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Comment la nature des métabolites primaires exsudés par les plantes régule la dénitrification dans la rhizosphère ?

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**Comment la nature des métabolites
primaires exsudés par les plantes régule
la dénitrification dans la rhizosphère ?**

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Résumé

De nos jours, pour améliorer les rendements agricoles de plus en plus d'intrants azotés sont utilisés ce qui conduit à une élévation non négligeable de la concentration en protoxyde d'azote (N_2O) dans l'atmosphère. Le N_2O est un gaz à effet de serre dont l'impact est 300 fois supérieur à celui du dioxyde de carbone (CO_2) à quantité équivalente. Les émissions de N_2O proviennent principalement du processus de la dénitrification. En effet, la dénitrification est une respiration alternative uniquement réalisée par les microorganismes consistant en à réduire progressivement le nitrate (NO_3^-) en diazote (N_2) en absence d'oxygène. C'est également une source majeure de perte d'azote dans le sol affectant la productivité des exploitations agricoles en plus de l'émission importante de N_2O atmosphérique. De ce fait, comprendre comment la dénitrification est régulée dans la rhizosphère nous permettrait de limiter les émissions de N_2O ainsi que des perte d'azote dans les sols. La dénitrification nécessite l'absence d'oxygène, la présence d'une source d'azote comme le NO_3^- mais aussi la présence d'une source de carbone facilement assimilable (composés organiques). A ce jour, de nombreuses études se sont intéressées à la régulation de la dénitrification par l'oxygène et par le nitrate mais peu d'informations sont connues sur le lien avec le métabolisme du carbone.

Dans le cycle du Carbone, l'exsudation racinaire retrouvée dans la rhizosphère, représente jusqu'à 20% du carbone fixé par les plantes durant la photosynthèse. Il va représenter une source non négligeable d'énergie pour l'établissement et l'activité des communautés microbiennes du sol. En présence de différents substrats organiques, le mécanisme de la répression catabolique du carbone (CCR) permet aux bactéries de hiérarchiser l'assimilation de ces différents substrats carbonés de façon à optimiser leur croissance. De plus la CCR régule également d'autres fonctions physiologiques de la bactérie telles que la virulence, la formation de biofilm ainsi que la colonisation des racines. Dans ce contexte, l'objectif général de cette étude est d'explorer l'influence de la plante *via* l'exsudation racinaire sur la physiologie des microorganismes qui lui sont associées, et notamment le rôle de la CCR dans la régulation de la dénitrification. La modulation de cette activité microbienne aura en retour une conséquence sur la croissance et la santé de la plante.

Afin de répondre à l'objectif de cette thèse, nous avons dans un premier temps étudié le rôle de la CCR dans la régulation de la dénitrification. Les résultats obtenus en utilisant la souche modèle *Pseudomonas fluorescens* F113, bactérie dénitrifiante et colonisatrice des racines de plantes, ainsi que de la plante modèle *Arabidopsis thaliana* Col-0 nous ont permis de mettre en évidence le rôle répresseur de la CCR sur l'expression des gènes de la dénitrification chez F113. L'utilisation de lignées mutantes de Col-0 exprimant des profils d'exsudations différents ont également permis de montrer qu'en présence d'une exsudation enrichie en sucre, les gènes de la dénitrification étaient surexprimés. Durant la deuxième partie de cette étude, nous avons étudié l'influence des exsudats racinaires sur le recrutement du microbiote de la plante et le retour du microbiote sur la balance hormonale de la plante. Nos résultats démontrent une communication active entre la plante et son microbiote où l'exsudation racinaire va moduler l'influence du microbiote sur la physiologie de la plante.

Ainsi au terme de cette étude, nous démontrons l'importance du rôle des exsudats racinaires dans l'établissement des interactions plantes-bactéries et dans la régulation de la dénitrification. Dans la continuation, l'élargissement de ces études en présence de communauté complexe du sol nous permettra d'établir des stratégies de limitation de l'émission atmosphérique de N_2O et donc de la dénitrification dans les sols.

Abstract

Nowadays, in order to improve crop yields, more and more nitrogenous inputs are used, which leads to a significant increase in the concentration of nitrous oxide (N₂O) in the atmosphere. N₂O is a greenhouse gas whose impact is 300 times greater than that carbon dioxide (CO₂) in equivalent quantities. N₂O emissions come in majority from the process of denitrification. The denitrification is an alternative microbial respiration process consisting in the progressive reduction of nitrate (NO₃⁻) to nitrogen (N₂) in the absence of oxygen. It is also a major source of nitrogen loss in the soil affecting plant productivity in addition to the significant emission of atmospheric N₂O. Therefore, understanding how denitrification is regulated in the rhizosphere would allow us to limit N₂O emissions as well as nitrogen loss in soils. The denitrification is induced in absence of oxygen, and in presence of a nitrogen source such as NO₃⁻ and an easily assimilable carbon source of (organic compounds). To date, many studies have focused on the regulation of denitrification by oxygen and nitrate but little information is known about the link with carbon metabolism. Root exudation in the rhizosphere, representing up to 20% of the carbon fixed by plants during photosynthesis, represent a significant source of energy for the establishment and activity of soil microbial communities. In the presence of different organic substrates, the mechanism of catabolic carbon repression (CCR) allows the bacteria to prioritize the assimilation of these substrates in order to optimize their growth. In parallel, there is a repression of the catabolism of the non-preferred substrates. Moreover, CCR also regulates other physiological functions of the bacteria such as virulence, biofilm formation and root colonization. In this context, the general objective of this study is to explore the influence of the plant by their root exudation on the physiology of its associated microorganisms, and in particular the role of CCR in the regulation of denitrification and the consequence of this influence on the growth and health of the plant.

In order to meet the objective of this thesis, we first studied the role of CCR in the regulation of denitrification. The results obtained by using the model strain *Pseudomonas fluorescens* F113, a denitrifying bacterium that colonizes the roots of plants, and the model plant *A. thaliana* Col-0 allowed us to highlight the repressive role of the CCR on the expression of denitrification genes in F113. Furthermore, the use of Col-0 mutant lines expressing different exudation profiles also allowed us to show that in the presence of sugar-enriched

exudation, denitrification genes were overexpressed suggesting a better denitrification activity. In the second part of this study, we investigated the influence of root exudates on the recruitment of the plant microbiota and what the feedback of the microbiota will be on the hormonal balance of the plant. Our results demonstrate an active communication between the plant and its microbiota where root exudation will modulate the influence of the microbiota on the plant physiology.

In the end, this study demonstrates the importance of the role of root exudates in the establishment of plant-bacteria interactions and in the regulation of denitrification. As a next step, we plan to extend this study to complex rhizosphere context in order to truly understand the regulation of denitrification in soils in order to establish appropriate strategies to limit the atmospheric emission of N₂O.

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Cette thèse n'aurait jamais pu être menée à terme sans l'aide et le soutien de certaines personnes.

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Introduction Générale

Un récent rapport de l'ONU annonce que la population mondiale devrait atteindre le seuil des 8 milliards de personnes d'ici la fin de l'année 2022 avec, notamment une estimation avoisinant les 10 milliards de personnes pour l'an 2050 (Buettner, 2020; Gaigbe-Togbe et al., 2022). La surpopulation humaine, croissante d'année en année, entraîne une augmentation en demande alimentaire. Une récente méta-analyse estime que pour répondre à ce besoin, il faudra une augmentation de plus de 50 % de la production alimentaire pour atteindre les demandes de 2050 (Alexandratos and Bruinsma, 2012; Dijk et al., 2021) De ce fait, l'agriculture intensive, caractérisée par l'usage important d'intrants azotés, s'est intensifiée afin d'augmenter les rendements agricoles. Ce phénomène est responsable de l'élévation non négligeable de la concentration de protoxyde d'azote (N_2O) dans l'atmosphère, un gaz à effet de serre dont l'impact est 300 fois supérieur à celui du dioxyde de carbone (CO_2) à quantité équivalente (Galloway et al., 2008). En conséquence les émissions de N_2O anthropogène sont passées de 5,6 TgN/an dans les années 80 à 7,3 TgN/an en 2016, correspondant en une augmentation de 32% de N_2O émis depuis les années 80 (Tian et al., 2020). Parmi les émissions de N_2O anthropogène, la moitié provient de l'agriculture où l'émission résulte principalement du processus de dénitrification (Tian et al., 2020). La dénitrification, un processus microbien qui consiste en la réduction progressive du nitrate (NO_3^-) en diazote (N_2), est également une source majeure de perte d'azote dans le sol (25-90%) (Radersma and Smit, 2011) qui affecte la productivité végétale (van Der Heijden et al., 2008) et augmente les émissions atmosphériques de N_2O (Hu et al., 2015). En effet, malheureusement, le taux de réduction du N_2O est inférieur à celui de la réduction des nitrates et des nitrites, ce qui entraîne un ratio N_2O/N_2 atmosphérique plus élevé (Philippot et al., 2011). **Ainsi, comprendre comment le processus microbien de dénitrification est régulé dans l'environnement permettrait la réduction des émissions de N_2O dans l'atmosphère et limiterait les pertes d'N à partir des sols.**

La dénitrification est **une fonction inductible**, qui nécessite une **faible concentration en oxygène, des composés organiques facilement assimilables** comme source d'énergie et la **présence de nitrate (NO_3^-)** (Henry et al., 2008; Salles et al., 2009). La dénitrification est fortement stimulée dans la rhizosphère, la zone du sol sous l'influence des racines de plantes (Hiltner, 1904), par la présence des exsudats racinaires (source de carbone), de nitrate et le faible taux d'oxygène dû à la respiration racinaire. De nombreuses études se sont intéressées

à la régulation de la dénitrification par la présence de nitrate et la concentration en oxygène. Mais à ce jour la régulation par le métabolisme du carbone (C) reste clairement non élucidée.

Dans la rhizosphère, la plante exsude jusqu'à 20% du C fixé durant la photosynthèse (Bais et al., 2006; Haichar et al., 2008). L'exsudation racinaire de molécules carbonées représente une source d'énergie potentielle pour le développement et l'activité des communautés microbiennes du sol, dont les bactéries dénitrifiantes (Haichar et al., 2012; Guyonnet et al., 2017a; Guyonnet et al., 2018a). Ainsi, si la quantité du C disponible apparaît comme un élément important dans la régulation de la dénitrification (Philippot et al., 2008), la nature de la source de C est également déterminante. De fait, nous proposons de **comprendre l'effet régulateur de la nature de la source en carbone et notamment la nature des métabolites primaires exsudés par la plante dans la régulation de la dénitrification.**

Il est connu que les bactéries sont dotées d'un **métabolisme polyvalent** qui leur permet d'utiliser de nombreux composés différents comme source de carbone et d'énergie. Cela facilite la colonisation de niches écologiques différentes et leur adaptation à des conditions environnementales changeantes. Les bactéries peuvent soit co-métaboliser différentes sources de carbone, soit **assimiler de manière préférentielle une classe spécifique de composés** qui assurent leur croissance de la façon la plus efficace. Simultanément à l'assimilation de composés préférés, les bactéries déclenchent l'inhibition de nombreuses fonctions dont celles impliquées dans le catabolisme des composés non préférés. Ce processus est nommé la répression catabolique du carbone (**Carbon Catabolite Repression (CCR)**). Jusqu'à présent, deux types de CCR ont été décrits dans la littérature : la CCR classique pour une préférence pour des sucres, comme décrit chez *Escherichia coli* (Monod, 1949; Görke and Stülke, 2008a; Rojo, 2010), et la CCR dite inverse (revCCR) pour une préférence pour des acides organiques, comme décrite chez *Pseudomonas* (Rojo, 2010). En plus de la stimulation des gènes impliqués dans l'assimilation du substrat préféré et l'inhibition de ceux qui régulent l'assimilation des substrats non-préférés, la CCR impacte la régulation d'autres gènes impliqués dans d'autres fonctions physiologiques de la bactérie. En effet, Il est estimé que la régulation de 5 à 10% des gènes bactériens sont soumis à la CCR (Görke and Stülke, 2008b) comme par exemple le quorum sensing (Nasser et al., 1998; Reverchon et al., 1998) ou la formation de biofilm (Chakravarthy et al., 2017a; Habib et al., 2017).

L'objectif général de cette thèse est **d'explorer l'influence de la plante *via* l'exsudation racinaire sur la physiologie des microorganismes qui lui sont associés, et notamment le rôle de la CCR dans la régulation de la dénitrification et la conséquence de cette influence sur la croissance et la santé de la plante.** Récemment, plusieurs études ont suggéré un lien entre le métabolisme du C et la dénitrification (Takaya et al., 2003; Otani et al., 2004; SALles et al., 2009). Cependant, le mécanisme de régulation reste encore inconnu. L'hypothèse proposée est que la **nature de la source de carbone utilisée par la bactérie est un facteur important contrôlant la régulation des gènes de la dénitrification *via* le mécanisme de répression catabolique.** Nous visons donc dans une première étude à : (i) identifier et valider la régulation de la dénitrification par la CCR *in vitro* en utilisant une souche modèle *Pseudomonas fluorescens* F113 ; (ii) mettre en évidence l'effet régulateur de la nature des exsudats racinaires sur le contrôle de la dénitrification *in planta* en utilisant le modèle *Arabidopsis thaliana* et des mutants affectés dans l'exsudation racinaire. Puis dans une dernière étude (iii), comprendre comment la plante *via* l'exsudation racinaire affecte la diversité et le fonctionnement du microbiote et d'étudier l'impact de ce dernier sur la croissance et la santé de la plante.

Ce manuscrit sera organisé en quatre chapitres. Le premier chapitre consiste à une synthèse de la littérature associée aux enjeux de cette thèse dont une partie est déjà publiée dans la revue *Plant Communication* (Franzino et al., 2022). Un second chapitre, sous un format d'article scientifique en cours de soumission, présentera comment la nature des métabolites primaires exsudés par les plantes régule la dénitrification *via* la CCR chez la bactérie *P. fluorescens* F113 et l'importance de la CCR dans l'interaction *P. fluorescens* F113 et *A. thaliana*. Un troisième chapitre évoquera les études sur l'effet des exsudats racinaires d'*A. thaliana* sur la diversité et le fonctionnement du microbiote, ainsi que leur impact sur la physiologie de la plante (en cours de finalisation au niveau des résultats). Enfin un dernier chapitre de discussion générale soulignera les résultats obtenus au cours de cette thèse et les perspectives de cette étude.

Chapitre 1 :

Synthèse Bibliographique :

Régulation des activités microbiennes par les Exsudats Racinaire ; cas de la dénitrification

Préambule à la synthèse bibliographique

Dans ce premier chapitre, une synthèse des connaissances autour du contexte de la thèse est présentée afin de contextualiser l'ensemble de l'étude et ses enjeux. Dans une première partie, nous parlerons du rôle de l'exsudation racinaire dans la modulation des interactions établies par la plante et son microbiome, puis une deuxième partie s'intéressera à l'effet du microbiote sur la physiologie de la plante. Enfin une dernière partie présentera le mécanisme de répression catabolique du carbone sous forme de revue déjà publiée dans Plant Communications cette année (Franzino et al., 2022).

A. Rôle des exsudats racinaires dans la régulation des interactions plante - microorganismes

I. Les exsudats racinaires

La photosynthèse, du grec phos-synthesis = lumière-combinaison, est un processus bioénergétique permettant à différents organismes, notamment les plantes, de synthétiser de la matière organique. Lors de la photosynthèse, il y a conversion du dioxyde de carbone (CO_2) présent dans l'atmosphère et de l'eau (H_2O) en composés organiques, tel que les sucres, et en oxygène (O_2). L'énergie des rayons du soleil est la source énergétique permettant de réaliser cette conversion. Ces composés organiques produits par anabolisme vont être ensuite redistribués aux différents organes de la plante (racines, tiges, feuilles, fleurs...) permettant ainsi leur développement et leur bon fonctionnement. De plus, tout au long de son développement, la plante va accumuler et sécréter au niveau de ses racines une partie importante de cette matière organique, c'est ce que l'on nomme la rhizodéposition (Shamoot et al., 1968). Il est estimé que la plante va investir et sécréter dans le sol jusqu'à 20% du C fixé durant sa photosynthèse, démontrant ainsi l'importance de la rhizodéposition dans l'interaction de la plante avec son environnement (Haichar et al., 2008). Parmi la matière

organique sécrétée par les racines, on retrouve ce que l'on appelle « les exsudats racinaires » qui vont représenter des molécules simples, facilement assimilables et de faibles poids moléculaires. Parmi les exsudats racinaires, deux classes de métabolites sont retrouvées : les métabolites primaires (acides aminés, acides organiques, sucres et protéines) et les métabolites secondaires (phénols, vitamines, hormones). Ces métabolites vont être les acteurs principaux de l'interaction de la plante avec son microbiome.

Les métabolites primaires et secondaires

Les métabolites primaires sont des composés résultant directement de la photosynthèse tels que des sucres (glucose, galactose, fructose, xylose...), des acides aminés (asparagine, alanine, glutamine, leucine...) et d'acides organiques (acide aspartique, acide succinique, acide citrique...). Les métabolites secondaires sont des composés plus complexes nécessitant un acheminement métabolique plus approfondi que les précédents. On retrouve entre autres des composés phénoliques, des stérols, des hormones et des vitamines. Ces métabolites primaires et secondaires sont sécrétés dans la rhizosphère par deux systèmes de sécrétion : actif ou passif. La sécrétion passive peut s'effectuer de différentes manières suivant la masse molaire de la molécule et de la perméabilité de la membrane de la cellule, mais aussi en fonction de facteurs abiotiques tel que le pH. Des mécanismes de diffusion, de transport par canaux ioniques et aussi de transport vésiculaire sont impliqués (Baetz and Martinoia, 2014). La sécrétion active, quant à elle, s'effectue par le biais de complexes protéiques transmembranaires que l'on nomme ABC transporteur (ATP Binding Cassette) qui requiert donc l'utilisation d'énergie sous forme d'ATP pour son fonctionnement afin de véhiculer les molécules. Cette classe de protéine est l'une des plus répandue chez les organismes vivants. De plus, ils sont trois à quatre fois plus nombreux chez les plantes terrestres ; on en dénombre pas moins de 130 ABC transporteurs chez la plante *Arabidopsis thaliana* (Kang et al., 2011; Hwang et al., 2016). Parmi les ABC transporteurs on retrouve différentes classes de protéines : Multidrug Resistance-related Protein (MRP, transport vacuolaire de composé xenobiotic), Pleiotropic Drug Resistance protein (PDR, transporteurs pouvant intervenir en cas de stress biotiques ou abiotiques) et les multidrug resistance P-glycoproteins (PGP, transporteurs intervenant dans le transport d'hormones de type auxines)(Kang et al., 2011; Hwang et al., 2016). Des études de mutants de plantes dans des gènes codants pour les ABC transporteurs

ont montré leur rôle dans l'exsudation des métabolites primaires et secondaires (Badri et al., 2008; Cox et al., 2019). Un second type de transporteur actif est les transporteurs Multi-AnTIMicrobial Extrusion protein (MATE, transporteurs de métabolites secondaires et participent également dans la détoxification de certains composés) qui fonctionnent comme antiporteurs réalisant le transport des molécules en utilisant un gradient électrochimique de H^+ ou Na^+ à partir d'une pompe à proton. Les MATE sont par exemple les transporteurs exclusifs des proanthocyanines (Zhao, 2015).

II. Facteurs influençant l'exsudation racinaire

C'est donc par ces différents systèmes de sécrétions que les exsudats racinaires vont être rendus disponibles dans la rhizosphère. Cependant, de nombreux facteurs à la fois biotiques et abiotiques vont influencer la composition et la quantité des exsudats racinaires. Parmi les différents facteurs dits biotiques qui influencent l'exsudation racinaire, on trouve les interactions positives ou négatives possibles entre la plante et les organismes vivant dans son environnement, mais aussi la physiologie de la plante comme les différents stades de son développement et également sa stratégie de gestion de ressources. Les facteurs abiotiques sont l'ensemble des facteurs physico-chimiques qui sous forme de stress vont influencer l'écosystème et la réponse des organismes qui y sont présents tels que : la texture du sol, le pH, la salinité, la température, l'humidité, la disponibilité en métaux ou encore en matière organique.

1. Les facteurs Biotiques

Développement de la plante

Tout au long de son développement, la plante va synthétiser et donc exsuder différents composés afin de répondre à ses besoins suivant sa croissance.

Le cycle de vie de la plante, c'est-à-dire ses différents stades de développement, va influencer son exsudation (Jones et al., 2004). De plus, tout au long de sa croissance, la plante va exsuder dans le sol de moins en moins de C qu'elle aura fixé durant la photosynthèse (Meharg and Killham, 1990). En effet, lors des premières étapes de sa croissance, la plante *via* son exsudation va modifier son environnement et le rendre plus favorable pour son développement (pH du sol favorable, augmentation de la disponibilité en minéraux...). Une fois bien installée, la plante va donc moins investir dans l'exsudation racinaire. En effet, il a été montré que le *Lolium perenne*, lors de son premier mois de développement, sécrète 64% du C assimilé contre seulement 14% après 5 mois de croissance une fois bien établi (Meharg and Killham, 1990). Aulakh et ses collègues (2001) ont montré également que le riz exsudait de plus en plus d'acides organiques jusqu'à la floraison. La baisse d'exsudation est corrélée avec le stade de maturité de la plante où elle va produire son grain. La plante va donc moins investir dans son exsudation favorisant l'utilisation du C fixé pour son rendement. L'exsudation racinaire est également corrélée avec la mise en place du microbiote racinaire. Les différents exsudats vont permettre le recrutement et la stimulation du microbiome racinaire (Chaparro et al., 2013). Ce microbiote va en retour participer à la nutrition et la défense de la plante dont nous reparlerons dans les paragraphes suivants. Chez *Arabidopsis thaliana*, le stage de développement est un facteur déterminant dans la proportion en métabolite primaire dans son exsudation. Ainsi on passe d'une forte quantité de sucre et d'une faible quantité en acides aminés, pendant sa première semaine de croissance, à des taux inversés dès la troisième semaine de développement (Chaparro et al., 2013) (Figure 1). Ce changement de profil d'exsudation a été démontré comme étant corrélé à un changement au niveau de la racine dans l'expression des gènes impliqués dans les voies de biosynthèse et de transport des exsudats racinaires, notamment les transporteurs ABC (Chaparro et al., 2013). Chez *A. thaliana* il a été montré qu'en mutant les gènes *AtPDR2*, *AtPDR6*, *AtMRP2* codant pour des transporteurs ABC, les profils d'exsudation en étaient modifiés (Badri et al., 2008). Le mutant *AtMRP2* exsude plus d'acides aminés comparé à la lignée sauvage, tandis que le mutant *AtPDR6* une plus faible exsudation d'acides organiques. Egalement chez la tomate, les mutations des gènes *ABC-C6* et *ABC-G33* (codant pour des ABC transporteurs) modifient l'exsudation de composés semi volatiles (Cox et al., 2019). Ainsi l'exsudation racinaire suit un modèle de développement dont une part est programmée génétiquement.

