moonglow'	151-178	touchdown 63-58
CH04g09	LG5	for: TTGTCGCACAAGCCAGTTTA rev: GAAGACTCATGGGTGCCATT	complex	--	--	--
CH05e06	LG5	for: ACACGCACAGAGACAGAGACAT rev: GTTGAATAGCATCCCAAATGGT	00xab	LG5 on 'Moonglow'	109-131	touchdown 63-58
CH05f06	LG5	for: TTAGATCCGGTCACTCTCCACT rev: TGGAGGAAGACGAAGAAGAAAG	abxcd	LG5 on PEAR3 and 'Moonglow'	158-182	touchdown 63-58
CN444636	LG2	for: CACCACTTGAGTAATCGTAAGAGC rev: GTTTGCCAGTTAAGGACCACAAGG	abxcd	LG2 on PEAR3 and 'Moonglow'	238-255	touchdown 63-58
CN445599	LG5	for: TCAAATGGGTTTCGATCTTCAC rev: GTTTGCCTGGCTGTAAGTGTGG	abxab	not mapped	128-130	touchdown 63-58
CN493139	LG2	for: CACGACCTCCAAACCTATGC rev: GTTTATGAAAGTACGGCACCCATC	efxeg	LG2 on PEAR3 and 'Moonglow'	135-148	touchdown 63-58
CN581493	LG2	for: GCTTTTCATGGTGAAAACTG rev: GTTTGACTCTCCGCTCTGATGGAC	aaxbc	LG2 on 'Moonglow'	182-200	touchdown 63-58
Hi02a07	LG2	for: TTGAAGCTAGCATTTGCCTGT rev: TAGATTGCCCAAAGACTGGG	aaxbc	LG2 on 'Moonglow'	227-288	touchdown 61-56
Hi04d02	LG5	for: TTCGTGGCTGAGAAAGGAGT rev: GTTTGTACGGTGCATTGTGAAAG	abxcd	LG5 on PEAR3 and 'Moonglow'	159-199	touchdown 61-56
Hi05g12	LG2	for: TCTCTAGCATCCATTGCTTCTG rev: GTTTGTGTGTCTCTCATCGGATTC	no amplification	--	--	--
Hi08g12	LG2	for: AGTTCGGTCCGGTCCGTAAT rev: GTTTAGGGCAAGGGGAAAGAAGT	efxeg	LG2 on PEAR3 and 'Moonglow'	179-196	touchdown 63-58
Hi11a03	LG5	for: GGAATTGGAGCTTGATGCAG	complex	--	132-139	touchdown 61-56

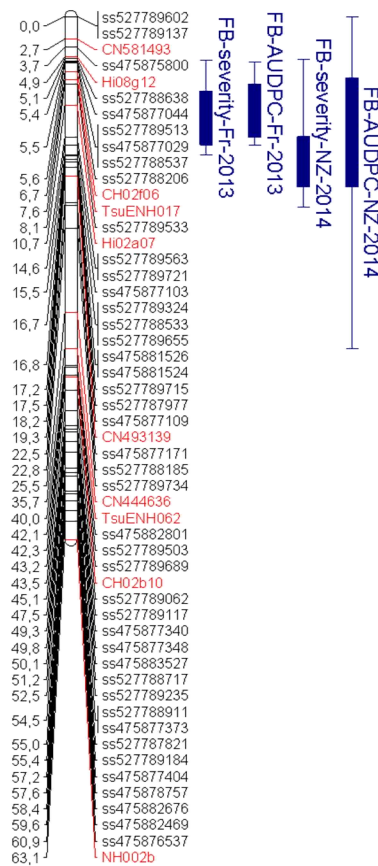
		rev: GTTTCATACGGAATGGCAAATCG				
Hi21c08	LG5	for: TTCTTCTCCTCCACCACCTC rev: GTTTGTCAC TGAGAAGGCGGTAGC	monomorphic	--	212-223	touchdown 61-56
Hi24f04	LG2	for: CCGACGGCTCAAAGACAAC rev: TGAAAAGTGAAGGGAATGGAAG	abxcc	LG2 on PEAR3	130-144	touchdown 61-56



