



**HAL**  
open science

# Genetic and chemical genomic dissection of the cell adhesion mechanisms in plants

Stephane Verger

► **To cite this version:**

Stephane Verger. Genetic and chemical genomic dissection of the cell adhesion mechanisms in plants. Cellular Biology. Université Paris Sud - Paris XI, 2014. English. NNT: 2014PA112255 . tel-01252736

**HAL Id: tel-01252736**

**<https://theses.hal.science/tel-01252736>**

Submitted on 8 Jan 2016

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

UNIVERSITE PARIS-SUD

ÉCOLE DOCTORALE : SCIENCES DU VÉGÉTAL

Institut Jean-Pierre Bourgin, UMR1318 INRA-AgroParisTech

*DISCIPLINE : BIOLOGIE*

THÈSE DE DOCTORAT

Soutenance prévue le 3 octobre 2014

par

**Stéphane VERGER**

Genetic and chemical genomic dissection of the  
cell adhesion mechanisms in plants

**Composition du jury :**

*Directeur de thèse :*

*Rapporteurs :*

*Examineurs :*

Grégory MOUILLE

Simone FERRARI

Hoai-Nam TRUONG-CELLIER

Jérôme PELLOUX

Jacqui SHYKOFF

Sebastian WOLF

Directeur de recherche INRA, IJPB, Versailles

Assistant Professor, Università Di Roma La Sapienza

Chargée de recherche INRA, Dijon

Professeur à l'UPJV, Amiens

Directeur de recherche CNRS, Orsay

Group Leader, COS, Heidelberg

UNIVERSITE PARIS-SUD

ÉCOLE DOCTORALE : SCIENCES DU VÉGÉTAL

Institut Jean-Pierre Bourgin, UMR1318 INRA-AgroParisTech

*DISCIPLINE : BIOLOGIE*

THÈSE DE DOCTORAT

Soutenance prévue le 3 octobre 2014

par

**Stéphane VERGER**

Genetic and chemical genomic dissection of the  
cell adhesion mechanisms in plants

**Composition du jury :**

*Directeur de thèse :*

*Rapporteurs :*

*Examineurs :*

Grégory MOUILLE

Simone FERRARI

Hoai-Nam TRUONG-CELLIER

Jérôme PELLOUX

Jacqui SHYKOFF

Sebastian WOLF

Directeur de recherche INRA, IJPB, Versailles

Assistant Professor, Università Di Roma La Sapienza

Chargée de recherche INRA, Dijon

Professeur à l'UPJV, Amiens

Directeur de recherche CNRS, Orsay

Group Leader, COS, Heidelberg



## **Summary: Genetic and chemical genomic dissection of the cell adhesion mechanisms in plants**

Cell to cell adhesion in plants is mediated by the cell wall in which the components are cross-linked in order to create a continuum of polysaccharides linking the cells together. However the cell wall is a dynamic compartment that participates in growth and development through its constant loosening and remodeling and it is not very clear how cell adhesion is actually maintained in these conditions. In order to get a better understanding of the mechanisms that control cell adhesion in plants we used a combination of a forward genetic suppressor screen and a chemical genomic suppressor screen on the cell adhesion defective and pectin synthesis deficient mutants *quasimodo1* and *quasimodo2*, and have isolated a number of suppressor mutants and molecules implicated in cell adhesion. The genetic screen led to the identification and study of a suppressor mutated in the gene *ESMERALDA1*, an uncharacterized putative O-fucosyltransferase. The genetic study of cell adhesion including another putative O-fucosyltransferase *FRIABLE1* showed that the disruption of *ESMD1* was sufficient to suppress the cell adhesion defect of *qua1*, *qua2* and *frb1*, making it a major player of the pathway. The chemical genomic screen has revealed the implication of auxin transport and pectin methyl esterase activity in the process of cell adhesion.

Based on these new information we have established a model explaining the loss of cell adhesion in the *quasimodo* and *friable1* mutants, and from this model we have inferred the existence of the mechanisms that dynamically allow the maintenance of cell adhesion in plants during growth and development.

## **Résumé: Dissection génétique et chemogénomique des mécanismes d'adhésion cellulaire chez les plantes**

L'adhésion cellulaire chez les plantes est permise par la présence de la paroi dont les composants sont réticulés afin de former un réseau de polysaccharides liant les cellules entre elles. Cependant, la paroi est un compartiment cellulaire dynamique qui participe à la croissance et au développement de la plante, notamment par son relâchement et sa réorganisation constante et nous ne savons pas exactement comment l'adhésion cellulaire est effectivement maintenue dans ces conditions. Afin d'obtenir une meilleure compréhension des mécanismes qui contrôlent l'adhésion cellulaire chez les plantes, nous avons utilisé une combinaison de crible génétique suppresseur et de crible chémozénomique suppresseur sur les mutants *quasimodo1* et *quasimodo2* présentant un défaut d'adhésion cellulaire accompagné d'une déficience de synthèse de pectine. Par ces approches nous avons pu isoler des mutants suppresseurs et des molécules chimiques impliquées dans l'adhésion cellulaire. Le crible génétique a conduit à l'identification et l'étude d'un suppresseur muté dans le gène *ESMERALDA1*, une O-fucosyltransférase putative non caractérisée. L'étude génétique du défaut d'adhésion cellulaire en incluant *friable1*, muté dans une autre O-fucosyltransférase putative, a montré que la mutation de *ESMD1* était suffisante pour supprimer le défaut d'adhésion cellulaire de *qua1*, *qua2* et *frb1*, ce qui en fait un acteur majeur de l'adhésion cellulaire. Le crible chemogénomique a montré l'implication du transport de l'auxine et de l'activité pectin méthylesterase dans le processus contrôlant l'adhésion cellulaire.

Sur la base de ces nouvelles informations, nous avons établi un modèle qui explique la perte de l'adhésion cellulaire chez les mutants *quasimodo* et *friable1*, et à partir de ce modèle, nous avons pu déduire l'existence de mécanismes qui permettent le maintien de l'adhésion cellulaire de façon dynamique au cours de croissance et de développement chez les plantes.



## **Acknowledgements/Remerciements**

First of all I would like to thank Drs Simone Ferrari, Jérôme Pelloux, Jacqui Shykoff, Hoai - Nam Truong-Cellier and Sebastian Wolf for accepting to review my PhD Thesis.

Je vais bien sur commencer par te remercier Grégory pour m'avoir accueilli dans l'équipe. Mais surtout merci pour ton enthousiasme, tes conseils, tout ce que tu m'as appris, la grande confiance que tu m'as accordée depuis que je suis arrivé en stage au labo, et l'autonomie que tu m'as laissée et qui m'a permis de m'investir pleinement dans ce sujet de recherche passionnant. Bref, faire ma thèse avec toi est une chance que j'ai su saisir !

Je te souhaite aussi bonne chance pour la suite et pour l'avenir de ta nouvelle équipe.

Un grand merci à Salem. J'ai énormément apprécié travailler avec toi, et ma thèse ne serait pas ce qu'elle est sans ton aide si précieuse.

Merci Herman pour ton accueil dans l'équipe paroi primaire. Merci pour les discussions scientifiques ou pas, toutes plus enrichissantes les unes que les autres, pour ta sympathie et ta franchise.

Un grand merci à toute l'équipe paroi primaire que je quitte avec tristesse. Marjolaine, Julien, Thierry, Martine et Samantha, merci pour votre amitié, et votre accueil au sein de l'équipe.

Merci aussi aux anciens membres de l'équipe que j'ai eu le plaisir de côtoyer au cours de ma thèse (Rodnay, Hélène, Zhenni, Fabien, Maxence, Michael, Jozef, Sebastian, Renaud, Volker, et les autres). De plus un grand merci à Thierry et Philippe qui m'ont quasiment tout appris au labo.

Je remercie également l'ensemble des membres des plate formes expérimentales de l'IJPB qui permettent l'accès à de nombreuses ressources indispensables (serre, microscopie, biochimie...). Un grand merci notamment à Gwendal pour ton aide à la plateforme de biochimie.

Merci a Stéphanie et bien sur Mateusz, Adeline, Qian, Lennard et Thomas pour votre accueil si chaleureux dans votre pays nordique et pour le super mois et demi passé en votre compagnie que ce soit au labo à Umea ou en voyage autour de la mer Baltique.

Je tiens aussi à remercier les personnes qui ont participé à mes comités de thèse, Patrick Laufs, Hoai-Nam Truong-Cellier, Sebastian Wolf, Kian Hématy, Catherine Perrot-Rechenmann et Jean Colcombet, pour les idées et les conseils apportés.

Merci à l'ensemble des membres de l'IJPB que j'ai eu l'occasion de côtoyer, et notamment tous les doctorants, stagiaires, post-doc et autres non permanents avec qui j'ai pu partager des moments de détente à l'INRA ou en dehors. Merci a ceux d'entre eux qui sont devenus mes amis, notamment, Laure, Marjolaine, Davy, Julien, Yannick, Camille (blonde), Camille (brune), Thomas (les collègues AA), Mika, Alban, Aude, Kat, Céline, Marie-Cap et tous les autres.

Et pour finir, un énorme merci à mes amis, à ma famille, et surtout à mes parents qui m'ont toujours soutenu et qui m'ont permis d'arriver jusque là.

## Table des matières

### CHAPTER I: WHAT CONTROLS CELL ADHESION IN PLANTS ?

#### PART I:

<b>ORIGINS, STRUCTURAL FEATURES AND CONTROL OF THE CELL ADHESION IN PLANTS .....</b>	<b>6</b>
I) INTRODUCTION .....	6
II) THE ORIGINS OF CELL ADHESION IN PLANTS .....	7
1) <i>Cell adhesion and multicellularity</i> .....	7
2) <i>Driving forces for the apparition of cell adhesion</i> .....	8
3) <i>Different mechanisms for cell adhesion</i> .....	9
4) <i>The origins of cell adhesion in plants</i> .....	10
5) <i>What made pectins the main determinant of cell adhesion in land plants?</i> .....	12
III) SET UP AND STRUCTURAL BASES OF THE CELL ADHESION IN PLANTS .....	13
1) <i>Ontogeny of the cell adhesion</i> .....	13
2) <i>The role of cell wall structure and composition in cell adhesion</i> .....	17
IV) WHAT CONTROLS CELL ADHESION IN PLANTS.....	23
1) <i>Developmentally regulated cell separation</i> .....	23
2) <i>Maintenance and modulation of cell adhesion during growth and development</i> .....	26
V) CONCLUSION .....	28

#### PART II :

<b>PROBLEMATIC, EXPERIMENTAL DESIGN AND OBJECTIVES OF THE THESIS. ....</b>	<b>30</b>
--	-----------

### CHAPTER II: GENETIC DISSECTION OF THE CELL ADHESION MECHANISMS

#### PART I :

<b>PLANT CELL ADHESION IS UNDER THE CONTROL OF PUTATIVE O-FUCOSYLTRANSFERASES .....</b>	<b>34</b>
SUMMARY .....	34
RESULTS .....	35
DISCUSSION .....	42
ACKNOWLEDGEMENTS .....	44
CONTRIBUTIONS .....	44
EXPERIMENTAL PROCEDURES .....	45

#### PART II:

<b>ADDITIONAL WORK ON ESERALDA1 AND FRIABLE1 FUNCTION.....</b>	<b>56</b>
I. ESERALDA1 AND FRIABLE1 ARE PUTATIVE O-FUCOSYLTRANSFERASES .....	56
1. <i>Are ESMD1 and FRB1 actual O-fucosyltransferases?</i> .....	56
2. <i>Are the WAKs the substrates of ESMD1 and FRB1?</i> .....	57
II. ADDITIONAL DATA ON THE MUTANT PHENOTYPES .....	71
1. <i>Characterisation of the cell wall modification in quasimodo and the suppressor lines</i> .....	71



2. <i>ESMD1</i> pattern of expression .....	75
3. Transcriptomic analysis.....	78
4. <i>qua2</i> and <i>esmd1</i> show an altered response to <i>OGA</i> .....	83
5. <i>qua2-1</i> is resistant to <i>B. cinerea</i> maceration and sensitivity is restored in the suppressor line.....	85
III. EXPERIMENTAL PROCEDURES .....	87
ACKNOWLEDGMENTS.....	100
CONTRIBUTIONS .....	100

## **CHAPTER III: CHEMICAL GENOMIC DISSECTION OF THE CELL ADHESION MECHANISMS AND SUCROSE SENSITIVITY**

<b>HIGH THROUGHPUT CHEMICAL GENOMICS HIGHLIGHTS THE ROLE OF AUXIN TRANSPORT AND PECTIN METHYL ESTERASE ACTIVITY IN PLANT CELL ADHESION AND CARBON/NITROGEN BALANCE PERCEPTION .....</b>	<b>104</b>
ABSTRACT.....	104
INTRODUCTION.....	105
RESULTS .....	108
DISCUSSION .....	121
MATERIALS AND METHODS .....	127
ACKNOWLEDGMENTS.....	131
CONTRIBUTIONS.....	131

## **GENERAL DISCUSSION**

<i>The power and the complementarity of the forward genetic and the chemical genomics.....</i>	<i>134</i>
<i>The potential role of O-fucosylation in cell adhesion.....</i>	<i>136</i>
<i>Plants can trigger cell separation throughout their tissues .....</i>	<i>140</i>
<i>A working model for the control of cell adhesion in plants.....</i>	<i>143</i>
<i>Why is quasimodo sensitive to sucrose? .....</i>	<i>148</i>
<i>Perspectives .....</i>	<i>149</i>

## **List of abbreviations**

## **References**



## **Chapter I:**

**What controls cell adhesion in plants?**

---

**Preamble:**

This chapter is the introduction of my thesis work. In the first part I review\* the knowledge that has been accumulated over the years on the different aspects relating to cell adhesion in plants: Its origins and its evolution toward the cell adhesion that we witness in today's land plants, the set up and the structural features that allow its maintenance, and the molecular mechanisms that dynamically control it. This bibliographic review mostly focuses on the fundamental understanding of the cell adhesion mechanisms in land plants and point out our lack of understanding of some of these aspects and the need to extend our knowledge on these questions.

In the second part, I introduce the problematic that motivated my work and the project that we set up to experimentally explore our question and investigate on the mechanisms that control cell adhesion in plants.

*\*The first part of this chapter is written as a review and is meant to be submitted for publication after reformatting and realization of original illustrations. Parts of the work presented in the other chapters of my thesis are also meant to be submitted for publication, and these publications should provide additional input to this review work. Thus this review may only be submitted after the acceptance for publication of these other publications and formatted consequently.*

## **Part I:**

# **Origins, structural features and control of the cell adhesion in plants**

## **I) Introduction**

Most of the plants that we see around us in nature and the plants that we study in the lab or in the field, whether it is for fundamental research or breeding and improvement of agriculture, share the fundamental feature of being multicellular organisms. It is such a fundamental feature of a complex organism that we tend to forget how important it can be. From a unique cell, the ovule, and after fertilization by the sperm cells, the developing embryo and future organism develops through clonal division and growth of cells that remain attached to each other. Except for a few cases such as the pollen adhesion to the stigma (Swanson et al., 2004), the pollen tube growth through the transmitting tract of the pistil (Jauh and Lord, 1996) and the rare cases of post embryonic organs fusions for which cell adhesion takes place independently from cell division and with a cell from an originally distant tissue (organ fusion) and often different tissue (self pollination and intrusive growth) or different individual (cross pollination and graft union). For the large majority of the cells in plants however, cell to cell adhesion is set up at cell division during the formation of a new cell wall between two daughter cells. The plant then has to accommodate with the fact that the large majority of its cells are fixed and will keep the same neighbor cells throughout their life. The consequence of this is that the morphogenesis of the plant then only results from the spatial and temporal control of cell division and cell expansion.

Although it may seem like after its set up, cell adhesion remains a very passive phenomenon, a number of crucial events in plant's development proves this wrong. Petal shedding, pod shatter, dehiscence, pollen release, root cap sloughing off, lateral root emergence... are all tightly controlled mechanisms of cell separation. And it is easy to realize that these events must be precisely controlled in order to trigger cell separation at a specific location and for a specific subset of cells at a specific time. In addition, one highly underestimated problem for the plant is to actually maintain cell adhesion in the rest of the plant during growth and

development, while the enzymes and mechanisms implicated in cell wall loosening necessary for cell expansion are the same as those implicated in cell separation.

But this complex process of cell adhesion and the emergence of complex organisms, takes its origins at the onset of cell adhesion between unicellular organisms. The selective pressure that favored organisms with a larger size led to the emergence of cell adhesion. And cell to cell adhesion is such a favorable adaptation that multicellularity has evolved multiple times, and the mechanisms that mediate cell adhesion are diverse.

In this review we focus on this type of cell adhesion that keeps the cells attached together after cell division and that ultimately turn a unicellular zygote into a complex multicellular plant. How did such a cell adhesion emerge? How is it set up? What are the structural components that hold the cells together? And finally how is the state of cell adhesion dynamically controlled in plants? are some of the questions that are covered in this review.

## **II) The origins of cell adhesion in plants**

### **1) Cell adhesion and multicellularity**

Cell adhesion is one of the most basic features of a multicellular organism (Abedin and King, 2010; Kirk, 2005). From what we know based on extant organisms, the development of a multicellular organism may result from two types of processes: an aggregative or a clonal development (Grosberg and Strathmann, 2007). In the first type the organism spends most of its life cycle under a unicellular form and then, when necessary, aggregates to form a multicellular migrating slug-like structure in order to move to a more favorable environment and develop a fruiting body (Bonner, 1998). These represent only a few groups of terrestrial and semi terrestrial microorganisms such as the dictyostelids, that didn't evolved into complex multicellular organisms (Crespi, 2001). The second type of process leads to the development independent multicellular organisms via the clonal multiplication of a unicellular spore or zygote in which the cells usually stay connected after cell division (Queller, 2000). This one represents the type of multicellularity that is observed in all of the marine and most of the terrestrial multicellular organisms. It is this type of development that has allowed the apparition of the complex multicellularity that is found in animals, fungi and plants (Bonner, 1998; Abedin and King, 2010).

Cell adhesion and multicellularity are tightly related since cell to cell adhesion is a requirement to make a multicellular organism, and cell to cell adhesion wouldn't be so wide spread if there wasn't a selective advantage for multicellularity.

## **2) Driving forces for the apparition of cell adhesion**

Probably the main driving force toward the apparition of cell to cell adhesion is the advantage that multicellularity can give. And likely the initial driving force toward the apparition of multicellularity is the advantage that an increase in size can give (Bonner, 2004; Grosberg and Strathmann, 2007). The living world was and is still largely dominated by unicellular organisms in terms of number and diversity. And in a unicellular world, having a larger size compared to the other unicellular organisms likely provides a considerable advantage. The apparition of phagotrophic organisms (Unicellular organisms that consume unicellular preys) may have started a race for an increase in size between predators and preys (Stanley, 1973). However increasing in cell size is not that simple and is subjected to physical constraints in order to maintain the homeostasis of the cell (Bonner, 2004), while a transition to multicellularity seems to represent a relatively simple way to overcome this problem. A number of studies of experimental evolution have demonstrated that unicellular organism could relatively quickly adopt a persistent undifferentiated multicellular form under a selective pressure (Boraas et al., 1998; Alegado et al., 2012; Koschwanez et al., 2013; Ratcliff et al., 2012; 2013). Experiments on a unicellular green algae and a unicellular choanoflagellate have pointed out the advantage of a larger size that multicellularity can give, in order to prevent the predation by other phagotrophic microorganisms (Boraas et al., 1998; Alegado et al., 2012). But simple multicellularity can provide other advantages as was shown in other experiments with yeast, for which it is the exposure to starvation that led to multicellularity (Ratcliff et al., 2012; 2013; Koschwanez et al., 2013). While these represent constraints that pushed the organisms to become multicellular, the state of multicellularity can then provide additional advantages. A multicellular organism can start to differentiate and compartmentalize (Bonner, 2004), in order to increase its complexity: a requirement for the further emergence of different tissue types such as the epidermis or the vascular tissues of today's land plants.

On an evolutionary point of view, the step necessary to develop multicellularity can in fact be effectively reached by the co-option of already existing genes and molecular mechanisms in order to develop cell adhesion. This was demonstrated by comparing the number of genes

within gene families and the number of different gene families in *Chlamydomonas reinhardtii* and *Volvox carteri*. These two organisms are very closely related but *Chlamydomonas* is unicellular while *Volvox* is multicellular. This study revealed that there was no increase in the number of different gene families, but an increase in the number of genes within families in the multicellular *Volvox*. This points out a role for the diversification of function of existing genes after duplication rather than the apparition of new genes or gene families dedicated to this new function (Prochnik et al., 2010; Gresham, 2013).

The driving forces toward the apparition of cell adhesion are considerable and the transition seems relatively simple. In fact multicellularity has evolved independently in at least 25 lineages (Grosberg and Strathmann, 2007), and the mechanisms of cell adhesion differ among the different lineages in which it has appeared (Abedin and King, 2010).

### 3) Different mechanisms for cell adhesion

In animals, the cells are directly attached to each other by mechanisms implicating protein-protein interaction with proteins such as the cadherins (Pokutta and Weis, 2007), or protein-extracellular matrix (ECM) involving proteins such as the integrins (Sebé-Pedrós et al., 2010). Cell adhesion in animals is under the form of punctuate junctions along the surface of contact between two adjacent cells (Abedin and King, 2010). Although they have a different type of multicellular development (aggregating as opposed to clonal in animals), the dictyostelids have a very similar type of cell adhesion (Harwood and Coates, 2004). In animal as in dictyostelids, the absence of a rigid cell wall allows dynamic attachment, detachment and migration of cells within the multicellular organism.

On the other hand, many multicellular lineages such as the plants and fungi, have developed a large cell wall, and their cell to cell adhesion is based on the presence of this cell wall. As opposed to the “dynamic” cell adhesion of animals and dictyostelids, cell adhesion here seems more passive. It largely results from the absence of separation of two daughter cells after cell division, often due to a partially incomplete cell division and the presence of an intact cell wall linking the two cells together. This cell wall forms a continuum also called apoplast or extracellular matrix, which is composed of glycoproteins and polysaccharides that are cross-linked to form a coherent rigid network. The plants also possess proteins homologous to the animal integrins (Laval et al., 1999), or other plant specific proteins such as the fasciclin-like arabinogalactan proteins with a GPI anchor (Johnson et al., 2003), the Wall-Associated Kinases (He et al., 1996) and other types of proteins (Kohorn, 2000) that mediate plasma



membrane-cell wall association (Canut et al., 1998; Kohorn, 2000; Baluška et al., 2003; Knepper et al., 2011; Lü et al., 2012; Shi et al., 2003). They have a role in attaching the plasma membrane to the cell wall and most of them are also likely implicated in signaling and in the perception of the cell wall integrity (Kohorn, 2000). However they are not directly implicated in cell to cell adhesion. Indeed, each plant cell produces its own cell wall and it is the continuity of the polysaccharides cross-linking between the two adjacent cells' cell walls that ensures cell to cell adhesion. ((Jarvis et al., 2003); detailed description in III.). Cell migration is not believed to occur with this type of cell adhesion, but there are cases of invasive cell growth (e.g. pollen through the pistil transmitting track;(Jauh and Lord, 1996)) and it doesn't prevent from later detachment via the active degradation of the cell wall (e.g.; organ abscission; (Roberts et al., 2002; Estornell et al., 2013)), or attachment via the cross-linking of directly exposed polysaccharides (Post embryonic organ fusion; (Pruitt et al., 2000)).

#### 4) The origins of cell adhesion in plants

Understanding the origin (as opposed to today's aggregating or clonal development) of cell adhesion in the different lineages that have evolved multicellularity remains very difficult (Abedin and King, 2010; Harwood and Coates, 2004; Niklas, 2014). Although most of these lineages still have extant unicellular and multicellular members, these have largely diverged, and the common ancestors of these relative multicellular and unicellular organisms of a same clade have disappeared long ago. The volvocine algae however, provide a very interesting insight into the origins of cell adhesion and the transition from unicellular to multicellular organism. This clade still has the unicellular (*Chlamydomonas*), the differentiated multicellular (*Volvox*) and four clearly distinct intermediate extant forms (*Gonium*, *pandorina*, *eudorina*, *pleodorina*) of increasing complexity (Kirk, 2005) which are believed to have diverged quite recently (Rausch et al., 1989). Interestingly, the study of these closely related unicellular and multicellular organisms indicates that the first steps toward the complexification of these organisms seems to be the occurrence of an incomplete cell division and further enlargement of the extracellular matrix to maintain cell adhesion and allow cell to cell communication (Sachs, 2008; Kirk, 2005). But the cell wall and extracellular matrix of the volvocine algae are mainly made of Hydroxyproline Rich GlycoProteins (HRGP) which makes them considerably different from the cell walls of land plants (Woessner and Goodenough, 1994).

The charophycean green algae (CGA) are the closest relatives of the land plants (Wodniok et al., 2011; Zhong et al., 2014). They are all aquatic, have unicellular and multicellular members and their cell walls closely resemble those of the land plants (Domozych et al., 2012; 2014; Sørensen et al., 2011; 2012; Sorensen et al., 2010). Land plants multicellularity (and thus cell adhesion), complexification and later colonization of the terrestrial habitat has likely emerged thanks to the apparition of plant-like cell walls in CGA (Sørensen et al., 2011). A number a recent studies have focus on the characterization of the cell walls of these green algae (Domozych et al., 2009; 2007; 2014; Sørensen et al., 2011) as well as their properties (Proseus and Boyer, 2012a; 2012b). Interestingly these studies have also brought very interesting insights about the evolution of the land plant cell walls and the properties and function of their constituting polysaccharides (Peaucelle et al., 2012). Recently the unicellular *Penium margaritaceum* has emerged as a powerful model for the study the cell walls of these CGA (Sørensen et al., 2014).

Although unicellular, *Penium* will likely also be a powerful tool to understand the origins of cell adhesion in land plants. The cell wall of *Penium* is made of three distinct layers: a cellulose rich inner layer, a HG rich outer layer, and a middle layer in which pectins embed cellulose (Domozych et al., 2007; 2014). The composition and structure of this cell wall has striking similarities with the cell wall of land plants. Although *Penium* is a unicellular organism and its cell wall doesn't mediate cell to cell adhesion, the HG enriched outer layer can easily be compared with the middle lamella of the multicellular land plants. This outer layer seem implicated in cell to substrate adhesion (Domozych et al., 2014) but such a layer may also have been the precursor of the land plants middle lamella mediating cell to cell adhesion.

Interestingly some work on *spyrogyra*, a filamentous multicellular CGA, has revealed the potential implication of a xyloglucan-like compound for cell adhesion in this species (Ikegaya et al., 2008). This could reveal a somewhat unexpected role of a xyloglucan-like compounds in the apparition of cell adhesion for land plants. Indeed, since the advent of *Arabidopsis thaliana* as the main model species for plants, most of the research indicates a major role of pectins in cell adhesion (Jarvis et al., 2003). However *arabidopsis* is genetically and physiologically largely distant from its earliest land plants ancestors. It is possible that the mechanisms that led to the apparition of cell adhesion in the ancestors of land plants was different from what cell adhesion is in the land plant that we study today. However such evidence could also be misleading since *Spyrogyra* has largely evolved independently from the land plants (as any other CGA) and have a cell wall structure likely less related to land

plants than other CGA such as *Penium* (Sørensen et al., 2011; 2012). The use of this xyloclucan-like compound for cell adhesion may have emerged in adaptation to their habitat and a different cell adhesion mechanism, implicating pectins, may have emerged for land plant.

We still clearly don't know very much on the origins of cell adhesion in land plants. Further work on these CGAs would bring very interesting insights on this question. In the meantime, the role of pectins at the origins of the cell adhesion in plants remains the most likely and seems to be the most parsimonious when comparing the cell wall structure and compositions of most of the CGA and land plants.

### **5) What made pectins the main determinant of cell adhesion in land plants?**

The land plants represent a single clade in which all the members are multicellular and terrestrial (except for some members that have re-adapted to aquatic environments). As briefly described above, land plants have a large polysaccharidic cell wall surrounding their protoplasts and within this cell wall pectins are thought to have a major role in cell adhesion. If we look far back into evolution, the first step toward this form of cell adhesion is the acquisition of a chloroplast by a eukaryotic protist. The incorporation of a cyanobacteria by endosymbiosis, which later evolved into a chloroplast, marked the formation of the Plantae kingdom. And as a consequence, the cyanobacteria has provided by horizontal gene transfer the cellulose synthases genes (CesA; (Nobles and Brown, 2004)) and the galacturonosyltransferases (GAUT; (Yin et al., 2010)) as well as the capacity to photosynthesize and produce a large excess of energy and carbohydrates (Keeling, 2010). This made possible the synthesis of a large polysaccharidic cell wall (Niklas, 2004).

The second major step is likely the adaptation to freshwater. Plantae members that are adapted to marine environment such as the red algae and the chlorophytes, have cell walls made of cellulose, hemicelluloses and a sulfated polysaccharide matrix (Domozych et al., 2012; Popper et al., 2011). However, it is likely that the biochemical properties of a sulfated matrix are not adapted to the low ionic conditions of the freshwater, thus to adapt and conquer this new habitat, the ancestors of the streptophytes (CGA and land plants) have gotten rid of their sulfated matrix and have adopted an only carboxylic matrix made of pectins (Michel et al., 2010). Furthermore a sulfated matrix is probably not adapted to the terrestrial environment since the availability of sulfate in terrestrial habitat (and freshwater) is likely too low for the

synthesis of a sulfated polysaccharide matrix. This has likely prevented the direct colonization of land by marine organisms (Raven, 1997) . The colonization of freshwater has certainly provided a smoother transition and made possible in a subsequent stage the colonization of the terrestrial habitat. It is after this diversification to a freshwater-adapted cell wall with a pectic matrix that the land plants-like cell walls and cell adhesion are believed to have emerged(Becker and Marin, 2009; Sørensen et al., 2011).

From that point, cell adhesion mediated by a pectin rich cell wall and the onset of multicellularity has provided the support for complexification, tissue specialization and compartmentalization while still in freshwater. The increased number and diversification of the cell wall synthesis genes (Atmodjo et al., 2013; McCarthy et al., 2014) and the formation of an epidermis covered with a hydrophobic cuticle, to control gas exchange and limit water loss have allowed survival in a terrestrial environment. Finally the formation of vascular tissues for long distance transport of water, nutrient and metabolites (Lucas et al., 2013), have further adapted the plants to dryer and dryer environments to further colonize the terrestrial habitat (Popper et al., 2011). It is likely this scenario, largely simplified here, that led to the apparition of complex land plants. In this process, the acquisition of a chloroplast allowing the synthesis of a large cell wall and the adaptation to fresh water that switched the matrix to pectins, are certainly the two most decisive events that made land plant cell adhesion what it is today.

### **III) Set up and structural bases of the cell adhesion in plants**

#### **1) Ontogeny of the cell adhesion**

##### *Cytokinesis and new cell wall formation*

Cell adhesion starts right after cell division. It is the formation of a new cell wall between the two daughter cells that keeps the two cell attached. Thus the correct formation and sequential deposition of the polysaccharides in this developing cell wall is crucial for cell adhesion.

At the end of anaphase, a group of vesicles guided by the phragmoplast starts to align at the center of the dividing cell. These vesicles start to fuse forming what is called the tubulo vesicular network (TVN). As more and more vesicles are added to the sides, the forming cell plate extends centrifugally toward the edges of the cell and the center of the cell plate starts to

organize into a tubular network (TN) and later a planar fenestrated sheet (PFS). Finally the cell plate reaches the edges of the cell and fuses with the parental plasma membrane to separate the two daughter cells with the newly formed cell wall (Samuels et al., 1995; Seguí-Simarro et al., 2004; Verma, 2001).

During the formation of the cell plate, polysaccharides are synthesized and added to form the future cell wall. First, callose is synthesized in quantity and fills the cell plate at the TN transition (Samuels et al., 1995). This synthesis of callose is believed to provide mechanical support to the cell plate and to promote the transition to the PFS. The study of callose synthesis defective mutants as well as the effect of the inhibition of callose synthesis have revealed that callose was also crucial for later stages of cell plate maturation (Thiele et al., 2009; Chen and Kim, 2009; Park et al., 2014).

Hemicelluloses and pectins are then added to the cell plate (Moore and Staehelin, 1988) and pectins tends to accumulate in the middle of the cell plate compared to the callose which is more present against the plasma membrane of the cell plate and xyloglucans which seem to be more homogeneously distributed (Moore and Staehelin, 1988; Samuels et al., 1995).

Cellulose appears to be a minor component of the cell plate (Samuels et al., 1995) but a recent study indicates that its synthesis likely starts very early, at the TVN stage as revealed by the presence of the cellulose synthase at these early stages of the cell plate formation (Miart et al., 2014). Cellulose synthesis, along with callose may have a role in the mechanical support and the formation of the TVN (Zuo et al., 2000; Miart et al., 2014). Callose finally disappears during the cell plate maturation and fenestrae closure as it is replaced by the other polysaccharides.

The cell plate formation is also the place of formation of primary plasmodesmata. Their structure and the connexion they create between two adjacent cells could make them good candidates for maintaining cell to cell contact and thus adhesion. However to our knowledge no evidence has demonstrated a structural role of plasmodesmata for maintaining cell adhesion.

The precisely orchestrated sequential delivery and synthesis of polysaccharides creates a cell wall structured with three layers. The middle lamella largely enriched in pectins (Knox, 2008), and on both sides, the two daughter cells' cell walls that are independently synthesized by each daughter cells and are composed of cellulose, hemicelluloses and pectins. The formation of the middle lamella may in a way have an active role in keeping the cells attached together as it is really the junction between the two adjacent cells. Its specific enrichment in

pectins could account for specific adhesive properties. But in fact it seems to mostly provide a continuum of polysaccharides between the two cell walls that is not degraded right after cell division. However the interesting point about this structure is that the absence of cellulose and hemicelluloses render this junction much easier to degrade by specific pectin directed enzymes for later cell separation while allowing the two adjacent cell walls to keep their integrity (Roberts et al., 2002; Estornell et al., 2013).

### *Middle lamella deposition*

The middle lamella is deposited during the cell plate formation by the addition of vesicle-derived polysaccharides. Although this fact is known for more than two decades (Samuels et al., 1995; Moore and Staehelin, 1988), the precise provenance of these vesicles is still not clear. The Golgi apparatus is well known to be the place where hemicelluloses and pectins are synthesized (Driouich et al., 2012), and the formation of the cell plate by incoming vesicles has been demonstrated to largely depend on Golgi derived vesicles (Reichardt et al., 2007). So it has long been stated that the cell plate's polysaccharides were only directly derived from the Golgi apparatus. However, other studies have shown that endocytosis and recycling of proteins and cell wall polysaccharides were also implicated in the process (Baluška et al., 2006; Van Damme et al., 2008). Moore et al. (1988) have shown that xyloglucan and pectins (RGI) were present in the Golgi apparatus near the cell plate, however only the xyloglucans derived from these Golgi vesicles were shown to be later present at the cell plate. In addition Baluska et al. (Baluška et al., 2005; 2002) and Dhonuske et al. (Dhonushe et al., 2006) have demonstrated that pectins along with xyloglucans, could be internalized by endocytosis from the parental cell walls (Baluška et al., 2002) and that the recycled polysaccharides accumulated at the forming cell plate (Baluška et al., 2005; Dhonushe et al., 2006). Homogalacturonans with a low degree of esterification (see structural description of the pectins in part III) 2)) were found in the cell plate while this form of pectin is typical of a mature cell wall and not of usually newly deposited pectin.

Interestingly, the study of some mutants also seems to point out the implication of polysaccharide recycling, from the parental cell wall to the forming cell plate. GNOM/emb30 is a protein that has been shown to be implicated in the secretion of endocytosis-derived vesicles (recycling endosome via the trans Golgi network), including the secretion of PIN1 to the apical part of the cell and to the cell plate (Richter et al., 2010). Interestingly the

disruption of this gene impairs normal pectin localization in the cell wall, which is accompanied by a cell adhesion defect (Shevell et al., 2000; 1994). Although it could be an indirect effect and it would need more experimental evidences, this pectin localization and cell adhesion defect could be due to a defect of the GNOM-mediated mature pectin recycling and deposition at the cell plate. On the other hand, ECHIDNA, a trans-Golgi network localized protein (Gendre et al., 2011) was shown to be responsible for the secretion of Golgi-derived polysaccharides (Gendre et al., 2013). Although the disruption of this protein revealed a large defect in pectin secretion, to our knowledge it is not accompanied with a cell adhesion defect (McFarlane et al., 2013). Despite a potential functional redundancy, the absence of cell adhesion defect in *echidna* may indicate that the correct deposition of newly synthesized Golgi-derived pectins during cell plate formation is not crucial for cell adhesion while the deposition of endocytosis-recycled mature pectin is. However the absence of a cell adhesion defect in *echidna* should be more thoroughly checked and the existence of a functional redundancy of this function for cell plate formation is unknown. The role of endocytosis-recycled vs Golgi-derived pectin deposition during cell plate formation for cell adhesion should be further studied to give a clear answer.

#### *Middle lamella expansion*

The middle lamella is set up at cell division, but then during plant growth the cells usually undergo a dramatic anisotropic expansion. This growth is accompanied with a large production of polysaccharides: Cellulose is synthesized as microfibrils perpendicular to the growth direction to allow anisotropy while pectins and hemicelluloses are continuously deposited. However due to the width of the cell wall, the middle lamella is distant from the place of deposition of the polysaccharides, and the amount of pectin that is laid down at the middle lamella prior to cell expansion is likely insufficient to allow such a growth while still maintaining the integrity of the middle lamella and thus the cell adhesion. Interestingly a very recent work on the unicellular CGA *Penium* brought an interesting insight on how pectins could be continuously added to the middle lamella during cell expansion (Domozych et al., 2014). As described above (*The origins of cell adhesion in plants*, II.), the cell wall structure and composition of *Penium* have striking similarities with the cell wall of land plants. However it is a unicellular organism, which makes its cell wall directly accessible for observation. In addition, compared to the diffuse growth of plant cells, *Penium* cell expansion

takes place at a central isthmus zone. Thanks to these properties, it was shown that pectins are deposited at the plasma membrane, in the inner layer, and progressively migrates toward the outer layer during cell extension. During this migration the HGs are progressively de-methyl esterified so that they can form Ca<sup>2+</sup> mediated cross-links when they reach the outer layer. A similar process of secretion, de-methyl esterification and migration to the middle lamella could occur in plants, and explain how the middle lamella and thus cell adhesion is maintained during cell expansion.

Interestingly the complex regulation of cell wall deposition during cell plate formation and cell expansion indicates that cell adhesion does not simply happen by a very passive lack of cell wall degradation, but a specific layer is laid down and maintained to allow the maintenance of cell adhesion and even more importantly the potential for later controlled cell separation.

## **2) The role of cell wall structure and composition in cell adhesion**

### *Pectin cross-linking*

The pectins are the main constituents of the middle lamella. They are also the main gel-forming polysaccharide due to their hydrophilic and cross-linking properties. The pectins in fact designates a group of polysaccharide which is recognized as the most complex (Caffall and Mohnen, 2009; Atmodjo et al., 2013). It is composed of three main forms: 1) The homogalacturonan (HG) which is the simplest and most abundant form. It is a linear chain of galacturonic acids partially substituted with methyl ester groups and sometimes acetyl, xylose and apiose. 2) The rhamnogalacturonan I is a fraction of the pectins composed of an alternating rhamnose and galacturonic acid backbone, likely continuous to the homogalacturonan, and largely substituted with arabinan, galactan and arabinogalactan side chains on the rhamnose residues. 3) The rhamnogalacturonan II is an extremely complex and conserved domain based on an HG backbone substituted with four side chains comprising 12 different sugars (Bar-Peled et al., 2012). These three different forms of pectins are linked together by glycosidic linkages (Vincken et al., 2003). But the most interesting aspect of the pectins in regard to the maintenance of cell adhesion is their potential to be actively remodel after deposition at the cell wall and to form intermolecular cross-linking (Sénéchal et al.,



2014). These remodeling can usually radically change the physico-chemical properties of the pectins (Willats, 2001). The most studied is the non-covalent cross-linking of HG. HG is synthesized under a highly methyl esterified form (Atmodjo et al., 2013) and Pectin Methyl esterases (PMEs) can later remove the methyl ester groups leaving a negatively charged residue (Micheli, 2001; Pelloux et al., 2007). Depending on the pattern of de-methyl esterification this can have two opposite consequences (Willats, 2001). If de-methyl esterification happens in a block wise manner, and long enough stretches of de-methyl esterified galacturonic acids are present (>10) (Liners et al., 1989), two independent HG chains can cross-link via the formation of calcium bridges on the negatively charged galacturonic acids (Jarvis and Apperley, 1995) which in turn leads to a stiffening of the cell wall (Willats et al., 2001). However, if de-methyl esterification happens in a random manner this renders the HG sensitive to polygalacturonases (PGs) and pectate lyase (PLs), leading to a softening of the cell wall (Moustakas et al., 1991). Pectins are also well known to mediate cross-linking by the borate mediated dimerization of the RGII (O'Neill et al., 1996; 2001), however this dimerization takes place before secretion which seems to make it less relevant as a cross-linking related with cell to cell adhesion (Chormova et al., 2014). There are also some evidences for the existence of covalent ester cross-linking between HG chains as some of the HG can only be extracted under alkali condition (Jarvis, 2009).

Thus this very complex polysaccharide can be synthesized by two independent cells, deposited throughout the cell wall and could even migrate to the middle lamella and cross-link to form a gel-structure that maintains the cell adhesion between the two adjacent cells. It is the HG that is mostly present in the middle lamella and is largely recognized as the most important polysaccharide for cell adhesion. Among pectins, it is also the most suited polysaccharide for post deposition cross-linking. However it is also the cross-linking of all the components of the cell wall that creates the continuum between the cell that really holds the cells together.

#### *Other cross-linking*

Other cross-linking take place between the different polysaccharides and glycoproteins of the cell wall. One of the most studied is the non-covalent interaction between xyloglucans and cellulose (Levy et al., 1997; Scheller and Ulvskov, 2010). Pectins have been shown be covalently cross-linked with xyloglucan (Cumming et al., 2005; Marcus et al., 2008; Popper

and Fry, 2007) and recent evidences show that some xylan is covalently linked to RGI side chain and that this xylan can mediate non-covalent interaction with cellulose (personal comm. Ralet & North). A recent work identified the ARABINOXYLAN-PECTIN-ARABINOGALACTAN PROTEIN 1 (APAP1) which revealed the existence of pectin and hemicellulose covalently linked with an arabinogalactan protein (Tan et al., 2013). Another recent work revealed the non-covalent interaction of AGP31 with RGI galactan branches, polygalacturonic acid and itself (Hijazi et al., 2014). These interactions reveal that pectin, hemicellulose, cellulose and glycoproteins may all be intercross-linked in the cell wall.

The cell wall compositions and possibilities of cross-linking can greatly vary between different species or for example between monocots and dicots (Carpita, 1996), and the potential importance of the different type of cross-linking in regard to cell adhesion should greatly differ. For example monocots have a much lower pectin content (Vogel, 2008) and in some species at least, cell adhesion may be maintained in large part by arabinoxylan diferulic acid cross-linking (Ng et al., 1997). Despite the low pectins content, other species like rice still rely on the pectins for cell adhesion (Liu et al., 2014).

Additionally the developmental transition from primary to secondary cell wall marks the arrest of pectins synthesis and the onset of lignin synthesis. Very little is known about the consequences that it has on the presence of pectin, but it is likely that lignin largely replaces the pectins in the middle lamella and that lignin polymerization throughout the primary cell wall takes over the role of cell adhesion in a cell wall that in turns becomes static (Li and Chapple, 2010; Tobimatsu et al., 2013).

#### *Experimental insights on the polysaccharides responsible for cell adhesion*

The cross-links that exist in the cell wall are certainly not all equally implicated in maintaining cell adhesion. Experimental approaches to decipher which polysaccharide or cross-linking are the most important are based on two strategies: The specific dissolution or digestion of certain cell wall polymers in order to determine whether it affects cell adhesion or not. Or the forward genetic study of cell adhesion defective mutants and the structural changes in their cell wall that may be responsible for the loss of cell adhesion.

The first approach has revealed that the pectins, in most dicot species, could alone be responsible for most of the cell adhesion. Treatments with a chelating agent disrupting Ca<sup>2+</sup> HG cross-linking as well as borate RGII dimer, usually leads to cell separation, pointing out a primordial role of these cross-linking in cell adhesion (McCartney and Knox, 2002; Ehwald et

al., 2002). However adhesion is not always completely lost but in many cases the remaining linkages could also be attributed to the covalent ester cross-linking of the HG (McCartney and Knox, 2002; Jarvis, 2009). Enzymatic solubilization of HG by endo polygalacturonases also usually allows cells separation in dicots (Ramana and Taylor, 1994; Zhang et al., 2000), while the degradation of galactan side chains does not (Redgwell and Harker, 1995).

In most grass species and monocot cell separation does not occur consequently to these treatments. This observation correlates with the fact that the diferulic acid cross-linking of arabinoxylans is probably involved (Ng et al., 1997).

The study of cell adhesion defective mutants was also very informative on the implication of pectins but has mostly only been carried out on *Arabidopsis* or other dicots such as tomato and tobacco. A number of mutants have been shown to present specific defects in cell adhesion or separation. Most of them are defective in cell separation during developmentally regulated events and are either upstream receptor of an endogenous signal for triggering cell separation (Estornell et al., 2013), downstream regulators of the response such as transcription factors (Shi et al., 2011), or actual cell wall remodeling enzymes implicated in cell separation. Among these last ones are for example *quartet1, 2 and 3*, respectively mutated in a PME and two PGs, which disruption prevents the separation of the four pollen grains after meiosis, by indirectly (PME) or directly (PG) preventing the degradation of the HG between the pollen grains (Rhee et al., 2003; Francis et al., 2006; Ogawa et al., 2009). In all such cases, only mutants implicated in homogalacturonan modification or degradation have been identified, which would point out the HG as the only structural determinant of cell adhesion. However other approaches such as transcriptomic analyses of the abscission zones have revealed the potential implication of other enzymes acting on cellulose and/or xyloglucans as well (Cai and Lashbrook, 2008; Lashbrook and Cai, 2008; Meir et al., 2010; Estornell et al., 2013).

On the other hand, some mutants show a defect in cell adhesion when the cells should remain attached. The mutagenesis of haploid tobacco callus led to the identification of the Non-Organogenic callus with Loosely Attached Cells (nolac) H14 and H18. Nolac-H14 was shown to have an absence of arabinan side chains in the pectic fraction (Iwai et al., 2001). Nolac-H18 is mutated in npGUT1 a glucuronosyltransferase, implicated in RGII synthesis (Iwai et al., 2002). Tobacco plants expressing an antisense copy of this gene had crumbled shoots (revealing a cell adhesion defect) and largely reduced dimerization of the RGII. These observations reveal the importance of RGI and RGII pectin fraction in cell adhesion. The overexpressor of an endogenous polygalacturonase in apple tree lead to cell adhesion defect

and premature leaf shedding (Atkinson et al., 2002). Similarly the overexpression of the rice polygalacturonase subunit *osBURP16* led to the over activation of the polygalacturonase activity and defects in cell adhesion (Liu et al., 2014). The mutations in QUASIMODO1/GAUT8 and QUASOMODO2/TSD2/OSU1 genes, respectively a putative galacturonosyltransferase of the GT8 family of glycosyltransferases, and a putative pectin methyltransferase, lead in both cases to a 50% reduction in HG content in the cell wall and a clear cells detachments phenotype (Bouton et al., 2002; Mouille et al., 2007; Orfila et al., 2005; Leboeuf et al., 2005). Recently a new cell adhesion defective mutant was described. It is affected in FRIABLE1, a member of the family of putative O-fucosyltransferase (Neumetzler et al., 2012). Although the precise function of the gene could not be elucidated, the mutant presents various modifications in its cell wall structure and composition with one of the major features being a higher PME activity and a lower degree of pectin methyl esterification. *GNOM/emb30*, as previously described, is likely affected in pectin recycling, and shows an abnormal pectin pattern in the cell wall, which is associated with a cell adhesion defect (Shevell et al., 2000). However this mutant is affected in embryo development and does not develop into a proper seedling, which makes it difficult to compare with *qual*, *qua2* or *frb1* in *Arabidopsis*. The study of the different cell adhesion defective mutants however, seems to confirm the major implication of the pectins in cell adhesion

### *Cell wall spatial organisation*

As described above, in the context of the primary cell wall, the continuum of cross-linked polysaccharides can actually be subdivided into three layers: the two cell walls of the adjacent cells and the middle lamella. The middle lamella can be clearly distinguished by its higher density to electrons when observed under a transmission electron microscope (Orfila et al., 2001), by its composition as observed using confocal Raman microscopy (Gierlinger and Schwanninger, 2006; Zeng et al., 2010) and by immunolabeling with different antibodies recognizing cell wall epitopes (Orfila et al., 2001). These observations reveal that this layer is highly enriched in HG harboring a low degree of methyl esterification, and seems more or less depleted of most of the other polysaccharides (Orfila et al., 2001; Guillemin et al., 2005; Knox, 2008). An additional structural characteristic of the cell wall that is related with cell adhesion is the formation of intercellular spaces. Indeed, just after cell division, the cells present very sharp edges. But the growth of the plant relies on the building up of a very high

intracellular turgor pressure in order to drive the cell expansion. This high internal turgor pressure forces the cells to adopt a more spherical or cylindrical shape. This leads to the separation of the cells at these sharp edges, thus forming the intercellular spaces (Jarvis, 1998). While the middle lamella is important for cell adhesion, it is actually at the middle lamella that the cells separate here. Although very little is known about it, this release of the tension could be highly controlled. Indeed, in order to prevent complete cell separation, the cell wall is specifically reinforced at this tricellular junction. Immunolocalization studies have revealed that HG with a very low degree of esterification and with de-methyl esterified stretches longer than 34 consecutive galacturonic acid residues (PAM1 epitope; (Willats et al., 1999; Manfield et al., 2005)), as well as HG with a very low degree but a more random pattern of esterification (LM7 epitope; (Willats, 2001; Clausen et al., 2003)) were specifically present throughout this reinforcing zone. In addition, the corners of the intercellular spaces represent the limits of cell separation and in some species they can even be identified prior to cell separation (Kollöffel and Linssen, 1984). Secondary ion Mass Spectroscopy (SIMS) and Electron-energy-loss spectroscopic (EELS) imaging of calcium in the cell wall revealed that the calcium is mainly concentrated at these corners of the tricellular junction where cell separation is blocked (Huxham et al., 1999; RIHOUEY et al., 1995). Mathematical modeling seems to point out the necessity for a tight reinforcement at this tricellular junction in order to prevent complete cell separation (Jarvis, 1998). Overall, since this reinforcing zone likely provides a tight attachment between the adjacent cells, extending this observation at the tissue level seems to make this reinforcement one of the main actors in the maintenance of cell adhesion throughout the plant (Jarvis, 1998).

The actual composition and cross-links that are created in this reinforcing zone vary between species just like the rest of the cell wall. It results that there should not be a “universal” cell adhesion polysaccharide or cross-link. But rather the presence of a continuum of polysaccharides between the cells, supported by such reinforcing zones at the points of tension, whatever their composition or the cross-linking that are taking place (Jarvis, 1998; Jarvis et al., 2003).

## IV) What controls cell adhesion in plants

### 1) Developmentally regulated cell separation

#### *The different cell separation events*

The cell separation events are processes during which individual cells, groups of cells or even entire organs, partially or completely separate as a consequence of the dissolution of the middle lamella originally holding them together. Controlled cell separation happens at different times and places during plant development. The most common are:

- The abscission of the floral organs, leaves, fruit pedicels and seeds.
- The dehiscence of the seed pod and anther.
- The fruit ripening that involves cell separation as part of its maturation and softening process.
- The lateral root emergence.
- The border cells and border-like cells sloughing off.
- The intercellular space formation.

The most studied of these events are the abscission, dehiscence and fruit ripening since the understanding of these processes have potential applications for agricultural as well as ornamental purposes. In agriculture the control of pod shatter has probably been among the earliest traits selected for, since limiting seed shedding greatly simplifies the harvesting process (Paterson et al., 1995). Yield losses due to pod shatter are still considerable and the potential for its amelioration is a good example of one of the goals that drives the research on the cell separation events (Østergaard et al., 2006). There are many other examples in which research on cell separation processes should help find solutions for some of today's agriculture problems (Estornell et al., 2013) and for this reason the study of cell separation events has and still represents a very active field of research. A large body of knowledge starts to accumulate on the different mechanisms and pathways that are implicated in these different processes, including cell separation events that may not directly concern agriculture. These have been reviewed in details by (Patterson, 2001; Roberts et al., 2002; Lewis et al., 2006; Driouich et al., 2007; Péret et al., 2009; Seymour et al., 2013; Estornell et al., 2013; Nakano and Ito, 2013; Niederhuth et al., 2013) and others. Thus the purpose here is not to

describe all this knowledge in detail, but rather to give a condensed description and the common themes and mechanisms that are at play in cell separation. Also, although some precious information come from the study of cultivated species, the large majority of the recent advances come from studies carried out on *Arabidopsis thaliana*, and as a consequence most of the work reported here focuses on *Arabidopsis*. Thanks to this model species, the events and the molecular pathway that control floral organ abscission are starting to be resolved in great details and recent investigations tend to show that similar (homologous or analogous) pathways could be implicated in most of the other cell separation events (Aalen et al., 2013).

### *The common themes and mechanisms in developmentally controlled cell separation*

Most of the studies converge toward a chain of event including four main steps. 1) Definition of the future cell separation zone. 2) Activation of a signaling cascade for the initiation of cell separation with an hormonal control. 3) Cell wall loosening, dissolution of the middle lamella and mechanically induced cell separation. 4) Formation of a protective layer and activation of the defenses.

All these steps are not respected in all the separation events. Such as in the case of lateral root development for which there may not be a zone defined early in advance by transcription factors, but a direct initiation of the second step with a timing and localization defined by the lateral root primordia initiation (Aalen et al., 2013). Moreover the control of some cell separation events is still not understood at all. Probably the best example for this is the formation of intercellular spaces at the tricellular junction. We have a detailed anatomical description of their development (Roland, 1978; Jeffree and Yeoman 1983; Jeffree et al., 1986; Kollöffel and Linssen, 1984), but to our knowledge nothing is clear about what controls where it takes place, what pathways are implicated, how cell separation is mediated in the end and even if this is actually a tightly controlled mechanism or if it may only originate in large part from self organization of the cell wall and mechanical forces inherent to the internal turgor pressure necessary for plant growth (Jarvis, 1998).

For the other ones however (at least those for which we have enough information) they seem to follow this scheme quite well with some variation. Step 1) is basically the definition of a zone in which the cells will later have the potential to separate and slightly reorganize. These are the abscission and dehiscence zone, border and border-like cells, the fleshy fruit pericarp,... Most of these zones are defined by transcription factors involved in patterning

such as *INDEHISCENT*, *SHATTERPROOF1*, *BLADE ON PETIOLE1* and *2*, *SEEDSTICK*,... (Estornell et al., 2013).

Step 2) involves hormonal signaling and a somehow hormone-independent signaling cascade (Butenko et al., 2006). Ethylene and auxin have been shown to have the main roles in almost all the separation events. They are able to directly trigger the expression of some of the genes that are directly implicated in cell separation such as cell wall remodeling enzymes. But most importantly they are implicated in defining the timing of cell separation (Meir et al., 2010). Other hormones such as abscisic acid (ABA) may also play a role but to a lower extent (Ogawa et al., 2009). Ethylene and auxin control the expression of a small peptide called INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) (Niederhuth et al., 2013). This peptide is perceived by the redundant set of leucine rich repeat receptor like kinases (LRR-RLK) HAESA (HAE) and HAESA-LIKE2 (HSL2) (Cho et al., 2008; Stenvik et al., 2008). Their ligand-induced dimerization triggers a MAP Kinase cascade including MKK4 and 5 followed by MPK3 and 6 (Cho et al., 2008) which finally inhibits the expression of the class I knotted1-like homeobox transcription factor BREVIPEDICELLUS/ KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1 (BP/KNAT1). This in turns de-repress the related transcription factors KNAT2 and KNAT6 (Shi et al., 2011). The activation of these transcription factors then triggers the expression of cell wall remodeling enzymes and other genes implicated in step 3). Other actors have been identified such as the ADP-ribosylation factor GTPase-activating protein (ARF-GAP) NEVERSHED (Liljegren et al., 2009), the LRR-RLK EVERSHED (Leslie et al., 2010) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) (Lewis et al., 2010), and the membrane associated cytoplasmic receptor like kinase CASTAWAY (Burr et al., 2011), however their role is less clear but they should be implicated in repressing the activation of the signaling cascade by inhibiting HAE and HSL2 signaling (Liljegren, 2012). This cascade has only been deeply studied in the case of floral organ abscission, but recent work shows that the same cascade with the same molecular players is implicated in the cell separation for lateral root emergence and gene expression studies have revealed that it should also be implicated in mature silique dehiscence (Stenvik et al., 2008; Kumpf et al., 2013). Although investigation of the other cell separation events is required, this signaling cascade may actually be widely used for the induction of cell separation in plants (Aalen et al., 2013).

In step 3) the downstream targets of the signaling cascade, mostly cell wall remodeling enzymes, are expressed (Lashbrook and Cai, 2008; Cai and Lashbrook, 2008). Almost all the separation events involve the same type of enzymes. Usually expansins (EXP) and xyloglucan



endotransglucosydase/hyrolase (XTH) are expressed, promoting cell wall loosening which should have two effects: ease accessibility for PMEs, PGs and PLs to the middle lamella for its dissolution, and allow cell expansion to push the cells apart from each other and finally allow separation. Interestingly, although these enzymes are usually part of large gene families, a relatively small group of enzymes seem to be involved in the different separation events as revealed by genetic approaches. For example in the case of the PGs, QUARTET2 (QRT2) and QUARTET3 (QRT3) are required for pollen grain separation (Rhee et al., 2003; Ogawa et al., 2009), but QUARTET2, along with ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 and 2 (ADPG1 and ADPG2) are also involved in anther Dehiscence (Ogawa et al., 2009). ADPG1 and ADPG2 are also implicated in silique dehiscence, ADPG1 is implicated in seed abscission, and ADPG2, QRT2 and PGAZAT in floral organ abscission (Estornell et al., 2013). The implication of these enzymes in most of the cases have been identified by genetic approaches and interestingly, to our knowledge, only mutants defective in PG or PME (pectin degradation / middle lamella dissolution) have been reported to actually affect normal cell separation when mutated (Estornell et al., 2013). Finally in step 4), the “wound” that is left by cell separation, is healed by lignification, suberin deposition and expression of defense related genes to protect this potential point of pathogens intrusion and prevent water and nutrient loss (Sexton and Roberts, 1982). Although a lot remains to be understood, and a number of intermediate players are missing, the mechanisms and the pathways that control cell separation are now quite clear.

## **2) Maintenance and modulation of cell adhesion during growth and development**

Much less is known about how cells actually remain attached during plant growth and development. Indeed it is not such a trivial question, and it seems to face two major problems: The first problem is that the cell wall material deposited at the middle lamella during cell division may not be present at a quantity important enough to allow the large anisotropic cell expansion that cells undergo during growth without affecting the middle lamella to the point of probably losing cell adhesion. As described above (*middle lamella expansion*, III.) there is likely a mechanism of deposition of new pectic polysaccharides at the middle lamella. But the question remains as to what controls the synthesis and timing of deposition of this material. Probably an interesting mutant to study would be the mutant *echidna* (Gendre et al., 2013). In this mutant a large part of the polysaccharides are not deposited at the cell wall and the

mutant is very affected in cell elongation but not in cell adhesion (McFarlane et al., 2013). There may actually be a feedback mechanism limiting plant growth as long as polysaccharides are not correctly deposited to the cell wall in order to avoid problems including losing cell adhesion.

The second problem is that cell expansion as well as a many other developmental mechanisms, use cell wall remodeling enzymes, including XTH, EXP, PME, PG and PL that are also used in cell separation events. How does the cell control whether these enzymes will induce cell wall loosening for expansion or cell separation remains mysterious.

To our knowledge, only a few mutants in *Arabidopsis* actually show a dramatic cell adhesion defect phenotype that can be observed at the seedling level. These are *quasimodo1* (Bouton et al., 2002), *quasimodo2* (Mouille et al., 2007) and *friable1* (Neumetzler et al., 2012).

As described above (*Experimental insights on the polysaccharides responsible for cell adhesion*, III.) *quasimodo1* and *quasimodo2* mutations both affect putative HG synthesis enzymes. In addition another alleles of *qua2* was found in a genetic screen as *tumorous development2 (tsd2)* (Krupková et al., 2007), and another one as *oversensitive to sugar1 (osu1)* (Gao et al., 2008). The straightforward explanation for the cell adhesion defect of the *quasimodo* mutants is the potential disruption of the middle lamella due to the decreased HG synthesis and content. Differentiation leading to the tumorous development (*tsd2*) and metabolic stress revealed by the sucrose sensitivity (*osu1*) could originate from an impaired cell to cell communication, metabolite and signaling factors usually transmitted through the apoplasm or plasmodesmata. But the link between HG content and cell adhesion is not so clear and the decrease observed in *quasimodo* may not actually be directly responsible for the cell adhesion defect. Indeed other mutants are affected in HG content at a similar level and are not defective in cell adhesion, such as *pme3* (Guénin et al., 2011), *irx8* (Persson et al., 2007) and *PGXI* (Xiao et al., 2014). Additionally the other cell adhesion defective mutant *friable1* is not affected in HG content while its cell adhesion defect is strikingly similar to *quasimodo* (Neumetzler et al., 2012).

This mutant is affected in a putative o-fucosyltransferase and although the substrate of this putative glycosyl transferase has not been elucidated yet, its putative function is to transfer a fucose onto a protein. In the case of this mutant the cause of the cell adhesion defect remains mysterious but one of the main change observed is a lower degree of methyl esterification of the HG and higher PME activity.

We now begin to have a detailed knowledge of how cell separation is tightly controlled. Although the maintenance of cell adhesion seemed like a passive phenomenon, more and more evidences are starting to indicate that it is not, and that there is likely a tight control for the maintenance of cell adhesion. However we know very little about this point but future research on this aspect should start to resolve what dynamically maintain cell adhesion in plants.

## **V) Conclusion**

Cell adhesion in plants is generally seen as a very basic and static process. Cell adhesion is set up after cell division and the cells remain attached throughout the plants life, except for the few ones that undergo separation. The structural features of cell adhesion are starting to be well defined as well as the importance of the different polysaccharides forms and cross-links. The cell separation events are also starting to be very well understood on a molecular point of view. But probably two aspects that have been largely unexplored are the origins of plant cell adhesion, and the control of the maintenance of cell adhesion. Future research on these aspects promises to be very exciting and should greatly extend our understanding of cell adhesion in land plants.



## Part II:

### Problematic, experimental design and objectives of the thesis.

The focus of the research that is carried out in the laboratory that I integrated for my PhD consist in the study of the synthesis and remodeling of the primary cell wall of the plant and the role that it has in the growth and the development of the plant.

In the last couple of years, part of the research that has been carried out in this lab has focused on the characterization of two mutants affected in cell adhesion: *quasimodo1* and *quasimodo2*. As described in the first part of this chapter, in addition to the cell adhesion defect, these mutants have a reduced content of homogalacturonan (the main form of pectin) in their cell wall. The identification and the study of the genes affected in both of these mutants led to the conclusion that QUA1 is a putative galacturonosyltransferase of the GT8 family of glycosyltransferases (Bouton et al., 2002) and QUA2 a putative pectin methyltransferase (Mouille et al., 2007). Both mutants have a similar phenotype, and similarly affect HG content in the cell wall. In addition these types of enzyme are known to usually act in complex. These observations thus brought up a model in which QUA1 and QUA2 act in complex to synthesize highly or fully methyl esterified HG in the Golgi apparatus before deposition to the cell wall. The loss of cell adhesion was thus easily attributable to the defect in HG synthesis and the likely disruption of the middle lamella. Thus both establishing the function of these genes in pectin synthesis and confirming the importance of pectins in cell adhesion. However the *quasimodo* mutants are not only affected in cell adhesion. One allele of the gene *QUASIMODO2* was isolated in a genetic screen as *tumorous shoot development2* (Krupková et al., 2007) for developing an abnormal callus instead of the normal leaves and stem in certain conditions. Another allele of *QUASIMODO2* was identified in another genetic screen as *oversensitive to sugar1* (Gao et al., 2008). These pleiotropic phenotypes questioned us on the effect that the loss of cell adhesion or the deficiency in HG, could have on the plant's development and metabolism.

In addition, other mutants are deficient in HG content in their cell wall (Guénin et al., 2011; Persson et al., 2007; Xiao et al., 2014) but are not affected in cell adhesion, which questions the actual link between HG content and cell adhesion. The recent identification of *friable1* a mutant affected in cell adhesion, but not in HG content (Neumetzler et al., 2012), added even more consideration to the question.

Things may be more complicated than what the initial characterization of *QUA1* and *QUA2* predicted, and should implicate other molecular players responsible for the cell adhesion defect as well as for the other pleiotropic phenotypes.

Our main strategy to try to answer these questions was to use a classical but very powerful forward genetic approach. An EMS mutagenesis of the mutants *qual* and *qua2* was carried out and a screening for suppressor mutant was undertaken before I integrated the lab. The main part of my PhD has consisted in the identification of the causal mutation that was responsible for the suppression of the cell adhesion defect and sucrose sensitivity in a couple of the lines isolated from the screen. I then undertook the characterization of the function of one of these genes and set up experiments to try to understand its role in cell adhesion.

A second approach added during the course of my PhD was the realization of a chemical genomic suppressor screen. The purpose of the screen was the identification of small molecules that could restore either the cell adhesion defect, the sensitivity to sucrose or both phenotypes observed in the *quasimodo* mutants. This approach can be considered as complementary to the classical genetic approach since the small molecules can affect the function of genes that are not usually isolated in genetic screening due to functional redundancy or lethality. The goal then was to try to identify what were the protein targets of these small molecules and similarly to the genetic screen, identify their role in cell adhesion.

In conclusion, the general objective of my thesis was to identify the function of new molecular player implicated in cell adhesion and understand how affecting their function impact cell adhesion in plants.

## **Chapter II:** Genetic dissection of the cell adhesion mechanisms

---

**Preamble:**

As the main part of my PHD project I undertook the identification of the suppressor loci of the mutants *quasimodo1* and *quasimodo2* that were isolated from a genetic suppressor screen. The screening consisted in the isolation of suppressor lines that restored the sensitivity of the mutants to a high sucrose content in the medium, with the idea that these suppressor line would also have a restoration of the cell adhesion or not. I carried out the partial genetic mapping and sequencing of 7 of the suppressor lines and finally focused on two of these lines that were allelic. This second chapter focuses on the identification and characterization of *ESMERALDA1* (*ESMD*), a genetic suppressor locus of the mutants *quasimodo1* and *quasimodo2*.

In the first part of this chapter I describe the genetic suppressor screen, the identification of *ESMD1* and a genetic analysis of the different mutants affected in cell adhesion, which led us to the conclusion that the cell adhesion in plants should be under the control of a feedback from the state of the pectin in the cell wall. \*

In the second part I report a number of additional work that I have carried out in order to unravel the molecular function of *ESMD1*, as well as a number of experiments and phenotypic analyses of *quasimodo* and its suppressor that were performed in order to better understand the pleiotropic phenotypes of *quasimodo* and how the suppressor mutation can restore cell adhesion.

*\*The first part of this part of the chapter is written under the form of a publication and will be submitted for publication after minor changes and the addition of a complementary experiment.*



## Part I:

# Plant cell adhesion is under the control of putative O-fucosyltransferases

Stéphane Verger<sup>1,2</sup>, Salem Chabout<sup>1,2</sup> and Grégory Mouille<sup>1,2</sup> \*

<sup>1</sup> INRA, Institut Jean-Pierre Bourgin, UMR1318 Saclay Plant Sciences, 78026 Versailles, France

<sup>2</sup> AgroParisTech, Institut Jean-Pierre Bourgin, UMR1318 Saclay Plant Sciences, 78026 Versailles, France

\* Correspondence to: [gregory.mouille@versailles.inra.fr](mailto:gregory.mouille@versailles.inra.fr)

*To be submitted for publication*

## Summary

Cell to cell adhesion in plants is mediated by a polysaccharidic cell wall and the presence of a pectin-rich middle lamella (Jarvis et al., 2003). The deficiency in pectin synthesis was previously shown to lead to a loss of cell adhesion (Bouton et al., 2002; Mouille et al., 2007). However recent work indicated that the loss of cell adhesion could happen without a pectin synthesis deficiency (Neumetzler et al., 2012) and that the pectin deficiency did not always lead to a loss of cell adhesion (Persson et al., 2007; Xiao et al., 2014; Guénin et al., 2011). We carried out a genetic suppressor screen of the pectin deficient and cell adhesion defective mutants *quasimodo1* and *2*, and a genetic analysis of the different mutants affected in cell adhesion. Our results demonstrate that indeed the loss cell adhesion is not directly linked with a decreased pectin content in the cell wall and instead is correlated with an altered pectin-related signaling. Two of the mutants studied affect putative O-fucosyltransferase that may directly affect the function of pectin sensing receptor-like kinases. Our results suggest that the state of cell adhesion is under the control of a feedback loop positively or negatively

influencing cell adhesion. Such a mechanism could act in the modulation of the cell adhesion strength in order to ease the correct coordination of growth and development.

## Results

### **Forward genetic suppressor screen for the restoration of cell adhesion in the *quasimodo* mutants**

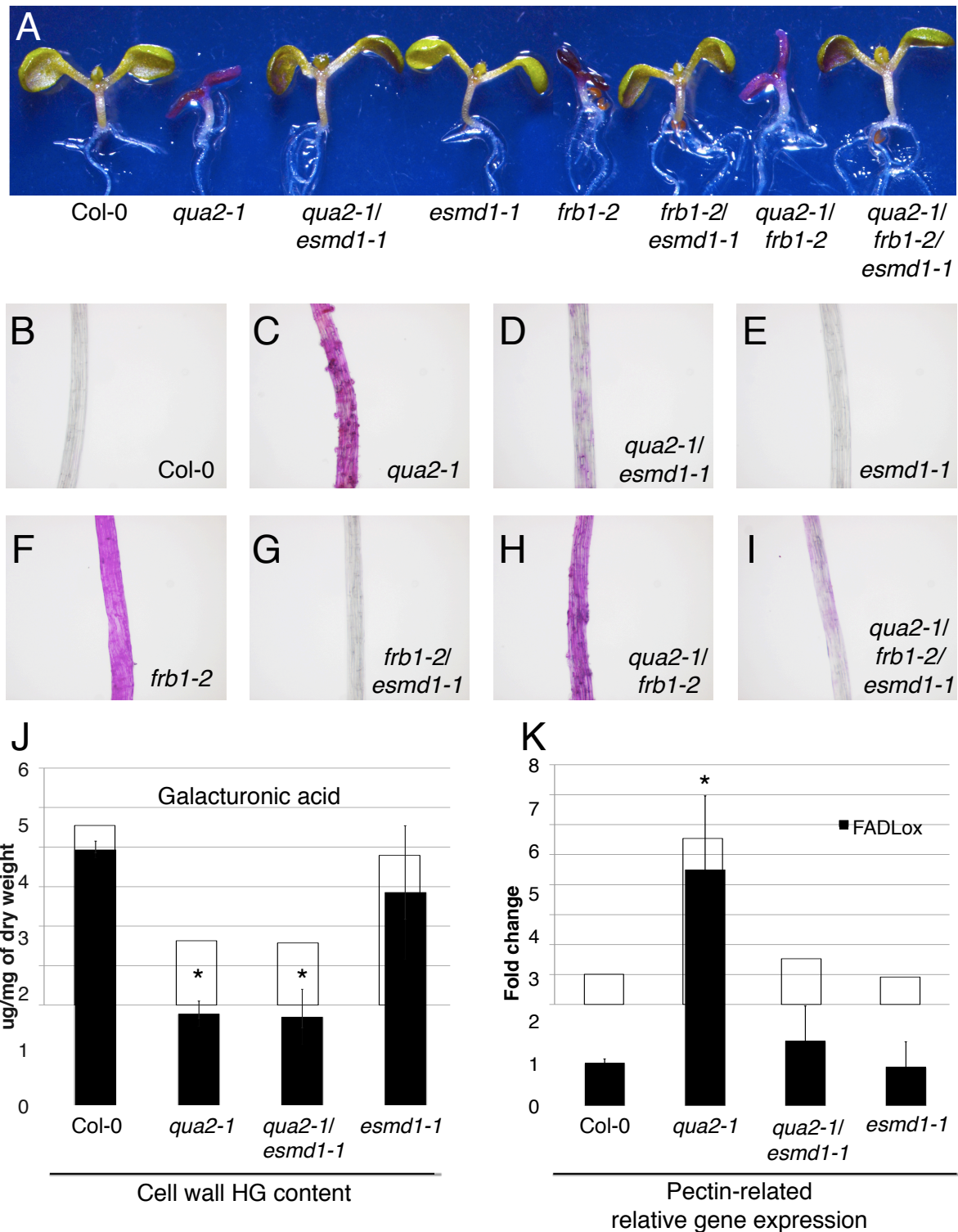
Compared to animals, cell adhesion in plants is usually described as a relatively passive phenomenon. A new cell wall is set during cytokinesis between two daughter cells, and from that point cell adhesion may be maintained thanks to the crosslinking of the polysaccharides forming a continuum between the two adjacent cells (Jarvis et al., 2003). Pectins, and particularly homogalacturonan (HG) may play an important role in cell adhesion since it is the main component of the middle lamella, the interface between the two adjacent cell's cell walls, and it's structure is particularly suited for cross linking (Willats, 2001). Mutations in *QUASIMODO1/GAUT8* and *QUASOMODO2/TSD2/OSUI* genes, respectively a putative galacturonosyltransferase of the GT8 family of glycosyltransferases ([www.cazy.org](http://www.cazy.org)), and a putative pectin methyltransferase, lead in both cases to a 50% reduction in HG content and a clear cells detachments phenotype (Bouton et al., 2002; Mouille et al., 2007). Additionally these two mutants are very sensitive to carbon/nitrogen imbalance (Bouton et al., 2002; Gao et al., 2008), and have a tendency to develop callus instead of normal leaves and stems (Krupková et al., 2007). But the link between HG content and cell adhesion is not so clear and the decrease observed in *quasimodo* may actually not be responsible for the cell adhesion defect directly. Indeed other mutants or overexpressor lines are affected in HG content at a similar level and are not defective in cell adhesion such as *pme3* (Guénin et al., 2011), *irx8* (Persson et al., 2007) and *PGX1* (Xiao et al., 2014). Additionally another cell adhesion defective mutant, *friable1* is not affected in HG content while its cell adhesion defect is strikingly similar to the one of *Quasimodo* (Neumetzler et al., 2012). In order to better understand what controls cell adhesion in plants and to identify new molecular players implicated in this process, we have carried out a forward genetic suppressor screen of the cell adhesion defective mutants *quasimodo*. *qua1-1* and *qua2-1* mutant lines exhibiting a similar phenotype of cell adhesion defect and a high sensitivity to sucrose were EMS mutagenized. As a primary screening, M2 seeds of both of these lines were grown on a high sucrose (3%)

medium and suppressor lines were screened for a restored growth and greening under these conditions (figure 1A, Col-0, *qua2-1*, *qua2-1/esmd1-1*). We then checked for restored cell adhesion among the different suppressor lines (figure 1 B, C, D), consistency of the phenotype at the M3 generation, and the presence of a single recessive suppressor locus. We have isolated a set of *esmeralda* mutants affecting different loci. Here we report the characterization of *ESMERALDA1*, one of the suppressor locus, and its implication, along with other previously described molecular players, in the control of cell adhesion.