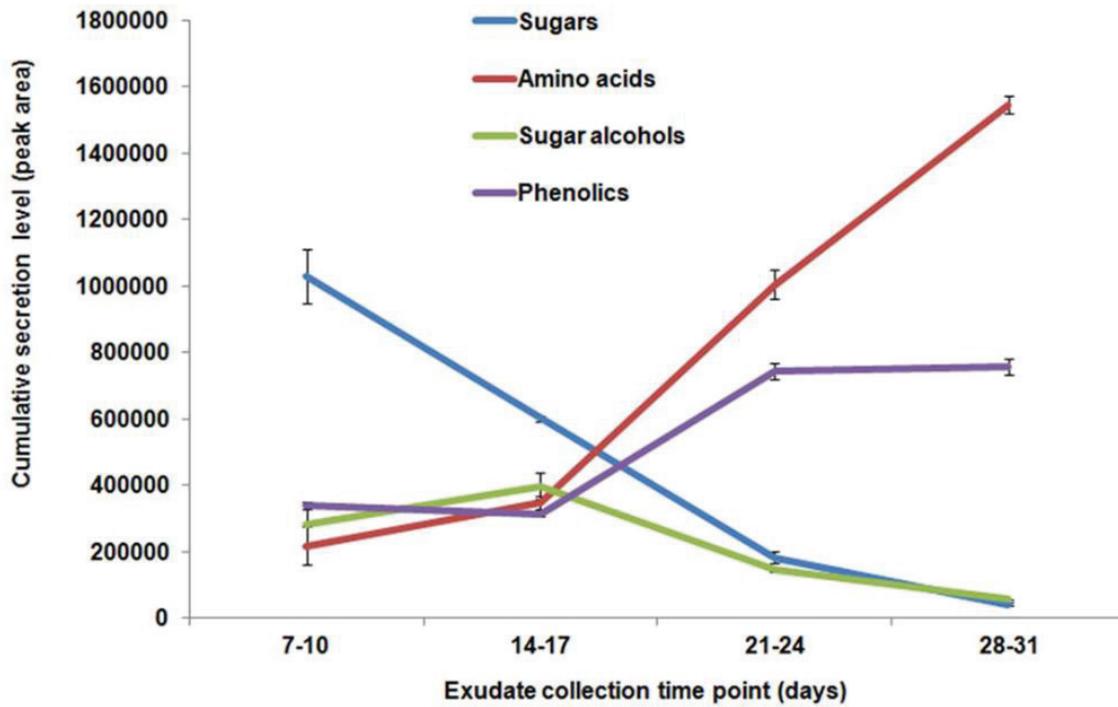


Figure 1 : Composition des exsudats racinaires d'*Arabidopsis thaliana* au cours de son développement. Les composés identifiés ont été regroupés dans différentes classes chimiques. Les niveaux de sécrétion ont été calculés sur la base de la surface cumulée des pics après normalisation. Le temps de collection est exprimé en jour après semis. D'après Chaparro *et al.* (2013).



Figure 2 : Représentation schématique des deux types d'espèces « conservatrices » et « exploitatrices » et leurs caractéristiques. Les plantes dites « conservatrices » possèdent une forte densité tissulaire, mais possèdent une faible surface foliaire et racinaire, ainsi qu'une faible exsudation. Contrairement aux plantes dites « exploitatrices » qui possèdent une forte surface foliaire et racinaire, ainsi qu'une exsudation élevée mais possèdent une faible densité tissulaire.

Les caractéristiques physiologiques de la plante vont également influencer l'exsudation racinaire. C'est ce que l'on appelle **les traits fonctionnels** définis par « toutes caractéristiques morphologiques, physiologiques ou phénologiques qui ont un effet indirect sur l'avantage adaptative (fitness) de l'individu de par leurs effets sur la croissance, la reproduction et la survie » (Violle et al., 2007). De ce fait, les caractéristiques physiques telles que l'architecture racinaire et la surface foliaire vont permettre d'établir un schéma de différenciation entre les différentes espèces. On distingue ainsi différents types de stratégie de gestion des ressources au sein du règne végétal (Michalet et al., 2011). D'un côté, il y a les plantes dites « exploitatives », telle que *Dactylis glomerata*, qui possèdent une surface foliaire élevée, de longues racines, un taux élevé d'exsudation racinaire et un faible poids (Guyonnet et al., 2018a) traduisant un plus grand investissement dans l'exsudation racinaire et une forte

interaction de la plante avec son microbiome (Guyonnet et al., 2018a) (Figure 2). De l'autre côté, il y a les plantes dites « conservatrices », telle que *Festuca paniculata*, qui possèdent une surface foliaire et un système racinaire de faible taille, un faible taux d'exsudation racinaire, mais avec une teneur en matière sèche plus élevée soulignant leur capacité de stockage des nutriments (Figure 2). Les études réalisées par Guyonnet et ses collègues (2017; 2018) ont mis en avant les différences dans la nature et la quantité d'exsudats racinaires entre les différentes stratégies. Il a été montré que les plantes exploitatrices exsudent plus d'acide fumarique et de glutamine afin d'augmenter son interaction avec son microbiome en lui fournissant du C et de l'N (Grassein et al., 2015; Guyonnet et al., 2017a). L'exsudation racinaire est donc un trait fonctionnel de la plante corrélé à la stratégie de gestion de ressource mais aussi au développement (Guyonnet et al., 2018a).

Modulation bactérienne de l'exsudation racinaire

En dehors de facteurs intrinsèques à la plante pour moduler son exsudation, la plante va également réguler son exsudation en fonction de son interaction avec son microbiome comme vu plus haut. Par exemple, *A. thaliana* présente *in vitro* un profil d'exsudation racinaire différent lorsqu'elle est en présence de *Pseudomonas putida* KT2440 comparé à une condition sans inoculum (Matilla et al., 2010). En présence de la bactérie, la plante exsude plus de composés organiques dont un certain composé de masse de 163 Da, présentant un noyau aromatique. Ce composé interviendrait dans la réponse de résistance systémique induite (ISR), mécanisme mis en place permettant d'améliorer la capacité de défense de la plante au contact de la bactérie non pathogène. En effet, en présence d'un mutant de *P. putida* KT2440 incapable de déclencher la réponse ISR, ce composé de 163 Da est 30 fois moins retrouvé dans les exsudats de la plante inoculé (Matilla et al., 2010).

Les bactéries sont capables également de moduler directement l'exsudation racinaire. En effet, *via* la sécrétion de diverses molécules telles que la phenazine et le DAPG (2,4-diacetylphloroglucinol) les bactéries dites PGPR (Plant Growth Promoting Rhizobacteria) comme certaines *Pseudomonas* sont capables de moduler l'exsudation racinaire. Outre leur action contre divers phytopathogènes, ces produits microbiens vont également modifier le flux entrant et sortant d'acides aminés chez *Medicago truncatula*, le blé, mais aussi chez le

maïs (Phillips et al., 2004). Par exemple, le DAPG va bloquer l'importation en Alanine de certains transporteurs non précisés durant l'étude.

2. Influence des facteurs abiotiques sur l'exsudation racinaire

En plus des facteurs biotiques, comme nous venons de voir, des facteurs abiotiques comme la texture du sol, le pH, la salinité, la température, l'humidité, la disponibilité en métaux ou encore en matière organique sont également responsables de la modification de l'exsudation racinaire. Nous allons maintenant aborder comment la plante réagit au niveau de son exsudation face à des stress physiques et chimiques souvent étudiés et en lien direct avec les problématiques environnementales d'aujourd'hui.

Stress physiques

La quantité d'eau apportée, notamment pendant l'arrosage, est un facteur important pour la vie de la plante et pour ses racines. Un trop faible apport entraîne un dessèchement tandis qu'un apport trop élevé va entraîner leur pourrissement. Dans les deux cas les racines sont affectées ce qui affecte également l'exsudation racinaire. Par exemple, *Agropyron cristatum* (l'agropyre à crête) va augmenter son exsudation de 45 % en composé organique tel que l'acide malonique et l'acide oxalique en cas d'inondation, tandis qu'en cas de sécheresse l'augmentation en composé organique atteint 71 % avec, notamment une augmentation en acides organiques comme le succinate, le fumarate ou encore le malate (Henry et al., 2007). Il est connu que les acides organiques entraînent une solubilisation de certains minéraux et sont également capables de détoxifier le sol de métaux lourds par chélation (Adeleke et al., 2017). En cas de stress dû à une teneur élevée en sel dans le milieu, le soja tout comme le haricot ont tendance à exsuder plus de flavonoïdes (Dardanelli et al., 2010; Dardanelli et al., 2012). Les flavonoïdes sont connus comme étant des composés chimioattractants, afin de permettre l'établissement d'interaction bénéfique avec des microorganismes qui auront comme rôle d'aider la plante à résister face au stress.

La température joue également un rôle dans l'exsudation étant donné son importance pour la croissance des organismes. Le concombre en cas d'élévation de la température va exsuder plus d'acide benzoïque, phtalique et palmitique (Pramanik et al., 2000). Le cycle jour/nuit, qui influence la photosynthèse et donc le CO₂ fixé puis transformé en C assimilable qui sera secrété dans la rhizosphère, va également jouer un rôle. L'enrichissement en acide organique de l'exsudation du concombre est d'autant plus important en cas de cycle « jour long » (cycle 14h de jour 10h de nuit) (Pramanik et al., 2000). Dans le cas inverse d'une période prolongée de nuit, Martin et ses collègues (2018) ont montré une altération dans l'exsudation racinaire de trois plantes vertes aquatiques, *Halophila ovalis*, *Halodule uninervis* et *Cymodocea serrulata*. Il en résulte une baisse d'exsudation de composé azoté et une augmentation dans l'exsudation de protéines organiques. De plus, cette altération dans l'exsudation a également révélé une réduction dans la diversité de leur microbiote associé (Martin et al., 2018).

Stress Chimique - limitation en ressource nutritive

La disponibilité des divers nutriments dans la rhizosphère va également influencer la composition des exsudats racinaires. En cas de faible disponibilité en Phosphore (P) dans son environnement, la betterave, par exemple, va augmenter son exsudation en acide organique (acide citra-malique ou encore le précurseur de l'aspirine l'acide salicylique), ce qui va avoir pour conséquence d'acidifier le sol (baisse du pH) et ainsi rendre plus soluble le phosphore et donc plus assimilable (Khorassani et al., 2011). Carvalhais et ses collaborateurs (2011) ont publié une étude sur les différents profils d'exsudation du maïs dans différentes conditions de croissance en présence de carences en certains nutriments. En cas de faible disponibilité en phosphore, il a été montré une exsudation plus importante de sucre, cas contraire en condition limitante en potassium où celle-ci y est justement limitée en glycérol, ribitol, fructose ou encore en maltose (Carvalhais et al., 2011). Une faible présence d'azote conduit à une baisse d'exsudation en acide aminé. Enfin en cas de limitation en Fer, une hausse de l'exsudation en acide organique (glutamate, citrate) et en sucre (glucose, ribitol) a été constatée (Carvalhais et al., 2011). En cas de limitation en Fer, les plantes de la famille des graminoides sont capables de sécréter des phytosidérophores, pouvant chélater le fer insoluble afin de le rendre plus disponible et donc plus assimilable pour la plante (Römheld

and Marschner, 1986; Chen et al., 2017). Autre exemple pour l'acquisition du fer, des riboflavines sont également sécrétées par la betterave pour faciliter la chélation du fer, mais aussi faciliter sa solubilisation (Siso-Terraza et al., 2015). Du côté d'*A. thaliana*, il a été démontré qu'en présence d'un sol très alcalin (pH élevé), l'arabette sécrète des composés phénoliques de la classe des coumarines tels que la scopolamine afin d'acquérir le fer plus facilement (Sisó-Terraza et al., 2016).

Il est donc clair que les plantes sont capables d'exsuder une multitude de composés qui vont changer les propriétés physico-chimiques du sol et augmenter la disponibilité de certains nutriments faiblement concentrés. On peut donc conclure que la modulation de la balance de l'exsudation des métabolites primaires et secondaires est une stratégie que possède les plantes pour s'adapter aux différents stress abiotiques.

L'exsudation racinaire peut être également manipuler par les microorganismes (comme énoncé plus haut) pour exsuder de façon spécifique des composés dans la rhizosphère. Il est bien établi que la plante via l'exsudation racinaire interagit avec son microbiome et que ce dernier va avoir un impact positif ou négatif sur sa croissance et sa santé.

III. Les interactions plante-microorganisme médiées par l'exsudation racinaire

De par leur nature riche en C et facilement métabolisable, les exsudats racinaires représentent une source de nutriments pour les organismes vivants aux alentours de la plante. La rhizosphère est considérée comme un « hotspot » où la diversité et l'activité des communautés microbiennes sont variées et intenses conduisant à l'établissement d'interactions complexes où l'exsudation racinaires en plus de son rôle trophique, sert également de molécules signales pour initier plusieurs types d'interactions. Dans cette section, nous nous focaliserons sur les interactions médiées par les exsudats racinaires entre la plante et son microbiote (bactéries de la rhizosphère). Plus particulièrement, nous allons

traiter différents exemples où la plante va pouvoir moduler le microbiote mais aussi certaines fonctions de ce dernier à travers ses exsudats.

1. Interactions négatives

La plante est capable de libérer certains composés qui vont avoir un impact négatif sur certaines communautés. Par exemple elle va inhiber les fonctions de la dénitrification et de la nitrification, mais aussi perturber la communication cellulaire de certains pathogènes afin de limiter l'agression subie.

Pour sa croissance, la plante va assimiler l'azote sous forme d'ammonium (NH_4^+) ou de nitrate (NO_3^-). Cependant, certaines rhizobactéries du sol utilisent également le NH_4^+ et le NO_3^- pour réaliser la nitrification et la dénitrification respectivement. Il va donc y avoir compétition entre la plante et son microbiote pour la même ressource. Ainsi la plante a également dû développer une stratégie pour limiter cette compétition et donc inhiber la dénitrification, c'est ce qu'on appelle l'inhibition biologique de la dénitrification (BDI : Biological Denitrification Inhibition). Le BDI a été mis en évidence chez la plante *Fallopia japonica* (renouée du Japon), comme son nom l'indique, originaire d'Asie orientale et invasive en occident. Le BDI est médié par un métabolite secondaire exsudé à savoir les procyanidines. Ces dernières inhibent spécifiquement la réductase NarG catalysant la réduction du NO_3^- en NO_2^- (Bardon et al., 2014; Bardon et al., 2016)(Figure 3). L'utilisation des procyanidines peut donc être une piste à envisager en agronomie afin de limiter la compétition pour N entre la plante et son microbiote. Une étude en champs sur la laitue *Lactuca sativa* menées par Galland et ses collègues (2019), a révélé que l'utilisation des procyanidines pouvait non seulement réduire l'activité de dénitrification du sol, mais aussi augmenter les rendements. Dans le même principe, on retrouve l'inhibition biologique de la nitrification (BNI : Biological Nitrification Inhibition). Comme le BDI, le BNI consiste en l'inhibition de la nitrification qui consiste à la transformation du NH_4^+ en NO_3^- , pour limiter la compétition pour l'azote. De nombreuses études se sont intéressées au BNI exercé par le sorgho sur *Nitrosomas* sp. (Subbarao et al., 2007; Subbarao et al., 2013) Des exsudats, tels que le methyl 3-(3-hydroxyphenyl) propionate et le sorgoleone (p-benzoquinone), ont été identifiés comme responsable de cette action

(Subbarao et al., 2013). Le sorgoléone va bloquer l'activité enzymatique de l'ammonium monooxygénase et l'hydroxylamine oxydoréductase entraînant l'inhibition de la nitrification (Subbarao et al., 2013) (Figure 3). On retrouve le BNI également chez le riz (*Oryza sativa*) qui est réalisé *via* le 1,9-decanediol exsudé par la plante (Sun et al., 2016). Il est donc évident que la plante est capable d'inhiber certaines fonctions microbiennes afin de limiter la compétition entre elle et son microbiote pour une même ressource.

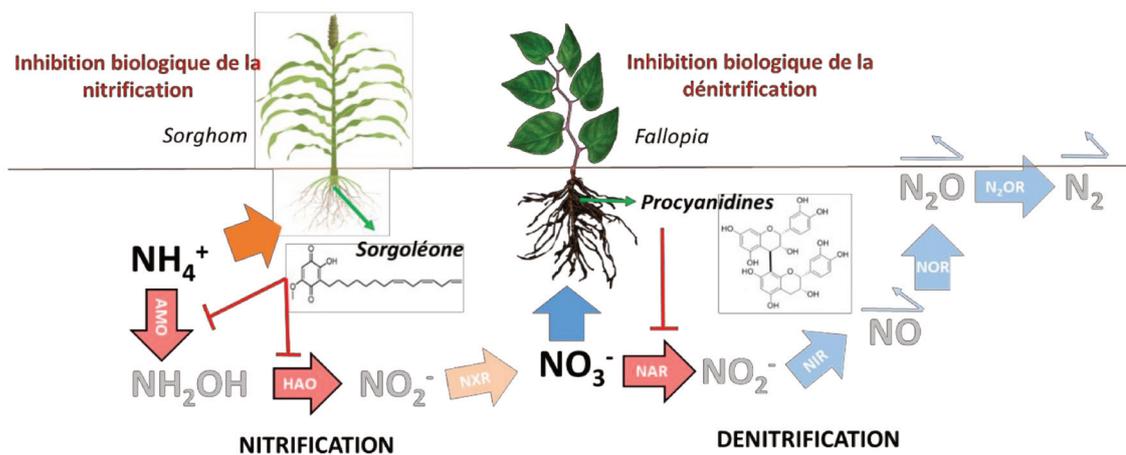


Figure 3 : Schéma de l'inhibition biologique de la nitrification (BNI for Biological Nitrification Inhibition) et de la dénitrification (BDI for Biological Denitrification Inhibition). Chez le *Sorghom*, la plante exsude du sorgoléone qui va bloquer les enzymes AMO et HAO inhibant ainsi la nitrification et permettant l'acquisition du NH_4^+ par la plante. L'exsudation de procyanidines par *Fallopia* va inhiber la NAR empêchant ainsi la dénitrification d'être réalisée. AMO : ammonium monooxygénase ; HAO : hydroxylamine oxydoréductase ; NXR : nitrite oxydoréductase ; NAR : nitrate réductase ; NIR : nitrite réductase ; NOR oxyde nitrique réductase ; N₂OR : oxyde nitreux réductase. D'après Subbarao *et al.* (2013) et Bardon *et al.* (2014, 2016).

Les racines de la plante sont constamment attaquées par de nombreuses bactéries dites phytopathogènes attirées par les exsudats racinaires. Les plantes ont su développer différentes stratégies pour se défendre contre ces agresseurs (Figure 4). Une des stratégies est la synthèse directe de composés antimicrobiens. Ce sont principalement des métabolites secondaires tels que des flavonoïdes, terpenoïdes et des indoles, retrouvés chez les plantes modèles *A. thaliana* et *M. truncatula* mais aussi chez le soja et le riz (Bais et al., 2006). L'expression des gènes de virulence chez certains phytopathogènes est régulée par le

mécanisme du Quorum Sensing (QS) : un mode de communication cellulaire utilisé par les bactéries (Miller and Bassler, 2001). Cette communication repose sur une évaluation de la densité cellulaire. Les bactéries diffusent dans le milieu des molécules de la famille des N-Acyl homosérines lactones (AHLs). Plus il y a de bactéries et plus il y aura de AHLs sécrétés. Une fois un certain seuil de densité atteint, il y a perception de ce seuil par des récepteurs de ces molécules et déclenchement de l'expression des gènes régulé par le QS dont ceux impliqués dans la virulence. L'une des stratégies de défense de la plante est de perturber ce système de communication. Pour y arriver plusieurs choix possibles : **le Quorum-Quenching** où la plante va exsuder des composés tels que des enzymes pouvant dégrader la molécule signal AHLs et ainsi empêcher la perception du signal du côté de la bactérie. Autre option, la possibilité de **sécréter des leurres dit « AHLs mimics »**. Ce sont des molécules proches de la structure des AHLs mais qui perturbent la régulation du QS en le déclenchant de façon prématurée, c'est-à-dire sans que le seuil de la densité cellulaire ne soit atteint. Parmi les phytopathogènes utilisant le QS pour réguler leurs gènes de virulence, on compte par exemple, le genre *Dickeya*, *Pseudomonas* ou encore *Agrobacterium*. Il a été montré que l'acide rosmarinique exsudé par les plantes est capable d'interférer avec le QS de *P. aeruginosa* PAO1 (Corral-Lugo et al., 2016). En effet cet acide mime l'action de l'AHL et se lie avec le récepteur RhIR (régulateur de réponse du QS ayant un rôle de facteur de transcription des gènes de virulence) de PAO1. Ceci déclenche prématurément la virulence de la bactérie avant que le QS soit atteint. Les défenses de la plante seront alors activées afin de se protéger face aux pathogènes avant que celui-ci ne produisent des facteurs de virulence QS dépendant (Corral-Lugo et al., 2016). Un autre exemple de perturbation du QS avec les acides gallique et la p-coumarique qui à des concentrations de 120 et 240 $\mu\text{mol.L}^{-1}$, respectivement, inhibent la synthèse de AHLs chez *P. fluorescens* KM120 et par conséquent le QS (Myszka et al., 2016).

Une autre stratégie pour améliorer la défense de la plante face à divers envahisseurs est de sécréter des composés qui vont promouvoir l'interaction avec d'autres bactéries PGPR (Plant Growth Promoting Rhizobacteria), stratégie aussi appelée « cry for help » (Bakker et al., 2018). C'est notamment le cas avec le recrutement de bactéries ayant un pouvoir de biocontrôle sur les espèces phytopathogène en inhibant leur croissance par la sécrétion de composés antimicrobiens par exemple. La plante va donc mettre en place des interactions dites positives qui seront abordées au paragraphe suivant.

2. Interactions positives

La plante va également promouvoir d'autres types d'interactions qui seront positives pour les différents partenaires. La plante va ainsi sécréter des molécules qui vont avoir un fort pouvoir attractif sur ses futurs partenaires et aussi des composés qui vont représenter une source nutritive de choix (Figure 4).