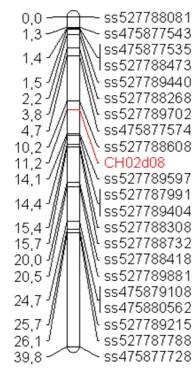
'Moonglow'_LG1



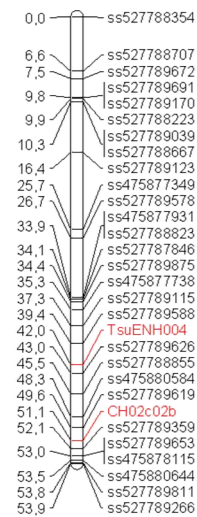
'Moonglow'_LG2



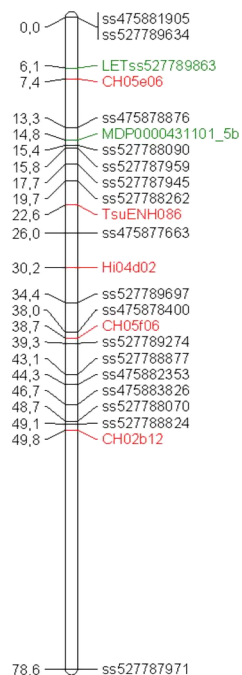
'Moonglow'_LG3



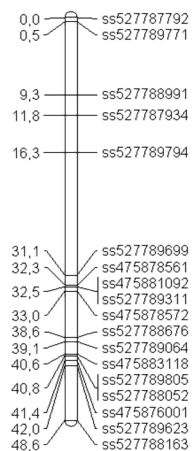
'Moonglow'_LG4



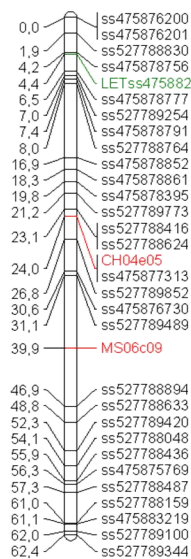
'Moonglow'_LG5



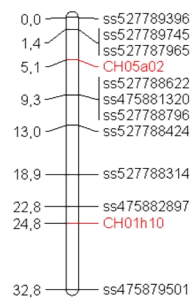
'Moonglow'_LG6



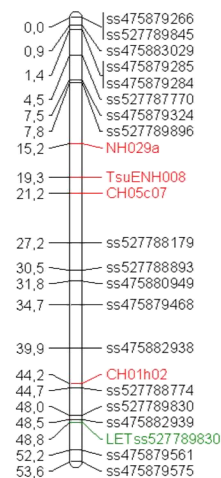
'Moonglow'_LG7



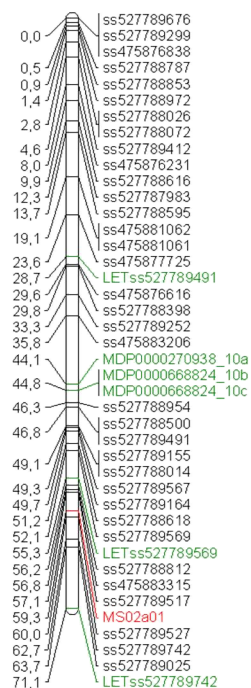
'Moonglow'_LG8



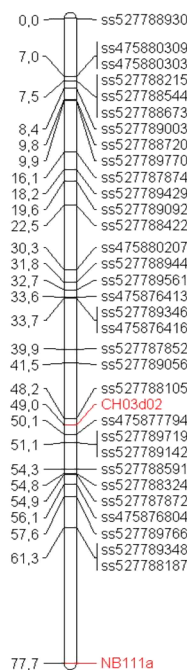
'Moonglow'_LG9



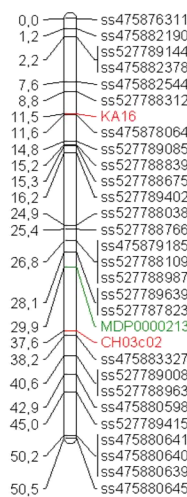
'Moonglow'_LG10



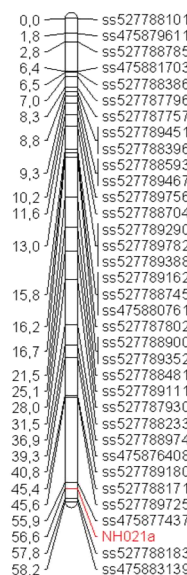
'Moonglow'_LG11



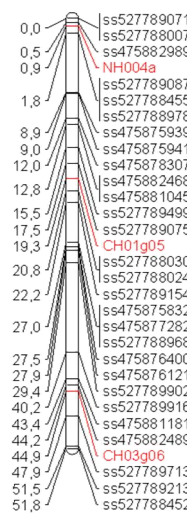
'Moonglow'_LG12



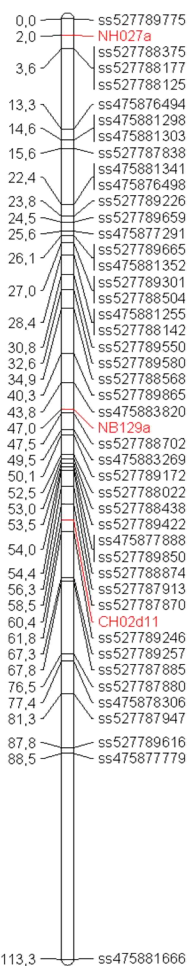
'Moonglow'_LG13



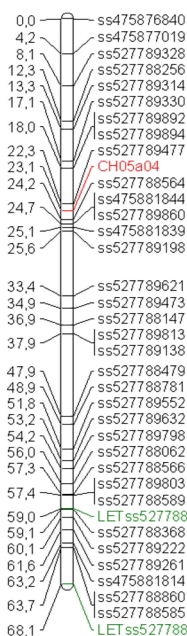
'Moonglow'_LG14



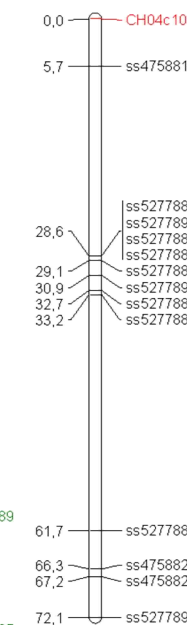
'Moonglow'_LG15



'Moonglow'_LG16



'Moonglow'_LG17



Thèse de Doctorat

Sara MONTANARI

Identification et cartographie de régions du génome contrôlant la résistance au feu bactérien et au psylle et la nécrose hybride chez le poirier

Identification and mapping of genomic regions controlling fire blight and psylla resistance and hybrid necrosis in pear

Résumé

Le feu bactérien et le psylle causent d'importantes pertes économiques dans les zones de production du poirier dans le monde entier. Le développement de nouvelles variétés de poirier résistantes à ces bio-agresseurs constitue un enjeu majeur dans le cadre d'un programme de lutte intégrée. L'objectif de ce projet de thèse est l'étude du déterminisme génétique de la résistance vis-à-vis de ces deux bio-agresseurs. La thèse a été réalisée dans le cadre d'une collaboration internationale entre Fondazione Edmund Mach (Italie), Institut de Recherches en Horticulture et Semences (France) et Plant & Food Research (Nouvelle-Zélande). Une descendance interspécifique de poirier PEAR3 x 'Moonglow' a été développée avec pour objectif de cumuler les résistances au feu bactérien et au psylle provenant de variétés asiatiques et européennes de *Pyrus*. Deux cartes génétiques ont été élaborées pour PEAR3 et 'Moonglow' sur la base de marqueurs SNP (Single Nucleotide Polymorphism) et SSR (microsatellite), et la cartographie de QTLs (Quantitative Trait Loci) a permis de démontrer le déterminisme polygénique de la résistance à ces bio-agresseurs. Une