### **ESMERALDA1 and FRIABLE1 are putative O-fucosyltransferases with antagonistic effects on cell adhesion**

A combination of genetic mapping and whole genome sequencing of the different suppressor lines allowed us to identify two independent alleles of a suppressor locus that we named *ESMERALDA1* (Figure S1 and S2). One of the alleles (*esmd1-2*) was found as a suppressor of *qua1-1* and the other one (*esmd1-1*) as a suppressor of *qua2-1*. *ESMERALDA1* belongs to a group of 39 arabidopsis proteins possessing a predicted O-fucosyltransferase domain (Pfam ID number PF10250) related to the GT65 family of glycosyltransferases (Hansen et al., 2009; 2012; Neumetzler et al., 2012; Wang et al., 2012). *In vivo* observation of a GFP tagged version of the *ESMD1* protein as well as colocalization experiments revealed a Golgi localization of the protein, consistent with a glycosyltransferase function (Figure S3). This is also consistent with other members of this putative O-fucosyltransferase family that have been experimentally demonstrated to localize to the Golgi apparatus (Parsons et al., 2012; Dunkley et al., 2004; 2006; Neumetzler et al., 2012; Wang et al., 2012).

*FRIABLE1* (At5g01100) is another member of the putative O-fucosyltransferase family. Interestingly, as mentioned above, the *friable1* mutant reveals a cell adhesion defect at the seedling level strikingly similar to that of *quasimodo* (Neumetzler et al., 2012). To our knowledge this is the only other *arabidopsis* mutant published to date, along with *qua1* and *qua2*, described with such a striking cell adhesion defect phenotype at the seedling level. We thus decided to test whether the defects observed in *frb1* and *quasimodo* were genetically related and if *esmd1* could also restore the *frb1* defects. Crosses were made to obtain the double and triple mutants (figure 1 A-I). *qua2-1*, *frb1-2* and *qua2-1/frb1-2* mutants show a clear cell adhesion defect as revealed by the Ruthenium red staining (figure 1 C, F and H) and a high sensitivity to sucrose compared to the wild type as revealed by the reduced growth and



**Figure 1: *qua2*, *frb1* and *esmd1* affect cell adhesion and pectin signaling in a same pathway and independently from HG content in the cell wall**

(A-I) Phenotype of *col-0*, *qua2-1*, *qua2-1/esmd1-1*, *esmd1-1*, *frb1-2*, *frb1-2/esmd1-1*, *qua2-1/frb1-2*, *qua2-1/frb1-2/esmd1-1* : (A) Light grown seedlings on a 0.2% agarose and 3% sucrose medium similar to the suppressor primary screening conditions and (B-I) Close up view of the dark grown hypocotyls stained with ruthenium red in order to reveal the cell adhesion defect. The principle of the Ruthenium red staining here is that the cell separation creates ruptures in the continuity of the hydrophobic cuticle, which directly exposes the cell wall and allows the entry and binding of the ruthenium red, thus highlighting the cell adhesion defect. *esmd1-1* suppresses the phenotype of *qua2-1*, *frb1-2* and the double mutant *qua2-1/frb1-2*, and *qua2-1/frb1-2* does not seem to show an additive phenotype compared to either *qua2-1* or *frb1-2* alone. (J) Galacturonic acid content

(constitutive monomer of homogalacturonan (HG)) measured on a HG enriched cell wall extracts from *col-0*, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1*. The HG deficiency of the *qua2-1* mutant is not restored by *esmd1-1*. (K) Basal expression levels of FADLox in *col-0*, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1* seedlings. All relative expression is expressed in fold change relative to *col-0*. These relative expression levels of FADLox indicate that there is a pectin related signal induced in *qua2-1* that is restored to a wild type level by the suppressor *esmd1-1*. The values shown in (J) and (K) are average and standard deviation of three biological replicates. \* indicates a significant difference (t-Test,  $p < 0.05$ ) with the *col-0* sample.

---

anthocyanin accumulation (figure 1 A). Interestingly the double mutant *qua2-1/frb1-2* (figure 1 H) does not seem to show an additive phenotype compared to each single mutant (figure 1 A, C and F). Although these observations are not quantitative, they seem to indicate that *qua2-1* and *frb1-2* are likely affected in the same pathway. The double mutants *qua2-1/esmd1-1*, *frb1-2/esmd1-1* and the triple mutant *qua2-1/frb1-2/esmd1-1* showed a clear restoration of the phenotype (figure 1 A, D, G and I), indicating that a mutation in the *ESMD1* locus is sufficient to prevent the apparition of the cell adhesion defect and sucrose sensitivity induced by a mutation in *QUA2*, *FRB1* and even in the double mutant.

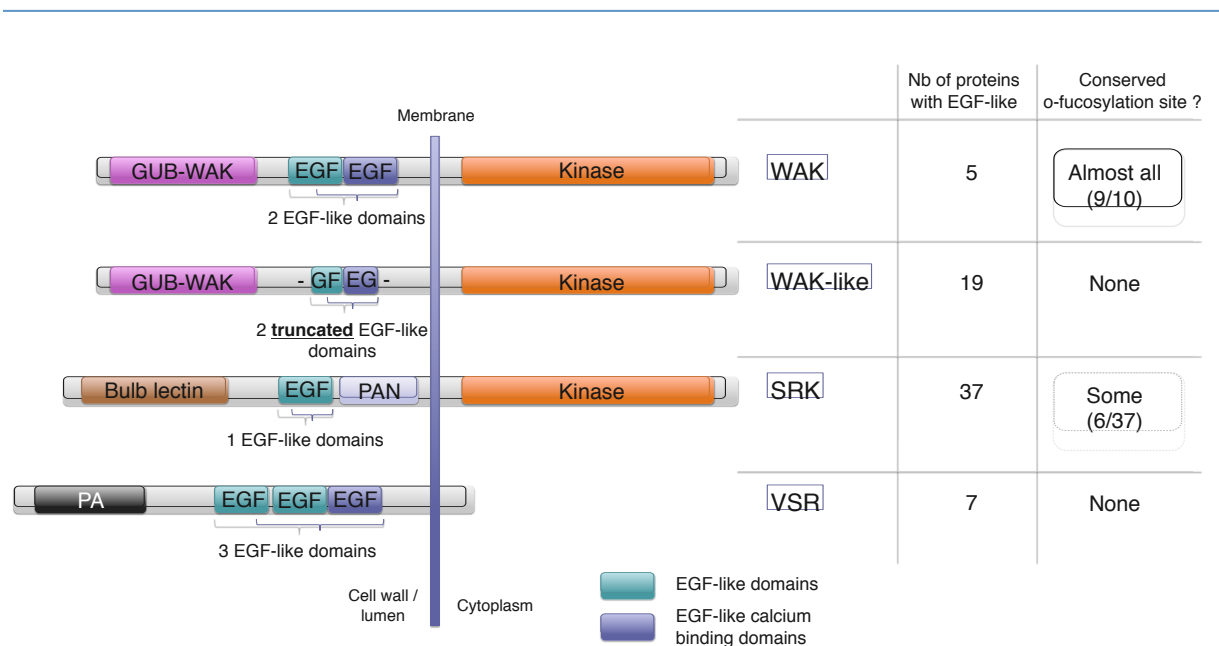
These results reveal an antagonistic effect of the mutation of these two putative O-fucosyltransferases on cell adhesion. They also reveal that *qua2*, *frb1*, *esmd1* and certainly *qual* affect cell adhesion in a same pathway.

### **Cell adhesion is uncoupled from HG content**

The *quasimodo* mutants are defective in HG content in their cell wall and this difference in quantity seemed to explain quite well the loss of cell adhesion. However it is not the case for the other cell adhesion defective mutant *frb1* as was previously demonstrated by Neumetzler et al (2012). We thus wanted to determine if the restoration of cell adhesion in the double mutant *qua2-1/esmd1-1* was accompanied with a restoration of HG quantity in the cell wall or not. Galacturonic acid content was measured in a HG enriched cell wall fraction from 5 days old dark grown hypocotyls of the different lines. This measurement revealed that the specific HG defect of *qua2* was not restored in the suppressor line (figure 1 J) indicating that the restoration of the cell adhesion is not due to a restoration of the HG content. Interestingly this result indicates that like in *frb1*, the decrease in HG content of the cell wall of *qua2* is not directly correlated with the loss of cell adhesion.

Overall *esmd1* and *frb1* mutants have an antagonistic effect on cell adhesion that is independent from the HG content in the cell wall. So the question is: What is the actual

function of these putative O-fucosyltransferases and how can we explain their role in cell adhesion?



**Figure 2: Potential substrates of ESMERALDA1 and FRIABLE1**

UniProtKB/Swiss-Prot database search reveal four classes of proteins harboring EGF-like domains: The Wall-Associated Kinases (WAK), the WAK-like, the S-domain RLK (SRK) and the Vacuolar Sorting Receptors (VSR). These EGF-like domains are the potential substrates of the putative O-fucosyltransferases ESMERALDA1 and FRIABLE1, however, only the WAKs and some of the SRK actually have the conserved site for O-fucosylation.

GUB-WAK: GalactUronic acid Binding domain - Wall-Associated Kinases; PA: Protease associated domain.

The drawing of each protein type/family is intended to be an average representative structure, but variations exist within families (see table S1). Also not all the proteins considered as part of these families had conserved EGF-like domains (e.g. there are 21 WAK-like but only 19 have EGF-like)

## ESMD1 and FRB1 putative function is to transfer fucose onto Epidermal Growth Factor-like repeats

O-fucosyltransferases in animals have been demonstrated to be responsible for adding a fucose to Epidermal Growth Factor (EGF)-like repeats (GT65, poFUT1) (Wang et al., 2001) or to ThromboSpondin type 1 Repeats (TSR) (GT68, poFUT2) (Luo et al., 2006). In a number of proteins containing these domains, the presence of this glycosylation is necessary for the correct function of the protein. It participates in protein-protein interaction and is the substrate for further glycosylation affecting the specificity of interaction with one protein partner or another (Takeuchi and Haltiwanger, 2014). These EGF and TSR domains have six

conserved cysteines that are required for disulfide bridges formation and provide the three dimensional structure to the domains. This structure is necessary for the glycosyltransferase recognition and fucose transfer (Lira-Navarrete et al., 2011). O-fucosylation happens on a serine or a threonine, and the conserved sequences found around the glycosylation site have been identified. For example in the case of the EGF-like repeats, the conserved sequence is C2-X(3-5)-S/T-C3, where C2 and C3 are the second and the third conserved cysteines of the domain, X can be any amino acid and O-fucosylation occurs on the conserved serine or threonine next to the third conserved cysteine (Shao and Haltiwanger, 2003; Takeuchi and Haltiwanger, 2014).

ESMD1 and FRB1 are predicted as putative O-fucosyltransferases for two reasons: They are part of the superfamily of fucosyltransferases (Hansen et al., 2009), which is characterized by the conservation of three peptide domains which seem to be relevant for the specificity of the protein for transferring fucose, and not another sugar (Martinez-Duncker et al., 2003) (figure S2). The second reason is that they have a conserved GDP-fucose protein O-fucosyltransferase signature (IPR019378; figure S2) that indicates that they should indeed glycosylate the same type of substrate as poFut1 or poFut2 (EGF, TSR, or other similar domains that could be found in plant proteins). ESMD1 and FRB1 are in a subgroup of the arabidopsis family of putative O-fucosyltransferases that is more related to the GT65 (Hansen et al., 2012; Wang et al., 2012) so they are more likely to be able to glycosylate EGF-like repeats.

Interestingly, while we were not able to find arabidopsis proteins containing TSR domains, we found a number of proteins containing EGF-like repeats (figure 2 and table S1). Based on the manually annotated and reviewed protein database UniProtKB/SwissProt ([www.uniprot.org](http://www.uniprot.org)), these domains seem to be only present on proteins belonging to the very large family of the Receptor-Like Kinases (RLK) and Receptor-Like Proteins (RLP). There are EGF-like repeats on the Wall-Associated Kinases (WAKs) (5 members with 2 domains each), the WAK-like (19 members with usually 2 truncated domain each, and 2 member without EGF-like domains), the G-type lectins S-domain RLK (SRK) (37 members with 1 domain each), and on the Vacuolar Sorting Receptors (VSR) (7 members with 3 domains each) (Figure 2). All these domains are located in the N-terminal extracellular part of the proteins, which makes them accessible to the membrane bound, and Golgi localized putative O-fucosyltransferases. In order to narrow down which of these proteins could be the substrate of our two glycosyltransferases we analyzed the different EGF-like sequences found on these proteins (figure 2 and file S1). Only a few of these domains actually have the conserved C2-

X(3-5)-S/T-C3 O-fucosylation site that is the potential substrate for the putative O-fucosyltransferases. Interestingly, along with some of the SRK, the 5 WAKs could be the substrates of ESMD1 and FRB1 since nine out of ten of these EGF-like domains present on the WAK1 to 5 have the conserved O-fucosylation site (figure 2). In addition the two EGF-like sites present on the WAKs are slightly different. The one on the N-terminal side is a classical EGF-like domain, while the one on the C-terminal side is annotated as a calcium binding EGF-like domain. This could correlate with two different enzymes to glycosylate the two different sites.

The WAKs are also particularly good candidates in our case since they are known to bind to the pectins (Wagner and Kohorn, 2001; Decreux and Messiaen, 2005; Decreux et al., 2006) and to transduce a signal upon treatment with small pectin fragments (Kohorn et al., 2006; 2009; Brutus et al., 2010). In addition an overexpressed dominant active WAK2 construct was shown to lose its activity when the construct was modified by a point mutation in a cysteine affecting one of its EGF-like domains (Kohorn et al., 2012). Finally, the absence of fucose in the plant was shown to affect the intracellular trafficking and secretion of a WAK1-GFP fusion protein at the plasma membrane in leaf mesophyll protoplasts (Kohorn, 2006).

### ***quasimodo* and *esmeralda1* mutations affect pectin related signaling**

Due to the atypical biochemical properties of the WAKs (tight linkage to the cell wall, as reported in He et al., 1996) we have not been able to characterize the glycosylation state of the WAKs yet, neither to determine whether ESMD1 and FRB1 were the O-fucosyltransferases of these domains. In addition, the five WAKs have been shown to be at least partially redundant in function since single mutants have barely any phenotype, while the knockdown of the five WAKs impairs growth (Wagner and Kohorn, 2001; Lally et al., 2001; Kohorn et al., 2009). The fact that they are arranged in tandem on the genome (He et al., 1996), makes it impossible to create multiple mutant lines and study by basic genetic approaches their implication in our case. However we wanted to test if the mutations in these genes could affect the signaling that is mediated by the WAKs. To determine this we analyzed the expression levels of a gene known to be responsive to pectins via a perception of a cell wall signal by the WAKs: *FAD-LINKED OXYDASE (FADLox)* (Denoux et al., 2008; Kohorn et al., 2014). We thus looked at the expression of this gene in our wild type, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1* lines (Figure 1 K). The results show that *FADLox* expression is



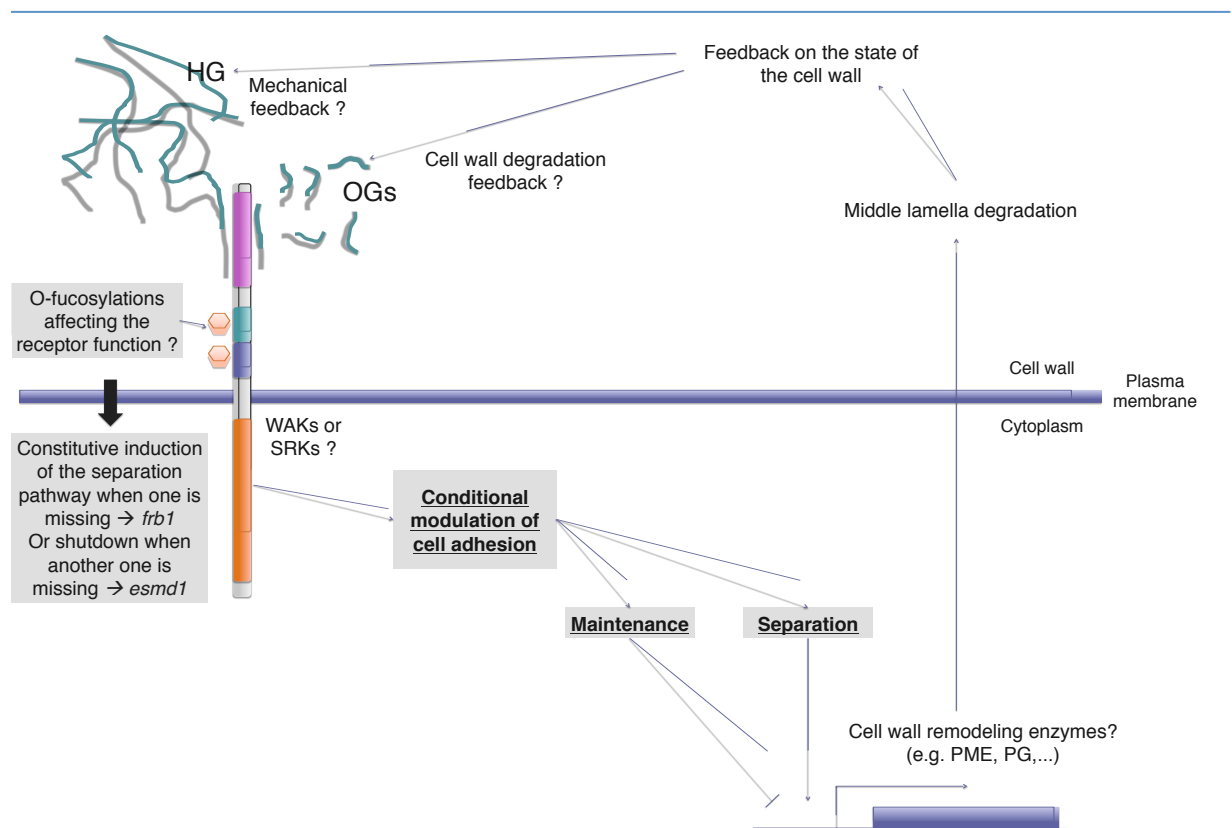
approximately 5 fold upregulated in *qua2-1* compared to the wild type, the suppressor line and *esmd1-1* (Figure 1 K). Interestingly it is a comparable level to what was observed in the dominant active WAK2cTAP overexpressor line reported in Kohorn et al. (2014). Similarly they showed that suppressor mutations of the phenotype of this overexpressor lines had a level of FADLox back to a wild type level. This result indicates that there is actually a constitutive pectin-related signal induced in *quasimodo* and that it is likely through the WAKs. The suppressor mutation in *ESMD1* also suppresses the induction of this signal, and *ESMD1* may directly affect the function of the WAKs. In such a case the absence of the glycosylation mediated by *ESMD1* on a WAK EGF-like domain, could affect the capacity of the WAKs to normally transduce the signal.

## Discussion

Our original understanding of the cell adhesion defect in *quasimodo* was that the decreased HG content in the cell wall was responsible for the disruption of the middle lamella which as a consequence led to the loss of cell adhesion. However mutation in *ESMD1*, a putative O-fucosyltransferase can restore the cell adhesion without restoring the HG content. Using a genetic analysis including *friable1*, a mutant defective in cell adhesion and also affected in a putative O-fucosyltransferase, we were able to demonstrate that the *esmd1* and *frb1* mutations induces an antagonistic effect on cell adhesion. It also indicates that *qua1*, *qua2*, *frb1* and *esmd1* affect cell adhesion via a same pathway that is independent from the HG content in the cell wall.

Investigation on the putative function of *ESMD1*, and *FRB1* led us to the hypothesis that they should be O-fucosyltransferase of the EGF-like domains that are present of the WAK receptor like kinases (RLK). Another hypothesis is that the SRK RLK could also be the substrate of this kind of putative O-fucosyltransferases since some of the SRK members also have a conserved O-fucosylation site. However the WAKs are better candidates in our case due to their implication in pectin perception. Although we have not been able yet to characterize the glycosylation state of the WAKs, neither to determine whether *ESMD1* and *FRB1* were the O-fucosyltransferases of these domains, we did show that a pectin stress related signal typical of what is perceived and transduced by the WAKs, was induced in *qua2* and restored by the suppressor *esmd1-1*. On this basis we speculate that it is the activation of a WAK-mediated

signaling pathway related to the pectin defect in *quasimodo* that in the end induces cell separation in the mutant.



**Figure 3: cell adhesion control feedback loop: A working model**

The state of cell adhesion is suggested to be under the control of a feedback loop positively or negatively influencing cell adhesion. Such a feedback mechanism could be mediated by the WAKs, sensing mechanical cues by their tight attachment to the HG, as well as perceiving the degradation state of the cell wall in the form of Oligogalacturonans. This perception may induce the expression of cell wall remodeling enzymes in order to degrade the middle lamella and lead to cell separation, or repress their expression in order to maintain cell adhesion. The consequence of these cell wall modifications can then be perceived by the WAKs thus closing the loop. In this model the function of ESMD1 and FRB1 at a molecular level remains to be fully understood, but their disruption affect the signaling of the WAKs (*frb1*) transducing a signal leading to cell separation or negatively (*esmd1*) preventing the induction of cell separation

Interestingly this situation is reminiscent of recent studies that have shown that some of the phenotypes primarily described in cell wall mutants were actually the result of cell wall integrity sensing leading to a disturbed growth and development (Hématy et al., 2007; Wolf et al., 2012b). For example the mutant *procuste1* that is affected in the cellulose synthase CesA6 and is deficient in cellulose shows a striking reduction of growth (Fagard et al., 2000). The decreased cellulose content in the cell wall seemed to explain very well a direct physical limitation of growth. However it was shown that a mutation in the *THESEUS1* receptor like kinase could suppress this growth defect, and that it was in fact the perception of the cell wall

state by this receptor that was inducing the inhibition of growth (Hématy et al., 2007). It demonstrated that the cellulose deficiency in the mutant was not the limiting factor and that in fact there was a true control and regulation of the growth by a cell wall integrity sensing mechanism. Our observations imply that similarly to the existence of an elongation control mechanism by the feedback of the cell wall state, there seems to be a cell adhesion control by the feedback of the pectin state. Such a feedback mechanism mediated by the WAKs, sensing mechanical cues by their tight attachment to the HG, as well as perceiving the degradation state of the cell wall under the form of Oligogalacturonans (small pectin fragments), could prevent cell separation when it is not wanted or trigger it when necessary (figure 3). In this model the function of ESMD1 and FRB1 at a molecular level remains to be fully understood, but their disruption should affect the signaling of the WAKs by inducing a signaling leading to cell separation (*frb1*) or by preventing the induction of cell separation (*esmd1*). The fact that in a *quasimodo* or *frb1* background there is an induction of cell separation, reveals the capacity of the plant to trigger cell separation throughout its tissues. We can assume that the decrease in HG content in *quasimodo* is very likely a situation that the plant (*Arabidopsis thaliana*) faces only in specific situations. This modification of the cell wall state could mimic a situation where such a cell separation mechanism is necessary. This mechanism could allow the modulation of cell adhesion strength between adjacent cells in order to ease the correct coordination of growth and development for morphogenesis.

## Acknowledgements

This work was supported by a PhD fellowship of the French Ministry of Higher Education and Research and the INRA. We thank T. Desprez for the general advices on the realization of the ESMD1:GFP construct and G.V. James (Max Planck Institute for Plant Breeding Research, Department for Plant Developmental Biology, Cologne, Germany) for the help and advices on the use of the SHORE and SHOREmap WGS analysis pipeline.

## Contributions

G.M. and S.V. conceived and designed the experiments. S.C. and S.V. performed most of the experiment with inputs from G.M.. S.V. and G.M. wrote the manuscript.

## Experimental procedures

### *Plant material and growth conditions*

*Arabidopsis thaliana* seedlings were grown at 20°C on solid custom made Duchefa (www.duchefa-biochemie.com/) medium with various agarose and sucrose concentration depending on the experiment. The seeds were cold-treated for 48 h to synchronize germination, and the plants were grown in a 16 h light/8 h dark cycle. For dark growth conditions, seeds were exposed to light for 4 h to induce germination, after which, the plates were wrapped in three layers of aluminum foil. Seedling age was counted starting from the light exposure.

The *qua1-1* (Bouton et al., 2002) and *frb1-2* (Neumetzler et al., 2012) T-DNA insertion lines were genotypes using the primers described in their reference publications. *qua2-1*, *esmd1-1* and *esmd1-2* EMS induced SNPs were genotyped by High Resolution Melt analysis (HRM) (Vossen et al., 2009) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) with a 0.2°C resolution for the melt curve acquisition. The PCR amplification was carried out prior to the fluorescence detection, with a classical thermocycler, PCR reaction mix and protocol, but supplemented with the Evagreen® intercalating fluorescent dye (Bio-Rad).

*qua2-1* H FW (5'-TGTACCTCTGGACGAAGCAA-3')

*qua2-1* H RV (5'-GCCCCGAAAAACATGTCTAC-3')

*esmd1-1* H FW (5'-TCTGTGTTTCAGAATTTCTCTTCCA-3')

*esmd1-1* H RV (5'-TGCGTGGTCACAAATACCTC-3')

*esmd1-2* H FW (5'-TTGCCTGAGTCAAACGGTTA-3')

*esmd1-2* H RV (5'-ATCAACAAACCCACCGATGT-3')

### *Mutagenesis and genetic suppressor screen*

Approximately 15.000 seeds of *quasimodo1-1* (Bouton et al., 2002) and 15.000 seeds of *quasimodo2-1* (Mouille et al., 2007) were EMS mutagenized (Kim et al., 2006). M1 plants were bulked in groups of 100-150 and M2 seed were screened on a low agarose (0.2%) and high sucrose (3%) medium. Clear suppressors of the *quasimodo* phenotype were transplanted to the greenhouse. Suppressor lines were further genotyped for the presence of the *quasimodo1-1* or *quasimodo2-1* mutation and progeny was tested for the consistency of the suppressor phenotype. Suppressor lines were backcrossed twice with either *qua1-1* or *qua2-1*

and F2 progeny's segregation of the suppressor phenotype was tested after the second backcross.

#### *Genetic mapping, whole genome sequencing and identification of *ESMD1* causal mutation*

Genetic mapping was carried out using an outcross population with an allelic *quasimodo* mutant of a diverging ecotype. *qual-1* (WS) suppressors were outcrossed with *qual-3* (col-0, EMS), and *qua2-1* (col-0) suppressors were outcrossed with *qua2-3* (WS, flag\_256B11). Populations of 96 F2 suppressors were used to map the localization of the suppressor loci. Whole genome sequencing was either carried out on homozygous non backcrossed lines or on a pool of F2 suppressors seedlings from a backcross population (with *qual-1* or *qua2-1* depending on the suppressor line) (James et al., 2013). The DNA of about 200 pooled F2 suppressor seedlings was extracted using a Cetyltrimethylammonium Bromide (CTAB) extraction. RNA was degraded using RNase and the DNA was further purified using the DNeasy plant mini kit (qiagen). Sequencing of the DNA was carried out at The Genome Analysis Centre (TGAC, Norwich, UK). We then proceeded to the analysis of the sequencing data using the SHORE (Ossowski et al., 2008) and SHOREmap (Schneeberger et al., 2009) pipelines for the identification of the SNPs, indels and the analysis of their frequency for the bulk segregant analysis. Mapping of the short reads on the reference genome of col-0 TAIR10 (<http://www.arabidopsis.org/>) was processed by the GenomeMapper algorithm. The background mutations from the *qual-1* or *qua2-1* line used for mutagenesis were removed from the analysis by subtracting the common mutations identified after sequencing of at least two independent suppressor lines. The SNP output was first filtered for EMS induced specific mutations (transitions) with a >8x coverage, >0.75 frequency and >25 SHORE score. The genetic mapping interval was thoroughly checked for potential mutations of interest filtered out, such as low coverage mutations, non EMS specific SNPs and indels. Relevant SNPs were further confirmed by sanger sequencing of an amplicon surrounding the SNP.

In an initial attempt to determine the locus of *ESMERALDA1* by genetic mapping we were able to localize the locus to a 485Kb zone at the top of chromosome 2 between the positions 208.000 and 693.000. Whole genome sequencing of pooled F2 suppressors from a *qua2-1*-based backcross population led to the identification of seventeen homozygous mutations at the top of chromosome 2, consistent with the classical mapping approach (figure S1). Only two of the identified mutations were within the restricted genetic mapping interval and only one was intragenic and non synonymous. The combination of both approaches led to the

identification of At2g01480, a putative O-fucosyltransferase, as the best candidate for the suppressor locus of *qua2-1* (figure S1). The mutation is a G to A transition at the position +1363 of the coding sequence resulting in an Alanine to Threonine change of the 455th amino acid of the protein.

Whole Genome sequencing of other suppressor lines from the *qua1-1* and *qua2-1* suppressor screen identified a second allele of this gene in a *qua1-1* suppressor line. Rough genetic mapping of this suppressor line indicated that the suppressor locus segregated with the top arm of chromosome 2, consistent with the mutation in *ESMD1*. The *esmd1-2* mutation is a G to A transition at the position +509 of the coding sequence resulting in an Arginine to Glutamine change of the 170th amino acid of the protein (figure S2).

A ConSeq analysis (Berezin et al., 2004) of the conserved structural and functional domains of the protein, based on the 13 closest related members of the putative O-fucosyltransferase family of *Arabidopsis thaliana*, as selected by the “homolog search algorithm” of the server and based on the full length amino acid sequence of *ESMD1*, indicated that both mutations found in *esmd1-1* and *esmd1-2* affect very conserved domains of the protein with respectively a predicted structural and functional role for the protein (figure S2). Additionally *esmd1-1* mutation falls within the third conserved peptide domain of the fucosyltransferase superfamily (Hansen et al., 2009; figure S2) and *esmd1-2* mutation is adjacent to a conserved signature motif of the O-fucosyltransferase family (PF10250) (Hansen et al., 2012; figure S2). Both mutations are thus very likely to affect the structure or catalytic activity of the protein, confirming the implication of these mutations in at2g01480 as the causal mutations.

#### *Generation of p35S::ESMD1:GFP, N.Benthamiana leaf infiltration and confocal microscopy.*

Constructions were made following the gateway strategy (Invitrogen, <http://www.invitrogen.com>) as described in the *gateway technology* instruction manual from the Gateway cloning kit. Full length genomic sequence from the translation initiation site to codon just before the stop codon of *ESMD1* was PCR amplified from col-0 using attB flanked primers and the Phusion High-fidelity DNA polymerase (Thermo Fischer Scientific; Cat. No. F-530S).

ESMD1attB1 (5'-  
GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGCTAGCGAAGAATCGG-3')

ESMD1attB2

(5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTTGGTGGCAGGAGGTGGTCTC-3')

The PCR product was first recombined into the pDONR207 donor vector and later in the pGWB5 destination vector placing the gene under the CMVp35S promoter and in frame with a C-terminal GFP. After each steps the constructs were transformed into *E. coli* for amplification and the sequences were verified. Finally they were transformed into the C58 strain of *A. tumefaciens* for transient expression in *N. benthamiana* as described in (Voinnet et al., 2003). Co-infiltrations were made with a MannosidaseI:mCherry construct (Nelson et al., 2007), and GFP and mRFP fluorescence was observed with a confocal microscope after 48h. Images were collected using a spectral Leica SP2 AOBS confocal microscope (Leica Microsystems, <http://www.leica-microsystems.com>) equipped with an argon laser. For GFP and mCherry constructs, excitation was performed using the argon ion laser at 488 nm, and fluorescence was detected at 495–540 nm (GFP) and 560–620 nm (mCherry).

#### *Galacturonic acid quantification*

Four biological replicates of about 350 dark grown hypocotyls per genotypes were dissected to remove the root and cotyledons in order to obtain about 1 mg of dry weight of hypocotyl. They were incubated in 100% ethanol at 100°C for 10 minutes twice and then incubated in 100% acetone for 5 min at room temperature and air dried for 24h. Dry weight was measured and samples were ground in acetone and air dried. Samples were treated with 0.1 M NaOH at 4°C overnight. After centrifugation for 10 minutes at max speed the supernatant was discarded and the pellet was washed twice with water. Pectins were extracted with 0.5% ammonium oxalate at room temperature for 2 hours. Uronic acid content was measured as described in (Blumenkrantz and Asboe-Hansen, 1973).

#### *RNA extraction, cDNA synthesis and qPCR quantification of gene expression*

Seedlings were light grown in vitro without sucrose. After 8 days the seedlings were flash frozen in liquid nitrogen. Three biological replicates were collected. Total RNA isolation was carried out with the RNeasy plant mini kit (qiagen) according to the manufacturer's instruction and with the on column DNA digestion using Rnase-Free DNase (qiagen). cDNA was synthesized using 1 µg of total RNA and the SuperScript® II Reverse Transcriptase

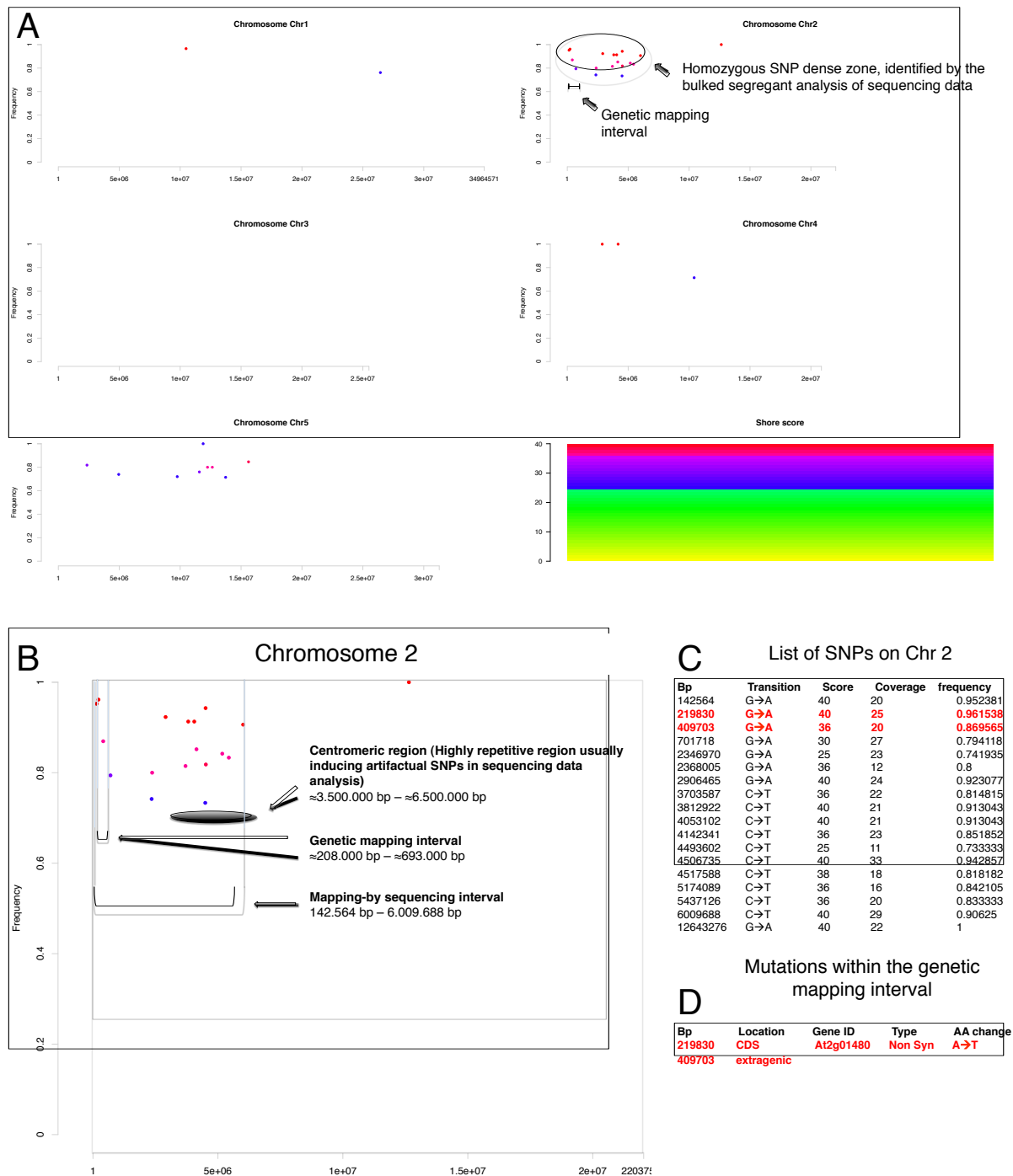
(Invitrogen) according to the manufacturer's instructions. Real time PCR reactions were performed in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using the SsoAdvanced™ Universal SYBR® Green Supermix, 25-100 ng cDNA template and 250 nM of each gene-specific primer in a final reaction volume of 15 µL. The primers used for FADLox (At1g26380) are those described in (Denoux et al., 2008). GADPH (At1g13440) and UBQ10 (At4g05320) were used as reference genes with the primers described in (Czechowski et al., 2005). Each sample reaction was run in three technical replicates. The Ct values of the tested genes were normalized to the reference genes and the reference-normalized samples were then normalized to the col-0 samples and the fold change in expression level were calculated. Relative expression of the RT-PCR products was determined using the  $\Delta\Delta\text{Ct}$  method (Livak and Schmittgen, 2001).

#### *Plants EGF sequences analysis*

*Arabidopsis thaliana* proteins containing EGF-like repeats were identified from the UniProtKB/SwissProt manually annotated and reviewed protein database ([www.uniprot.org](http://www.uniprot.org)). The sequences annotated as EGF were retrieved and their structure were analyzed based on cysteine and O-fucosylation site conservation to determine whether they were the potential substrates for our O-fucosyltransferases.







**Figure S1: Mapping and sequencing strategy, and the identification of *esmd1-1***

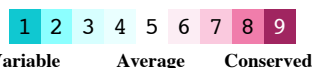
The graphs and annotations are the data obtained for the mapping and sequencing of a bulk of the segregant F2 suppressors of the *qua2-1/esmd1-1* line. The graphs are SHOREmap outputs (schneeberger et al., xxx) with added annotations relative to the genetic mapping done in parallel. (A) shows all the EMS canonical SNPs filtered for a >8x coverage, >0.75 frequency and >25 SHORE score. Each dot represents one mutation. The Shore score gives an idea of the quality of the sequenced reads on which the SNP call is based (25-40 are good to very good quality). The five graphs represent the five chromosomes of *Arabidopsis thaliana*, with on the x axis the position on the chromosome from the top to the bottom, and on the y axis the frequency of the mutation. The coverage is of an average of 28x. SNPs with a frequency of 1 (or almost 1) are likely linked with the suppressor loci. Thus the “cloud” of near-homozygous SNPs at the top of chromosome 2 defines the mapping-by-sequencing interval. The genetic mapping interval colocalizes with the mapping-by-sequencing interval. (B) is a close up on chromosome 2 with both genetic mapping and mapping-by-sequencing intervals. The mapping

by sequencing interval is much less restrictive and contains the large centromeric region of chromosome 2. Centromeric regions contain highly repetitive sequences which tend to induce errors in the mapping of the short sequenced reads on the reference genome. This usually leads to a number of artifactual SNP called by the sequencing data analysis pipeline. (C) Eighteen mutations were identified on chromosome 2: Seventeen mutations that we consider forming the mapping-by-sequencing interval (142.564 to 6.009.688) and two of them are within the genetic mapping interval. Bp: Localisation of the mutation on the chromosome, based on the TAIR10 reference sequence, and counted in base pairs from the top of the chromosome. Transition: nucleotide change induced by the mutation. Score: Shore score as described above. Coverage: Number of reads that were sequenced that cover this base, and on which the SNP call is based. Frequency: Frequency of those sequenced read that have the mutation. (D) Only one of the two mutations affect a gene and it creates an amino acid change of an Alanine to Threonine at the 455th amino acid of the protein sequence of At2g01480. Location: Type of sequence surrounding the mutation (CDS: Coding sequence). Gene ID: atg ID of the gene that is affected. Type: Synonymous vs Nonsynonymous. AAchange: Consequent amino acid change in the protein sequence.

## ConSurf Results



The conservation scale:



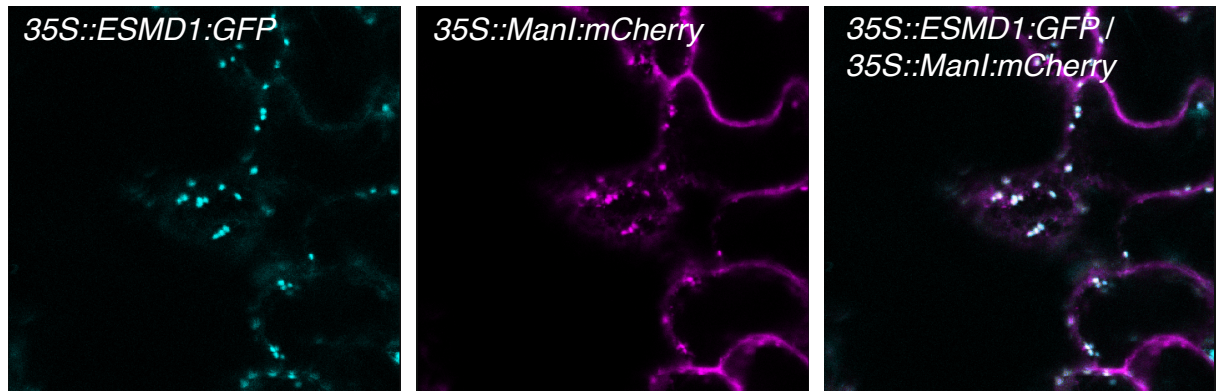
- Fucosyltransferase superfamily conserved peptide I, II and III
- GDP-fucose protein O-fucosyltransferase signature (IPR019378)
- ★ Amino acid affected by the mutant allele

- e - An exposed residue according to the neural-network algorithm.
- b - A buried residue according to the neural-network algorithm.
- f - A predicted functional residue (highly conserved and exposed).
- s - A predicted structural residue (highly conserved and buried).
- x - Insufficient data - the calculation for this site was performed on less than 10% of the sequences.

**Figure S2: *ESMD1* mutant alleles and the structural and functional domains conservation**

ConSeq analysis (Berezin et al., 2004) of the conserved structural and functional domains of the protein, based on the 13 closest related members of the putative O-fucosyltransferase family of *arabidopsis thaliana*, as selected by the “homolog search algorithm” of the server and based on the full length amino acid sequence of *ESMD1*. Color scale for the highlighted amino acids represent their degree of conservation among the 13 related proteins. The letter below is a prediction of whether the amino acid is buried (b) within the 3D structure of the protein, or exposed (e), and when conserved, whether the amino acid may have a functional (f) or structural (s) role for the protein. The frames around successive amino acids show the conserved motifs of signature relevant for the predicted function of the protein. The red are for the GDP-fucose protein O-fucosyltransferase signature (IPR019378) (Hansen et al., 2012). And the blue are the peptides domains I, II and III characteristic of the fucosyltransferase superfamily (hansen et al., 2009). The red stars mark the two alleles *esmd1-1* and *esmd1-2* position and the amino acid change.

---



**Figure S3: *ESMD1:GFP* fusion protein accumulates in the Golgi apparatus**

Imaging of *ESMD1:GFP* (cyan) and the Golgi marker mannosidase I–*mCherry* (magenta, Nelson et al., 2007), co-infiltrated and transiently expressed in epidermal cells of *N. benthamiana*. *ESMD1:GFP* and our Golgi marker colocalize, indicating a Golgi localization of *ESMD1*.



## Part II:

### Additional work on **ESMERALDA1** and **FRIABLE1** function

Stéphane Verger<sup>1,2</sup>, Salem Chabout<sup>1,2</sup>, Zhenni Li<sup>1,2</sup>, Cécile Labrune<sup>3</sup> and Grégory Mouille<sup>1,2</sup>

<sup>1</sup> INRA, Institut Jean-Pierre Bourgin, UMR1318 Saclay Plant Sciences, 78026 Versailles, France

<sup>2</sup> AgroParisTech, Institut Jean-Pierre Bourgin, UMR1318 Saclay Plant Sciences, 78026 Versailles, France

<sup>3</sup> Unité de Recherche en Génomique Végétale (URGV), Plateforme Transcriptome, UMR INRA 1165 - Université d'Evry Val d'Essonne - ERL CNRS 8196, 91057 Evry, France

#### I. **ESMERALDA1** and **FRIABLE1** are putative **O-fucosyltransferases**

After the identification of the *esmeralda1* locus as a putative O-fucosyltransferase, I undertook a number of experiments to try to demonstrate the catalytic activity of the protein and its homolog FRIABLE1. I also tried to determine whether the WAKs were actually O-fucosylated on their EGF-like domains and whether they were the substrate of the two putative O-fucosyltransferases. Setting up and performing these experiments have taken a considerable amount of time, and although I did not succeed to demonstrate the catalytic function and identify the substrate of these proteins during the course of my PhD, this work has generated useful biological material and negative results. Hopefully this work will soon yield clear answers on the catalytic function and substrates of ESMD1 and FRB1.

##### 1. Are **ESMD1** and **FRB1** actual **O-fucosyltransferases**?

In order to determine the catalytic function of ESMD1 and FRB1 we chose to produce and purify the recombinant proteins to test their activity *in vitro*.

Neumetzler et al (2012) had produced a full length version of FRIABLE1 from insect cells and did incorporation assays on membrane protein preparations with UDP-Glc, UDP-Gal,

UDP-GlcA, UDP-GalA, UDP-GlcNac, GDP-Man and GDP-Fuc, but did not observe incorporation. Lira-Navarrete et al. (2011) had produced a soluble truncated version of the *Caenorhabditis elegans* POFUT1 (Animal O-fucosyltransferase) in *Pichia pastoris* purified it and assayed its hydrolase and glycosyltransferase activity. They were able to show that this enzyme transfers fucose from GDP-fucose, onto EGF-like domains.

I thus constructed plasmids containing a truncated (without the transmembrane domain) tagged version of ESMD1 and another of FRB1. Codon optimized sequences for the expression in *Pichia pastoris* were ordered from life technologies (<http://www.lifetechnologies.com/>) and inserted into a *Pichia pastoris* recombinant protein expression vector with a N-terminal secretion factor and a C-terminal polyhistidine and c-myc tag (see III. Experimental procedure).

We started a collaboration with Ramón Hurtado Guerrero's lab (that performed the work described in Lira-Navarrete et al. (2011) on the catalytic activity of cePOFUT1) and sent them the two *Escherichia coli* clones containing the verified plasmids as well as the extracted plasmids for the ESMD1 and FRB1 constructs. They carried out the transformation of *Pichia pastoris*, the selection of transformant strains and assayed the secretion of the recombinant protein in the medium for 5 to 10 transformant strains per constructs. However they did not obtain strains secreting the soluble protein.

The proteins may either be produced in a too small quantity, may not be secreted in the medium or may be aggregated in inclusion bodies. We will test whether the protein is produced in small quantities by determining its presence or not using a western blot analysis with either a polyhistidine or c-myc directed antibodies, and also check the presence of inclusion bodies. Based on these information we will determine which strategy to adopt for future work on this.

## **2. Are the WAKs the substrates of ESMD1 and FRB1?**

Our objective was to overexpress the different WAK isoforms (WAK1 to 5) in different *Arabidopsis thaliana* mutants backgrounds. This, in order to extract and purify the overexpressed WAKs and determine by mass spectrometry whether or not the WAKs are O-fucosylated on their EGF-like domain, and whether or not ESMD1 and FRB1 are the protein



mediating this glycosylation. Finally, if it is indeed glycosylated, the objective is to try to characterize the glycan(s).

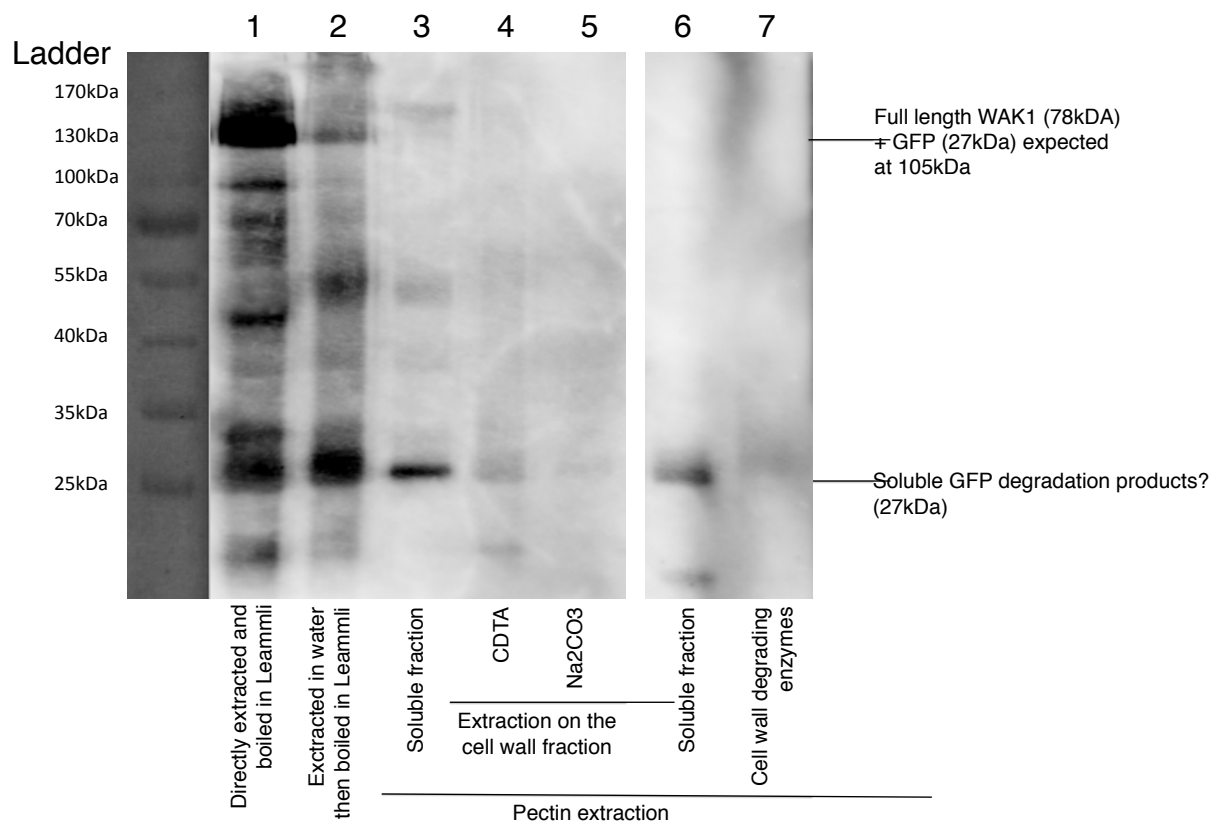
*Overexpression, extraction and purification of the WAKs in different genetic background*

Gateway constructs containing the full length genomic sequence with a C-terminal GFP tag fusion and placed behind a 35S promoter (35S::WAK:GFP) were made for the WAK1 to 4 (I did not succeed to amplify the full length version of WAK5). Transformation was done on the wild type col-0 and the mutant lines *qua2-1/esmd1-1*, *frb1-2* and *mur1-1* (mutant deficient in GDP-fucose synthesis). The idea being that after extraction and purification, we could determine by mass spectrometry the presence or absence of glycosylation in the different genetic background. We would expect no O-fucosylation in the *mur1-1* mutant due to the lack of fucose. Potentially two O-fucosylations, one on each EGF-like domains in the wild type. And the absence of one O-fucosylation or the other (in the case of two O-fucosylations), in *qua2-1/esmd1-1* and *frb1-2*. This could have demonstrated the existence of O-fucosylation in plants, the presence of these O-fucosylations on the WAKs' EGF-like domains and it would have allowed us to genetically and biochemically demonstrate that ESMD1 and FRB1 are O-fucosyltransferases of the WAKs' EGF-like domains.

However, two major problems seem to have prevented us from demonstrating this by this approach:

- The overexpression of the WAKs is relatively low: 4 to 6 transformant lines were tested per construction and genetic background. Specific WAK:GFP bands were detectable in western blot with analysis using an anti GFP antibody (as in figure 1, 2 or 3 for example in a Col-0 background). However, no additional band corresponding to WAK overexpression was detectable on coomassie blue stained SDS-PAGE gels (data not shown). This may reveal a relatively low overexpression, a high degradation rate, or a low extractability. The problem of the extractability is addressed below. The potential degradation of the protein during the extraction process and handling of the protein extract could be a problem. A relatively high amount of WAK:GFP digestion product was consistently observed (figure 1, 2 and 3). These degradation products are at about 25 kDa, 35 kDa, and some higher molecular weight bands, while WAK:GFP fusion protein should be around 105 kDa. As shown in figure 1 the degradation of the high molecular weight product and the accumulation of an approximately

25 kDa form is considerable when the sample is first ground in water with no particular precautions (figure 1 lane 1 and 2). For this reason the subsequent experiments were performed on ice, from the tissue grinding to the extract denaturation, and the extraction buffer was supplemented with an anti-proteases cocktail. But even when the sample was directly flash frozen at harvesting, ground in Laemmli and directly boiled for ten minutes there was still a considerable amount of degradation product (figure 1 lane 1). This observation suggested that at least some of the proteins were likely degraded in the plant prior to extraction.



**Figure 1: *WAK:GFP* construct expression and extraction**

Western Blot of different extracts for *35S::WAK1:GFP* transformed plants.

In lane 1 the leaf samples were directly ground in Laemmli and boiled for 10 minutes, while in lane 2 they were first ground in water and then boiled in Laemmli for 10 minutes. The difference in GFP fusion protein profile reveals a degradation of the protein in the latter situation. In lane 3, 4 and 5 the leaf samples were first ground in a simple Tris-HCl extraction buffer containing an anti-protease cocktail. A first soluble fraction was obtained after centrifugation of the extract at 10,000g for 10 minutes. Then the pellet was treated with 0.05M CDTA for 2h at room temperature, and the extract was centrifuged at 10,000g for 10 minutes to obtain the second fraction. Finally the pellet was treated with 0.05M Na<sub>2</sub>CO<sub>3</sub> for 14h at room temperature, and the extract was centrifuged at 10,000g for 10 minutes to obtain the third fraction. In lane 6 and 7 leaf samples were first ground in a simple Tris-HCl extraction buffer containing an anti-protease cocktail. A first soluble fraction was obtained after centrifugation of the extract at 10,000g for 10 minutes. Then the pellet was treated with the protoplast cell wall degradation cocktail for 16h at room temperature and the extract was centrifuged at 10,000g for 10 minutes to obtain the second fraction. Finally the samples that were not directly ground in Laemmli were supplemented with 1 volume of 2X Laemmli, and all the samples were boiled for 10 minutes before gel migration. The membrane was probed with an anti-GFP antibody and reveal no detectable full length WAK fusion protein in the soluble or cell wall extracted fractions.

It is likely that the plant does not tolerate high overexpression of the WAKs, and only transformant with low overexpression of the WAKs or that degrade the surplus of WAK can survive.

- The full length WAKs are tightly bound to the cell wall: Indeed as previously reported (He et al., 1996) only boiling in 4% SDS and 50mM DTT can efficiently extract the protein from the cell wall. Since no additional band corresponding to WAK overexpression was detectable on coomassie blue stained SDS-PAGE gels we wanted to determine whether purification of the GFP-tagged protein could work. Unfortunately the 4% SDS and 50mM DTT extraction is not compatible with a classical GFP-trap technique and did not allow the purification of the protein (figure 2 lane 2). We thus attempted to use different extraction buffers that could have been compatible with the purification technique.

*Can pectin extraction or degradation release the WAKs?*

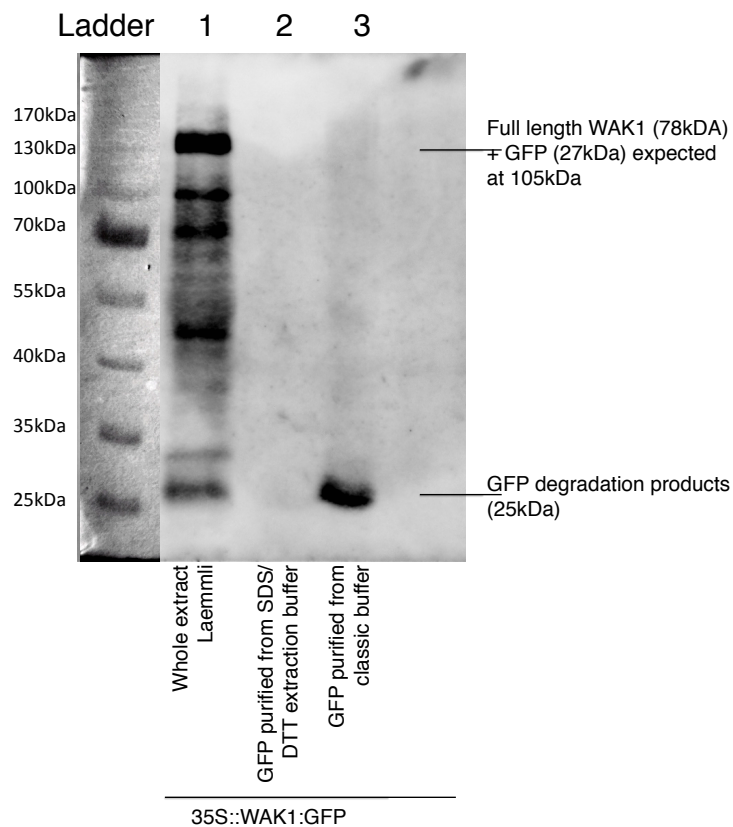
Digestion with pectin degrading enzymes has also been reported to work (Wagner et al., 2001), but to a fairly lower extent than with the 4% SDS/50mM DTT buffer. Extraction of the pectins with CDTA and Na<sub>2</sub>CO<sub>3</sub> on the cell wall fraction of the extract did not seem to release the WAKs in either the soluble or the different extract fraction (figure 1 lane 3, 4 and 5). Similarly, degradation of the cell wall fraction with a mix of protoplast preparation enzymes (Cellulase, driselase and macerozyme), didn't release detectable full length WAK (figure 1 lane 6 and 7). Only what probably correspond to the GFP degradation band was found in the soluble fraction.

It is however possible that the proteins were degraded during the treatments. Also the release of the WAKs by pectin degradation was shown to work using purified polygalacturonase, which may be more specific than the protoplast cell wall degradation cocktail.

*Does lower concentration of SDS and DTT allow the release of the WAKs?*

In addition, I tested the extraction with lower SDS or DTT concentration but it didn't release detectable full length WAK (data not shown). I also attempted to reduce the SDS and DTT concentration of the extract with a protein purification amicon column and subsequently perform GFP-trap purification but I did not obtain purified proteins probably due to the incomplete removal of SDS and DTT (data not shown). For all the above extraction buffer tested I also attempted GFP-trap purification after extraction, although very little or no full length protein was detected in western blot analysis of the extracts, and as expected I did not

obtain purified proteins. Protein purification usually only yielded an approximately 25 kDa band likely corresponding to the GFP fragment alone (figure 2 lane 3).



**Figure 2: GFP-trap purification of the WAK:GFP fusion proteins**

Lane 1 is the extract from leaves directly ground and boiled in Laemmli. In lane 2 the leaf samples were first ground in a 4% SDS / 50mM DTT extraction buffer, and GFP-trap purification was carried out on this extract. In lane 3, the leaf samples were first ground in a simple TrisHCl extraction buffer containing an anti-protease cocktail and GFP-trap purification was carried out. The presence of the GFP fusion proteins was probed with an anti-GFP antibody. In lane 2 no purification at all was observed due to the incompatibility of the SDS/DTT extraction buffer with the GFP-trap. In lane 3 only the approximately 25 kDa GFP fragment was observed likely because the full length version of the fusion protein didn't detach from the cell wall with the extraction buffer used.

*Can we extract the WAKS from freshly prepared protoplast?*

Finally I attempted to extract the WAKs from freshly prepared protoplasts. The principle was to prepare protoplast (as described in III. Experimental procedure) such that the cell wall would be removed and the newly synthesized overexpressed WAKs would be more easily extractable. I also prepared microsomal fractions of these protoplasts to increase the chance of purifying the WAKs. Western Blot analysis of the different fractions did not reveal the

presence of the full length GFP-tagged WAK in any of the GFP-trap compatible fraction (data not shown).

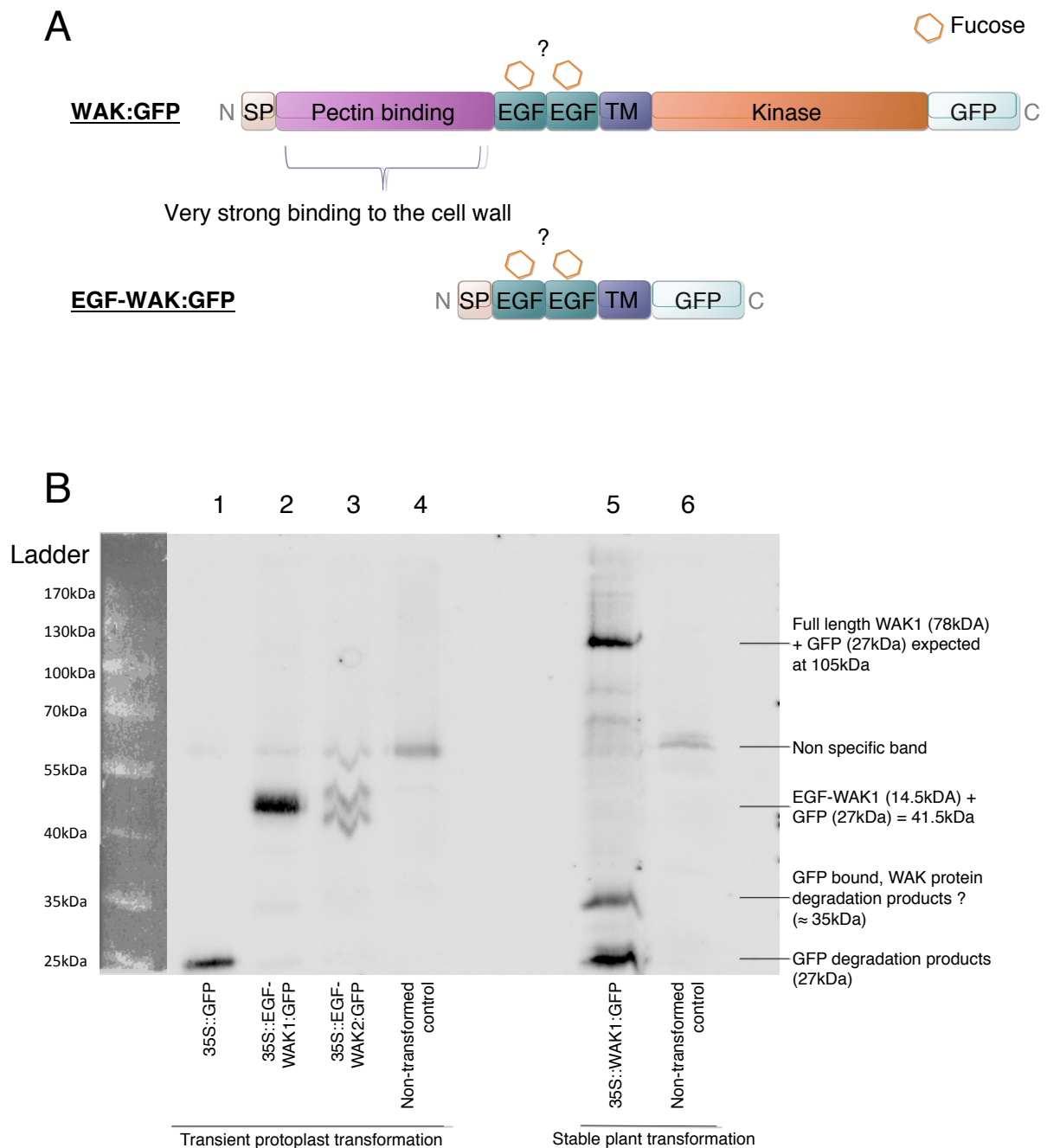
Overall it appeared to us that this approach was not suitable for our purpose.

It is interesting however to note that the highest band detected by western blot, that we suppose correspond to the full length version of the fusion protein, is of approximately 130 kDa while its expected size is 105 kDa. This probably reveals post-transcriptional modification of the protein such as glycosylations. The O-fucosylation however would likely generate a much smaller glycan, based on what is described in the animal system, and this difference in size likely does not correspond to the presence of O-fucosylation but probably another glycosylation. As expected there was no detectable difference in the size of this band between the different genetic backgrounds (data not shown).

*Click chemistry approach to reveal the presence of fucose on the WAKs*

Click chemistry, in this case, refers to the azide-alkyne cycloaddition. Azide and alkyne groups can very specifically react in the presence of copper. One can provide alkynylated fucose to the plant, which will incorporate it in its metabolism, such as on its polysaccharides. Another molecule such as an azide modified alexa fluorescent probe can then be provided to the plant, and in the presence of copper the fluorescent probe will covalently bind specifically to the modified fucose. This provides a mean to directly observe the presence of fucose since the reaction happen after incorporation when a fluorescently-tagged fucose wouldn't be incorporated by the organism as was shown in animal cells (Rabuka et al., 2006).

A recent work from (Anderson et al., 2012) revealed that an alkynylated fucose analog (FucAL) could be metabolically incorporated into the RGI pectic polysaccharide as a terminal fucose on the arabinogalactan side chains. Surprisingly they showed that no fucose was incorporated on xyloglucans or RGII while these are believed to be the main fucose-containing cell wall polysaccharides. Instead, FucAL would be incorporated as a terminal fucose on the RGI arabinogalactan side chain. Although it was reported to exist in soybean (Nakamura et al., 2001), to our knowledge such a terminal fucose was not reported to exist in *Arabidopsis thaliana*. The experiments described did not excluded that the fucose could be incorporated onto a protein behaving like pectin. Taking into account that the WAKs are



**Figure 3: *35S::EGF-WAK:GFP* transient transformation in protoplasts: Tests**

(A) Schematic view of the EGF-WAK:GFP fusion protein compared to the WAK:GFP. SP: Signal peptide; EGF: Epidermal Growth Factor; TM: Transmembrane domain. N: N-Terminal end of the protein; C: C-terminal end. (B) Western blot of different extracts from transiently expressed EGF-WAK:GFP fusion protein in col-0 cell cultures protoplasts and WAK1:GFP fusion protein in stably transformed plants.

Lane 1 is a positive control transformation with a simple *35S::GFP* construct. Lane 2 and 3 are the extracts from the transformations with *35S::EGF-WAK1:GFP* (lane 2) and *35S::EGF-WAK2:GFP* (lane 3). Lane 4 is a negative non transformed control. The proteins were extracted from equal amounts of transformed or control protoplasts with a simple lysis buffer (as described in the experiment procedure). The presence of the GFP fusion proteins was probed with an anti-GFP antibody. Lane 5 and 6 are for comparison with the stably transformed *35S::WAK1:GFP* construct. Interestingly the EGF-WAK1 construct (lane 2) yielded a nice band of the expected size of the fusion protein, while EGF-WAK2 (lane 3) yielded two bands with a lower intensity. These results show that at least for EGF-WAK1, the transient transformation works well, the expected size protein is produced, is easily extractable and produces barely any GFP degradation products.

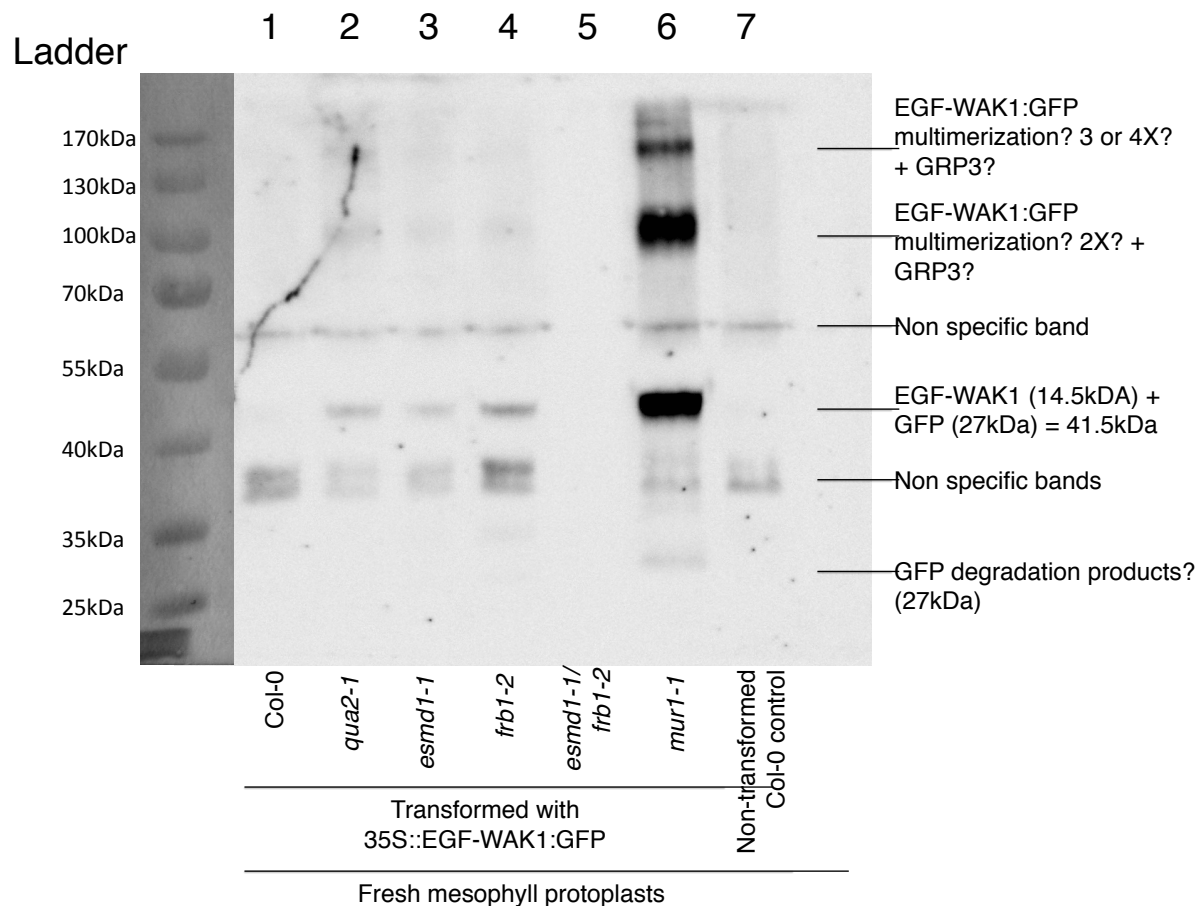
tightly bound to the pectin, the incorporated fucose that is described in Anderson et al (2012) could correspond to an O-fucose on the WAKs. We thus wanted to test whether that could be the case.

Our approach here was to try to determine by a western blot analysis coupled with click chemistry whether or not the WAKs were O-fucosylated. We thus used our 35S::WAK:GFP lines from the different mutant background and grew them on a medium containing FucAL. The proteins were extracted from 8 days old seedlings using the 4%SDS / 50mM DTT buffer and the click reaction was performed either on the protein extract or on the nitrocellulose membrane after protein migration and transfer. The azide containing marker used was either an alexa488 or a biotin. Observation under a fluorescence scanner for alexa488 or revelation using an HRP grafted streptavidin, did not reveal a specific signal corresponding to a WAK, or another protein. However no positive control was available to determine whether the click reaction had actually worked, which does not allow us to conclude on the absence of FucAL on the WAKs.

Further test should be done on this method to determine whether it is a technical problem or not. This technique could also provide a highly valuable *a priori* approach to identify O-fucosylated protein that would be the potential substrates of the putative O-fucosyltransferases family members.

*Protoplast transformation with the EGF-WAK construct to overcome the low expression of stable transformant and the problem of WAK extractability*

Since the previous approach of overexpression of the full length WAK protein did not succeed due to technical problems, we undertook a different strategy. First, we wanted to overexpress a WAK peptide without the pectin binding domain to avoid the problem of the tight binding to the cell wall. We thus decided to make a construction containing only the signal peptide, the two EGF-like domains, the transmembrane domain and a C-terminal GFP-tag (EGF-WAK:GFP; figure 3 A). The constructions were done for WAK1 and WAK2. The idea was also to have a much smaller fusion protein that would be easier to study by mass spectrometry. Then we wanted to try to overexpress the protein transiently in protoplasts, since the overexpression could affect the development of the plant and prevent the existence of actual overexpressing plants.



**Figure 4: 35S::EGF-WAK1:GFP transient transformation in leaf mesophyll protoplasts from different genetic backgrounds : Western blot**

The same 35S::EGF-WAK1:GFP construct as in figure 3 was transformed into col-0 (lane 1), *qua2-1* (lane 2), *esmd1-1* (lane 3), *frib1-2* (lane 4), *esmd1-1/frib1-2* (lane 5) and *mur1-1* (lane 6) leaf mesophyll protoplasts, or not transformed (lane 7). The expression level of the fusion protein was assayed by western blot. The presence of the GFP fusion proteins was revealed with an anti-GFP antibody. Interestingly *mur1-1* transformation efficiency was much higher than the other genotypes as revealed by the presence of a band at the expected size (approximately 42 kDa) of a much higher intensity than the other genotypes. The higher molecular weight bands at approximately 100 and 170 kDa could be multimers of the EGF-WAK1:GFP fusion protein potentially also associated with GRP3, a peptide known to interact with WAK1 and to promote its multimerization (Park et al., 2001).

#### *Transformation of Col-0 cell suspension culture*

The first tests were carried out on Col-0 cell suspension culture available at the institute, following the protocol described in (AXELOS et al., 1992). These provided encouraging results (figure 3 B). Western blot analyses were carried out on whole protoplast lysate approximately 16h after transformation. The 35S::EGF-WAK1:GFP construct produced a single band at approximately 42 kDa as expected, in relatively high amount and with almost no visible degradation product (figure 3 B lane 2). The 35S::EGF-WAK2:GFP construct however produced two specific bands around the expected size and in a lower quantity compared to EGF-WAK1 (figure 3 B lane 3).