De nombreux phénomènes de coopération entre la plante et les bactéries du sol ont été décrits. Par exemple, les bactéries dites PGPR vont jouer un rôle de phytostimulation et de phytoprotection pour la plante en échange de sources nutritives fournies par la plante. La première étape de la mise en place de ces interactions bénéfiques va être de sécréter des molécules qui vont avoir un pouvoir chimioattractant afin de recruter spécifiquement certains partenaires (Figure 4). Chez les PGPR, on retrouve majoritairement les genres *Bacillus* et *Pseudomonas*. *Bacillus velezensis* SQR9 est une PGPR isolée de la rhizosphère du concombre. Une étude s'est intéressée au pouvoir chimioattractant des métabolites primaires exsudés par le concombre sur *B. velezensis* SQR9 et a démontré que sur une centaine d'exsudats, près de la moitié possède un effet attractant sur cette bactérie (Feng et al., 2018). Chez la tomate (*Solanum lycopersicum*), il a été reporté que l'exsudation de malate ou de citrate attirait la bactérie *P. fluorescens* WCS365 en stimulant la production du flagelle de la bactérie et ainsi favoriser sa mobilité pour la colonisation des racines de la plante (De Weert et al., 2002). Les métabolites primaires ne sont pas les seuls à avoir un effet chimioattractant, les métabolites secondaires ont également un rôle attractif. Dans les métabolites secondaires exsudés par le maïs, on retrouve le DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) exerçant un pouvoir attractant sur *P. putida* KT2440 (Neal et al., 2012). Les flavonoïdes, grande classe de métabolites secondaires, ont également un rôle dans la mise en place d'interaction bénéfique pour la plante. En effet, les flavonoïdes vont être reconnus et induire l'expression des facteurs Nod des rhizobactéries. Ces derniers seront reconnus par la plante et ainsi déclencher le processus symbiotique permettant l'attraction et l'internalisation de la rhizobactérie pour ensuite l'héberger dans organe végétal néoformé à savoir la nodosité (Cooper, 2004). Selon la nature des flavonoïdes, cela va permettre de sélectionner

spécifiquement certaines espèces de rhizobactéries par rapport à d'autres. Par exemple les flavonoïdes du soja, daidzéine et genistéine, induisent les gènes *nod* chez *Bradyrhizobium japonicum* et répriment ceux de *Sinorhizobium meliloti* qui eux sont stimulés par la luteolin exsudée par *M. sativa* (Cooper, 2007). Autre exemple de relation spécifique : la mise en place de la symbiose entre l'actinobactérie *Frankia* et plantes actinorhiziennes où également l'attraction de la bactérie se fait *via* les flavonoïdes (Cooper, 2004) mais par contre, la nature du facteur bactérien reste à déterminer (Cissoko et al., 2018). En conclusion, la plante est capable de sélectionner spécifiquement son ou ses partenaires *via* ses exsudats et d'y établir une interaction très étroite.

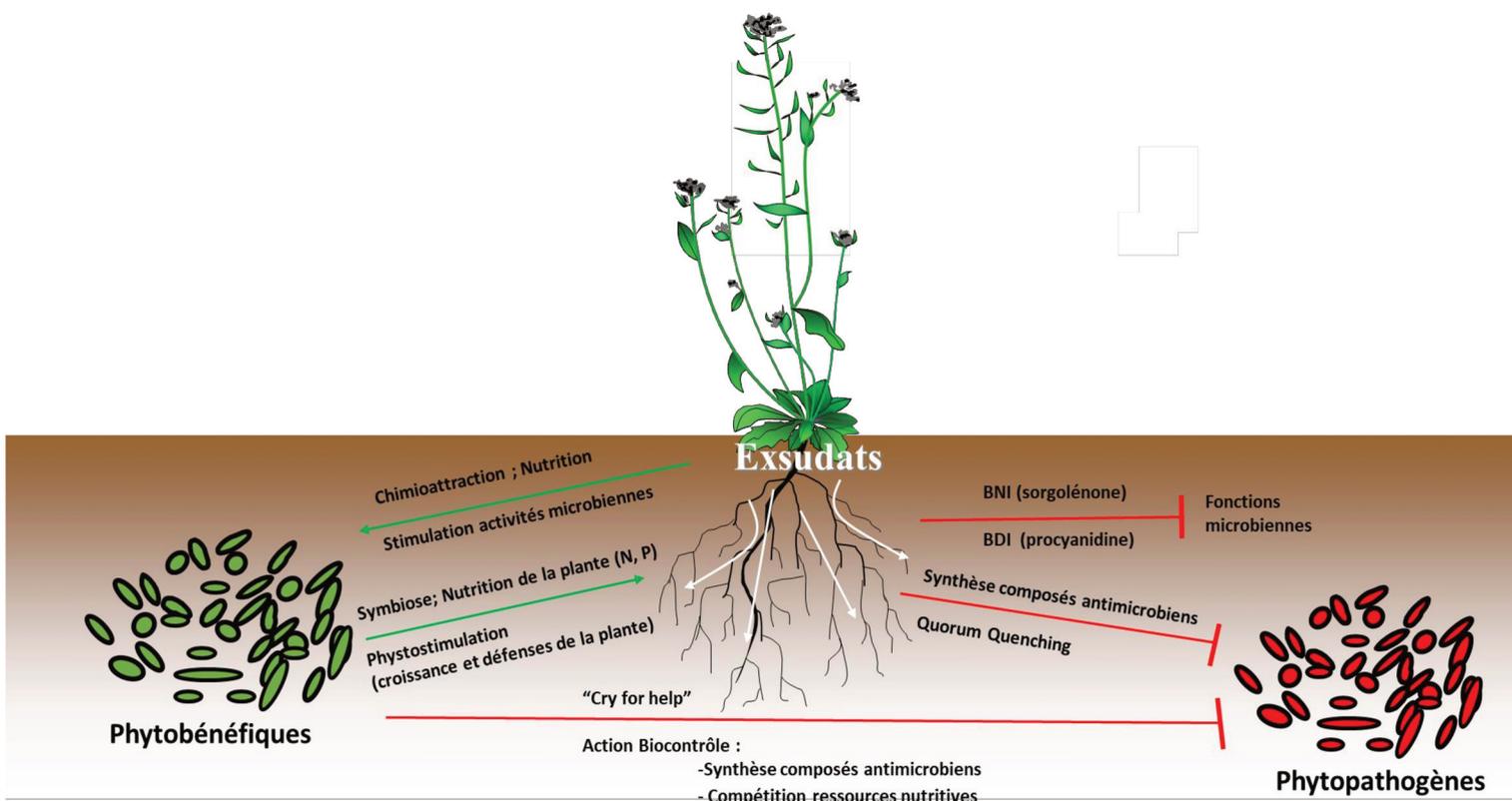


Figure 4 : Représentation schématique des exemple d'interactions plante – bactéries médiées par les exsudats racinaires. Les flèches vertes indiquent une interaction positive. Les flèches barrées en rouge indiquent une interaction négative. De par son exsudation, la plante va pouvoir inhiber certaines fonctions microbiennes (BNI et BDI) mais aussi perturber la communication bactérienne par Quorum Quenching. La plante va aussi exsuder des composés antimicrobiens afin d'inhiber certaines espèces pathogènes. De l'autre coté la plante va exsuder des molécules ayant un effet attracteur sur certaines espèces, représenter une source nutritive de choix et aussi permettre la stimulation de différentes activités microbiennes. En retour, les organismes phytobénéfiques vont en retour participer dans la nutrition de la plante, sa phyostimulation mais aussi lutter contre les différents organismes phytopathogènes. Modifié de Haichar et al., 2014.

Si les exsudats racinaires participent à la mise en place des interactions plante-bactéries, ils peuvent également moduler certaines fonctions microbiennes. De nombreuses études s'intéressent à la bioremédiation des sols en cherchant les conditions optimales pour dépolluer. Récemment, (Wang et collègues (2021) ont montré qu'en présence de la plante *Koeleria paniculata* (Savonnier de Chine) la dégradation du phenanthrène (PHE), qui est un hydrocarbure aromatique polycyclique polluant l'environnement, est activée, en comparaison à un sol nu. La stimulation de la dégradation du PHE a également été retrouvée par ajout d'un cocktail d'exsudats synthétiques mimant la composition de l'exsudation de la plante. Ainsi les exsudats racinaires présentent également un intérêt biotechnologique dans la lutte contre la pollution des sols. Autre exemple de modulation d'activité microbienne du sol est le cas de la dénitrification. Il a été démontré qu'en comparaison avec un sol nu, l'activité potentielle de dénitrification était significativement supérieure en présence de la plante (Smith and Tiedje, 1979). Guyonnet et ses collègues (2007) ont montré que les différences observées dans l'exsudation racinaire (qualité et quantité) étaient corrélées à une différence dans l'activité microbienne de dénitrification.

B. Retour vers la plante

Si la plante est capable d'influencer le développement de son microbiome par ses exsudats racinaires, le microbiome va en retour influencer la croissance et le développement de la plante. Nous allons donc voir dans les prochains paragraphes comment les bactéries influencent la croissance des plantes. Les bactéries PGPR vont pouvoir stimuler la croissance de différentes façons que cela soit par une stimulation directe *via* la sécrétion de phytohormones comme des auxines ou des gibbérellines (Drogue et al., 2012) ou par l'amélioration de la disponibilité de certains nutriments pour faciliter leur assimilation par la plante, comme par exemple le phosphate ou le nitrogène (Richardson et al., 2009) (Figure 4). Nous retrouvons également des agents de biocontrôle qui vont prévenir les agressions et les infections de la plante provenant de diverses pathogènes en contrôlant leur présence par

compétition et par la production de composés antimicrobiens, mais aussi en stimulant les défenses de la plante (Pieterse et al., 2014) (Figure 4).

I. Stimulation directe de la croissance de la plante

La production de phytohormones exogènes à la plante par les PGPR va principalement avoir pour effet d'augmenter la surface racinaire de la plante en favorisant l'apparition des poils racinaires (Grover et al., 2021). Cette augmentation va ainsi permettre à la plante d'exploiter une plus grande surface de sol pour sa nutrition. Parmi les phytohormones, on retrouve principalement les auxines qui sont des régulateurs de croissance (Napier, 2016). De façon non exhaustive, les principales rhizobactéries étudiées pour leur production d'auxines sont les *Azospirillum*, *Pseudomonas* et les *Bacillus*. Les auxines sont principalement retrouvées dans la plante dans les méristèmes en division au niveau des apex racinaires. L'auxine la plus étudiée est l'acide indole-3-acétique (IAA) produite par un très grand nombre de rhizobactéries (Spaepen and Vanderleyden, 2011). Chez *Azospirillum brasilense* Sp245, *IpdC* est une enzyme importante intervenant dans la voie de biosynthèse de l'IAA. Le mutant Sp245 *ipdC* est incapable de produire de l'IAA et de ce fait perd son pouvoir phytostimulant. En effet, aucun changement racinaire n'a été observé suite à l'inoculation de ce mutant sur des plantules de blé contrairement à la souche Sp45 sauvage (Dobbelaere et al., 1999). D'autres phytohormones produits par les PGPR sont également responsables de la phytostimulation de la plante on parle alors d'effet additives entre les différentes molécules. Les gibbérellines, acides tetracyclique diterpenoïdes, sont d'autres phytohormones participant à la régulation du développement de la plante comme la division cellulaire, la production du fruit ainsi que la senescence (Gomi and Matsuoka, 2003). L'inoculation d'*A. lipoferum* sur des plantules de maïs a permis l'augmentation significative de la taille et du poids des racines de la plante d'environ un facteur 2. Cette augmentation est également observable en utilisant une dose de gibbérellines de concentration équivalente à celle produite par *A. lipoferum* (Fulchieri et al., 1993). D'autres PGPR sont également productrices de gibbérellines telles que *B. licheniformis* et *B. pumilus* (Gutie and Talon, 2001). Si les PGPR sont connues pour synthétiser des phytohormones et améliorer la croissance de la plante lors de relations bénéfiques, des

bactéries phytopathogènes peuvent détourner ce processus pour leur avantage comme par exemple chez *Agrobacterium fabrum* C58. Cette bactérie est porteuse du plasmide pTi, responsable de la maladie de la galle du collet chez la plante. Ce plasmide possède un ADN-T dit de transfert capable de s'intégrer dans l'ADN de la plante après infection. Suite à cela, la plante va exprimer des gènes codant pour la synthèse d'auxine dérégulant ainsi sa balance hormonale et provoquant l'apparition de la galle. En retour, la bactérie nichant dans la galle va se retrouver dans un environnement fortement propice pour son développement par la présence d'une multitude de composés organiques fournis par la plante à son détriment (Gohlke and Deeken, 2014).

II. Amélioration de la nutrition de la plante

Les bactéries bénéfiques pour les plantes sont donc capables de produire des phytohormones qui vont promouvoir l'expansion racinaire de la plante, ce qui va donc avoir pour conséquence d'améliorer ses apports nutritifs. Mais elles peuvent également directement participer à cette amélioration en rendant certains nutriments plus disponibles pour la plante dans le sol. Nous allons voir dans le paragraphe suivant deux exemples du rôle que peuvent jouer les bactéries du sol : la participation à la solubilisation du phosphate (P) dans le sol et la fixation d'azote (Figure 4).

Le P dans le sol peut être un facteur limitant dans la croissance de la plante dû au fait de sa présence sous forme non soluble. De nombreux microorganismes, notamment les bactéries du genre *Bacillus*, *Pseudomonas* et *Rhizobium*, vont permettre de rendre ce P accessible pour la plante. Divers mécanismes pour rendre le P soluble sont connus et bien étudiés comme l'acidification du sol, la chélation du P ou encore sa minéralisation (Kalayu, 2019). Une façon de diminuer le pH du sol va être la sécrétion de nombreux acides organiques dans le milieu. Comme exemple, nous pouvons retrouver l'acide gluconique sécrété par des *Pseudomonas* et l'acide 2-ketogluconique produit par des *Rhizobium* (Rodríguez and Fraga, 1999). Liu et ses collègues (2019) ont récemment montré qu'en utilisant la souche PGPR *Pseudomonas* sp. P34, du blé inoculé présentait une augmentation significative dans

l'accumulation de P au niveau de ses feuilles mais aussi une augmentation de tout le système racinaire de la plante comparé à la condition non inoculée. En ce qui concerne la chélation du P cela se passe par l'utilisation d'acides comprenant des groupements hydroxyles et carboxyles. Il va y avoir chélation de l'élément associé au P, comme le calcium, et ainsi rendre le P plus accessible pour son assimilation par la plante. On retrouve également l'acide gluconique comme chélateur de calcium (Song et al., 2008). Ainsi, en présence de Phosphate de calcium ($\text{Ca}_3(\text{PO}_4)_2$), l'acide gluconique va venir capturer les ions calcium libérant ainsi le P sous forme organique augmentant ainsi sa disponibilité. La minéralisation du P organique présent dans le sol passe par l'utilisation de phosphatase alcaline ou acide dont les plus connus sont les phytases retrouvées notamment chez les *Bacillus* (Jorquera et al., 2010). Ces phosphatases vont couper les formes organiques du P comme le phytate et ainsi libérer le P qui sera ensuite assimilé par la plante.

En ce qui concerne la fixation d'N nous allons nous intéresser aux bactérie PGPR dite diazotrophe comme *Azospirillum* (Fukami et al., 2018). Il a été estimé que jusqu'à 70% de l'apport en N lors de culture en champs était dû aux PGPR fixatrices de N (Kuan et al., 2016). Ces bactéries vont pouvoir fixer le N_2 atmosphérique et le redistribuer à la plante hôte. En utilisant l'isotope ^{15}N en serre, il a été montré que la stimulation de la croissance du riz par *A. amazonense* était principalement due à la fixation de l'azote par la bactérie (Rodrigues, 2008).

En dehors des actions directes dont nous venons de parler, les bactéries sont capables d'améliorer la croissance des plantes en les rendant plus résistantes en cas d'attaques venant de phytopathogènes. De nombreuses bactéries PGPR sont capables d'induire le système de résistance systémique (ISR) et ainsi favoriser la vigueur et la santé de la plante (Pieterse et al., 2014). Elles peuvent également servir en tant que « agent de biocontrôle », en contrôlant la présence des pathogènes par des phénomènes de compétitions ou par la production de molécules antimicrobiennes.

III. Rôle antagoniste

Les *Pseudomonas* sont très connus pour la synthèse de l'antibiotique : 2,4-diacetylphloroglucinol (DAPG), ce composé va avoir un effet délétère direct sur les organismes pathogènes. De nombreuses études se sont intéressées à l'effet du DAPG étant donné son large spectre d'action sur les phytopathogènes bactériens mais aussi les champignons ainsi que les nématodes (Cronin et al., 1997a; Cronin et al., 1997b; Souza et al., 2003). Prenons comme exemple l'action du DAPG sur le genre de champignon phytopathogène *Pythium*. De Souza et ses collègues (2003) se sont intéressés à l'effet du DAPG pendant les différents stades du cycle de vie de *Pythium ultimum* var. *sporangiferum* où le stade de mycélium apparaît être le plus résistant contrairement au stade de zoospores. De plus, ils ont également montré que l'activité du DAPG dépend du pH avec une activité supérieure à faible pH. Par observation en microscopie électronique à transmission, Souza et ses collègues (2003) ont observé que le DAPG entraînait une altération de la membrane plasmique, mais aussi la désintégration du contenu cellulaire provoquant une désorganisation dans les apex des hyphes du champignon. Ainsi par utilisation du DAPG, les *Pseudomonas* vont pouvoir réduire significativement la prolifération des pathogènes. Les *Bacillus* sont également des bactéries productrices de peptides antimicrobiens. Les *Bacillus* produisent des lipopeptides qui vont avoir une action antagoniste envers les pathogènes (Fira et al., 2018). Parmi les lipopeptides, on retrouve différentes familles de molécules telles que les surfactines, iturines et fengycines (Ongena and Jacques, 2007). Les surfactines sont connues pour l'établissement du biofilm robuste chez les *Bacillus*. Cependant, il a été montré qu'elles possèdent également un rôle dans le biocontrôle contre *P. syringae* lors de l'infections d'*A. thaliana* (Bais et al., 2004). L'iturine A et aussi des surfactines produits par *B. subtilis* RB14 interviennent dans la lutte contre *Rhizoctonia solani* responsable de la fonte des semis de la tomate (Asaka and Shoda, 1996). En dehors de la synthèse de composés ayant des propriétés antimicrobiennes directe, certaines bactéries sont également capables d'exercer un rôle antagoniste sur les autres organismes présents par une autre méthode. Certaines ressources nutritives essentielles au bon développement des organismes se retrouvent facilement limitante dans le sol. Cette limitation en ressource va entraîner des phénomènes de compétition. Par exemple le fer, faiblement disponible dans le

sol, est facilement limitant et entraîne donc une compétition pour son acquisition et assimilation. Pour avoir facilement accès au fer, les bactéries peuvent produire des sidérophores qui vont chélater le fer et ainsi le rendre plus disponible (Andrews et al., 2003). Le genre *Pseudomonas* est également bien connu pour la production de sidérophores. Il a été montré qu'en conditions limitantes en fer, *P. fluorescens* SPs9 et SPs20, produisent des sidérophores et inhibent le développement du champignon phytopathogène responsable du flétrissement de la tomate *Fusarium oxysporum* f.sp. *lycopersici* (Arya et al., 2018). Cette inhibition diminue lorsque le fer n'est plus limitant dans le milieu car il a également été observé que la quantité de sidérophores produit diminue lorsque la concentration en fer augmente, montrant ainsi le rôle clef des sidérophores dans le biocontrôle de phytopathogènes (Arya et al., 2018).

IV. Stimulation des défenses de la plante

Ainsi les bactéries bénéfiques à la plante possèdent tout un arsenal de molécules pour défendre la plante contre les pathogènes de façon directe. Mais elles peuvent également aider la plante en améliorant ses défenses naturelles. Nous allons à présent voir comment les défenses de la plante sont stimulées par les bactéries. Comme annoncé précédemment, l'induction de la résistance systémique (ISR pour induced systemic resistance) par les bactéries stimulent les défenses de la plante et donc sa vigueur et sa santé face à divers agresseurs. Le système immunitaire de la plante (SI : système immunitaire) est activé par les récepteurs capables de reconnaître des motifs précis (PRR pour pattern-recognition receptors). Ces motifs moléculaires associés aux microbes (ou MAMPs pour « microbe-associated molecular pattern » en anglais) peuvent correspondre à différentes protéines microbiennes tels que la flagelline, le peptidoglycane, les lipopolysaccharides. Les MAMPs vont pouvoir activer l'ISR. L'ISR fait intervenir deux hormones clefs dans la réponse : l'éthylène (ET) et l'acide jasmonique (JA). Le déclenchement de l'ISR va avoir pour conséquence la synthèse de différents composés ayant des propriétés antimicrobiennes. Pour exemple, *P. putida* est capable de déclencher l'ISR du haricot, il en résulte une augmentation de la synthèse de hexenal un antifongique (Ongena et al., 2004). Le DAPG est également capable de déclencher l'ISR lors de l'interaction

P. fluorescens CHA0 et *A. thaliana*, le mutant CHA0 *phl* (gène codant pour la synthèse du DAPG) est beaucoup moins efficace dans la protection de la plante contre le pathogène *Peronospora parasitica* (Iavicoli et al., 2003).

De manière non exhaustive, nous venons de voir qu'il existait différentes méthodes pour une même bactérie de promouvoir la croissance de la plante. En agronomie, de plus en plus d'études s'intéressent à l'utilisation d'inoculum microbien pour limiter les pertes mais aussi augmenter les rendements agricoles. Il serait donc judicieux d'utiliser des souches capables de jouer sur tous les fronts pour améliorer la croissance et le rendement des plantes tout en limitant l'apparition de pathogène ce qui aurait un lourd impact économique en cas de perte. Cependant les études présentées ont été réalisées en condition contrôlée de manière in vitro et in planta. Afin d'étendre ces connaissances dans les domaines pratiques, il serait donc judicieux de poursuivre ces études en utilisant dans un premier temps des communautés complexes à l'image de celles retrouvées dans les sols puis réaliser des études en champs.

Dans la prochaine partie de ce chapitre, nous allons nous intéresser à la fonction de la dénitrification. Comme indiqué précédemment, la dénitrification est une fonction microbienne, pouvant être retrouvée dans la rhizosphère des plantes. Nous avons vu également que c'était une fonction pouvant être inhibée (BDI) ou stimulée par les exsudats racinaires lors des interactions plante-bactéries. De plus la dénitrification va présenter des enjeux écologiques et agronomiques. En effet, lors de la dénitrification, il y a libération du N₂O, gaz à effet de serre et perte d'azote dans le sol renforçant la compétition pour l'azote entre les microbes et la plante pouvant affecter la productivité des plantes. Ainsi, comprendre comment la dénitrification est mise en place dans l'environnement permettrait d'élaborer de nouvelles stratégies en agroécologie dans le but réduire l'émission de N₂O, l'utilisation des intrants azotés et de limiter la perte de N dans les sols.