sélection assistée par marqueurs (MAS) peut donc être engagée pour ces deux caractères. Des incompatibilités génétiques ont aussi été observées dans une partie de la descendance, ce qui a permis de cartographier pour la première fois chez le poirier les zones du génome liées au phénomène de « nécrose hybride ». Le développement de marqueurs liés aux gènes létaux devrait permettre aux sélectionneurs d'éviter les combinaisons incompatibles en croisement qui peuvent impacter certains caractères agronomiques co-ségrégant avec ces gènes létaux.

Mots clés

***Pyrus x bretschneideri*; *Pyrus communis*; cartographie génétique; détection de QTL; *Cacopsylla pyri*; *Erwinia amylovora*; gènes létaux; incompatibilités génétiques**

Abstract

The goal of this PhD project was to study the genetic architecture of pear resistance to two of its most significant diseases and pests, fire blight and psylla, which cause severe yield losses in all the main pear production regions worldwide. The development of new pear varieties with resistance against these two biotic stresses is of major interest for Integrated Pest Management. This project was designed in a joint collaboration among Fondazione Edmund Mach (Italy), Institut de Recherches en Horticulture et Semences (France) and Plant & Food Research (New Zealand). The interspecific pear F1 progeny PEAR3 x 'Moonglow' was developed with the purpose of cumulating resistances to fire blight and psylla deriving from Asian and European pear cultivars. Single nucleotide polymorphism (SNP) and simple sequence repeat (SSR)-based genetic maps were built for PEAR3 and 'Moonglow'. Quantitative Trait Loci (QTLs) were detected for the resistances, demonstrating their polygenic nature. Marker-assisted selection (MAS) can now be applied for these two traits. Furthermore, the segregating population exhibited genetic incompatibilities, and the genomic regions associated with hybrid necrosis were mapped for the first time in pear. Development of molecular markers linked to the lethal genes should allow breeders to avoid crosses leading to incompatible combinations that could affect the expression of important agronomic traits co-segregating with these genes.

Key Words

***Pyrus x bretschneideri*; *Pyrus communis*; genetic mapping; QTL detection; *Cacopsylla pyri*; *Erwinia amylovora*; lethal genes; genetic incompatibility**