### *Transformation on freshly prepared mesophyll protoplasts*

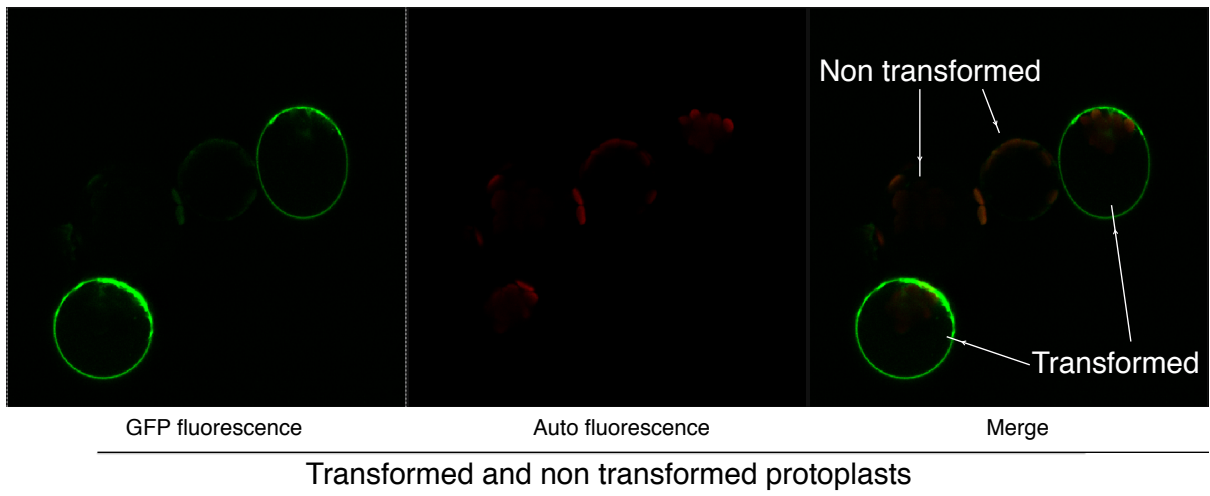
Since we wanted to study the protein in different genetic backgrounds, the use of such a cell line was not applicable, so we switched to a different approach. Transformation of freshly prepared mesophyll protoplasts was performed following the protocol described in (Yoo et al., 2007). The protoplasts were prepared from the wild type col-0, and the mutants *qua2-1*, *esmd1-1*, *frb1-2*, *esmd1-1/frb1-2* and *mur1-1*. Interestingly, although all the transformations were carried out in the exact same condition and following the exact same protocol, the transformation yield was largely variable between the different genetic backgrounds (figure 4). The genetic background *mur1-1* yielded a much higher transformation rate. Approximate estimation under epifluorescence microscope indicated that roughly 40-60% of the protoplasts were transformed and expressing the construct in this background (figure 5 A), whereas almost no transformed protoplasts were found in samples observed for col-0, *qua2-1*, *esmd1-1* and *qua2-1/esmd1-1* and relatively little were found in *frb1-2*, which largely reflect the western blot results in figure 4.

Two specific bands were also clearly observable at approximately 100kDa and between approximately 150 and 200 kDa. These could correspond to multimerization of the EGF-WAK:GFP along with the GLYCINE RICH PROTEIN3 (GRP3), a small peptide known to bind to the WAKs. This peptide was shown to associate to the extracellular domain of the WAK1, 3 and 5, likely on the EGF-like domains (Park, 2001) and to induce the multimerization of the receptor WAK1.

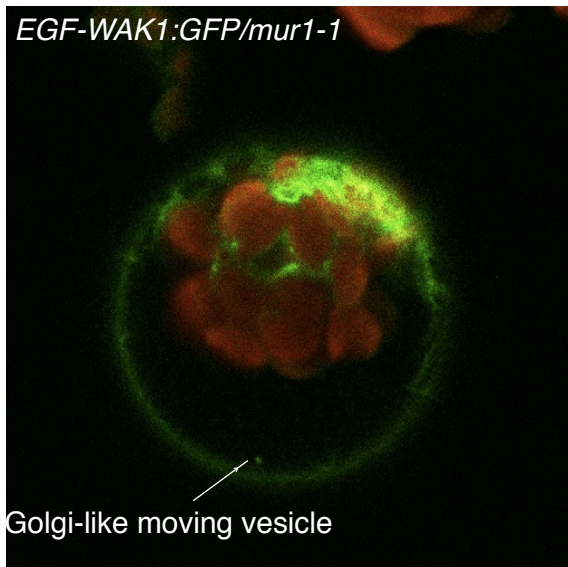
### *Subcellular localization of EGF-WAK1:GFP in different genetic background*

Interestingly confocal microscopy observations revealed a different localization of the EGF-WAK1:GFP construct in the different genetic background observable. Only for *mur1-1* and *frb1-2* enough transformed protoplast were seen after transformation to comment on the subcellular localization of the fusion protein at this point. In *mur1-1* the signal seemed to be localized throughout the secretion system (Endoplasmic reticulum and Golgi), and sometimes at what appears to be the plasma membrane or the cytoplasm (figure 5 B-E). These assumptions are based on the observation of the fluorescence in intracellular compartments resembling the network-like shape of the ER (figure 5 B and C), moving Golgi-like vesicles (figure 5 C), and the presence of fluorescence at the surface of the cell, similar to the membrane or the cytoplasm between the vacuole and the plasma membrane. These results are similar to the results reported in Kohorn et al. (2006a) where a WAK1:GFP (full length)

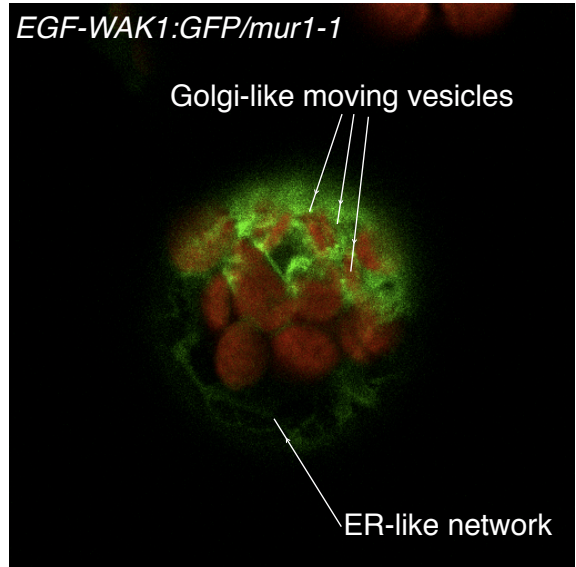
A



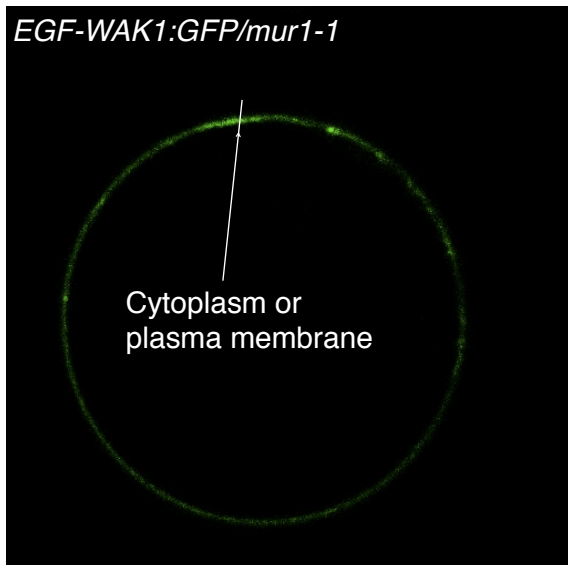
B



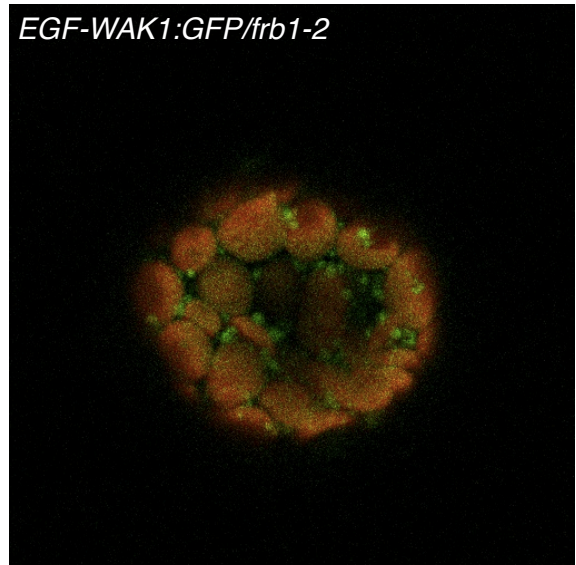
C



D



E



**Figure 5: 35S::EGF-WAK1:GFP transient transformation in leaf mesophyll protoplasts from different genetic backgrounds : Confocal microscopy**

(A) Example of transformed and non-transformed protoplasts expressing the EGF-WAK1:GFP fusion protein. (B-D) *mur1-1* protoplasts transformed with the EGF-WAK1:GFP construct showing subcellular localization of the fusion protein that likely correspond to the ER and Golgi apparatus (B and C) and the plasma membrane or the protoplast (D). (E) *frb1-2* protoplast expressing the EGF-WAK1:GFP fusion protein and showing a subcellular localization of the fusion protein that could correspond to the endosome.

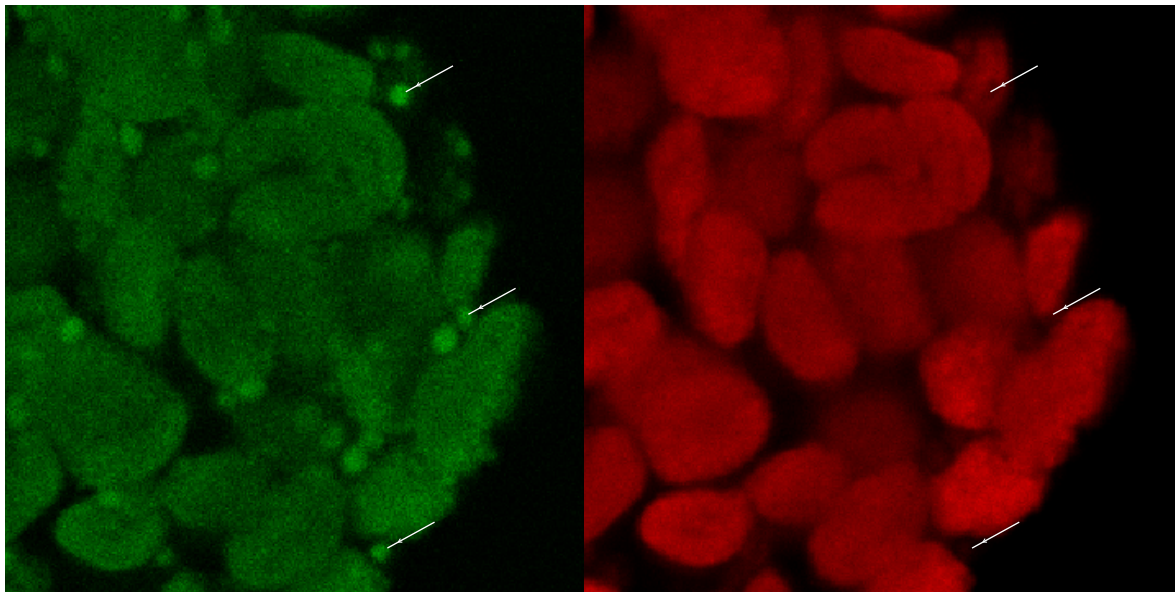
The green channel on the pictures correspond to the GFP signal (detected between 495 and 540 nm), and the red channel corresponds to the autofluorescence of the chloroplasts (detected between 560 and 620 nm).

---

construct was transformed into mesophyll protoplast following the same protocol. Interestingly in our case some of the protoplast had most of the fluorescence in intracellular compartments, while other had the fluorescence at what may be the membrane. We did our observations 16-24 hours after transformation. Interestingly in Kohorn et al (2006a) they noticed that the WAK1:GFP fusion protein took longer than that to reach the plasma membrane, and that 1 day after transformation most of the protoplasts had an intracellular localization of the signal while after 5 days almost all of the protoplast had a plasma membrane localization of the GFP signal.

In *frb1-2* however the localization of the EGF-WAK1:GFP fusion protein was strikingly different. most of the signal seemed to localize to relatively small intracellular compartments that did not exactly resemble Golgi apparatus (figure 5 E). Short staining of the protoplasts with FM4-64 to stain the plasma membrane as well as the endocytic compartments, revealed a colocalization of the internalized fm4-64 with the EGF-WAK1:GFP intracellular compartment after a couple of minutes (figure 6). This seems to indicate that in a *frb1-2* background the EGF-WAK1:GFP fusion proteins undergoes a high rate of endocytosis since in addition no signal is detected at the plasma membrane. Interestingly internalization of plasma membrane receptor upon binding of the substrate to amplify signalization is a well described phenomenon. It has been shown for example for the receptors BRI1 (Rusina et al., 2004; Geldner et al., 2007) and FLS2 (Robatzek et al., 2006). Internalization of the activated receptor is supposed to increase the signal response by increasing the surface bearing activated receptor: those internalized, and new one reaching the plasma membrane to be activated. It is also proposed that it physically facilitates the signaling to the nucleus as the endocytosed compartments can move around the cell and get closer to the nucleus (Geldner and Robatzek, 2008). Another interesting observation on the *frb1-2* transformed protoplasts is that a considerable amount of the GFP signal seems to be located in the chloroplasts. Indeed the exact same imaging settings as for *mur1-1* were used but in *frb1-2* clearly some signal can be observed in the chloroplasts (figure 5 E and figure 6) while it is not the case in *mur1-1*

A

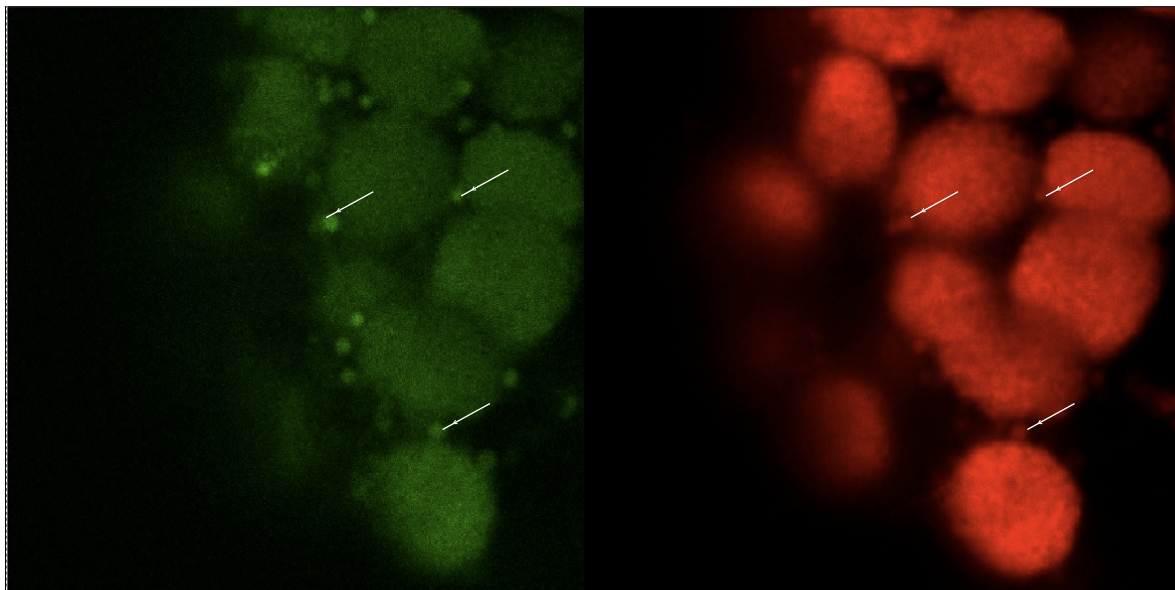


GFP fluorescence

"Red" fluorescence/auto fluorescence

EGF-WAK1:GFP/*frb1-2*

B



GFP fluorescence

"Red" fluorescence/auto fluorescence

EGF-WAK1:GFP/*frb1-2*  
+ fm4-64

**Figure 6: Endosomal localization of EGF-WAK1:GFP in *frb1-2* leaf mesophyll protoplasts**

*frb1-2* protoplasts transformed with the EGF-WAK1:GFP construct were observed with (B) and without (A) fm4-64 staining. When stained the observation was done about 10 minutes after the addition of the fm4-64. Interestingly the vesicle like localization of the fusion protein in *frb1-2* colocalizes with the fm4-64 internalization. This suggests that the protein is indeed located in the endosome. Interestingly there is also a GFP signal in the chloroplast in *frb1-2* compared to *mur1-1* (figure 5). The images were taken with the exact same settings which tend to indicated that the signal is indeed specific to GFP.

The green channel on the pictures correspond to the GFP signal (detected at 495–540 nm), and the red channel corresponds to the fm4-64 signal as well as the autofluorescence of the chloroplasts (detected at 560–620 nm).

(figure 5 B and C). Although this is surprising, this observation is actually reminiscent of the original work on WAK1 where the authors found that WAK1 was a potential interactor of a thylakoid membrane associated protein (Kohorn et al., 1992).

Overall, these observations of localization are preliminary results and are based on a probably too small number of observations in the case of *frb1-2*. The actual compartments observed in *mur1-1* should be determined by colocalization with different subcellular organelles markers to confirm the localization. The subcellular localization in *mur1-1* reveals that likely a considerable amount of the EGF-WAK:GFP had not reached the Golgi apparatus at the time of observation, which is not surprising, but should be taken into account when analyzing the potential glycosylation state of the proteins, since the putative O-fucosyltransferases are located in the Golgi.

Although these are preliminary results, it seems to indicate that a mutation in *FRB1* does affect WAK1, reinforcing the idea that *FRB1* could actually be a glycosyltransferase of the WAKs EGF-like domains. It also seems to indicate that it affects the behavior of the receptor in a way similar to what a constitutive induction of the receptor would be. This tend to correlate with our observation and hypothesis (described in part I figure 3 and general conclusion and discussion) suggesting that the missing glycosylation in the mutant *frb1* could constitutively induce a signaling pathway leading to cell separation, even in the absence of a specific inductive signal.

These observations and interpretation are however to be taken with caution since they are done in a protoplast system which may largely affect the behavior of the receptors. Also the observed constructs are largely truncated version of the receptor, which may also differ in behavior from the native protein.

### *Perspectives*

The transformation and extraction of proteins from these protoplast were done in a too small scale at this time, did not allow a sufficient extraction of protein for purification and analysis before the end of my PhD. However it seems to be the most promising approach for future work in order to reveal whether or not these proteins are O-fucosylated. We should be able to make use of the high transformation yielding *mur1-1* complemented or not with fucose to mimic the wild type. Also empirical adaptation of the transformation protocol could allow higher transformation yield in the other genetic backgrounds.

Finally the constructs were also used to stably transform *Arabidopsis* in the different genetic background described above. Preliminary results seem to indicate a relatively low expression of the fusion protein as observed by confocal microscopy (data not shown). However the protein should be more easily extractable and purifiable, and it would provide an alternative way to obtain reasonable amounts of purified proteins for mass spectrometry analysis.

To conclude on this work, it hasn't yet provided the information that we were looking for. Also it is a strategy based on the *a priori* that ESMD1 and FRB1 are O-fucosyltransferases of the WAKs while this function is only based on predictions from the protein sequence, and the potential of the WAK as substrates is only based on homologies with the system described in animals. A no *a priori* approach was imagined consisting in a proteomic identification of the proteins differentially glycosylated between a *col-0* and a *mur1-1* background. This could have revealed a list of fucosylated proteins potentially the substrates of ESMD1 and FRB1. However very early it appeared that the WAKs were the most likely substrate of these O-fucosyltransferases and early experiments on the overexpression of the WAK:GFP construct, which were assumed to provide a "quick" answer actually pointed out the difficulty to analyse the WAKs by biochemical approaches. It was thus highly likely that our favorite *a priori* substrate would actually be excluded from such approach. However if the work on the WAKs finally proves that they are not the substrates, this kind of approach could be undertaken.

## II. Additional data on the mutant phenotypes

The *quasimodo* mutants present a number of pleiotropic phenotypes in addition to the cell adhesion defect: Sucrose sensitivity, tumorous development, hyperhydricity and reduced hypocotyl elongation are some of the most striking. The study of the mutants and suppressors has led us to search in different directions to characterize and better understand the link between these various phenotypes.

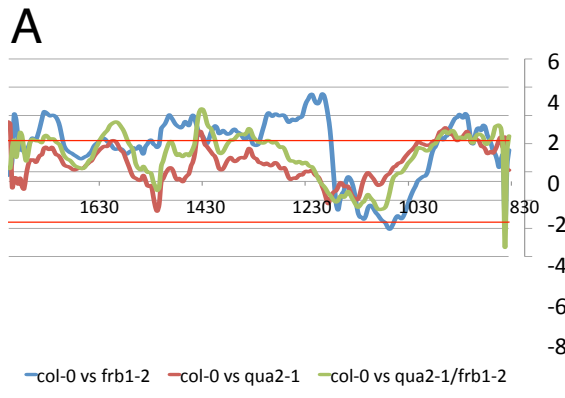
### 1. Characterization of the cell wall modification in *quasimodo* and the suppressor lines

*quasimodo1* and *quasimodo2* are mutated in putative HG synthesis genes (Bouton et al., 2002; Mouille et al., 2007) and are deficient in homogalacturonan content in their cell wall

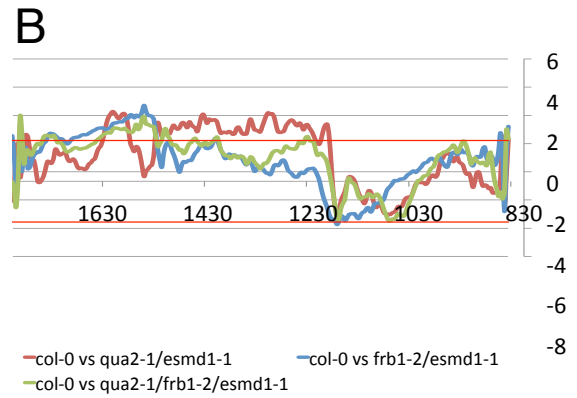
(Leboeuf et al., 2005; Orfila et al., 2005; Ralet et al., 2008). Previous reports did not identify other major changes in the cell wall composition; however the *qual* mutation was shown to alter xylan synthase activity in addition to the galacturonan synthase activity in intact microsomal membranes (Orfila et al., 2005). Thus we wanted to investigate on the modifications that may take place as a consequence of the disruption of *ESMD1* and that could explain the restoration of the cell adhesion defect.

### *FTIR analysis*

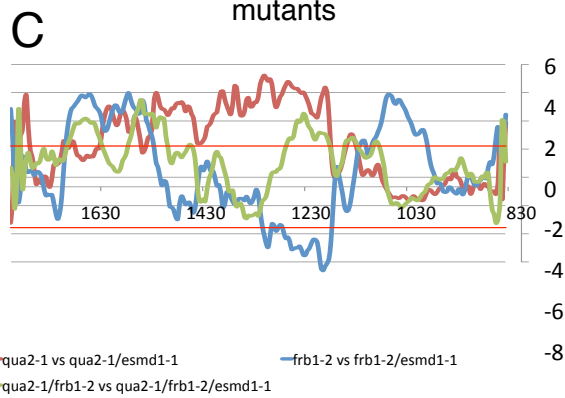
In order to characterize the suppressor line as well as the different lines that are studied in part 1 of this chapter we undertook a Fourier Transformed Infrared spectroscopy (FTIR) analysis of the modifications in the cell wall structure and composition of these different lines (Figure 7). The absorption spectra obtained for the different genotypes were compared to each other with a t-test. This analysis outputs a plot of the t value for each wavenumber, highlighting the significant differences in the cell wall structure and composition of the two compared genotypes. The graphs in figure 7 represent the results of these t-tests, and the red horizontal bars are the significance thresholds of the statistical analysis. If the t value remains within the threshold bars, there are no significant differences between the two initially compared spectra. If the t passes the threshold, there is a significant difference of absorbance at this wavenumber suggesting a difference in structure and composition between the two tested lines. This modified absorbance can sometimes be attributed to a specific variation in a cell wall component. Most of the time however these differences are very complex to interpret and simply point out a significant difference in the cell wall structure and composition between the two tested lines. For clarity, the plots of the t values were put in different groups. The *col-0* vs cell adhesion defective mutants (figure 7 A), *col-0* vs suppressed lines (figure 7 B) and cell adhesion defective vs suppressed lines (figure 7 C) respectively, were put together in three separate groups in order to determine if there were clear changes in the spectra that could be attributed to the loss or the restoration of cell adhesion. The changes detected by the t-test are relatively weak and in most of the case the significant peaks do not clearly correlate for the different mutant lines. The main clear difference between the wild type and either the cell adhesion defective (figure 7 A) or the suppressor lines (figure 7 B) is the large negative peak between approximately 1200  $\text{cm}^{-1}$  and 1000  $\text{cm}^{-1}$ . This peak is within the so-called “polysaccharide fingerprint” domain of the spectra. The variations of absorption intensity in this zone are very complex to analyze and as such cannot give any clear information. But the



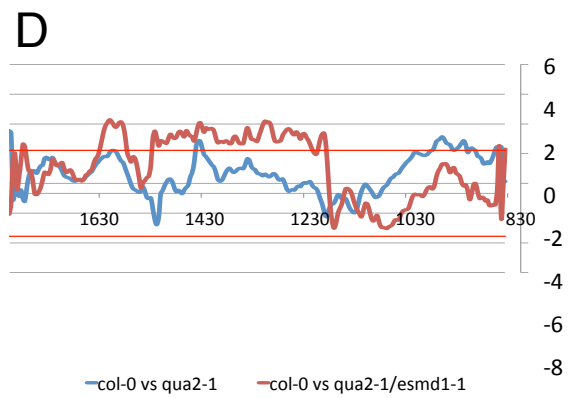
Col-0 vs cell adhesion defective mutants



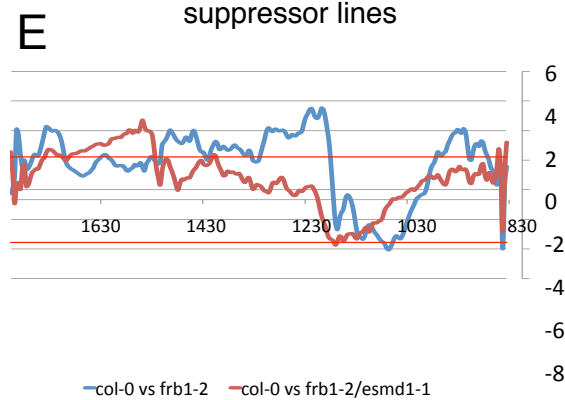
Col-0 vs suppressor lines



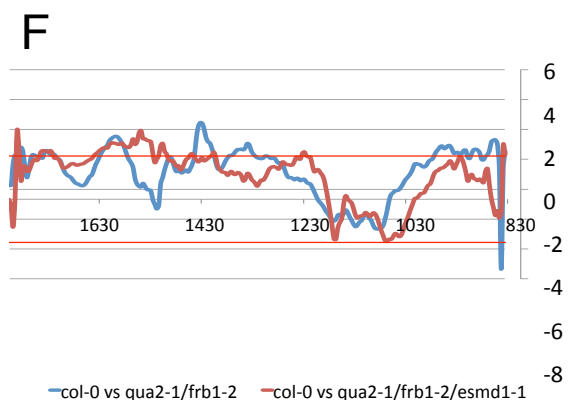
Cell adhesion defective vs suppressor lines



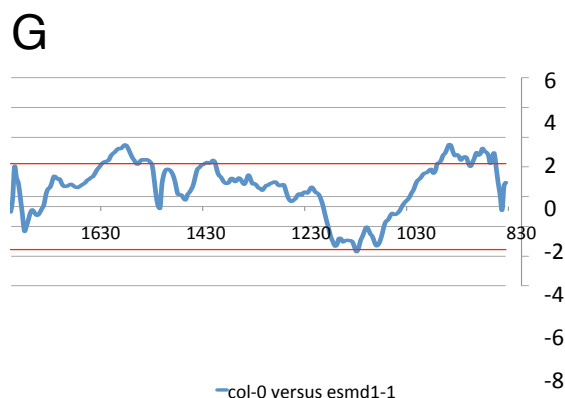
*qua2-1* and suppressor



*frb1-2* and suppressor



*qua2-1/frb1-2* and suppressor



Col-0 vs *esmd1-1*



**Figure 7: Characterisation of the cell wall modification in *quasimodo* and the suppressor lines: FTIR analysis**

These spectra are t-tests results of the comparison of two absorption spectra corresponding to two different genotypes. This analysis highlights the significant differences in the cell wall structure and composition of the two compared genotypes. The red horizontal bars are the significance threshold of the statistical analysis. If t-test spectra remains within the threshold bars, there is no significant differences between the two initially compared spectra. If a peak of the t-test spectra passes the threshold, there is a significant difference in structure and composition that can sometimes be attributed to a specific variation in a cell wall component.

The abscissa corresponds to the wave number of absorption in cm<sup>-1</sup> and the ordinate corresponds to the value of t in the t-test.

---

fact that this peak is conserved in both cell adhesion defective and suppressor compared to the wild type indicates that this is a specific cell wall modification induced in the cell adhesion defective mutants, which is not restored by the suppressor mutation. The comparison of the cell adhesion defective mutants with the suppressor lines does not reveal any clear common pattern (figure 7 C). This does not allow us to identify a specific modification that could take place in the suppressor line and that could explain from a structural point of view, the restoration of the cell adhesion by *esmd1-1*. In graph D to F we can also see that there are differences in the structure of the cell wall induced by the suppressor line but these are not clearly identifiable.

Finally the comparison of *col-0* with *esmd1-1* shows weak but significant modification of the cell wall structure and composition, indicating that this mutation by itself affects the cell wall. But overall the t-test spectra are very noisy (most of the peaks are small and irregular), and not very significant (statistically) meaning that most of the observed variations observed here may not be relevant or may not clearly point out a specific modification of the cell wall.

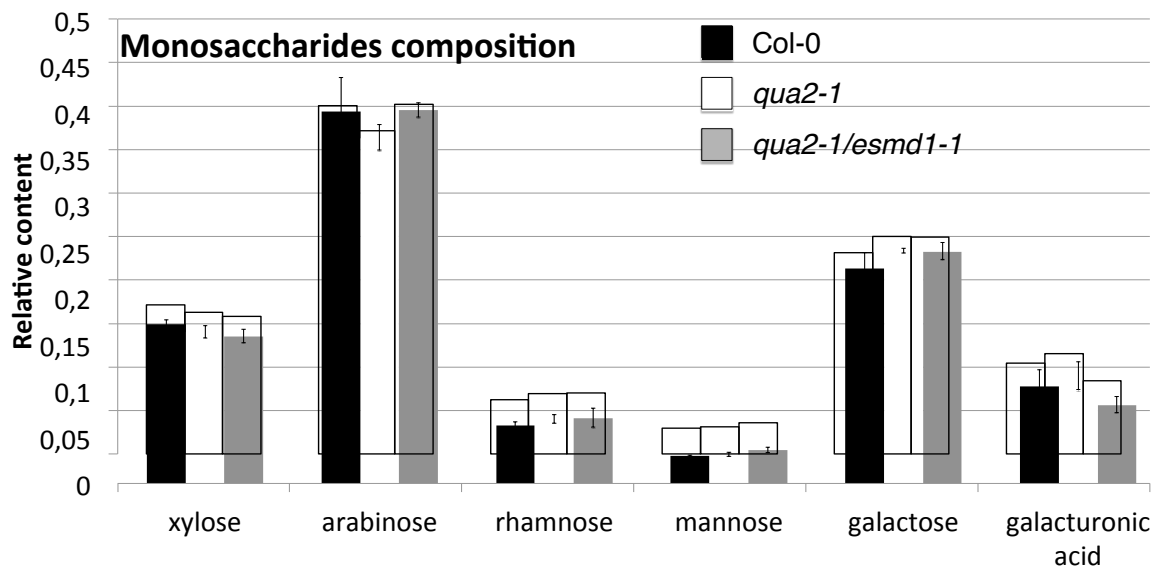
*Cell wall monosaccharides composition*

We also carried out a more detailed characterization of the cell wall of the mutant *qua2-1* and its suppressor *esmd1-1*. The analysis of the monosaccharides composition of *col-0*, *qua2-1* and *qua2-1/esmd1-1* did not reveal any major changes in the cell wall content of the sugars analyzed here (Figure 8).

Quite surprisingly these cell wall analyses are not very informative on the causes of the restoration of cell adhesion, at least at the cell wall level, but indicate that the suppression is not due to a restoration of the HG content. More detailed analyses such as linkages analyses or the monosaccharide composition analysis of additional cell wall fraction could provide more informations.

However in depth characterization of the cell wall has been carried out in the case of the mutant *frb1* (Neumetzler et al., 2012) and although they were able to point out that the low degree of pectin methyl esterification and higher PME activity were likely responsible for the loss of cell adhesion, a number of other modifications are taking place in the cell wall of this mutant and a clear picture could not be drawn either. This also answer the concern of a potential direct role of ESMD1 and FRB1 on the cell wall structure and composition instead of signaling, since they are glycosyltransferases and could glycosylate structural components of the cell wall. None of the cell wall analyses that were carried out on *frb1* could clearly point out a cell wall component that could be the substrate of FRB1. Overall this seems to correlate with the fact that these enzymes affect the signaling and that the downstream modifications that they induce in the cell wall are complex.

---



**Figure 8: Characterisation of the cell wall modification in *quasimodo* and the suppressor lines: Monosaccharide composition analysis**

Monosaccharide composition of *col-0*, *qua2-1* and *qua2-1/esmd1-1*. No major changes are observed between the different genotypes.

---

## 2. *ESMD1* pattern of expression

A GUS construct harbouring the 2168 pb upstream promoter region of *ESMD1* was used to study the expression pattern of *ESMD1*. GUS expression seems to be present almost throughout the seedling and to a stronger degree in the vascular tissues (figure 9). In dark grown hypocotyls the expression is present in the root but absent in the root cap (figure 9 D). The GUS staining is present in the hypocotyl and seems to be more intense in the already

elongated region of the hypocotyl (basal to median part), less intense in the elongating (upper part) and absent in the apical hook as well as the very base of the hypocotyl (figure 9 B and C). The GUS staining is also highly present at the apex of the cotyledons but absent from their base and the meristem zone (figure 9 C). Interestingly the localization on the hypocotyl seems to nicely correlate with the region where the cell adhesion defect is usually the most obvious (Cell detachment and outward curving) in the *quasimodo* mutants. In the light grown seedlings, the expression appears to be much stronger in the root than in the aerial parts but seems to be present everywhere (figure 9 A).

To get further information on what may affect the expression of *ESMDI*, we used the web based application of gene expression analysis, Genevestigator ([www.genevestigator.com](http://www.genevestigator.com)). Interestingly it reveals that *ESMDI* is upregulated by sucrose. Another interesting regulating factor of *ESMDI* expression, as revealed by the Genevestigator data, is the bacterial typeIII effector HrpA. Indeed the presence of the wild type version of this effector upregulates *ESMDI*, while the mutated version down regulates it.

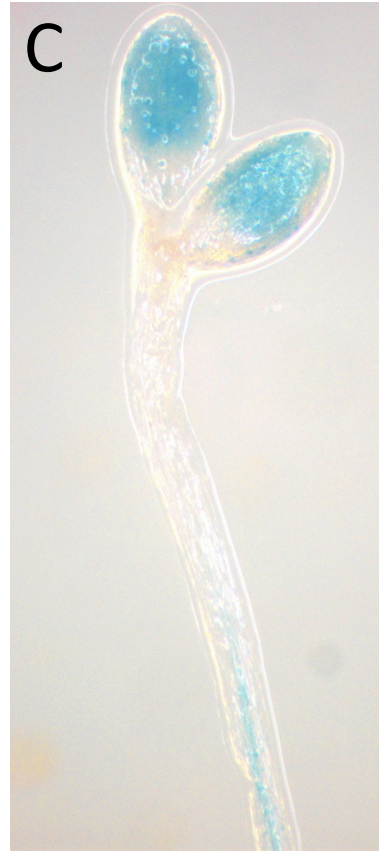
We tested the responsiveness to sucrose with our GUS line and could indeed see that the staining was more intense in seedlings grown with increasing sucrose concentration (preliminary data, not shown). The fact that *quasimodo* is sensitive to sucrose and that this sensitivity is suppressed by the mutation *ESMDI* could be explained by the responsiveness of *ESMDI* to sucrose. Indeed the potential upregulation of *ESMDI* in the presence of sucrose could be responsible for the high sensitivity observed in *quasimodo*. The disruption of *ESMDI* and thus the absence of response to sucrose could explain the suppression of the sucrose sensitivity.



Seedling grown on a medium containing 1% sucrose



Dark grown hypocotyl, without sucrose



**Figure 9: Histochemical staining for GUS activity in arabidopsis seedlings expressing a *pESMD1::GUS* transgene**

Expression of the uidA reporter gene driven by the 2kb upstream region of *ESMD1* in: (A) lightgrown seedling grown on 0.8% agarose and 1% sucrose. (B-D) Darkgrown hypocotyl grown on 0.8% agarose and without sucrose. (C) is a close up of the cotyledons and the apical part of the hypocotyl and (D) is a close up of the root. In (A) the seedlings shows a GUS staining throughout its tissue with a stronger intensity in the root. In (B-D) the dark grown hypocotyl shows a staining in the already elongated part of the hypocotyl (basal to median part), a less intense staining in the elongating (upper part) and is not stained in the apical hook as well as the very base of the hypocotyl (B and C). It is also highly stained at the apex of the cotyledons but absent from their base and the meristem (C).

---

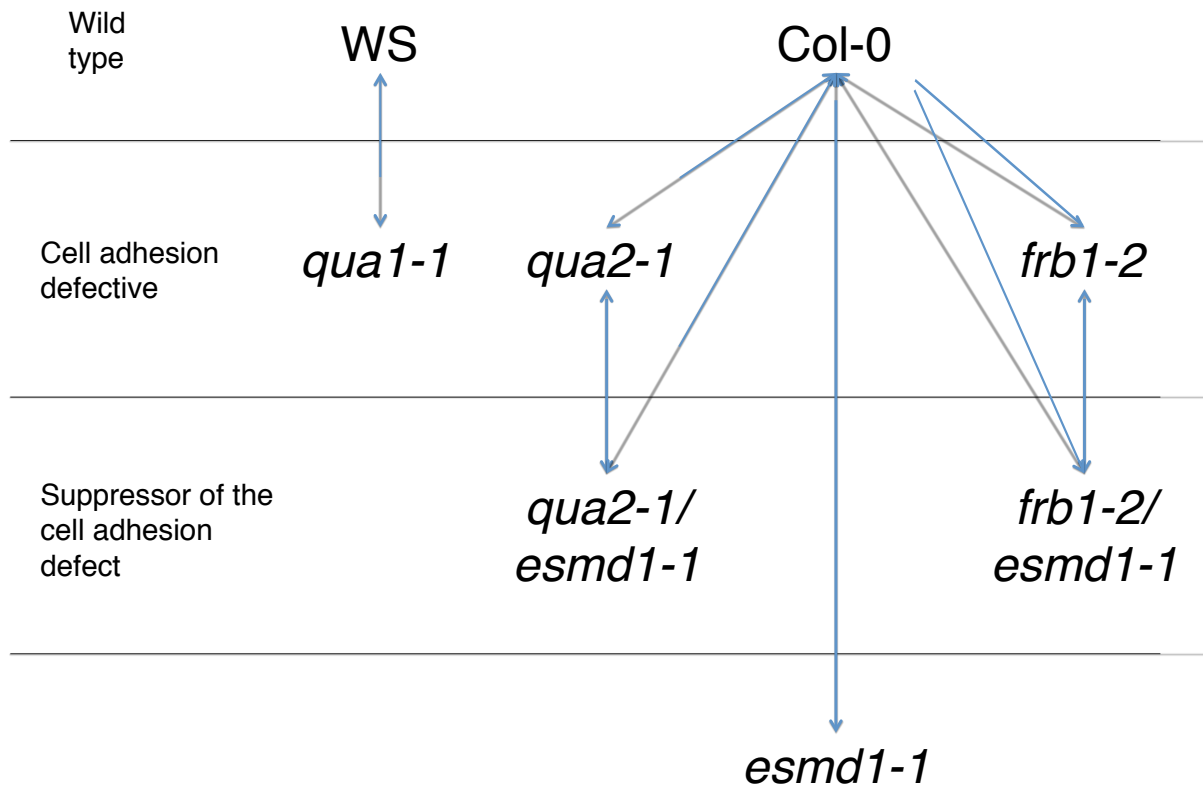
### 3. Transcriptomic analysis

The pleiotropic phenotypes of *quasimodo* seems to indicate that a number of secondary responses are activated in the mutant. In order to identify these different responses we performed a transcriptome analysis. The idea was also to identify genes specifically induced by the loss of cell adhesion and genes which expression is specifically restored by the suppressor *esmd1-1*, in order to identify which genes are specifically regulated downstream of *ESMD1*. This analysis was done on 8 days old seedlings grown in a medium without sucrose and compared wild type, cell adhesion defective and suppressor lines as described on figure 10. Briefly, the cell adhesion defective lines were compared to their wild type, and the suppressors compared to their cell adhesion defective relative. In addition Col-0 was compared with all the other lines in a col-0 background. The overall analysis identified a large number of genes differentially regulated between the different genotypes but did not allow us to draw very clear picture of the affected pathways. However the goal then was to compile the data from the different genotypes and determine a list of gene specifically affected in the cell adhesion defective mutants compared to the wild type (figure 10 B) and in the suppressor compared to the cell adhesion defective (figure 10 C).

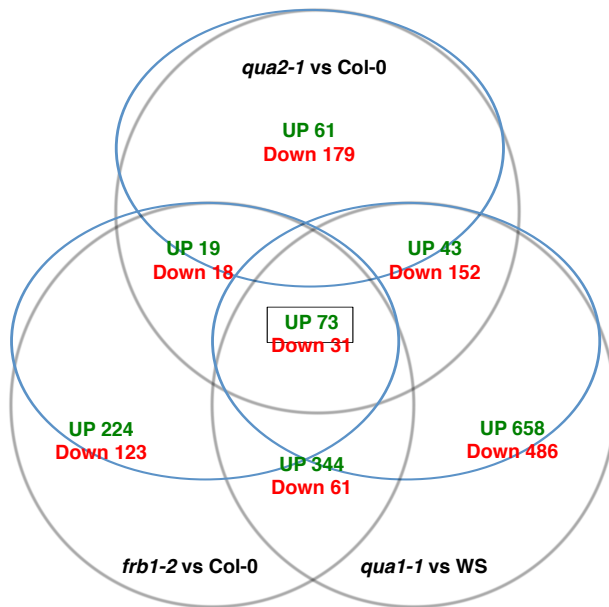
The intersection of the genes differentially regulated in *qua1-1*, *qua2-1* and *frb1-2* compared to their wild type background, identified 73 genes up regulated in common and 31 genes down regulated in common (figure 10 B and table 1 and 2). Gene Ontology (GO) enrichment as determined by the agriGO tool (<http://bioinfo.cau.edu.cn/agriGO/index.php>) (Du et al., 2010), reveals mostly an up-regulation of the “response to stimulus” related genes (Only up regulated genes were analyzed because down regulated genes didn’t provide enough significant Gene Ontology terms; figure 11).

The similar analysis was carried out for the genes differentially regulated in both *qua2-1/esmd1-1* vs *qua2-1* and *frb1-2/esmd1-1* vs *frb1-2*. We found 105 genes up regulated in common and 17 genes down regulated in common (figure 10 C and table 3 and 4). GO also

A

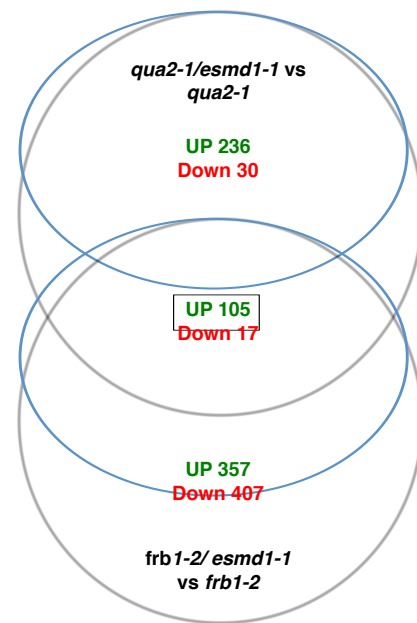


B



Cell adhesion defect specific genes

C



*esmd1-1* cell adhesion restoration specific genes

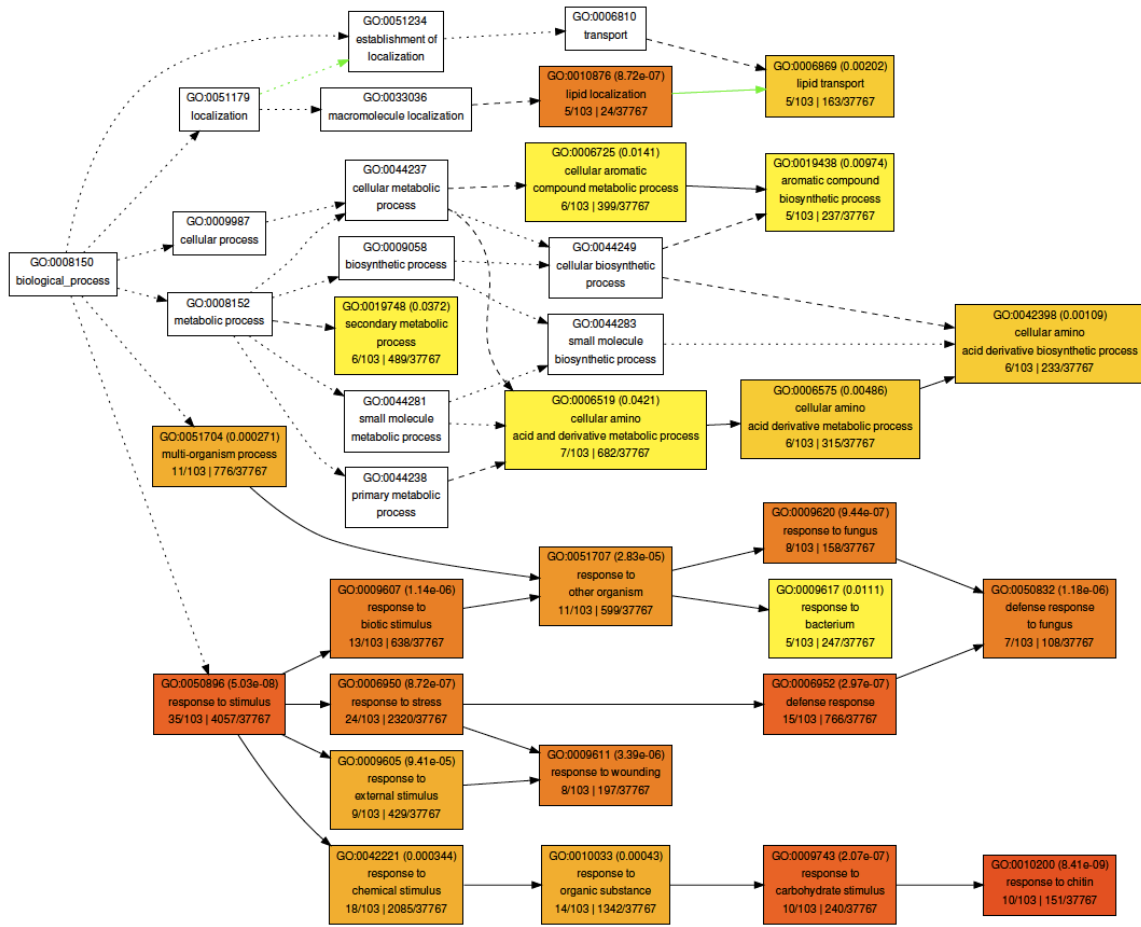
### **Figure 10: Transcriptomic analysis**

(A) Schematic representation of the comparisons of gene expression, that were carried out by microarray CATMA v7 analysis, between the different genotypes. The cell adhesion defective lines were compared to their wild type, and the suppressors compared to their cell adhesion defective relative. In addition Col-0 was compared with all the other lines in a Col-0 background. (B and C) are Venn diagrams that represent the number of genes up and down regulated in the different genetic background. The intersection of the genes differentially regulated in *qua1-1*, *qua2-1* and *frb1-2* compared to their wild type background, identified 73 genes up regulated in common and 31 genes down regulated in common (B). The similar analysis was carried out for gene differentially regulated in both *qua2-1/esmd1-1* vs *qua2-1* and *frb1-2/esmd1-1* vs *frb1-2* identified 105 genes up regulated in common and 17 genes down regulated in common (C).

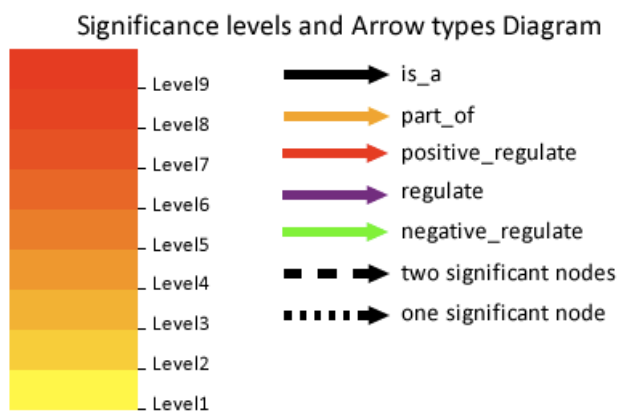
---

reveals a clear up-regulation of the “response to stimulus” related genes as well as genes implicated in the “indole and derivative metabolic process” (Similarly only the up regulated genes were analyzed; figure 12).

Overall a stress response seems to be induced in the mutant, which is not surprising. The most surprising however is that a large number of genes are also up regulated in the suppressor lines compared to their relative cell adhesion defective mutants. We would have expected the opposite since they are suppressor line and a lot of the secondary responses induce in the mutant should be shut down in the suppressor. In addition based on the Gene ontology it seems to be the same types of genes that are upregulated in both cases. Although this analysis gives us a list of potentially interesting genes, it provides relatively little information at this stage of the analysis. Finally we identified the genes that are differentially regulated in cell adhesion defective mutants, and restored in the suppressor line. Three genes were up regulated in common in the cell adhesion defective mutants *frb1-2* and *qua2-1* and down regulated in their suppressor lines (At1g12610, At2g38530 and At3g20470) and one gene was down regulated in common in the cell adhesion defective mutants *frb1-2* and *qua2-1* and up regulated in their suppressor lines (At5g03545). These should represent genes regulated or belonging to the downstream genes which expression is specifically affected by *ESMD1*. At1g12610 is DWARFED AND DELAYED FLOWERING 1 (DDF1) an ERF/AP2 transcription factor. The over expression of this genes results in a delayed flowering and dwarfism, but also reduces gibberellic acid biosynthesis and increases the tolerance to high levels of salt (Kang et al., 2011). At2g38530 is LIPID TRANSFER PROTEIN 2 (LTP2). Overexpression of this gene in tobacco led to an increased resistance to *Pseudomonas syringae* (Molina and García-Olmedo, 1997). Interestingly it was also shown to induce cell wall loosening in tobacco (Nieuwland et al., 2005). At3g20470 is GLYCINE RICH PROTEIN 5, a structural protein of the cell wall. It also has a potential role in response to the pathogenic invasion mediated by the cutin monomer (Park et al., 2008). Finally, At5g03545 is INDUCED BY PI STARVATION 2 (ATIPS2) and seems to be a regulator of phosphate



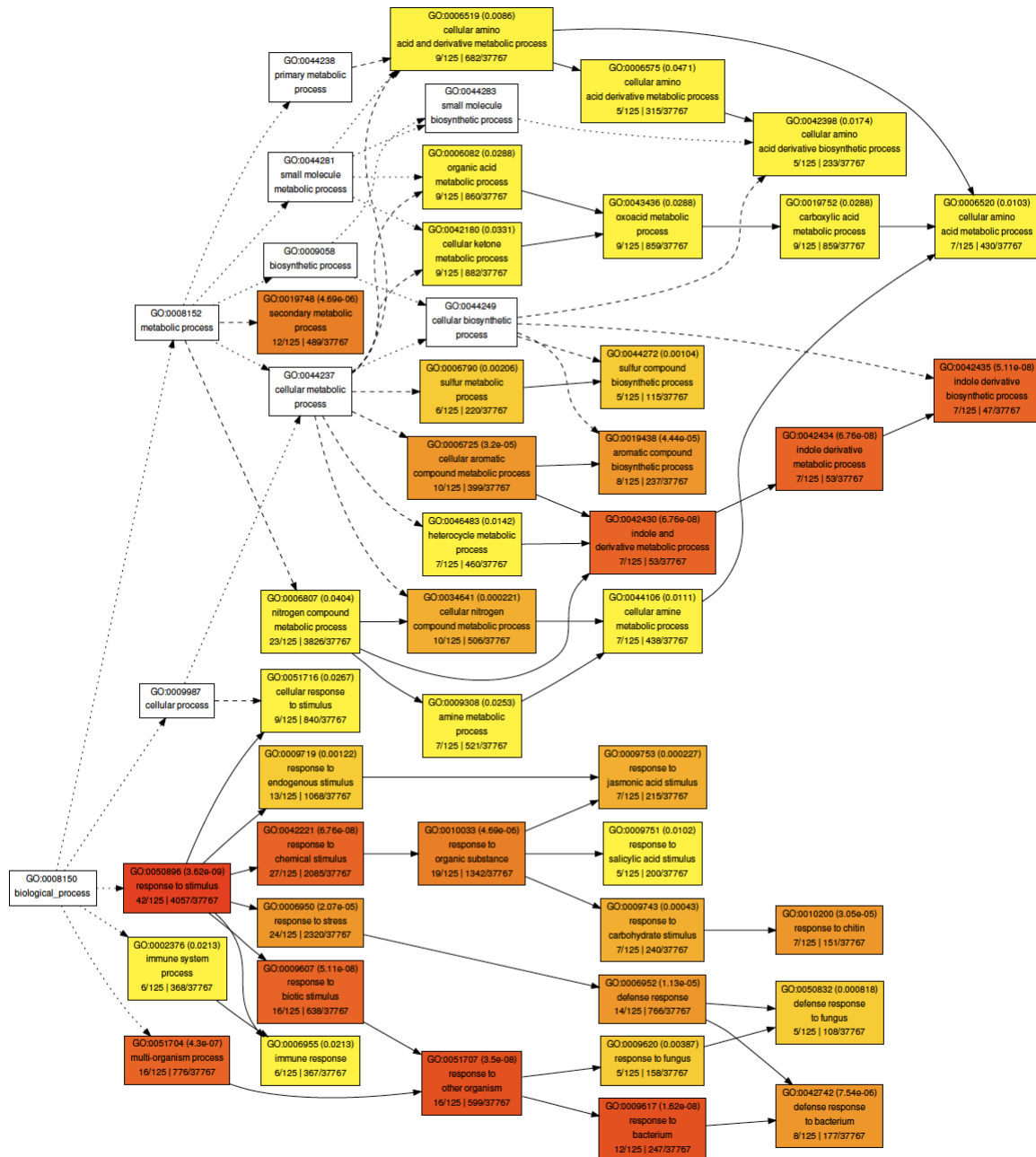
Gene Ontology analysis for genes specific of the cell adhesion defect



**Figure 11: Gene Ontology of the genes up regulated in the cell adhesion defective mutants**

AgriGO tool (<http://bioinfo.cau.edu.cn/agriGO/index.php>; Du et al., 2010) Gene ontology analysis output reveals mostly an up-regulation of the “response to stimulus” related genes.





Gene Ontology analysis for genes specific of *esmd1-1* cell adhesion restoration

**Figure 12: Gene Ontology of the genes up regulated in the *esmd1-1* restored line**

AgriGO tool (<http://bioinfo.cau.edu.cn/agriGO/index.php>; Du et al., 2010) Gene ontology analysis output reveals mostly an up-regulation of the “response to stimulus” related genes as well as genes implicated in the “indole and derivative metabolic process”.

homeostasis (Shin et al., 2006). The functions of these genes and the phenotypes that they induce when they are differentially expressed could be responsible for some of the pleiotropic phenotypes of *quasimodo*.

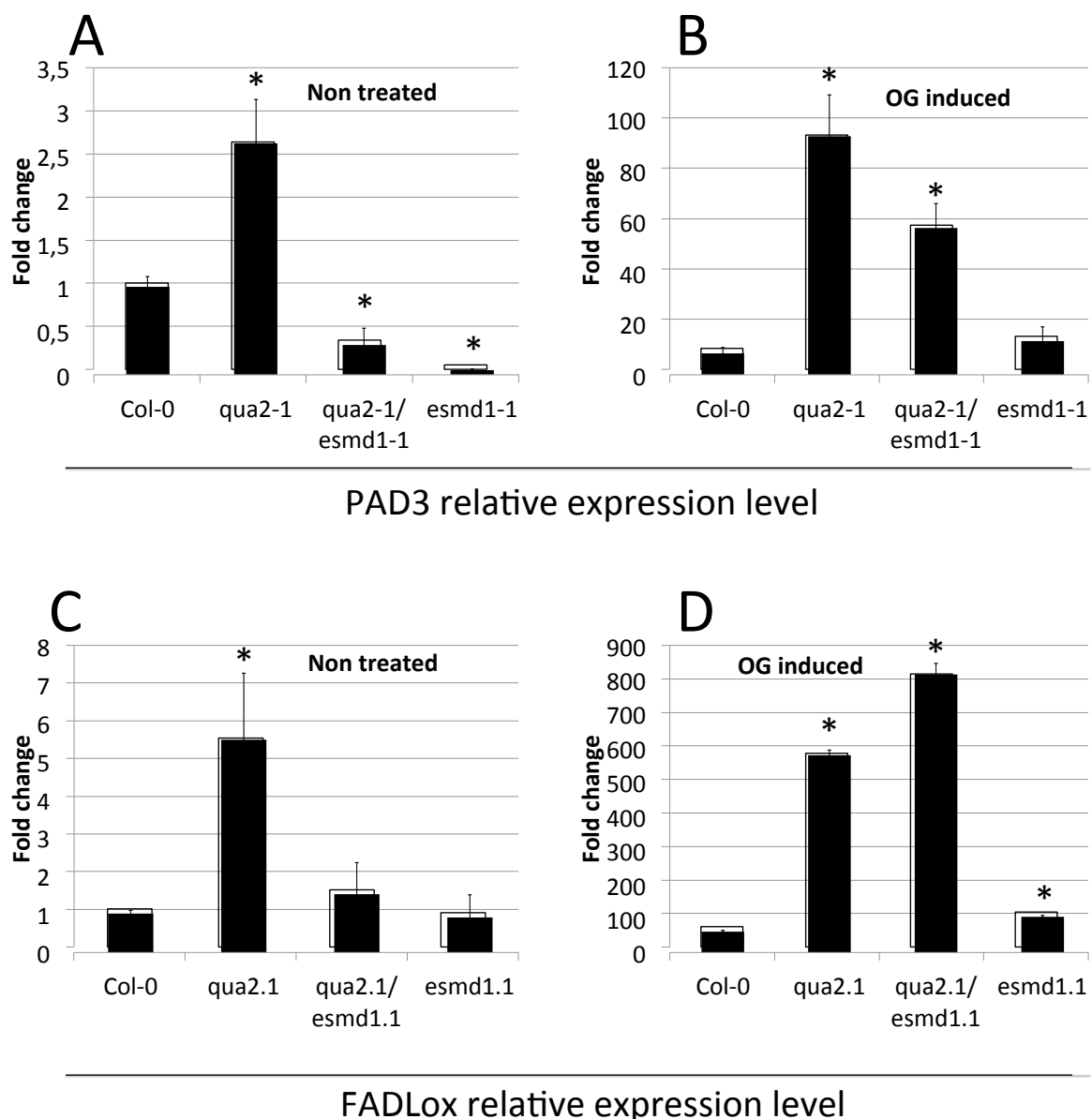
Although I only performed a very basic analysis of the data and didn't use any of its outcome for my PhD work since this analysis was performed around the end of my PhD, further work on the genes identified here could reveal very interesting information for the understanding of cell adhesion in plants as well as the occurrence of the pleiotropic phenotypes of *quasimodo*.

#### 4. *qua2* and *esmd1* show an altered response to pectin

The putative function of ESMD1, based on the homology with the O-fucosyltransferases in animals would be to fucosylate protein domains called Epidermal Growth Factor (EGF) like repeats. EGF-like repeats in plants happen to be found on Receptor-Like Kinases (RLK), including receptors which have been shown to bind to pectin and respond to the treatment by oligogalacturonides (OGs). The absence of such a glycosylation could affect the function of the receptor. We hypothesized that the phenotypes observed in *quasimodo* could be the consequence of an over-activation of a pectin mediated stress response due to the pectin defect, and that the disruption of *ESMD1* would prevent this response by preventing a normal functioning of the receptors. To test this hypothesis we wanted to determine whether the perception and response to OGs were affected in our different lines. Eight days old seedlings were treated for three hours with a final concentration of 50  $\mu\text{g}\cdot\text{ml}^{-1}$  of OGs or were not treated. The expression of PAD3 and FADLox, two genes responsive to OGs (Denoux et al., 2008), was assayed. First of all, as reported in part 1 of this chapter, the non-treated samples revealed that the expression of these two genes is already significantly upregulated in *qua2-1* compared to *col-0*, *qua2-1/esmd1-1* and *esmd1-1* (figure 13 A and C). Interestingly this may reveal a constitutive basal activation of a response to the pectin defect of the *quasimodo* mutants.

The OG treatment triggers a very high induction of the genes. About 9 and 60 folds for the OG treated *col-0* compared to the non-treated *col-0* respectively for PAD3 (figure 13 A and B) and FADLox (figure 13 C and D). But it goes up to about 100 and almost 600 folds for OG treated *qua2-1* compared to non-treated *col-0*. These genes are ten times more upregulated in *quasimodo* than in *col-0* when treated with OG which reveals a specific sensitivity to OGs in *qua2-1* and would be consistent with our first hypothesis. However it gets more complicated and confusing when it comes to *qua2-1/esmd1-1*. FADLox is significantly more induced in the suppressor line than in *qua2-1*, while PAD3 is significantly less induced. Finally *esmd1-1* has a response indistinguishable from *col-0* for PAD3, and has a slightly upregulated response for FADLox when treated with OG.

Overall this demonstrates that the mutation in *ESMD1* does not abolish the response to OGs as we would have expected. But it still clearly affects the response as observed with the two genes that we tested. We expected that *PAD3* and *FADLox* would behave similarly in response to OG, and it does for *col-0* and *qua2-1*, but it is not the case in the suppressor line. This may actually mean that the potential presence or absence of the O-fucosylation may not have a radical effect such as permitting or abolishing the signaling but may affect the specificity of the response and the downstream signaling pathway.



**Figure 13: *quasimodo* and *esmd1* have an altered response to pectin**

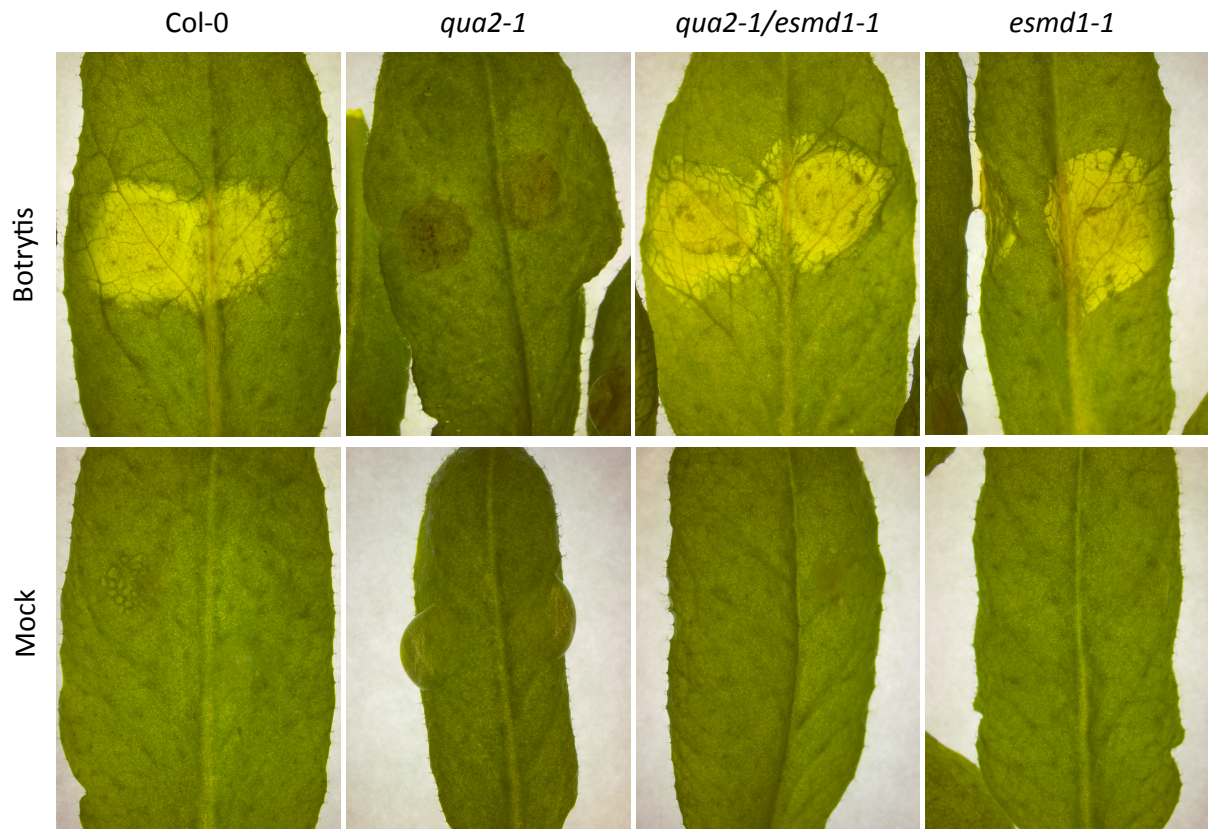
Expression levels of *PAD3* (A and C) and *FADLox* (B and D), of either non treated (A and B) or OG treated (C and D) seedlings of *col-0*, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1*. Values shown are average and standard deviation of three biological replicates. All relative expression is expressed in fold change relative to the untreated *col-0* (A). However, \* indicates independently in each graph a significant difference (t-Test,  $p < 0.05$ ) with the *col-0* sample in this graph.

The higher responsiveness in *qua2-1* and *qua2-1/esmd1-1* could actually be explained by a higher permeability of the cell wall to the OG due to the HG deficiency, allowing much more cell to perceive the signal and in higher quantity. Based on this we can likely interpret the changes in ratio of gene expression, such as the fact that FADLox is more induced in the suppressor line than in *qua2-1* while PAD3 behaves in the opposite direction. However we are unable to clearly conclude on these results at this stage.

### **5. *qua2-1* is resistant to *B. cinerea* maceration and sensitivity is restored in the suppressor line**

OGs are specific signals that are well characterized as elicitors characteristic of the necrotrophic fungal attacks. Since our mutant and suppressor lines had a perturbed response to these OGs we wanted to test whether our mutants and suppressor had a different response to the fungal necrotrophic pathogen *B. cinerea*. This kind of pathogen acts by killing the cells and triggers a characteristic maceration of the tissues. So we inoculated col-0, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1* with a *Botrytis* spore suspension to see whether the lines that were more responsive to OG were more resistant to *Botrytis*. Two drops of germinated spores in PDB, or PDB alone (mock) were placed on 6 weeks old leaves, and the apparition of maceration was observed after 48H (figure 14). The maceration can be clearly observed since it makes the leaf translucent around the site of inoculation as observed for col-0, *qua2-1/esmd1-1* and *esmd1-1*. *Qua2-1* however shows no maceration at all after 48h, but a browning just under the *Botrytis* suspension drop. Even after 96h (data not shown) while the leaves of the other genotypes were completely macerated, *qua2-1* showed no maceration. Interestingly, although *qua2-1/esmd1-1* had clearly an increased response to OG compared to col-0, it wasn't resistant to *Botrytis* maceration as was *qua2-1*. The resistance to botrytis maceration seems more correlated with the higher basal expression of defense response genes in *qua2-1* than to the higher level of response to OGs in both *qua2-1* and *qua2-1/esmd1-1*.

Some of these experiments are inconclusive and would need additional work to be exploited and be informative for the characterization of the cell adhesion defect and its suppression by *esmd1*. But overall these additional phenotypic characterizations bring interesting insights and ideas to set up future experiments.



**Figure 14: *qua2-1* is resistant to *B. cinerea* maceration and sensitivity is restored in the suppressor line**

Six weeks old leaves of col-0, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1* inoculated with two drops of germinated spores in PDB, or PDB alone (mock) and observed after 48H. The maceration makes the leaf translucent around the site of inoculation. *Qua2-1* doesn't show any signs of maceration 48h after inoculation compared to the other lines.