C. La dénitrification

Ainsi nous venons de voir que la plante pouvait inhiber ou stimuler l'activité microbienne du sol dont la dénitrification. Cette dernière est sûrement la voie respiratoire alternative la plus étudiée à ce jour. En effet, elle est retrouvée chez de nombreux microorganismes disséminés sur toute la planète, à la fois dans les sols terrestres, mais également dans les milieux aquatiques. Bien qu'elle ait permis un avantage évolutif aux microorganismes pour l'adaptation à des environnements en absence d'oxygène, elle comporte cependant un contre coup écologique. En effet la dénitrification est la principale source d'émission de l'oxyde nitrique (N_2O), un gaz à effet de serre ayant un pouvoir réchauffant 300 fois supérieur à celui du CO_2 à quantité équivalente (Galloway et al., 2008). Sa principale source anthropogénique est l'agriculture à cause notamment de l'utilisation intensive d'engrais azoté stimulant la croissance des plantes, mais également de l'activité des microorganismes dénitrifiants. Bien que la dénitrification permet la réduction du NO_3^- en N_2 , elle est souvent inachevée. En effet, la dernière étape réduisant le N_2O en N_2 est souvent manquante, ce qui conduit à un rejet de N_2O et non du N_2 entraînant ainsi une augmentation de l'effet de serre et la perte en N du système. Ayant un grand intérêt écologique, nous allons dans la section qui suit faire un point sur ce qu'est la dénitrification, comment elle est orchestrée et régulée dans la rhizosphère.

I. Fonctionnement de la dénitrification

La dénitrification fait partie du cycle de l'azote (N) (Figure 5). L'azote est le quatrième élément le plus abondant dans les organismes vivants après l'oxygène, le carbone et l'hydrogène, il est donc d'autant plus intéressant d'en étudier son rôle et son cycle. Le cycle de l'azote est un cycle biogéochimique composé de différentes étapes, permettant la transformation du diazote présent dans l'atmosphère en azote assimilable par les organismes avant d'être de nouveau transformé et relargué dans l'atmosphère sous forme de diazote, et ainsi de suite. Il est principalement réalisé par les microorganismes du sol et, comme on l'a vu précédemment, influencé par la plante *via* l'exsudation racinaire. Avant de faire un focus sur la dénitrification

qui est l'une des parties importantes de cette étude, nous allons énoncer les différentes étapes du cycle de l'N en guise de présentation. Les étapes du cycle de l'N sont les suivantes : la fixation d'N, la minéralisation de l'N, l'anammox, la nitrification et la dénitrification (Figure 5). La fixation d'azote permet l'assimilation du N sous forme d'ammonium (NH_4^+), cette réduction est accomplie par diverses bactéries (et également des archées), possédant des gènes codant pour des enzymes dites nitrogénases. Cette étape est principalement retrouvée lors de symbiose plante-bactérie avec notamment des bactéries dites fixatrice d'azote (Mylona et al., 1995; Bruijn, 2015). La minéralisation de l'azote va permettre la conversion du N organique en NH_4^+ (Cabrera et al., 2005). L'étape suivante est la nitrification, fonction permettant la transformation de l'ammonium en nitrite (NO_2^-) ou nitrate (NO_3^-). Ensuite nous retrouvons l'anammox qui permet la formation de diazote suite à l'oxydation de l'ammonium en condition anaérobie ou partir du NO_2^- et du NO. Cependant, la réalisation de l'anammox passe par l'intermédiaire de l'hydrazine (N_2H_4), un composé toxique (Naeher et al., 2015).

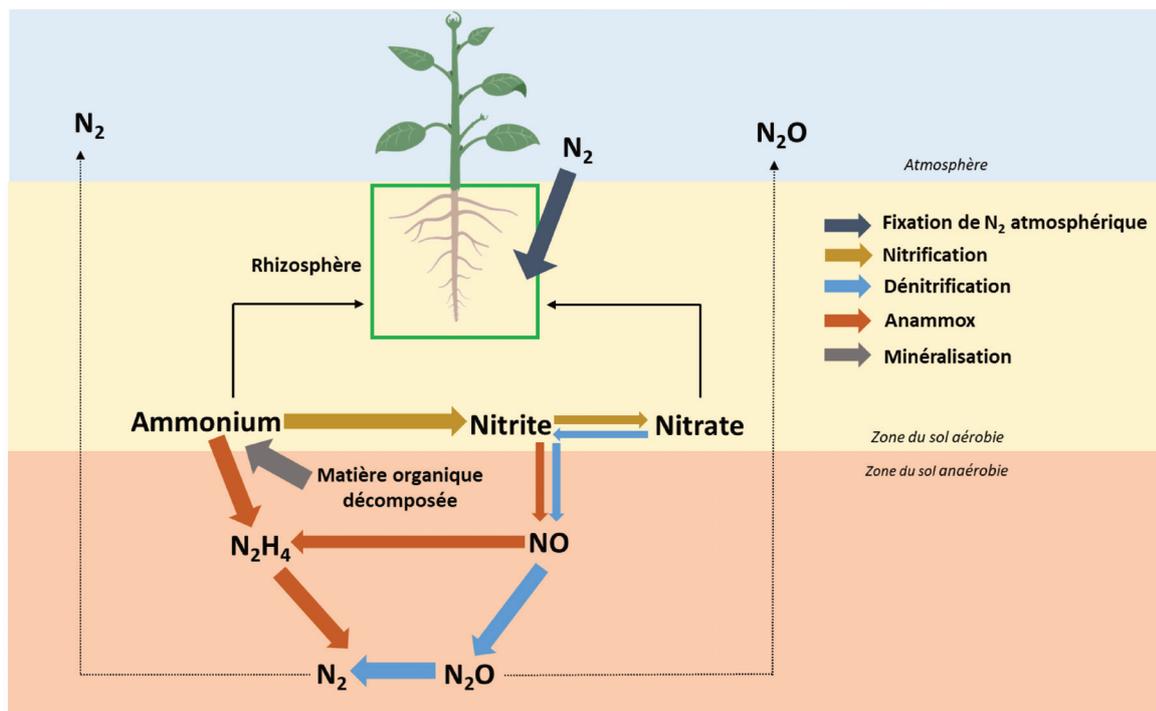


Figure 5 : Schéma du cycle de l'azote dans la rhizosphère. Les flèches colorées représentent les différentes étapes enzymatiques du cycle de l'azote. Adapté de Coskun *et al.* (2017). La fixation de l'azote permet l'assimilation du N_2 atmosphérique sous forme d'ammonium par la plante. La nitrification permet la transformation de l'ammonium en nitrite ou nitrate par oxygénation. La dénitrification réduit le nitrate en N_2O ou N_2 . L'anammox oxyde l'ammonium en N_2 . La minéralisation permet la conversion de la matière organique en ammonium.

En fin, la dénitrification est une succession de quatre réductions enzymatiques permettant la réduction progressive du nitrate en diazote ou en N_2O si la dénitrification est incomplète (Zumft, 1997) (Figure 6).

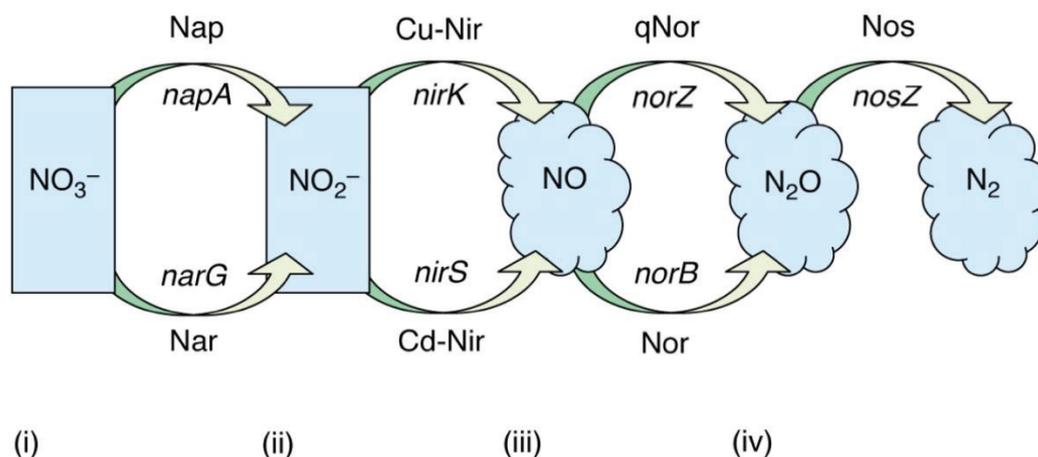


Figure 6 : Voie métabolique de la dénitrification. (i) La réduction du nitrate soluble en nitrite est catalysée soit par une nitrate réductase liée à la membrane (Nar), soit par une nitrate réductase periplasmique (Nap). (ii) La réduction des nitrites en oxyde nitrique gazeux est catalysée par une nitrite réductase liée au cuivre (Cu-Nir) ou au cytochrome cd (Cd-Nir). (iii) La réduction du NO en N_2O est catalysée par une oxyde nitrique réductase à deux composants (Nor) ou un composant (qNor). (iv) la réduction de l'oxyde nitreux en diazote est catalysée par l'oxyde nitreux réductase. D'après Philippot et Hallin (2005).

La dénitrification est une fonction régulée nécessitant la présence d'une source d'azote (nitrate ou nitrite), un très faible taux voire une absence d'oxygène, ainsi qu'une source de C tels que les exsudats racinaires. La dénitrification a surtout été étudiée chez *Paracoccus denitrificans* et dans le genre *Pseudomonas* (principalement chez *P. aeruginosa* PAO1). Nous allons prendre ces organismes comme référence. La première étape de la dénitrification consiste en la première réduction du NO_3^- en NO_2^- par une nitrate réductase. Cette réduction enzymatique peut être réalisée par différentes enzymes suivant leur localisation dans la cellule. On retrouve principalement la nitrate-réductase periplasmique (NapA) qui comme son nom l'indique est localisée dans le périplasma, ainsi que la nitrate-réductase respiratoire

(NarG) qui, elle, est transmembranaire (Figure 6). NarG et NapA sont deux réductases participant à la réduction du nitrate fournissant ainsi de l'énergie à la cellule par génération d'ATP. Les gènes *narG* et *napA* sont regroupés en opéron avec d'autres gènes codant des protéines participant à la maturation et au bon fonctionnement de NarG et NapA (Figure 7). Ensuite vient la réduction du NO_2^- en oxyde nitrique (NO) réalisée par une nitrite réductase. Il existe deux nitrite réductases, NirS et NirK (Figure 6). NirK est une nitrite réductase homotrimérique à cuivre tandis que NirS est une nitrite réductase homodimérique à cytochrome C (Philippot, 2002). Les deux Nir sont des enzymes périplasmiques et réalisent la réduction après transfert d'électron depuis le cytochrome C ou de l'unité cuivreux. NirS est souvent retrouvée chez les bactéries à Gram négatif, tandis que NirK est retrouvée chez les bactéries à Gram négatif et positif. Dans les premières études, il a été considéré que la présence de l'une excluait la présence de l'autre, étant donné leur évolution indépendante (Philippot, 2002). Toutefois, de récentes études génomiques ont révélé la présence simultanée de ces deux Nir dans certaines bactéries, notamment chez *Pseudomonas stutzeri* et *Bradyrhizobium oligotrophicum*, où un rôle redondant entre NirS et NirK est supposé (Graf et al., 2014; Sanchez and Minamisawa, 2018; Wittorf et al., 2018). A forte concentration, le NO est toxique pour les êtres vivants. Mais à faible concentration, il peut servir de molécule signal dans le cadre des interactions plante bactéries et favoriser l'activité de dénitrification en anaérobiose (Bakken et al., 2012). Ainsi pour éviter l'accumulation du NO, sa réduction doit être rapide et efficace (Van Spanning et al., 2005). Il existe deux types de NO réductases en fonction du donneur d'électron, cNOR reçoit l'électron depuis un cytochrome C et qNOR depuis des quinols (Lecomte et al., 2018). cNOR est composé de deux sous-unités NorC et NorB. NorC donne l'électron à NorB qui réduit le NO en N_2O (Figure 6). Chez *Agrobacterium fabrum* C58 il a été récemment découvert l'intervention d'un petit ARN non codant NorR régulant positivement *norCB* (Lecomte et al., 2021). La dernière étape de la dénitrification consiste en la réduction du N_2O en N_2 . La réaction est catalysée par l'enzyme NosZ (Figure 6). NosZ est une réductase homodimérique à cuivre présent dans le périplasma. Bien que l'étape de réduction du N_2O en N_2 permet de boucler le cycle de l'azote, cette dernière réduction est cependant absente chez de nombreuses bactéries dénitrifiantes ce qui aboutit à un processus de dénitrification incomplet. Approximativement 40% des bactéries dénitrifiantes ne possèdent pas NosZ (Philippot et al., 2011; Graf et al., 2014). Cette absence de réduction du

N₂O conduit ainsi à un ratio plus élevé d'émission de N₂O que de N₂ et contribue donc à la fois au réchauffement climatique mais aussi à la perte d'azote dans les agrosystèmes (Philipot et al., 2011). Ce ratio N₂O/N₂ est également dépendant de facteurs abiotiques comme le pH où à un faible pH du sol l'activité de NosZ est inhibée empêchant ainsi la réduction du N₂O en N₂ (Dannenmann et al., 2008; Bakken et al., 2012).

II. Régulation de la dénitrification

Il est évident que la régulation de la dénitrification, pour aboutir à une dénitrification complète, est un aspect écologique important. Comme annoncé plus haut, la dénitrification est une fonction régulée par la présence d'une source d'azote, une source de C et l'absence d'oxygène. Nous allons à présent aborder comment la dénitrification est régulée par ces différentes molécules.

1. Régulation par l'oxygène

La dénitrification est une fonction induite en l'absence d'O₂. L'oxygène va donc avoir un rôle important dans sa régulation. On retrouve chez *P. denitrificans* le régulateur FnrP qui possède un cluster de type [4Fe-4S] répondant à l'oxygène et contrôle la transcription des gènes tels que l'opéron *nar* en fonction de la déplétion en oxygène du milieu (Van Spanning et al., 2005). Toujours chez *P. denitrificans*, NNR possède un groupement hème sensible à l'oxygène (et au NO) et régule l'expression de *nir* et *nor* (Van Spanning et al., 2005). Chez *P. aeruginosa* PAO1, deux facteurs de transcription de type FNR vont fonctionner de paire afin de réguler positivement l'expression des gènes codant pour la dénitrification : ANR (régulateur de la réduction de l'arginine et du nitrate) et DNR (régulateur de la dénitrification) (Schreiber et al., 2007). Le premier : ANR va percevoir l'appauvrissement en oxygène du milieu et réguler positivement l'expression de *narXL* et de *narGHI* (Figure 7). De plus, ANR va réguler l'expression de DNR. DNR, sous la régulation de ANR et du NO, va réguler l'expression des

gènes codants pour toutes les réductases de la dénitrification et ainsi induire la réduction du nitrate en N_2O/N_2 (Schreiber et al., 2007).

2. Régulation par l'azote

La première étape, consistant en la réduction du nitrate en nitrite par NarG, fait intervenir au départ un système senseur/régulateur qui va sentir la présence du nitrate dans le milieu puis induire une cascade de régulation. Ce système à deux composantes est NarXL présent chez les *Pseudomonas* où NarX, le senseur, va percevoir la présence du NO_3^- dans le périplasma de la cellule et par une cascade de phosphorylation active le régulateur de réponse NarL qui à son tour active la transcription de l'opéron *narGHJ* (Figure 7). NarL est aussi capable de réprimer l'expression de l'opéron *nap* au profit de celle de l'opéron *nar* en condition d'anaérobiose stricte, étant donné son apport en énergie plus important (Alst et al., 2009). De plus, NarXL contrôle également l'expression d'un petit ARN régulateur Pail qui régule également la nitrite réductase chez *P. aeruginosa* PA14 (Tata et al., 2017). Pour *P. denitrificans*, la régulation des nitrate réductases s'effectue grâce au régulateur NarR qui se trouve être une protéine de type FNR (Fumarate and Nitrate reduction Regulator). En présence de NO_3^- , NarR va activer l'expression de *narGHJ*. En effet, cet opéron est sous exprimé chez une souche mutante $\Delta narR$ (Gaimster et al., 2018).

Bien que le NO puisse servir de molécule signal au cours de l'interaction plante-bactérie et l'activation des défenses de la plantes (Vaishnav et al., 2018), à forte concentration, il en devient toxique pour les organismes. C'est pourquoi il faut éviter qu'il s'accumule. Le NO est formé suite à la réduction du nitrite par NirS/K. Il est utilisé comme molécule signal pour la régulation de la dénitrification afin qu'il soit vite réduit et donc éviter sa toxicité. Le NO régule sa propre réduction et va donc jouer un rôle régulateur central dans l'expression des gènes de la dénitrification. Le NO régule l'expression des facteurs de transcription de type FNR : Nnr (nitrite and nitric oxide gene regulator) chez *P. denitrificans* ; DNR (denitrification regulator) chez *P. aeruginosa* ; DnrD chez *P. stutzeri* qui vont réguler l'expression des gènes de la dénitrification (Van Spanning et al., 2005) (Figure 7). Le NO régule également le régulateur NosR qui régule NosZ chez les *Pseudomonas* (Cuypers et al., 1992; Arai et al., 2003) (Figure 7).

De plus il a été montré chez *P. stutzeri* que l'expression des gènes *nirS*, *norCB* et *nosZ* est dépendante de la concentration en NO (Vollack and Zumft, 2001).

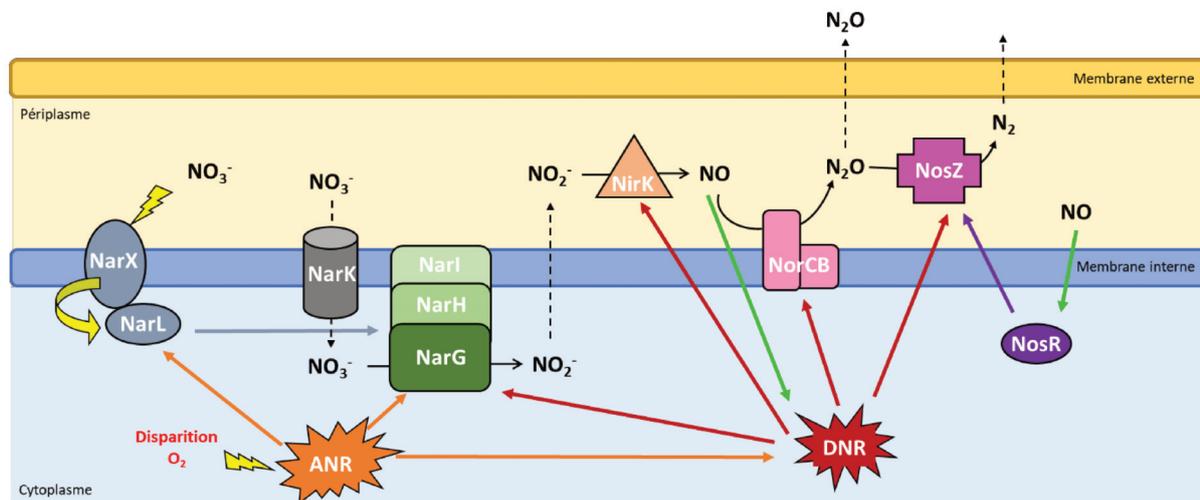


Figure 7 : Représentation schématique de la dénitrification et ses régulateurs chez *P. aeruginosa* PAO1. Les flèches segmentées correspondent aux transports des différentes molécules au travers des membranes. Les flèches noires pleines correspondent aux réactions enzymatiques. Les flèches de couleurs correspondent à une régulation positive apportée par le régulateur.

3. Régulation par le carbone

La matière organique (source de C), servant de fournisseur d'électron pour les réactions enzymatiques, va également jouer un rôle régulateur sur cette fonction. Des études ont montré que la présence de C augmentait la production de N_2O et donc l'activité de dénitrification (Morley and Baggs, 2010). Durant les dernières décennies, des chercheurs ont mené des études en sol ou en bioréacteur pour s'intéresser à la régulation de la dénitrification suivant la nature de la source de C donné. En plus de montrer l'effet positif de l'ajout d'exsudat racinaire artificiel (source de C) sur l'activité potentielle de dénitrification, Henry et al (2008) ont également mis en lumière que la composition de l'exsudation impactait le taux de dénitrification. Ainsi, en jouant sur la composition de différents cocktails d'exsudats, ils ont montré qu'avec une dominance de sucre par rapport aux acides organiques et aminés (80% contre 10% et 10%), l'activité potentielle de dénitrification de leur sol était significativement

plus élevée comparé à d'autres variations de composition en métabolites primaires. Guyonnet et ses collègues (2017) ont montré que l'activité de dénitrification était positivement corrélée avec l'exsudation de succinate et d'acide butanoïque contrairement au xylose. Une autre étude de Xu et al (2018), cette fois-ci en bioréacteur, montre une activité de dénitrification plus importante en présence de source de C simple (glucose, acétate de sodium) par rapport à des sources de C complexe (le polymère de poly (3-hydroxybutyrate-co-3-hydroxyvalerate) /poly (lactic acid) (PHBV/PLA)). Il est également intéressant de noter que l'activité varie également en fonction du ratio C/N du milieu. En effet avec un ratio C/N de 6:1, l'activité de dénitrification est plus élevée en présence d'acétate de sodium qu'en présence de glucose en tant que source de carbone. Cependant, avec un ratio C/N de 3:1 c'est en présence de glucose comme source de carbone que l'activité en est supérieure (Xu et al., 2018). Bien que l'on montre l'effet régulateur de la source de C sur l'activité de la dénitrification, il est important de noter que cette étude a été réalisée en communauté complexe où des phénomènes de compétition pour une même ressource de C peuvent être observés.

Plusieurs études se sont intéressées à l'influence d'une source de C sur l'activité potentielle de dénitrification, toutefois, très peu se sont intéressées à l'effet régulateur du C sur la dénitrification d'un point de vue moléculaire.

Il est bien connu que les microorganismes possèdent un métabolisme très versatile leur permettant de s'adapter à de nombreux environnements en fonction des ressources disponibles. Bien qu'ils soient capables d'utiliser diverses ressources, il existe un mécanisme permettant de réguler la hiérarchisation d'utilisation de ces diverses ressources de C. C'est le mécanisme de répression catabolique du carbone (CCR pour carbon catabolic repression) permettant de prioriser l'acquisition d'une source par rapport à d'autres. Dans la partie suivante, nous allons explorer le rôle de la CCR dans la régulation des gènes impliqués dans les interactions plantes bactéries dans la rhizosphère (revue publiée dans Plant Communications en 2022) et probablement dans la régulation de la dénitrification (objectif de la thèse).

D. La Répression Catabolique du Carbone et son rôle régulateur dans les interactions plante-bactéries

Dans cette partie, une synthèse bibliographique a été réalisée sur les différents types de CCR connus à ce jour dans la littérature chez les bactéries ainsi que leur rôle dans la régulation des interactions plantes-bactéries. Une présentation du mécanisme chez les levures et les champignons filamenteux est également présentée afin de montrer que la CCR est également présente chez les eucaryotes (une partie rédigée en anglais dans le cadre de la revue mais non publiée afin de faire un focus sur les bactéries uniquement).

I. CCR chez les Procaryotes



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Implications of carbon catabolite repression for plant–microbe interactions

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ABSTRACT

Carbon catabolite repression (CCR) plays a key role in many physiological and adaptive responses in a broad range of microorganisms that are commonly associated with eukaryotic hosts. When a mixture of different carbon sources is available, CCR, a global regulatory mechanism, inhibits the expression and activity of cellular processes associated with utilization of secondary carbon sources in the presence of the preferred carbon source. CCR is known to be executed by completely different mechanisms in different bacteria, yeast, and fungi. In addition to regulating catabolic genes, CCR also appears to play a key role in the expression of genes involved in plant–microbe interactions. Here, we present a detailed overview of CCR mechanisms in various bacteria. We highlight the role of CCR in beneficial as well as deleterious plant–microbe interactions based on the available literature. In addition, we explore the global distribution of known regulatory mechanisms within bacterial genomes retrieved from public repositories and within metatranscriptomes obtained from different plant rhizospheres. By integrating the available literature and performing targeted meta-analyses, we argue that CCR-regulated substrate use preferences of microorganisms should be considered an important trait involved in prevailing plant–microbe interactions.

Key words: carbon catabolite repression, CCR, carbon utilization, plant–microbe interaction, rhizosphere, substrate-use preferences

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INTRODUCTION

Plants are usually inhabited by a large number of different microorganisms. The interactions within the microbial community and the plant host are driven by different survival strategies of the organisms involved. For instance, some microorganisms may live in close symbiotic associations and are thus reliant on distinct partners in the plant holobiont. Conversely, others may engage in ferocious competition, resulting in a relentless war to win finite resources such as nutrients, light, or territory (Bauer et al., 2018). Competition is often linked to the acquisition of nutritional resources that contribute to the improved development of the organism. To ensure their survival in the presence of competitors, some organisms have developed specific strategies that enable them to gain an advantage over others. Prominent examples of such adaptations are bacteria from the genus *Pseudomonas*, which are known to frequently occur in the plant rhizosphere (Haichar et al., 2012) and to produce

siderophores with various affinities as well as receptors for xenosiderophores produced by other species, thereby ensuring the efficient acquisition of iron (Taguchi et al., 2010). While the efficient acquisition of nutrients is a crucial prerequisite for microbial development, microorganisms are also able to make use of their highly versatile metabolic capacities. This allows them to fulfil their nutritional needs via a wide range of resources present in the environment. However, this functional versatility comes with high energetic costs for the cell (Carlson and Taffs, 2010). To reduce the effects of such energy constraints, microorganisms have developed a variety of metabolic regulatory mechanisms. This allows them to thrive at the lowest possible cost. Catabolic regulation primarily involves

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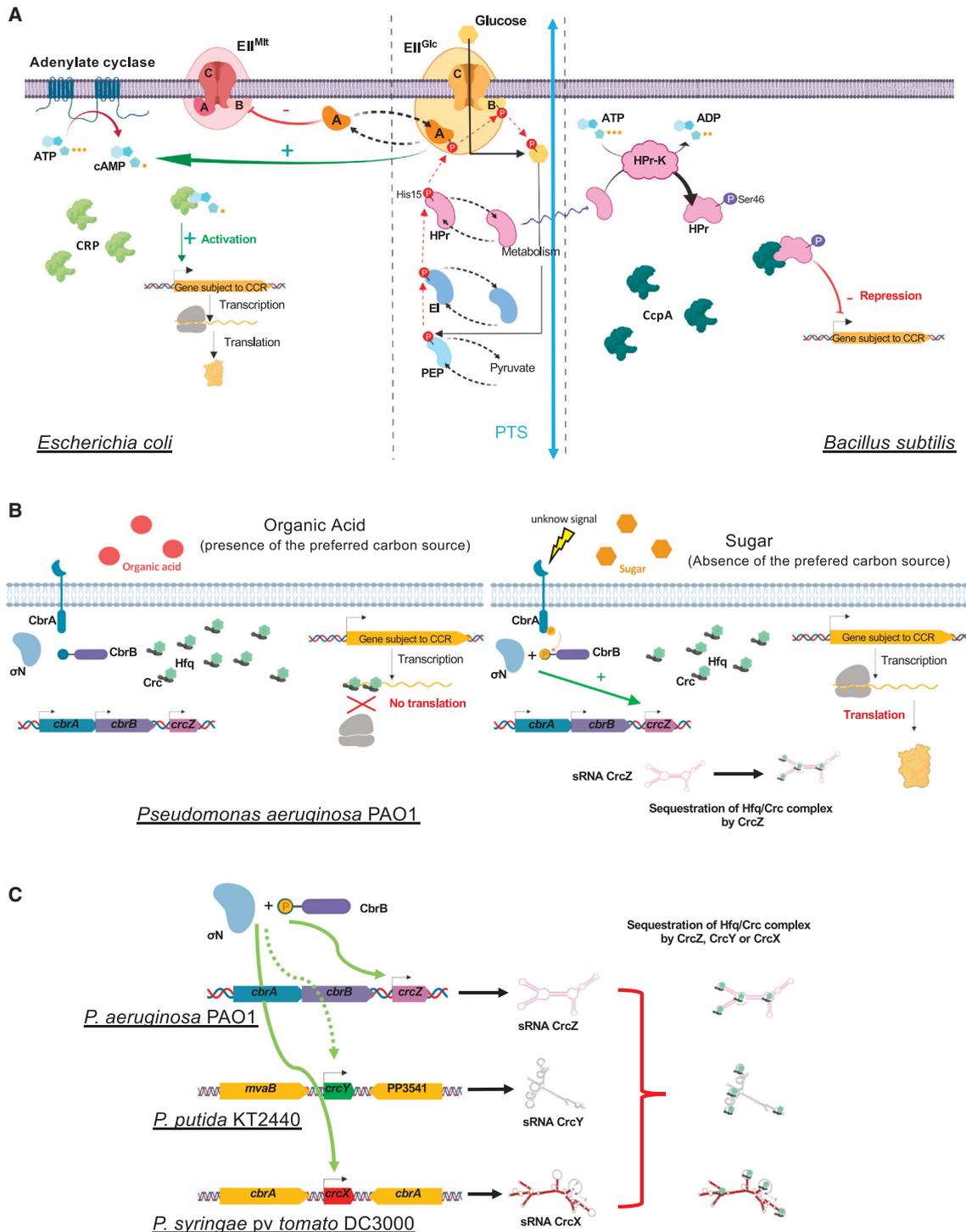


Figure 1. Schematic representation of the CCR system in Enterobacteriaceae (*E. coli*) and Firmicutes (*B. subtilis*) and of the revCCR system in pseudomonads.

(A) The PTS phosphorylation cascade allows the transport and metabolism of different sugars within the cell. The PTS consists of a set of phosphotransferases, which can include enzyme I (EI), histidine-containing protein (HPr), and phosphotransferases like enzyme II (EII). The EII subunits exhibit different localizations (EIIA and EIIB, cytoplasmic; EIIC and EIID, transmembrane). EII enzymes are substrate specific and thus synthesized only in the presence of the substrate. The first reaction step of this system is the phosphorylation of EI by phosphoenolpyruvate (PEP) (Alpert et al., 1985); phosphorylation is then transferred from protein to protein (from EI to HPr at the His-15 position to EIIA) to the B subunit of EII^{Glc}, which, once phosphorylated, allows the transport of glucose from the outside to the inside of the cell via the C subunit. For Enterobacteriaceae, inducer exclusion (legend continued on next page)

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directing available energy into a distinct metabolic pathway that facilitates utilization of the available resource that yields the most energy in return. In short, catabolic regulation is generally directed toward providing the best return on investment.

The best-studied catabolic regulatory mechanism in bacteria is arguably carbon catabolite repression (CCR); this mechanism enables the preferential assimilation of one carbon source rather than another (Monod, 1949; Deutscher, 2008). For this reason, when glucose and lactose are present in equal amounts, *Escherichia coli* first metabolizes the glucose and only later the lactose present in the local environment. Following this crucial observation, numerous studies focused on catabolic repression in different organisms (Deutscher, 2008; Görke and Stülke, 2008; Rojo, 2010; Iyer et al., 2016). To date, two different CCR types have been discovered. Classical CCR regulates the preference for sugars, such as glucose, not only in Enterobacteriaceae and Firmicutes but also in eukaryotes such as yeasts and filamentous fungi (Deutscher, 2008; Görke and Stülke, 2008). Reverse CCR (revCCR) determines the preference of organic acids, such as succinate, in pseudomonads and rhizobia (Rojo, 2010; Iyer et al., 2016). revCCR has mainly been studied in *Pseudomonas aeruginosa* PAO1, which shows post-transcriptional repression, whereas classic CCR is regulated by transcriptional repression (Rojo, 2010). It is not fully clear whether, in addition to regulating catabolic genes, CCR can also control other genes. Moreno et al. (2001) suggested that 5%–10% of bacterial genes are subject to CCR regulation, including those involved in virulence or biofilm formation (Zheng et al., 2004; Rojo, 2010; Chen et al., 2020).

In this review, we address the central role of CCR in plant–microbe interactions in the rhizosphere. First, we provide a condensed overview of the different CCR mechanisms that have been characterized to date in various bacterial models. Then we continue to focus on microbial functions that are involved in interactions with plants and regulated by CCR. In addition, we perform a meta-analysis using available genomic and metatranscriptomic datasets to investigate the distribution of genes involved in bacterial catabolic repression within the plant rhizosphere. The results support our initial hypothesis that this mechanism is widespread among bacteria, specifically rhizobacteria. We use these new insights to discuss the ecological and environmental importance of CCR mechanisms in the rhizosphere.

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MECHANISM OF CARBON CATABOLITE REPRESSION IN BACTERIA

In this first section, we focus on mechanistic aspects of CCR that can be found in different bacteria, such as members of Enterobacteriaceae (*E. coli*), Firmicutes (*Bacillus subtilis*), Rhizobia, and pseudomonads (*P. aeruginosa*). In general, CCR in bacteria is directly linked to carbohydrate transport into the cell. Notably, this system also acts as a regulatory system for nutrient uptake and certain physiological processes, such as virulence and biofilm formation (Görke and Stülke, 2008). Most of the mechanisms have been described using isolated microbes under laboratory conditions. However, it is likely that many of these mechanisms also play an important role in some host-metabolite-dominated environments, such as the plant rhizosphere. The mechanisms of CCR differ substantially among bacteria. Glucose and other sugars are the preferred carbon sources of enteric, Gram-positive bacteria and Firmicutes, the mechanism referred to as CCR, whereas organic acids are the repressing carbon sources in pseudomonads and rhizobia. The latter mechanism is commonly referred to as revCCR; we propose the term “organic acid-mediated CCR” for increased clarity.

CCR mechanisms in Enterobacteriaceae

In Enterobacteriaceae, such as *E. coli*, the main CCR regulator is known as glucose-specific component EIIA (EIIA^{Glc}). EIIA is a component of the phosphotransferase system (PTS) with phosphotransferases like enzyme I (EI), a histidine-containing protein (HPr), and different phosphotransferases (EII) (Figure 1A1). Depending on the phosphorylation state of EIIA^{Glc}, CCR is either activated or inactivated. In the presence of glucose, EIIA^{Glc} is in a non-phosphorylated state and cannot stimulate adenylate cyclase (CyaA), but it can inhibit the transport of several non-PTS sugars by interacting with their transporter; this is known as induced exclusion (IE). IE can be considered a crucial component of CCR in Enterobacteriaceae and also in Firmicutes, but it involves different mechanisms in the latter. One of the best examples of IE in *E. coli* is the repression of the lac operon, in which EIIA^{Glc} binds to the LacY transporter of lactose, preventing the uptake of the sugar and therefore the formation of allolactose required for dismissal of the lac repressor. In addition, CyaA is stimulated by P-EIIA^{Glc} and results in cAMP production (Figure 1A) (Harwood et al., 1976; Feucht and Saier, 1980). cAMP can bind to and activate the cAMP receptor protein (Crp) as the major transcriptional activator of CCR (Figure 1A). Crp-cAMP then activates the transcription of different catabolic genes

is caused by the phosphorylation state of the EIIA^{Glc} subunit. When EIIA^{Glc} is not phosphorylated, i.e., during glucose transport, EIIA^{Glc} interacts with and inactivates enzymes and transporters of secondary carbon sources. CCR is therefore active. Conversely, when EIIA^{Glc} is phosphorylated, i.e., in the absence of glucose transport, EIIA^{Glc} interacts with and activates adenylate cyclase for cAMP formation from ATP. Once formed, cAMP interacts with the cAMP receptor protein (Crp) and allows the transcription of genes associated with non-preferred carbon sources (and other functions that are also regulated) because the repression has been lifted. For Firmicutes, the CCR is orchestrated by the protein HPr. HPr can be phosphorylated at serine 46 by HPr-kinase (HPr-K). The CcpA-HPr-Ser-46 complex represses the transcription of genes associated with non-preferred carbon sources (and other functions that are also regulated).

(B) For pseudomonads, in the presence of the preferred carbon source (succinate), free Crc protein coupled with Hfq binds to the mRNAs of genes associated with non-preferred carbon sources (and other functions that are also regulated), thus preventing proper ribosome binding and mRNA translation. In the absence of the preferred carbon source, an unknown signal is transmitted to the CbrAB two-component system, which, together with the σ^N factor, allows the transcription of the small CrcZ non-coding RNA. CrcZ traps the Crc/Hfq couple, thus freeing the ribosome binding site of the Crc-regulated mRNAs. mRNAs are translated again, and repression is therefore lifted.

(C) Other small non-coding RNAs can lift the repression in other *Pseudomonas* species. Green arrows indicate positive control of RNA synthesis from CbrB. The green dotted arrow highlights a weaker control of CbrB on CrcY in *Pseudomonas putida*.

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and other operons. Several studies show that Crp–cAMP regulates the transcription of numerous *E. coli* genes (Shimada et al., 2011), suggesting a more complex role for this regulator than just CCR, such as involvement in biofilm formation and virulence in Enterobacteriaceae (El Mouali et al., 2018).

CCR mechanisms in Firmicutes

In Firmicutes, such as *B. subtilis*, HPr is the main regulator of CCR, by analogy to EIIA^{Glc} in *E. coli*. The uptake of a preferred carbon source mediates the phosphorylation state of HPr through the bifunctional enzyme HPr-kinase/phosphorylase (HPr-K/P). HPr is phosphorylated on Ser-46 by HPr-K/P after being activated by glycolysis following glucose uptake (Mijakovic et al., 2002; Nessler et al., 2003) (Figure 1A). P-Ser-HPr can then bind to catabolic control protein A (CcpA) and repress the transcription of other catabolic genes by binding to them (Schumacher et al., 2004). The P-Ser-HPr/CcpA complex binds to DNA at a specific promoter region called the catabolic-response element (*cre*) to inhibit transcription of the targeted gene (Miwa et al., 2000) (Figure 1A). For instance, CcpA negatively regulates ribose transporter genes or *chiB*, a gene necessary for chitinase production in *Bacillus thuringiensis* (Antunes et al., 2012; Jiang et al., 2015), in the presence of glucose. In addition, CcpA can positively regulate some genes required for acetate production, like *ackA* and *pta*, by binding to their upstream promoter regions (Presecan-Siedel et al., 1999). Other catabolic control proteins, including CcpB, CcpC, and CcpN, have analogous regulatory functions (Chauvaux et al., 1998; Kim et al., 2003; Servant et al., 2005).

CCR mechanisms in rhizobia

Carbohydrate transport in rhizobia differs from that in Enterobacteriaceae and Firmicutes, as carbon transport is not mediated by PTS. For example, fructose enters *Rhizobium leguminosarum* in an unphosphorylated form, whereas mannose is phosphorylated by the mannose uptake system (Arias et al., 1982; Glenn et al., 1984). In rhizobia, the transport of some organic acids, like succinate, is orchestrated by a dicarboxylate transport system encoded by *dct* genes (Ronson et al., 1987; Watson et al., 1993). In contrast to their utilization by the aforementioned bacteria, sugars are secondary carbon sources for rhizobia, and organic acids are preferred (Ucker and Signer, 1978); thus, their catabolic repression is named revCCR or succinate-mediated catabolite repression (SMCR), as described by Mandal and Chakrabarty (1993). Several studies have shown that succinate and other organic acids repress the activity of sugar transporters and major metabolic pathways like Entner–Doudoroff and Embden Meyerhof Parnas (Arias et al., 1982; Mukherjee and Ghosh, 1987). Interestingly, *Sinorhizobium meliloti* possesses the PTS protein Hpr by analogy to Enterobacteriaceae and the kinase/phosphatase HprK as found in Gram-positive bacteria. According to Pinedo et al. (2008), Hpr plays a role in SMCR, as an *hpr* mutant showed a decrease in SMCR for raffinose but not for lactose. We still lack a detailed understanding of SMCR regulation in rhizobia in terms of how organic acids induce repression and which regulators are implemented (Iyer et al., 2016). revCCR has been described mainly for *Pseudomonas*, in which the fine-tuning of repression is well described, even though the system appears to be different.

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CCR mechanisms in pseudomonads

Sugar uptake in different members of the genus *Pseudomonas* is mediated by ABC transporters, such as the GtsA protein involved in glucose transport in *Pseudomonas stutzeri* A1501 (Liu et al., 2020), and by outer and inner membrane proteins (Raneri et al., 2018). Moreover, organic acids are the preferred carbon source for pseudomonads. Consequently, their CCR is considered revCCR (Collier et al., 1996; Rojo, 2010). revCCR, or organic acid-mediated CCR, is mediated at a post-transcriptional level; the regulation is orchestrated by the master regulator protein catabolic repression control (Crc). Crc first stabilizes the interaction between Hfq and mRNA and subsequently stabilizes the post-transcriptional repression of genes associated with the use of a non-preferred carbon source through the inability of the ribosome to bind to mRNA and initiate translation (Sonnleitner et al., 2012, 2017, 2018; Sonnleitner and Bläsi, 2014) (Figure 1B). Indeed, Hfq was first described in Gram-negative bacteria as a global post-transcriptional regulator based on its RNA binding properties (Sonnleitner and Bläsi, 2014). Thus, under organic acid-mediated CCR conditions, translation of aliphatic amidase (*amiE*), autotransporter esterase (*estA*), and phenazine-specific methyltransferase (*phzM*) mRNAs is repressed by the Crc/Hfq complex in *P. aeruginosa* PAO1 (Sonnleitner et al., 2012). In addition, the two-component system (TCS) CbrAB, which belongs to the NtrBC family, was found to regulate the assimilation of C sources, but its activation is still unclear. CbrAB and NtrBC (a TCS that enables assimilation of nitrogen sources) have been found to act together to maintain a C/N balance (Nishijyo et al., 2001; Li and Lu, 2007; Naren and Zhang, 2021). CbrAB and NtrBC TCSs have been shown to participate in a CCR mechanism for histidine utilization in *Pseudomonas fluorescens* SBW25 by interacting with each other to maintain C/N homeostasis (Naren and Zhang, 2021).

In the absence of the preferred carbon source, the repression is lifted. The CbrAB TCS induces the expression of CrcZ, a small RNA (sRNA), with the help of the sigma factor RpoN. CrcZ has different binding sites with high affinity for Crc. CrcZ sequesters Crc, and thus the Crc/Hfq complex can no longer bind to the mRNA, and the repression is no longer effective (Sonnleitner et al., 2017) (Figure 1B). Interestingly, some *Pseudomonas* species have more than one sRNA that can control the level of free Crc. Whereas *P. aeruginosa* possesses only CrcZ, *Pseudomonas syringae* and *Pseudomonas putida* possess CrcZ/CrcX and CrcZ/CrcY, respectively (Moreno et al., 2012; Filiatrault et al., 2013) (Figure 1C). Their functions seem to be redundant, although their regulation may differ between species. Moreover, CbrAB appears to regulate CrcZ and CrcX transcription in *P. syringae* pv. tomato DC300 (Filiatrault et al., 2013), but it has little effect on CrcY transcription in *P. putida* (Moreno et al., 2012) (Figure 1C).

BENEFICIAL AND DELETERIOUS CCR-MEDIATED INTERACTIONS BETWEEN MICROBES AND PLANTS

Microorganisms associated with distinct plants not only rely on adaptation mechanisms that are required in the local environment but also must adapt to the metabolic activity of other members of the microbiota as well as the host plant itself. It is known that 5%–10% of all bacterial genes are subject to CCR

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(Görke and Stülke, 2008; Rojo, 2010). We argue that a certain proportion of these genes participate in the regulation of interactions within the plant holobiont. These regulations can be either direct, such as by improving plant growth, or indirect, by shielding plants from pathogens. The latter effect can be achieved if CCR affects competition between beneficial microorganisms and pathogens. On the other hand, pathogens also rely on various CCR mechanisms when infecting their hosts. A non-exhaustive list of the different functions regulated by Crc, CcpA, and Crp that are involved in nutrient acquisition or other relevant plant–bacteria interactions is presented in Table 1. Specific CCR-related functions were selected and are subjected to a deeper review below.

Improved use of carbon sources during N fixation or root exudate utilization is regulated by the CCR system

In the free-living (N)-fixing bacterium *Azotobacter vinelandii*, CCR occurs under both diazotrophic and non-diazotrophic conditions (Quiroz-Rocha et al., 2017; Martínez-Valenzuela et al., 2018). For example, the preference for acetate assimilation over glucose has been shown in *A. vinelandii* grown under N-fixing conditions (Taichert et al., 1990). Quiroz-Rocha et al. (2017) provided evidence for the role of CbrAB and Crc/Hfq in the control of CCR processes, specifically the catabolic repression of glucose consumption, under diazotrophic conditions. *A. vinelandii* imports glucose using a GluP transporter (annotated as an H⁺-coupled glucose–galactose symporter), a protein that is absent in most *Pseudomonas* spp. Levels of *gluP* mRNA were shown to be reduced in the presence of acetate and increased 19-fold during glucose uptake. Moreover, *gluP* mRNA levels were reduced when Crc was overexpressed, suggesting that *gluP* may be one of the targets of Crc-Hfq for CCR control. Furthermore, Martínez-Valenzuela et al. (2018) demonstrated that CCR is also controlled by CbrAB and Crc/Hfq under non-diazotrophic conditions.