Gene ID	CATMA annotation	<i>qua1-1</i> vs WS	<i>qua2-1</i> vs col-0	<i>frb1-2</i> vs Col-0
AT4G31970	"CYP82C2 (cytochrome P450, family 82, subfamily C, polypeptide 2); oxygen binding"	3,33	2,28	4,96
AT4G12490	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	4,17	1,47	3,33
AT1G19610	"LCR78/PDF1.4 (Low-molecular-weight cysteine-rich 78)"	3,48	1,52	3,73
AT4G22470	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	3,96	1,18	3,33
AT3G23250	"AtMYB15/AtY19/MYB15 (myb domain protein 15); DNA binding"	3,79	0,74	3,82
AT1G67980	"CCoAMT (caffeoyl-CoA 3-O-methyltransferase)"	3,74	1,48	3,07
AT2G17740	"DC1 domain-containing protein"	3,55	1,14	3,58
AT4G31950	"CYP82C3 (cytochrome P450, family 82, subfamily C, polypeptide 3); oxygen binding"	2,34	1,83	3,91
AT1G56240	"ATPP2-B13 (Phloem protein 2-B13)"	3,65	0,77	3,57
AT4G12480	"pEARL1 1; lipid binding"	4,17	1,75	1,90
AT4G12500	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	4,02	1,23	2,21
AT5G39580	peroxidase	3,39	0,94	2,96
AT4G31940	"CYP82C4 (cytochrome P450, family 82, subfamily C, polypeptide 4); oxygen binding"	1,74	1,58	3,87
AT3G20470	pseudogene	2,45	1,77	2,93
AT2G38530	"LTP2 (LIPID TRANSFER PROTEIN 2); lipid binding"	2,87	1,06	2,90
AT1G51820	"leucine-rich repeat protein kinase, putative"	3,09	0,96	2,78
AT2G22880	"VQ motif-containing protein"	2,81	0,81	3,02
AT5G24160	"squalene monooxygenase 1,2 / squalene epoxidase 1,2 (SQP1,2)"	2,06	1,25	3,14
AT4G25810	"XTR6 (XYLOGLUCAN ENDOTRANGLYCOSYLASE 6) /XTH23; hydrolase, acting on glycosyl bonds"	2,49	0,92	2,89
AT5G36925	"unknown protein"	2,55	1,09	2,45
AT2G02930	"ATGSTF3 (GLUTATHIONE S-TRANSFERASE 16); glutathione transferase"	2,63	0,82	2,28
AT1G27730	"STZ (SALT TOLERANCE ZINC FINGER); nucleic acid binding / transcription factor/ zinc ion binding"	1,91	1,09	2,61
AT1G65500	"unknown protein"	2,02	1,62	1,95
AT1G72920	"disease resistance protein (TIR-NBS class), putative"	2,20	0,83	2,55
AT3G44300	"NIT2 (NITRILASE 2)"	2,18	0,77	2,53
AT5G64120	"peroxidase, putative"	2,90	0,79	1,79
AT3G26230	"CYP71B24 (cytochrome P450, family 71, subfamily B, polypeptide 24); oxygen binding"	1,36	1,69	2,37
AT3G21080	"ABC transporter-related"	1,49	1,22	2,63
AT5G11410	"protein kinase family protein"	1,86	0,87	2,52
AT3G44260	"CCR4-NOT transcription complex protein, putative"	1,32	1,41	2,50
AT1G19380	"unknown protein"	2,48	1,03	1,71
AT3G46080	"zinc finger (C2H2 type) family protein"	2,46	0,78	1,94
AT5G51190	"AP2 domain-containing transcription factor, putative"	1,36	1,08	2,53
AT1G80840	"WRKY40 (WRKY DNA-binding protein 40); transcription factor"	1,59	0,83	2,51
AT5G08760	"unknown protein"	2,56	1,01	1,29
AT1G07135	"glycine-rich protein"	1,73	1,07	2,02
AT4G17500	"ATERF-1 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1); DNA binding / transcription factor/ transcriptional activator"	1,94	0,74	2,09
AT4G11650	"ATOSM34 (OSMOTIN 34)"	1,65	1,31	1,77
AT3G46280	"protein kinase-related"	1,77	0,80	2,08
AT5G44568	"unknown protein"	2,13	1,06	1,45
AT4G11890	"protein kinase family protein"	2,53	0,94	1,14
AT1G25400	"unknown protein"	1,43	1,06	2,05
AT1G64160	"disease resistance-responsive family protein / dirigent family protein"	1,83	0,82	1,89
AT4G22470	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	1,94	0,80	1,80
AT2G46750	"FAD-binding domain-containing protein"	1,71	1,02	1,69
AT1G10340	"ankyrin repeat family protein"	2,41	0,82	1,18
AT1G10070	"ATBCAT-2; branched-chain-amino-acid transaminase/ catalytic"	1,57	0,93	1,78
AT2G41100	"TCH3 (TOUCH 3)"	2,16	0,75	1,35
AT1G10070	"ATBCAT-2; branched-chain-amino-acid transaminase/ catalytic"	1,56	0,82	1,84
AT5G46050	"ATPTR3/PTR3 (PEPTIDE TRANSPORTER PROTEIN 3);	1,53	0,98	1,71

	transporter"			
AT3G26220	"CYP71B3 (cytochrome P450, family 71, subfamily B, polypeptide 3); oxygen binding"	1,36	0,89	1,84
AT3G46090	"ZAT7; nucleic acid binding / transcription factor/ zinc ion binding"	1,59	1,05	1,45
AT5G17350	"unknown protein"	0,76	1,22	2,11
AT5G38200	catalytic	1,65	1,09	1,31
AT4G31800	"WRKY18 (WRKY DNA-binding protein 18); transcription factor"	1,40	0,74	1,89
AT3G45140	"LOX2 (LIPOXYGENASE 2)"	0,96	1,04	1,99
AT3G55980	"zinc finger (CCCH-type) family protein"	1,10	0,72	2,16
AT3G26210	"CYP71B23 (cytochrome P450, family 71, subfamily B, polypeptide 23); oxygen binding"	1,58	1,07	1,22
AT5G61600	"ethylene-responsive element-binding family protein"	1,09	1,09	1,69
AT1G65390	"ATPP2-A5; transmembrane receptor"	1,67	0,88	1,24
AT4G27280	"calcium-binding EF hand family protein"	0,98	1,03	1,75
AT1G76600	"unknown protein"	1,19	0,75	1,78
AT3G49780	"ATPSK4 (PHYTOSULFOKINE 4 PRECURSOR); growth factor"	1,39	0,80	1,44
AT3G17050	0	1,25	0,77	1,56
AT1G18300	"ATNUDT4 (Arabidopsis thaliana Nudix hydrolase homolog 4); hydrolase"	0,75	0,90	1,88
AT4G23030	"MATE efflux protein-related"	1,01	0,82	1,45
AT4G24570	"mitochondrial substrate carrier family protein"	0,90	0,75	1,60
AT2G41640	"unknown protein"	0,74	0,75	1,74
AT2G28630	"beta-ketoacyl-CoA synthase family protein"	1,07	0,88	1,27
AT1G73500	"ATMKK9 (Arabidopsis thaliana MAP kinase kinase 9); kinase"	0,87	0,74	1,55
AT4G01250	"WRKY22 (WRKY DNA-binding protein 22); transcription factor"	1,14	0,82	1,13
AT1G76650	"calcium-binding EF hand family protein"	0,87	0,91	1,18

**Table 1: List of genes upregulated in common in the cell adhesion defective mutants**

Each gene is represented by its gene ID and the annotation of its function as provided by the analysis of the CATMAv7 chip. The numbers in the right column are the fold changes in expression of the gene between the two compared lines

Gene ID	CATMA annotation	<i>qua1-1</i> vs WS	<i>qua2-1</i> vs col-0	<i>frb1-2</i> vs Col-0
AT4G27670	"HSP21 (HEAT SHOCK PROTEIN 21)"	-0,75	-1,27	-4,71
AT5G37970	"S-adenosyl-L-methionine:carboxyl methyltransferase family protein"	-1,97	-2,63	-1,31
AT5G37990	S-adenosylmethionine-dependentmethyltransferase	-1,84	-2,52	-1,17
AT4G14060	"major latex protein-related / MLP-related"	-1,92	-2,07	-1,35
AT2G39510	"nodulin MtN21 family protein"	-1,64	-1,81	-1,49
AT1G77530	"O-methyltransferase family 2 protein"	-1,18	-2,25	-1,16
AT1G08090	"ATNRT2:1 (Arabidopsis thaliana high affinity nitrate transporter 2.1); nitrate transporter"	-1,35	-1,19	-2,03
AT4G22214	"unknown protein"	-1,97	-1,07	-1,34
AT4G11210	"disease resistance-responsive family protein / dirigent family protein"	-2,08	-1,02	-1,25
AT3G48940	"remorin family protein"	-1,69	-1,21	-1,33
AT5G35940	"jacalin lectin family protein"	-1,26	-0,87	-2,09
AT5G37980	"NADP-dependent oxidoreductase, putative"	-1,22	-1,61	-1,35
AT2G22930	"glycosyltransferase family protein"	-1,69	-1,18	-1,19
AT4G33790	"acyl CoA reductase, putative"	-1,46	-0,98	-1,57
AT5G53250	"AGP22/ATAGP22 (ARABINOGLACTAN PROTEINS 22)"	-1,69	-0,78	-1,47
AT5G03545	"unknown protein"	-1,63	-0,98	-1,28
AT4G15390	"transferase family protein"	-1,10	-0,90	-1,69
AT1G35250	"thioesterase family protein"	-1,21	-1,07	-1,40
AT3G31415	"terpene synthase/cyclase family protein"	-0,86	-1,54	-1,28
AT2G19060	"GDSL-motif lipase/hydrolase family protein"	-1,44	-0,72	-1,41
AT5G47450	"AtTIP2;3 (Arabidopsis thaliana tonoplast intrinsic protein 2;3); water channel"	-1,07	-1,09	-1,41
AT3G30280	"transferase family protein"	-1,02	-0,91	-1,62
AT3G09680	"40S ribosomal protein S23 (RPS23A)"	-0,73	-1,19	-1,54
AT4G25250	"invertase/pectin methylsterase inhibitor family protein"	-1,35	-0,79	-1,17
AT5G48070	"ATXTH20 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 20); hydrolase, acting on glycosyl bonds"	-0,73	-1,25	-1,23

AT5G52260	"AtMYB19 (myb domain protein 19); DNA binding / transcription factor"	-0,88	-0,84	-1,44
AT1G34040	"alliinase family protein"	-1,02	-0,77	-1,26
AT5G25880	"ATNADP-ME3 (NADP-MALIC ENZYME 3); malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+)/ malic enzyme/ oxidoreductase, acting on NADH or NADPH, NAD or NADP as acceptor"	-0,96	-0,76	-1,22
AT4G25760	"unknown protein"	-0,83	-0,88	-1,22
AT3G06035	"unknown protein"	-0,93	-0,74	-1,22

**Table 2: List of genes downregulated in common in the cell adhesion defective mutants**  
Similar to table 1

Gene ID	CATMA annotation	<i>qua2-1/esmd1-1 vs qua2-1</i>	<i>frb1-2/esmd1-1 vs frb1-2</i>
AT3G49620	"DIN11 (DARK INDUCIBLE 11); oxidoreductase"	5,02	0,94
AT2G29460	"ATGSTU4 (GLUTATHIONE S-TRANSFERASE 22); glutathione transferase"	3,11	1,93
AT3G26830	"oxygen binding"	2,03	2,60
AT2G30770	"CYP71A13 (cytochrome P450, family 71, subfamily A, polypeptide 13); oxygen binding"	2,16	2,46
AT2G23270	"unknown protein"	2,39	1,92
AT2G28210	"carbonic anhydrase family protein"	1,15	3,15
AT3G45060	"ATNRT2.6 (Arabidopsis thaliana high affinity nitrate transporter 2.6); nitrate transporter"	2,69	1,57
AT3G44860	"S-adenosyl-L-methionine:carboxyl methyltransferase family protein"	2,68	1,53
AT1G21525	pseudogene	3,02	0,91
AT2G44578	"protein binding / zinc ion binding"	1,88	1,96
AT1G56240	"ATPP2-B13 (Phloem protein 2-B13)"	2,87	0,84
AT5G40990	"GLIP1 (GDSL LIPASE1); carboxylic ester hydrolase"	0,95	2,74
AT1G69930	"ATGSTU11 (Arabidopsis thaliana Glutathione S-transferase (class tau) 11); glutathione transferase"	2,56	1,11
AT1G05880	"zinc ion binding"	0,92	2,52
AT3G26200	"CYP71B22 (cytochrome P450, family 71, subfamily B, polypeptide 22); oxygen binding"	1,14	2,30
AT5G25250	"unknown protein"	2,41	0,99
AT4G37290	"unknown protein"	1,89	1,51
AT1G68390	"unknown protein"	1,48	1,92
AT5G40590	"DC1 domain-containing protein"	0,90	2,50
AT4G14630	"GLP9 (GERMIN-LIKE PROTEIN 9); manganese ion binding / metal ion binding / nutrient reservoir"	0,84	2,52
AT2G44581	"unknown protein"	1,52	1,81
AT5G56960	"basic helix-loop-helix (bHLH) family protein"	1,35	1,92
AT4G16820	"lipase class 3 family protein"	1,82	1,41
AT5G64905	"PROPEP3 (Elicitor peptide 3 precursor)"	2,08	1,10
AT1G53625	"unknown protein"	2,04	1,08
AT3G15356	"legume lectin family protein"	1,91	1,21
AT1G56060	"unknown protein"	2,11	0,98
AT3G29000	"calcium-binding EF hand family protein"	2,20	0,87
AT3G60120	"glycosyl hydrolase family 1 protein"	1,99	1,08
AT2G35980	"YLS9 (YELLOW-LEAF-SPECIFIC GENE 9)"	1,86	1,21
AT4G28460	"unknown protein"	1,73	1,30
AT5G24110	"WRKY30 (WRKY DNA-binding protein 30); transcription factor"	1,12	1,90
AT5G26920	"calmodulin binding"	1,84	1,16
AT5G22520	"unknown protein"	1,74	1,24
AT1G35210	"unknown protein"	1,96	0,99
AT4G11170	"disease resistance protein (TIR-NBS-LRR class), putative"	1,96	0,97
AT5G39670	"calcium-binding EF hand family protein"	1,71	1,19
AT1G26380	"FAD-binding domain-containing protein"	1,55	1,31
AT2G40740	"WRKY55 (WRKY DNA-binding protein 55)"	1,15	1,70
AT3G19615	"unknown protein"	1,79	1,02
AT4G22030	"F-box family protein"	1,33	1,48
AT3E13410		1,54	1,22



AT3G23550	"MATE efflux family protein"	1,89	0,84
	"CYP81F2 (cytochrome P450, family 81, subfamily F, polypeptide 2); oxygen binding"	1,45	1,25
AT5G57220	"glutaredoxin family protein"	1,41	1,29
AT1G28480	"VQ motif-containing protein"	1,33	1,30
AT5G03545	"unknown protein"	1,52	1,10
AT4G00130	"unknown protein"	1,30	1,30
AT5G64890	"PROPEP2 (Elicitor peptide 2 precursor)"	1,42	1,17
AT2G24850	"TAT3 (TYROSINE AMINOTRANSFERASE 3); transaminase"	1,14	1,44
	"ATPCA/ATPRX33/PRX33/PRXCA (PEROXIDASE 33); peroxidase"	1,33	1,22
AT3G49110	"unknown protein"	1,16	1,39
	"CYP71A12 (cytochrome P450, family 71, subfamily A, polypeptide 12); oxygen binding"	0,94	1,61
AT2G30750	"YLS5 (yellow-leaf-specific gene 5)"	1,50	1,04
AT2G38860	"unknown protein"	1,48	1,05
AT5G22530	"curculin-like (mannose-binding) lectin family protein"	1,37	1,14
AT5G18470		1,65	0,83
AT1E38580	pseudogene	1,58	0,87
AT3G48650	"MYB51 (myb domain protein 51); DNA binding / transcription factor"	1,48	0,97
AT1G18570	"ethylene-responsive factor, putative"	1,41	1,04
AT3G23230	"FAD-binding domain-containing protein"	1,39	1,06
AT1G26400	"AACT1 (ANTHOCYANIN 5-AROMATIC ACYLTRANSFERASE 1); transferase"	1,26	1,17
AT5G61160	"glutamate decarboxylase, putative"	0,96	1,46
AT2G02010	"unknown protein"	1,33	1,08
AT1G06137	"ATNRT2.6 (Arabidopsis thaliana high affinity nitrate transporter 2.6); nitrate transporter"	1,04	1,36
AT3G45060	"unknown protein"	1,19	1,20
AT5G44990	"zinc finger (C3HC4-type RING finger) family protein", "unknown protein"	1,15	1,23
AT2G18670	"ASB1 (ANTHRANILATE SYNTHASE BETA SUBUNIT 1); anthranilate synthase"	1,32	1,05
AT1G25220	"unknown protein"	1,27	1,07
AT1G13520	"transcription factor"	1,05	1,29
AT1G10585	"heavy-metal-associated domain-containing protein"	1,51	0,82
AT5G52750	"disease resistance-responsive family protein / dirigent family protein"	1,10	1,21
AT1G64160	"zinc finger (C2H2 type) family protein (ZAT11)"	1,48	0,82
AT2G37430	"ATGSTF6 (EARLY RESPONSIVE TO DEHYDRATION 11); glutathione transferase"	1,46	0,83
AT1G02930	"TSA1 (TRYPTOPHAN SYNTHASE ALPHA CHAIN); tryptophan synthase"	1,25	1,03
AT3G54640	"CYP71A18 (cytochrome P450, family 71, subfamily A, polypeptide 18); oxygen binding"	0,87	1,41
AT1G11610	"sulfotransferase family protein"	1,19	1,08
AT1G74100	"O-methyltransferase, putative"	0,97	1,25
AT1G21120	"NIT4 (NITRILASE 4)"	1,19	1,01
AT5G22300	"anthranilate synthase beta subunit, putative", "anthranilate synthase beta subunit, putative", "anthranilate synthase beta subunit, putative"	1,10	1,09
AT1G24807	"AIG2 (AVRRPT2-INDUCED GENE 2)"	1,20	0,97
AT3G28930	"lipase class 3 family protein"	1,10	1,04
AT1G30370	"FAD-binding domain-containing protein"	1,27	0,84
AT1G26420	"protein phosphatase 2C, putative / PP2C, putative"	0,97	1,13
AT2G40180	"unknown protein"	1,02	1,06
AT5G19240	"unknown protein"	1,13	0,93
AT4G40020	"PBP1 (PINOID-BINDING PROTEIN 1); calcium ion binding"	1,22	0,83
AT5G54490	"cinnamoyl-CoA reductase"	1,11	0,94
AT1G76470	"AGC2-1 (OXIDATIVE SIGNAL-INDUCIBLE1); kinase"	1,08	0,96
AT3G25250	"unknown protein"	1,12	0,91
AT3G48640	"ATGSTU12 (Arabidopsis thaliana Glutathione S-transferase (class tau) 12); glutathione transferase"	0,90	1,12
AT1G69920	"DSBA oxidoreductase family protein"	1,16	0,85
AT5G38900	"AAA-type ATPase family protein"	0,84	1,17
AT5G40000			

AT3G53600	"zinc finger (C2H2 type) family protein"	0,94	1,06
AT5G13490	"AAC2 (ADP/ATP CARRIER 2); ATP:ADP antiporter/ binding"	1,06	0,92
AT3G02800	"phosphoprotein phosphatase"	1,03	0,92
AT5G37490	"U-box domain-containing protein"	0,91	1,04
AT5G65600	"legume lectin family protein / protein kinase family protein"	0,99	0,92
AT5G17990	"TRP1 (TRYPTOPHAN BIOSYNTHESIS 1); anthranilate phosphoribosyltransferase"	1,01	0,89
AT1G47510	"endonuclease/exonuclease/phosphatase family protein"	0,97	0,88
AT3G56400	"WRKY70 (WRKY DNA-binding protein 70); transcription factor"	0,83	1,02
AT5G51830	"pfkB-type carbohydrate kinase family protein"	0,99	0,82
AT5G54710	"ankyrin repeat family protein"	0,91	0,88
AT2G31345	"unknown protein"	0,85	0,94
AT2G34500	"CYP710A1 (cytochrome P450, family 710, subfamily A, polypeptide 1); C-22 sterol desaturase/ oxygen binding"	0,87	0,89

**Table 3: List of genes upregulated in common specific to the restoration of the cell adhesion defect by *esmd1-1***

Similar to table 1

Gene ID	CATMA annotation	<i>qua2-1/esmd1-1</i> vs <i>qua2-1</i>	<i>frb1-2/esmd1-1</i> vs <i>frb1-2</i>
	"oxidoreductase, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors"	-1,00	-2,43
AT3G60290		-1,24	-2,24
AT2G38530	"LTP2 (LIPID TRANSFER PROTEIN 2); lipid binding"	-2,25	-2,15
AT3G20470	pseudogene	-1,12	-1,28
AT2G42530	"cold-responsive protein / cold-regulated protein ( <i>cor15b</i> )"	-0,88	-1,16
AT2G46970	"PIL1 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 1); transcription factor"	-0,92	-1,10
AT1G12610	"DDF1 (DWARF AND DELAYED FLOWERING 1); DNA binding / transcription factor"	-0,95	-1,09
AT2G42530	"cold-responsive protein / cold-regulated protein ( <i>cor15b</i> )"	-1,03	-1,06
AT3G16670	"unknown protein"	-1,14	-0,85
AT1G18710	"AtMYB47 (myb domain protein 47); DNA binding / transcription factor"	-0,84	-0,83
AT3G28270	"unknown protein"	-0,85	-0,81
AT4G12545	"protease inhibitor/seed storage/lipid transfer protein (LTP) family protein"	-1,19	-0,79
AT5G44440	"FAD-binding domain-containing protein"	-0,87	-0,78
AT1G78490	"CYP708A3 (cytochrome P450, family 708, subfamily A, polypeptide 3); oxygen binding"	-1,01	-0,74
AT3G14210	"ESM1 (EPITHIOSPECIFIER MODIFIER 1); carboxylic ester hydrolase"	-1,41	-0,71
AT5G03350	"legume lectin family protein"	-0,84	-0,61
AT1G01600	"CYP86A4 (cytochrome P450, family 86, subfamily A, polypeptide 4); oxygen binding"	-1,04	-0,55
AT1G10640	"polygalacturonase, putative / pectinase, putative"		

**Table 4: List of genes downregulated in common specific to the restoration of the cell adhesion defect by *esmd1-1***

Similar to table 1

### III. Experimental procedures

#### *Plant material and growth conditions*

*Arabidopsis thaliana* seedlings were grown at 20°C on solid custom made Duchefa ([www.duchefa-biochemie.com/](http://www.duchefa-biochemie.com/)) medium with various agarose and sucrose concentration depending on the experiment. The seeds were cold-treated for 48 h to synchronize germination, and the plants were grown in a 16 h light/8 h dark cycle. For dark growth conditions, seeds were exposed to light for 4 h to induce germination, after which plates were wrapped in three layers of aluminum foil. Seedling age was counted starting from the light exposure.

Adult plants were grown either in a greenhouse or a growth chamber at 20°C in a 16 h light/8 h dark cycle.

#### *Generation of the ESMD1 and FRB1 Pichia expression constructs.*

Codon optimized sequences for the expression in *Pichia pastoris* were ordered from life technologies (<http://www.lifetechnologies.com/>). These sequences were designed similarly for ESMD1 and FRB1 and correspond to the coding sequence of the gene without the peptide signal and without the C-terminal transmembrane domain. Cloning was done following the instructions from the “EasySelect *Pichia* Expression Kit” (<http://www.lifetechnologies.com/>). Both constructs were cloned into the pPICZ $\alpha$ A expression vector using the 5' EcoRI site and 3' XbaI site to create an in frame fusion with the N-terminal secretion a-factor and the C-terminal c-myc tag followed by the polyhistidine tag. The plasmids were first transformed into *E.coli* for amplification and the sequence and frame were verified. The plasmids were then extracted, purified and transformed into *Pichia* as described in the “EasySelect *Pichia* Expression Kit”. Five to ten transformed strains per construct were assayed for the secretion of the protein in the medium by SDS-PAGE.

#### *Generation of the 35S::WAK:GFP and 35S::EGF-WAK:GFP constructs and transgenic plants*

Constructions were made following the gateway strategy (Invitrogen, <http://www.invitrogen.com>) as described in the *gateway technology* instruction manual from the Gateway cloning kit.

For the *35S::WAK:GFP* constructs, the full length genomic sequences from the traduction initiation site to the codon just before the stop codon of *WAK1-4* were PCR amplified from col-0 using attB flanked primers and the Phusion High-fidelity DNA polymerase (Thermo Fischer Scientific; Cat. No. F-530S).

WAK1attB1 (5'-  
GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGAAGGTGCAGGAGGGTT-3')

WAK2attB1 (5'-  
GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGAAGGTACAGGAGGGTT-3')

WAK3attB1 (5'-  
GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGAAGTTCCAGGAGGGTG-3')

WAK4attB1 (5'-  
GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGAAAGTGCAGCGTCTGT-3')

WAK1/3attB2 (5'-  
GGGGACCACTTTGTACAAGAAAGCTGGGTAGCGGCCAGTTTCAATGTCC-3')

WAK2attB2 (5'-  
GGGGACCACTTTGTACAAGAAAGCTGGGTAACGGCCAGCTTCAATGTCC-3')

WAK4attB2 (5'-  
GGGGACCACTTTGTACAAGAAAGCTGGGTAGCGGCCTGCTTCAATGTCC-3')

The PCR products were first recombined into the pDONR207 donor vector and later in the pGWB5 destination vector placing the gene under the CMVp35S promoter and in frame with a C-terminal GFP. After each steps the constructs were transformed into *E. coli* for amplification and the sequences were verified. Finally they were transformed into the C58 strain of *A. tumefaciens* and used for the transformation of *Arabidopsis thaliana* plants by the floral dip method (Clough and Bent, 1998). T1 transformants were selected on 50 mg mL<sup>-1</sup> hygromycin and T2 plants were used for the experiments after the selection by western blot analysis with an anti-GFP antibody of the plants expressing the fusion protein.

For *35S::EGF-WAK:GFP* constructs, the signal peptide and the EGF1-EGF2-transmembrane domain were first amplified independently. The construct was done for WAK1 and WAK2. Using an attB1 flanked primer in 5' of the signal peptide, an attB2 flanked primer in 3' of the EGF1-EGF2-TM, and overlapping primers were designed that overlap the 3' of the signal peptide and the 5' of the EGF1-EGF2-TM.

SP-WAK1attB1 (*W1sigFW* *attB1*) (5'-  
 GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGAAGGTGCAGGAGGGTTT-3')  
 SP-EGF-WAK1overlapRV (*W1sigRV*) (5'-  
 GTCTGATTTCCAACAGACCACCCCTTCACCAGCTGCGTACA-3')  
 SP-EGF-WAK1overlapFW (*W1egfFW*) (5'-  
 TGTACGCAGCTGGTGAAGGGGTGGTCTGTTGGAAATCAGAC-3')  
 EGF-TM-WAK1attB2 (*W1egfRV* *attB2*) (5'-  
 GGGGACCACTTTGTACAAGAAAGCTGGGTACAGGTGCTTCATTCTCTGT-3')  
 SP-WAK2attB1 (*W2sigFW* *attB1*) (5'-  
 GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGAAGGTACAGGAGGGTTT-3')  
 SP-EGF-WAK2overlapRV (*W2sigRV*) (5'-  
 GTCTTGTCTCCGATAGACCACCCCTTGACTAGCTGCGTAT-3')  
 SP-EGF-WAK2overlapFW (*W2egfFW*) (5'-  
 ATACGCAGCTAGTCAAGGGGTGGTCTATCGGAGACAAGAC-3')  
 EGF-TM-WAK2attB2 (*W2egfRV* *attB2*) (5'-  
 GGGGACCACTTTGTACAAGAAAGCTGGGTATGTGTTCTTCCGGTGCTTA-3')

The reverse overlapping primer was used to amplify the signal peptide with the attB1 primer. And forward overlapping primer was used to amplify the EGF1-EGF2-TM with the attB2 primer. Independent PCR amplification of the two fragments was done on col-0 cDNA for 35 cycles. A subset of the product of both reactions was then pool and used as template for PCR amplification with the attB1 and attB2 primers, in order to obtain the chimeric fragment containing the signal peptide followed by the EGF1-EGF2-TM. PCR amplifications were done using the Phusion High-fidelity DNA polymerase (Thermo Fischer Scientific; Cat. No. F-530S).

Then a similar strategy was used as for the *35S::WAK:GFP* constructs except that the destination vector used was the pMDC83 which also places the gene under the CMVp35S promoter and in frame with a C-terminal GFP, but is overall a smaller plasmid more suited for transient protoplast transformation.

#### *Generation pEMSD1::GUS constructs, and GUS staining*

Constructions were made following the gateway strategy as described above.

The *ESMD1* promoter was amplified using the gateway primers ESMD1 attB1 promoter (*esm attB1pr*) (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGGACGAGGACATCCTTGGTA-3') and ESMD1 attB2 promoter (*esm attB2pr*) (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTATCGACAGATCTCAATCTC-3') as described above into the pGWB3 destination vector placing the *uidA* reporter gene under the control of the *ESMD1* 2168pb upstream sequence. T2 plants were used for the experiments after the selection of a representative line in terms of GUS staining pattern and with a 3:1 segregation of the GUS coloration indicating a single insertion locus of the construct. GUS assays were performed as described in Jefferson et al (1987) on seedlings, and fixed in 100% ethanol.

#### *Protein extraction and buffers*

Protein extraction was carried out either on seedlings or on adult plant leaf discs. In the case of adult plants leaf discs, they were harvested in the greenhouse and immediately frozen in liquid nitrogen. Seedlings were also flash frozen in liquid nitrogen before grinding. From the harvest point, the samples were handled in liquid nitrogen, and on ice after grinding. The tissues were ground using a mortar and pestle. Depending on the experiments, the tissues were either ground in 50mM TrisHCl pH6.8, 50mM TrisHCl pH6.8 supplemented with anti-protease cocktail (cOmplete ULTRA Tablets, mini, EDTA-free, EASYpack; <http://lifescience.roche.com/>), directly into 1X Laemmli, or 1X modified Laemmli for WAK extraction (see below for composition). When different fractions were separated, the samples were first ground in 50mM TrisHCl pH6.8 supplemented with anti-protease cocktail, centrifuge at 10,000g for 10 minutes and the cell wall fraction was additionally extracted with either the 4%SDS / 50mM DTT WAK extraction buffer and boiled for 10 minutes, 0.05M CDTA (1,2-diaminocyclohexanetetraacetic acid) for 2h at room temperature, 0.05M Na<sub>2</sub>CO<sub>3</sub> for 14h at room temperature, or the protoplast cell wall degradation cocktail for 16h at room temperature. The samples were then re-centrifuged at 10,000g for 10 minutes and the different fraction separated. Finally the samples that were not directly ground in Laemmli were supplemented with 1 volume of 2X Laemmli, and all the samples were boiled for 10 minutes before gel migration.

Laemmli1X: 50mM TrisHCl pH6.8, 2% SDS, 10% glycerol, 0,01% Bromophenol Blue

Laemmli2X: 50mM TrisHCl pH6.8, 4% SDS, 20% glycerol 0,02% Bromophenol Blue

WAK extraction buffer: 50mM TrisHCl pH6.8, 50mM DTT, 4% SDS, 10% glycerol

Laemmli 1X WAK: Same as 4%SDS/50mM DTT WAK extraction buffer with 0,01% Bromophenol Blue

Protoplast cell wall degradation cocktail: 50mM TrisHCl pH6.8, 0.1% Onozuka cellulase R10 (Duchefa), 0.015% Macerozyme R10 (Duchefa) and 0.02% driselase (Sigma-Aldrich)

Protoplastes (see preparation and transient transformation below) were lysed by adding 800µl of lysis buffer to 200µl protoplast suspension.

Protoplast lysis buffer : 10mM TrisHCl pH8, 1mM EDTA, 150mM NaCl and 1,25% (V/V) Triton X100.

Microsomes were prepared by centrifugation of the lysate for 10 min at 10,000G. The supernatant was then ultracentrifuged for 1h at 100,000g. 1X Laemmli was then added to the microsomal pellet and the samples were boiled for 10 minutes before gel migration.

### *GFP trap*

Immunoprecipitation of the GFP-fusion protein was done with the GFP-Trap<sup>®</sup> \_M kit from Chromotek ([www.chromotek.com](http://www.chromotek.com)), following the instructions provided in the manual.

### *Western blot analysis*

The extracts were run on 10% acrylamide gels for various times depending on the expected size of the protein of interest, variable voltage and 60mA intensity. The PageRuler protein ladder (Thermo scientific) was used. Semi dry transfer of the protein was carried out onto nitrocellulose c-hybond extra membrane (Amersham) using a caproic acid three phase buffer for 1 h and with various voltage and amperage depending on the surface of transfer (Kurien and Scofield, 2003). The membrane was then saturated in 5% milk powder in phosphate-buffered saline (MPBS) for 1h at room temperature. Primary antibody incubation was done in MPBS containing 1/5000 AbCAM A290 anti-GFP antibody (<http://www.abcam.com/>) for 1h at room temperature. The membrane was washed 3 times 10 minutes in PBS. Secondary antibody incubation was done in MPBS containing 1/10000 Santa Cruz Biotechnology anti-

rabbit HRP grafted antibody (<http://www.scbt.com/>) for 1h at room temperature. The membrane was washed 3 times 10 minutes in PBS. The ECL Select GE Healthcare (<http://www.gelifesciences.com/>) was used for revelation and imaging of the membranes was done with the ImageQuant LAS4000.

### *Click chemistry*

The seedlings were grown on a medium containing 2.5  $\mu$ M Fucose Alkyne (<https://www.lifetechnologies.com/>) as described in Anderson et al (2012). The click reaction was done either on a protein extract or on a blot membrane after transfer.

Seventy six microliter of 1M TrisHCl pH8 and 1.5  $\mu$ l of Alexa Fluor  $\text{\textcircled{R}}$  488 Azide (<https://www.lifetechnologies.com/>) or Biotin Azide (<https://www.lifetechnologies.com/>) were added to 50  $\mu$ l of the protein extract. Seven and a half microliter of 10 mM CuSo<sub>4</sub> was then added and the mix was vortexed. Seven and a half microliter of 20 mM TBTA (Tris(benzyltriazolylmethyl)amine) was then added and the mix was vortexed. After 2-3 minutes incubation at room temperature, 7,5  $\mu$ L of 40mM Ascorbic acid was then added and the mix was vortexed. The mix was then incubated a in the dark at room temperature for 20 minutes.

After boiling in laemmli the extracts were migrated on gel and transferred onto a nitrocellulose membrane. In the case of the Alexa Fluor  $\text{\textcircled{R}}$  488 Azide the membrane was then scanned in a fluorescence scanner. In the case of the Biotin Azide, the signal was detected with a HRP grafted streptavidin and as described in the *western blot* section.

For the click reaction directly on membrane the same reagents were used but in a single mix and with the addition of 50  $\mu$ l water (instead of the 50  $\mu$ l protein extract), spread on the blot membrane and incubated a in the dark at room temperature for 20 minutes. Signal observation was done as described above.

### *Protoplast preparation and transient transformation*

The protoplasts were prepared and transformed following either Axelos et al (1992) on a Col-0 cell suspension culture available at the institute or following Yoo et al. (2007) on rosette leaves from 4 to 5 weeks old plants grown in the greenhouse in short days conditions.



Confocal microscopy observation or western blot analyses were performed approximately 16h-24h after transformation.

#### *FTIR*

Four-day-old dark grown hypocotyls were squashed between two BaF<sub>2</sub> windows and abundantly rinsed in distilled water for 2 min. The samples were then dried on the window at 37°C for 20 min. An area of 50 mm X 50 mm halfway the hypocotyl, on the side of the central cylinder, was selected for spectra collection. Spectra were collected using a ThermoNicolet Nexus spectrometer with a Continuum microscope accessory. Fifty interferograms were collected in transmission mode with 8 cm<sup>-1</sup> resolution and co-added to improve the signal-to-noise ratio of the spectrum.

#### *Cell wall monosaccharides composition and uronic acid content*

The polysaccharides analyses were performed on an alcohol insoluble material prepared as follows. Hundred mg (FW) of ground 8d old light grown seedlings grown on a medium without sucrose, were washed twice in 4 volumes of absolute ethanol for 15 min, then rinsed twice in 4 volumes of acetone at room temperature for 10 min and left to dry under a fume hood overnight at room temperature. Neutral monosaccharide composition was performed on 5 mg of dried alcohol insoluble material after hydrolysis in 2.5 M trifluoroacetic acid (TFA) for 1.5 h at 100 °C as described in (Harholt et al., 2006). Quantification was performed using HPAEC-PAD chromatography as described in (Harholt et al., 2006).

Galacturonic acid content was measured as described in Part I of this chapter and following the quantification method described in Blumenkrantz & Asboe-Hansen (1973).

#### *RNA preparation for the microarray analysis*

The seedlings were grown in vitro in a liquid medium without sucrose. After 8 days about 20 seedlings per genotype and biological replicate, were flash frozen in liquid nitrogen. Three biological replicates were done. Total RNA isolation was carried out with the RNeasy plant mini kit (qiagen) according to the manufacturer's instruction and with the on column DNA digestion using Rnase-Free DNase (qiagen). The biological replicates were then pooled for

economic reasons. The microarray hybridation and data extraction were performed at the URGV transcriptomic platform (<http://www-urgv.versailles.inra.fr/microarray/>), on CATMA V7 chips. All raw and normalized data will be available through the CATdb database (Gagnot et al., 2008) and from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) (Barrett et al., 2007) when deposited.

#### *OG induction, RNA extraction, cDNA synthesis and qPCR quantification of gene expression*

Seedlings were grown in vitro in a liquid medium without sucrose. After 8 days the seedling were treated by adding oligogalacturonides (OGs) degree of polymerization >9 to a final concentration of 50  $\mu\text{g}\cdot\text{ml}^{-1}$ , or were not treated. After three hours induction, the plants, about 20 seedlings per conditions and biological replicate, were flash frozen in liquid nitrogen. Three biological replicates were done. Total RNA isolation was carried out with the RNeasy plant mini kit (qiagen) according to the manufacturer's instruction and with the on column DNA digestion using Rnase-Free DNase (qiagen). cDNA was synthesized using 1  $\mu\text{g}$  of total RNA and the SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Real time PCR reactions were performed in CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using the SsoAdvanced™ Universal SYBR® Green Supermix, 25-100ng cDNA template and 250 nM of each gene-specific primer in a final reaction volume of 15  $\mu\text{L}$ . The primers used for FADLox (At1g26380) and PAD3 (At3g26830) are those described in Denoux et al. (2008). GADPH (At1g13440) and UBQ10 (At4g05320) were used as reference genes with the primers described in Czechowski et al (2005). Each sample reaction was run in three technical replicates. The Ct values of the tested genes were normalized to the reference genes and each reference-normalized samples were then normalized to the wild type non treated samples and the fold change in expression level were calculated. Relative expression of the RT-PCR products was determined using the DDCT method (Livak and Schmittgen, 2001).

#### *Botrytis maceration assay*

*B. cinerea*, strain B05, spores were harvested in a Potato Dextrose Broth (PDB) liquid medium from a 10d old culture on a petri dish. The harvested spores were filtered on a 25  $\mu\text{m}$  nylon mesh, diluted to a concentration of  $5 \cdot 10^5$  spores/ml in PDB and germinated for 3h. Inoculation with two drops of 10  $\mu\text{l}$  of spore suspension per leaf was done on 6 weeks old

leaves from four independent plants per genotypes, placed in a petri dish on a wet whatman paper. The mock treatment was done with PBD alone. Observations were made 48 and 96 hours after inoculation.

#### *Laser scanning confocal microscopy*

Images were collected using a spectral Leica SP2 AOBS confocal microscope (Leica Microsystems, <http://www.leica-microsystems.com>) equipped with an argon laser. For GFP, fm4-64 and chlorophyll autofluorescence observation, excitation was performed using the argon ion laser at 488 nm, and fluorescence was detected at 495–540 nm (GFP) and 560–620 nm (fm4-64 and chlorophyll autofluorescence).

#### **Acknowledgments**

I am thankful to Thierry Desprez for the training and advices for the design of the different constructs, to Gwendal Cueff for the training and advices on the western blot analysis, to Elodie Akary for the training and advices on the protoplast transformation and to Marie Christine Soulié for the training and advices on the *Botrytis* maceration assay. The Oligogalacturonans were kindly provided by P. Van Cutsem (University of Namur, Namur, Belgium), and the B05 *B. cinerea* strain was kindly provided by Marie Christine Soulié.

#### **Contributions**

G.M. and S.V. conceived and designed the experiments. S.C. and S.V. performed most of the experiment with inputs from G.M. The monosaccharide composition analysis of neutral sugar was performed by Z.L., and the microarray chip hybridization and data treatment were done by C.L..



**Chapter III:**  
Chemical genomic dissection of the cell  
adhesion mechanisms and sucrose  
sensitivity

---

**Preamble:**

The second part of my PhD project consisted in the screening and the characterization of small molecules with a suppressor effect on the cell adhesion defect and/or sucrose sensitivity of the mutants *quasimodo1* and *quasimodo2*.

In order to uncouple the two most prominent phenotypes of the *quasimodo* mutants, I realized two screens in parallel. The screening consisted in the identification of small molecules that restored specifically the cell adhesion defect of *quasimodo* in one screen and molecules that restored specifically the sucrose sensitivity of *quasimodo* in the other screen. The rationale of this approach was to isolate the chemical tools to dissect the phenotypes of *quasimodo*.

For this work I had the opportunity to realize the chemical genomic screening in Stéphanie Robert's lab (UPSC, Umeå, Sweden). I then undertook the characterization of the small molecules. In the context of this work I supervised Clément Férésini, a Master1 student that carried out some of the experiment that are reported in this chapter.

In this chapter\* I describe the chemical genomic screening, secondary screenings, and the identification of the target (or putative target for some) of some of the molecules that were isolated in the screen. These result reveal that the inhibition of auxin transport as well as the inhibition of the pectin methyl esterase activity are sufficient to restore the phenotype of *quasimodo*.

*\*This chapter is written under the form of a publication and will be submitted for publication after minor changes, replication of some experiments and the addition of some of the complementary experiment that are suggested as "interesting" in the results and discussion.*

# High throughput chemical genomics highlights the role of auxin transport and pectin methyl esterase activity in plant cell adhesion and carbon/nitrogen balance perception

Stéphane Verger<sup>1,2</sup>, Clément Feresini<sup>1,2</sup>, Mélanie L'Enfant<sup>3</sup>, Stéphanie Robert<sup>4</sup>, Jérôme Pelloux<sup>3</sup>, Grégory Mouille<sup>1,2</sup> \*

<sup>1</sup> INRA, Institut Jean-Pierre Bourgin, UMR1318 Saclay Plant Sciences, 78026 Versailles, France

<sup>2</sup> AgroParisTech, Institut Jean-Pierre Bourgin, UMR1318 Saclay Plant Sciences, 78026 Versailles, France

<sup>3</sup> EA3900 BIOPI Biologie des Plantes et Innovation, Université de Picardie Jules Verne, F-80039 Amiens, France

<sup>4</sup> Umeå Plant Science Center, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83, Umeå, Sweden

\* Correspondence to: [gregory.mouille@versailles.inra.fr](mailto:gregory.mouille@versailles.inra.fr)

*To be submitted for publication*

## abstract

A number of studies on the processes of cell separation in developmentally regulated events such as floral organs abscission have shed some light on what controls cell separation in these specific events. On the other hand, we have a rough idea of how cells stay attached to each other in plants, but the precise mechanisms and the molecular players that regulate and maintain cell adhesion during growth and development, when the cell wall is constantly synthesized and remodeled are still not clear. *quasimodo1* and *quasimodo2* are *Arabidopsis thaliana* mutants that are deficient in pectin synthesis and that are affected in cell adhesion. Here we report the chemical genomics screening for suppressor molecules that are able to restore *quasimodo* cell adhesion defect and *quasimodo* sensitivity to carbon/nitrogen (C/N) imbalance. We were able to efficiently identify the putative activity of some of these suppressor compounds and confirmed, among other hypotheses, that the inhibition of auxin

transport and pectin methyl esterase (PME) activity were both sufficient to suppress the phenotype of *quasimodo*. We also tested the direct inhibitory effect of our hit compounds on the activity of purified PMEs, and have found that approximately one third of them were direct inhibitors of PME activity *in vitro* and did induce an increase in methyl esterification of the cell wall upon treatment. While this approach allowed us to identify the actual target of a number of the suppressor compounds, and leaves us with a shorter list of compounds to focus on for future work, it also provides interesting insights on the role of auxin transport and PME activity on the mechanisms of cell adhesion and C/N balance perception in plants.

## Introduction

Cell adhesion in plants seems to depend mostly on the accumulation of pectin in the cell plate during the cell division (Jarvis et al., 2003). The retention of cell adhesion after cell division seems then be due to a lack of active degradation of this intercellular interface, the middle lamella, as well as a specific stiffening of the cell wall at the junction of three adjacent cells (Jarvis et al., 1998; Willats et al., 2001). Although cell adhesion is maintained in most tissues throughout plant's development, cell separation occurs in certain organs or for certain specific events in plant development such as the pollen grain separation, petal shedding, fruit maturation, dehiscence, lateral root emergence and root cap detachment (Robert et al., 2002; estornell et al., 2013). These cell separation events are starting to be well understood and usually implicate ethylene and auxin, antagonistically inducing a peptide ligand triggered signaling pathway leading to the expression of cell wall remodeling enzymes to dissolve the middle lamella and promote cell separation (Aalen et al., 2013).

The homogalacturonan (HG), a homopolymer of (1→4)- $\alpha$ -linked galacturonic acids, is the simplest and most abundant form of pectin (Voragen et al., 2009). Its interesting cross linking properties and the fact that it is mostly this type of pectin that accumulates at the middle lamella, makes it a particularly good candidate for being responsible for cell adhesion. HG is synthesized under a highly methyl esterified form and the key in the functional properties of HG is its ability to be actively modified and remodeled by endogenous apoplastic enzymes after it has been deposited to the cell wall (Sénéchal et al., 2014). HGs are mostly subjected to the Pectin Methyl Esterases (PME) (Micheli et al., 2001; Pelloux et al., 2007) that can remove the methyl ester groups in a random or in a blockwise manner. Long-enough stretches of de-



methyl esterified HG can lead to the formation of crosslinks between two HG chains via the formation of calcium bridges (Willats et al., 2001), potentially leading to the stiffening of the cell wall. However, a more random pattern of de-methyl esterification renders the HG more sensitive to degradation by polygalacturonases (PG), potentially leading to the loosening of the cell wall. In Addition, HG degradation by PG should lead to the formation of oligogalacturonans (OGs). These small pectin fragments are known elicitors of the defense responses, and act antagonistically to auxin to influence plant's growth and development (Savatin et al., 2011; Ferrari et al., 2013). The modifications catalyzed by the PGs and PMEs along with the pectin acetyl esterases (PAE) and pectate lyases (PL), have a huge impact on the pectin structure and physicochemical properties (Willats et al., 2001), as well as on signaling (Wolf et al., 2012a). Interestingly these modifications in structure, physicochemical properties and signaling play a major role in controlling plant growth and development (Wolf et al., 2009) and also seem to be implicated in the control of cell adhesion.

A number of mutants have been shown to present specific defects in cell adhesion or separation. Most of them are defective in cell separation during developmentally regulated events such as the floral organ abscission or the pollen grain separation. These mutants are usually affected in either upstream receptor of an endogenous signal for triggering cell separation (Stenvik et al., 2008), downstream regulators of the response (Cho et al., 2008; Shi et al., 2011), or actual cell wall remodeling enzymes implicated in cell separation. Among these last ones are for example *quartet1, 2 and 3*, respectively mutated in a PME and in two PGs, in which disruption of the activity prevents the separation of the four pollen grains after meiosis, by indirectly (PME) or directly (PG) preventing the degradation of the HG between the pollen grains (Rhee et al., 2003; Francis et al., 2006; Ogawa et al., 2009).

On the other hand, some mutants show a defect in cell adhesion when the cells should remain attached. Mutations in *QUASIMODO1/GAUT8* and *QUASOMODO2/TSD2/OSU1* genes, respectively a putative galacturonosyltransferase of the GT8 family of glycosyltransferases, and a putative pectin methyltransferase, lead in both cases to about 50% reduction in HG content and a clear cells detachments phenotype (Bouton et al., 2002; Orfila et al., 2005; lebeouf et al., 2005; Mouille et al., 2007). In addition these two mutants are very sensitive to C/N imbalance (Gao et al., 2008), and have a tendency to develop callus instead of normal leaves and stems (Krupkova et al., 2007). Another cell adhesion defective mutant is *friable1*. It is affected in a putative O-fucosyltransferase (Neumetzler et al., 2012) and although the precise function of the gene affected could not be elucidated, the mutant presents various

modifications in the cell wall structure and composition with one of the major feature being a higher PME activity and a lower degree of pectin methyl esterification (Neumetzler et al., 2012).

Classical forward genetic screening has proven very powerful in order to isolate important genes implicated in cell adhesion, as is exemplified above. However it is usually limited to the identification of non redundant genes and non lethal mutations. In this regard, chemical genomics provides a way to circumvent these limitations (for reviews see (Robert et al., 2009; Tóth and van der Hoorn, 2010; Hicks and Raikhel, 2012)). The principle behind this approach is that small organic compounds with the capacity to pass through the membranes, can bind and disrupt the function of proteins (e.g. inhibition of catalytic activity or protein-protein interaction), with an effect comparable to a mutation. The advantages are that it may affect all or part of a redundant family of proteins, it can be added at any time point to induce its effect, the intensity of its effect should be dose dependent, and it can be removed by washing off of the molecule to stop the effect. The idea then is to screen for the effect of a large number of highly diverse drug-like compounds looking for those that induce the phenotype of interest. Overall it both provides a complementary approach to the genetic screening by affecting different types of genes, and it provides very useful tools for the study of a given pathway. The problem however, is that finding an interesting chemical doesn't directly inform on the protein target that it binds to or the pathway that it affects. Biochemical approaches to purify the target protein and/or insensitivity genetics screening, among other approaches (Dejonghe and Russinova, 2014) are usually necessary to characterize the compound. Keeping in mind that the goal is either to use the molecule as a tool or to answer the biological question that was originally asked by doing the screening, it can turn out to be quite a lengthy process before being informative. An additional problem is that the chemical genomic screening may yield a large number of potentially interesting molecules with various structures. It may be difficult to decide which molecule to focus on for in depth characterization or in other words, which one will be the best choice and should give new and interesting output given that further work on it will be time consuming. The classical answer to this problem is to do a secondary screening, focusing on another aspect of the studied phenotype in order to refine which subset of the hit compounds may be the most interesting. But other approaches can be useful too. In addition to a large scale screening (with a large library of diverse compounds), the screening of a small, high-content, prescreened and annotated library of compounds can provide very useful information. Chemical genomics has relatively recently started to interest

plants biologist for basic research, but the literature already describes a large number of molecules for which the targets and pathways affected have been elucidated. Looking for the structure similarities with already characterized compounds represent another strategy that can help elucidate the pathway affected by a hit compounds.

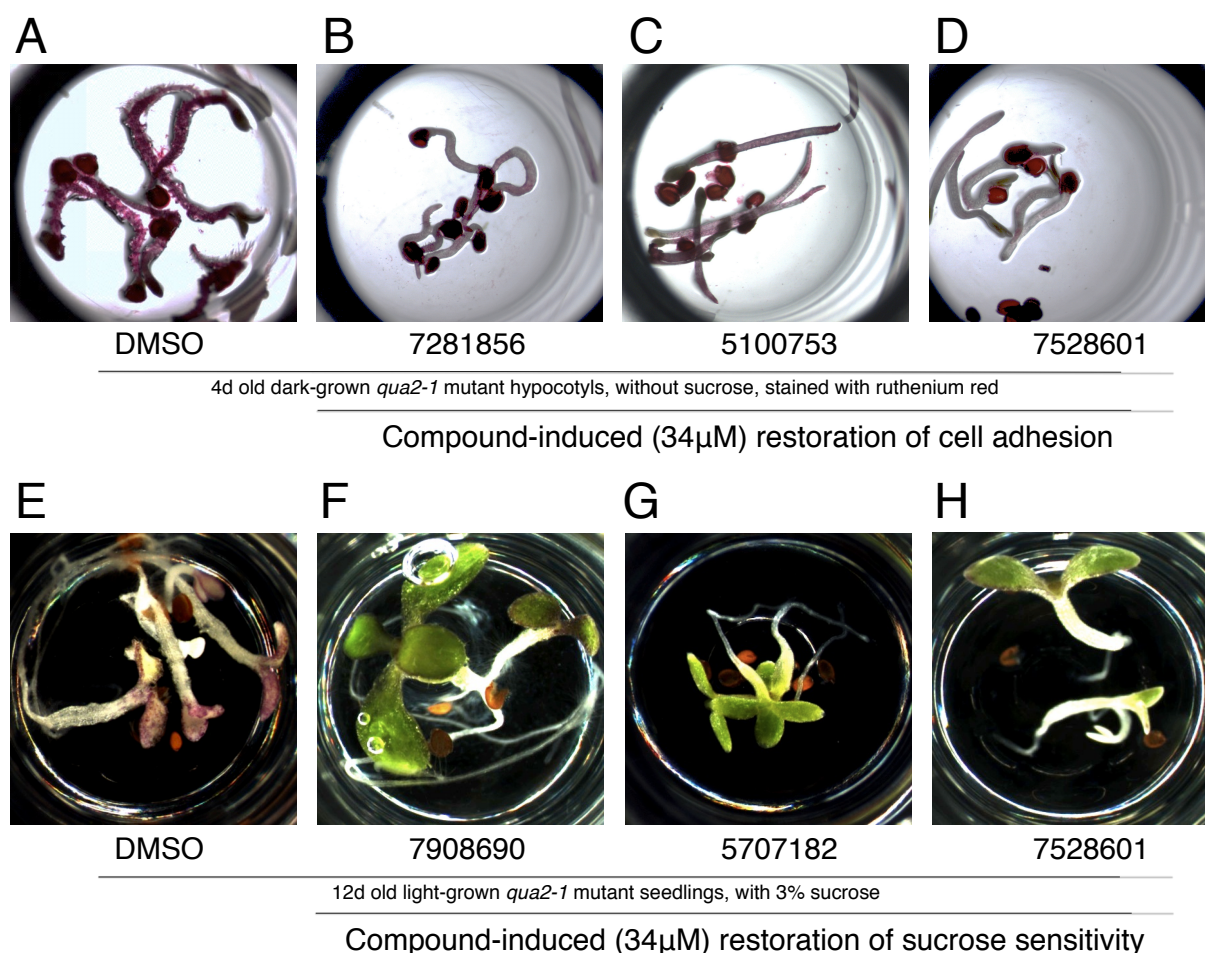
In order to get a better understanding of the mechanisms of cell adhesion and C/N balance perception in plants, we have carried out a chemical genomics suppressor screen for molecules that were able to restore *quasimodo2* cell adhesion defect and *quasimodo2* high sensitivity to sucrose in two independent screens. The high throughput screening led to the identification of 49 suppressor compounds. We then identified the putative function of a number of these compounds based the information inferred from molecules screened from a small annotated library of compounds, the similarity of structure with other characterized compounds and a well chosen high throughput secondary screening. Among other hypotheses, we then focused on the putative effect of auxin transport inhibition and PME activity inhibition and confirmed their implication in the restoration of cell adhesion and sensitivity to C/N imbalance in *quasimodo2*. We also tested the *in vitro* inhibitory effect of our hit compounds on purified PMEs, and have found that 17 of the 49 compounds were indeed direct inhibitors of PME activity *in vitro* and did induce an increase in methyl esterification of the cell wall *in vivo*.

## Results

### **High throughput chemical genomic screening for restored cell adhesion and carbon/nitrogen imbalance sensitivity in the pectin deficient mutant *quasimodo2***

We used the library of 17,500 diverse synthetic compounds from the chemical screening platform of the laboratory for chemical biology Umea (LCBU, Umea University, Sweden) as well as a small library of 360 compounds that were selected for their inhibitor effect on germination and growth of tobacco pollen (PMRA/PMRP) (Robert et al., 2008; Drakakaki et al., 2011).

On a technical point of view, our aim was to carry out a very high throughput primary screening for molecules of interest and try to uncouple the two phenotypes that are the consequence of the *quasimodo* mutation. For this latter reason, we did two screens in parallel:



**Figure 1: High throughput chemical genomic screening for the restoration of cell adhesion and sucrose sensitivity**

(A-D) Four days old dark grown hypocotyls of the *qua2-1* mutants treated with DMSO for control (A) or with suppressor compounds (B-D). The seedlings were stained with ruthenium red to reveal the presence or restoration of the cell adhesion defect. A characteristic outward-curving of the epidermal cells can also be seen in the *quasimodo* mutant, which is characteristic of the cell adhesion defect when observed on the dark grown hypocotyl. This phenotype is restored by the suppressor compounds as well, showing actual restoration of cell adhesion and not simply the inhibition of the ruthenium red staining. (E-H) Twelve days old light grown seedlings of the *qua2-1* mutant grown with 3% sucrose, treated with DMSO for control (E) or with suppressor chemicals (F-H). The mutant is very sensitive to C/N imbalance in the growth medium, accumulates anthocyanin (not to be confused with ruthenium red staining) and has no chlorophyll (E), while the suppressor compounds induce a suppression of these phenotypes (F-H). Note that compound 7528601 (D and H) has an effect on both phenotypes while the other four compounds taken as example here (B, C, F and G) were specific for either cell adhesion or sucrose sensitivity. The chemicals' names correspond to their ChemBridge ID (<http://www.hit2lead.com/>)

One was based on the restoration of cell adhesion and the second one on the restoration of sucrose sensitivity.

In order to achieve a high throughput screening, the *qua2-1* seeds were sown by pipetting from a liquid medium into 96-well plates with 5 to 10 seeds and 150μl of medium per well. The chemicals were then added to a final concentration of 34μM by an automated workstation

(Biomek NX, BeckmanCoulter), and the plants were grown as described on figure 1 and in the “material and methods” section for each screening conditions.

Efficient screening for the restoration of cell adhesion was done on dark grown hypocotyls stained with ruthenium red. The principle is that the cell separation creates ruptures in the continuity of the hydrophobic cuticle, which directly exposes the cell wall and allows the entry and binding of the ruthenium red, thus highlighting the cell adhesion defect. We screened for seedlings that didn't stain red as opposed to the *quasimodo* mutant (figure 1 A-D). In addition, a characteristic outward-curving of a number of the epidermal cells appears in the *quasimodo* mutants' hypocotyl, due the cell adhesion defect and likely to the high expansion rate of the hypocotyl. These curved cells appear clearly at the surface of the cell adhesion defective mutant as can be seen on figure1 A and give an impression of a “hairy” and irregular hypocotyl. Restoration of this phenotype was also checked to ensure that the effect was specific to the restoration of cell adhesion and not only the inhibition of ruthenium red binding to the cell wall. The suppression of the C/N imbalance sensitivity of light grown *qua2-1* seedlings was determined by the restoration of growth and greening of the mutant seedlings in a high sucrose (3%) medium (figure 1 E-H). The high C/N ratio in the growth medium leads to the accumulation of anthocyanins in the cotyledons, the loss of chlorophyll and the arrest of growth in the mutant. We thus screened for the suppression of the sensitivity to a high sucrose concentration but that should account for the restoration C/N imbalance sensitivity.

This high throughput screening strategy allowed us to screen twice (once for each condition), the 17,500 LCBU compounds and the 360 PMRA/PMRP compounds. Hit compounds found in the initial screening were confirmed by retesting their effect with the same chemicals but from a copy of the library used for the initial screening, and those that were confirmed were finally re-confirmed by testing their effect from pure powder of the compound freshly solubilized in DMSO, to ensure that the effect is caused by the pure molecule and not another one, contaminating from an adjacent well in the screening plate, the mix of two or more molecules, or the degradation product of the original molecule.

In the end 15 molecules were found in the screen for the suppression of *quasimodo2* sucrose sensitivity, and 35 in the screen for the suppression of the cell adhesion defect. Only 1 molecule was found in common in both screening conditions. In total 49 molecules were found as active for suppressing *qua2* phenotype in one way or another. However, since for technical reasons the screening process was visual and did not include an objective and

quantitative phenotyping method, we placed the threshold for selection of hits very high and may have missed some less active suppressor compounds. Also some molecules found in one screening condition may have an effect on the phenotype of the other screening condition but may not have been found as a hit in this screen due to the high selective threshold. We thus re-tested all the compounds found in each screens for both phenotypes, with three biological replicates. We then determined whether the compounds had an effect specifically on cell adhesion, sucrose sensitivity, or both phenotypes. Interestingly 31 of the compounds only restored cell adhesion, 7 only restored sucrose sensitivity, and 11 restored both phenotype (table 1). However it is noteworthy that only 4 of these compounds that had an effect on both phenotypes, were very clear for both effect. The 7 other compounds usually had a strong effect for the phenotype that they were screened for and a mild effect on the other phenotype. This interestingly proves that both phenotypes can be uncoupled, and that thanks to this double screening approach we have found molecules that likely have an upstream effect (compound that alter both phenotypes), and some with a more downstream effect (compounds that affect specifically C/N imbalance sensitivity or cell adhesion) which will be very useful tools to molecularly dissect and understand the phenotype of the *quasimodo* mutants.

### **Determining the putative bioactivity of the compounds based on available resources**

The screening yielded a large number of compounds, some that we could group based on their similar structure (Visually and using the “binning clustering” ChemMine Tool, <http://chemmine.ucr.edu/>, see figure 2, 5 and table 1 “Structural cluster”), and others with very diverse structures. However at this point it is extremely difficult to choose one compound and focus on it for further in-depth work. So our aim here was to determine whether some of the compounds that we had found as active, or close analogs of these compounds, had already been identified as active for another phenotype, and characterized for their target and the pathway they affect. The goal being to avoid working on the characterization of compounds that have already been characterized, and at the same time using this resource to quickly identify the protein target or pathway affected by some of the chemicals that we found and that are relevant to our biological question. We screened and found some active compounds from the PMRA/PMRP library. The great advantage of screening this library is that it has been secondarily screened and carefully annotated for various disruptions of the plasma membrane endocytic trafficking by looking at markers such

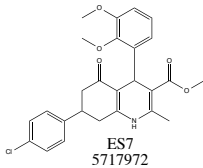
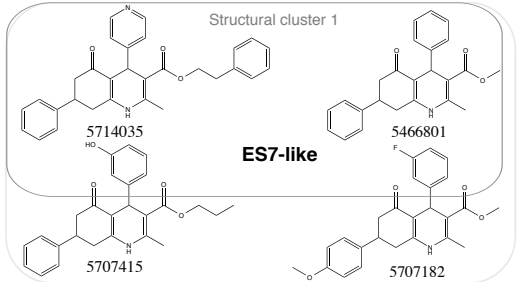

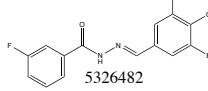
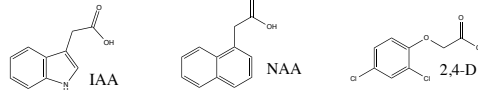
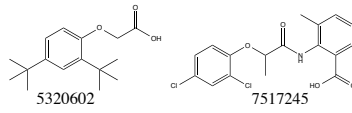
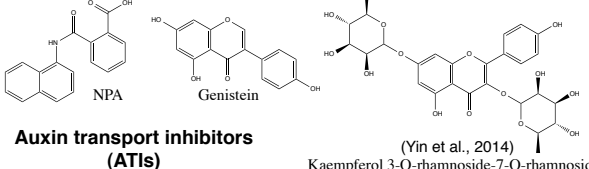
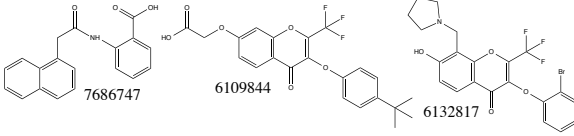

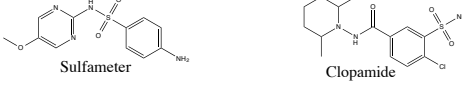
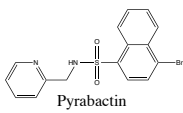
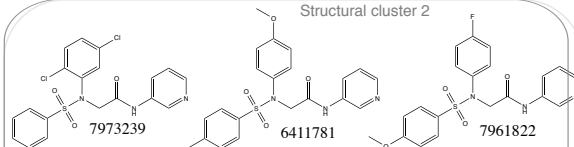
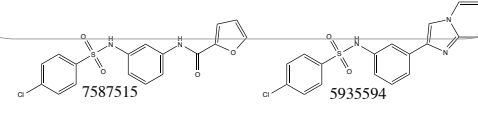
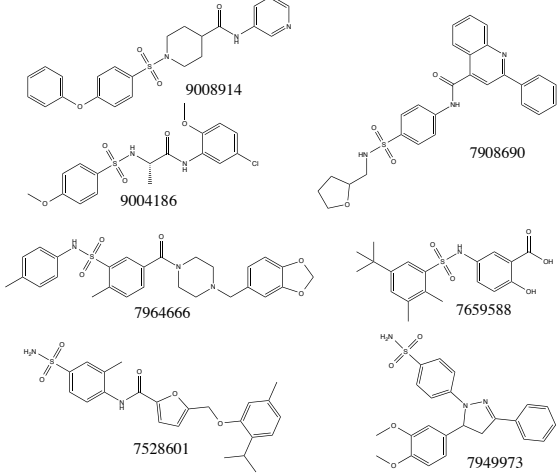
as BRI1:GFP, PIN1:GFP and PIN2:GFP (Drakakaki et al., 2011). Four hits were found from this library (5326482, 5673258, 6740368 and 5714035), among which three had an effect on trafficking (5326482, 5673258 and 5714035), and one did not (6740368), as reported in Drakakaki et al. (2011).

- Interestingly compound 5714035 is very similar in structure with Endosidin7 (ES7; figure 2), also originating from the PMRA/PMRP library, which was recently characterized as a callose synthase inhibitor (Park et al., 2014). Annotation for the disruptions of the plasma membrane endocytic trafficking indicates that, like ES7, compound 5714035 affects cell plate formation, which is characteristic of callose synthesis deficiency (Chen et al., 2009; Thiele et al., 2009). However, unlike ES7, compound 5714035 also affects BRI1, PIN1 and PIN2 trafficking. Screening of the LCBU library also identified 3 very similar compounds (5466801, 5707182 and 5707415; figure 2). In our screen, these four compounds are active in restoring both cell adhesion and sucrose sensitivity.

- Compound 5326482 has some similarities with ES3 (figure 2). In addition clustering based on the phenotypic annotations from Drakakaki et al (2011), groups it with the Endosidin-like compounds including ES3 and ES1. ES1 was shown to induce the accumulation of PIN2, AUX1 and BRI1 into endomembrane compartment called “endosidin-bodies” (Robert et al., 2008). ES1 also represses brassinosteroid induced gene expression and induces a *brassinosteroid insensitive1 (bri1)* loss-of-function mutant phenotype. ES3 was shown to affect the localization to the plasma membrane of ROP6, a Rho-GTPase implicated in PIN1 and PIN2 subcellular localization and polarity (Xu et al., 2010; Chen et al., 2012). Like ES1 and ES3, compound 5326482 affects BRI1, PIN1 and PIN2 trafficking. Finally it is also quite similar with RAB2 effector1 (RAE1), a compound that affects RAB2 trafficking (Ung et al., 2013).

- Compound 5673258 however did not cluster into an already distinct class, but was annotated as affecting BRI1, PIN1 and PIN2 trafficking like the two other hit compounds (table 1).

Although this “resource” secondary screening is focused mainly on BRI1 and PIN trafficking and does not interrogate many other potential bioactivities, these compounds (the 3 hit described above) do have an effect on the trafficking of PIN1, PIN2 and BRI1 that is not negligible and that could account for the restoration of *quasimodo* phenotype. BRI1 is the receptor of the brassinosteroid phytohormone and PIN1 and PIN2 are well characterized auxin efflux transporter. The fact that our hit compounds affect the trafficking of these proteins could indicate that a perturbation of the phytohormones homeostasis is the cause of the phenotype of *quasomodo*.

Known compounds	Hit compounds
 <p>ES7 5717972</p> <p>Inhibition of callose synthesis (Park et al., 2014)</p>	 <p>Structural cluster 1</p> <p>ES7-like</p> <p>5714035, 5466801, 5707415, 5707182</p>
 <p>ES3 5279218</p> <p>Affects BRI1, PIN1 and PIN2 trafficking (Drakakaki et al., 2011)</p> <p>RAE1 5271226</p> <p>Affects RAB2 trafficking (Ung et al., 2013)</p>	 <p>5326482</p> <p><b>Endosidin-like</b></p>
 <p>IAA, NAA, 2,4-D</p> <p><b>Auxins</b></p>	 <p>5320602, 7517245</p> <p><b>2-4-D-like</b></p>
 <p>NPA, Genistein, Kaempferol 3-O-rhamnoside-7-O-rhamnoside</p> <p><b>Auxin transport inhibitors (ATIs)</b></p> <p>(Yin et al., 2014)</p>	 <p>7686747, 6109844, 6132817</p> <p><b>ATI-like</b></p>
<p><b>Sulfonamides</b></p>  <p>Sulfamethazine, Sulfamethoxazole</p> <p>Suppression of epigenetic silencing, inhibition of folate synthesis (Zhang et al., 2012)</p> <p><i>Pseudomonas syringae</i> resistance (Schreiber et al., 2008)</p>  <p>Sulfamer, Clopamide</p> <p>Plant immune-priming (Noutoshi et al., 2012b)</p> <p>Plant immune-priming (Noutoshi et al., 2012a)</p>  <p>Pyrabactin</p> <p>ABA signaling (Park et al., 2009)</p>	<p><b>Sulfonamides 1</b></p>  <p>Structural cluster 2</p> <p>7973239, 6411781, 7961822</p> <p><b>Sulfonamides 2</b></p>  <p>7587515, 5935594</p>  <p>9008914, 7908690, 9004186, 7964666, 7659588, 7528601, 7949973</p> <p><b>Other Sulfonamides</b></p>



**Figure 2: Structural comparison of known compounds with hit compounds suppressor of *quasimodo***

Some of the hit compounds (suppressor of either cell adhesion or sucrose sensitivity *qua2-1* phenotype; See table1 for the detailed effect of each molecules), are represented in the right column. These compound are represented here because they have interesting structural similarities with known compounds, and some have similar effects on endocytic trafficking (Drakakaki et al., 2011). The known compounds are represented in the left column next to the hit compounds with similarities. In bold is the typical description of the designated type of molecule, for known compounds (left column), and the description that we gave to these compounds for the hit compounds (right column). The structural clusters 1 and 2 were determined using the online ChemMineTool, but the clustering was only carried out on hit compounds. The sulfonamides 1 and 2 groups were determined visually. The chemicals' names correspond to their ChemBridge ID (<http://www.hit2lead.com/>)

---

We then took a look at the other hit compounds and their similarities in structure with known and characterized chemicals. Two of the hits (7517245 and 5320602) have interesting similarities with the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) (figure 2), one hit (7686747) looks similar to the auxin transport inhibitor (ATI) 1-N-Naphthylphthalamic acid (NPA), and two hits (6109844, 6132817) have similarities with endogenous flavonoid ATIs (figure 2).

Another important class of hit compounds is the sulfonamides. Twelve out of the 49 hit compounds have a sulfonamide group, however, although structural subgroups can be made (sulfonamides 1 and 2; figure 2), the others have quite different overall structures. In addition, sulfonamide containing compounds have been shown to be implicated in diverse pathways such as folate biosynthesis (Yun et al., 2012), plant immune priming (Schreiber et al., 2008; Noutoshi et al., 2012a; 2012b), epigenetic silencing (Zhang et al., 2012) and abscisic acid (ABA) signaling (Park et al., 2009). Our hit compounds are structurally different from these known sulfonamide-containing molecules and seem to lack some of the structural requirements for the activities described (figure 2).

Overall there is a lot of preliminary information that can inform on the potential bioactivity of the hit compounds. Clearly the similarities do not demonstrate that the hit compounds have similar activities nor that it is this activity that affects the phenotype of interest, but it provides highly interesting information to set up hypotheses and experiments.

In this case, based on the annotations of the disruptions of the plasma membrane endocytic trafficking that shows perturbation of PIN1 and PIN2 trafficking, and the fact that a number of hit compounds have auxin-like or ATI-like structures, we speculate that auxin transport or signaling is implicated in the phenotype of the *quasimodo* mutants.

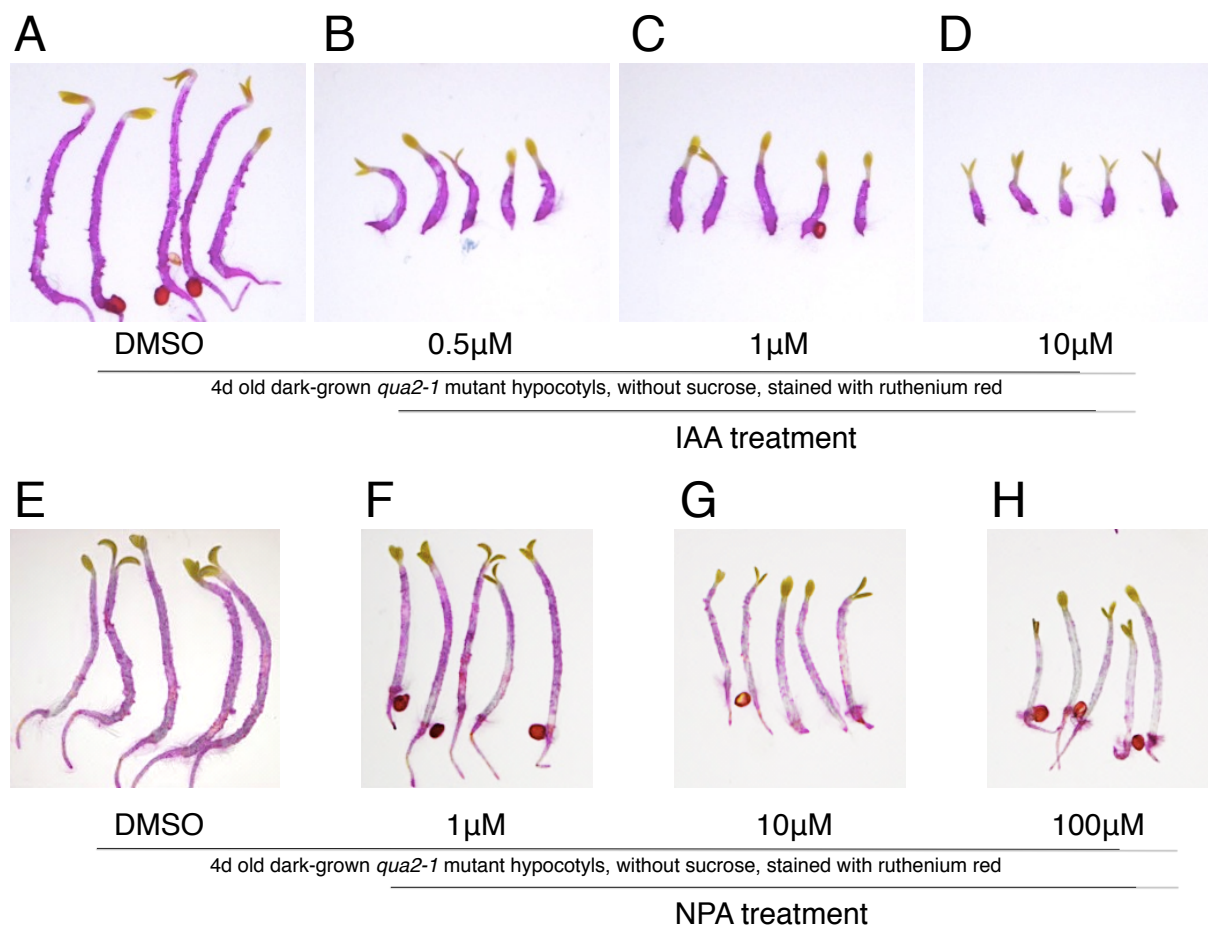
## **Inhibition of auxin transport prevents cell separation in *quasimodo***

Auxin-related chemical biology is probably by far the best described and most studied field of chemical genetics in plants (De Rybel et al., 2009; Ma and Robert, 2014) with a large number of thoroughly described and characterized related compounds.

We thus tested the effect of the well characterized auxins Indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4 D) at a 0.5, 1 and 10 $\mu$ M concentration, and the ATI 1-N-Naphthylphthalamic acid (NPA) at a 1, 10 and 100  $\mu$ M concentration. While the different auxins induced a clear inhibition of the hypocotyl elongation at these concentrations, it did not restore the cell adhesion defect of *qua2-1*, as revealed by the ruthenium red staining (Figure 3 (A-D); data not shown for NAA and 2,4-D but similar to IAA). NPA however was clearly able to restore cell adhesion on the dark grown hypocotyl, in a dose-effect manner, leaving only small patches of cell separation at 100 $\mu$ M (figure 3 E-H). This nicely shows that auxin transport is implicated in the phenotype of the *quasimodo* mutants, since inhibition of auxin transport is sufficient to prevent or limit cell separation.

## **High throughput secondary screening for cell wall structure modifications**

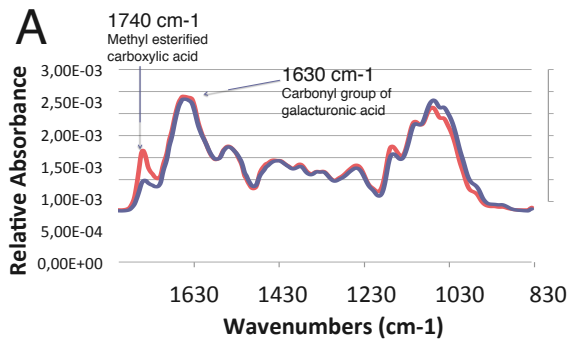
To answer the problem of the large number of hit compounds found and the difficulty to focus on one of them, we decided to carry out a secondary screening looking for compounds that specifically induce changes in the cell wall structure and composition. *quasimodo* mutants are affected in HG synthesis, and it is likely that some of the suppressor hit compounds induce modifications of the cell wall upon treatment that lead to the suppression of the cell adhesion defect. We used the high throughput Fourier Transformed InfraRed spectroscopy (FTIR) fingerprinting technique on dark grown hypocotyl of *qua2-1* treated with DMSO or the suppressor compounds, to determine whether the hit compounds found in the screen could induce cell wall modifications or not. One striking observation that can be made when comparing the DMSO-treated (control) *qua2-1* FTIR spectra with some of the chemically suppressed ones is that some of these show a clear increase of the peak intensity at 1740  $\text{cm}^{-1}$  (figure 4 A and B). Interestingly the 1740  $\text{cm}^{-1}$  peak is known to correspond to the vibrational stretching frequency of methyl esterified carboxylic acid, and an increase of this peak intensity should reveal an increase in HG methyl esterification. As described in



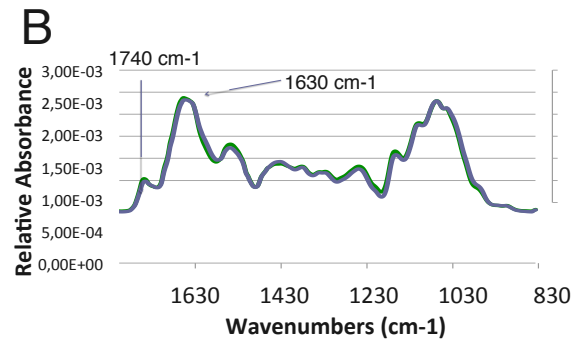
**Figure 3: Inhibition of auxin transport can prevent cell separation in *quasimodo***

(A-H) Four days old dark grown hypocotyls of the *qua2-1* mutants treated with DMSO for control (A and E), with the auxin IAA at increasing concentrations (B-D), or with the auxin transport inhibitor NPA at increasing concentrations (F-H). The seedlings were stained with ruthenium red to reveal the presence or restoration of the cell adhesion defect. Auxin treatment induces an inhibition of growth but does not prevent ruthenium red staining. In contrast the inhibition of auxin transport by NPA only slightly reduces growth while it clearly limits ruthenium red staining in a dose-response manner. The characteristic outward-curving of the epidermal cells of the *quasimodo* mutant dark grown hypocotyl is also restored by increasing concentrations of NPA, showing a clear effect of NPA on the restoration of the *quasimodo* mutant's cell adhesion defect.