The plant-growth-promoting bacterium *P. fluorescens* SBW25 can grow on different substrates, including xylose. Xylose is the backbone monomer of hemicellulose, which is a major structural component of the plant cell wall. Xylose also accumulates on plant surfaces colonized by microorganisms and has been identified as a dominant constituent of root exudates in a wide range of plant species (Guyonnet et al., 2017). Many plant-associated bacteria such as *P. fluorescens* SBW25 are able to grow on xylose as a carbon and energy source (Zhang and Rainey, 2008; Liu et al., 2015). In an interesting study, Liu et al. (2017) performed transposon mutagenesis of $\Delta cbrB$ and selected suppressors of xylose utilization (*xut* gene). They provided evidence that Crc/Hfq sequentially binds (and represses) transcriptional activator mRNAs and structural genes involved in xylose catabolism. However, in the absence of succinate, repression is relieved through the competitive binding of two sRNAs, CrcY and CrcZ, whose expression is activated by CbrAB.

Plant-colonizing bacteria continually alter gene expression patterns, particularly those involved in nutrition acquisition, as root exudation is dynamic (Haichar et al., 2014). Interestingly, among *P. fluorescens* SBW25 genes with elevated expression during plant colonization, Gal et al. (2003) and Giddens et al. (2007) found implications of *dctA* for succinate uptake and *xutA* for xylose catabolism. These findings strongly implicate

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succinate and xylose as two carbon substrates frequently encountered *in planta*. Liu et al. (2017) demonstrated that a wild-type strain in which CCR is intact outcompeted a CCR-defective strain when succinate was present at concentrations above 20 μ M; but more significantly, it imposed a fitness burden under conditions of low succinate (<20 μ M). These results suggest that the ecological significance of CCR depends on fluctuating concentrations of the preferred carbon source and may confer a selective disadvantage when succinate is present at low concentrations together with xylose.

Catabolic repression is involved in symbioses with legumes for nitrogen fixation

The ability to utilize a broad range of carbon sources is an important trait that allows adaptability to growth habitats. Rhizobia are found in various associations with plants, as well as in the soil as free-living organisms. To fix nitrogen, these bacteria can utilize the carbon sources available in the plant's root nodules. In exchange, the bacteria provide a constant supply of ammonia to the plant (O'Gara et al., 1989). C4-dicarboxylates, the preferred C source for rhizobia, appear to play a central role in symbiosis. They are the major C source provided to bacteroids by the host plant. These compounds have been shown to support the highest levels of nitrogen fixation in isolated bacteroids *in vitro*. Relevant work on soybean, pea, alfalfa, and lupine bacteroids showed that N₂ fixation in isolated bacteroids was highly stimulated by C4-dicarboxylic acids but not by sucrose (Yurgel and Kahn, 2004). Moreover, C4-dicarboxylates are also chemoattractants for rhizobia. Mutants with defective dicarboxylate transporters generally retain the ability to nodulate a host plant, but the nodules formed are unable to fix nitrogen (Ronson et al., 1981; Arwas et al., 1985). In addition, successful establishment of a symbiosis via penetration of the infection thread by rhizobia requires both continuous biosynthesis of Nod factors and biosynthesis of symbiotic exopolysaccharide (EPS) (Jones et al., 2007). EPS-deficient mutants of *S. meliloti* either fail to nodulate their host plants or induce the formation of ineffective nodules (Leigh et al., 1985; Rolfe et al., 1996; Skorupska et al., 2006). Among the known EPS molecules, succinoglycan plays a critical role in the *S. meliloti* symbiosis with alfalfa (Cheng and Walker, 1998; Mendis et al., 2016). Mutations in components of the PTS-like system (*hpr* and *ellA*) have shown negative effects on EPS production (Pinedo et al., 2008). Deletion of HPr led to impaired control of succinoglycan synthesis. By contrast, a Δ manX (an EIIA^{Man}-type enzyme) strain showed accumulation of high-molecular-weight succinoglycan. In addition to negative effects on EPS production, *manX* null mutants also showed an inability to grow using different carbon sources (Bélanger et al., 2009) and exhibited lower expression levels of genes involved in raffinose and lactose utilization (Bringhurst and Gage, 2002). It therefore seems that, depending on the nature of the carbon source exuded by leguminous plants, the SMCR system of rhizobia can be activated to induce EPS production, which is indispensable for successful infection of the host plant roots leading to ammonia production and hence to plant nutrition.

Catabolic repression mediates host protection against pathogens

In addition to regulation of the primary metabolite, CCR also regulates the production of secondary metabolites involved in

	Bacteria versus plant	Gene/sRNA targeted	Regulatory mechanism	Physiological effect	References
Metabolism/nutrition	<i>Pseudomonas putida</i>	Crc	Crc regulates the expression of branched-chain keto acid dehydrogenase	Complex branched-chain keto acid dehydrogenase	Hester et al. (2000)
	<i>P. putida</i> GPo1	Crc	Crc reduces the expression of the AlkS regulator, which activates alkane degradation	Alkane degradation	Yuste and Rojo (2001)
	<i>Pseudomonas fluorescens</i> SBW25	Hfq/Crc/sRNA and CbrAB	Crc/Hfq sequentially bind to (and repress) mRNAs of both the transcriptional activator and the structural genes involved in xylose catabolism; in the absence of succinate, repression is relieved through competitive binding by two ncRNAs, CrcY and CrcZ, whose expression is activated by CbrAB	Xylose and histidine utilization	Liu et al. (2017) and Zhang and Rainey (2008)
	<i>Azotobacter vinelandii</i>	CbrA/CbrB, Hfq, Crc, CrcZ, and CrcY	Crc-Hfq proteins recognize the <i>gluP</i> A-rich Hfq-binding motif, reducing translation in a Crc-dependent manner; CbrB and CrcZ/Y are essential for GluP expression	Glucose uptake through GluP transporter	Quiroz-Rocha et al. (2017) and Martínez-Valenzuela et al. (2018)
Symbiosis	<i>Sinorhizobium meliloti</i> versus alfalfa	Hpr and EIIAMan-type enzyme	Hpr and EIIAMan-type enzyme positively regulate EPS production; EIIAMan-type enzyme positively regulates growth using different carbon sources: succinate, glucose, glycerol, raffinose, lactose, and maltose	Mutants unable to transport C4 dicarboxylic acids are able to nodulate plants, but the bacteroids do not fix N; mutations in components of the PTS-like system (Hpr and EIIAMan-type enzyme) showed dramatic effects on EPS production	Finan et al. (1983), Ronson and Astwood (1985), Bélanger et al. (2009), and Pinedo et al. (2008)
Antibiotic and antifungal compounds	<i>Pseudomonas chlororaphis</i>	ND	ND	Phenazine 1-carboximide production using L-pyroglutamic acid and glucose; fructose, sucrose, and ribose repress phenazine 1-carboximide production	Van Rij et al. (2004)

Table 1. Carbon catabolite repression-mediated gene regulation in various bacterial and fungal species during interactions with plants.

(Continued on next page)

	Bacteria versus plant	Gene/sRNA targeted	Regulatory mechanism	Physiological effect	References
	<i>P. fluorescens</i> F113	ND	ND	Sucrose, fructose, and mannitol promoted high yields of 2,4-diacetylphloroglucinol (DAPG) by strain F113, whereas glucose and sorbose resulted in very poor DAPG production	Shanahan et al. (1992)
	<i>Streptomyces coelicolor</i>	cAMP-CRP system	<i>cya</i> mutants and strains defective in CRP showed a lack of germination and actinorhodin production	Spore germination and actinorhodin antibiotic production	Süsstrunk et al. (1998)
	<i>Streptomyces griseus</i>	ND	Glucose suppresses streptomycin production by mannosido streptomycinase repression	Spore germination and actinorhodin antibiotic production	(Demain and Inamine, 1970)
Biofilm formation	<i>Bacillus subtilis</i>	CcpA ^a	The expression of the <i>gan</i> operon was significantly induced in <i>ccpA</i> mutants; a putative high-affinity <i>cre</i> box was identified within the <i>ganS</i> promoter region	CcpA regulates the galactan utilization pathway, which results in production of UDP-Gal and UDP-Glu, two sugar nucleotides that are essential precursors for EPS biosynthesis during biofilm formation	Stanley et al. (2002), Marciniak et al. (2012), and Habib et al. (2017)
	<i>Pseudomonas syringae</i> pv. tomato DC3000 versus <i>Arabidopsis thaliana</i>	Crc	Crc inhibits biofilm formation	The Δ <i>crc</i> strain showed enhancement of biofilm formation compared with the wild-type strain	Chakravarthy et al. (2017)
Virulence	<i>P. syringae</i> pv. tomato DC3000	Crc, CrcZ, CrcX	During plant infection, when T3SS is active, the expression of CrcZ and CrcX supports utilization of fructose and citrate (poor C sources)	Sugars such as glucose, sucrose, and fructose are known to be inducers of the <i>P. syringae</i> TTSS genes, whereas tricarboxylic acid intermediates can suppress T3SS <i>in vitro</i> ; fructose and citrate utilization pathways are upregulated when cells are exposed to tomato apoplast extracts	Rico and Preston (2007), Chakravarthy et al. (2017), and Filiatrault et al. (2013)
	<i>Dickeya dadantii</i> versus chicory leaves	Crp-AMPc system	CAMP-CRP positively regulate pectate lyase production, virulence, and pathogenicity	<i>crp</i> mutation has serious consequences for the virulence of <i>D. dadantii</i> , as it strongly decreases pectate lyase production	Reverchon et al. (1991), Nasser et al. (1994), Hugouvieux-Cotte-Pattat (2016), Nasser et al. (1997), and Reverchon et al. (1997)

Table 1. Continued

(Continued on next page)

	Bacteria versus plant	Gene/sRNA targeted	Regulatory mechanism	Physiological effect	References
	<i>Xanthomonas campestris</i> versus Chinese	HprK	HprK regulates genes that make a positive contribution to virulence, extracellular polysaccharides, extracellular enzymes, motility, and stress tolerance	Deletion of <i>hprK</i> demonstrated its requirement for virulence and other associated diverse cellular processes, including extracellular enzyme activity, extracellular polysaccharide production, and cell motility	Li et al. (2019)
Quorum sensing	<i>D. dadantii</i> 3937	Crp–AMPc system	The cAMP–Crp system induces an increase in AHL production when less of the preferred substrate is present; CRP activates <i>expR</i> expression and represses <i>expl</i> transcription	CRP induces virulence	Nasser et al. (1998) and Reverchon et al. (1998)

Table 1. Continued

AHL, N-acyl-homoserine lactone; EPS, exopolysaccharide; T3SS, type III secretion system; ND, not determined.

host–microbe and microbe–microbe interactions. Various rhizobacteria produce bioactive compounds that can protect plants against disease and thus improve plant health. The production of antifungal and antibacterial metabolites is also considered an important prerequisite for optimal performance of biocontrol agents (Chin-A-Woeng et al., 2003). *Pseudomonas* species have been shown to commonly produce several types of antifungal compounds, the most studied of which are pyoluteorin, pyrrolnitrin, phenazines, and 2,4-diacetylphloroglucinol (DAPG) (Chin-A-Woeng et al., 2003). In several *Pseudomonas* species, phenazine production is affected by the available carbon source. For example, Van Rij et al. (2004) tested the effects of different sources of carbon present in tomato root exudates on phenazine production in a *Pseudomonas chlororaphis* strain. The highest production of phenazine 1-carboximide was obtained in the presence of L-pyroglutamic acid and glucose. However, fructose, sucrose, and ribose had negative effects on phenazine 1-carboximide production. Similar results were observed in a *gacA*-inactivated *Pseudomonas* sp. M18G, in which the highest production of phenazine 1-carboxylic acid was achieved with glucose and ethanol as carbon sources (Li et al., 2008). Moreover, DAPG production has also been shown to be regulated by the carbon source in *P. fluorescens* F113 (Shanahan et al., 1992). Sucrose, fructose, and mannitol promoted high yields of DAPG, whereas glucose and sorbose resulted in drastically reduced DAPG production. Thus, catabolic repression in *Pseudomonas* species inhabiting the plant rhizosphere may play a crucial role in the expression of genes involved in the control of plant root diseases, depending on the nature of exudates released by the plants during plant–bacteria interactions.

The production of secondary metabolites, including antibiotics and bioactive compounds, is one of the main characteristics of

members of *Streptomyces*. Glucose is generally the preferred carbon source for *Streptomyces* growth. However, when used in high concentrations, it also interferes with the formation of secondary metabolites (Demain, 1989). For example, glucose suppresses streptomycin production by *Streptomyces griseus*, known for plant growth promotion, by repressing mannosidostreptomycinase (Demain and Inamine, 1970). In addition, production of the actinorhodin antibiotic has been shown to depend on the cAMP–CRP system in the filamentous soil bacterium *Streptomyces coelicolor* (Süsstrunk et al., 1998).

The roles of catabolic repression in biofilm formation

Biofilms are structured communities of microorganisms in which cells are embedded in an extracellular matrix (Flemming and Wuertz, 2019), generally formed to promote bacterial survival in harsh environments (Flemming et al., 2016). Although biofilm formation is a complex process regulated by several different factors in various bacteria, CCR seems to be an important global regulator involved in biofilm production. Plant-beneficial representatives of *B. subtilis* can utilize polysaccharides and other carbohydrate substances present in the rhizosphere as major carbon sources, many of which are derived from the decomposition of plant tissues (Ochiai et al., 2007). Plant polysaccharides have been shown to stimulate the formation of root-associated multicellular communities, or biofilms. In *B. subtilis*, a five-gene *gan* operon (*ganSPQAB*) has been shown to participate in the utilization of galactan, a plant-derived polysaccharide (Habib et al., 2017). Interestingly, plant-derived galactan was shown to promote biofilm formation in *B. subtilis* (Habib et al., 2017). A putative high-affinity *cre* box was identified in the promoter region of *ganS*, with strong repression upon CcpA induction (Marciniak et al., 2012). In addition, by quantifying biofilm formation at

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different glucose concentrations, Stanley et al. (2003) showed that biofilm formation by *B. subtilis* is repressed by catabolites through the transcription factor CcpA. The same observation was also made in *P. syringae* DC3000; a Δ *crc* mutant showed enhanced biofilm formation compared with the wild-type strain (Chakravarthy et al., 2017). *P. syringae* is considered to be a hemibiotrophic pathogen that can cause diseases in both tomato and *Arabidopsis*. When conditions are optimal, the bacteria invade plant tissues via wounds or natural openings like stomata, colonize the apoplastic space, and metabolize host nutrients in order to multiply and survive. The observed increase in biofilm formation may be detrimental for the *crc* mutant once it is inside the plant cell because of its inability to spread within the plant and hence grow and infect the plant.

Catabolic repression mediating quorum sensing regulation

Bacterial species employ a complex communication mechanism termed quorum sensing (QS) that is used to link cell density to gene expression. In this process, bacteria secrete chemical signaling molecules, called autoinducers (AIs), that accumulate as cell density increases. Once the AI level reaches a threshold, signaling a “quorum” of cells, AI signals are transported into cells, where they activate gene expression and enable coordinated phenotypic responses in the population (Ha et al., 2018). The QS system contributes to different processes in plant–microbe interactions, such as cell density maintenance, biofilm formation, antibiotic production, natural competence, sporulation, and pathogenicity (Pena et al., 2019). Some rhizobacteria and phytopathogenic bacteria have been shown to express several important genes under QS control. Few studies have focused on how the cell regulates QS processes, for example, based on the availability of substrates like glucose, which provides a link between CCR and QS. The most-studied model is *E. coli*, in which there is evidence that the QS system mediated by the autoinducer AI-2 is partially regulated by substrate availability and cellular metabolism. LuxS synthesizes AI-2, after which AI-2 accumulates extracellularly. AI-2 is imported by LsrACDB and phosphorylated by the kinase LsrK, which sequesters it within the cell. The phosphorylated AI-2 relaxes LsrR-mediated repression of the *lsr* operon, allowing transcription of *lsr* genes and accelerated AI-2 uptake. Several studies suggest that the bidirectional *lsr* operon, in addition to being regulated by LsrK and LsrR, is also subject to CCR. For example, activation of the *lsr* promoter does not occur in the presence of glucose (Wang et al., 2005) or glycerol (Xavier and Bassler, 2005) and requires the global regulators cAMP and CRP (Wang et al., 2005). Moreover, cAMP–Crp was shown to induce the *lsr* AI-2 uptake system gene by binding to its promoter (Xavier and Bassler, 2005). In addition, cAMP–Crp stimulates the production of the Hfq-binding sRNA CyaR, which can bind to and destabilize luxS mRNA, thereby reducing LuxS and AI-2 levels (De Lay and Gottesman, 2009). Ha et al. (2018) reported that HPr of *E. coli* co-purifies with LsrK. LsrK activity is inhibited when bound to HPr, revealing novel links between QS activity and sugar metabolism. A role for cAMP–Crp in the regulation of QS genes was also identified in the phytopathogen *Dickeya dadantii*, which contains the QS signal generator (*expI*) and the response regulator (*expR*). ExpR activates virulence genes in response to ExpI-made N-acyl-homoserine lactone (AHL)

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(Nasser et al., 1998; Reverchon et al., 1998). *In vivo* and *in vitro* studies have revealed that CRP functions as an activator of *expR* expression but as a repressor of *expI* transcription. This could explain the observation that the production of AHL decreases after quorum has been reached and when bacteria enter the stationary phase (Nasser et al., 1998). cAMP–Crp appears to be a modulator of QS gene expression, causing an increase in AHL production when less-preferred substrates are present. A regulatory effect of catabolic repression on QS signaling has been extensively studied in human-pathogenic bacteria such as *P. aeruginosa* PAO1, demonstrating a substantial impact of the Crc protein on QS-related social behavior, such as synthesis of virulence factors, biofilm formation, and fitness (Linares et al., 2010; Sonnleitner et al., 2012; Zhang et al., 2013). We hypothesize that the same could apply to rhizobacteria and that the dynamic nutritional environment of the host may have an impact via CCR on the cross talk between rhizosphere processes such as plant colonization and infection.

Catabolic repression-mediated control of virulence in plant pathogenic bacteria

In many pathogenic microorganisms, the CCR mechanism is crucial for virulence-gene expression and thus pathogenicity (Table 1). It should be noted that the primary aim of pathogenic bacteria is to access nutrients rather than to damage the host, and the expression of virulence genes is mostly linked to the nutrient supply of the bacteria (Görke and Stülke, 2008). For most pathogens, penetration through plant cell walls is of major importance for the invasion of host tissue and the acquisition of nutrients (Collmer and Keen, 1986). As a plant pathogen, *Dickeya* is capable of catabolizing a wide range of plant oligosaccharides and glycosides. The pectate lyases secreted by the bacterium provoke general disorganization of the plant cell wall and also release oligosaccharides, which are used as carbon sources by various *Dickeya* strains. Specific and global regulators serve to tailor pectate lyase (*pel*) gene expression to available substrates. Among them, the cAMP–CRP system is the best characterized (Nasser et al., 1997; Reverchon and Nasser, 2013). A *crp* mutation has serious consequences for the virulence of *D. dadantii*, in that it strongly decreases pectate lyase production (Reverchon et al., 1997). The *D. dadantii crp* mutant retains the ability to grow only on glucose, fructose, or sucrose, three efficiently metabolized carbon sources that are highly abundant in plant tissues (Hugouvieux-Cotte-Pattat and Charaoui-Boukerzaza, 2009). Thus, Crp plays a crucial role in the pathogenesis of *D. dadantii* by tuning the expression of virulence genes to the nutrient conditions encountered during plant infection.

The Crc/CrcZX system of *P. syringae* DC3000 is involved in the regulation of the type III protein secretion system (T3SS) encoded by *hpr* genes that deliver effectors into plant cells (Filiatrault et al., 2013). Indeed, sugars such as glucose, sucrose, and fructose are known to be inducers of the *P. syringae* T3SS genes, whereas intermediate tricarboxylic acids can suppress the T3SS *in vitro* through catabolite repression. The fructose and citrate utilization pathways used by *P. syringae* are upregulated when cells are exposed to tomato apoplast extracts (Rico and Preston, 2008). Filiatrault

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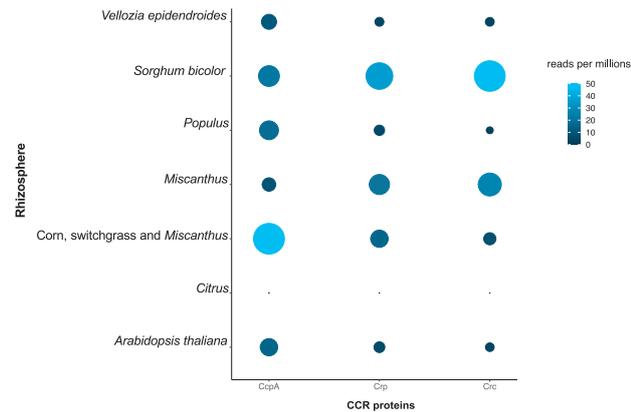


Figure 2. Carbon catabolite repression (CCR) gene expression in different plant rhizospheres.

The level of gene expression is quantified as the number of hits per 10^6 reads. Target genes are *crc* from *Pseudomonas fluorescens* F113, *ccpA* from *Bacillus subtilis*, and *crp* from *E. coli*. The rhizosphere metatranscriptomics bioproject accession numbers are, for *Vellozia epidendroides*, PRJNA441428; *Sorghum bicolor*, PRJNA406786; *Populus*, PRJNA375667; *Miscanthus*, PRJNA337035; corn, switchgrass, and *Miscanthus*, PRJNA365487; and *Arabidopsis thaliana*, PRJNA366978, PRJNA366977, and PRJNA336798.

et al. (2013) hypothesized that when the T3SS is active, expression of *CrcZ* and *CrcX* sRNAs may promote the utilization of carbon sources that are abundant in the plant apoplast. Moreover, Chakravarthy et al. (2017) provided evidence for a clear link between *Crc* and virulence in *P. syringae* DC3000. In this work, a Δ *crc* mutant caused fewer disease symptoms compared with the wild type in tomato and *Arabidopsis thaliana*. In addition, the *crc*-deficient mutant showed a delayed hypersensitive response when used to infiltrate *Nicotiana benthamiana* and tobacco. Taken together, these results demonstrated the direct and indirect roles played by *Crc* in nutrient acquisition and virulence-related traits.