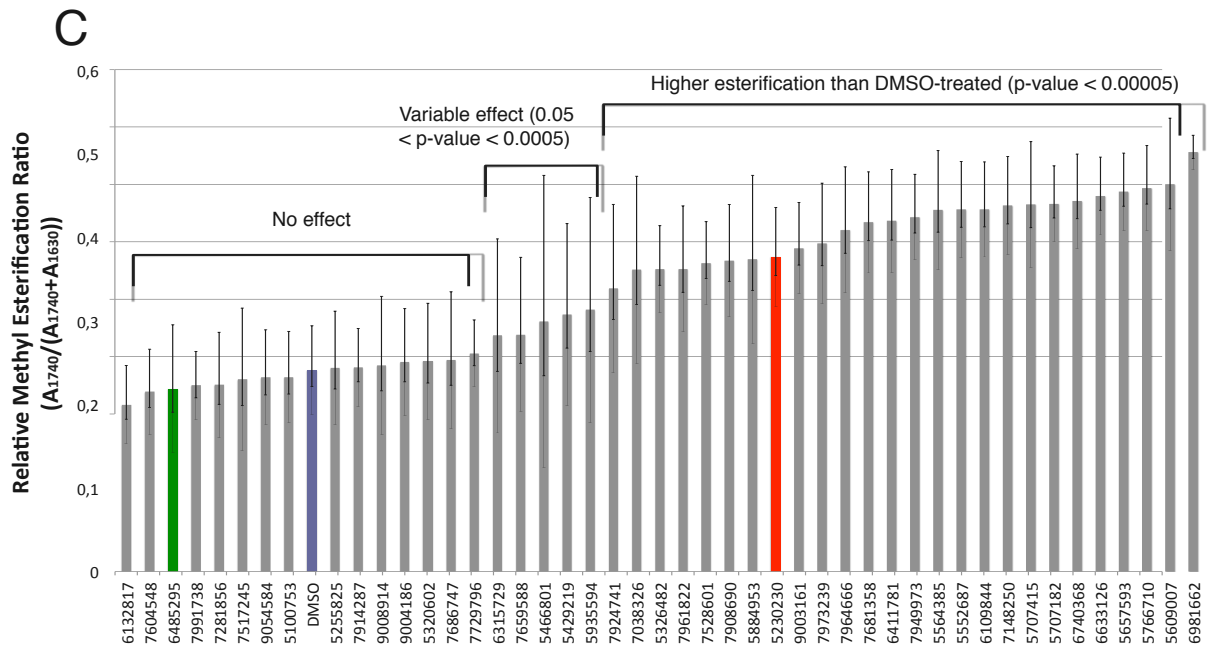
(Manrique and Lajolo, 2002) we evaluated the relative degree of methyl esterification of the HG in the *qua2-1* mutant treated with the suppressor compounds or with DMSO for control. This measure is based on the value of the ratio between the peak intensity at the 1630  $\text{cm}^{-1}$  band, assigned to the stretching frequency for the carbonyl group of galacturonic acid, and the peak intensity at the 1745  $\text{cm}^{-1}$  band, assigned to the methyl ester groups (ME ratio =  $\text{Abs}_{1740\text{cm}^{-1}}/(\text{Abs}_{1470\text{cm}^{-1}} + \text{Abs}_{1630\text{cm}^{-1}})$ ). This ratio is in theory linearly proportional to the actual degree of methyl esterification of the HG (Manrique et al., 2002). T-test on these ratio comparing the compound-treated samples with the DMSO-treated samples were used to determine whether some of the chemical significantly induced an increase in the methyl



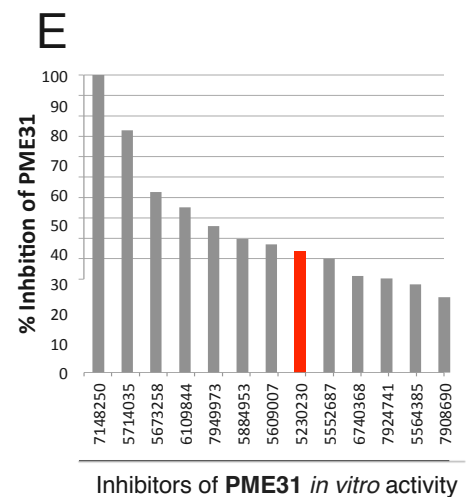
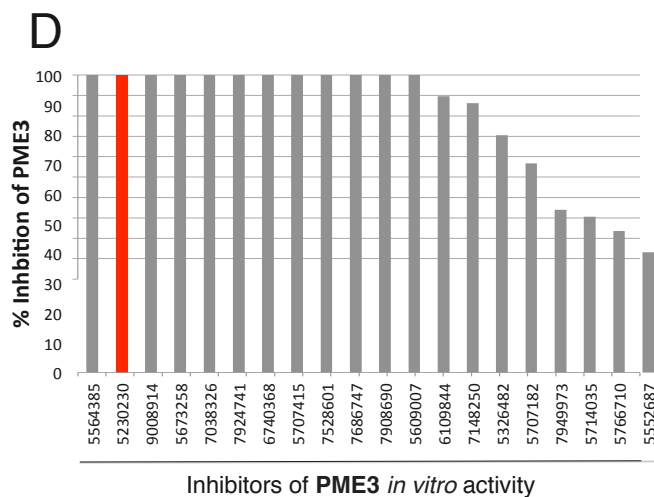
FTIR spectra of  
**5230230** vs **DMSO** treated *qua2-1* hypocotyls



FTIR spectra of  
**6485295** vs **DMSO** treated *qua2-1* hypocotyls



Effect of the chemical treatment on the degree of methyl esterification of HG in *qua2-1* hypocotyls



**Figure 4: Increase in HG methyl esterification and inhibition of PME activity can suppress the phenotypes of *quasimodo***

(A and B) Examples of FTIR spectra of *qua2-1* mutant dark grown hypocotyls either treated with a suppressor compound or DMSO for control: red spectra for compound 5230230 (A), green spectra for compound 6485295 (B), and in both case for comparison, blue spectra for DMSO control (A and B). In (B) control and treated spectra completely overlap indicating no detectable modification in cell wall structure and composition, while in (A), the treated sample shows a clear higher intensity peak at 1740 cm<sup>-1</sup>. This peak being assigned to the methyl esterified carboxylic acid vibrational stretching frequency, its increase reveals an increase in methyl esterification of HG. (C) Relative methyl esterification ratio of the compound and DMSO treated *qua2-1* dark-grown hypocotyls, based on FTIR spectra. The ratio is calculated from the peak intensity of the 1740 cm<sup>-1</sup> band (A1740) assigned to the methyl esterified carboxylic acid, and the peak intensity of the 1630 cm<sup>-1</sup> band (A1630) assigned to the carbonyl group of galacturonic acid. This ratio is in theory linearly proportional to the actual degree of methyl esterification of the HG (Manrique et al., 2002). Averages and standard deviations result from calculations on about 20 spectra of 20 individual hypocotyl from four biological replicates. T-test were then used to determine similarity or not of the compound-treated samples with the DMSO-treated. Samples with “no effect” have no change in methyl esterification (p-value > 0.05). “Variable effect” samples had large variations in the ratio value within the different replicate spectra used for calculation but were significantly different from the DMSO-treated (0.05 < p-value < 0.0005). The other samples have a largely significant higher degree of methyl esterification of the HG (p-value < 0.00005) than the DMSO-treated and have relatively narrow variations. (D and E) Compound that were found as *in vitro* inhibitors of either PME3 (D) or PME31 (E) and the intensity of their inhibitory effect in our assay conditions. All treatments (A-C) and *in vitro* inhibition assay (D and E) were done at a final concentration of 34μM of the compound. In (C-E), red bar is for the 5230230 treated sample, green for the 6485295 treated sample and blue is for the DMSO-treated sample, corresponding to the example spectra in (A and B).

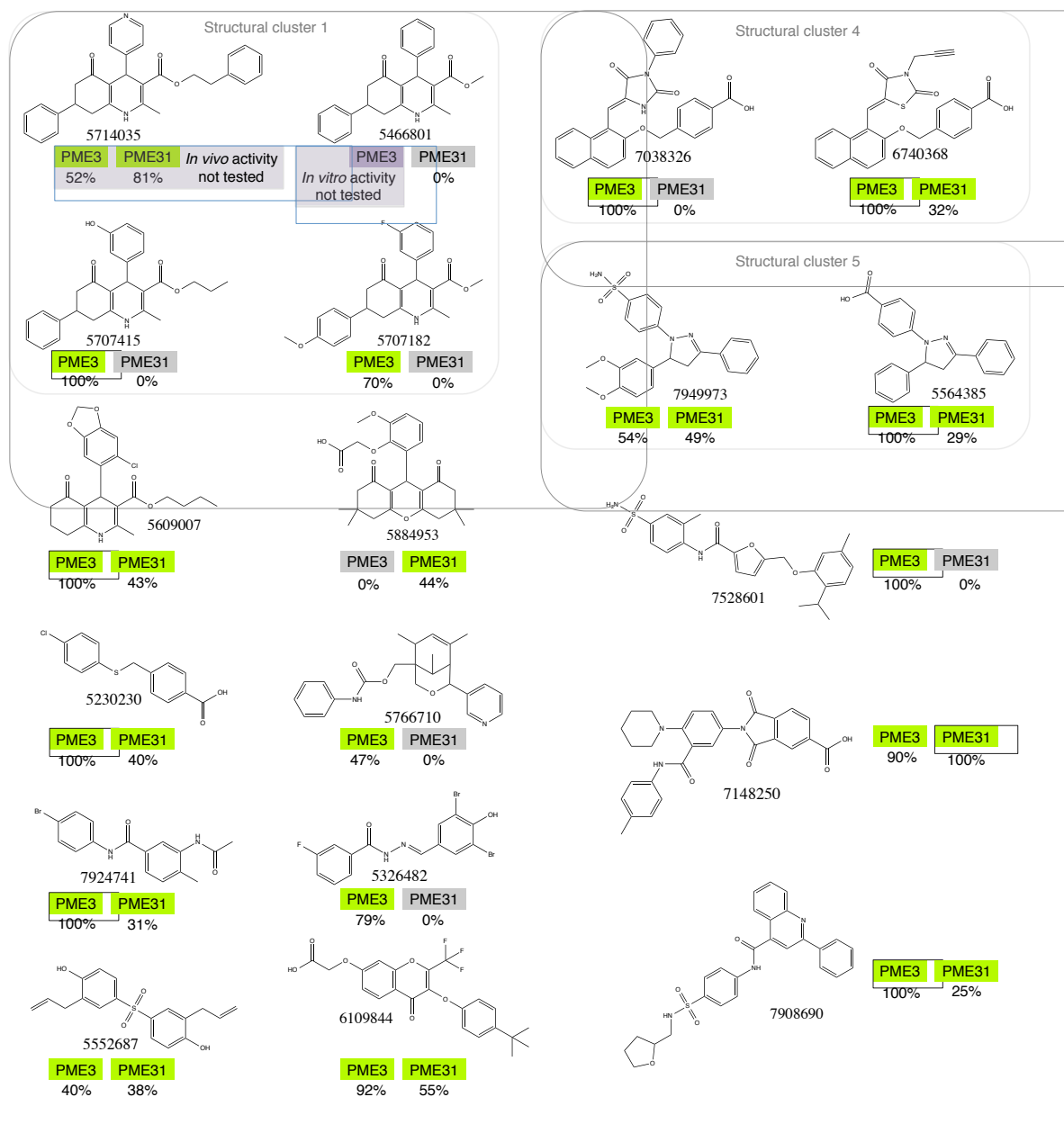
---

esterification ratio (figure 4 C). Twenty-six of the compounds clearly increased the degree of methyl esterification of the HG (p-value < 0.00005), while 5 had a more variable effect (larger standard deviation, and lower significance, 0.05 < p-value < 0.0005). Among the remaining compounds, 19 had no effect on the methyl esterification, one showed a slight decrease in esterification, and 3 have not been measured yet.

The observation that more than half of the suppressor compounds increase the methyl esterification of HG provides a very good evidence that modulating the degree of methyl esterification of HG is a key mechanism in controlling cell adhesion and C/N balance perception and that increasing methyl esterification of HG can suppress the phenotype of *quasimodo*.

**Direct inhibition of pectin methyl esterase activity can suppress the phenotype of *quasimodo***

FTIR analyses revealed that a number of our hit compounds induced an increase in the degree of methyl esterification of the HG. This could be the consequence of the direct inhibition of PME activity by the compounds. To determine if and which of our compounds have a direct effect on PME activity, we tested their inhibitory effect *in vitro* on two different purified PME



### *In vivo* and *in vitro* PME inhibitor hit compounds

**Figure 5: List of PME inhibitor hit compounds**

All of these hit compounds were found as suppressors of *qua2* and are both *in vitro* inhibitors of PME3 and/or PME31, and induce *in vivo* an increase of the degree of methyl esterification of HG compared to the DMSO-treated *qua2*. Except for 5714035 for which the *in vivo* activity was not tested and 5466801 for which *in vitro* inhibition of PME3 was not tested. The percentage below the PME names indicates the percentage of inhibition *in vitro* in our assay conditions. Green outline of the PME names indicates that the molecule is inhibitor of the PME, black edge indicate complete inhibition, while gray outline indicates no inhibitory effect.

isoforms: PME3 and PME31. Interestingly PME3 is an isoform that can be inhibited by endogenous proteinaceous PMEI (Sénéchal et al., unpublished), while PME31 is not (Dedeurwaerder et al., 2009). Our results show that 21 out of our 49 compounds actually have a direct inhibitory effect on these PME isoforms (figure 4 D and E and table 1). Twelve of the compounds affect the activity of both PME, 8 are specific to PME3 and 1 is specific to PME31. In general PME31 is only partially inhibited (most induce less than 50% inhibition; figure 4 E and table 1), while PME3 is mostly completely inhibited (figure 4 D and table 1). Except for two of the compounds (and two other compounds with no FTIR measurements), the *in vitro* inhibitory effect measured here is correlated with an increased degree of methyl esterification of the HG *in vivo* as measured by FTIR (Table 1). It results that 17 of our compounds have a direct *in vitro* inhibitory effect on PME, and upon treatment are able to induce an increase of the degree of methyl esterification *in vivo*, which makes them *bona fide* PME inhibitors (figure 5 and table 1).

Nine of these PME inhibitor compounds restore specifically the cell adhesion defect, 4 specifically the sucrose sensitivity and 4 both phenotype (table 1). These compounds that can be considered true PME inhibitors, represent quite a substantial fraction of the *qua2* suppressor. They are also quite diverse in structure, but all have in common the PME inhibitor effect that likely excludes the hypothesis that it is the disruption of another target than the PMEs that is responsible for the restoration of the phenotype in these cases. Also the fact that we tested two different PME isoforms and that some of the compounds are specific to one isoform or another demonstrates that, at least for these compounds the inhibitory effect happen via a direct targeting of the PME protein and is not an indirect consequence such as a perturbation of the HG substrate. This provides a confirmation that it is the inhibition of PME activity and the increase in the degree of methyl esterification that is responsible for the suppression of the *qua2* phenotypes.

## Discussion

Our objective in this work was to develop a very high throughput approach to identify a number of interesting compounds relevant to the study of our biological question. We also wanted to demonstrate that the study of these compounds could quickly yield very interesting insights on this biological question, both by exploiting already available data on the chemicals, and by performing a well-chosen secondary screening.

Finally, this chemical genomic screen aimed at providing a complementary approach to classical genetic by providing very useful tools as well as the possibility to circumvent lethality and redundancy that can be limiting for gene discovery in classical genetic approaches.

### **High throughput screening and putative activity searching**

The screening process was fast and yielded a large number of compounds of interest. The two screenings in parallel allowed us to separate the cell adhesion defect of *quasimodo* from its sensitivity to C/N imbalance. Interestingly the fact that these phenotypes can be uncoupled and that we have compounds specific for each or both phenotype will be very helpful in future work in order to dissect in more details the pathways affected in the mutant (figure 6) and to get a better understanding of the cell adhesion mechanisms and C/N balance perception in general.

Some of the compounds were also screened from a high-content, prescreened, and annotated library of compounds (Drakakaki et al., 2011). This represents a great advantage as it directly provides a good source of information on the bioactivity of the hit compounds and can help set up hypotheses and experiments.

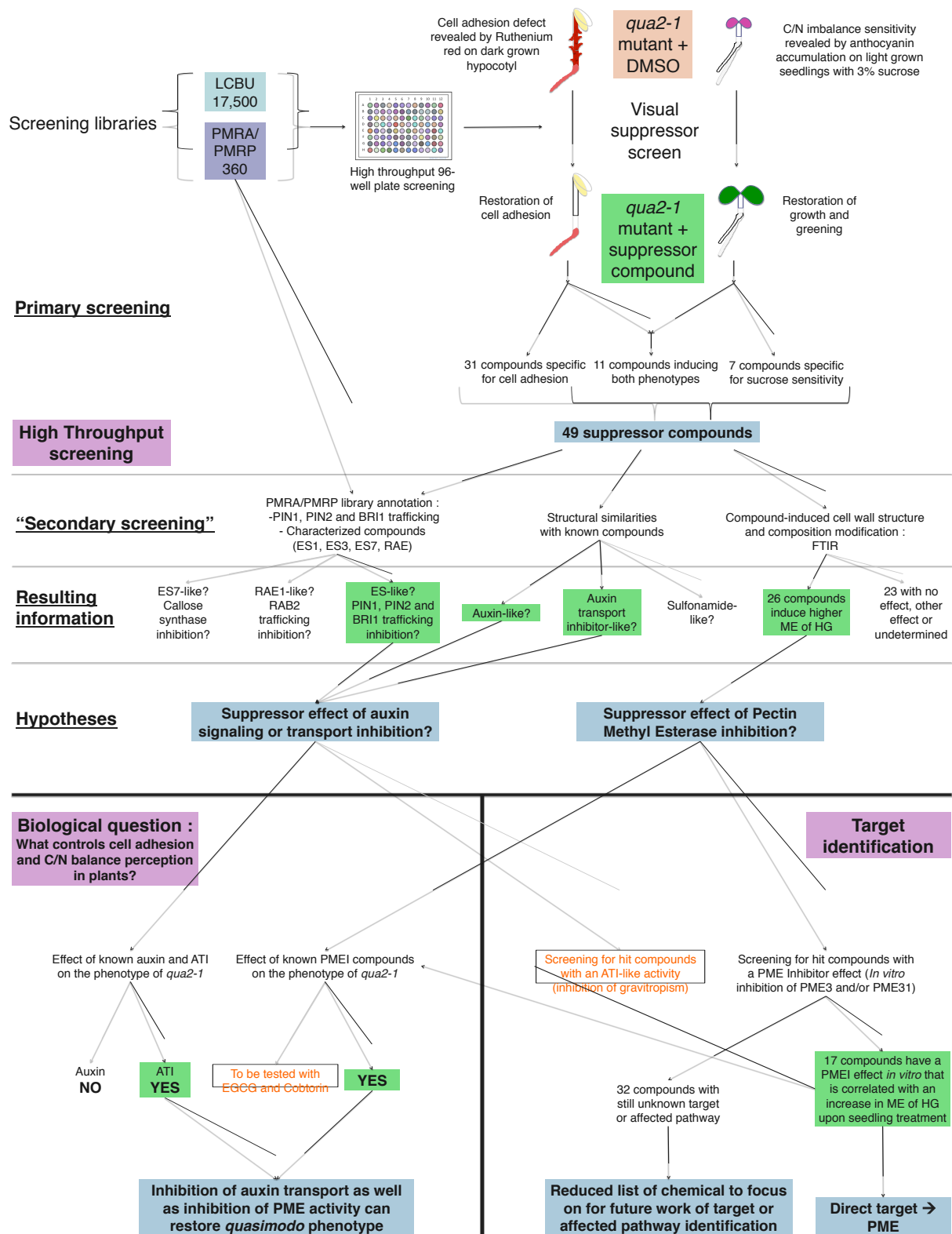
We found that one of our hit compounds from this library was very similar in structure and in annotations (from Drakakaki et al., 2011) with ES7, another compound of this library which was recently shown to be a direct inhibitor of callose synthesis (park et al., 2014). Although we didn't follow further on this hypothesis here, callose synthesis defect could actually play a role in cell adhesion since correct callose deposition is important during cell plate formation, when cell adhesion is set up. This compounds was also annotated as affecting the normal trafficking of PIN1, PIN2 and BRI1, while ES7 did not, but it will be interesting to test whether the specific callose synthase inhibitor ES7 can actually restore the phenotype of



*quasimodo*. This could quickly indicate us whether or not the inhibition of callose synthesis plays a role here and could lead to other experiments to further demonstrate and try to understand what role it plays.

We went in a different direction since three of our hit compounds were affecting the normal trafficking of PIN1, PIN2 and BRI1, which seemed to indicate that a perturbation of the normal signaling or transport of hormones could be implicated in the restoration of the phenotype of *quasimodo*. The additional observation that some of our compounds had striking similarities with the synthetic auxin 2,4-D and with ATIs led us to think that auxin signalling or transport was certainly implicated. The experiment revealed that inhibition of auxin transport by NPA could indeed suppress the cell adhesion defect of *quasimodo*. NPA has been shown to affect PIN trafficking at high concentrations (Geldner et al., 2001), but more importantly to prevent the association of the ABCB / P-GLYCOPROTEIN / MULTIDRUG RESISTANCE (ABCB/PGP/MDR) auxin efflux carrier with its activating partner TWISTED DWARF1 (TWD) (Bailly et al., 2008). But it is not negligible that NPA could have another activity on a different target that would be the cause of restoration of the cell adhesion defect.

To answer this concern it will be interesting to make use of the large number of the well characterized synthetic auxins and ATI compounds (Ma and Robert, 2014). The compound BUM (Kim et al., 2010) was shown to be highly specific for the dissociation of the ABCB/PGP/MDR-TWD complex and to block auxin efflux at a much lower concentration than NPA. On the other hand, TIBA (2,3,5-Triiodobenzoic acid) was shown to block auxin transport by affecting the actin mediated trafficking and polar localization of auxin transporters (Geldner et al., 2001; Dhonukshe et al., 2008). The effect of these two compounds with different protein targets, but both blocking auxin efflux, should help us determine if it is actually auxin transport inhibition, and not another activity, that restores the cell adhesion defect of the *quasimodo* mutants. It will also be interesting to test the effect of auxin biosynthesis inhibitors (L-Kynurenine and/or Naxillin), auxin influx inhibitors (2-NOA), and anti auxin compounds (Auxinole) on the phenotype of *quasimodo* to determine which aspect of auxin transport or maybe signaling is implicated in the cell adhesion defect of *quasimodo*. We should also screen among our 49 compounds, which of them have such an ATI activity, and for which it could be accounted that their suppressor effect is due to their ATI activity. Secondary screening for such compounds could be done by looking for compounds that inhibit the gravitropic response of the seedling since both phenomena are tightly linked (Rojas-Pierce et al., 2007; Ueda et al., 2014). By putting aside these ATI



**Figure 6: Workflow of the high throughput chemical genomics screening and efficient identification of the implication of auxin transport and pectin methyl esterification in cell adhesion.**

This scheme summarizes the work reported in this article. The text written in orange reports to work to be done.

compounds, it will provide with us a shorter list of uncharacterized compounds to focus on for future work.

In a parallel approach using FTIR fingerprinting of the cell wall structure and composition, we were able to identify a group of compounds that induce an increase in the degree of methyl esterification of HG in *quasimodo*. Screening of our compounds for the *in vitro* inhibition of two PME isoforms revealed that 17 of our compounds were actual PME inhibitors (figure 5). It will be interesting to additionally confirm the effect of PME inhibition with other characterized compounds such as the well described direct inhibitor of PME, epigallocatechin gallate (EGCG) (Lewis et al., 2008) as well as the probably more indirect inhibitor, cobtorin (Yoneda et al., 2010). However our data already provide a good proof that an increased degree of methyl esterification mediated by a direct inhibition of the PME activity can restore the phenotype of *quasimodo*.

Two of our compounds have an *in vitro* inhibitory effect on PME but surprisingly do not induce an *in vivo* increased in methyl esterification. One hypothesis to explain this phenomenon is that PME3 and PME31 that are produced and purified from *E.coli* for the *in vitro* assay could be slightly different (e.g. post-translational modifications) from the plant endogenous PME3 and PME31. The compound could thus be active on the bacteria-produced PME, and not the plant-produced PME, and have different activity *in vivo* for the suppression of *quasimodo*. Another hypothesis is that the chemicals could actually be modified by the plant when taken up, as has been described for some compounds with the process of glycoactivation (Zhao et al., 2007). In our case such a modification would prevent the PME inhibitor effect of the compound while giving it a different activity. PGs are active on a relatively similar substrate as PME. In the hypothesis that the inhibitor blocks the substrate recognition site it could also be an inhibitor of the PG and become specific of the PG upon *in vivo* modification for example.

The spectrum of protein affected by these compounds could be wide since different types of HG modifying enzymes exist and they are all part of large gene families (senechal et al., 2014). Interestingly one of our PME inhibitor compound (6740368; figure 5) contains an alkyne group. Using “Click chemistry” (Kaschani et al., 2009) to couple this compound to a Biotin Azide and performing an affinity purification of the associated proteins could allow us to identify the spectrum of protein targets of this inhibitor.

Finally it remains some compounds that have an *in vivo* effect on methyl esterification of HG but not a direct effect on PME. This is much less surprising and could indicate that these

compounds affect other PME3 and PME 31, or that they are upstream regulators of PME and PME1 gene expression or activity.

The chemical genomics approach allowed us to identify the role of PME that are usually not isolated by genetic approaches due to the large size of the gene family and their high redundancy (sénéchal et al., 2014).

It remains a shorter list of uncharacterized hit compounds that will be the focus of future work.

### **The role of auxin transport and pectin methyl esterification in cell adhesion and carbon/nitrogen balance perception**

Our strategy allowed us to quickly demonstrate that the inhibition of auxin transport as well as the inhibition of PME activity were sufficient to suppress the phenotype of *quasimodo*. Then what could be the implication of these two processes in the suppression of the phenotype of *quasimodo* and what insight does it give on the mechanisms of cell adhesion and C/N balance perception?

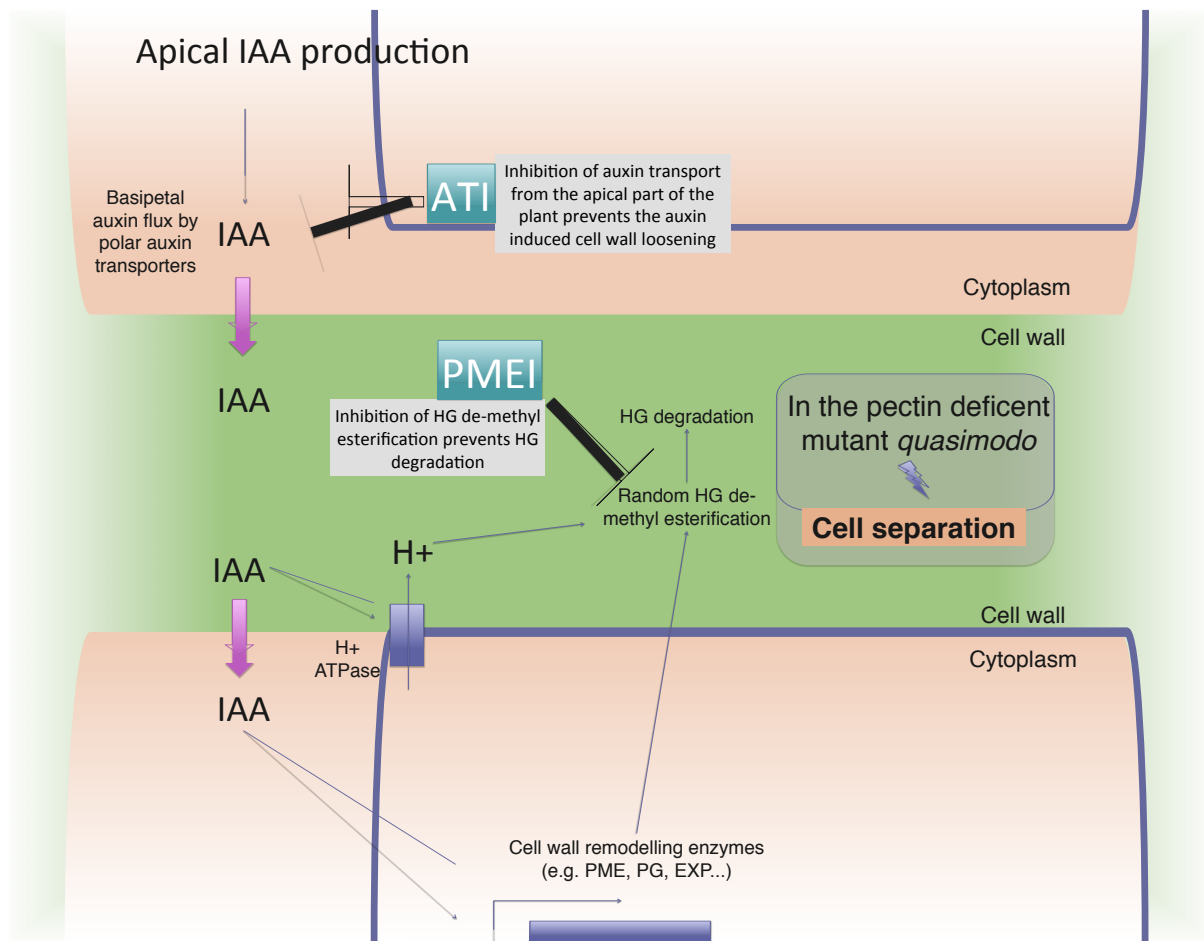
Auxin and auxin transport are well known players implicated in the events of cell separation. Auxin acts antagonistically to ethylene in the induction of a signaling cascade leading to cell separation. In the case of floral organ abscission, auxin treatment tends to delay the process. In the root however, auxin has been shown to trigger the induction of a small peptide called INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), which is the initiator signal for the induction of cell separation (Kumpf et al., 2013). This peptide is perceived by a pair of homologous leucine-rich repeat receptor-like kinase (LRR-RLK) HAESA (HAE) and HAESA-LIKE2 (HSL2), that trigger a MAP kinase signaling cascade upon binding (Cho et al., 2008; Shi et al., 2011). This signaling activates the expression of cell wall remodeling and degrading enzymes such as xyloglucan endotransglucosylase/hydrolases (XTHs), expansins (EXPs), PMEs and PGs (cai and lashbrook, 2008) finally leading to cell separation (Aalen et al., 2013). Independently from IDA, auxin is able to induce a loosening of the cell wall by acting both on the expression of cell wall remodeling enzymes and the pH of the apoplasm (Rayle and Cleland, 1992). Auxin can induce the acidification of the cell wall by promoting the efflux of protons, and in these conditions the auxin induced PMEs tend to process demethyl esterification in a more random manner, forming an HG substrate more suitable for

the degradation by PGs (Raiola et al., 2004; Sénéchal et al., 2014). This process along with the action of EXPs, leads to the loosening of the cell wall and a rapid growth (Rayle and Cleland, 1992). The phenotype of *quasimodo* and the fact that it is suppressed by the inhibition of auxin transport could be linked with one of these processes. The implication of the IDA triggered cell separation pathway here is questionable since to be activated it needs the HAE and HLS2 LRR-RLK to be expressed. However these are usually only transiently and very locally expressed, and do not seem to be inducible by auxin. The hypothesis of a somewhat “uncontrolled” acid growth leading to cell separation, could actually make sense in the context of a pectin deficient mutant (figure 7). It will be interesting to test whether preventing the acidification of the apoplast could also restore cell adhesion, or if the IDA triggered cell separation pathway is implicated in the process.

In this context, the finding that the inhibition of PME activity can restore the cell adhesion defect is much more intuitive. It is consistent with the precedent finding of the implication of auxin transport and potentially auxin induction of PMEs and PGs expression. It is also interesting that in *friable1*, another mutant defective in cell adhesion, it was pointed out that an increased level of PME activity and a decreased level of methyl esterification of HG were the most likely explanations for the loss of cell adhesion (Neumetzler et al., 2012).

Upon treatment with a PME inhibitor compound, the higher level of esterification could prevent the degradation of the HG by polygalacturonases, and thus prevent cell separation (figure 7). This scenario is reminiscent of what is observed in the *quartet* mutants (Rhee et al., 2003; Francis et al., 2006; Ogawa et al., 2009). It will be interesting to test whether there is actually an increase in the expression of PME and PGs in *quasimodo* and whether this induction can be prevented by a treatment with an auxin transport inhibitor. This should tell us if auxin, PME and PG act sequentially in the induction of cell separation in *quasimodo*.

It is surprising however that some of the PME inhibitors can specifically suppress the sucrose sensitivity of *quasimodo* and not its cell adhesion defect. The question of the C/N imbalance sensitivity in *quasimodo* is still very puzzling to us. By adopting a double screening approach in this work we were able to find some compounds specific to the restoration of sucrose sensitivity but also some compounds specific to the restoration of cell adhesion and not sucrose sensitivity, indicating that this sensitivity to C/N imbalance is probably not simply the consequence of the cell adhesion defect. This set of chemicals will be very useful to study further the question of the cell adhesion defect and C/N imbalance sensitivity in this mutant to get a better understanding of these phenomena in general.



**Figure 7: Model of auxin and PME mediated cell separation in the pectin deficient mutants *quasimodo***

Schematic representation of our hypothesis concerning the phenomenon leading to the loss of cell adhesion in *quasimodo*. Auxin is synthesised at the apex of the plant and is transported to the rest of the plant thanks to the basipetal transport of auxin mediated by polar auxin transporters. When entering a cell, auxin can trigger the expression of cell wall remodeling (CWR) enzymes. In addition it can activate the efflux of proton into the apoplast. In acidic conditions, PMEs act in a more random manner and produce HG substrate suitable for degradation by PGs. In wild type plant, that process is known to lead to cell wall loosening and growth. However in the context of the pectin deficient mutant *quasimodo*, it may lead to cell separation. Thus inhibiting or limiting the transport of auxin may prevent the induction of CWR enzymes and further degradation of the cell wall leading to cell separation. Similarly, inhibiting the de-methyl esterification of HG should prevent its degradation by PGs leading to cell separation. These events may thus represent an upstream and a downstream step in the process leading to the loss of cell adhesion in *quasimodo*.

ChemBridge ID	Sup. of cell adh.	Sup of suc. Sens.	HG ME ratio	Inhib. PME3	Inhib. PME31	cluster	annotation
7908690	no	yes	yes***	100.00%	25.27%		Sulfonamide
5564385	yes	no	yes***	100.00%	29.6%	5	
7924741	no	yes	yes***	100.00%	31.59%		
6740368	yes	yes	yes***	100.00%	32.42%	4	
5230230	yes	no	yes***	100.00%	40.79%		
5673258	yes	no	N/A	100.00%	60.65%		
5707415	yes (mild)	yes	yes***	100.00%	no	1	ES7-like
7038326	yes	no	yes***	100.00%	no	4	
7528601	yes	yes	yes***	100.00%	no		Sulfonamide
7686747	yes (mild)	no	no	100.00%	no		ATI-like
9008914	no	yes	no	100.00%	no		Sulfonamide
5552687	yes	no	yes***	40.48%	38.27%		
5766710	no	yes	yes***	47.62%	no		
5714035	yes	yes	N/A	52.38%	81.41%	1	ES7-like
7949973	no	yes	yes***	54.76%	49.28%	5	Sulfonamide
5707182	yes (mild)	yes	yes***	70.24%	no	1	ES7-like
5326482	yes	no	yes***	79.76%	no		Endosidin-like
7148250	yes	no	yes***	90.48%	100.00%		
6109844	yes	no	yes***	92.86%	55.6%		ATI-like
5609007	yes	no	yes***	99.92%	43.14%		
5884953	yes	no	yes***	no	44.95%		
7973239	yes (mild)	yes	yes***	no	no	2	Sulfonamide 1
6411781	yes	yes (mild)	yes***	N/A	no	2	Sulfonamide 1
7961822	yes (mild)	yes (mild)	yes***	N/A	no	2	Sulfonamide 1
7964666	yes	yes	yes***	no	no		Sulfonamide
5657593	yes	no	yes***	no	no		
6981662	yes	no	yes***	no	no		
7681358	yes	yes (mild)	yes***	N/A	no		
6633126	yes	no	yes***	no	no		
9003161	yes	no	yes***	no	no		
5935594	yes	no	no**	N/A	no	2	Sulfonamide 2
5429219	yes	no	no**	no	no		
5466801	yes (mild)	yes	no*	N/A	no	1	ES7-like
6315729	yes (mild)	no	no*	N/A	no		
7659588	yes	no	no*	no	no		Sulfonamide
9054584	yes	no	no	no	no	3	
7281856	yes (mild)	no	no	no	no	3	
7604548	yes (mild)	no	no	no	no		
7729796	yes	no	no	no	no		
6485295	yes	no	no	N/A	no		
7517245	yes	no	no	no	no		2,4-D-like
7914287	yes	no	no	no	no		
6132817	yes	no	no	N/A	no		ATI-like
5100753	yes (mild)	no	no	no	no		
5320602	yes	no	no	no	no		2,4-D-like
7991738	yes	no	no	no	no		
5255825	no	yes	no	no	no		
9004186	no	yes	no	no	no		Sulfonamide
7587515	yes	no	N/A	no	no	2	Sulfonamide 2

**Table1: List of hit compounds and annotations**

This table gather all the compounds identified as suppressor of esmeralda in this work and the phenotypic and structural annotations associated with each of them.

## Materials and methods

### *Plant material and growth conditions*

*Arabidopsis thaliana qua2-1* seedlings (Mouille et al., 2007) were grown at 20°C on liquid custom made Duchefa ([www.duchefa-biochemie.com/](http://www.duchefa-biochemie.com/)) medium with 0% or 3% sucrose concentration depending on the experiment. Seeds were surface sterilized. For sowing, *qua2-1* seed were continuously homogenized in liquid culture medium in order to allow pipetting of 150µl medium containing 5 to 10 seed per tips with a multi channel pipette. Sowing of the seed and growth of the seedling was done in 96 round-bottom-wells plates. The addition of the chemicals was performed by an automated workstation (Biomek NX, BeckmanCoulter) for the screening of the 17,500 compounds LCBU library, and by manual pipetting for the rest of the experiments. The various compounds added to the medium in the different experiments were added at the concentration stated for each case. The volume of DMSO or DMSO diluted chemicals however, was of 1µl for 150µl of growth medium, meaning that adequate dilution in DMSO were done prior to the addition to the medium. Seed were then cold-treated for 48h to synchronize germination, and grown in a 16h light / 8h dark cycle. For dark growth conditions, seeds were exposed to light for 4h to induce germination, after which plates were wrapped in three layers of aluminum foil. Seedling age was counted starting from the light exposure.

### *Primary chemical genomics screening*

The largest part of the screening was done on the 17,500 diverse synthetic compounds plus DMSO controls from the chemical screening platform of the laboratory for chemical biology Umea (LCBU, Umea University, Sweden, <http://www.chemistry.umu.se/english/research/facilities/lcbu/screening-platform/>) distributed on 220 96-well plates. The LCBU library is composed of synthetic compounds purchased from ChemBridge (<http://www.chembridge.com/>) and assembled to contain the most diverse and drug-like compounds possible. Along with this library, the small library of 360 compounds that were selected for their inhibitor effect of germination and growth of tobacco pollen (PMRA/PMRP, Robert et al., 2008, Drakakaki et al., 2011) were tested for both suppression of the cell adhesion defect on dark grown hypocotyls in a liquid medium with 0%



sucrose and suppression of the sucrose sensitivity in light grown seedlings in a liquid medium with 3% sucrose. The compounds were diluted in DMSO at a concentration 5mM. For screening, 1 $\mu$ L of compound was added to the 150 $\mu$ L medium, leading to a final concentration of 34 $\mu$ M.

For cell adhesion defect suppression, after 4 days in the dark, the liquid medium was removed and replaced by 150 $\mu$ L ruthenium red at a 0,05% concentration. Submersion of each hypocotyl was checked to prevent false negative of non-staining hypocotyls. After about 30s the RR was removed and the hypocotyls washed twice with water. Hypocotyls were then visually screened for absence or largely reduced red coloration.

For the suppression of sucrose sensitivity, after 12 days, light grown seedlings were visually screened for greening and absence of anthocyanin accumulation.

The 214 initial hits in total were then retested using a copy of the screening library. The 83 retested molecules were finally confirmed by using fresh dilution in DMSO of the pure powder of the compound and by confirming their effect on three independent biological replicates.

#### *Structural analysis of the compounds*

Binning clusters grouping compounds with similar structures were obtained from the online ChemMineTool (<http://chemmine.ucr.edu/>). The similarity cutoff of the binning clustering was set to 0.4.

#### *Auxin and auxin transport inhibitor treatments*

Seedlings were grown in the same condition as for the screening for suppression of the cell adhesion defect. Seedlings were treated with Indole-3-acetic acid (IAA), 1-Naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4 D) at a 0.5, 1 and 10 $\mu$ M concentration, and N-1-Naphthylphthalamic acid (NPA) at a 1, 10 and 100  $\mu$ M concentration.

#### *FT-IR analysis*

The liquid growth medium of 4d old, DMSO and compound-treated, dark-grown hypocotyls, was removed and replaced by 100% ethanol. Hypocotyls were kept in ethanol at 4 $^{\circ}$ C for 24h. They were then rehydrated in water for 2h and carefully placed on gold coated glass slides.

The samples were then dried on the slides at 37°C for 20 min. For each DMSO or compound-treated condition, 1 spectra per hypocotyl was collected from 20 individual hypocotyls, from four independent cultures (five seedlings from each culture), as described by (Mouille et al., 2003). An area of 30  $\mu\text{m}$  x 30  $\mu\text{m}$  was selected for FT-IR microspectroscopy, using a Thermo-Nicolet Nexus iN 10 MX spectrometer equipped with a continuum microscope accessory (Thermo Scientific). Normalization of the data and the discriminant variable selection method were performed as described by Mouille *et al.* (2003)

### *PMEI inhibition assay*

The inhibition assay was carried out on recombinant PME3 and PME31 proteins produced in *E.coli*. The PME activity was assayed using the alcohol oxidase coupled assay (Klavons and Bennett, 1986) in the presence or absence of the small molecules at 34  $\mu\text{M}$  . The data are the means of two replicates.

### **Acknowledgments**

This work was supported by a PhD fellowship of the French Ministry of Higher Education and Research and the INRA. We are thankful to Per-Anders Enquist for his advices on the use of the screening library at the LCBU and his comments on some of the small molecules. We are also thankful to the other member of the LCBU for their help and advices for the screening and the use of the automated workstation.

### **Contributions**

G.M. and S.V. conceived and designed most of the experiments with input from S.R. and J.P. The experiments were carried out by S.V., C.F. and M.L. C.F. performed the FTIR spectra acquisition, M.L. performed the PME inhibition assay, and S.V. performed the screening and the other experiments. S.V. and G.M. wrote the manuscript.

## General discussion

---



## The power and the complementarity of the forward genetic and the chemical genomics

The goal of the work presented in this thesis was to get a better understanding of the mechanisms that control cell adhesion in plants. In order to achieve this goal we chose to base our strategy on two *a priori* approaches to identify the molecular players that are involved in this process. We used a combination of a forward genetic screen and a chemical genomic screen. The true power of these two approaches is their exploratory nature and the fact that they are based on no *a priori* on the pathway that may be involved in the process (Vidaurre and Bonetta, 2012). The classical forward genetic screens on *Arabidopsis thaliana* have been used for decades now and have been proven powerful to isolate uncharacterized genes and identify their function based on the process that they affect or phenotype that their disruption induces. EMS mutagenesis combined with a well-designed phenotypic screen usually allows the identification of the main genes implicated in a given process. More recently the “suppressor approach” has started to be widely used for further dissecting given pathways, and have uncovered even more genes and functions. In the last few years the advent of the Next Generation Sequencing (Ossowski et al., 2008) and Next Generation Mapping (Schneeberger et al., 2009, James et al., 2013) have largely accelerated the identification of the causal mutations needed after a genetic screen. Finally one of the advantages of these forward genetic approaches is that they generate mutants that affect genes that usually have major functions. Crosses between these types of mutants for the creation of double, triple, or more... mutants and the study of the genetic interactions provide invaluable information on the pathways that they affect or the interaction with other processes.

However one of the major disadvantages of the forward genetic screens in the discovery of new genes is that it is usually limited to the identification of non redundant genes and often non lethal mutations. In this regard, chemical genomics provides a way to circumvent these limitations (Robert et al., 2009; Toth et al., 2010; Hicks et al., 2012, and as described in chapter III). The disruption of the function of proteins by small molecules provides the advantages that it could affect all or part of a redundant family of proteins, their effect is dose dependent and inducible or reversible at any time. In addition to providing a complementary approach to the genetic screening by affecting different types of genes, it provides very useful tools for the study of a given pathway. Indeed the study of their effect and the interaction with other pathway is much more direct than when studying mutants since it doesn't require crosses and the time consuming wait for the second generation and selection of double mutants. One difficulty however may be the identification of the protein (or proteins) target.

Given that the target can be a unique gene product, one of the approaches that has proven useful is the screening for mutants insensitive to the effect of the molecule. The high throughput screening of insensitive mutants combined with NGS can relatively quickly lead to the identification of the target. It is not always the case and many other strategies exist (Dejonghe et al., 2014) but can often be more complicated to set up for the regular plant biologist and can require the help of chemists. However as exemplified in Chapter III of this thesis there are ways to quickly and efficiently identify the target when these are already described. Although this latter approach does not lead to the identification of new uncharacterized genes, it leads a better understanding of the pathway by incorporating already described and characterized components.

Ultimately the combination of forward genetic and chemical genomics supported by NGS provides a very powerful strategy in order to identify new uncharacterized gene, study their function and thus genetically dissect a given pathway.

The work presented in this thesis exemplifies the use of the combination of these two approaches. We have carried out a genetic suppressor screen and a chemical genomic suppressor screen of the cell adhesion defective mutants *quasimodo*, and have isolated a number of suppressor mutants and molecules implicated in cell adhesion and sucrose sensitivity.

The genetic screen led to the identification and study of a suppressor affected in *ESMD1*, an uncharacterized putative O-fucosyltransferase. The genetic study of cell adhesion including the other putative O-fucosyltransferase *FRB1* showed that the disruption of *ESMD1* was able to suppress the cell adhesion defect of *qua1*, *qua2* and *frb1*, making it a major player of the pathway. Although we have not yet fully unravel it's role, future work on this gene will surely prove highly significant for the understanding of cell adhesion in plants.

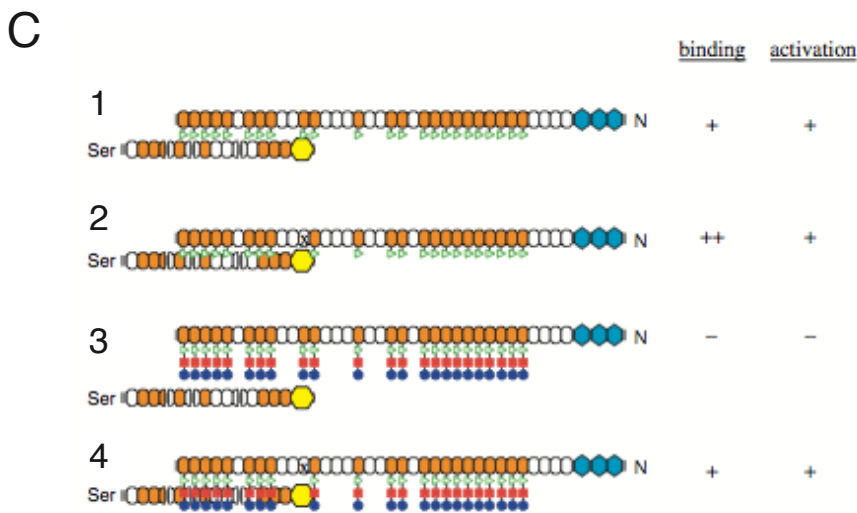
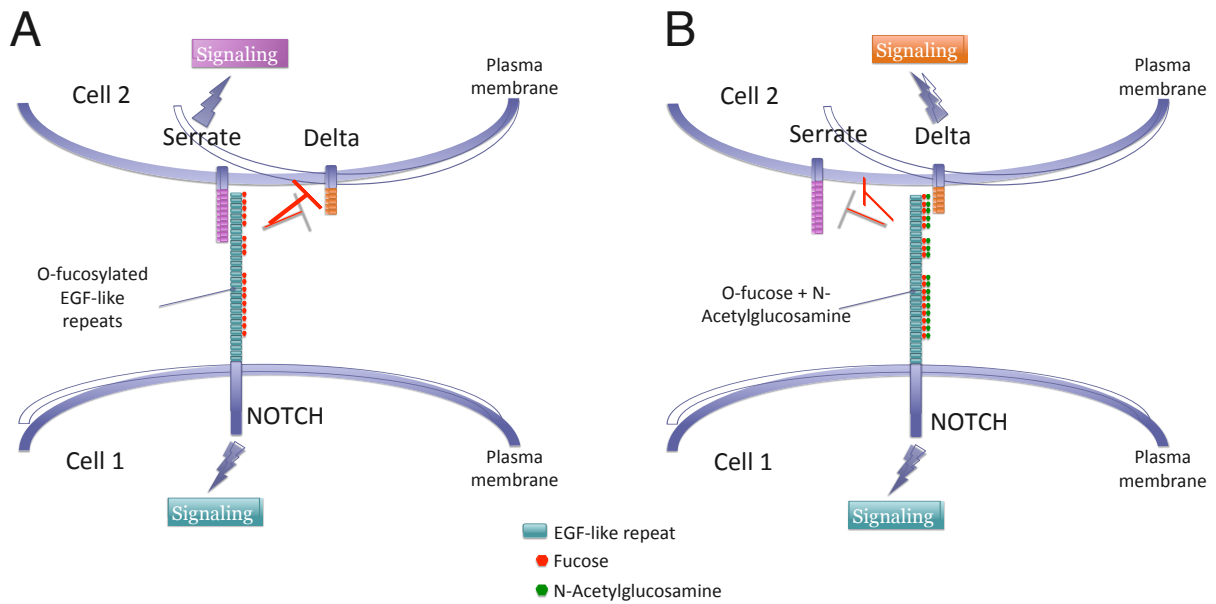
The chemical genomic screen has revealed the implication of auxin transport and pectin methyl esterase activity in the process of cell adhesion.

Although in a way we have just started to identify and study some of the molecular players that we isolated, these are already changing our view and our understanding to the mechanisms of cell adhesion in plants.

## The potential role of O-fucosylation in cell adhesion

Despite many efforts, we have not been able to clearly identify the function of ESMD1 and FRB1. However based on their putative function we have speculated that they should be O-fucosyltransferases of the WAKs' EGF-like domains. In addition to the predictions from the putative function of these O-fucosyltransferases, the WAKs themselves are particularly suited candidates in our case due to their pectin binding and signaling function. The function that such a glycosylation may have on the WAKs would also makes sense, but necessitate some explanation.

To understand how the O-fucosylation of the WAKs could affect their signaling, we have to take into account that the signal transduction by the RLKs in general happens upon homo- or hetero- dimerization (or oligomerization) of the receptors (Heldin, 1995; Han et al., 2014). Usually it is the presence of a peptide, a small molecules or any other specific signal that promotes or stabilizes the interaction between two receptors. This physical interaction brings together the cytoplasmic kinase domains of the receptors that can then autophosphorylate and initiate downstream signaling (often the induction of a MAP kinase cascade). Interestingly, in animals, the EGF-like domains and their O-fucosylation have been shown to regulate protein-protein interaction (Takeuchi et al., 2014). Usually they are necessary and promote the interactions between the proteins, but the system works in such a way that it can also limits the interaction with one protein or another. An additional glycosylation such as the addition of an N-acetylglucosamine to the fucose will change the affinity of the protein with its original partner, and a different partner will associate with it. This is exemplified in the case of the protein NOTCH in *drosophila* (figure1 A-C). This protein can associate with the protein called SERRATE when its EGF-like repeats are only O-fucosylated, or with DELTA when they are additionally N-acetylglucosaminated. The state of glycosylation specific to one of the proteins also excludes the interaction with the other. There seem to be some specificity in the function of different EGF-like domains on a protein, with some promoting the specific interaction, and others inhibiting the non specific interaction. Indeed, the glycosylation of one of these EGF-like repeat was shown to be specifically inhibitor of the non specific protein interaction (figure 1 B) (Lei et al., 2003) and the absence of O-fucosylation of this site led to the constitutive interaction of two proteins despite the glycosylation state of the rest of the EGF-like domains indicating otherwise.



From Lei et al. (2003). *An O-fucose site in the ligand binding domain inhibits Notch Activation.*

**Figure 1: O-fucosylation in the notch signalling pathway**

(A-B) Schematic representation of the Notch signalling pathway that exist in animals, and the role of the O-fucosylation of the EGF-like repeats in this pathway. Notch can either interact with a ligand called Delta or another called Serrate. When the protein Notch is only O-fucosylated on it's EGF-like repeats (A), it can only interact with Serrate, and not with Delta. This interaction triggers a specific signalling in cell 2. When the EGF-like repeats have an additional N-Acetylglucosamine on top of the fucoses (B), then Notch can only interact with Delta, and not with Serrate anymore. A different signal is triggered in cell 2.

(C) is a figure from Lei et al. (2003). It shows the influence of O-fucosylation of EGF12 on Notch- Serrate interactions. (1) In the absence of N-Acetylglucosamine, Serrate binds to and activates Notch. (2) When the EGF12 cannot be O-fucosylated, the Notch-Serrate interaction is enhanced. (3) When N-acetylglucosamine is added to O-fucose, Serrate cannot bind or activate Notch (The O-fucose glycan is shown extended to the trisaccharide which is the case in certain species (Chen et al., 2001)). (4) When the O-fucose site in EGF12 is mutant, Serrate can still bind despite the presence of elongated O-fucose glycans at other sites, and consequently can still activate Notch. Green triangles, fucose; red squares, N-acetylglucosamine; blue circles, galactose.

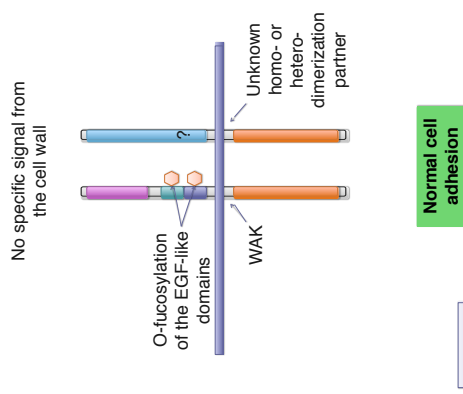


Based on this knowledge and the phenotypes observed in our cell adhesion defective and suppressed mutants, we propose a working model of the O-fucosylation of the WAKs, that could explain what happens at a molecular level (figure 2).

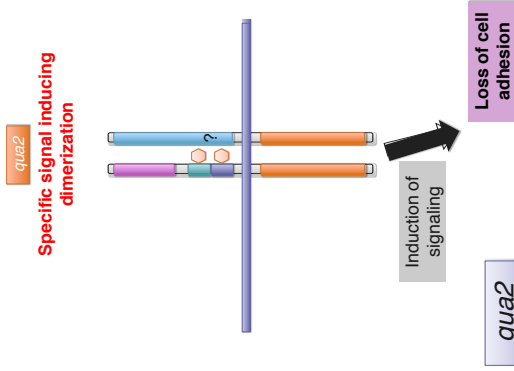
In the case of the HG deficient mutants *quasimodo*, a specific signal originating from the affected cell wall (maybe related to OGs), could promote the specific homo- or heterodimerization of the WAKs and trigger a downstream signaling leading to cell separation (figure 2 B). The disruption of FRB1 leading to the absence of a O-fucosylation necessary for the inhibition of non specific protein-protein interaction (in analogy with the O-fucosylation inhibiting nonspecific interaction in animals, figure 1 C), could lead to a constitutive dimerization of the receptors, and to a signaling leading to cell separation, even in the absence of a specific signal from the cell wall (figure 2 C and D). The disruption of ESMD1 leading to the absence of a O-fucosylation necessary for protein-protein interaction could inhibit the dimerization of the receptors and prevent the induction of the signaling leading to cell separation (*qua2/esmd1*, *frb1/esmd1*, *qua2/frb1/esmd1*, figure 2 respectively E, F and G).

This is only a model and so far we have not proven the existence of O-fucosylation on these domains neither of the implication of the WAKs. However at the current state of our investigation this seems to be a valid model to explain the phenotypes that we encounter: It can nicely explain the antagonism of the effect of the respective disruption of the two putative O-fucosyltransferases ESMD1 and FRB1 and it also seems to correlate with the affected subcellular localization of our EGF-WAK:GFP construct as observed in *frb1-2* mesophyll protoplasts. O-fucosylation of the WAKs EGF-like domains could represent a so far not described mechanism of regulation and determination of the specificity of interaction between receptor like kinases. The decisive point in the validation of this hypothesis will be the biochemical characterization of the WAK in the different genetic background in order to determine whether they are O-fucosylated or not, and whether ESMD1 and FRB1 are the O-fucosyltransferases of their EGF-like domains. This model also opens new perspectives such as the determination of the partner receptors in the case of a heterodimerization. Interestingly the small GLYCINE RICH PROTEIN 3 (GRP3) has been shown to interact with the extracellular domain of WAK1, 3 and 5 through its C-terminal cysteine rich domain and it was speculated that this interaction could be with one of the EGF like domains of the WAKs. Although GRP3 is not a receptor like kinase, its presence *in vitro* was shown to induce the homo-oligomerization of WAK1 (Park et al., 2001). Such an interaction and subsequent oligomerization of the WAKs could be under the control of the O-fucosylation state of the

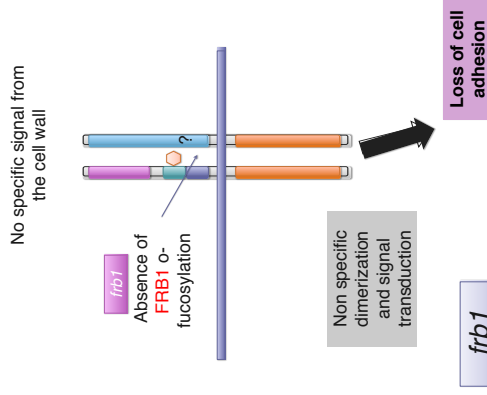
A



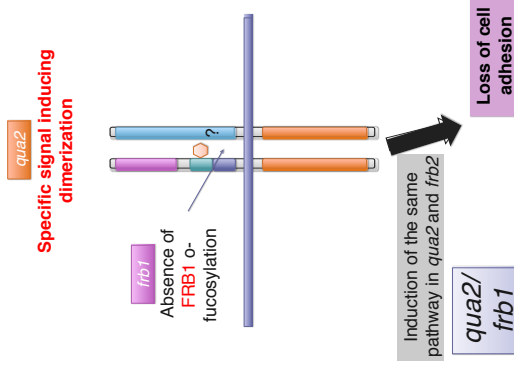
B



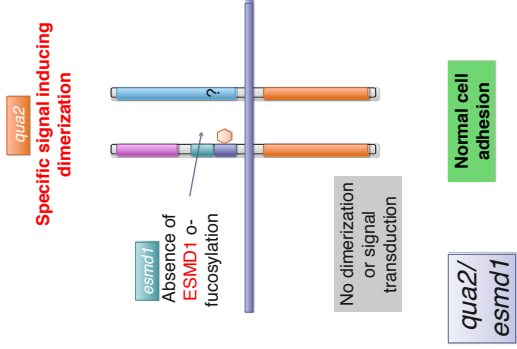
C



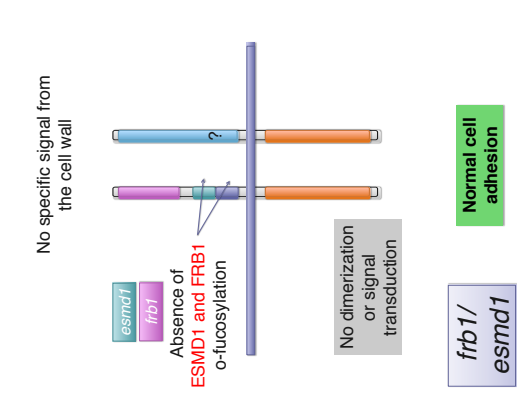
D



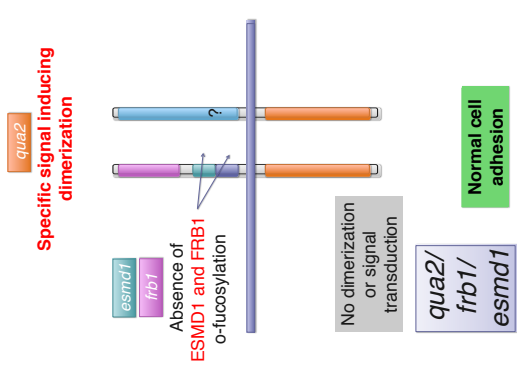
E



F



G



**Model of the O-fucosylation of the WAKs and the impact on WAK signaling**

### **Figure 2: Model of the O-fucosylation of the WAKs and the impact on WAK signaling**

(A-G) Schematic model of the potential role of the O-fucosylation on the WAKs and their impact on signaling for the control of cell adhesion. (A) In this model we speculate that in a wild type background the WAKs are O-fucosylated on both EGF-like domains and that no specific signal in the cell wall active the signaling by the WAKs. In case of a signal, the WAKs could interact with on other WAK or another RLK. (B) in a *qua2* background, there is a specific, unknown signal in the cell wall that induces the dimerization of the receptors and trigger a signal leading to cell separation. The presence of the O-fucoses on both EGF-like domains allow the signaling leading to cell separation. (C) in a *frb1* background, the lack of the O-fucosylation mediated by FRB1 induces a constitutive dimerization of the receptors and signal leading to cell separation, even in the absence of a specific signal. This is in analogy with the case presented in figure 1 C. (D) If both *QUA2* and *FRB1* are disrupted, there is both a signal from the cell wall and a constitutive dimerization of the receptor, which are equivalent and thus do not have additive effect. In all of the previous cell adhesion defective background (B-D) suppressed by *esmd1* (E-G), the lack of the O-fucosylation mediated by ESMD1 prevents the dimerization of the receptors and prevent the induction of the signaling leading to cell separation even in the presence of a specific signal from the cell wall.

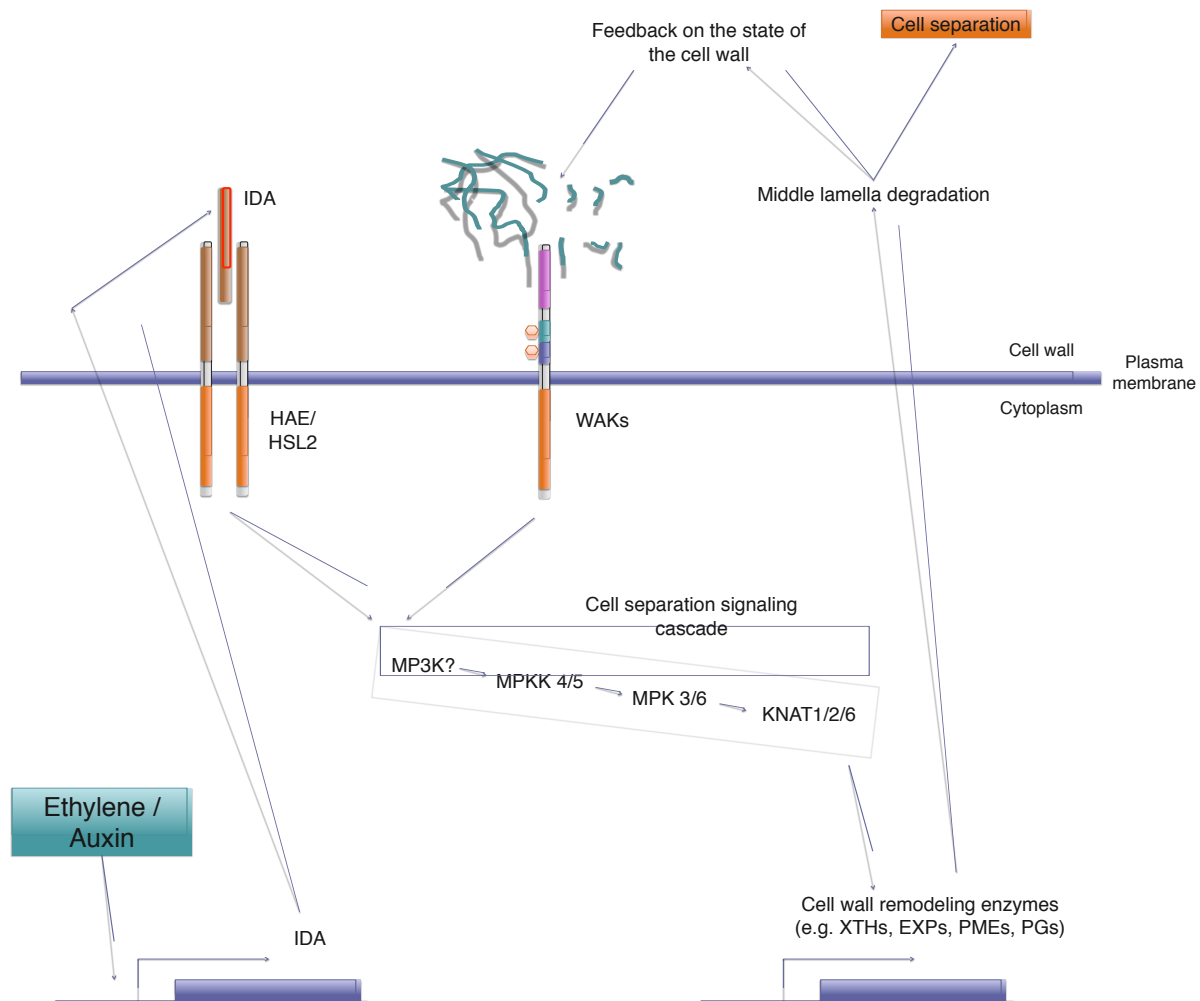
---

EGF-like domains. The identification of the other *ESMERALDA* loci could identify a major interacting protein, a RLK for example, given that there is indeed heterodimerization and that the interacting protein would not be part of a redundant family or its disruption lethal. In the latter cases maybe the molecules from the chemical genomic screen could help identify such a target.

In the subsequent part of the discussion, for simplification purpose I state that the WAKs are the potential substrate of ESMD1 and FRB1, although it is not proven and we do not ignore that it could be a different substrate. Also for simplification I do not mention these potential mechanisms and the potential homo- or heterodimerization in the different scheme, but it should be applicable to the different cases presented.

### **Plants can trigger cell separation throughout their tissues**

The study of *quasimodo*, *esmeralda1* and *friable1* in the first part of the second chapter of this thesis led to the conclusion that the cell adhesion defect of the *quasimodo* mutants is not directly the consequence of a decreased HG content in the cell wall but is more likely due to the induction of a signaling pathway leading to cell separation. Developmentally controlled cell separation is a process that so far is thought to be limited to very specific events such as the abscission, dehiscence, fruit ripening, lateral root emergence, and others (Roberts et al., 2002; Estornel et al., 2013). The molecular mechanisms that take place during these events are now quite well described and recent studies on floral organ abscission have uncovered a number of interesting players in this pathway (as described in the first chapter of this thesis and figure 3).



**Figure 3: Plant cell separation pathways**

Schematic representation of the cell separation pathway that is described for the developmentally controlled cell separation events (abscission, dehiscence,...) and the potential interaction with the cell separation that is triggered in *quasimodo*. An interplay between auxin and ethylene triggers the expression of the small peptide IDA. This peptide is then perceived by the pair of LRR-RLK receptor HAE/HSL2 and triggers a MAP kinase cascade leading to the repression and derepression of KNAT transcription factors. Finally the transcription factors induce the expression of cell wall remodeling enzymes such as XTHs, EXPs, PME and PGs that mediate the degradation of the middle lamella leading to cell separation. In parallel the perception of the cell wall pectins by the WAKs could have the potential to induce this same MAP kinase cascade leading to cell separation.

Interestingly *tsd2*, an allele of *QUA2* (Krupkova et al., 2007) was isolated in a genetic screen for its ability to develop a callus like tissue instead of a normal shoot apical meristem (SAM), a phenotype that is also observed in the other alleles of *qua2* as well as *qua1* and *friable1*. This phenotype and the disruption of *QUA2* were correlated with the overexpression of the class I knotted1-like homeobox transcription factors KNAT1 and KNAT2 (Frank et al., 2002; Krupková et al., 2007). Interestingly, among other function, KNAT1 and KNAT2, along with KNAT6 were shown to be implicated in the signaling pathway leading to cell separation during abscission (Shi et al., 2011). In addition the cell separation pathway that is described for abscission implicates MPK3 and 6 (Cho et al., 2008). These MAP kinases were also

shown to be downstream elements of the OG and WAK signaling pathway which seem to be implicated in our case (Galletti et al., 2011; Kohorn et al., 2012). Finally the chemical genomic suppressor screen showed that inhibiting the transport of auxin transport could restore the phenotype of *quasimodo*. The auxin transport was shown to be one of the crucial events in triggering the expression of the IDA peptide and inducing cell separation (Péret et al., 2013; Kumpf et al., 2013).

From these observations we question the implication of this cell separation pathway in the case of *quasimodo* cell adhesion defect. One hypothesis could be that in a *quasimodo* mutant background, the expression of IDA, HAE and HSL2 is upregulated throughout the plant, leading to the induction of this cell separation pathway. In that regard testing the effect of the disruption of *IDA* on the suppression of the cell adhesion defect on *quasimodo* phenotype could help us determine whether this pathway is implicated or not. Although this hypothesis is unlikely due to the very restricted expression of HAE and HSL2, these observations shows that a number of links or similarities seem to exist between this well described abscission pathway, and the loss of cell adhesion in our case.

Another hypothesis however, is that these two pathways function in parallel, but using the same downstream signaling pathway. As schematized in figure 3, these two pathways could use the same downstream MAP kinases cascades, transcription factors and induction of the same kind of cell wall remodeling enzymes, finally leading to cell separation. However, upstream this cascade could be either induced by the presence of the peptide signal IDA and perception by HAE/HSL2, or respond to feedback from the state of the cell wall via a perception by the WAKs. Interestingly when looking at this putative interaction between these pathways one could hypothesize that the existence of the WAKs' pectin perception and signalization could serve as some kind of amplification of the abscission signal leading to cell separation, in order to actually ensure cell separation when it is necessary.

These are hypotheses that could be verified by testing the genetic interactions between these two pathways. Does the mutation in *ESMDI* limit organ abscission? Would the disruption of the MAP kinase cascade, or the KNAT transcription factors affect cell adhesion in *quasimodo* or *esmdi*? Such observations could effectively indicate us whether the two phenomena are related. They could reveal common downstream events or otherwise reveal independent cascade of events.

## A working model for the control of cell adhesion in plants

The work presented in this thesis has led to the identification of *ESMD1* a putative O-fucosyltransferase, as well as the auxin transport and the PME's as new players implicated in cell adhesion. In fact auxin transport and the PME's may not be considered as really new players in cell adhesion in general since their role was already described in the case of cell separation. However we identified them in a different context, and as such they provide new information on the mechanisms of cell adhesion. We have also incorporated *frb1* in our study, and the genetic interactions with *qua2* and *esmd1* has provided even more new information on the mechanisms of cell adhesion. In this paragraph we try to compile this knowledge and integrate the new molecular players, in order to propose a new model explaining the control of cell adhesion in plants.

*We have shown that cell adhesion phenotype of quasimodo can be suppressed through action on PME's and Auxin transport:*

-The PME's are cell wall remodeling enzymes and their action, in conjunction with PGs, can lead to the degradation of the pectins (Micheli et al., 2001; Pelloux et al., 2007; Senechal et al., 2014). Thus preventing their action with small molecule inhibitors can prevent the degradation of the pectins that otherwise leads to the loss of cell adhesion in *quasimodo*.

-Auxin is known to cause a loosening of the cell wall by the concerted action of the expression of cell wall remodeling enzymes (XTHs, EXPS, PME's, PGs) and the acidification of the cell wall (Rayle and Cleland 1992; Senechal et al., 2014). In acidic condition the action of the XTHs and EXPs can ease the accessibility of the PME and PG to the middle lamella for its dissolution. Thus preventing the transport of auxin from its apical place of synthesis and preventing its action on the other cells can explain the absence of cell wall loosening and pectin degradation that otherwise leads to the loss of cell adhesion in *quasimodo*.

*But why would these well-known mechanisms lead to cell separation in quasimodo while they do not in the wild type?*

A straightforward explanation could be that the decreased HG content in *quasimodo* cell wall makes it more sensitive to these cell wall remodeling enzymes and instead of simply

loosening the cell wall, it leads to cell separation. However the genetic study has also pointed out the role of ESMD1 and FRB1 in cell adhesion. *frb1* undergoes the same type of cell separation as *quasimodo* but it is not affected in pectin content, and *esmd1* restores the cell adhesion defect of *quasimodo* but does not restore its HG content in the cell wall. The genetic analysis demonstrated that *quasimodo*, *esmd1* and *frb1* affect cell adhesion in the same pathway. ESMD1 and FRB1 are putative O-fucosyltransferases and the WAKs EGF-like domains are most likely their substrates.

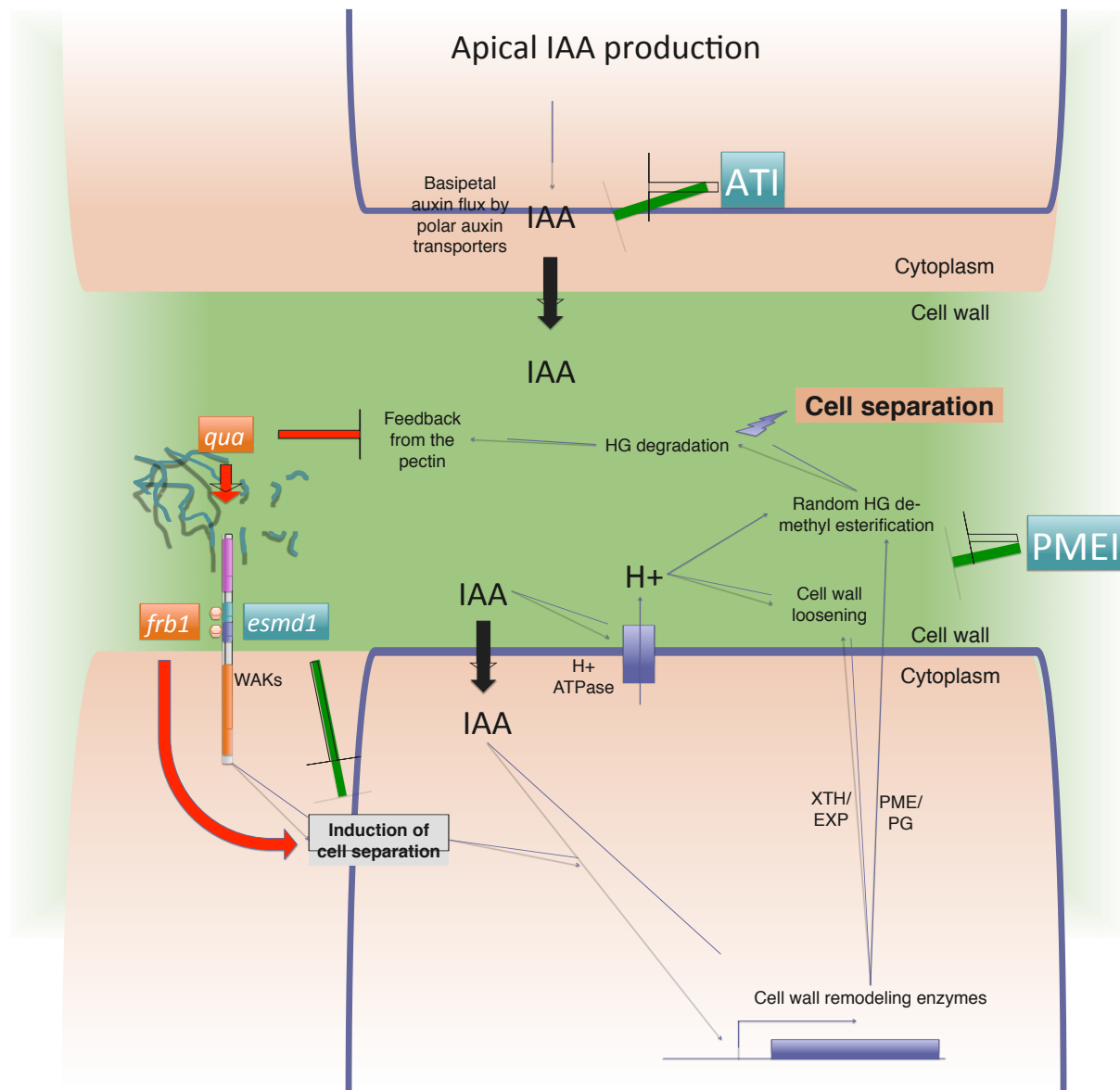
*So how can we integrate these molecular players with the role of auxin and PMEs?*

The mutation in *ESMD1* could affect the WAKs' downstream signaling in such a way that it would prevent the cell wall loosening action of auxin that otherwise leads to cell separation. On the other hand the mutation in *FRB1* could affect the WAKs' downstream signaling in such a way that it would promote the action of auxin and induce a cell separation that is similar to that of *quasimodo* except that *frb1* is not deficient in HG.

This hypothesis implies that it is not simply a higher susceptibility of the *quasimodo* cell wall that leads to cell separation, but that there is in fact the induction of a signaling pathway likely mediated by the WAKs, that leads to cell separation via the action of auxin and PMEs. This model reconciles the fact that auxin transport inhibition, PME inhibition and the disruption of ESMD1 can all individually prevent the induction of cell separation in *quasimodo*, and that the disruption of FRB1 can induce this cell separation independently of a HG deficiency.

*But what is the signal perceived by the WAKs that induces this cell separation pathway?*

Interestingly the WAKs are known to bind to the HG (Wagner et Kohorn, 2001; Decreux and Messiaen 2005; Decreux et al., 2006) and to transduce a signal upon treatment with small pectin fragments (Kohorn et al., 2006b, Kohorn et al., 2009; Brutus et al., 2010). *quasimodo* is affected in its HG content and thus the specific state of the pectins in its cell wall could be perceived by the WAKs and transduced as a signal leading to cell separation. The WAKs could thus induce the degradation of the cell wall. But the interesting thing is that they should also be able to perceive the degradation of the cell wall due to the consequent formation of OGs. There should thus exist a feedback indicating to the plants the level of degradation of the pectins. This feedback could then be used to induce or repress further cell wall degradation depending on the situation. We can imagine that such a feedback loop should



**Figure 4: Induction of a cell separation pathway in the cell adhesion defective mutants**

Schematic representation of the cell separation pathway that seems to be induced in *quasimodo* and *frb1*.

In *quasimodo* the specific state of the pectins is perceived by the WAKs. This signal leads to the induction of a cell separation pathway that acts by promoting the cell wall loosening action of auxin. Auxin is transported throughout the plant and can triggers cell wall loosening by the concerted action of the expression of cell wall remodeling enzymes (XTHs, EXPS, PMEs, PGs) and the acidification of the cell wall (Rayle and Cleland 1992; Senechal et al., 2014). In these acidic condition the action the XTHs and EXPs can ease the accessibility of the PME and PG to the middle lamella for its dissolution. Due to the particular state of the pectins in the cell wall of *quasimodo*, the massive degradation of the cell wall is either not perceived or induces even more degradation, finally leading to the loss of cell adhesion. The mutation in *FRB1* affects the WAKs' downstream signalling in such a way that it promotes the action of auxin and induce the same cell separation pathway as *quasimodo* except that *frb1* is not deficient in HG. On the other hand, the mutation in *ESMD1* affects the WAKs' downstream signalling in such a way that it prevents the cell wall loosening action of auxin that otherwise leads to cell separation in *frb1* or *quasimodo*. The inhibition of auxin transport prevents the accumulation of auxin that is necessary for the induction of the pathway. And finally the inhibition of the PME activity prevents the formation of the HG substrate susceptible to the degradation by the PG and thus limits the cell wall degradation that would otherwise lead to cell separation.

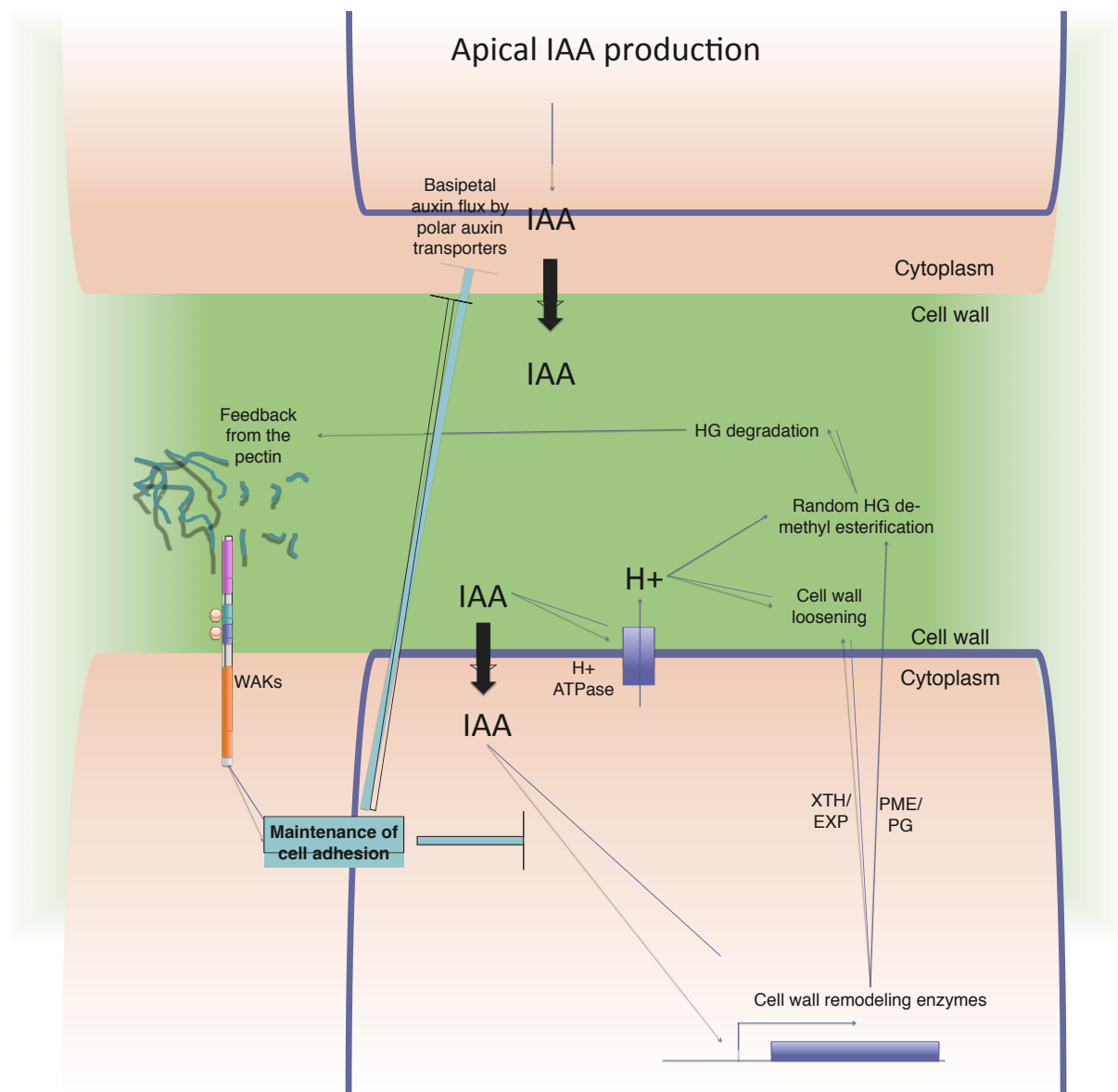


normally work as a negative feedback in a wild type plants, limiting the induction of the cell wall loosening when the cell wall is already undergoing too much degradation, in order to prevent cell separation. But that doesn't seem to be the case in *quasimodo*. What may happen in *quasimodo* is that the already modified state of the pectin in the cell wall affects this feedback in such a way that it either turns it into a positive feedback loop or that it simply prevents the perception of the cell wall degradation such that the initial "quasimodo signal" is not repressed by the OG negative feedback (figure 4).

Interestingly we can find similarities with this pathway in mechanism already described in the literature. The OGs were shown to have an antagonistic effect against auxin signalling (Bellincampi et al., 1993; Savatin et al., 2011; Ferrari et al., 2013; Branca et al., 1988). Although their mechanism of action in this process is still not clear (Savatin et al., 2011), some lines of evidence have shown that the OGs could trigger the production of flavonoids with a known auxin transport inhibition effect (Hernández-Mata et al., 2010). Inhibition of auxin transport would then be at least one of the explanations of the OG-auxin antagonism. Given that the auxin-induced loosening of the cell wall promotes the formation of OGs (Senechal et al., 2014), the apparition of OGs should then antagonize the action of auxin and prevent a too massive degradation of the cell wall, making it a negative feedback loop. Since the WAKs are the only demonstrated receptors of the OGs (brutus et al., 2011), the action of the OGs in this feedback loop may in fact be mediated by the WAKs.

Finally the pathway that we have identified by studying cell adhesion may use the same mechanism as this negative feedback loop described in the literature, except that in *quasimodo* it is perturbed in such a way that it leads to cell separation instead of preventing it. Overall these observations indicate that there is a modulable feedback loop implicating the WAKs, auxin, and PMEs at least, that may represent a so far not described dynamic mechanism for the maintenance and control of cell adhesion in plants.

This relatively complex mechanism / feedback loop is schematized in figure 4 and 5. Figure 4 represents the pathway that seems to be induced in *quasimodo* and *frb1* and indicates where the different mutant and suppressor would affect the pathway. Figure 5 represent the pathway as it may exist in the wild type plant and as it may normally function in order to prevent cell separation.



**Figure 5: Maintenance and control of cell adhesion in plants**

Schematic representation of the regulatory feedback loop that allows the maintenance and control of the cell adhesion in plants.

Auxin is transported throughout the plant and can triggers cell wall loosening by the concerted action of the expression of cell wall remodeling enzymes (XTHs, EXPS, PMEs, PGs) and the acidification of the cell wall (Rayle and Cleland 1992; Senechal et al., 2014). In these acidic conditions the action of the XTHs and EXPs can ease the accessibility of the PME and PG to the middle lamella for its dissolution. The degradation of the cell wall is perceived by the WAKs which subsequently induce the synthesis of endogenous flavonoid auxin transport inhibitors, and inhibit the cell wall loosening action of auxin. A too massive degradation of the cell wall is thus avoided and the cell adhesion is maintained.

Although a lot remains to be understood on the precise role of some of these molecular players, based on these new information we have established a new model explaining the loss of cell adhesion in the *quasimodo* and *friable1* mutants, and from this model we have inferred the existence of the mechanisms that dynamically allow the maintenance of cell adhesion in

plants during growth and development. Further work on the molecular player that we have identified here as well as the other ones isolated in our genetic and chemical genomic screen will continue to improve our understanding of this pathway and in general of the mechanisms that control cell adhesion in plants

### Why is *quasimodo* sensitive to sucrose?

Although the work presented in this thesis is largely focused on cell adhesion, the sensitivity of *quasimodo* to the carbon/nitrogen imbalance is not a negligible aspect of the mutants phenotype. The isolation of *oversensitive to sugar 1 (osu1)* (Gao et al., 2008), an allele of *qua2* identified in a genetic screen specifically directed at identifying mutants affected in the response to sucrose, indicates that the disruption of *quasimodo* has a significant impact on the plants' metabolism.

Since the function of QUA1 and QUA2 seems clearly defined as homogalacturonan synthesis enzymes (Bouton et al., 2002; Mouille et al., 2007), the most likely hypothesis for the presence of this phenotype is that it is a secondary effect of either the cell adhesion defect or the HG synthesis deficiency.

The cell adhesion defect itself could explain this phenotype by the fact that the loss of cell to cell adhesion should disrupt the connectivity between certain cells, and thus limit the exchange of molecules including metabolites. The sucrose may thus accumulate in certain cells and induce a stress. Another hypothesis is based on another phenotype of *quasimodo* that has not been described in this thesis. The mutant *quasimodo* tend to show a hyperhydric phenotype, that translate by the translucence of the tissues due to the fact that the intercellular spaces are filled with water or polysaccharides. This should reflect the affected state of the pectin and probably a high porosity of the cell wall in *quasimodo*. Such a porosity may easily allow the uptake of sucrose and the sucrose may easily diffuse throughout the plant by the apoplast. The uncontrolled accumulation of sucrose in the tissue may thus trigger a stress. Another hypothesis is that this phenotype is induced by the deficiency in HG. The direct effect of the inhibition of pectin synthesis in the *quasimodo* mutants could be that the carbohydrates that should ultimately be incorporated in the cell wall are not, and instead accumulate in the cell. Thus making the plant more sensitive to additional carbon input. Finally this higher sensitivity to sucrose could be a side effect of the signaling pathway that is activated and that leads to cell separation.

The fact that some of the chemical suppressors can restore the cell adhesion defect without restoring the sucrose sensitivity indicates that it is not a consequence of the cell adhesion defect. In addition the fact that *esmdl* can suppress the sucrose sensitivity without restoring the HG synthesis should indicate that it is not a problem of sucrose accumulation consequent to the HG synthesis inhibition either. The existence of this phenotype in *quasimodo* remains mysterious for now, but the study of the different suppressor specific for the restoration of the sucrose sensitivity, that are already isolated, should explain the existence of this phenotypes and unravel the molecular players responsible for it.

## Perspectives

The objectives of this work was to identify the molecular players implicated in cell adhesion, and get a better understanding of the cell adhesion mechanisms.

By the use of no *a priori* approaches our goal was to try to identify the function of new uncharacterized genes. We have isolated and identified *ESMD1* which that regard represent an uncharacterized gene member of a gene family within which none of the genes have been characterized for their catalytic function in plants.

The study of the putative function of *ESMD1* and *FRB1* has led us to the hypothesis that the O-fucosyltransferases are implicated in the O-fucosylation of the WAKs EGF-like domains. But of course other proteins could in fact be the substrates of these putative O-fucosyltransferases such as the *SRK* which for some of them have a conserved O-fucosylation sites. But there could be other substrates. The family of the putative O-fucosyltransferases in *Arabidopsis* contains 39 members, and based on our prediction inspired from the animal system, there would be only 15 conserved O-fucosylation sites on *Arabidopsis* protein. Such a higher number of enzymes compared to the putative target could be explained by a high redundancy of some of the members, or very distinct patterns of expression. But it also very likely indicates that there are more potential substrates than what we estimated. It will remain challenging to actually determine those but looking for protein domains resembling the EGF, forming disulfide bridges and having a three dimensional structure containing conserved serine or threonine at a given location in the domain could be a good starting point. In addition, no *a priori* proteomic approaches designed to identify O-fucosylated proteins could reveal very informative. The study of the plant putative O-fucosyltransferases, the

characterization of their catalytic function and the role that they play in plant development should reveal very interesting for future research.



## List of abbreviations

---

2,4 D	2,4-Dichlorophenoxyacetic acid
aa	amino acid
ABA	Abscisic acid
AGP	ArabinoGalactan Protein
AO	Ammonium Oxalate
bp	base pair
BRI	BRassinolide-Insensitive1
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CDS	CoDing Sequence
CDTA	CyclohexyleneDinitriloTetraacetic Acid
CESA	Cellulose Synthase Catalytic Subunit
CGA	Charophycean Green Algae
CMV	Cauliflower Mosaic Virus
CTAB	CetylTrimethylAmmonium Bromide
CWR	Cell Wall Remodeling
C/N	Carbon/Nitrogen
DMSO	DiMethyl SulphOxide
DNA	DeoxyriboNucleic Acid
DTT	DiThioThreitol
ECM	ExtraCellular Matrix
EDTA	Ethylene Diamine Tetra acetic Acid
EELS	Electron Energy Loss Spectroscopy
EGCG	EpiGalloCatechin Gallate
EGF	Epidermal Growth Factor



EMS	Ethyl MethaneSulfonate
EXP	Expansin
E.coli	Escherichia coli
FTIR	Fourier Transform Infra-Red
Fuc	Fucose
FucAL	Fucose Alkyne
Gal	Galactose
GalA	Galacturonic Acid
GAUT	GalactUronosylTransfearse
GDP	Guanosine DiPhosphate
GFP	Green Fluorescent Protein
Glc	Glucose
GlcA	Glucuronic Acid
GlcNac	N-AcetylGlucosamine
GO	Gene Ontology
GRP	Glycine-Rich Protein
GT	GlycosylTransferase
GUB	GalactUronic acid Binding
GUS	$\beta$ -glucuronidase
HG	HomoGalacturonan
HRGP	Hydroxyproline Rich GlycoProtein
HRM	High Resolution Melt
HRP	HorseRadish Peroxidase
IAA	Indole Acetic Acid
kDa	kiloDalton

LRR	Leucine-Rich Repeat
Man	Mannose
MAP	Mitogen activated Protein
ME	Methyl Esterification
NAA	1-Naphthaleneacetic acid
NGS	Next Generation Sequencing
NPA	1-N-Naphthylphthalamic acid
OG	OligoGalacturonan
PA	Protease Associated domain
PAE	Pectin Acetyl Esterase
PAGE	PolyAcrylamide Gel Electrophoresis
PCR	polymerase chain reaction
PDB	Potato Dextrose Broth
PFS	Planar Fenestrated Sheet
PG	PolyGalacturonase
PL	Pectate Lyase
PME	Pectin MethylEsterase
PMEI	Pectin MethylEsterase Inhibitor
POFUT	Protein O-FucosylTransferase
RFP	Red Fluorescent Protein
RGI	RhamnoGalacturonan I
RGII	RhamnoGalacturonan II
RLK	Receptor-like Kinase
RLP	Receptor-like Protein
RNA	RiboNucleic Acid

RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDS	Sodium DodecylSulphate
SIMS	Secondary Ion Mass Spectroscopy
SNP	Single Nucleotide Polymorphism
SP	signal peptide
SRK	S-locus Receptor Kinase
TBTA	Tris(benzyltriazolylmethyl)amine
TFA	TriFluoroacetic Acid
TIBA	2,3,5-triiodobenzoic acid
TM	TransMembrane domain
TN	Tubuar Network
TSR	ThromboSpondin Repeat
TVN	TubuloVesicular Network
T-DNA	Transfer DNA
UDP	Uridine DiPhosphate
VSR	Vacuolar Sorting Receptor
WAK	Wall associated kinase
WGS	Whole Genome sequencing
WS	A.thaliana ecotype Wassilevskija



## References

---

- Aalen, R.B., Wildhagen, M., Stø, I.M., and Butenko, M.A.** (2013). IDA: a peptide ligand regulating cell separation processes in Arabidopsis. *Journal of Experimental Botany* **64**: 5253–5261.
- Abedin, M. and King, N.** (2010). Diverse evolutionary paths to cell adhesion. *Trends in Cell Biology* **20**: 734–742.
- Alegado, R.A., Brown, L.W., Cao, S., Dermenjian, R.K., Zuzow, R., Fairclough, S.R., Clardy, J., and King, N.** (2012). A bacterial sulfonolipid triggers multicellular development in the closest living relatives of animals. *Elife* **1**.
- Anderson, C.T., Wallace, I.S., and Somerville, C.R.** (2012). Metabolic click-labeling with a fucose analog reveals pectin delivery, architecture, and dynamics in Arabidopsis cell walls. *Proc. Natl. Acad. Sci. U.S.A.* **109**: 1329–1334.
- Atkinson, R.G., Schröder, R., Hallett, I.C., Cohen, D., and MacRae, E.A.** (2002). Overexpression of polygalacturonase in transgenic apple trees leads to a range of novel phenotypes involving changes in cell adhesion. *PLANT PHYSIOLOGY* **129**: 122–133.
- Atmodjo, M.A., Hao, Z., and Mohnen, D.** (2013). Evolving Views of Pectin Biosynthesis. *Annu. Rev. Plant Biol.* **64**: 747–779.
- AXELOS, M., Curie, C., Mazzolini, L., Bardet, C., and Lescure, B.** (1992). A protocol for transient gene expression in Arabidopsis thaliana protoplasts isolated from cell suspension cultures. *Plant Physiology and Biochemistry* **30**: 123–128.
- Bailly, A., Sovero, V., Vincenzetti, V., Santelia, D., Bartnik, D., Koenig, B.W., Mancuso, S., Martinoia, E., and Geisler, M.** (2008). Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. *Journal of Biological Chemistry* **283**: 21817–21826.
- Baluška, F., Hlavacka, A., Šamaj, J., Palme, K., Robinson, D.G., Matoh, T., McCurdy, D.W., Menzel, D., and Volkmann, D.** (2002). F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. *PLANT PHYSIOLOGY* **130**: 422–431.
- Baluška, F., Liners, F., Hlavačka, A., Schlicht, M., Van Cutsem, P., McCurdy, D.W., and Menzel, D.** (2005). Cell wall pectins and xyloglucans are internalized into dividing root cells and accumulate within cell plates during cytokinesis. *Protoplasma* **225**: 141–155.
- Baluška, F., Menzel, D., and Barlow, P.W.** (2006). Cytokinesis in plant and animal cells: endosomes 'shut the door'. *Dev. Biol.* **294**: 1–10.
- Baluška, F., Šamaj, J., Wojtaszek, P., Volkmann, D., and Menzel, D.** (2003). Cytoskeleton-plasma membrane-cell wall continuum in plants. Emerging links revisited. *PLANT PHYSIOLOGY* **133**: 482–491.
- Bar-Peled, M., Urbanowicz, B.R., and O'Neill, M.A.** (2012). The Synthesis and Origin of the Pectic Polysaccharide Rhamnogalacturonan II - Insights from Nucleotide Sugar Formation and Diversity. *Front Plant Sci* **3**: 92.

- Barrett, T., Troup, D.B., Wilhite, S.E., Ledoux, P., Rudnev, D., Evangelista, C., Kim, I.F., Soboleva, A., Tomashevsky, M., and Edgar, R.** (2007). NCBI GEO: mining tens of millions of expression profiles--database and tools update. *Nucleic Acids Res.* **35**: D760–5.
- Becker, B. and Marin, B.** (2009). Streptophyte algae and the origin of embryophytes. *Annals of Botany* **103**: 999–1004.
- Bellincampi, D., Salvi, G., Lorenzo, G., Cervone, F., Marfà, V., Eberhard, S., Darvill, A., and Albersheim, P.** (1993). Oligogalacturonides inhibit the formation of roots on tobacco explants. *The Plant Journal* **4**: 207–213.
- Berezin, C., Glaser, F., Rosenberg, J., Paz, I., Pupko, T., Fariselli, P., Casadio, R., and Ben-Tal, N.** (2004). ConSeq: the identification of functionally and structurally important residues in protein sequences. *Bioinformatics* **20**: 1322–1324.
- Blumenkrantz, N. and Asboe-Hansen, G.** (1973). New method for quantitative determination of uronic acids. *Anal. Biochem.* **54**: 484–489.
- Bonner, J.T.** (2004). PERSPECTIVE: THE SIZE-COMPLEXITY RULE. *Evolution* **58**: 1883–1890.
- Bonner, J.T.** (1998). The origins of multicellularity. *Integrative Biology Issues News and Reviews* **1**: 27–36.
- Boraas, M.E., Seale, D.B., and Boxhorn, J.E.** (1998). Phagotrophy by a flagellate selects for colonial prey: A possible origin of multicellularity. *Evolutionary Ecology* **12**: 153–164.
- Bouton, S., Leboeuf, E., Mouille, G., Leydecker, M.-T., Talbotec, J., Granier, F., Lahaye, M., Höfte, H., and Truong, H.N.** (2002). QUASIMODO1 encodes a putative membrane-bound glycosyltransferase required for normal pectin synthesis and cell adhesion in Arabidopsis. *The Plant Cell* **14**: 2577–2590.
- Branca, C., Lorenzo, G.D., and Cervone, F.** (1988). Competitive inhibition of the auxin-induced elongation by  $\alpha$ -D-oligogalacturonides in pea stem segments. *Physiologia Plantarum* **72**: 499–504.
- Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G.** (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* **107**: 9452–9457.
- Burr, C.A., Leslie, M.E., Orlowski, S.K., Chen, I., Wright, C.E., Daniels, M.J., and Liljegren, S.J.** (2011). CAST AWAY, a membrane-associated receptor-like kinase, inhibits organ abscission in Arabidopsis. *PLANT PHYSIOLOGY* **156**: 1837–1850.
- Butenko, M.A., Stenvik, G.-E., Alm, V., Sæther, B., Patterson, S.E., and Aalen, R.B.** (2006). Ethylene-dependent and-independent pathways controlling floral abscission are revealed to converge using promoter:: reporter gene constructs in the ida abscission mutant. *Journal of Experimental Botany* **57**: 3627–3637.
- Caffall, K.H. and Mohnen, D.** (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate Research* **344**: 1879–1900.

- Cai, S. and Lashbrook, C.C.** (2008). Stamen abscission zone transcriptome profiling reveals new candidates for abscission control: enhanced retention of floral organs in transgenic plants overexpressing Arabidopsis ZINC FINGER PROTEIN2. *PLANT PHYSIOLOGY* **146**: 1305–1321.
- Canut, H., Carrasco, A., Galaud, J.P., Cassan, C., Bouyssou, H., Vita, N., Ferrara, P., and Pont-Lezica, R.** (1998). High affinity RGD-binding sites at the plasma membrane of Arabidopsis thaliana links the cell wall. *Plant J.* **16**: 63–71.
- Carpita, N.C.** (1996). STRUCTURE AND BIOGENESIS OF THE CELL WALLS OF GRASSES. *Annu. Rev. Plant Biol.* **47**: 445–476.
- Chen, X., Naramoto, S., Robert, S., Tejos, R., Löffke, C., Lin, D., Yang, Z., and Friml, J.** (2012). ABP1 and ROP6 GTPase Signaling Regulate Clathrin-Mediated Endocytosis in Arabidopsis Roots. *Current Biology* **22**: 1326–1332.
- Chen, X.-Y. and Kim, J.-Y.** (2009). Callose synthesis in higher plants. *psb* **4**: 489–492.
- Cho, S.K., Larue, C.T., Chevalier, D., Wang, H., Jinn, T.-L., Zhang, S., and Walker, J.C.** (2008). Regulation of floral organ abscission in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U.S.A.* **105**: 15629–15634.
- Chormova, D., Messenger, D.J., and Fry, S.C.** (2014). Rhamnogalacturonan-II cross-linking of plant pectins via boron bridges occurs during polysaccharide synthesis and/or secretion. *psb* **9**: e28169.
- Clausen, M.H., Willats, W.G., and Knox, J.P.** (2003). Synthetic methyl hexagalacturonate hapten inhibitors of anti-homogalacturonan monoclonal antibodies LM7, JIM5 and JIM7. *Carbohydrate Research* **338**: 1797–1800.
- Clough, S.J. and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**: 735–743.
- Crespi, B.J.** (2001). The evolution of social behavior in microorganisms. *Trends Ecol. Evol. (Amst.)* **16**: 178–183.
- Cumming, C.M., Rizkallah, H.D., McKendrick, K.A., Abdel-Massih, R.M., Baydoun, E.A.-H., and Brett, C.T.** (2005). Biosynthesis and cell-wall deposition of a pectin-xyloglucan complex in pea. *Planta* **222**: 546–555.
- Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.-R.** (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *PLANT PHYSIOLOGY* **139**: 5–17.
- De Rybel, B., Audenaert, D., Beeckman, T., and Kepinski, S.** (2009). The past, present, and future of chemical biology in auxin research. *ACS chemical biology* **4**: 987–998.
- Decreux, A. and Messiaen, J.** (2005). Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. *Plant and Cell Physiology* **46**: 268–278.
- Decreux, A., THOMAS, A., SPIES, B., BRASSEUR, R., CUTSEM, P., and MESSIAEN, J.** (2006). In vitro characterization of the homogalacturonan-binding domain of the wall-



- associated kinase WAK1 using site-directed mutagenesis. *Phytochemistry* **67**: 1068–1079.
- Dejonghe, W. and Russinova, E.** (2014). Target Identification Strategies in Plant Chemical Biology. Name: *Frontiers in Plant Science*.
- Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., Ferrari, S., Ausubel, F.M., and Dewdney, J.** (2008). Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. *Molecular Plant* **1**: 423–445.
- Dhonukshe, P. et al.** (2008). Auxin transport inhibitors impair vesicle motility and actin cytoskeleton dynamics in diverse eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* **105**: 4489–4494.
- Dhonukshe, P., Baluška, F., Schlicht, M., Hlavacka, A., Šamaj, J., Friml, J., and Gadella, T.W.J.** (2006). Endocytosis of cell surface material mediates cell plate formation during plant cytokinesis. *Developmental Cell* **10**: 137–150.
- Domozych, D.S., Ciancia, M., Fangel, J.U., Mikkelsen, M.D., Ulvskov, P., and Willats, W.G.T.** (2012). The Cell Walls of Green Algae: A Journey through Evolution and Diversity. *Front Plant Sci* **3**: 82.
- Domozych, D.S., Serfis, A., Kiemle, S.N., and Gretz, M.R.** (2007). The structure and biochemistry of charophycean cell walls: I. Pectins of *Penium margaritaceum*. *Protoplasma* **230**: 99–115.
- Domozych, D.S., Sørensen, I., and Willats, W.G.T.** (2009). The distribution of cell wall polymers during antheridium development and spermatogenesis in the Charophycean green alga, *Chara corallina*. *Annals of Botany* **104**: 1045–1056.
- Domozych, D.S., Sørensen, I., Popper, Z.A., Ochs, J., Andreas, A., Fangel, J.U., Pielach, A., Sacks, C., Brechka, H., Ruisi-Besares, P., Willats, W.G.T., and Rose, J.K.C.** (2014). Pectin metabolism and assembly in the cell wall of the charophyte green alga *Penium margaritaceum*. *PLANT PHYSIOLOGY* **165**: 105–118.
- Drakakaki, G. et al.** (2011). Clusters of bioactive compounds target dynamic endomembrane networks in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **108**: 17850–17855.
- Driouich, A., Durand, C., and Vitré-Gibouin, M.** (2007). Formation and separation of root border cells. *Trends in Plant Science* **12**: 14–19.
- Driouich, A., Follet-Gueye, M.-L., Bernard, S., Kousar, S., Chevalier, L., Vitré-Gibouin, M., and Lerouxel, O.** (2012). Golgi-mediated synthesis and secretion of matrix polysaccharides of the primary cell wall of higher plants. *Front Plant Sci* **3**: 79.
- Du, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z.** (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Res.* **38**: W64–70.
- Dunkley, T.P.J. et al.** (2006). Mapping the *Arabidopsis* organelle proteome. *Proc. Natl. Acad. Sci. U.S.A.* **103**: 6518–6523.

- Dunkley, T.P.J., Watson, R., Griffin, J.L., Dupree, P., and Lilley, K.S.** (2004). Localization of organelle proteins by isotope tagging (LOPIT). *Mol. Cell Proteomics* **3**: 1128–1134.
- Ehwald, R., Fleischer, A., Schneider, H., and O’Neill, M.** (2002). Stability of the Borate-ester Cross-link in Rhamnogalacturonan II at Low pH and Calcium Activity in muro and in vivo. In (Springer US: Boston, MA), pp. 157–166.
- Estornell, L.H., Agustí, J., Merelo, P., Talón, M., and Tadeo, F.R.** (2013). Elucidating mechanisms underlying organ abscission. *Plant Science* **199**: 48–60.
- Fagard, M., Desnos, T., Desprez, T., Goubet, F., Refregier, G., Mouille, G., McCann, M., Rayon, C., Vernhettes, S., and HÖFTE, H.** (2000). PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of Arabidopsis. *The Plant Cell* **12**: 2409–2424.
- Ferrari, S., Savatin, D.V., Sicilia, F., Gramegna, G., Cervone, F., and Lorenzo, G.D.** (2013). Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Front Plant Sci* **4**: 49.
- Francis, K.E., Lam, S.Y., and Copenhaver, G.P.** (2006). Separation of Arabidopsis pollen tetrads is regulated by QUARTET1, a pectin methyltransferase gene. *PLANT PHYSIOLOGY* **142**: 1004–1013.
- Frank, M., Guivarc’h, A., Krupková, E., Lorenz-Meyer, I., Chriqui, D., and Schmölling, T.** (2002). Tumorous shoot development (TSD) genes are required for co-ordinated plant shoot development. *Plant J.* **29**: 73–85.
- Gagnot, S., Tamby, J.-P., Martin-Magniette, M.-L., Bitton, F., Taconnat, L., Balzergue, S., Aubourg, S., Renou, J.-P., Lecharny, A., and Brunaud, V.** (2008). CATdb: a public access to Arabidopsis transcriptome data from the URGV-CATMA platform. *Nucleic Acids Res.* **36**: D986–90.
- Galletti, R., Ferrari, S., and De Lorenzo, G.** (2011). Arabidopsis MPK3 and MPK6 play different roles in basal and oligogalacturonide-or flagellin-induced resistance against *Botrytis cinerea*. *PLANT PHYSIOLOGY* **157**: 804–814.
- Gao, P., Xin, Z., and Zheng, Z.-L.** (2008). The OSU1/QUA2/TSD2-Encoded Putative Methyltransferase Is a Critical Modulator of Carbon and Nitrogen Nutrient Balance Response in Arabidopsis. *PLoS ONE* **3**: e1387.
- Geldner, N. and Robatzek, S.** (2008). Plant receptors go endosomal: a moving view on signal transduction. *PLANT PHYSIOLOGY* **147**: 1565–1574.
- Geldner, N., Friml, J., Stierhof, Y.-D., Jürgens, G., and Palme, K.** (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**: 425–428.
- Geldner, N., Hyman, D.L., Wang, X., Schumacher, K., and Chory, J.** (2007). Endosomal signaling of plant steroid receptor kinase BRI1. *Genes & Development* **21**: 1598–1602.
- Gendre, D., McFarlane, H.E., Johnson, E., Mouille, G., Sjödin, A., Oh, J., Levesque-Tremblay, G., Watanabe, Y., Samuels, L., and Bhalerao, R.P.** (2013). Trans-Golgi

network localized ECHIDNA/Ypt interacting protein complex is required for the secretion of cell wall polysaccharides in Arabidopsis. *The Plant Cell* **25**: 2633–2646.

- Gendre, D., Oh, J., Boutté, Y., Best, J.G., Samuels, L., Nilsson, R., Uemura, T., Marchant, A., Bennett, M.J., Grebe, M., and Bhalerao, R.P.** (2011). Conserved Arabidopsis ECHIDNA protein mediates trans-Golgi-network trafficking and cell elongation. *Proc. Natl. Acad. Sci. U.S.A.* **108**: 8048–8053.
- Gierlinger, N. and Schwanninger, M.** (2006). Chemical imaging of poplar wood cell walls by confocal Raman microscopy. *PLANT PHYSIOLOGY* **140**: 1246–1254.
- Gresham, D.** (2013). A sticky solution. *Elife* **2**.
- Grosberg, R.K. and Strathmann, R.R.** (2007). The evolution of multicellularity: a minor major transition? *Annual Review of Ecology, Evolution, and Systematics*: 621–654.
- Guénin, S. et al.** (2011). Identification of pectin methylesterase 3 as a basic pectin methylesterase isoform involved in adventitious rooting in Arabidopsis thaliana. *New Phytol.* **192**: 114–126.
- Guillemin, F., Guillon, F., Bonnin, E., Devaux, M.-F., Chevalier, T., Knox, J.P., Liners, F., and Thibault, J.-F.** (2005). Distribution of pectic epitopes in cell walls of the sugar beet root. *Planta* **222**: 355–371.
- Han, Z., Sun, Y., and Chai, J.** (2014). Structural insight into the activation of plant receptor kinases. *Current Opinion in Plant Biology* **20**: 55–63.
- Hansen, S.F., Bettler, E., Wimmerová, M., Imberty, A., Lerouxel, O., and Breton, C.** (2009). Combination of Several Bioinformatics Approaches for the Identification of New Putative Glycosyltransferases in Arabidopsis. *J. Proteome Res.* **8**: 743–753.
- Hansen, S.F., Harholt, J., Oikawa, A., and Scheller, H.V.** (2012). Plant Glycosyltransferases Beyond CAZy: A Perspective on DUF Families. *Front Plant Sci* **3**: 59.
- Harwood, A. and Coates, J.C.** (2004). A prehistory of cell adhesion. *Curr. Opin. Cell Biol.* **16**: 470–476.
- He, Z.-H., Fujiki, M., and Kohorn, B.D.** (1996). A cell wall-associated, receptor-like protein kinase. *Journal of Biological Chemistry* **271**: 19789–19793.
- Heldin, C.H.** (1995). Dimerization of cell surface receptors in signal transduction. *Cell* **80**: 213–223.
- Hernández-Mata, G., Mellado-Rojas, M.E., Richards-Lewis, A., López-Bucio, J., Beltrán-Peña, E., and Soriano-Bello, E.L.** (2010). Plant immunity induced by oligogalacturonides alters root growth in a process involving flavonoid accumulation in Arabidopsis thaliana. *Journal of Plant Growth Regulation* **29**: 441–454.
- Hématy, K., Sado, P.-E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J.-P., and Höfte, H.** (2007). A Receptor-like Kinase Mediates the Response of Arabidopsis Cells to the Inhibition of Cellulose Synthesis. *Current Biology*

17: 922–931.

**Hicks, G.R. and Raikhel, N.V.** (2012). Small molecules present large opportunities in plant biology. *Annu. Rev. Plant Biol.* **63**: 261–282.

**Hijazi, M., Roujol, D., Nguyen-Kim, H., del Rocio Cisneros Castillo, L., Saland, E., Jamet, E., and Albenne, C.** (2014). Arabinogalactan protein 31 (AGP31), a putative network-forming protein in *Arabidopsis thaliana* cell walls? *Annals of Botany*.

**Huxham, I.M., Jarvis, M.C., Shakespeare, L., Dover, C.J., Johnson, D., Knox, J.P., and Seymour, G.B.** (1999). Electron-energy-loss spectroscopic imaging of calcium and nitrogen in the cell walls of apple fruits. *Planta* **208**: 438–443.

**Ikegaya, H., Hayashi, T., Kaku, T., Iwata, K., Sonobe, S., and Shimmen, T.** (2008). Presence of xyloglucan-like polysaccharide in *Spirogyra* and possible involvement in cell–cell attachment. *Phycological research* **56**: 216–222.

**Iwai, H., Ishii, T., and Satoh, S.** (2001). Absence of arabinan in the side chains of the pectic polysaccharides strongly associated with cell walls of *Nicotiana plumbaginifolia* non-organogenic callus with loosely attached constituent cells. *Planta* **213**: 907–915.

**Iwai, H., Masaoka, N., Ishii, T., and Satoh, S.** (2002). A pectin glucuronyltransferase gene is essential for intercellular attachment in the plant meristem. *Proc. Natl. Acad. Sci. U.S.A.* **99**: 16319–16324.

**James, G.V., Patel, V., Nordström, K.J., Klasen, J.R., Salomé, P.A., Weigel, D., and Schneeberger, K.** (2013). User guide for mapping-by-sequencing in *Arabidopsis*. *Genome Biol.* **14**: R61.

**Jarvis, M.C.** (1998). Intercellular separation forces generated by intracellular pressure. *Plant, Cell & Environment* **21**: 1307–1310.

**Jarvis, M.C.** (2009). Plant cell walls: supramolecular assembly, signalling and stress. *Struct Chem* **20**: 245–253.

**Jarvis, M.C. and Apperley, D.C.** (1995). Chain conformation in concentrated pectic gels: evidence from <sup>13</sup>C NMR. *Carbohydrate Research* **275**: 131–145.

**Jarvis, M.C., Briggs, S., and Knox, J.P.** (2003). Intercellular adhesion and cell separation in plants. *Plant, Cell & Environment* **26**: 977–989.

**Jauh, G. and Lord, E.** (1996). Localization of pectins and arabinogalactan-proteins in lily (*Lilium longiflorum* L.) pollen tube and style, and their possible roles in pollination. *Planta* **199**.

**Jeffree, C.E. and YEOMAN, M.M.** (1983). Development of Intercellular Connections Between Opposing Cells in a Graft Union. *New Phytologist* **93**: 491–509.

**Jeffree, C.E., Dale, J.E., and Fry, S.C.** (1986). The genesis of intercellular spaces in developing leaves of *Phaseolus vulgaris* L. *Protoplasma* **132**: 90–98.

**Johnson, K.L., Jones, B.J., Bacic, A., and Schultz, C.J.** (2003). The fasciclin-like

- arabinogalactan proteins of Arabidopsis. A multigene family of putative cell adhesion molecules. *PLANT PHYSIOLOGY* **133**: 1911–1925.
- Kang, H.-G., Kim, J., Kim, B., Jeong, H., Choi, S.H., Kim, E.K., Lee, H.-Y., and Lim, P.O.** (2011). Overexpression of FTL1/ DDF1, an AP2 transcription factor, enhances tolerance to cold, drought, and heat stresses in Arabidopsis thaliana. *Plant Science* **180**: 634–641.
- Kaschani, F., Verhelst, S.H.L., van Swieten, P.F., Verdoes, M., Wong, C.-S., Wang, Z., Kaiser, M., Overkleeft, H.S., Bogyo, M., and van der Hoorn, R.A.L.** (2009). Minitags for small molecules: detecting targets of reactive small molecules in living plant tissues using 'click chemistry'. *The Plant Journal* **57**: 373–385.
- Keeling, P.J.** (2010). The endosymbiotic origin, diversification and fate of plastids. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **365**: 729–748.
- Kim, J.-Y., Henrichs, S., Bailly, A., Vincenzetti, V., Sovero, V., Mancuso, S., Pollmann, S., Kim, D., Geisler, M., and Nam, H.-G.** (2010). Identification of an ABCB/P-glycoprotein-specific inhibitor of auxin transport by chemical genomics. *Journal of Biological Chemistry* **285**: 23309–23317.
- Kim, Y., Schumaker, K.S., and Zhu, J.-K.** (2006). EMS mutagenesis of Arabidopsis.: 101–103.
- Kirk, D.L.** (2005). A twelve-step program for evolving multicellularity and a division of labor. *Bioessays* **27**: 299–310.
- Klavons, J.A. and Bennett, R.D.** (1986). Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. *J. Agric. Food Chem.* **34**: 597–599.
- Knepper, C., Savory, E.A., and Day, B.** (2011). Arabidopsis NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. *PLANT PHYSIOLOGY* **156**: 286–300.
- Knox, J.P.** (2008). Revealing the structural and functional diversity of plant cell walls. *Current Opinion in Plant Biology* **11**: 308–313.
- Kohorn, B.D.** (2000). Plasma membrane-cell wall contacts. *PLANT PHYSIOLOGY* **124**: 31–38.
- Kohorn, B.D.** (2006). Wall-associated kinase 1 (WAK1) is crosslinked in endomembranes, and transport to the cell surface requires correct cell-wall synthesis. *Journal of Cell Science* **119**: 2282–2290.
- Kohorn, B.D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., and Obregon, P.** (2009). Pectin activation of MAP kinase and gene expression is WAK2 dependent. *The Plant Journal* **60**: 974–982.
- Kohorn, B.D., Kobayashi, M., Johansen, S., Riese, J., Huang, L.-F., Koch, K., Fu, S., Dotson, A., and Byers, N.** (2006). An Arabidopsis cell wall-associated kinase required for invertase activity and cell growth. *The Plant Journal* **46**: 307–316.

- Kohorn, B.D., Kohorn, S.L., Saba, N.J., and Martinez, V.M.** (2014). Requirement for Pectin Methyl Esterase and Preference for Fragmented over Native Pectins for Wall-associated Kinase-activated, EDS1/PAD4-dependent Stress Response in Arabidopsis. *J. Biol. Chem.* **289**: 18978–18986.
- Kohorn, B.D., Kohorn, S.L., Todorova, T., Baptiste, G., Stansky, K., and McCullough, M.** (2012). A Dominant Allele of Arabidopsis Pectin-Binding Wall-Associated Kinase Induces a Stress Response Suppressed by MPK6 but Not MPK3 Mutations. *Molecular Plant* **5**: 841–851.
- Kohorn, B.D., Lane, S., and Smith, T.A.** (1992). An Arabidopsis serine/threonine kinase homologue with an epidermal growth factor repeat selected in yeast for its specificity for a thylakoid membrane protein. *Proc. Natl. Acad. Sci. U.S.A.* **89**: 10989–10992.
- Kollöffel, C. and Linssen, P.** (1984). The formation of intercellular spaces in the cotyledons of developing and germinating pea seeds. *Protoplasma* **120**: 12–19.
- Koschwanez, J.H., Foster, K.R., and Murray, A.W.** (2013). Improved use of a public good selects for the evolution of undifferentiated multicellularity. *Elife* **2**: e00367.
- Krupková, E., Immerzeel, P., Pauly, M., and Schmülling, T.** (2007). The TUMOROUS SHOOT DEVELOPMENT2 gene of Arabidopsis encoding a putative methyltransferase is required for cell adhesion and co-ordinated plant development. *The Plant Journal* **50**: 735–750.
- Kumpf, R.P., Shi, C.-L., Larrieu, A., Stø, I.M., Butenko, M.A., Péret, B., Riiser, E.S., Bennett, M.J., and Aalen, R.B.** (2013). Floral organ abscission peptide IDA and its HAE/HSL2 receptors control cell separation during lateral root emergence. *Proc. Natl. Acad. Sci. U.S.A.* **110**: 5235–5240.
- Lally, D., Ingmire, P., Tong, H.Y., and He, Z.H.** (2001). Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. *The Plant Cell* **13**: 1317–1331.
- Lashbrook, C.C. and Cai, S.** (2008). Cell wall remodeling in Arabidopsis stamen abscission zones: Temporal aspects of control inferred from transcriptional profiling. *psb* **3**: 733–736.
- Laval, V., Chabannes, M., Carrière, M., Canut, H., Barre, A., Rougé, P., Pont-Lezica, R., and Galaud, J.-P.** (1999). A family of Arabidopsis plasma membrane receptors presenting animal  $\beta$ -integrin domains. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1435**: 61–70.
- Leboeuf, E., Guillon, F., Thoiron, S., and Lahaye, M.** (2005). Biochemical and immunohistochemical analysis of pectic polysaccharides in the cell walls of Arabidopsis mutant QUASIMODO 1 suspension-cultured cells: implications for cell adhesion. *Journal of Experimental Botany* **56**: 3171–3182.
- Lei, L., Xu, A., Panin, V.M., and Irvine, K.D.** (2003). An O-fucose site in the ligand binding domain inhibits Notch activation. *Development* **130**: 6411–6421.
- Leslie, M.E., Lewis, M.W., Youn, J.-Y., Daniels, M.J., and Liljegren, S.J.** (2010). The

- EVERSHED receptor-like kinase modulates floral organ shedding in Arabidopsis. *Development* **137**: 467–476.
- Levy, S., Maclachlan, G., and Staehelin, L.A.** (1997). Xyloglucan sidechains modulate binding to cellulose during in vitro binding assays as predicted by conformational dynamics simulations. *Plant J.* **11**: 373–386.
- Lewis, K.C., Selzer, T., Shahar, C., Udi, Y., Tworowski, D., and Sagi, I.** (2008). Inhibition of pectin methyl esterase activity by green tea catechins. *Phytochemistry* **69**: 2586–2592.
- Lewis, M.W., Leslie, M.E., and Liljegren, S.J.** (2006). Plant separation: 50 ways to leave your mother. *Current Opinion in Plant Biology* **9**: 59–65.
- Lewis, M.W., Leslie, M.E., Fulcher, E.H., Darnielle, L., Healy, P.N., Youn, J.-Y., and Liljegren, S.J.** (2010). The SERK1 receptor-like kinase regulates organ separation in Arabidopsis flowers. *The Plant Journal* **62**: 817–828.
- Li, X. and Chapple, C.** (2010). Understanding lignification: challenges beyond monolignol biosynthesis. *PLANT PHYSIOLOGY* **154**: 449–452.
- Liljegren, S.J.** (2012). Organ abscission: exit strategies require signals and moving traffic. *Current Opinion in Plant Biology* **15**: 670–676.
- Liljegren, S.J., Leslie, M.E., Darnielle, L., Lewis, M.W., Taylor, S.M., Luo, R., Geldner, N., Chory, J., Randazzo, P.A., Yanofsky, M.F., and Ecker, J.R.** (2009). Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein. *Development* **136**: 1909–1918.
- Liners, F., Letesson, J.J., Didembourg, C., and Van Cutsem, P.** (1989). Monoclonal Antibodies against Pectin: Recognition of a Conformation Induced by Calcium. *PLANT PHYSIOLOGY* **91**: 1419–1424.
- Lira-Navarrete, E., Valero-González, J., Villanueva, R., Martínez-Júlvez, M., Tejero, T., Merino, P., Panjkar, S., and Hurtado-Guerrero, R.** (2011). Structural Insights into the Mechanism of Protein O-Fucosylation. *PLoS ONE* **6**: e25365.
- Liu, H., Ma, Y., Chen, N., Guo, S., Liu, H., Guo, X., Chong, K., and Xu, Y.** (2014). Overexpression of stress-inducible OsBURP16, the  $\beta$  subunit of polygalacturonase 1, decreases pectin content and cell adhesion and increases abiotic stress sensitivity in rice. *Plant, Cell & Environment* **37**: 1144–1158.
- Livak, K.J. and Schmittgen, T.D.** (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the  $2^{-\Delta\Delta CT}$  Method. *methods* **25**: 402–408.
- Lucas, W.J. et al.** (2013). The plant vascular system: evolution, development and functions. *Journal of Integrative Plant Biology* **55**: 294–388.
- Luo, Y., Nita-Lazar, A., and Haltiwanger, R.S.** (2006). Two distinct pathways for O-fucosylation of epidermal growth factor-like or thrombospondin type 1 repeats. *Journal of Biological Chemistry* **281**: 9385–9392.
- Lü, B., Wang, J., Zhang, Y., Wang, H., Liang, J., and Zhang, J.** (2012). AT14A mediates

the cell wall-plasma membrane-cytoskeleton continuum in *Arabidopsis thaliana* cells. *Journal of Experimental Botany* **63**: 4061–4069.

- Ma, Q. and Robert, S.** (2014). Auxin biology revealed by small molecules. *Physiologia Plantarum* **151**: 25–42.
- Manfield, I.W., Bernal, A.J., Møller, I., McCartney, L., Riess, N.P., Knox, J.P., and Willats, W.G.** (2005). Re-engineering of the PAM1 phage display monoclonal antibody to produce a soluble, versatile anti-homogalacturonan scFv. *Plant Science* **169**: 1090–1095.
- Manrique, G.D. and Lajolo, F.M.** (2002). FT-IR spectroscopy as a tool for measuring degree of methyl esterification in pectins isolated from ripening papaya fruit. *Postharvest Biology and Technology* **25**: 99–107.
- Marcus, S.E., Verhertbruggen, Y., Hervé, C., Ordaz-Ortiz, J.J., Farkas, V., Pedersen, H.L., Willats, W.G.T., and Knox, J.P.** (2008). Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. *BMC Plant Biol* **8**.
- Martinez-Duncker, I., Mollicone, R., Candelier, J.-J., Breton, C., and Oriol, R.** (2003). A new superfamily of protein-O-fucosyltransferases, alpha2-fucosyltransferases, and alpha6-fucosyltransferases: phylogeny and identification of conserved peptide motifs. *Glycobiology* **13**: 1C–5C.
- McCarthy, T.W., Der, J.P., Honaas, L.A., dePamphilis, C.W., and Anderson, C.T.** (2014). Phylogenetic analysis of pectin-related gene families in *Physcomitrella patens* and nine other plant species yields evolutionary insights into cell walls. *BMC Plant Biol* **14**: 79.
- McCartney, L. and Knox, J.P.** (2002). Regulation of pectic polysaccharide domains in relation to cell development and cell properties in the pea testa. *Journal of Experimental Botany* **53**: 707–713.
- McFarlane, H.E., Watanabe, Y., Gendre, D., Carruthers, K., Levesque-Tremblay, G., Haughn, G.W., Bhalerao, R.P., and Samuels, L.** (2013). Cell wall polysaccharides are mislocalized to the Vacuole in echidna mutants. *Plant and Cell Physiology* **54**: 1867–1880.
- Meir, S., Philosoph-Hadas, S., Sundaresan, S., Selvaraj, K.S.V., Burd, S., Ophir, R., Kochanek, B., Reid, M.S., Jiang, C.-Z., and Lers, A.** (2010). Microarray analysis of the abscission-related transcriptome in the tomato flower abscission zone in response to auxin depletion. *PLANT PHYSIOLOGY* **154**: 1929–1956.
- Miart, F., Desprez, T., Biot, E., Morin, H., Belcram, K., Höfte, H., Gonneau, M., and Vernhettes, S.** (2014). Spatio-temporal analysis of cellulose synthesis during cell plate formation in *Arabidopsis*. *The Plant Journal* **77**: 71–84.
- Michel, G., Tonon, T., Scornet, D., Cock, J.M., and Kloareg, B.** (2010). The cell wall polysaccharide metabolism of the brown alga *Ectocarpus siliculosus*. Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. *New Phytol.* **188**: 82–97.
- Micheli, F.** (2001). Pectin methylesterases: cell wall enzymes with important roles in plant



- physiology. *Trends in Plant Science* **6**: 414–419.
- Molina, A. and García-Olmedo, F.** (1997). Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant J.* **12**: 669–675.
- Moore, P.J. and Staehelin, L.A.** (1988). Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways. *Planta* **174**: 433–445.
- Mouille, G., Ralet, M.-C., Cavelier, C., Eland, C., Effroy, D., Hématy, K., McCartney, L., Truong, H.N., Gaudon, V., Thibault, J.-F., Marchant, A., and Höfte, H.** (2007). Homogalacturonan synthesis in *Arabidopsis thaliana* requires a Golgi-localized protein with a putative methyltransferase domain. *The Plant Journal* **50**: 605–614.
- Mouille, G., Robin, S., Lecomte, M., Pagant, S., and Höfte, H.** (2003). Classification and identification of *Arabidopsis* cell wall mutants using Fourier-Transform InfraRed (FT-IR) microspectroscopy. *The Plant Journal* **35**: 393–404.
- Moustacas, A.M., Nari, J., Borel, M., Noat, G., and Ricard, J.** (1991). Pectin methylesterase, metal ions and plant cell-wall extension. The role of metal ions in plant cell-wall extension. *Biochem. J.* **279 ( Pt 2)**: 351–354.
- Nakano, T. and Ito, Y.** (2013). Molecular mechanisms controlling plant organ abscission. *Plant Biotechnology* **30**: 209–216.
- Nelson, B.K., Cai, X., and Nebenführ, A.** (2007). A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants. *Plant J.* **51**: 1126–1136.
- Neumetzler, L., Humphrey, T., Lumba, S., Snyder, S., Yeats, T.H., Usadel, B., Vasilevski, A., Patel, J., Rose, J.K.C., Persson, S., and Bonetta, D.** (2012). The FRIABLE1 Gene Product Affects Cell Adhesion in *Arabidopsis*. *PLoS ONE* **7**: e42914.
- Ng, A., Greenshields, R.N., and Waldron, K.W.** (1997). Oxidative cross-linking of corn bran hemicellulose: formation of ferulic acid dehydrodimers. *Carbohydrate Research* **303**: 459–462.
- Niederhuth, C.E., Cho, S.K., Seitz, K., and Walker, J.C.** (2013). Letting go is never easy: abscission and receptor-like protein kinases. *Journal of Integrative Plant Biology* **55**: 1251–1263.
- Nieuwland, J., Feron, R., Huisman, B.A.H., Fasolino, A., Hilbers, C.W., Derksen, J., and Mariani, C.** (2005). Lipid transfer proteins enhance cell wall extension in tobacco. *The Plant Cell* **17**: 2009–2019.
- Niklas, K.J.** (2004). The cell walls that bind the tree of life. *BioScience* **54**: 831–841.
- Niklas, K.J.** (2014). The evolutionary-developmental origins of multicellularity. *American Journal of Botany* **101**: 6–25.

- Nobles, D.R. and Brown, R.M.** (2004). The pivotal role of cyanobacteria in the evolution of cellulose synthases and cellulose synthase-like proteins. *Cellulose* **11**: 437–448.
- Noutoshi, Y., Ikeda, M., and Shirasu, K.** (2012a). Diuretics Prime Plant Immunity in *Arabidopsis thaliana*. *PLoS ONE* **7**: e48443.
- Noutoshi, Y., Ikeda, M., Saito, T., Osada, H., and Shirasu, K.** (2012b). Sulfonamides identified as plant immune-priming compounds in high-throughput chemical screening increase disease resistance in *Arabidopsis thaliana*. *Front Plant Sci* **3**: 245.
- O'Neill, M.A., Eberhard, S., Albersheim, P., and Darvill, A.G.** (2001). Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* **294**: 846–849.
- O'Neill, M.A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A.G., and Albersheim, P.** (1996). Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. In vitro conditions for the formation and hydrolysis of the dimer. *Journal of Biological Chemistry* **271**: 22923–22930.
- Ogawa, M., Kay, P., Wilson, S., and Swain, S.M.** (2009). ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are Polygalacturonases required for cell separation during reproductive development in *Arabidopsis*. *The Plant Cell* **21**: 216–233.
- Orfila, C., Seymour, G.B., Willats, W.G., Huxham, I.M., Jarvis, M.C., Dover, C.J., Thompson, A.J., and Knox, J.P.** (2001). Altered Middle Lamella Homogalacturonan and Disrupted Deposition of (1→5)- $\alpha$ -l-Arabinan in the Pericarp of *Cnr*, a Ripening Mutant of Tomato. *PLANT PHYSIOLOGY* **126**: 210–221.
- Orfila, C., Sørensen, S.O., Harholt, J., Geshi, N., Crombie, H., Truong, H.N., Reid, J.S.G., Knox, J.P., and Scheller, H.V.** (2005). QUASIMODO1 is expressed in vascular tissue of *Arabidopsis thaliana* inflorescence stems, and affects homogalacturonan and xylan biosynthesis. *Planta* **222**: 613–622.
- Ossowski, S., Schneeberger, K., Clark, R.M., Lanz, C., Warthmann, N., and Weigel, D.** (2008). Sequencing of natural strains of *Arabidopsis thaliana* with short reads. *Genome Res.* **18**: 2024–2033.
- Park, A.R.** (2001). Interaction of the *Arabidopsis* Receptor Protein Kinase Wak1 with a Glycine-rich Protein, AtGRP-3. *Journal of Biological Chemistry* **276**: 26688–26693.
- Park, E., Díaz-Moreno, S.M., Davis, D.J., Wilkop, T.E., Bulone, V., and Drakakaki, G.** (2014). Endosidin 7 Specifically Arrests Late Cytokinesis and Inhibits Callose Biosynthesis, Revealing Distinct Trafficking Events during Cell Plate Maturation. *PLANT PHYSIOLOGY* **165**: 1019–1034.
- Park, J.H., Suh, M.C., Kim, T.H., Kim, M.C., and Cho, S.H.** (2008). Expression of glycine-rich protein genes, AtGRP5 and AtGRP23, induced by the cutin monomer 16-hydroxypalmitic acid in *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* **46**: 1015–1018.

- Park, S.-Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., and Tsz-fung, F.C.** (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071.
- Parsons, H.T. et al.** (2012). Isolation and Proteomic Characterization of the Arabidopsis Golgi Defines Functional and Novel Components Involved in Plant Cell Wall Biosynthesis. *PLANT PHYSIOLOGY* **159**: 12–26.
- Paterson, A.H., Lin, Y.R., Li, Z., Schertz, K.F., Doebley, J.F., Pinson, S.R.M., Liu, S.C., Stansel, J.W., and Irvine, J.E.** (1995). Convergent Domestication of Cereal Crops by Independent Mutations at Corresponding Genetic Loci. *Science* **269**: 1714–1718.
- Patterson, S.E.** (2001). Cutting loose. Abscission and dehiscence in Arabidopsis. *PLANT PHYSIOLOGY* **126**: 494–500.
- Peaucelle, A., Braybrook, S., and Höfte, H.** (2012). Cell wall mechanics and growth control in plants: the role of pectins revisited. *Front Plant Sci* **3**: 121.
- Pelloux, J., Rustérucci, C., and Mellerowicz, E.J.** (2007). New insights into pectin methylesterase structure and function. *Trends in Plant Science* **12**: 267–277.
- Persson, S., Caffall, K.H., Freshour, G., Hilley, M.T., Bauer, S., Poindexter, P., Hahn, M.G., Mohnen, D., and Somerville, C.** (2007). The Arabidopsis irregular xylem8 mutant is deficient in glucuronoxylan and homogalacturonan, which are essential for secondary cell wall integrity. *The Plant Cell* **19**: 237–255.
- Péret, B. et al.** (2013). Sequential induction of auxin efflux and influx carriers regulates lateral root emergence. *Molecular Systems Biology* **9**: 699.
- Péret, B., De Rybel, B., Casimiro, I., Benková, E., Swarup, R., Laplaze, L., Beeckman, T., and Bennett, M.J.** (2009). Arabidopsis lateral root development: an emerging story. *Trends in Plant Science* **14**: 399–408.
- Pokutta, S. and Weis, W.I.** (2007). Structure and mechanism of cadherins and catenins in cell-cell contacts. *Annu. Rev. Cell Dev. Biol.* **23**: 237–261.
- Popper, Z.A. and Fry, S.C.** (2007). Xyloglucan–pectin linkages are formed intraprotoplasmically, contribute to wall-assembly, and remain stable in the cell wall. *Planta* **227**: 781–794.
- Popper, Z.A., Michel, G., Hervé, C., Domozych, D.S., Willats, W.G.T., Tuohy, M.G., Kloareg, B., and Stengel, D.B.** (2011). Evolution and Diversity of Plant Cell Walls: From Algae to Flowering Plants. *Annu. Rev. Plant Biol.* **62**: 567–590.
- Prochnik, S.E., Umen, J., Nedelcu, A.M., Hallmann, A., Miller, S.M., Nishii, I., Ferris, P., Kuo, A., Mitros, T., and Fritz-Laylin, L.K.** (2010). Genomic analysis of organismal complexity in the multicellular green alga *Volvox carteri*. *Science* **329**: 223–226.
- Proseus, T.E. and Boyer, J.S.** (2012a). Calcium deprivation disrupts enlargement of *Chara corallina* cells: further evidence for the calcium pectate cycle. *Journal of Experimental Botany* **63**: 3953–3958.

- Proseus, T.E. and Boyer, J.S.** (2012b). Pectate chemistry links cell expansion to wall deposition in *Chara corallina*. *psb* **7**: 1490–1492.
- Pruitt, R.E., Vielle-Calzada, J.P., Ploense, S.E., Grossniklaus, U., and Lolle, S.J.** (2000). FIDDLEHEAD, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **97**: 1311–1316.
- Queller, D.C.** (2000). Relatedness and the fraternal major transitions. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **355**: 1647–1655.
- Rabuka, D., Hubbard, S.C., Laughlin, S.T., Argade, S.P., and Bertozzi, C.R.** (2006). A chemical reporter strategy to probe glycoprotein fucosylation. *J. Am. Chem. Soc.* **128**: 12078–12079.
- Raiola, A., Camardella, L., Giovane, A., Mattei, B., De Lorenzo, G., Cervone, F., and Bellincampi, D.** (2004). Two *Arabidopsis thaliana* genes encode functional pectin methylesterase inhibitors. *FEBS Letters* **557**: 199–203.
- Ralet, M.-C., Crépeau, M.-J., Lefèbvre, J., Mouille, G., Höfte, H., and Thibault, J.-F.** (2008). Reduced Number of Homogalacturonan Domains in Pectins of an *Arabidopsis* Mutant Enhances the Flexibility of the Polymer. *Biomacromolecules* **9**: 1454–1460.
- Ramana, S.V. and Taylor, A.J.** (1994). Effect of various agents on rheological properties of carrot cells and protoplasts. *J. Sci. Food Agric.* **64**: 519–525.
- Ratcliff, W.C., Denison, R.F., Borrello, M., and Travisano, M.** (2012). Experimental evolution of multicellularity. *Proc. Natl. Acad. Sci. U.S.A.* **109**: 1595–1600.
- Ratcliff, W.C., Herron, M.D., Howell, K., Pentz, J.T., Rosenzweig, F., and Travisano, M.** (2013). Experimental evolution of an alternating uni- and multicellular life cycle in *Chlamydomonas reinhardtii*. *Nature communications* **4**.
- Rausch, H., Larsen, N., and Schmitt, R.** (1989). Phylogenetic relationships of the green alga *Volvox carteri* deduced from small-subunit ribosomal RNA comparisons. *J Mol Evol* **29**: 255–265.
- RAVEN, J.A.** (1997). The role of marine biota in the evolution of terrestrial biota: gases and genes. *Biogeochemistry* **39**: 139–164.
- Rayle, D.L. and Cleland, R.E.** (1992). The Acid Growth Theory of auxin-induced cell elongation is alive and well. *PLANT PHYSIOLOGY* **99**: 1271–1274.
- Redgwell, R.J. and Harker, R.** (1995). Softening of kiwifruit discs: effect of inhibition of galactose loss from cell walls. *Phytochemistry* **39**: 1319–1323.
- Reichardt, I., Stierhof, Y.-D., Mayer, U., Richter, S., Schwarz, H., Schumacher, K., and Jürgens, G.** (2007). Plant cytokinesis requires de novo secretory trafficking but not endocytosis. *Curr. Biol.* **17**: 2047–2053.
- Rhee, S.Y., Osborne, E., Poindexter, P.D., and Somerville, C.R.** (2003). Microspore separation in the quartet 3 mutants of *Arabidopsis* is impaired by a defect in a

developmentally regulated polygalacturonase required for pollen mother cell wall degradation. *PLANT PHYSIOLOGY* **133**: 1170–1180.

- Richter, S., Anders, N., Wolters, H., Beckmann, H., Thomann, A., Heinrich, R., Schrader, J., Singh, M.K., Geldner, N., Mayer, U., and Jürgens, G.** (2010). Role of the GNOM gene in Arabidopsis apical-basal patterning--From mutant phenotype to cellular mechanism of protein action. *Eur. J. Cell Biol.* **89**: 138–144.
- RIHOUEY, C., JAUNEAU, A., CABINFLAMAN, A., DEMARTY, M., LEFEBVRE, F., and MORVAN, C.** (1995). Calcium and Acidic Pectin Distribution in Flax Cell-Walls - Evidence for Different Kinds of Linkages in the Cell Junction and Middle Lamella of the Cortical Parenchyma of Flax Hypocotyl. *Plant Physiology and Biochemistry* **33**: 497–508.
- Robatzek, S., Chinchilla, D., and Boller, T.** (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. *Genes & Development* **20**: 537–542.
- Robert, S., Chary, S.N., Drakakaki, G., Li, S., Yang, Z., Raikhel, N.V., and Hicks, G.R.** (2008). Endosidin1 defines a compartment involved in endocytosis of the brassinosteroid receptor BRI1 and the auxin transporters PIN2 and AUX1. *Proc. Natl. Acad. Sci. U.S.A.* **105**: 8464–8469.
- Robert, S., Raikhel, N.V., and Hicks, G.R.** (2009). Powerful Partners: Arabidopsis and Chemical Genomics. *The Arabidopsis Book* **7**: e0109.
- Roberts, J.A., Elliott, K.A., and Gonzalez-Carranza, Z.H.** (2002). Abscission, dehiscence, and other cell separation processes. *Annu. Rev. Plant Biol.* **53**: 131–158.
- Rojas-Pierce, M., Titapiwatanakun, B., Sohn, E.J., Fang, F., Larive, C.K., Blakeslee, J., Cheng, Y., Cuttler, S., Peer, W.A., Murphy, A.S., and Raikhel, N.V.** (2007). Arabidopsis P-Glycoprotein19 Participates in the Inhibition of Gravitropism by Gravacin. *Chemistry & Biology* **14**: 1366–1376.
- Roland, J.C.** (1978). Cell wall differentiation and stages involved with intercellular gas space opening. *Journal of Cell Science* **32**: 325–336.
- Russinova, E., Borst, J.-W., Kwaaitaal, M., Caño-Delgado, A., Yin, Y., Chory, J., and de Vries, S.C.** (2004). Heterodimerization and endocytosis of Arabidopsis brassinosteroid receptors BRI1 and AtSERK3 (BAK1). *The Plant Cell* **16**: 3216–3229.
- Sachs, J.L.** (2008). Resolving the first steps to multicellularity. *Trends Ecol. Evol. (Amst.)* **23**: 245–248.
- Samuels, A.L., Giddings, T.H., and Staehelin, L.A.** (1995). Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. *J. Cell Biol.* **130**: 1345–1357.
- Savatin, D.V., Ferrari, S., Sicilia, F., and De Lorenzo, G.** (2011). Oligogalacturonide-Auxin Antagonism Does Not Require Posttranscriptional Gene Silencing or Stabilization of Auxin Response Repressors in Arabidopsis. *PLANT PHYSIOLOGY* **157**: 1163–1174.
- Scheller, H.V. and Ulvskov, P.** (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* **61**: 263–289.

- Schneeberger, K., Ossowski, S., Lanz, C., Juul, T., Petersen, A.H., Nielsen, K.L., Jørgensen, J.-E., Weigel, D., and Andersen, S.U.** (2009). 2009\_NatureMethods\_Schneeberger(Weigel). Nature Publishing Group **6**: 550–551.
- Schreiber, K., Ckurshumova, W., Peek, J., and Desveaux, D.** (2008). A high-throughput chemical screen for resistance to *Pseudomonas syringae* in *Arabidopsis*. *Plant J.* **54**: 522–531.
- Sebé-Pedrós, A., Roger, A.J., Lang, F.B., King, N., and Ruiz-Trillo, I.** (2010). Ancient origin of the integrin-mediated adhesion and signaling machinery. *Proc. Natl. Acad. Sci. U.S.A.* **107**: 10142–10147.
- Seguí-Simarro, J.M., Austin, J.R., White, E.A., and Staehelin, L.A.** (2004). Electron tomographic analysis of somatic cell plate formation in meristematic cells of *Arabidopsis* preserved by high-pressure freezing. *The Plant Cell* **16**: 836–856.
- Sexton, R. and Roberts, J.A.** (1982). Cell Biology of Abscission. *Annu. Rev. Plant. Physiol.* **33**: 133–162.
- Seymour, G.B., Østergaard, L., Chapman, N.H., Knapp, S., and Martin, C.** (2013). Fruit development and ripening. *Annu. Rev. Plant Biol.* **64**: 219–241.
- Sénéchal, F., Wattier, C., Rustérucchi, C., and Pelloux, J.** (2014). Homogalacturonan-modifying enzymes: structure, expression, and roles in plants. *Journal of Experimental Botany*.
- Shao, L. and Haltiwanger, R.S.** (2003). O-fucose modifications of epidermal growth factor-like repeats and thrombospondin type 1 repeats: unusual modifications in unusual places. *CMLS, Cell. Mol. Life Sci.* **60**: 241–250.
- Shevell, D.E., Kunkel, T., and Chua, N.H.** (2000). Cell wall alterations in the *Arabidopsis* emb30 mutant. *The Plant Cell* **12**: 2047–2060.
- Shevell, D.E., Leu, W.M., Gillmor, C.S., Xia, G., Feldmann, K.A., and Chua, N.H.** (1994). EMB30 is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec7. *Cell* **77**: 1051–1062.
- Shi, C.-L., Stenvik, G.-E., Vie, A.K., Bones, A.M., Pautot, V., Proveniers, M., Aalen, R.B., and Butenko, M.A.** (2011). *Arabidopsis* class I KNOTTED-like homeobox proteins act downstream in the IDA-HAE/HSL2 floral abscission signaling pathway. *The Plant Cell* **23**: 2553–2567.
- Shi, H., Kim, Y., Guo, Y., Stevenson, B., and Zhu, J.-K.** (2003). The *Arabidopsis* SOS5 locus encodes a putative cell surface adhesion protein and is required for normal cell expansion. *The Plant Cell* **15**: 19–32.
- Shin, H., Shin, H.-S., Chen, R., and Harrison, M.J.** (2006). Loss of At4 function impacts phosphate distribution between the roots and the shoots during phosphate starvation. *Plant J.* **45**: 712–726.
- Sorensen, I., Domozych, D., and Willats, W.G.T.** (2010). How Have Plant Cell Walls Evolved? *PLANT PHYSIOLOGY* **153**: 366–372.

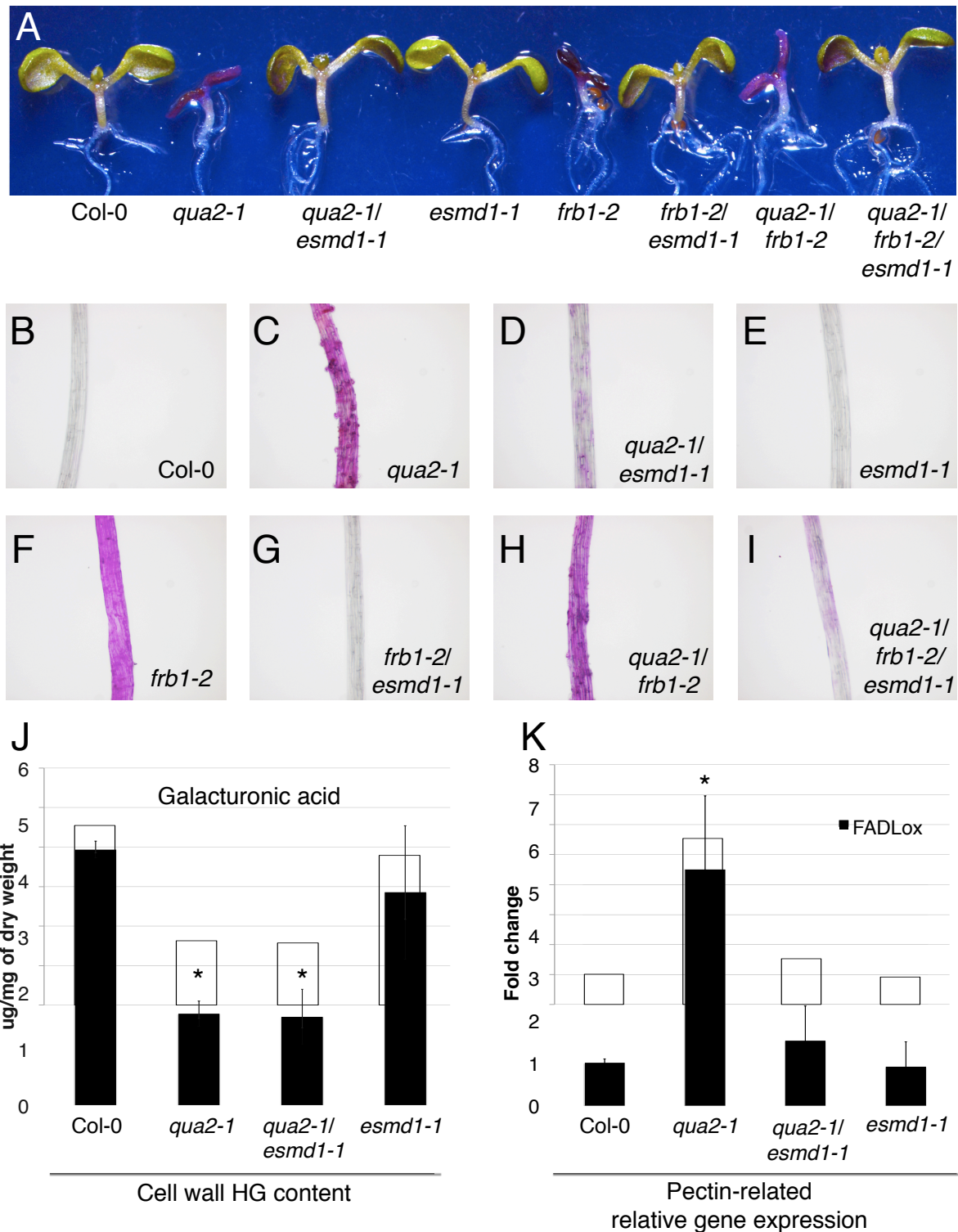
- Stanley, S.M.** (1973). An ecological theory for the sudden origin of multicellular life in the late precambrian. *Proc. Natl. Acad. Sci. U.S.A.* **70**: 1486–1489.
- Stenvik, G.-E., Tandstad, N.M., Guo, Y., Shi, C.-L., Kristiansen, W., Holmgren, A., Clark, S.E., Aalen, R.B., and Butenko, M.A.** (2008). The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2. *The Plant Cell* **20**: 1805–1817.
- Swanson, R., Edlund, A.F., and Preuss, D.** (2004). Species specificity in pollen-pistil interactions. *Annu. Rev. Genet.* **38**: 793–818.
- Sørensen, I., Fei, Z., Andreas, A., Willats, W.G.T., Domozych, D.S., and Rose, J.K.C.** (2014). Stable transformation and reverse genetic analysis of *Penium margaritaceum*: a platform for studies of charophyte green algae, the immediate ancestors of land plants. *The Plant Journal* **77**: 339–351.
- Sørensen, I., Pettolino, F.A., Bacic, A., Ralph, J., Lu, F., O'Neill, M.A., Fei, Z., Rose, J.K.C., Domozych, D.S., and Willats, W.G.T.** (2011). The charophycean green algae provide insights into the early origins of plant cell walls. *The Plant Journal* **68**: 201–211.
- Sørensen, I., Rose, J.K.C., Doyle, J.J., Domozych, D.S., and Willats, W.G.T.** (2012). The Charophycean green algae as model systems to study plant cell walls and other evolutionary adaptations that gave rise to land plants. *psb* **7**: 1–3.
- Takeuchi, H. and Haltiwanger, R.S.** (2014). Significance of glycosylation in Notch signaling. *Biochemical and Biophysical Research Communications*.
- Tan, L. et al.** (2013). An Arabidopsis Cell Wall Proteoglycan Consists of Pectin and Arabinoxylan Covalently Linked to an Arabinogalactan Protein. *The Plant Cell* **25**: 270–287.
- Thiele, K., Wanner, G., Kindzierski, V., Jürgens, G., Mayer, U., Pahl, F., and Assaad, F.F.** (2009). The timely deposition of callose is essential for cytokinesis in Arabidopsis. *The Plant Journal* **58**: 13–26.
- Tobimatsu, Y., Wagner, A., Donaldson, L., Mitra, P., Niculaes, C., Dima, O., Kim, J.I., Anderson, N., Loqué, D., Boerjan, W., Chapple, C., and Ralph, J.** (2013). Visualization of plant cell wall lignification using fluorescence-tagged monolignols. *The Plant Journal* **76**: 357–366.
- Tóth, R. and van der Hoorn, R.A.L.** (2010). Emerging principles in plant chemical genetics. *Trends in Plant Science* **15**: 81–88.
- Ueda, J., Miyamoto, K., Uheda, E., Oka, M., Yano, S., Higashibata, A., and Ishioka, N.** (2014). Close relationships between polar auxin transport and graviresponse in plants. *Plant Biol (Stuttg)* **16 Suppl 1**: 43–49.
- Ung, N., Brown, M.Q., Hicks, G.R., and Raikhel, N.V.** (2013). An approach to quantify endomembrane dynamics in pollen utilizing bioactive chemicals. *Molecular Plant* **6**: 1202–1213.