CATABOLITE REPRESSION: EXPLORATION OF PREVALENT MECHANISMS IN BACTERIAL GENOMES

The CCR system has been described in different model strains, but information on its distribution among microbial genomes is still limited (Warner and Lolkema, 2003; Filiatrault et al., 2013). Here, we investigated the presence of CCR systems in bacteria through BLAST analysis in two genomic databases (NCBI and MAGE) using different CCR models that are genetically well characterized (see the supplemental information). We focused on bacteria in which CCR master genes are well known. The presence and conservation of those master genes allowed us to assess their distribution among various available bacterial genomes. It is worth noting that such analyses are dependent on the availability as well as the quality of the sequenced genomes. Therefore, it can be assumed that future studies will unravel the presence of CCR in additional bacterial genera and species.

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Distribution of CCR among *Pseudomonas* and *Azotobacter* species

When assessing the CCR system in pseudomonads, we used several master genes that have been described as essential (Figure 2). We used the catabolic repression protein *Crc*, the global transcriptional regulator *Hfq*, the different sRNAs (*CrcZ*, *CrcY*, and *CrcX*), and the TCS *CbrAB*. Furthermore, we selected species with conserved homologous proteins (>40% identity in more than 80% of the aligned sequence, see supplemental information), synteny in the *cbrAB* genomic region, and potential involvement in plant–bacteria interactions, with the exception of *Pseudomonas oleovorans* T9AD, which was isolated from a marine environment. One or several genes in the CCR system have been functionally characterized in several *Pseudomonas* species, encompassing *P. putida*, *P. fluorescens*, *P. syringae*, and *P. aeruginosa* (Bharwad and Rajkumar, 2019). Our assessment indicated that the query proteins are widely distributed in this genus, suggesting their potential role in CCR (Table 2). Even though *Hfq* has a crucial function in CCR in *Pseudomonas*, its multiple functions in pleiotropic post-transcriptional regulation (Brennan and Link, 2007) may explain its high conservation among bacterial taxa (data not shown) and led us to not use it for the implemented screening. Thus, the genomic distribution of the CCR system was assessed using *CbrAB*, *Crc*, and sRNA (*CrcX/Y/Z*) sequences and showed high conservation only in pseudomonads (Table 2). Among the 26 species included here, genes from 5 had previously been described genetically as master genes and sRNAs involved in the CCR (Hester et al., 2000; Filiatrault et al., 2013; Liu et al., 2017; Quiroz-Rocha et al., 2017).

Azotobacter species, for example, *A. vinelandii*, also harbored the same, highly conserved CCR system found in *Pseudomonas* species (Table 2). Interestingly, it is known that this conservation is sustained, as the *Crc*-*Hfq* proteins from *A. vinelandii* and *P. putida* are functionally interchangeable (Quiroz-Rocha et al., 2017). In addition to these targeted proteins, CCR is mediated by sRNAs that antagonize the effect of *Crc*/*Hfq* proteins (Filiatrault et al., 2013). *CrcX*, *CrcY*, and *CrcZ* are the three sRNAs described to date. The *CrcZ*, *CrcZ/CrcY*, and *CrcZ/CrcX* sRNAs have been described in *P. aeruginosa* PAO1, *P. putida*, and *P. syringae*, respectively (Sonnleitner et al., 2009; Moreno et al., 2012; Filiatrault et al., 2013). Here, we combined BLAST and synteny analyses to identify and affiliate the best assignment of each sRNA (Table 2 and Supplemental Table 3). A more variable distribution of these sRNAs was observed among the *Pseudomonas* groups (Table 2). Three groups with or without *CrcX/CrcY* can be distinguished (Table 2). In the first group, which includes *P. syringae*, all three sRNAs are present. This suggests that in strains from the first group, all three sRNAs seem to be important and may work in concert, as suggested by Moreno et al. (2012). By contrast, strains in the second group, represented by *P. putida*, *P. fluorescens*, and *A. vinelandii*, harbored *CrcZ/CrcY* or *CrcZ/CrcX* sRNAs (Table 2). In *A. vinelandii*, both sRNAs (*CrcZ/CrcY*) play a key role in CCR (Martínez-Valenzuela et al., 2018), but this remains to be confirmed in the other species (Table 2). In the last group presented in Table 2, only *CrcZ* is present and seems to act solely as a unique sRNA in CCR, as demonstrated in

Strain ^a	Ref_Seq	Habitat	Crc			CbrA			CbrB			crcZ			crcY			crcX		
			Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val
<i>P. syringae</i> pv. tomato DC3000 ^{a,b}	NC_004578.1	Phytopathogen	100 (86)	0	99 (80)	0	99 (81)	0	94 (76)	0	31 (84)	3 e-22	100 (100) ^b	0	100 (95)	0	100 (100) ^b	0	100 (93)	0
<i>P. amygdali</i> pv. <i>lachrymans</i> NIM002	NZ_CP042804.1	Phytopathogen	100 (86)	0	99 (80)	0	99 (81)	0	94 (76)	0	100 (74)	5 e-30	100 (95)	0	100 (93)	0	100 (93)	0	100 (93)	0
<i>P. cerasi</i> isolate PL963	NZ_LT963395.1	Phytopathogen	100 (86)	0	99 (80)	0	99 (81)	0	96 (76)	0	100 (75)	2 e-38	100 (93)	0	100 (93)	0	100 (93)	0	100 (93)	0
<i>P. coronafaciens</i> pv. <i>oryzae</i> str. 1_6	NZ_CP046035.1	Phytopathogen	100 (87)	0	99 (79)	0	99 (82)	0	95 (77)	0	70 (78)	4 e-36	100 (90)	0	100 (90)	0	100 (90)	0	100 (90)	0
<i>P. asturiensis</i> CC1524	NZ_CP047265.1	Phytopathogen	100 (86)	0	99 (80)	0	99 (84)	0	94 (77)	0	100 (76)	7 e-39	98 (87)	0	98 (87)	0	98 (87)	0	98 (87)	0
<i>P. putida</i> KT2440 ^{a,b}	NP_747393.1	Soil bacteria	100 (86)	0	99 (81)	0	99 (84)	0	60 (78)	5-35	100 (100) ^b	0	No	-	No	-	No	-	No	-
<i>P. avellanae</i> R2leaf	NZ_CP026562.1	Phytopathogen	100 (86)	6 e-174	99 (79)	0	99 (82)	0	92 (76)	0	No	-	100 (99)	0	100 (99)	0	100 (99)	0	100 (99)	0
<i>P. viridiflava</i> TA043	GCA_000452485.1	Phytopathogen	100 (87)	1 e-171	99 (81)	0	99 (83)	0	92 (76)	6 e-45	No	-	99 (87)	3 e-112	99 (87)	3 e-112	99 (87)	3 e-112	99 (87)	3 e-112
<i>P. fragi</i> A22	GCA_000250595.1	Stored chilled meats	100 (87)	8 e-173	99 (81)	0	99 (82)	0	92 (75)	1 e-36	90 (74)	4 e-29	No	-	No	-	No	-	No	-
<i>P. extremotrais</i> 14-3	GCA_000242115.2	Highly stress-resistant Antarctic bacterium	100 (89)	6 e-175	99 (82)	0	100 (84)	0	93 (80)	3 e-68	90 (76)	1 e-43	No	-	No	-	No	-	No	-
<i>P. brassicaearum</i> NFM421	NC_015379.1	Soil bacteria PGPR	100 (88)	9 e-177	99 (81)	0	99 (83)	0	90 (76)	0	90 (77)	0	No	-	No	-	No	-	No	-
<i>P. fluorescens</i> F113 ^a	NC_016830.1	Soil-denitrifying bacteria	100 (88)	2 e-173	99 (82)	0	100 (84)	0	90 (78)	2 e-54	90 (78)	5 e-53	No	-	No	-	No	-	No	-
<i>P. protegens</i> CHA0	NC_021237.1	Soil bacteria PGPR	100 (89)	4 e-175	99 (83)	0	99 (85)	0	90 (78)	2 e-62	93 (75)	4 e-57	No	-	No	-	No	-	No	-
<i>P. chlororaphis</i> ATCC 17415	NZ_CP027714.1	Soil PGPR bacteria	100 (89)	6 e-175	99 (82)	0	100 (84)	0	90 (78)	1 e-56	94 (74)	2 e-31	No	-	No	-	No	-	No	-
<i>P. plecoglossicida</i> NyZ12	NZ_CP010359.1	Pathogen of fish	100 (86)	2 e-170	99 (82)	0	99 (84)	0	60 (78)	1 e-32	100 (97)	0	No	-	No	-	No	-	No	-
<i>P. entomophila</i> L48	NC_008027.1	Soil bacteria and entomopathogen	100 (85)	7 e-168	99 (82)	0	99 (84)	0	95 (73)	4 e-27	97 (89)	3 e-125	No	-	No	-	No	-	No	-

Table 2. Similarity of CCR systems between *Pseudomonas* and *Azotobacter* strains

(Continued on next page)

Strain ^a	Ref_Seq	Habitat	Crc		CbrA		CbrB		crcZ		crcY		crcX	
			Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val	Cov (ld)	E-val
<i>Azotobacter vinelandii</i> DJ ^a	NC_012560.1	Soil diazobacteria	100 (88)	1 e-176 (77)	98 (77)	0	99 (80)	0	90 (72)	2 e-42 (73)	94 (73)	1 e-46	No	-
<i>Azotobacter chroococcum</i> NCIMB 8003	NZ_CP010415.1	Soil diazobacteria	99 (86)	3 e-175 (78)	99 (78)	0	100 (81)	0	90 (71)	3 e-35 (72)	93 (72)	6 e-38	No	-
<i>Azotobacter salinestris</i> strain KACC 13899 ^c	CP037918.1	Soil diazobacteria	99 (84)	0	99 (80)	0	98 (83)	0	ND	ND	ND	ND	ND	ND
<i>P. aeruginosa</i> PAO1 ^b	NC_002516_2	Plant, animal, and human pathogen	100 (100) ^b	0	100 (100) ^b	0	100 (100) ^b	0	100 (100) ^b	0	No	0	No	-
<i>P. balearica</i> DSM6083	NZ_CP007511.1	Bioremediation (naphthalene)	100 (88)	5 e-176 (78)	99 (78)	0	99 (83)	0	27 (86)	1 e-27 (86)	No	-	No	-
<i>P. psychrotolerans</i> L19	GCA_000236825.2	Copper resistance	100 (84)	5 e-169 (75)	99 (75)	0	100 (81)	0	91 (72)	4 e-45 (72)	No	-	No	-
<i>P. stutzeri</i> DSM4166	NC_017532.1	Soil bacteria	100 (88)	8 e-175 (79)	99 (79)	0	100 (84)	0	92 (76)	6 e-45 (76)	No	-	No	-
<i>P. denitrificans</i> ATCC 13867	NC_020829.1	Soil-denitrifying bacteria (produce vitamin B12)	100 (89)	2 e-177 (89)	98 (89)	0	99 (89)	0	92 (86)	8 e-109 (86)	No	-	No	-
<i>P. alcaliphila</i> JAB1	NZ_CP016162.1	Bioremediation (phenol)	100 (89)	2 e-175 (83)	99 (83)	0	99 (87)	0	93 (80)	6 e-70 (80)	No	-	No	-
<i>P. oleovorans</i> T9AD	NZ_LR130779.1	Marine environment	100 (89)	8 e-175 (83)	99 (83)	0	99 (87)	0	93 (80)	6 e-70 (80)	No	-	No	-
<i>P. pseudoalcaligenes</i> CECT5344	NZ_HG916826.1	Bioremediation (cyanide)	100 (89)	3 e-175 (83)	99 (83)	0	99 (87)	0	93 (80)	5 e-66 (80)	No	-	No	-

Table 2. Continued

Coverage (Cov) and percentage of identity (ld) are indicated for each BLAST analysis.

^aSpecies are already genetically described in terms of functional CCR systems (Hester et al., 2000; Filiatrault et al., 2013; Liu et al., 2017; Quiroz-Rocha et al., 2017). Habitat, taxonomy, and other phenotype information, including pathogenicity, were obtained from the NCBI microbial genomes database (<http://www.ncbi.nlm.nih.gov/genomes/proks.cgi>).

^bAnalyses (PSI-BlastP for proteins and BlastN for sRNA and coding genes; see supplemental information) were performed using the sequences Crc (PA5332), CbrB (PA4726), CbrA (PA4725), and crcZ (PA4726.11) from *P. aeruginosa* PAO1; crcY (PP_mr44) from *P. putida* KT2440; and crcX (PSPTO_5669) from *P. syringae* pv. tomato DC3000 against references from MicroScope (<https://mage.genoscope.cns.fr/microscope/home/index.php>) and the NCBI database (<https://www.ncbi.nlm.nih.gov/genome>). Only species with conserved CbrAB synteny were selected for further analysis. Synteny was determined using MicroScope and the *Pseudomonas* browser (<https://beta.pseudomonas.com/blast/setnblast>).

^cThe percentage of identity, coverage, and E-value (E-val) were obtained by blastN. No proteins were annotated in the genome and, consequently, we could not affiliate sRNA found by blastN in NCBI (position 1, 1786973/1787336; position 2, 2234862/2235192) to the references (crcZ, crcY, or crcX) and also could not include them in the phylogeny analysis. ND, not determined.

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P. aeruginosa PAO1 (Sonnleitner et al., 2009), but this requires further investigation for all other species from this group. Possession of a single sRNA reflects a lesser need for fine-tuned regulation of metabolism compared with other pseudomonads, as previously suggested (Moreno et al., 2012).

Interestingly, the synteny of most sRNAs was conserved, as they harbored the same flanking gene in the left and right positions (Supplemental Table 1). CrcY is a unique sRNA with less conserved synteny. Indeed, 7 of the 13 assessed strains did not conserve Acyl-CoA dehydrogenase in the proximal region of CrcY, based on the use of *P. syringae* DC3000 as a reference. However, several of them conserved the same flanking gene as *crcY* of *P. putida* by harboring an MgtC transporter. Interestingly, *A. vinelandii* and *Azotobacter chroococcum* are the only species that also did not show conserved synteny in the downstream position of *crcY*. Indeed, the hydromethylglutaryl-CoA lyase (*mvaB*) gene was not present in the *crcY* right flanking region but rather in another locus of the *Azotobacter* genome (Supplemental Table 1). In the latter species, the affiliation of this second sRNA to *crcY* is questionable; however, it was retrieved with a high percentage of identity. Future experiments tracing the evolution of sRNAs in *Pseudomonas* and *Azotobacter* could reveal whether vertical or horizontal gene transmission has occurred between these species. Interestingly, the major *Pseudomonas* species shown to harbor this CRC system are not yet studied, although they are present in various ecological niches (Table 2) and associated with beneficial as well as pathogenic isolates. This reinforces the fact that the CRC system is involved in both beneficial and deleterious interactions. Remarkably, the strains that possess all the sRNAs are all pathogenic (Table 2), emphasizing that sRNA redundancy is likely to be important for this type of interaction. Even though the sRNAs are interchangeable for bacterial growth, as was demonstrated for CrcY and CrcZ in *P. putida* (Moreno et al., 2012), they may have different biological functions and confer distinct advantages or disadvantages under specific environmental conditions (Liu et al., 2017). Taken together, our results call for further investigations into the occurrence and redundancy of sRNAs that regulate CCR in phytopathogenic or beneficial strains as a response to plant exudate composition. This could facilitate optimization of their effects by triggering ecological functions such as QS, biofilm formation, and antibiotic or virulence factor production.

In addition, certain strains have been identified that are known to degrade xenobiotic compounds in soils (e.g., *P. pseudoalcaligenes* and *P. balearica*) (Table 2). Typically, these compounds are used as complex carbon sources, and their CCR-dependent degradation may reduce their ecological impacts on the environment. A good understanding of the fine regulation of the assimilation of these complex compounds by CCR could provide an ecological perspective for improving the biological remediation or assimilation of these pollutants in soil.

Distribution of pseudomonad CCR in other bacterial groups

Bioinformatics analysis using the same *Pseudomonas*-derived CCR sequences and synteny (CbrAB, Crc, and sRNAs) revealed

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the presence of this system in 98 other bacterial genera in addition to *Pseudomonas* and *Azobacter* (Supplemental Table 2). However, the percentage of sequence identity was substantially lower (40%–84%). Even though these proteins were annotated as homologous to those found in the pseudomonads group, functional experimentation must be performed to confirm their involvement in CCR. Interestingly, the distribution of Crc and CbrAB was found to be more restricted to γ -proteobacteria covering 11 different orders; there were only two exceptions, belonging to Firmicutes and to β -proteobacteria (Supplemental Table 2). Among all analyzed species, we did not find *cbrAB* in the genomes of *Acinetobacter baylyi*, *Acinetobacter baumannii*, or *Vibrio cholerae*, even though the roles of Crc and Hfq have been previously investigated, but the direct link to catabolic repression has not been demonstrated (Zimmermann et al., 2009; Vincent et al., 2012; Kuo et al., 2017). Notably, NtrBC, a TCS that plays an important role in N source utilization, has also been described as playing a pivotal role in the C/N balance in concert with the CbrAB system (Li and Lu, 2007). In addition, NtrBC was widely distributed in Proteobacteria, including the genomes of *A. baylyi*, *A. baumannii*, and *V. cholerae* (data not shown). Thus, in Proteobacteria that do not conserve CbrAB, it would be interesting to investigate the role of NtrBC with regard to the CCR system, as has already been characterized in *P. fluorescens* SBW25 for histidine utilization (Naren and Zhang, 2021). For the main species harboring the homologous CbrAB and Crc proteins (Supplemental Table 1), a blastN for all sRNAs (CrcZ/X/Y) was performed but provided no results. This result suggests the involvement of other divergent sRNAs, the activity of the CCR system in an sRNA-independent manner, or inefficiency of the CCR system owing to a lack of genetic regulation. Three species (*Paucimonas lemoignei*, *Priestia aryabhatai*, and *Stenotrophomonas rhizophila*) from different orders are exceptions, as they harbor one or two sRNAs in their genomes (Supplemental Table 1), suggesting possible horizontal gene transmission. Regarding their habitat, and unlike the pseudomonads groups (Table 2), the identified bacteria are found mostly in aquatic habitats (Supplemental Table 2), which offers a novel view of the implications of the CCR system for this less-investigated ecosystem. Certain bacteria that are capable of fixing N (e.g., *Teredinibacter turnerae*, *Azomonas agilis*) may rely on the CCR system, as has been found for telluric bacteria (e.g., *Azotobacter*). This suggests that CCR systems are not limited to carbon recycling, as observed in *Pseudomonas* species, but may also be involved in N assimilation. In a broader context, further study of these bacterial models, including aquatic strains, could provide evidence for other environmentally relevant functions regulated by CCR.

Distribution of catabolic repression mechanisms known from *E. coli*

To investigate the distribution of the *E. coli* CCR system across various bacterial taxa, we looked for high conservation (>40% identity in >80% of the aligned sequence) of the three master proteins in this system: Crp, CyaA, and the glucose-specific component EIIA. We documented the wide dispersal of this CCR system (Supplemental Table 3), and 54 different genera were shown to possess these three conserved proteins (Supplemental

Class	Strain	Ref_Seq	Habitat	Coverage (identity) ^a			Database
				Crp	CyA	EIA	
CCR system in Enterobacteriaceae							
δ-proteobacteria	<i>Escherichia coli</i> K12	ECK.1	Laboratory strain (reference)	100 (100)	100 (100)	100 (100)	MAGE
	<i>Atlantibacter subterranea</i> AS_373	RHXB01.1	Contaminated subsurface sediment	100 (99)	100 (92)	100 (92)	MAGE
	<i>Citrobacter koseri</i> ATCC BAA-895	NC_009792.1	Ubiquitous in soil and water (nitrogen fixing)	100 (99)	100 (96)	100 (98)	MAGE
	<i>Dickeya dadantii</i> 3937*	NC_014500.1	Phytopathogen	100 (99)	100 (83)	100 (95)	MAGE
	<i>Erwinia carotovora</i> subsp. <i>atroseptica</i> SCRI1043 (or <i>Pectobacterium carotovorum</i>) ^b	NC_004547.2	Phytopathogen	100 (99)	98 (83)	100 (95)	MAGE
	<i>Pantoea agglomerans</i> IG1	NZ_BAEF.1	Plant symbiont (N fixing)	100 (99)	99 (83)	100 (87)	MAGE
	<i>Brenneria nigrifluens</i> DSM 30175	NZ_CP034036.1	Phytopathogen	99 (99)	83 (97)	100 (94)	NCBI
	<i>Phaseolibacter flectens</i>	NZ_JAEE01000001.1	Phytopathogen	99 (94)	97 (67)	100 (73)	NCBI
	<i>Tolumonas auensis</i>	NC_012691.1	Toluene producer	99 (90)	96 (52)	100 (83)	NCBI
	<i>Zobellella maritima</i>	NZ_QCZE01000001.1	Sediments (degrades polycyclic aromatic hydrocarbons)	99 (88)	98 (50)	100 (78)	NCBI
	<i>Jejubacter calystegiae</i> strain KSNA2	NZ_CP040428.1	Stem tissue of <i>Calystegia soldanella</i> plant	99 (99)	100 (88)	100 (89)	NCBI
	<i>Lelliottia amnigena</i> strain NCTC12124	NZ_CP077331.1	Soil	99 (98)	100 (92)	100 (99)	NCBI
	<i>Kosakonia arachidis</i> strain KACC 18508	NZ_CP045300.1	Soil	99 (100)	100 (93)	100 (98)	NCBI
β-proteobacteria	<i>Thaueria selenatis</i> AX ATCC 55363	NZ_CACR.1	Sediments	100 (99)	100 (94)	100 (98)	MAGE
CCR system in Firmicutes							
Firmicutes	<i>Bacillus subtilis</i> 168	BSU.1	Laboratory strain	100 (100)	100 (100)	100 (100)	MAGE
	<i>Bacillus amyloliquefaciens</i> Bs006 ^b	LJAU.1	PGPR bacteria (<i>Physalis peruviana</i> roots)	100 (95)	100 (96)	100 (100)	MAGE
	<i>Peribacillus acanthi</i>	NZ_QBBX01000001.1	Rhizobacteria (<i>Acanthus ilicifolius</i>)	100 (75)	99 (78)	100 (70)	NCBI
	<i>Pullulanibacillus pueri</i>	NZ_BMFV01000001.1	Plant (tea)	98 (73)	96 (65)	100 (61)	NCBI
	<i>Pueribacillus theae</i>	NZ_QCZG01000001.1	Plant (tea)	98 (71)	99 (71)	97 (44)	NCBI
	<i>Ammoniphilus oxalaticus</i>	NZ_MCHY01000001.1	Rhizosphere (<i>Rumex acetosa</i>)	98 (64)	97 (60)	95 (46)	NCBI
	<i>Fontibacillus phaseoli</i>	NZ_QPJW01000001.1	Plant nodules (<i>Phaseolus vulgaris</i>)	98 (63)	97 (68)	97 (55)	NCBI
	<i>Saccharibacillus sacchari</i> DSM 19268	JFBU01.1	Endophyte (<i>Saccharum officinarum</i>)	98 (63)	98 (65)	98 (54)	MAGE

Table 3. CCR system comparison in *Escherichia coli* and *Bacillus* species.