- Van Damme, D., Inzé, D., and Russinova, E.** (2008). Vesicle trafficking during somatic cytokinesis. *PLANT PHYSIOLOGY* **147**: 1544–1552.
- Verma, D.P.S.** (2001). CYTOKINESIS AND BUILDING OF THE CELL PLATE IN PLANTS. *Annu. Rev. Plant Biol.* **52**: 751–784.
- Vidaurre, D. and Bonetta, D.** (2012). Accelerating forward genetics for cell wall deconstruction. *Front Plant Sci* **3**: 119.
- Vincken, J.-P., Schols, H.A., Oomen, R.J.F.J., McCann, M.C., Ulvskov, P., Voragen, A.G.J., and Visser, R.G.F.** (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *PLANT PHYSIOLOGY* **132**: 1781–1789.
- Vogel, J.** (2008). Unique aspects of the grass cell wall. *Current Opinion in Plant Biology* **11**: 301–307.
- Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D.** (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* **33**: 949–956.
- Voragen, A.G.J., Coenen, G.-J., Verhoef, R.P., and Schols, H.A.** (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Struct Chem* **20**: 263–275.
- Vossen, R.H., Aten, E., Roos, A., and Dunnen, den, J.T.** (2009). High-Resolution Melting Analysis (HRMA)—More than just sequence variant screening. *Human mutation* **30**: 860–866.
- Wagner, T.A. and Kohorn, B.D.** (2001). Wall-associated kinases are expressed throughout plant development and are required for cell expansion. *The Plant Cell* **13**: 303–318.
- Wang, Y., Mortimer, J.C., Davis, J., Dupree, P., and Keegstra, K.** (2012). Identification of an additional protein involved in mannan biosynthesis. *Plant J.* **73**: 105–117.
- Wang, Y., Shao, L., Shi, S., Harris, R.J., Spellman, M.W., Stanley, P., and Haltiwanger, R.S.** (2001). Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *Journal of Biological Chemistry* **276**: 40338–40345.
- Willats, W.G., Gilmartin, P.M., Mikkelsen, J.D., and Knox, J.P.** (1999). Cell wall antibodies without immunization: generation and use of de-esterified homogalacturonan block-specific antibodies from a naive phage display library. *Plant J.* **18**: 57–65.
- Willats, W.G.T.** (2001). Modulation of the Degree and Pattern of Methyl-esterification of Pectic Homogalacturonan in Plant Cell Walls. IMPLICATIONS FOR PECTIN METHYL ESTERASE ACTION, MATRIX PROPERTIES, AND CELL ADHESION. *Journal of Biological Chemistry* **276**: 19404–19413.
- Wodniok, S., Brinkmann, H., Glöckner, G., Heidel, A.J., Philippe, H., Melkonian, M., and Becker, B.** (2011). Origin of land plants: do conjugating green algae hold the key? *BMC Evol. Biol.* **11**: 104.



- Woessner, J.P. and Goodenough, U.W.** (1994). Volvocine cell walls and their constituent glycoproteins: an evolutionary perspective. *Protoplasma* **181**: 245–258.
- Wolf, S., Hématy, K., and Höfte, H.** (2012a). Growth Control and Cell Wall Signaling in Plants. *Annu. Rev. Plant Biol.* **63**: 381–407.
- Wolf, S., Mouille, G., and Pelloux, J.** (2009). Homogalacturonan Methyl-Esterification and Plant Development. *Molecular Plant* **2**: 851–860.
- Wolf, S., Mravec, J., Greiner, S., Mouille, G., and Höfte, H.** (2012b). Plant cell wall homeostasis is mediated by brassinosteroid feedback signaling. *Curr. Biol.* **22**: 1732–1737.
- Xiao, C., Somerville, C., and Anderson, C.T.** (2014). POLYGALACTURONASE INVOLVED IN EXPANSION1 Functions in Cell Elongation and Flower Development in Arabidopsis. *The Plant Cell* **26**: 1018–1035.
- Xu, T., Wen, M., Nagawa, S., Fu, Y., Chen, J.-G., Wu, M.-J., Perrot-Rechenmann, C., Friml, J., Jones, A.M., and Yang, Z.** (2010). Cell Surface- and Rho GTPase-Based Auxin Signaling Controls Cellular Interdigitation in Arabidopsis. *Cell* **143**: 99–110.
- Yin, Y., Chen, H., Hahn, M.G., Mohnen, D., and Xu, Y.** (2010). Evolution and function of the plant cell wall synthesis-related glycosyltransferase family 8. *PLANT PHYSIOLOGY* **153**: 1729–1746.
- Yoneda, A., Ito, T., Higaki, T., Kutsuna, N., Saito, T., Ishimizu, T., Osada, H., Hasezawa, S., Matsui, M., and Demura, T.** (2010). Cobtorin target analysis reveals that pectin functions in the deposition of cellulose microfibrils in parallel with cortical microtubules. *The Plant Journal* **64**: 657–667.
- Yoo, S.-D., Cho, Y.-H., and Sheen, J.** (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols* **2**: 1565–1572.
- Yun, M.-K., Wu, Y., Li, Z., Zhao, Y., Waddell, M.B., Ferreira, A.M., Lee, R.E., Bashford, D., and White, S.W.** (2012). Catalysis and sulfa drug resistance in dihydropteroate synthase. *Science* **335**: 1110–1114.
- Zeng, Y., Saar, B.G., Friedrich, M.G., Chen, F., Liu, Y.-S., Dixon, R.A., Himmel, M.E., Xie, X.S., and Ding, S.-Y.** (2010). Imaging lignin-downregulated alfalfa using coherent anti-Stokes Raman scattering microscopy. *Bioenergy Research* **3**: 272–277.
- Zhang, H., Deng, X., Miki, D., Cutler, S., La, H., Hou, Y.J., Oh, J., and Zhu, J.K.** (2012). Sulfamethazine Suppresses Epigenetic Silencing in Arabidopsis by Impairing Folate Synthesis. *The Plant Cell* **24**: 1230–1241.
- Zhang, J., Henriksson, G., and Johansson, G.** (2000). Polygalacturonase is the key component in enzymatic retting of flax. *J. Biotechnol.* **81**: 85–89.
- Zhao, Y., Chow, T.F., Puckrin, R.S., Alfred, S.E., Korir, A.K., Larive, C.K., and Cutler, S.R.** (2007). Chemical genetic interrogation of natural variation uncovers a molecule that is glycoactivated. *Nature Chemical Biology* **3**: 716–721.

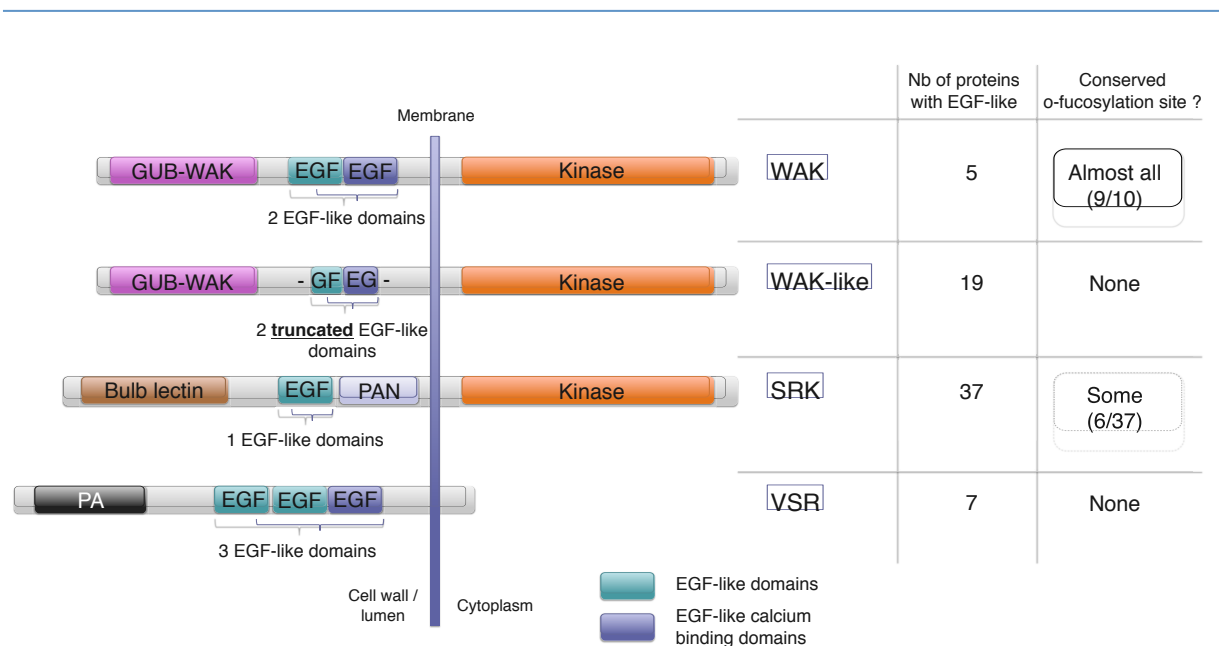
- Zhong, B., Xi, Z., Goremykin, V.V., Fong, R., Mclenachan, P.A., Novis, P.M., Davis, C.C., and Penny, D.** (2014). Streptophyte algae and the origin of land plants revisited using heterogeneous models with three new algal chloroplast genomes. *Mol. Biol. Evol.* **31**: 177–183.
- Zuo, J., Niu, Q.W., Nishizawa, N., Wu, Y., Kost, B., and Chua, N.H.** (2000). KORRIGAN, an Arabidopsis endo-1,4-beta-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. *The Plant Cell* **12**: 1137–1152.
- Østergaard, L., Kempin, S.A., Bies, D., Klee, H.J., and Yanofsky, M.F.** (2006). Pod shatter-resistant Brassica fruit produced by ectopic expression of the FRUITFULL gene. *Plant Biotechnology Journal* **4**: 45–51.



**Figure 1: *qua2*, *frb1* and *esmd1* affect cell adhesion and pectin signaling in a same pathway and independently from HG content in the cell wall**

(A-I) Phenotype of *col-0*, *qua2-1*, *qua2-1/esmd1-1*, *esmd1-1*, *frb1-2*, *frb1-2/esmd1-1*, *qua2-1/frb1-2*, *qua2-1/frb1-2/esmd1-1* : (A) Light grown seedlings on a 0.2% agarose and 3% sucrose medium similar to the suppressor primary screening conditions and (B-I) Close up view of the dark grown hypocotyls stained with ruthenium red in order to reveal the cell adhesion defect. The principle of the Ruthenium red staining here is that the cell separation creates ruptures in the continuity of the hydrophobic cuticle, which directly exposes the cell wall and allows the entry and binding of the ruthenium red, thus highlighting the cell adhesion defect. *esmd1-1* suppresses the phenotype of *qua2-1*, *frb1-2* and the double mutant *qua2-1/frb1-2*, and *qua2-1/frb1-2* does not seem to show an additive phenotype compared to either *qua2-1* or *frb1-2* alone. (J) Galacturonic acid content

function of these putative O-fucosyltransferases and how can we explain their role in cell adhesion?



**Figure 2: Potential substrates of ESMERALDA1 and FRIABLE1**

UniProtKB/Swiss-Prot database search reveal four classes of proteins harboring EGF-like domains: The Wall-Associated Kinases (WAK), the WAK-like, the S-domain RLK (SRK) and the Vacuolar Sorting Receptors (VSR). These EGF-like domains are the potential substrates of the putative O-fucosyltransferases ESMERALDA1 and FRIABLE1, however, only the WAKs and some of the SRK actually have the conserved site for O-fucosylation.

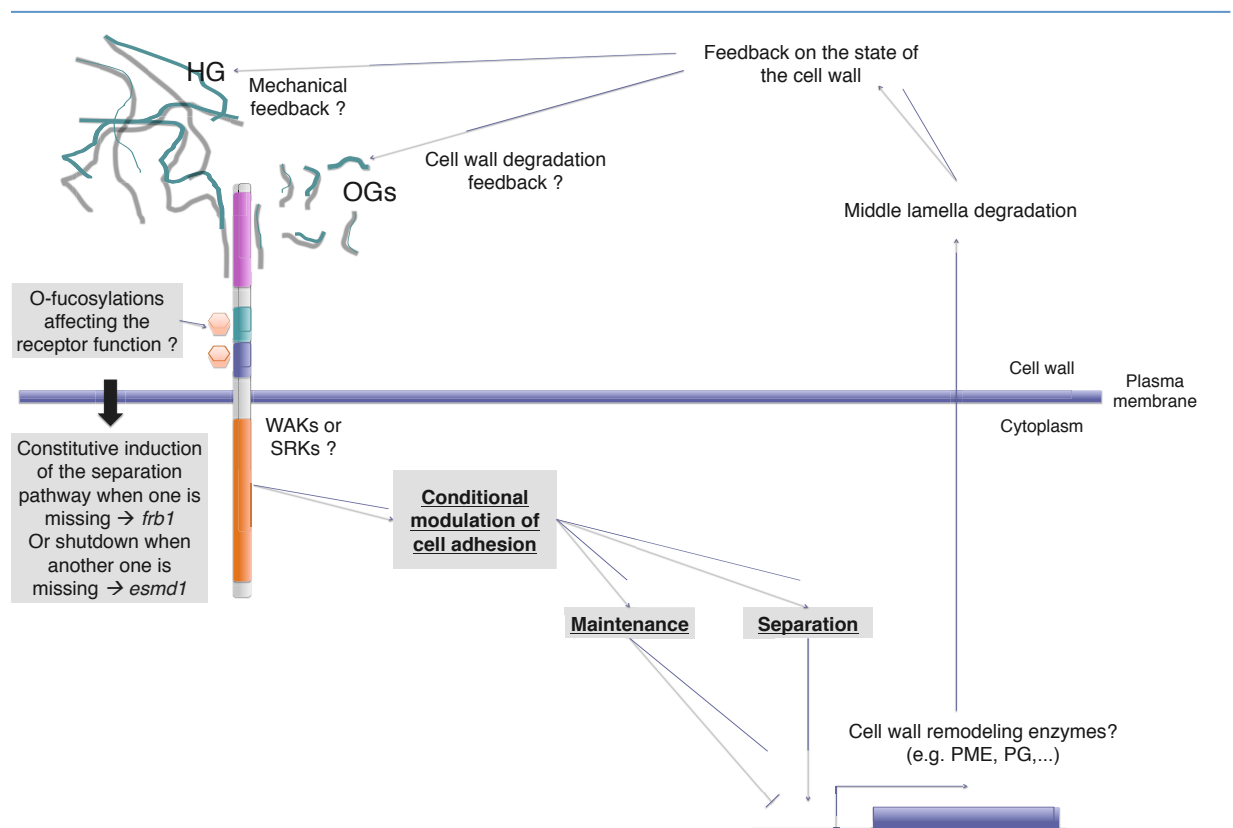
GUB-WAK: GalactUronic acid Binding domain - Wall-Associated Kinases; PA: Protease associated domain.

The drawing of each protein type/family is intended to be an average representative structure, but variations exist within families (see table S1). Also not all the proteins considered as part of these families had conserved EGF-like domains (e.g. there are 21 WAK-like but only 19 have EGF-like)

## ESMD1 and FRB1 putative function is to transfer fucose onto Epidermal Growth Factor-like repeats

O-fucosyltransferases in animals have been demonstrated to be responsible for adding a fucose to Epidermal Growth Factor (EGF)-like repeats (GT65, poFUT1) (Wang et al., 2001) or to ThromboSpondin type 1 Repeats (TSR) (GT68, poFUT2) (Luo et al., 2006). In a number of proteins containing these domains, the presence of this glycosylation is necessary for the correct function of the protein. It participates in protein-protein interaction and is the substrate for further glycosylation affecting the specificity of interaction with one protein partner or another (Takeuchi and Haltiwanger, 2014). These EGF and TSR domains have six

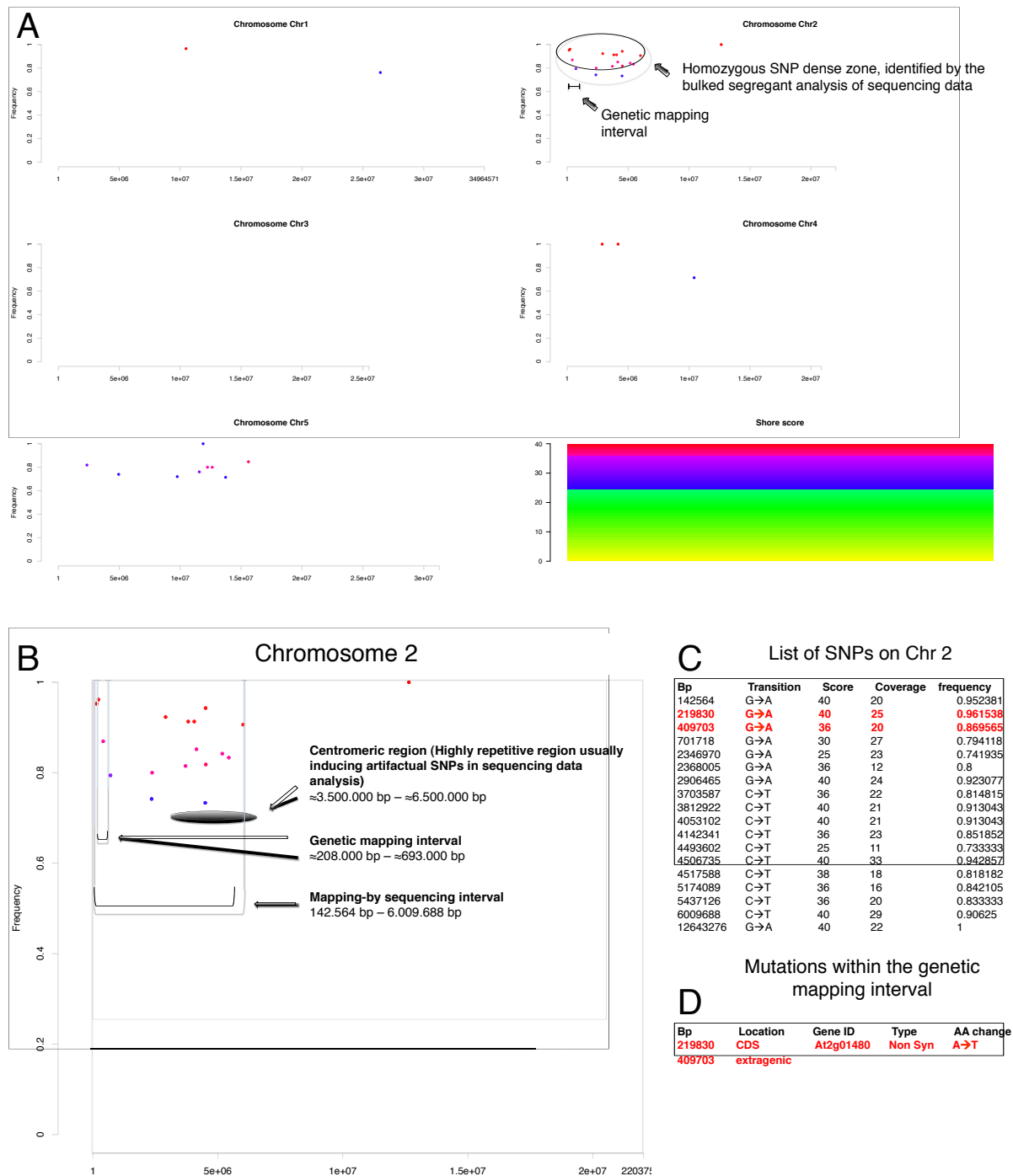
signaling pathway related to the pectin defect in *quasimodo* that in the end induces cell separation in the mutant.



**Figure 3: cell adhesion control feedback loop: A working model**

The state of cell adhesion is suggested to be under the control of a feedback loop positively or negatively influencing cell adhesion. Such a feedback mechanism could be mediated by the WAKs, sensing mechanical cues by their tight attachment to the HG, as well as perceiving the degradation state of the cell wall in the form of Oligogalacturonans. This perception may induce the expression of cell wall remodeling enzymes in order to degrade the middle lamella and lead to cell separation, or repress their expression in order to maintain cell adhesion. The consequence of these cell wall modifications can then be perceived by the WAKs thus closing the loop. In this model the function of ESMD1 and FRB1 at a molecular level remains to be fully understood, but their disruption affect the signaling of the WAKs (*frb1*) transducing a signal leading to cell separation or negatively (*esmd1*) preventing the induction of cell separation

Interestingly this situation is reminiscent of recent studies that have shown that some of the phenotypes primarily described in cell wall mutants were actually the result of cell wall integrity sensing leading to a disturbed growth and development (Hématy et al., 2007; Wolf et al., 2012b). For example the mutant *procuste1* that is affected in the cellulose synthase CesA6 and is deficient in cellulose shows a striking reduction of growth (Fagard et al., 2000). The decreased cellulose content in the cell wall seemed to explain very well a direct physical limitation of growth. However it was shown that a mutation in the *THESEUS1* receptor like kinase could suppress this growth defect, and that it was in fact the perception of the cell wall



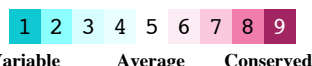
**Figure S1: Mapping and sequencing strategy, and the identification of *esmd1-1***

The graphs and annotations are the data obtained for the mapping and sequencing of a bulk of the segregant F2 suppressors of the *qua2-1/esmd1-1* line. The graphs are SHOREmap outputs (schneeberger et al., xxx) with added annotations relative to the genetic mapping done in parallel. (A) shows all the EMS canonical SNPs filtered for a >8x coverage, >0.75 frequency and >25 SHORE score. Each dot represents one mutation. The Shore score gives an idea of the quality of the sequenced reads on which the SNP call is based (25-40 are good to very good quality). The five graphs represent the five chromosomes of *Arabidopsis thaliana*, with on the x axis the position on the chromosome from the top to the bottom, and on the y axis the frequency of the mutation. The coverage is of an average of 28x. SNPs with a frequency of 1 (or almost 1) are likely linked with the suppressor loci. Thus the “cloud” of near-homozygous SNPs at the top of chromosome 2 defines the mapping-by-sequencing interval. The genetic mapping interval colocalizes with the mapping-by-sequencing interval. (B) is a close up on chromosome 2 with both genetic mapping and mapping-by-sequencing intervals. The mapping

## ConSurf Results



The conservation scale:



Fucosyltransferase superfamily conserved peptide I, II and III



GDP-fucose protein O-fucosyltransferase signature (IPR019378)



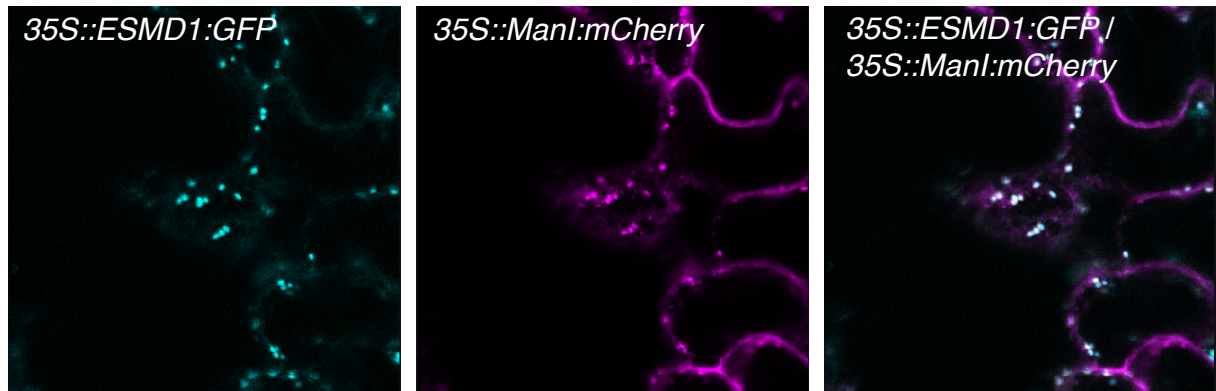
Amino acid affected by the mutant allele

- e** - An exposed residue according to the neural-network algorithm.
- b** - A buried residue according to the neural-network algorithm.
- f** - A predicted functional residue (highly conserved and exposed).
- s** - A predicted structural residue (highly conserved and buried).
- x** - Insufficient data - the calculation for this site was performed on less than 10% of the sequences.

**Figure S2: *ESMD1* mutant alleles and the structural and functional domains conservation**

ConSeq analysis (Berezin et al., 2004) of the conserved structural and functional domains of the protein, based on the 13 closest related members of the putative O-fucosyltransferase family of *arabidopsis thaliana*, as selected by the “homolog search algorithm” of the server and based on the full length amino acid sequence of *ESMD1*. Color scale for the highlighted amino acids represent their degree of conservation among the 13 related proteins. The letter below is a prediction of whether the amino acid is buried (b) within the 3D structure of the protein, or exposed (e), and when conserved, whether the amino acid may have a functional (f) or structural (s) role for the protein. The frames around successive amino acids show the conserved motifs of signature relevant for the predicted function of the protein. The red are for the GDP-fucose protein O-fucosyltransferase signature (IPR019378) (Hansen et al., 2012). And the blue are the peptides domains I, II and III characteristic of the fucosyltransferase superfamily (hansen et al., 2009). The red stars mark the two alleles *esmd1-1* and *esmd1-2* position and the amino acid change.

---

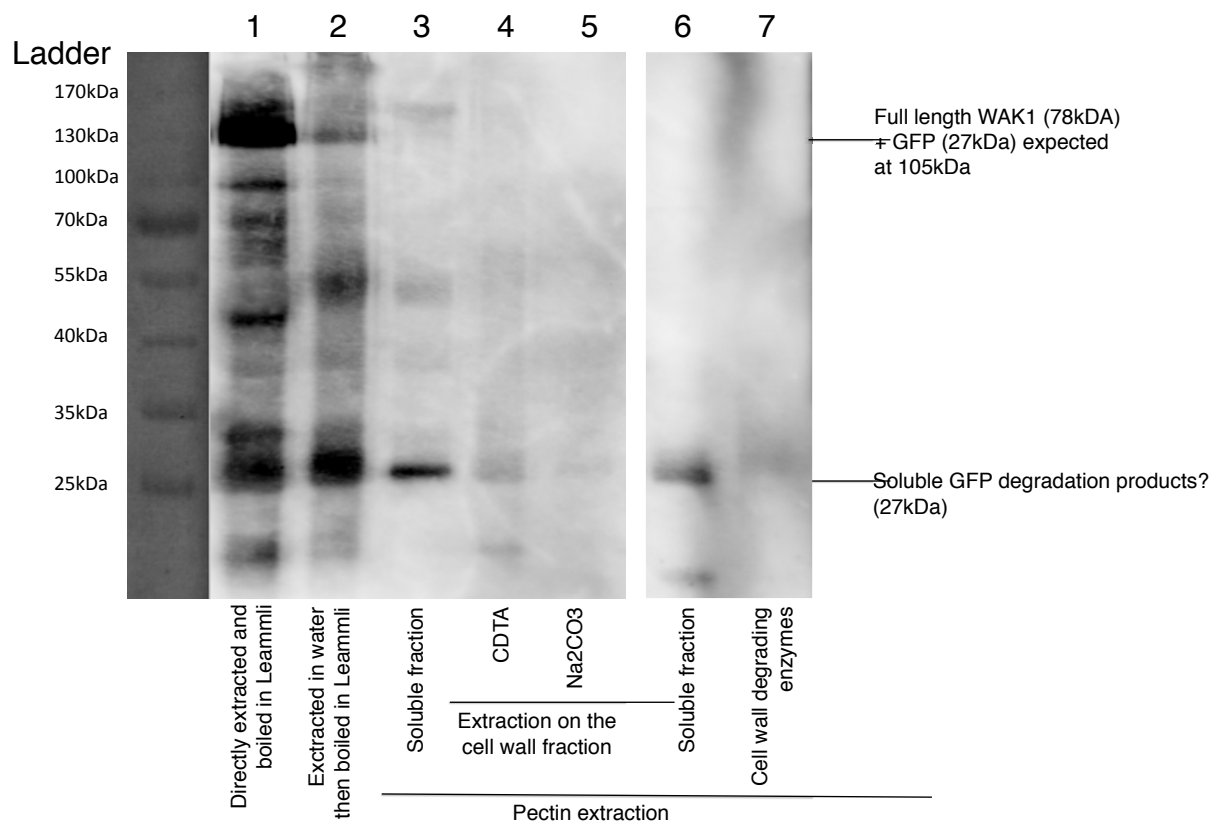


**Figure S3: *ESMD1:GFP* fusion protein accumulates in the Golgi apparatus**

Imaging of *ESMD1:GFP* (cyan) and the Golgi marker mannosidase I–*mCherry* (magenta, Nelson et al., 2007), co-infiltrated and transiently expressed in epidermal cells of *N. benthamiana*. *ESMD1:GFP* and our Golgi marker colocalize, indicating a Golgi localization of *ESMD1*.



25 kDa form is considerable when the sample is first ground in water with no particular precautions (figure 1 lane 1 and 2). For this reason the subsequent experiments were performed on ice, from the tissue grinding to the extract denaturation, and the extraction buffer was supplemented with an anti-proteases cocktail. But even when the sample was directly flash frozen at harvesting, ground in Laemmli and directly boiled for ten minutes there was still a considerable amount of degradation product (figure 1 lane 1). This observation suggested that at least some of the proteins were likely degraded in the plant prior to extraction.

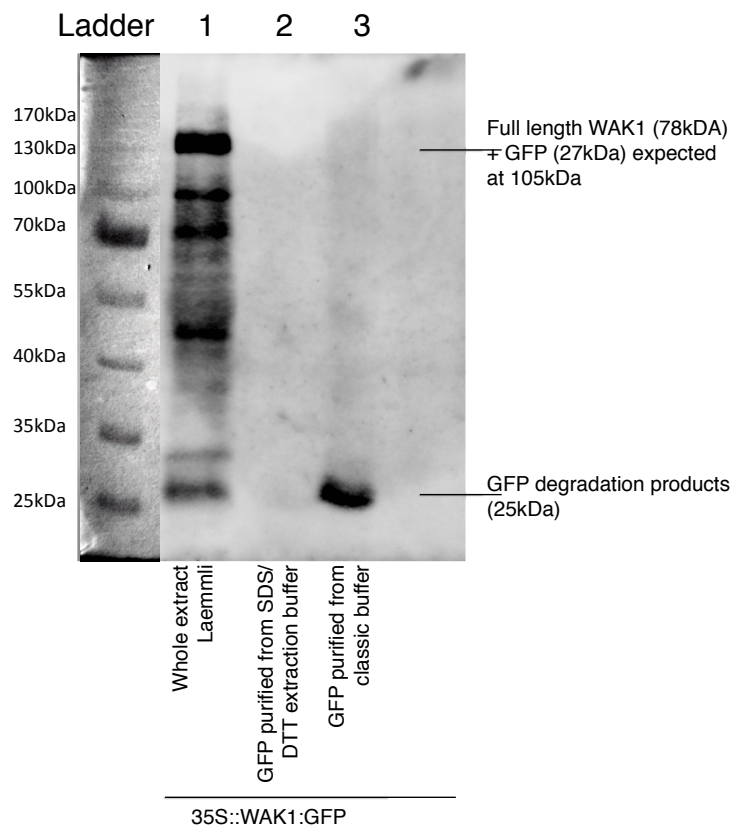


**Figure 1: *WAK:GFP* construct expression and extraction**

Western Blot of different extracts for *35S::WAK1:GFP* transformed plants.

In lane 1 the leaf samples were directly ground in Laemmli and boiled for 10 minutes, while in lane 2 they were first ground in water and then boiled in Laemmli for 10 minutes. The difference in GFP fusion protein profile reveals a degradation of the protein in the latter situation. In lane 3, 4 and 5 the leaf samples were first ground in a simple Tris-HCl extraction buffer containing an anti-protease cocktail. A first soluble fraction was obtained after centrifugation of the extract at 10,000g for 10 minutes. Then the pellet was treated with 0.05M CDTA for 2h at room temperature, and the extract was centrifuged at 10,000g for 10 minutes to obtain the second fraction. Finally the pellet was treated with 0.05M Na<sub>2</sub>CO<sub>3</sub> for 14h at room temperature, and the extract was centrifuged at 10,000g for 10 minutes to obtain the third fraction. In lane 6 and 7 leaf samples were first ground in a simple Tris-HCl extraction buffer containing an anti-protease cocktail. A first soluble fraction was obtained after centrifugation of the extract at 10,000g for 10 minutes. Then the pellet was treated with the protoplast cell wall degradation cocktail for 16h at room temperature and the extract was centrifuged at 10,000g for 10 minutes to obtain the second fraction. Finally the samples that were not directly ground in Laemmli were supplemented with 1 volume of 2X Laemmli, and all the samples were boiled for 10 minutes before gel migration. The membrane was probed with an anti-GFP antibody and reveal no detectable full length WAK fusion protein in the soluble or cell wall extracted fractions.

obtain purified proteins. Protein purification usually only yielded an approximately 25 kDa band likely corresponding to the GFP fragment alone (figure 2 lane 3).

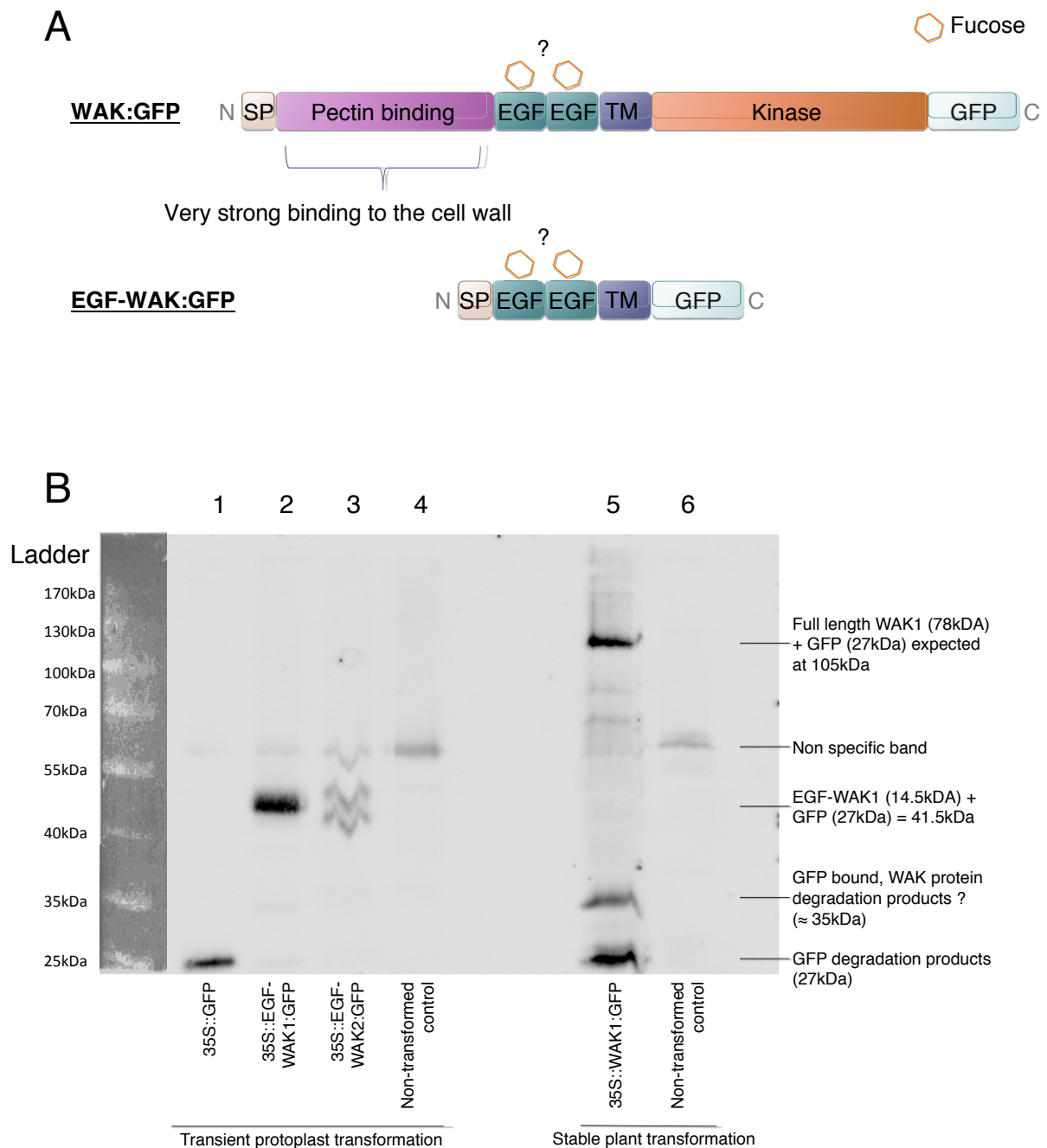


**Figure 2: GFP-trap purification of the WAK:GFP fusion proteins**

Lane 1 is the extract from leaves directly ground and boiled in Laemmli. In lane 2 the leaf samples were first ground in a 4% SDS / 50mM DTT extraction buffer, and GFP-trap purification was carried out on this extract. In lane 3, the leaf samples were first ground in a simple TrisHCl extraction buffer containing an anti-protease cocktail and GFP-trap purification was carried out. The presence of the GFP fusion proteins was probed with an anti-GFP antibody. In lane 2 no purification at all was observed due to the incompatibility of the SDS/DTT extraction buffer with the GFP-trap. In lane 3 only the approximately 25 kDa GFP fragment was observed likely because the full length version of the fusion protein didn't detach from the cell wall with the extraction buffer used.

*Can we extract the WAKs from freshly prepared protoplast?*

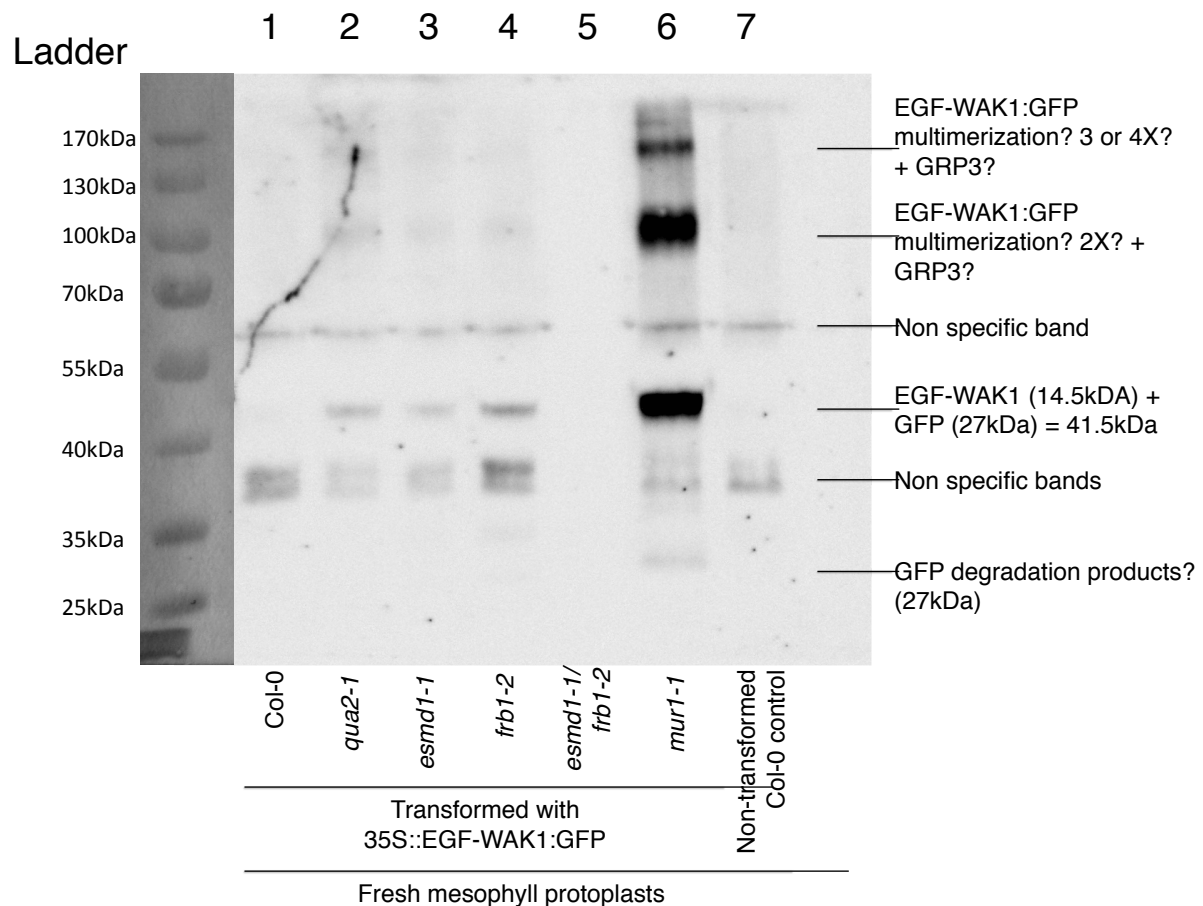
Finally I attempted to extract the WAKs from freshly prepared protoplasts. The principle was to prepare protoplast (as described in III. Experimental procedure) such that the cell wall would be removed and the newly synthesized overexpressed WAKs would be more easily extractable. I also prepared microsomal fractions of these protoplasts to increase the chance of purifying the WAKs. Western Blot analysis of the different fractions did not reveal the



**Figure 3: *35S::EGF-WAK:GFP* transient transformation in protoplasts: Tests**

(A) Schematic view of the EGF-WAK:GFP fusion protein compared to the WAK:GFP. SP: Signal peptide; EGF: Epidermal Growth Factor; TM: Transmembrane domain. N: N-Terminal end of the protein; C: C-terminal end. (B) Western blot of different extracts from transiently expressed EGF-WAK:GFP fusion protein in col-0 cell cultures protoplasts and WAK1:GFP fusion protein in stably transformed plants.

Lane 1 is a positive control transformation with a simple *35S::GFP* construct. Lane 2 and 3 are the extracts from the transformations with *35S::EGF-WAK1:GFP* (lane 2) and *35S::EGF-WAK2:GFP* (lane 3). Lane 4 is a negative non transformed control. The proteins were extracted from equal amounts of transformed or control protoplasts with a simple lysis buffer (as described in the experiment procedure). The presence of the GFP fusion proteins was probed with an anti-GFP antibody. Lane 5 and 6 are for comparison with the stably transformed *35S::WAK1:GFP* construct. Interestingly the EGF-WAK1 construct (lane 2) yielded a nice band of the expected size of the fusion protein, while EGF-WAK2 (lane 3) yielded two bands with a lower intensity. These results show that at least for EGF-WAK1, the transient transformation works well, the expected size protein is produced, is easily extractable and produces barely any GFP degradation products.



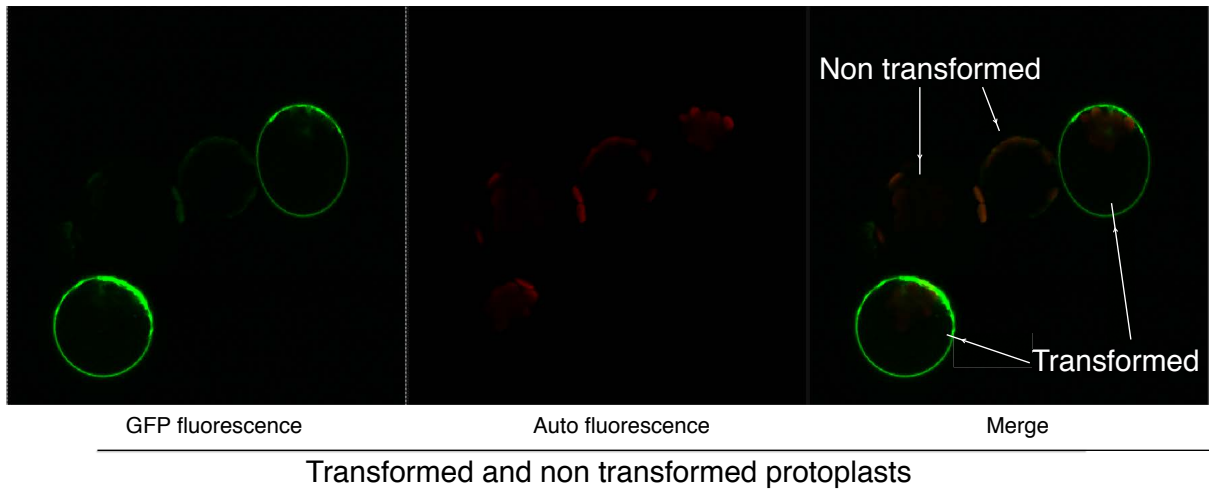
**Figure 4: *35S::EGF-WAK1:GFP* transient transformation in leaf mesophyll protoplasts from different genetic backgrounds : Western blot**

The same *35S::EGF-WAK1:GFP* construct as in figure 3 was transformed into *col-0* (lane 1), *qua2-1* (lane 2), *esmd1-1* (lane 3), *frib1-2* (lane 4), *esmd1-1/frib1-2* (lane 5) and *mur1-1* (lane 6) leaf mesophyll protoplasts, or not transformed (lane 7). The expression level of the fusion protein was assayed by western blot. The presence of the GFP fusion proteins was revealed with an anti-GFP antibody. Interestingly *mur1-1* transformation efficiency was much higher than the other genotypes as revealed by the presence of a band at the expected size (approximately 42 kDa) of a much higher intensity than the other genotypes. The higher molecular weight bands at approximately 100 and 170 kDa could be multimers of the EGF-WAK1:GFP fusion protein potentially also associated with GRP3, a peptide known to interact with WAK1 and to promote its multimerization (Park et al., 2001).

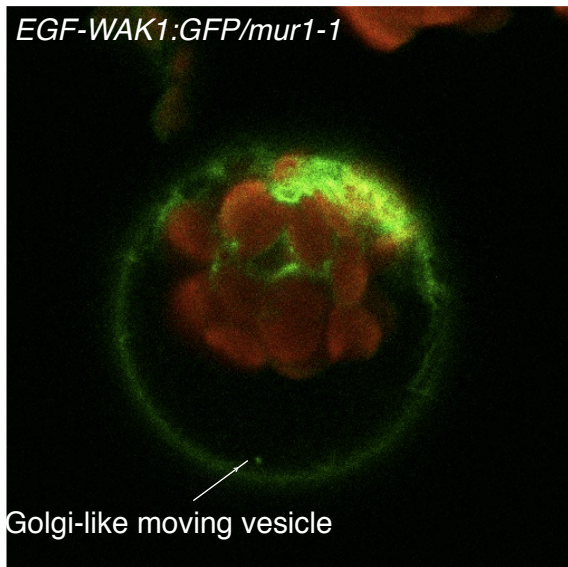
#### *Transformation of Col-0 cell suspension culture*

The first tests were carried out on *Col-0* cell suspension culture available at the institute, following the protocol described in (AXELOS et al., 1992). These provided encouraging results (figure 3 B). Western blot analyses were carried out on whole protoplast lysate approximately 16h after transformation. The *35S::EGF-WAK1:GFP* construct produced a single band at approximately 42 kDa as expected, in relatively high amount and with almost no visible degradation product (figure 3 B lane 2). The *35S::EGF-WAK2:GFP* construct however produced two specific bands around the expected size and in a lower quantity compared to EGF-WAK1 (figure 3 B lane 3).

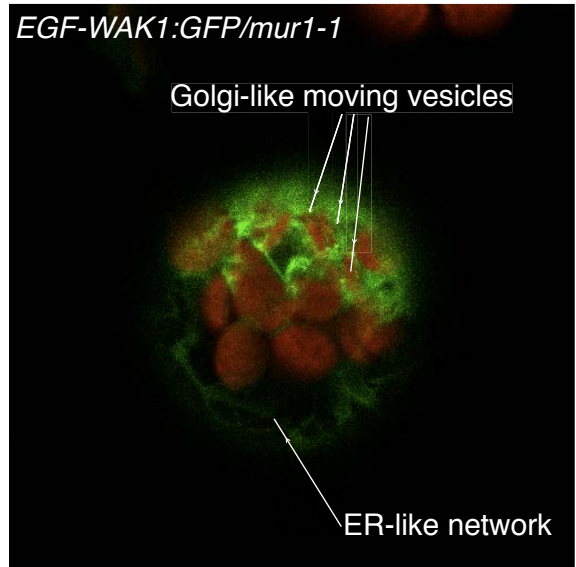
A



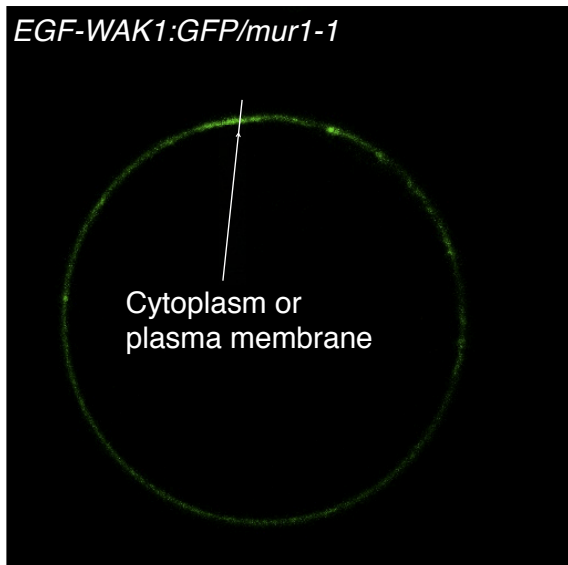
B



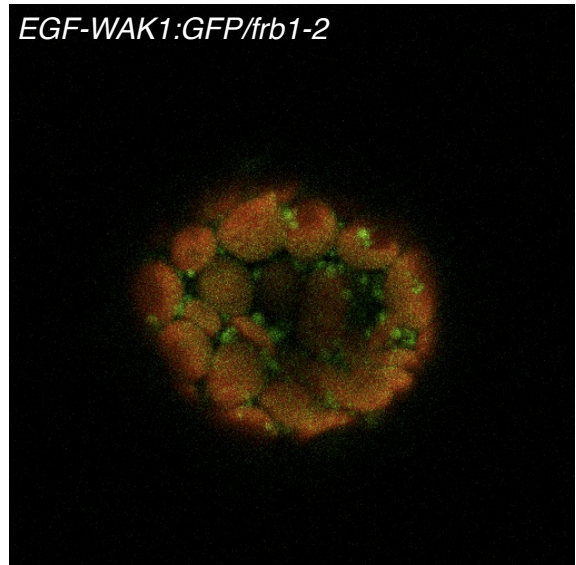
C



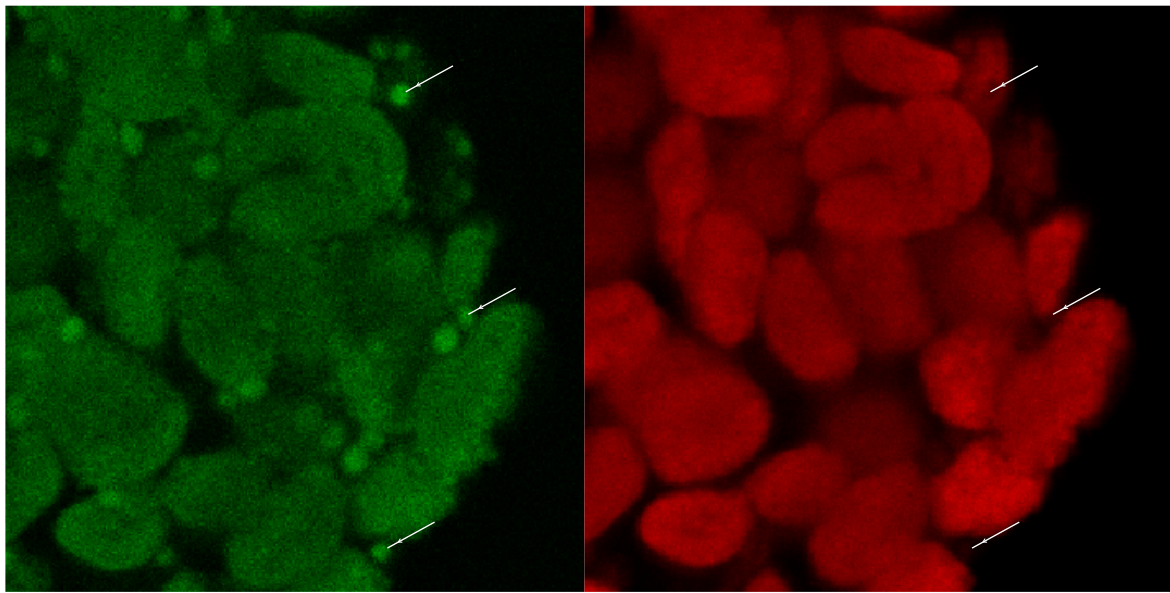
D



E



A

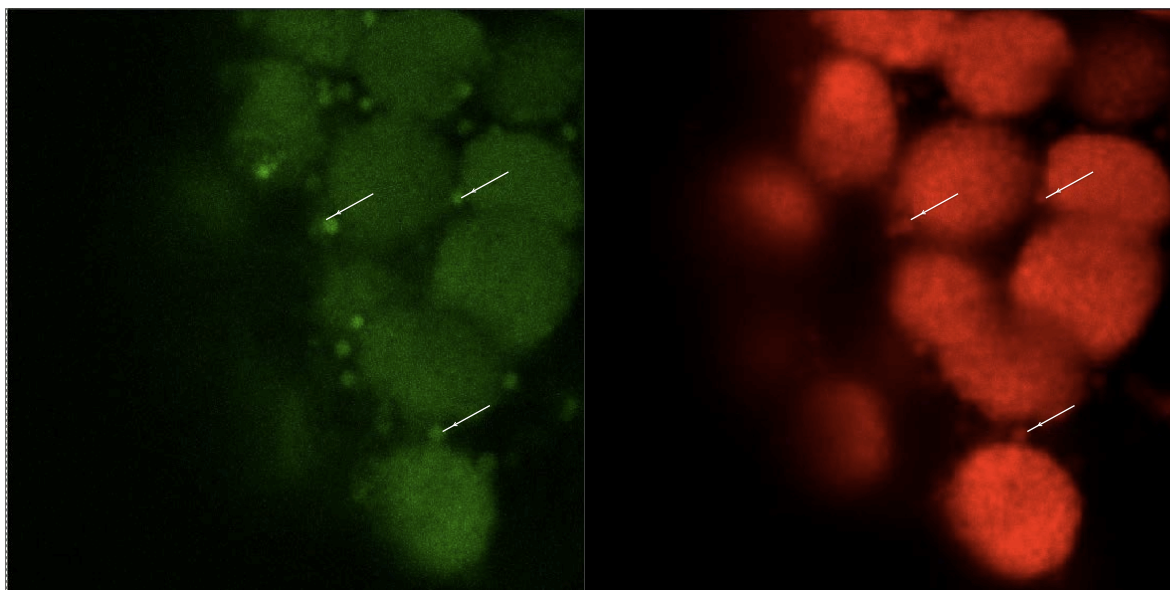


GFP fluorescence

"Red" fluorescence/auto fluorescence

EGF-WAK1:GFP/*frb1-2*

B



GFP fluorescence

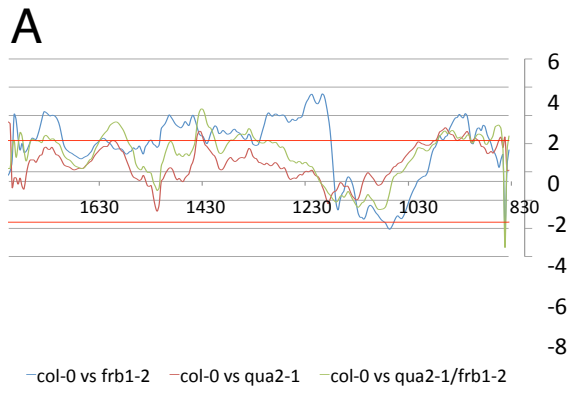
"Red" fluorescence/auto fluorescence

EGF-WAK1:GFP/*frb1-2*  
+ fm4-64

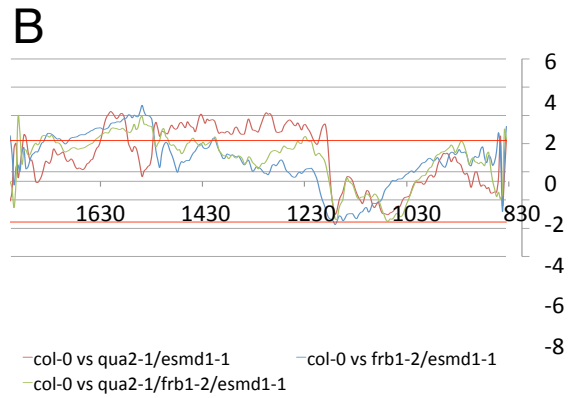
**Figure 6: Endosomal localization of EGF-WAK1:GFP in *frb1-2* leaf mesophyll protoplasts**

*frb1-2* protoplasts transformed with the EGF-WAK1:GFP construct were observed with (B) and without (A) fm4-64 staining. When stained the observation was done about 10 minutes after the addition of the fm4-64. Interestingly the vesicle like localization of the fusion protein in *frb1-2* colocalizes with the fm4-64 internalization. This suggests that the protein is indeed located in the endosome. Interestingly there is also a GFP signal in the chloroplast in *frb1-2* compared to *mur1-1* (figure 5). The images were taken with the exact same settings which tend to indicate that the signal is indeed specific to GFP.

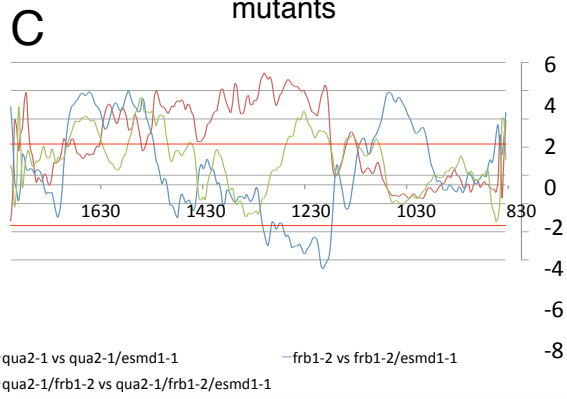
The green channel on the pictures correspond to the GFP signal (detected at 495–540 nm), and the red channel corresponds to the fm4-64 signal as well as the autofluorescence of the chloroplasts (detected at 560–620 nm).



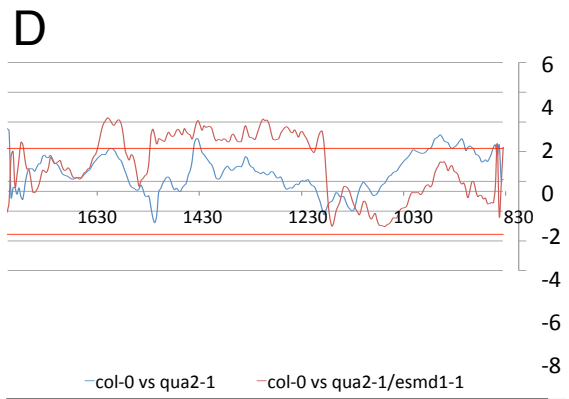
Col-0 vs cell adhesion defective mutants



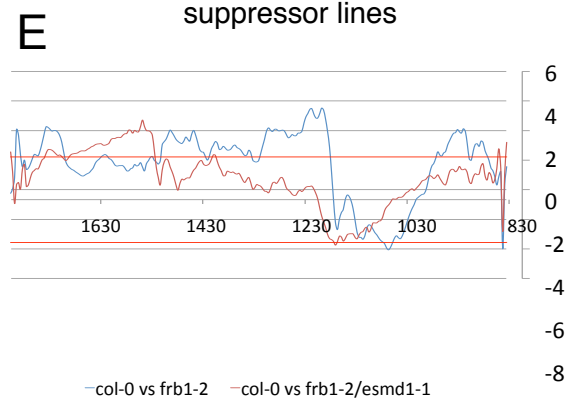
Col-0 vs suppressor lines



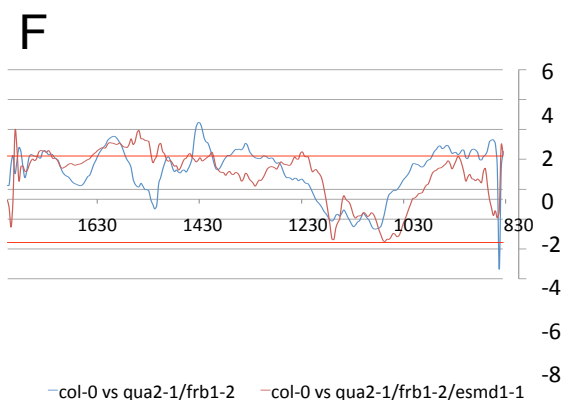
Cell adhesion defective vs suppressor lines



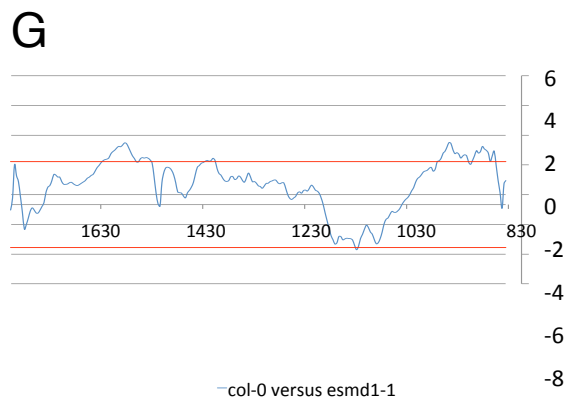
qua2-1 and suppressor



frb1-2 and suppressor



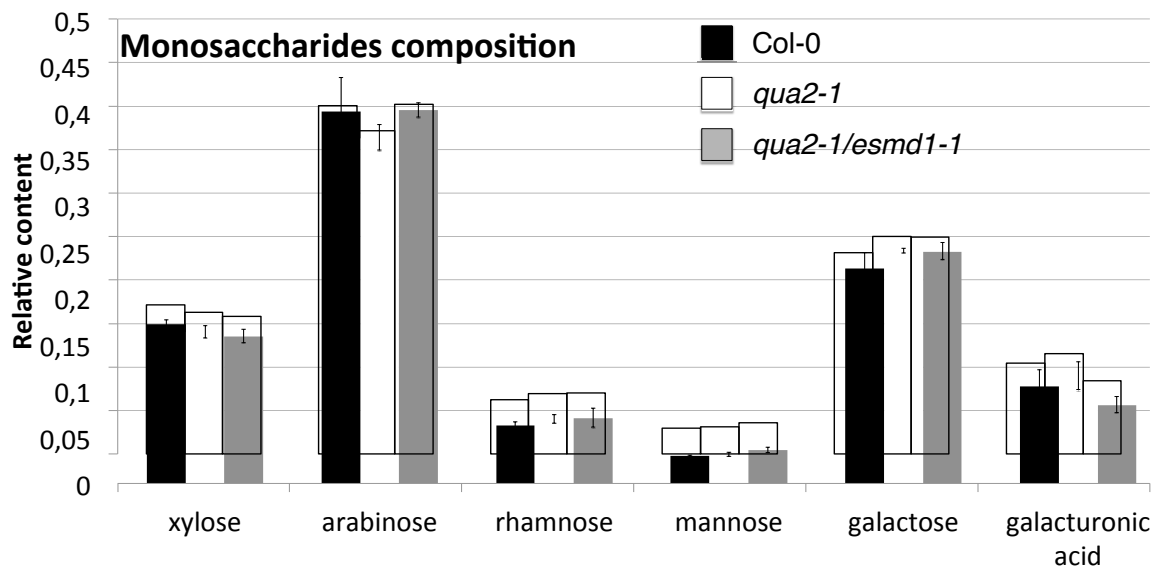
qua2-1/frb1-2 and suppressor



Col-0 vs esmd1-1

However in depth characterization of the cell wall has been carried out in the case of the mutant *frb1* (Neumetzler et al., 2012) and although they were able to point out that the low degree of pectin methyl esterification and higher PME activity were likely responsible for the loss of cell adhesion, a number of other modifications are taking place in the cell wall of this mutant and a clear picture could not be drawn either. This also answer the concern of a potential direct role of ESMD1 and FRB1 on the cell wall structure and composition instead of signaling, since they are glycosyltransferases and could glycosylate structural components of the cell wall. None of the cell wall analyses that were carried out on *frb1* could clearly point out a cell wall component that could be the substrate of FRB1. Overall this seems to correlate with the fact that these enzymes affect the signaling and that the downstream modifications that they induce in the cell wall are complex.

---



**Figure 8: Characterisation of the cell wall modification in *quasimodo* and the suppressor lines: Monosaccharide composition analysis**

Monosaccharide composition of *col-0*, *qua2-1* and *qua2-1/esmd1-1*. No major changes are observed between the different genotypes.

---

## 2. *ESMD1* pattern of expression

A GUS construct harbouring the 2168 pb upstream promoter region of *ESMD1* was used to study the expression pattern of *ESMD1*. GUS expression seems to be present almost throughout the seedling and to a stronger degree in the vascular tissues (figure 9). In dark grown hypocotyls the expression is present in the root but absent in the root cap (figure 9 D). The GUS staining is present in the hypocotyl and seems to be more intense in the already

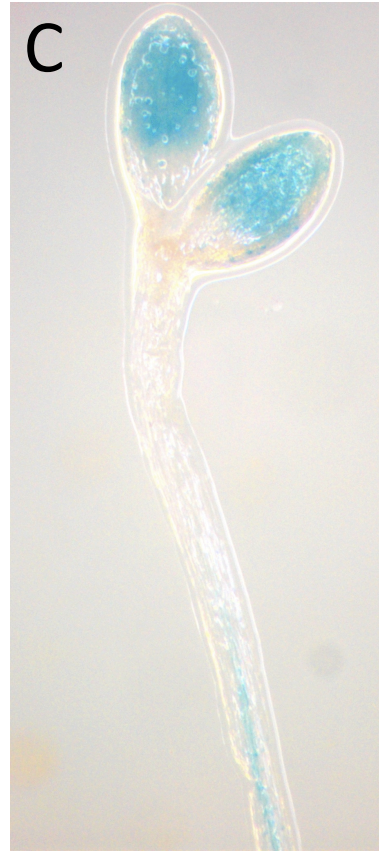




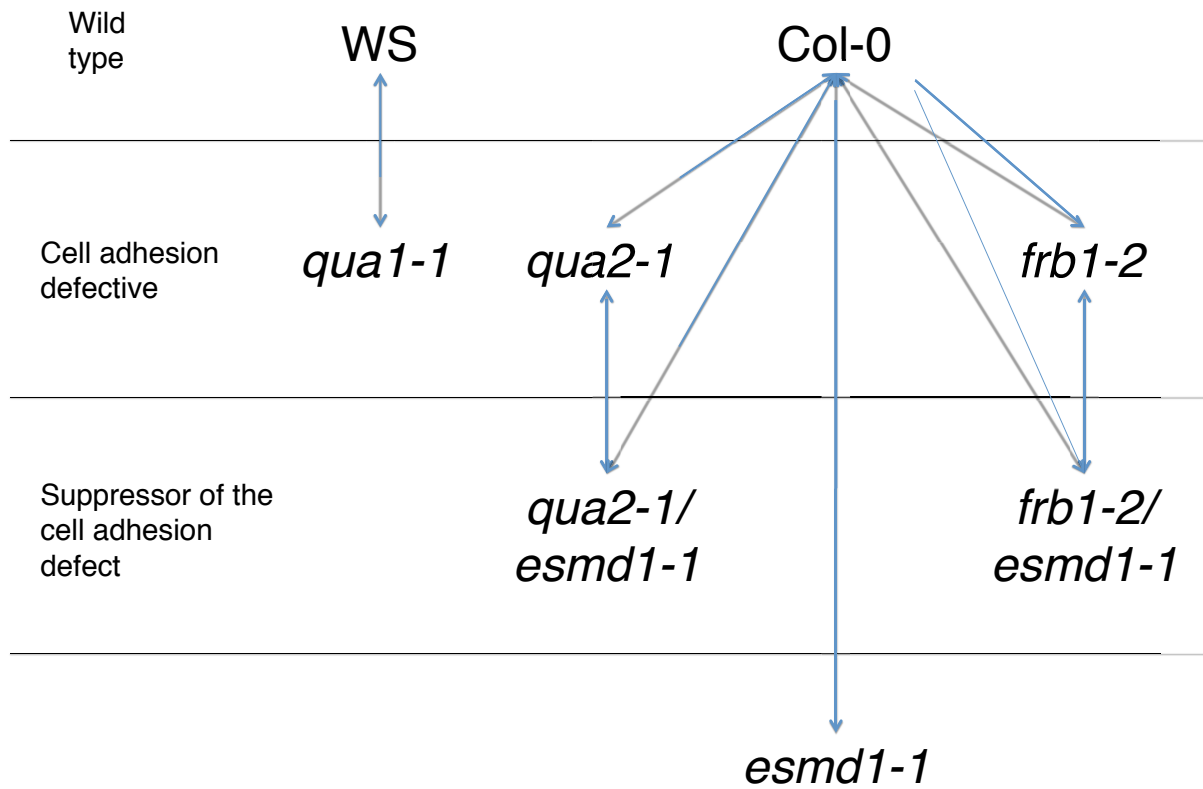
Seedling grown on a medium containing 1% sucrose



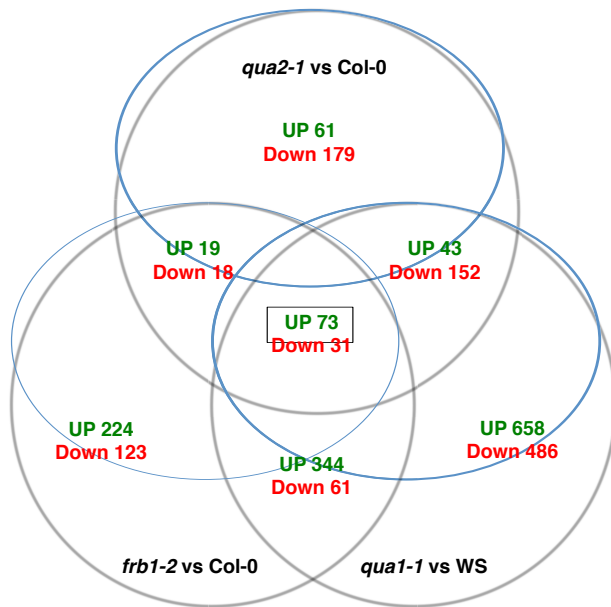
Dark grown hypocotyl, without sucrose



A

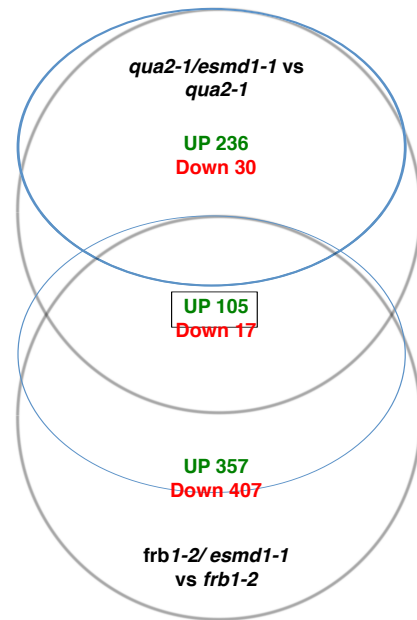


B

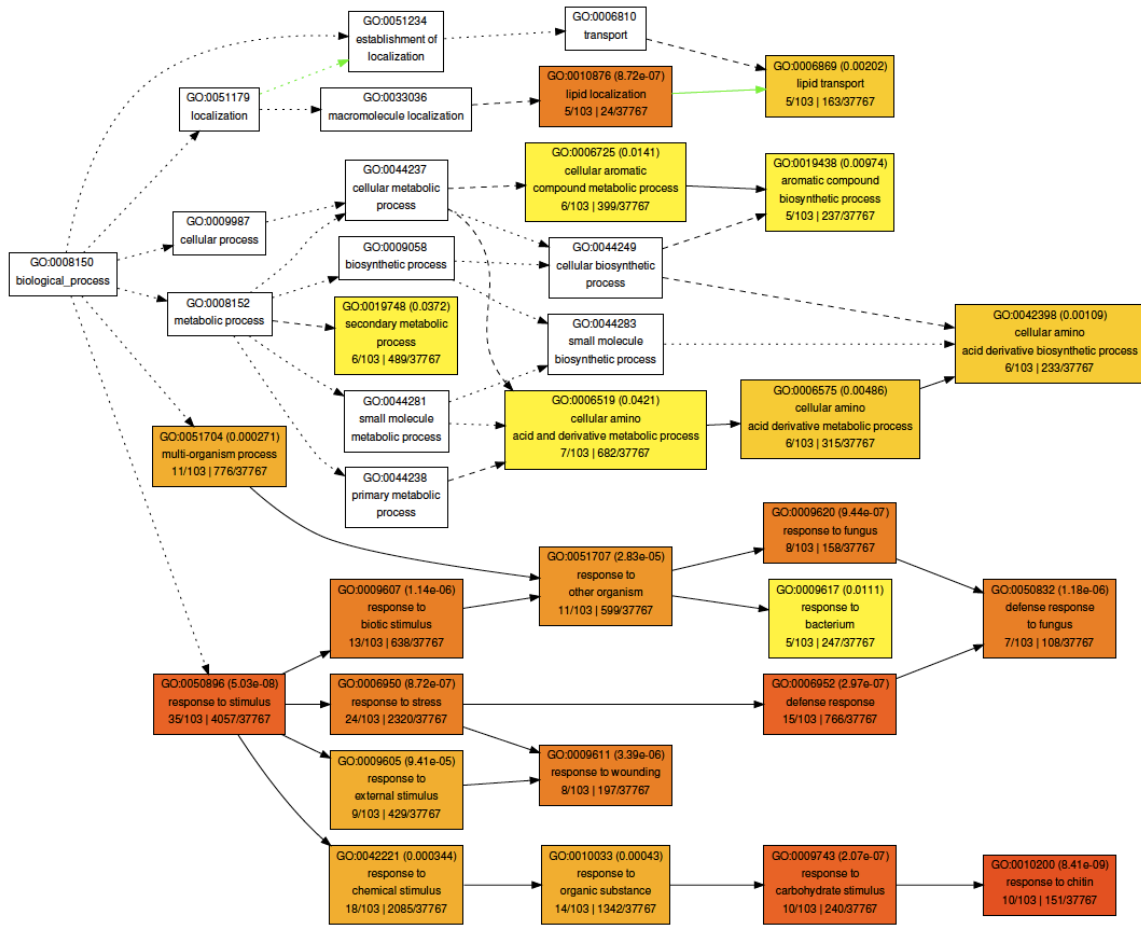


Cell adhesion defect specific genes

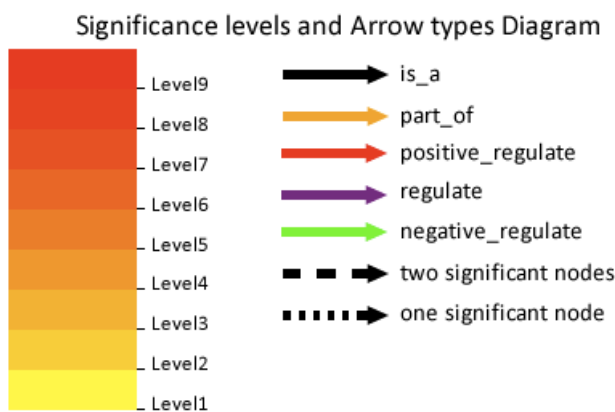
C



*esmd1-1* cell adhesion restoration specific genes

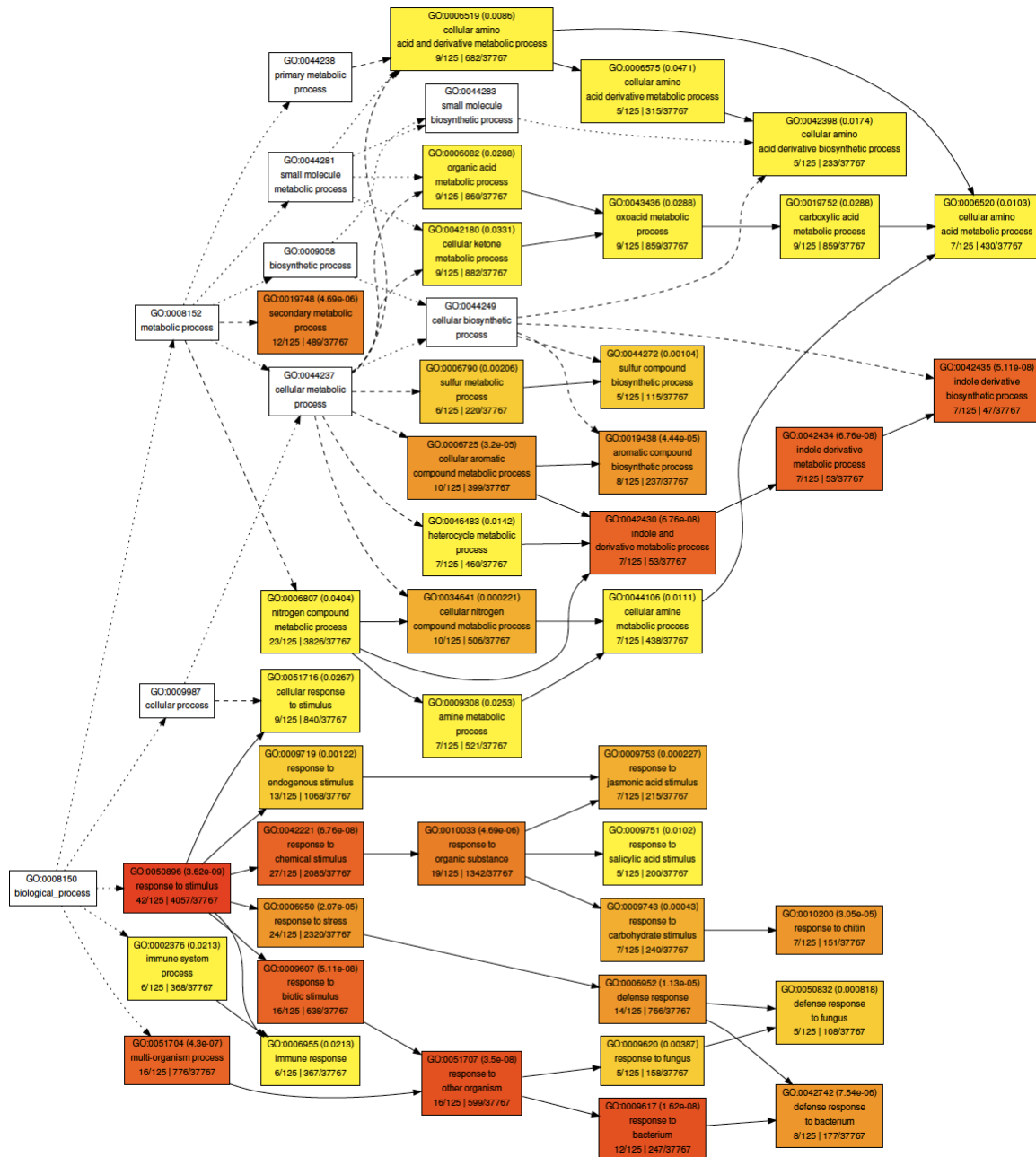


Gene Ontology analysis for genes specific of the cell adhesion defect



**Figure 11: Gene Ontology of the genes up regulated in the cell adhesion defective mutants**

AgriGO tool (<http://bioinfo.cau.edu.cn/agriGO/index.php>; Du et al., 2010) Gene ontology analysis output reveals mostly an up-regulation of the “response to stimulus” related genes.



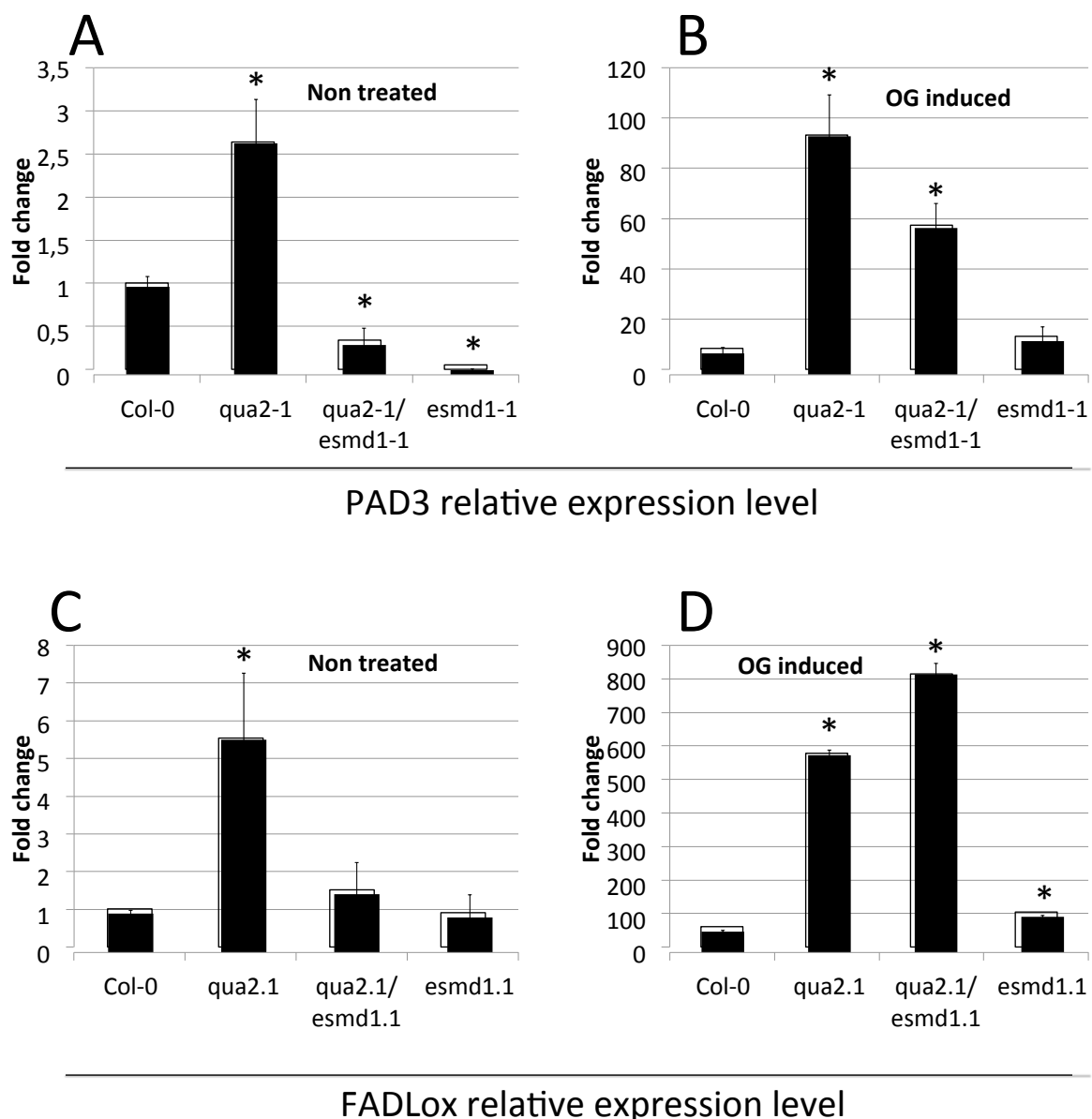
Gene Ontology analysis for genes specific of *esmd1-1* cell adhesion restoration

**Figure 12: Gene Ontology of the genes up regulated in the *esmd1-1* restored line**

AgriGO tool (<http://bioinfo.cau.edu.cn/agriGO/index.php>; Du et al., 2010) Gene ontology analysis output reveals mostly an up-regulation of the “response to stimulus” related genes as well as genes implicated in the “indole and derivative metabolic process”.

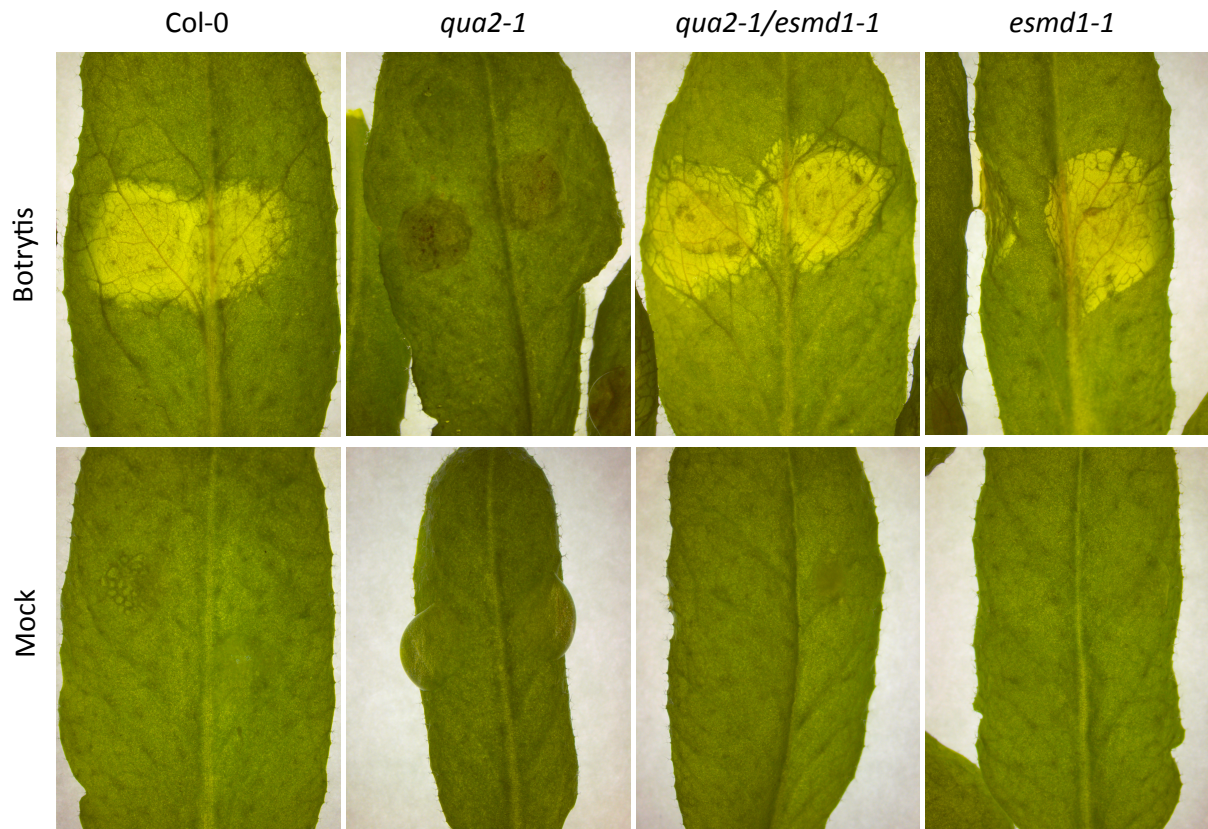
homeostasis (Shin et al., 2006). The functions of these genes and the phenotypes that they induce when they are differentially expressed could be responsible for some of the pleiotropic phenotypes of *quasimodo*.

Overall this demonstrates that the mutation in *ESMD1* does not abolish the response to OGs as we would have expected. But it still clearly affects the response as observed with the two genes that we tested. We expected that PAD3 and FADLox would behave similarly in response to OG, and it does for *col-0* and *qua2-1*, but it is not the case in the suppressor line. This may actually mean that the potential presence or absence of the O-fucosylation may not have a radical effect such as permitting or abolishing the signaling but may affect the specificity of the response and the downstream signaling pathway.



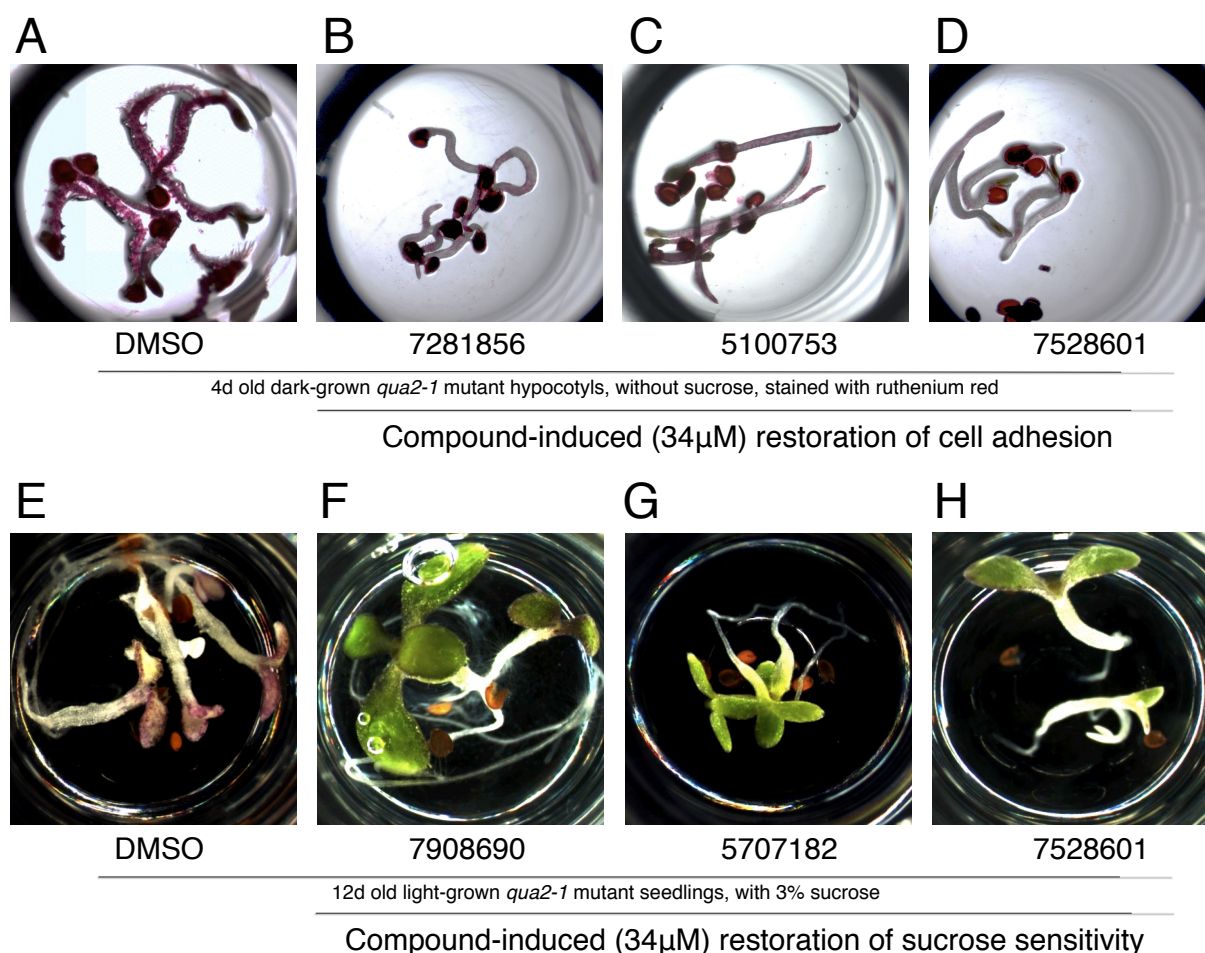
**Figure 13: *quasimodo* and *esmd1* have an altered response to pectin**

Expression levels of PAD3 (A and C) and FADLox (B and D), of either non treated (A and B) or OG treated (C and D) seedlings of *col-0*, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1*. Values shown are average and standard deviation of three biological replicates. All relative expression is expressed in fold change relative to the untreated *col-0* (A). However, \* indicates independently in each graph a significant difference (t-Test,  $p < 0.05$ ) with the *col-0* sample in this graph.



**Figure 14: *qua2-1* is resistant to *B. cinerea* maceration and sensitivity is restored in the suppressor line**

Six weeks old leaves of col-0, *qua2-1*, *qua2-1/esmd1-1* and *esmd1-1* inoculated with two drops of germinated spores in PDB, or PDB alone (mock) and observed after 48H. The maceration makes the leaf translucent around the site of inoculation. *Qua2-1* doesn't show any signs of maceration 48h after inoculation compared to the other lines.

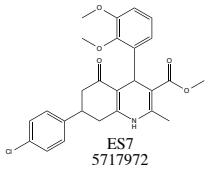
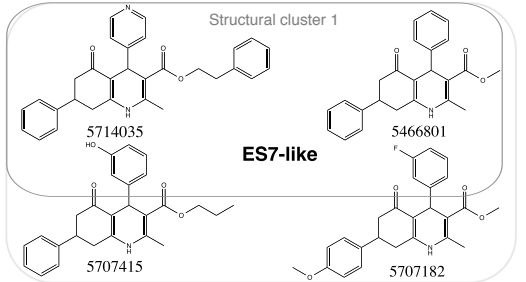

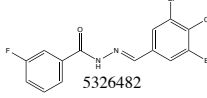
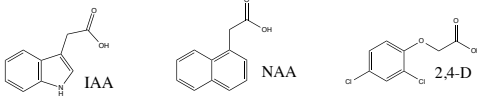
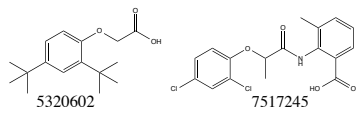
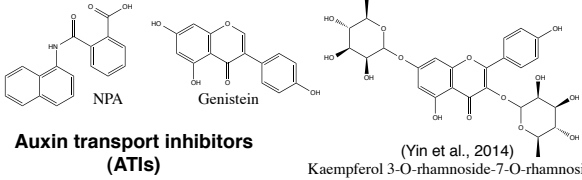
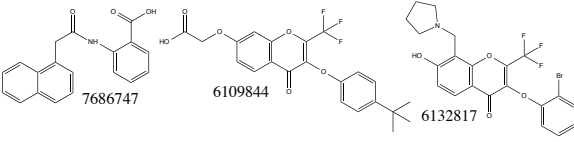


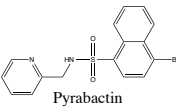
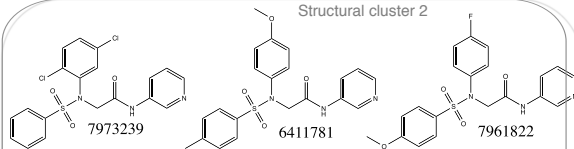
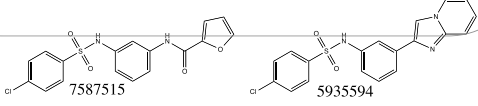
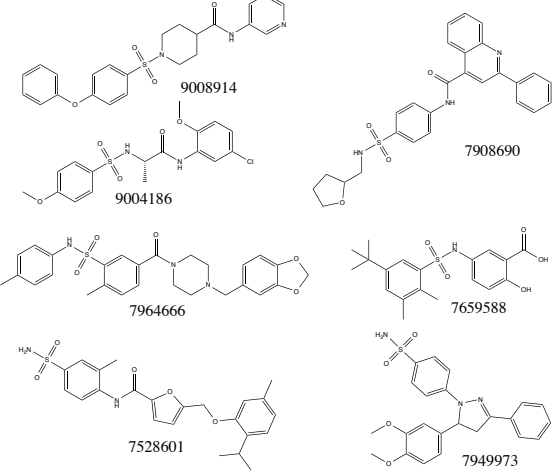


**Figure 1: High throughput chemical genomic screening for the restoration of cell adhesion and sucrose sensitivity**

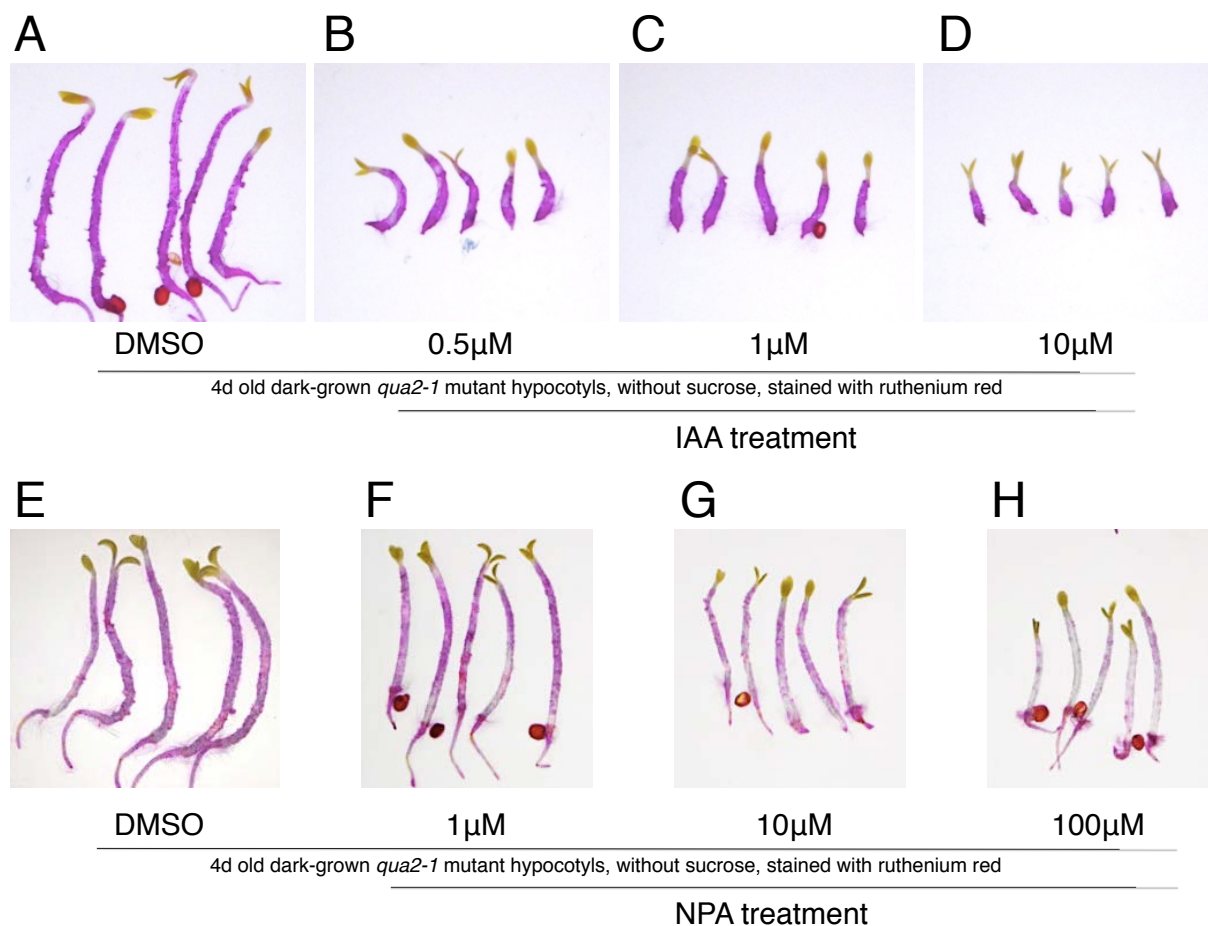
(A-D) Four days old dark grown hypocotyls of the *qua2-1* mutants treated with DMSO for control (A) or with suppressor compounds (B-D). The seedlings were stained with ruthenium red to reveal the presence or restoration of the cell adhesion defect. A characteristic outward-curving of the epidermal cells can also be seen in the *qua2-1* mutant, which is characteristic of the cell adhesion defect when observed on the dark grown hypocotyl. This phenotype is restored by the suppressor compounds as well, showing actual restoration of cell adhesion and not simply the inhibition of the ruthenium red staining. (E-H) Twelve days old light grown seedlings of the *qua2-1* mutant grown with 3% sucrose, treated with DMSO for control (E) or with suppressor chemicals (F-H). The mutant is very sensitive to C/N imbalance in the growth medium, accumulates anthocyanin (not to be confused with ruthenium red staining) and has no chlorophyll (E), while the suppressor compounds induce a suppression of these phenotypes (F-H). Note that compound 7528601 (D and H) has an effect on both phenotypes while the other four compounds taken as example here (B, C, F and G) were specific for either cell adhesion or sucrose sensitivity. The chemicals' names correspond to their ChemBridge ID (<http://www.hit2lead.com/>)

One was based on the restoration of cell adhesion and the second one on the restoration of sucrose sensitivity.

In order to achieve a high throughput screening, the *qua2-1* seeds were sown by pipetting from a liquid medium into 96-well plates with 5 to 10 seeds and 150μl of medium per well. The chemicals were then added to a final concentration of 34μM by an automated workstation

Known compounds	Hit compounds
 <p>ES7 5717972</p> <p>Inhibition of callose synthesis (Park et al., 2014)</p>	 <p>Structural cluster 1</p> <p>ES7-like</p> <p>5714035, 5466801, 5707415, 5707182</p>
 <p>ES3 5279218</p> <p>Affects BRI1, PIN1 and PIN2 trafficking (Drakakaki et al., 2011)</p> <p>RAE1 5271226</p> <p>Affects RAB2 trafficking (Ung et al., 2013)</p>	 <p>5326482</p> <p><b>Endosidin-like</b></p>
 <p>IAA, NAA, 2,4-D</p> <p><b>Auxins</b></p>	 <p>5320602, 7517245</p> <p><b>2-4-D-like</b></p>
 <p>NPA, Genistein, Kaempferol 3-O-rhamnoside-7-O-rhamnoside</p> <p><b>Auxin transport inhibitors (ATIs)</b></p> <p>(Yin et al., 2014)</p>	 <p>7686747, 6109844, 6132817</p> <p><b>ATI-like</b></p>
<p><b>Sulfonamides</b></p>  <p>Sulfamethazine, Sulfamethoxazole</p> <p>Suppression of epigenetic silencing, inhibition of folate synthesis (Zhang et al., 2012)</p> <p><i>Pseudomonas syringae</i> resistance (Schreiber et al., 2008)</p>  <p>Sulfamer, Clopamide</p> <p>Plant immune-priming (Noutoshi et al., 2012b)</p> <p>Plant immune-priming (Noutoshi et al., 2012a)</p>  <p>Pyrabactin</p> <p>ABA signaling (Park et al., 2009)</p>	<p><b>Sulfonamides 1</b></p>  <p>Structural cluster 2</p> <p>7973239, 6411781, 7961822</p> <p><b>Sulfonamides 2</b></p>  <p>7587515, 5935594</p>  <p>9008914, 7908690, 9004186, 7964666, 7659588, 7528601, 7949973</p> <p><b>Other Sulfonamides</b></p>

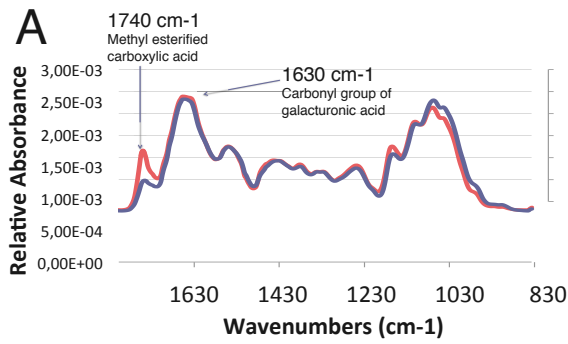




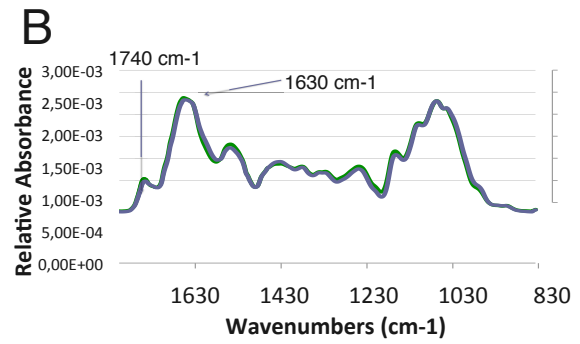
**Figure 3: Inhibition of auxin transport can prevent cell separation in *quasimodo***

(A-H) Four days old dark grown hypocotyls of the *qua2-1* mutants treated with DMSO for control (A and E), with the auxin IAA at increasing concentrations (B-D), or with the auxin transport inhibitor NPA at increasing concentrations (F-H). The seedlings were stained with ruthenium red to reveal the presence or restoration of the cell adhesion defect. Auxin treatment induces an inhibition of growth but does not prevent ruthenium red staining. In contrast the inhibition of auxin transport by NPA only slightly reduces growth while it clearly limits ruthenium red staining in a dose-response manner. The characteristic outward-curving of the epidermal cells of the *quasimodo* mutant dark grown hypocotyl is also restored by increasing concentrations of NPA, showing a clear effect of NPA on the restoration of the *quasimodo* mutant's cell adhesion defect.

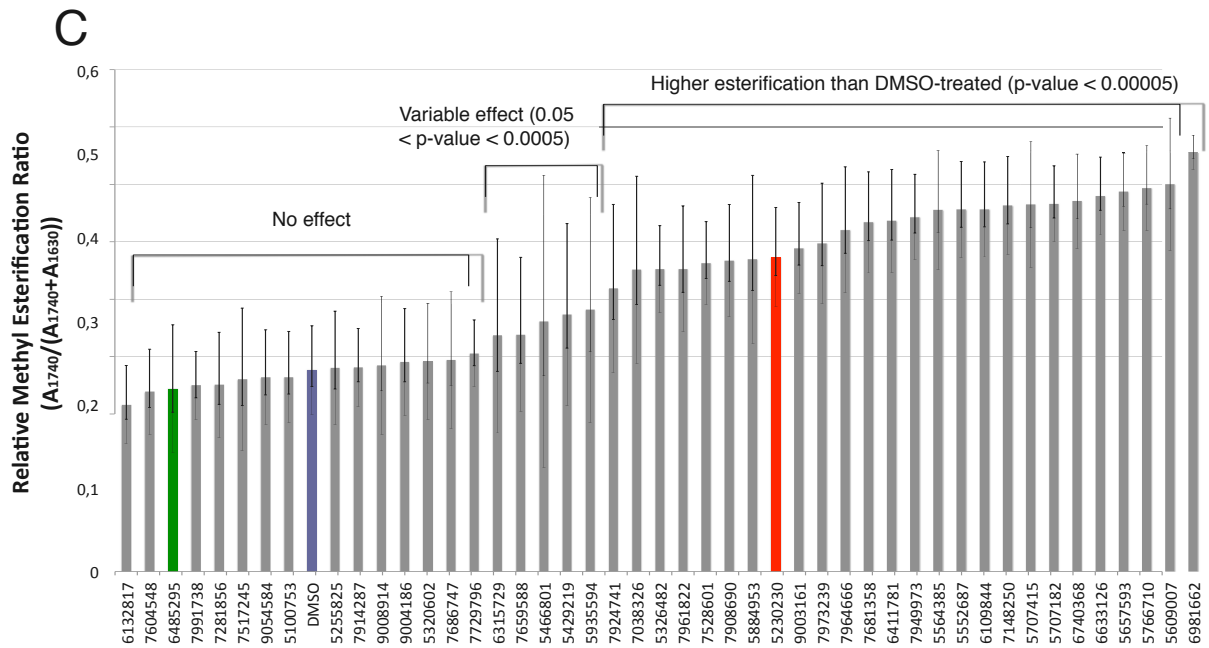
(Manrique and Lajolo, 2002) we evaluated the relative degree of methyl esterification of the HG in the *qua2-1* mutant treated with the suppressor compounds or with DMSO for control. This measure is based on the value of the ratio between the peak intensity at the 1630  $\text{cm}^{-1}$  band, assigned to the stretching frequency for the carbonyl group of galacturonic acid, and the peak intensity at the 1745  $\text{cm}^{-1}$  band, assigned to the methyl ester groups ( $\text{ME ratio} = \text{Abs}_{1740\text{cm}^{-1}} / (\text{Abs}_{1470\text{cm}^{-1}} + \text{Abs}_{1630\text{cm}^{-1}})$ ). This ratio is in theory linearly proportional to the actual degree of methyl esterification of the HG (Manrique et al., 2002). T-test on these ratio comparing the compound-treated samples with the DMSO-treated samples were used to determine whether some of the chemical significantly induced an increase in the methyl



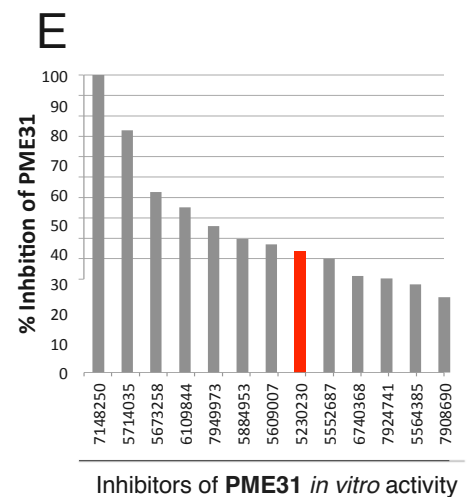
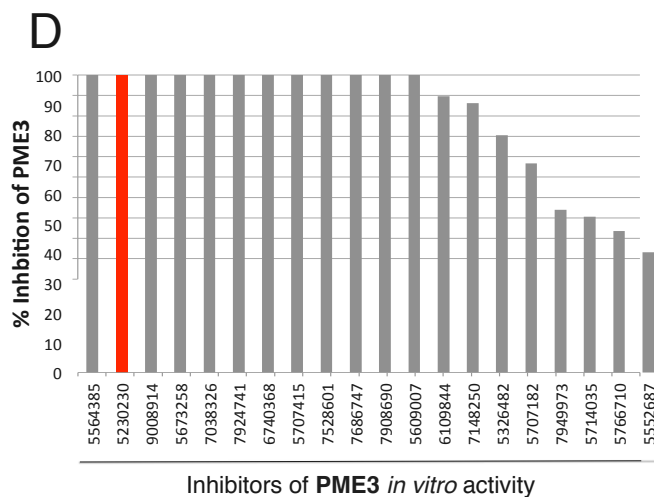
FTIR spectra of  
**5230230** vs **DMSO** treated *qua2-1* hypocotyls

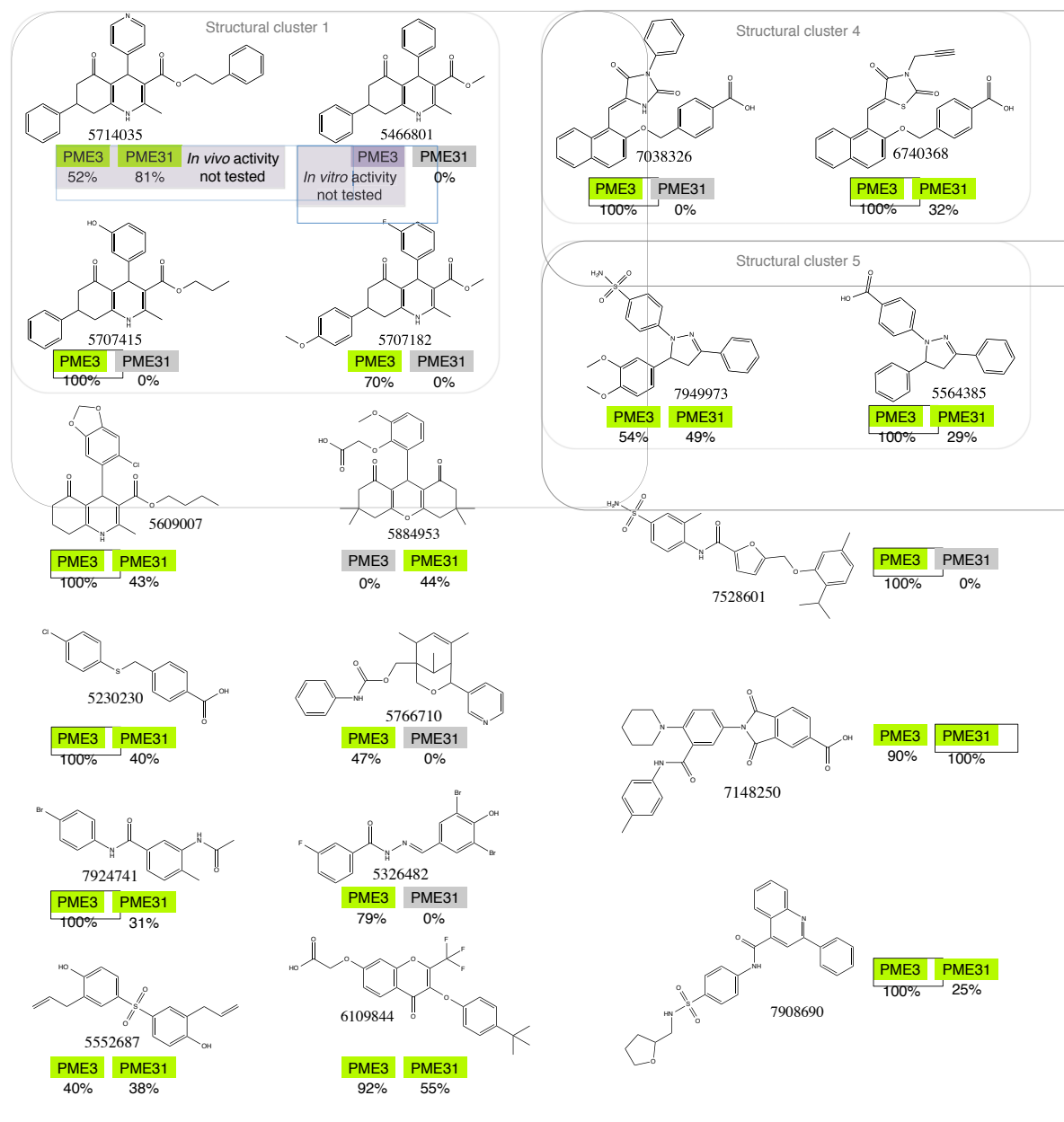


FTIR spectra of  
**6485295** vs **DMSO** treated *qua2-1* hypocotyls



Effect of the chemical treatment on the degree of methyl esterification of HG in *qua2-1* hypocotyls

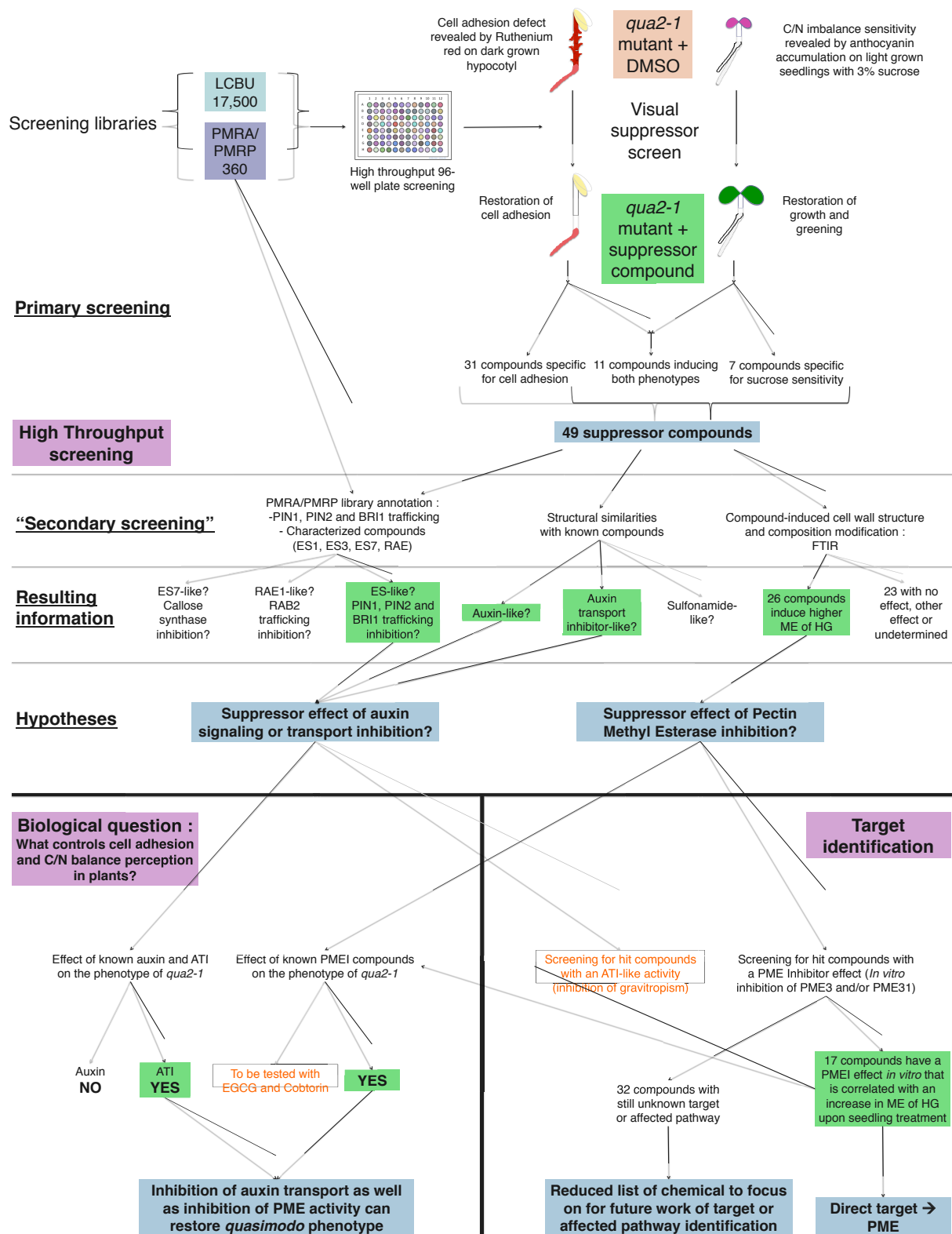




### *In vivo* and *in vitro* PME inhibitor hit compounds

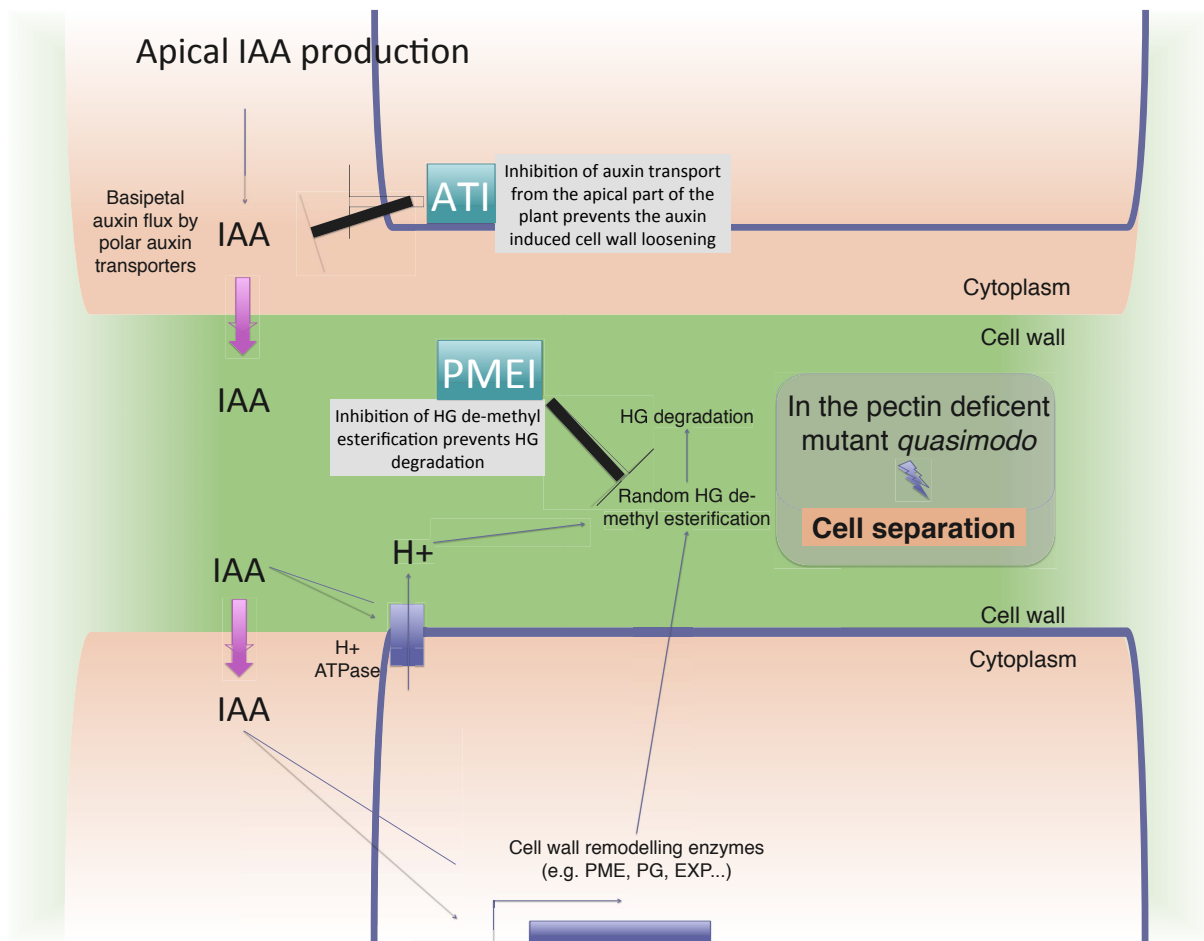
**Figure 5: List of PME inhibitor hit compounds**

All of these hit compounds were found as suppressors of *qua2* and are both *in vitro* inhibitors of PME3 and/or PME31, and induce *in vivo* an increase of the degree of methyl esterification of HG compared to the DMSO-treated *qua2*. Except for 5714035 for which the *in vivo* activity was not tested and 5466801 for which *in vitro* inhibition of PME3 was not tested. The percentage below the PME names indicates the percentage of inhibition *in vitro* in our assay conditions. Green outline of the PME names indicates that the molecule is inhibitor of the PME, black edge indicate complete inhibition, while gray outline indicates no inhibitory effect.



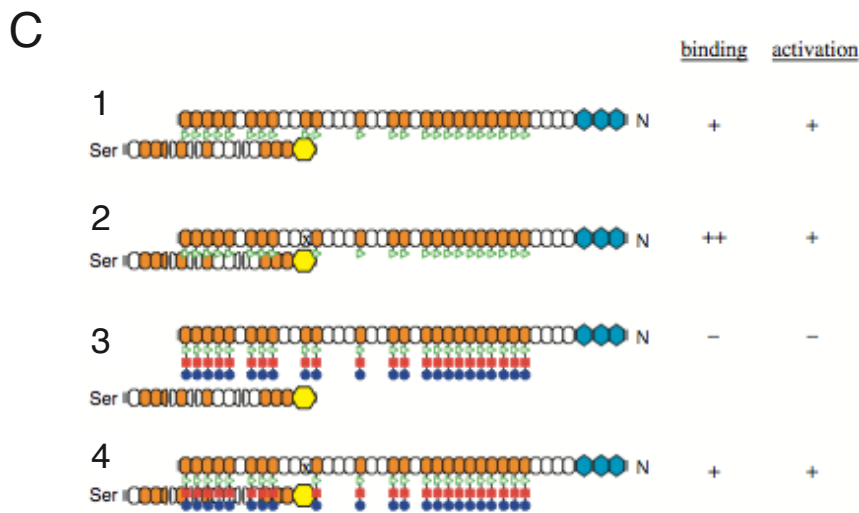
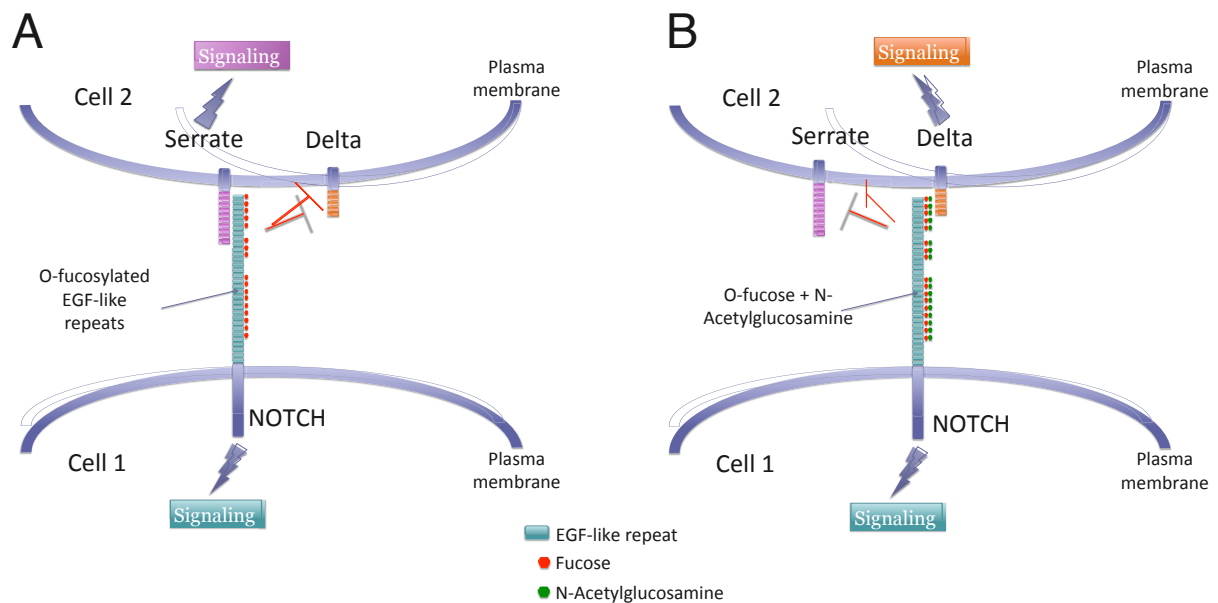
**Figure 6: Workflow of the high throughput chemical genomics screening and efficient identification of the implication of auxin transport and pectin methyl esterification in cell adhesion.**

This scheme summarizes the work reported in this article. The text written in orange reports to work to be done.



**Figure 7: Model of auxin and PME mediated cell separation in the pectin deficient mutants *quasimodo***

Schematic representation of our hypothesis concerning the phenomenon leading to the loss of cell adhesion in *quasimodo*. Auxin is synthesised at the apex of the plant and is transported to the rest of the plant thanks to the basipetal transport of auxin mediated by polar auxin transporters. When entering a cell, auxin can trigger the expression of cell wall remodeling (CWR) enzymes. In addition it can activate the efflux of proton into the apoplast. In acidic conditions, PMEs act in a more random manner and produce HG substrate suitable for degradation by PGs. In wild type plant, that process is known to lead to cell wall loosening and growth. However in the context of the pectin deficient mutant *quasimodo*, it may lead to cell separation. Thus inhibiting or limiting the transport of auxin may prevent the induction of CWR enzymes and further degradation of the cell wall leading to cell separation. Similarly, inhibiting the de-methyl esterification of HG should prevent its degradation by PGs leading to cell separation. These events may thus represent an upstream and a downstream step in the process leading to the loss of cell adhesion in *quasimodo*.



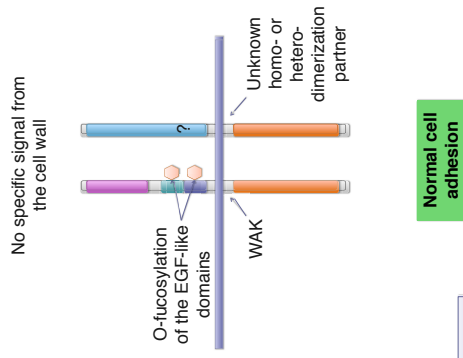
From Lei et al. (2003). *An O-fucose site in the ligand binding domain inhibits Notch Activation.*

### Figure 1: O-fucosylation in the notch signaling pathway

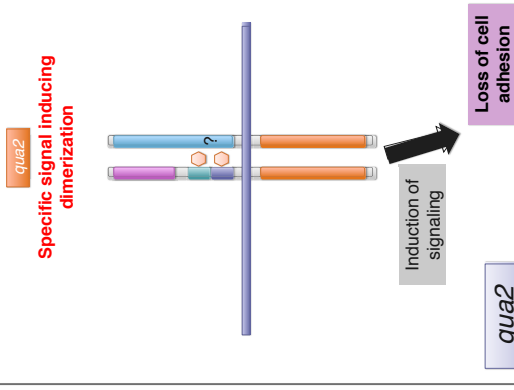
(A-B) Schematic representation of the Notch signalling pathway that exist in animals, and the role of the O-fucosylation of the EGF-like repeats in this pathway. Notch can either interact with a ligand called Delta or another called Serrate. When the protein Notch is only O-fucosylated on it's EGF-like repeats (A), it can only interact with Serrate, and not with Delta. This interaction triggers a specific signalling in cell 2. When the EGF-like repeats have an additional N-Acetylglucosamine on top of the fucoses (B), then Notch can only interact with Delta, and not with Serrate anymore. A different signal is triggered in cell 2.

(C) is a figure from Lei et al. (2003). It shows the influence of O-fucosylation of EGF12 on Notch- Serrate interactions. (1) In the absence of N-Acetylglucosamine, Serrate binds to and activates Notch. (2) When the EGF12 cannot be O-fucosylated, the Notch-Serrate interaction is enhanced. (3) When N-acetylglucosamine is added to O-fucose, Serrate cannot bind or activate Notch (The O-fucose glycan is shown extended to the trisaccharide which is the case in certain species (Chen et al., 2001)). (4) When the O-fucose site in EGF12 is mutant, Serrate can still bind despite the presence of elongated O-fucose glycans at other sites, and consequently can still activate Notch. Green triangles, fucose; red squares, N-acetylglucosamine; blue circles, galactose.

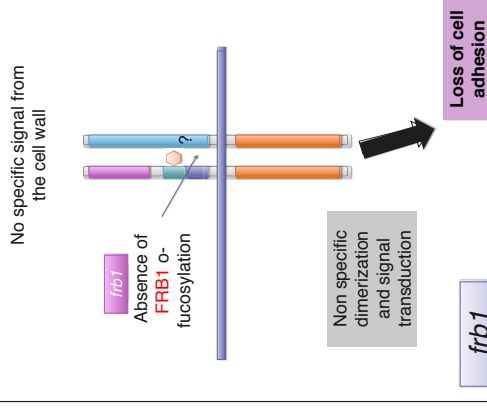
A



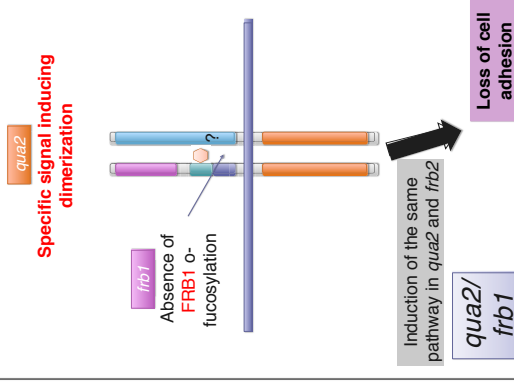
B



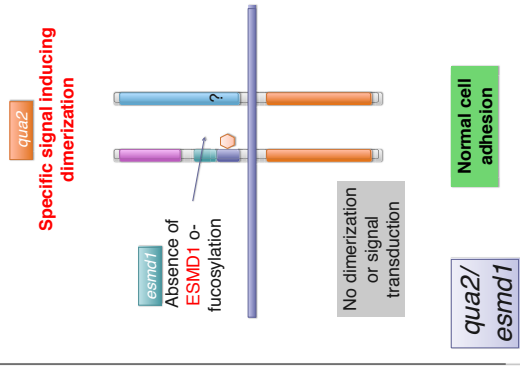
C



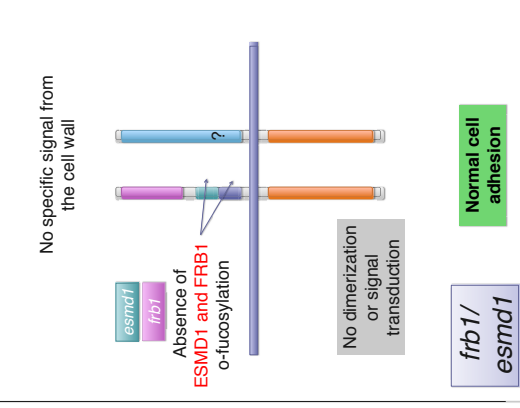
D



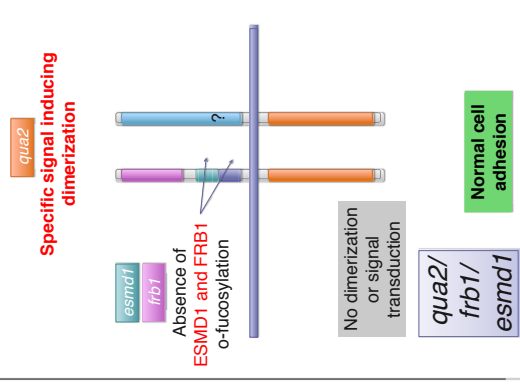
E



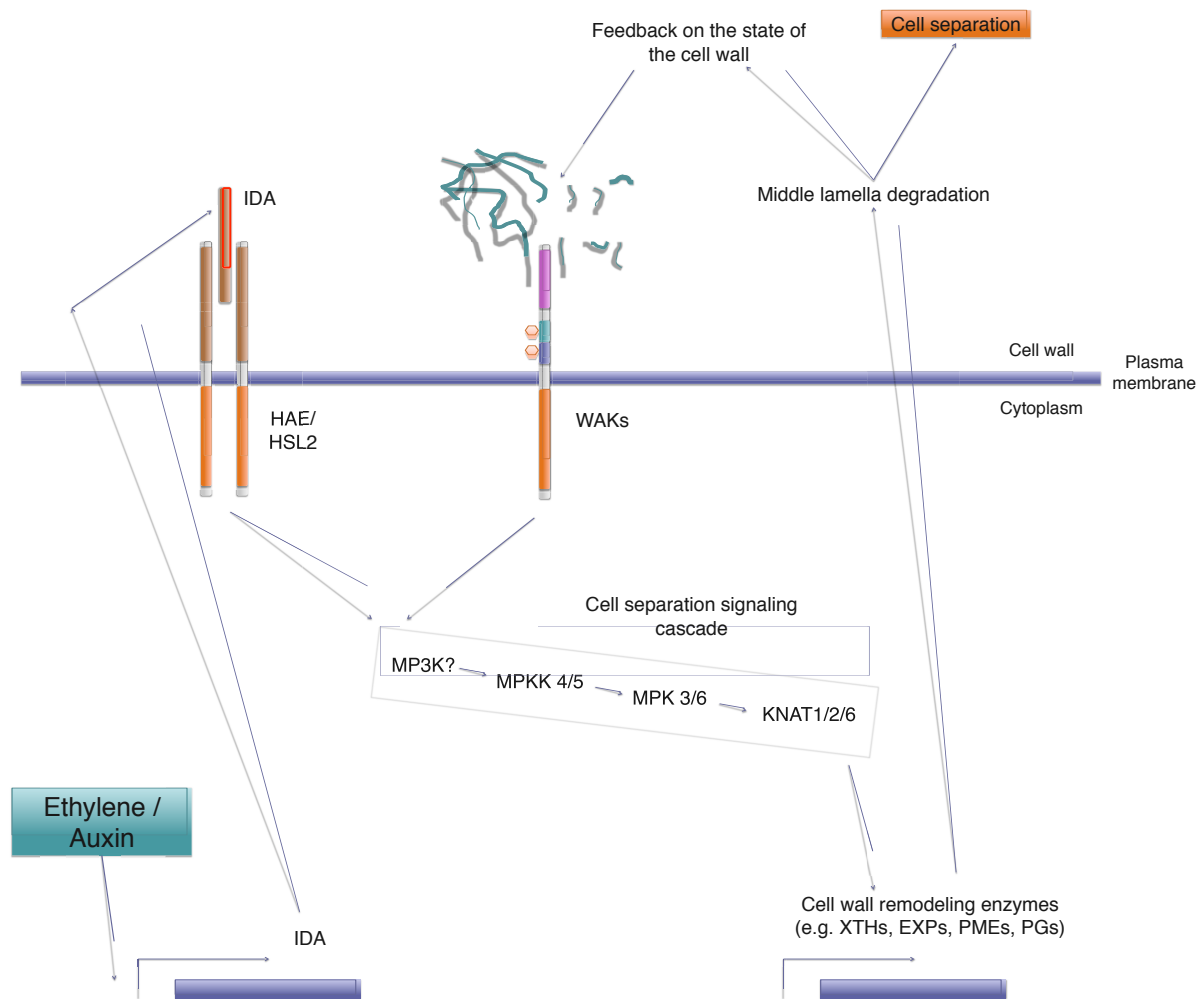
F



G



**Model of the O-fucosylation of the WAKs and the impact on WAK signaling**

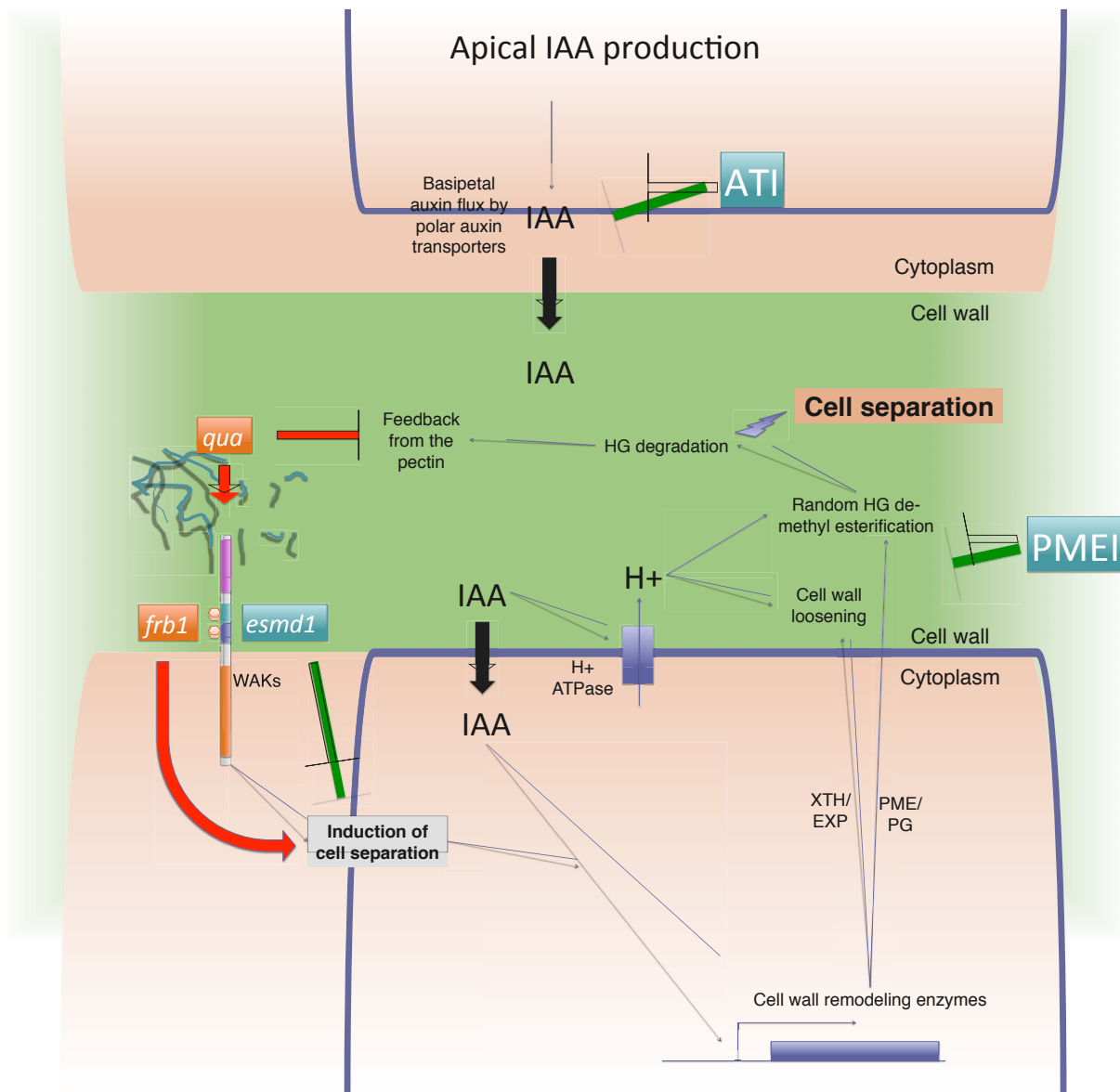


**Figure 3: Plant cell separation pathways**

Schematic representation of the cell separation pathway that is described for the developmentally controlled cell separation events (abscission, dehiscence,...) and the potential interaction with the cell separation that is triggered in *quasimodo*. An interplay between auxin and ethylene triggers the expression of the small peptide IDA. This peptide is then perceived by the pair of LRR-RLK receptor HAE/HSL2 and triggers a MAP kinase cascade leading to the repression and derepression of KNAT transcription factors. Finally the transcription factors induce the expression of cell wall remodeling enzymes such as XTHs, EXPs, PME and PGs that mediate the degradation of the middle lamella leading to cell separation. In parallel the perception of the cell wall pectins by the WAKs could have the potential to induce this same MAP kinase cascade leading to cell separation.

Interestingly *tsd2*, an allele of *QUA2* (Krupkova et al., 2007) was isolated in a genetic screen for its ability to develop a callus like tissue instead of a normal shoot apical meristem (SAM), a phenotype that is also observed in the other alleles of *qua2* as well as *qua1* and *friable1*. This phenotype and the disruption of *QUA2* were correlated with the overexpression of the class I knotted1-like homeobox transcription factors KNAT1 and KNAT2 (Frank et al., 2002; Krupková et al., 2007). Interestingly, among other function, KNAT1 and KNAT2, along with KNAT6 were shown to be implicated in the signaling pathway leading to cell separation during abscission (Shi et al., 2011). In addition the cell separation pathway that is described for abscission implicates MPK3 and 6 (Cho et al., 2008). These MAP kinases were also

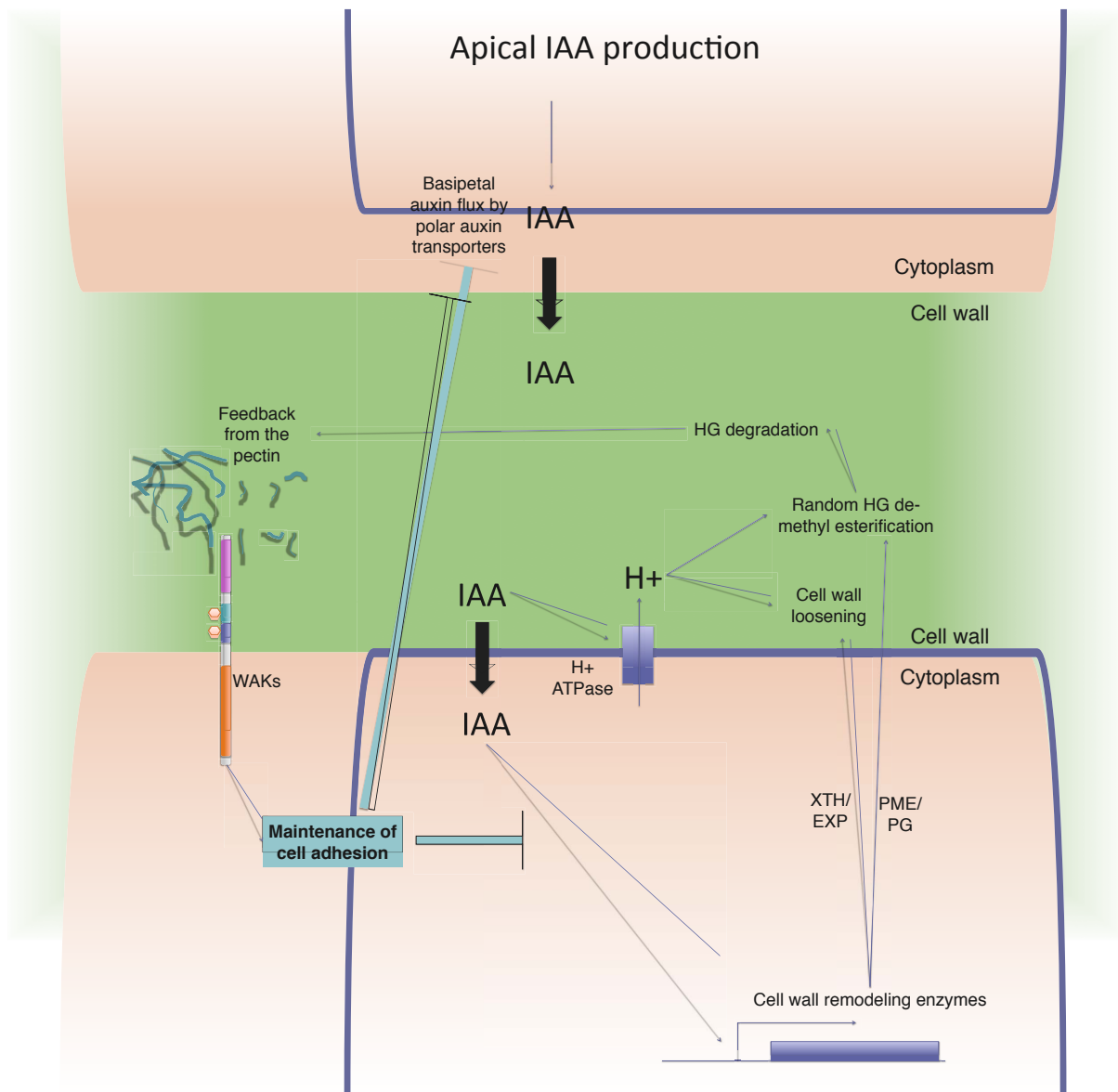




**Figure 4: Induction of a cell separation pathway in the cell adhesion defective mutants**

Schematic representation of the cell separation pathway that seems to be induced in *quasimodo* and *frb1*.

In *quasimodo* the specific state of the pectins is perceived by the WAKs. This signal leads to the induction of a cell separation pathway that acts by promoting the cell wall loosening action of auxin. Auxin is transported throughout the plant and can triggers cell wall loosening by the concerted action of the expression of cell wall remodeling enzymes (XTHs, EXPS, PMEs, PGs) and the acidification of the cell wall (Rayle and Cleland 1992; Senechal et al., 2014). In these acidic condition the action the XTHs and EXPs can ease the accessibility of the PME and PG to the middle lamella for its dissolution. Due to the particular state of the pectins in the cell wall of *quasimodo*, the massive degradation of the cell wall is either not perceived or induces even more degradation, finally leading to the loss of cell adhesion. The mutation in *FRB1* affects the WAKs' downstream signalling in such a way that it promotes the action of auxin and induce the same cell separation pathway as *quasimodo* except that *frb1* is not deficient in HG. On the other hand, the mutation in *ESMD1* affects the WAKs' downstream signalling in such a way that it prevents the cell wall loosening action of auxin that otherwise leads to cell separation in *frb1* or *quasimodo*. The inhibition of auxin transport prevents the accumulation of auxin that is necessary for the induction of the pathway. And finally the inhibition of the PME activity prevents the formation of the HG substrate susceptible to the degradation by the PG and thus limits the cell wall degradation that would otherwise lead to cell separation.



**Figure 5: Maintenance and control of cell adhesion in plants**

Schematic representation of the regulatory feedback loop that allows the maintenance and control of the cell adhesion in plants.

Auxin is transported throughout the plant and can triggers cell wall loosening by the concerted action of the expression of cell wall remodeling enzymes (XTHs, EXPS, PMEs, PGs) and the acidification of the cell wall (Rayle and Cleland 1992; Senechal et al., 2014). In these acidic conditions the action of the XTHs and EXPs can ease the accessibility of the PME and PG to the middle lamella for its dissolution. The degradation of the cell wall is perceived by the WAKs which subsequently induce the synthesis of endogenous flavonoid auxin transport inhibitors, and inhibit the cell wall loosening action of auxin. A too massive degradation of the cell wall is thus avoided and the cell adhesion is maintained.

Although a lot remains to be understood on the precise role of some of these molecular players, based on these new information we have established a new model explaining the loss of cell adhesion in the *quasimodo* and *friable1* mutants, and from this model we have inferred the existence of the mechanisms that dynamically allow the maintenance of cell adhesion in