(Continued on next page)

Class	Strain	Ref_Seq	Habitat	Coverage (identity) ^a			Database
	<i>Paenibacillus polymyxa</i> ATCC 15970	CP011420.1	PGPR bacterium (N fixing)	98 (62)	99 (65)	99 (54)	MAGE
	<i>Bhargavaea beijingensis</i>	NZ_FNAR01000001.1	Root of a ginseng plant	98 (59)	96 (64)	97 (48)	NCBI
	<i>Atopococcus tabaci</i>	NZ_AUCD00000000.1	Plant (tobacco)	99 (58)	96 (52)	100 (63)	NCBI

Table 3. Continued

PSI-BlastP analyses were conducted with the sequences Crp (ECK3345), CyaA (ECK3800), and PTS (ECK2412) proteins from *E. coli* (strain K12) or CcpA (BSU29740), HprK (BSU35000), and Hpr (BSU13900) proteins from *Bacillus subtilis* (strain 168) using MicroScope and NCBI genome databases. The *E. coli* CCR system was found in different Proteobacteria, and the *Bacillus* CCR system was present in members of Firmicutes. This list was extracted for Supplemental Tables 3 and 4 by selecting only telluric bacteria and/or bacteria associated with plants (endophytic, epiphytic, or rhizobacteria). Habitat, taxonomy, and other phenotype information, including pathogenicity, were obtained from the NCBI microbial genomes database (<http://www.ncbi.nlm.nih.gov/genomes/proks.cgi>). Information concerning strains used is provided according to the genome browser.

^aAll E-values are $<10 E^{-10}$ and are included in Supplemental Tables 3 and 4.

^bSpecies have already been described as harboring functional CCR systems (Tsuyumu, 1979; Reverchon et al., 1991; Chen et al., 2020).

Table 3). Interestingly, most bacteria were assigned to γ -proteobacteria, with only one exception (*Thauera selenatis*, assigned to β -proteobacteria) (Supplemental Table 3). Moreover, *Pseudomonas* (γ -proteobacteria) strains showed the presence of a conserved Crp protein in their genomes, but the other two proteins (CyaA/EIIA) were either not conserved or absent. The presence of Crp in *P. putida* has been reported previously, but the growth of a *crp* mutant was not affected by the carbon compounds tested and was impaired only by the use of various dipeptides as a nitrogen source (Milanesio et al., 2011). These results provided clear evidence that CCR is not Crp dependent in *Pseudomonas*.

Overall, we observed a high occurrence of this CCR system among Proteobacteria from diverse habitats. It is worth noting that a large proportion of the detected species are associated with animals or insects as pathogens, symbionts, or endophytes. CCR is poorly documented in these host-associated microbes. Among the detected species, some have been reported to degrade pollutants or fix nitrogen (Table 3), supporting previous suggestions related to the assimilation of complex carbon compounds and N by bacterial communities through fine regulation of CCR. Other detected species have been described as associated with plants, either as symbionts or pathogens, demonstrating once again the role of CCR in crucial ecological functions involved in plant–bacteria interactions.

Distribution of CCR mechanisms from *Bacillus* across other bacterial taxa

To investigate the distribution of CCR mechanisms known from *Bacillus* in other bacterial taxa, we searched for high conservation ($>40\%$ identity in $>80\%$ of the aligned sequence) of three master proteins: the phosphocarrier HPr, its kinase/phosphorylase HPr-K/P, and the catabolic control protein CcpA. This exploration resulted in the identification of 168 different genera (Supplemental Table 4) that almost all belong to the class Firmicutes. The two exceptions were assigned to *Haloplasma contractile* and *Mycobacteroides abscessus* subsp. *abscessus*, which showed variation in identity scores (46%–80%) for the query proteins. Similarly, *Rhizobiales* species are known to harbor the HPr-K

protein, but our analysis showed high divergence from the well-characterized proteins involved in the *Bacillus* CCR system (data not shown). Furthermore, *Rhizobiales* species possessed a gene encoding the glucose-specific component EIIA described in Proteobacteria, but this protein was also markedly divergent from the one found in *E. coli* (identity $<40\%$). The role of these proteins in the CCR of *Rhizobia* has already been demonstrated (Table 1). Remarkably, *Rhizobiales* combine genes encoding CCR from Proteobacteria and Firmicutes in their genomes, but their high divergence suggests a complex evolutionary history.

In Firmicutes, this system also showed a broad range of divergence (from 40% to 100% identity), regardless of the targeted proteins, suggesting a more relaxed pressure for substitutions in these proteins if they are functional (Supplemental Table 4). Bacteria possessing this CCR system were found to originate from various habitats, including soil and seawater. Several bacteria have been isolated from aliments and/or used for industrial applications such as fermentation to produce metabolites or degrade organic components. Thus, adjustments to their CCR systems could provide a viable strategy to optimize biomass production for industrial purposes. In terms of plant–bacteria associations, we identified 10 species isolated from plant tissues or from the rhizosphere (Table 3). Interestingly, all plant pathogens were less present, while human (e.g., *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus agalactiae*) and animal/insect (e.g., *Melissococcus plutonius*) pathogens were more represented (Supplemental Table 4). This could be explained by the fact that Firmicutes contain fewer representative phytopathogens compared with other classes. They more commonly include PGPR bacteria, such as *Bacillus amyloliquefaciens* and *Paenibacillus polymyxa*. Some of them are commonly used as biological control agents against phytopathogens in agriculture (e.g., *B. subtilis*).

In addition, several phylogenetic trees were constructed for each CCR system by including global master proteins (Crc, Crp, or CcpA; Supplemental Figures 1–3) in order to safely assign relationships, as well as reciprocal best hits. Globally, the trees confirmed the phylogenetic link between master proteins found

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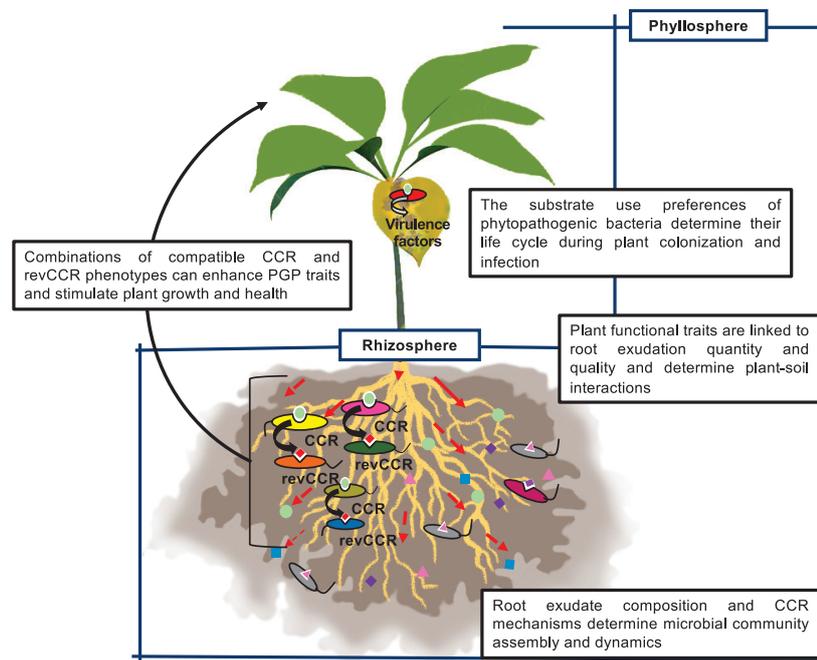


Figure 3. Carbon catabolite repression (CCR) mechanisms involved in plant–microbe interactions.

A schematic overview of how plant root exudates can regulate microbiome assembly, diversity, and function via CCR mechanisms. The quality and quantity of root exudates are linked to plant functional traits. Microorganisms that use CCR and revCCR coexist in the rhizosphere of plants. Those using revCCR may consume the by-products of other members of the plant microbiota that use CCR metabolism. Primary metabolites secreted by plants and present in vascular tissues may regulate the phytopathogenic and beneficial properties of bacteria via CCR.

and *crp* in *Sorghum bicolor*. Interestingly, bacteria using CCR and organic acid-mediated CCR coexist in the plant rhizosphere, as previously observed in medical environments, including chronic wounds and cystic fibrosis lungs (Orazi and O’Toole, 2017). Bacteria using revCCR may consume the by-products of CCR metabolism and thereby remove metabolites that could be a thermodynamic constraint on metabolism, as

in all strains, except for *Haemophilus influenzae* for *Crp*, as well as Enterobacteriaceae and *Haloplasma contractile* for *CcpA*. Sequences from Firmicutes, which generate long branches, appear to have a more complex evolutionary history, which will require deeper investigation in upcoming studies.

CATABOLITE REPRESSION: IDENTIFICATION OF MECHANISMS EXPRESSED IN PLANT RHIZOSPHERES

In a targeted approach, we explored whether genes encoding catabolite repression are expressed in the plant rhizosphere. We used different available rhizosphere metatranscriptomic datasets for BLAST analyses. Three protein sequences from three model strains were selected as queries: *Crc* from *P. fluorescens* F113, *CcpA* from *B. subtilis* (strain 168), and *Crp* from *E. coli* (strain K12). In total, 10 rhizosphere metatranscriptomic bioprojects available at NCBI were included (Supplemental Table 5). The datasets were obtained from rhizosphere soil and/or roots of *Arabidopsis*, corn, switchgrass, maize, canola, sorghum, *Miscanthus*, *Populus*, and *Vellozia epidendroides*. The reads were assembled, filtered, de-replicated, and screened for chimeras in order to obtain high-quality reads. We then compared the sequence similarity of the reference protein sequences against these unique reads by Diamond BLAST analysis (at least 40% identity and 80% coverage). We found that genes involved in catabolite repression are expressed in the rhizospheres of *A. thaliana*, *V. epidendroides*, *Populus*, *Miscanthus*, corn, switchgrass, and sorghum, suggesting a potentially important role for this mechanism in the regulation of plant–bacteria interactions in the rhizosphere (Figure 2). Notably, *ccpA* showed greater expression than *crp* and *crc* in corn, switchgrass, and *Miscanthus*, whereas *crc* showed greater expression than *ccpA*

well as being inhibitory (Brileya et al., 2014). This positive feedback mechanism would enable consortia to increase biomass productivity (Park et al., 2020). For example, *P. fluorescens* F113 (organic acid-mediated CCR) and *B. subtilis* (CCR) are excellent rhizosphere colonizers through root biofilm formation and are often isolated together. Their synergistic interactions in plant roots are likely to improve plant colonization through more efficient resource acquisition and greater efficiency of converting resources into biomass. At the bacterial community level, the complementary properties of the two strategies (CCR and organic acid-mediated CCR) can mitigate direct competition for energy and nutrients and instead establish a cooperative division of labor in the rhizosphere. We therefore suggest that CCR is a dynamic process that is important for bacterial community assembly and hence for ecosystem function. Interpretation of global regulation processes based on consortia rather than single cultures is needed for a better understanding of the plant rhizosphere microbiota and can contribute to future applications in agriculture.

FUTURE CONSIDERATIONS

Plants adopt different strategies to interact with their environment for the acquisition or conservation of nutrients. Many nutrient-use strategies can be explained by plant functional traits (Violle et al., 2007). Plants with higher photosynthetic capacity and efficient N uptake are referred to as exploitative (or fast-growing) plant species, whereas plants with lower nutrient uptake and photosynthetic activity but higher levels of leaf and root dry mass are considered conservative (or slow-growing) plant species (Aerts and Chapin, 1999). These contrasting strategies influence the input and output of C resources. Guyonnet et al. (2017, 2018) explored the influence of plant nutrient-use strategies on the level and quality of root exudation. They found that exploitative plant species exude more C in general, as well as more

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diversified C, than conservative species. This exudation plays a crucial role in shaping rhizosphere communities. Different studies have demonstrated the effects of root exudation on the diversity and function of the plant microbiome (Bulgarelli et al., 2013; Guyonnet et al., 2018), termed the “rhizosphere effect.” However, this effect has not, to date, been linked to CCR regulation. Until recently, no study had addressed the CCR regulatory effects of different root exudates in terms of the structure, diversity, assembly, and function of the microbiome. How does the nature of root exudates influence plant growth and health by shaping the diversity and function of the plant microbiota? How does the nature of primary metabolites contained within vascular tissues, such as xylem and apoplasts, regulate bacterial virulence? These questions could be addressed using *A. thaliana* mutants specifically altered in the exudation of sugars or organic acids. Analysis of the microbiome using metabarcoding approaches coupled with metatranscriptomics in isogenic mutants for a given sugar or organic acid could provide insight into the importance of CCR for rhizosphere functions. Approaches to accurately characterize the metabolite composition of root exudates under natural conditions, as done by Guyonnet et al. (2017), but coupled with metabarcoding and metatranscriptomics, are also needed for a better understanding of the plant’s capacity to shape the diversity and functions of its microbiota (Figure 3).

The substrate use preferences of rhizobacteria and phytopathogenic bacteria, regulated by CCR, determine the life cycle of bacteria during root colonization and infection. Analysis of transcriptomes from *D. dadantii* in synthetic cultures after exposure to different carbon sources mimicking the environment encountered in the apoplastic space during plant infection suggests that during the first stage of infection (asymptomatic phase), in the presence of sucrose and polygalacturonate (a pectin derivative), *D. dadantii* uses sucrose as a C source, and the *pel* genes encoding pectate lyases, responsible for soft rot symptoms, are repressed during the exponential phase of growth. At the end of the exponential growth phase (symptomatic phase), the *pel* genes are activated by the cAMP-CRP complex, and the bacteria begin to use polygalacturonate as a carbon source (Jiang et al., 2016). Analysis of the fate of strains mutated in genes encoding the CCR mechanism under natural conditions in the rhizosphere will be relevant to understanding trait regulation of bacteria during their life cycle. Are these mutants competitive, and do their beneficial or pathogenic functions continue to be expressed? Transcriptomic and metabolomic analysis of CCR mutants *in planta* is needed to respond to these questions. Beyond bacteria, these questions could also be applied to the whole plant microbiome, as its prokaryotic and eukaryotic members are constantly interacting with one another, resulting in specific adaptations.

Previous studies that have focused on improving plant growth and health have also contrasted single strains of rhizobacteria with different strain combinations as inoculum. Some of them demonstrated a significant improvement in plant growth and health, whereas others showed no effect (Oleńska et al., 2020). For example, Ansari and Ahmad (2019) observed a significant enhancement of vegetative growth and photosynthetic parameters of wheat seedlings growing in natural soil after co-inoculation with *P. fluorescens* strain FAP2 and *Bacillus licheniformis* B642 compared with single inoculation of each strain

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alone. The authors demonstrated a positive interaction between the two strains and showed that both harbored multiple plant growth-promoting traits, such as the production of auxin or siderophores and the solubilization of phosphate. The positive interaction between these bacteria may be explained by their substrate use preferences. Indeed, *Bacillus* species are known to prefer sugars; they primarily use glucose exuded by wheat seedlings, maximizing their growth rate and the production of secondary metabolites, which affects plant performance. By contrast, *Pseudomonas* prefers organic acids and can use acetate and other by-products produced by *Bacillus* to grow and produce secondary metabolites. It can therefore be assumed that combinations of compatible CCR and organic acid-mediated CCR phenotypes can enhance plant growth-promoting traits and stimulate plant growth and health by enabling cooperation via substrate allocation provided by the host plant. Future design of highly efficient rhizobacterial consortia will rely on a detailed understanding of their CCR mechanisms to ensure compatibility with plant root exudates and fully harness their potential for plant health and growth promotion.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.Z.H. and H.B.; methodology, T.F., D.A., H.B., and F.Z.H.; investigation, T.F., D.A., H.B., and F.Z.H.; writing – original draft, T.F., H.B., and F.Z.H.; writing – review & editing, T.F., W.A., T.C., W.N., S.R., H.B., and F.Z.H.; funding acquisition, F.Z.H.; resources, H.B. and F.Z.H.; supervision, F.Z.H.

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II. CCR chez les Eucaryotes (partie non publiée)

In this part, an overview of the CCR mechanism in yeast and filamentous fungi will be described showing how cellular localization and phosphorylation state of proteins contribute to CCR. With cellular compartmentalization, the complexification of CCR has increased. The CCR system now depends on the cellular localization of the main repressor. This localization depends on the phosphorylation state of the repressor. Between yeast and filamentous fungi, the mechanism of CCR is quite similar due to several homologies between the proteins acting in the system. Indeed, the main filamentous fungi repressor CreA is a functional homolog of Mig1, the main yeast repressor (Ries et al., 2016). They both have a C2H2 Zinc finger DNA binding domain motif and act as transcriptional factors (Ries et al., 2016). Like classical CCRs in bacteria, when glucose is present in high concentration, genes involved in the catabolism of other carbon sources are repressed (Amore et al., 2013).

1. CCR in Yeast

The most studied yeast is *Saccharomyces cerevisiae*, which has served as a model for cellular trafficking and regulation such as the CCR. The CCR can be divided into different interconnected parts. The first part consists in sensing the presence of glucose in the environment. Environmental glucose is sensed by the superfamily of G-protein coupled receptors (GPCRs) which represents the largest family of transmembrane receptors responsible for transmitting extracellular signals to intracellular responses (Xue et al., 2008). Among GPCRs, Gpr1 has been identified as involved in sensing fermentable sugars like glucose. The G α protein Gpa2 and the G protein Ras are activated after glucose is sensed by Gpr1. Ras proteins are regulated by either Ira (inhibitors of Ras), Cdc25 or Sdc25 (activators of Ras) (Tamanoi, 2011; Colombo et al., 2014) (Fig. 8). Activation of Ras proteins positively controls the adenylate cyclase (AC) to convert ATP into cAMP and thereby activate protein kinase A (PKA). The PKA is also negatively regulated by Krh and Sch9 proteins, but their actions are inhibited by the activated Gpa2 protein (Peeters et al., 2006; Peeters and Thevelein, 2014) (Fig. 8).

Glucose uptake into the cell is depends on the extracellular glucose concentration and is coordinated by different sensors and transporters displaying different affinities for glucose: on the one hand, for high glucose concentration, the low affinity hexose transporters HXT1, HXT3 and Rgt2 (Maier et al., 2002; Adnan et al., 2018) and on the other hand, for low glucose concentration, the high affinity hexose transporters HXT2, HXT4 and Snf3 (Boles and Hollenberg, 1997; Maier et al., 2002; Adnan et al., 2018). The transcriptional factor Rgt1 is a repressor of high affinity glucose transporters and is activated by low affinity transporters and PKA (i.e phosphorylated) (Kim and Johnston, 2006; Adnan et al., 2018). Glucose transport and signalling in glucose is therefore dependent on glucose concentration (Fig. 8).

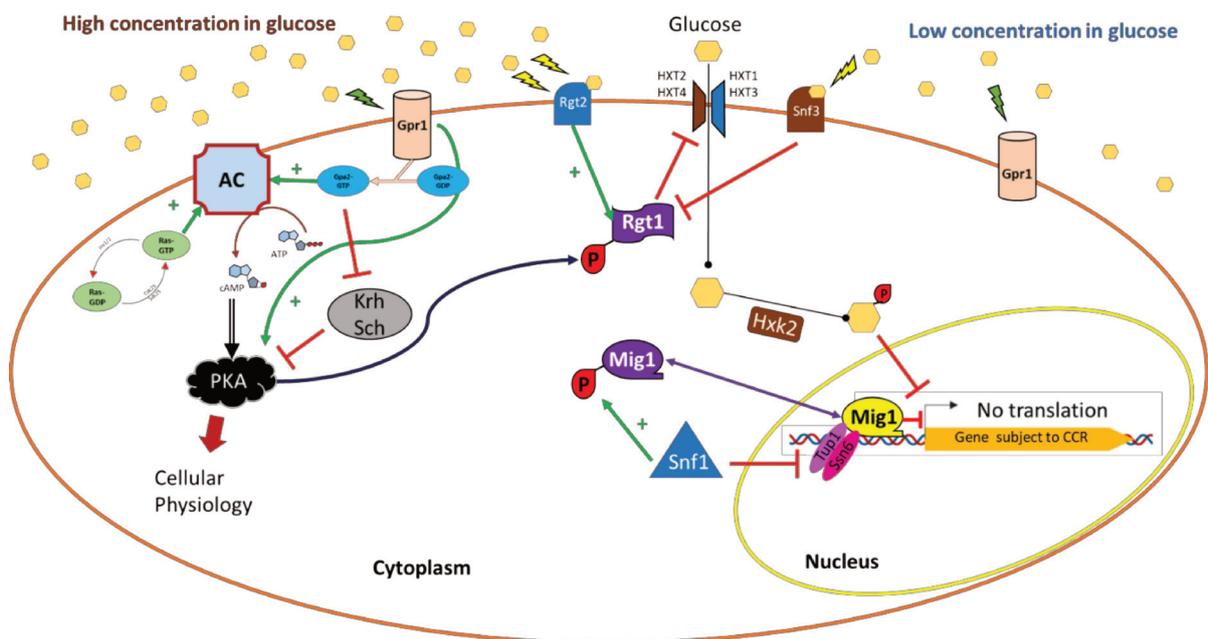


Figure 8: Schematic representation of the Glucose sensing, signaling and repression in Yeast (*S. cerevisiae*).

Glucose is sensed by the G protein-coupled receptor Gpr1. After that the $G\alpha$ protein Gpa2 is activated and with the activated form of Ras protein modulate Adenylate Cyclase (AC) which consist in an increase of cAMP level. ProteinKinaseA is indirectly activated by this pathway. In fact, PKA is inhibited by Krh and Sch which are inhibited by Gpa2. The glucose is signaled and transported into the cell by different affinities sensors and transporters. For high glucose concentration in media, glucose is sensed and transported by low affinity proteins Rgt2, HXT1 and HXT3. For low concentration in media, glucose is sensed and transported by high affinity proteins Snf3, HXT2 and HXT4. Increase of PKA activity during high glucose concentration leads to activation of Rgt1 by phosphorylation. Activated Rgt1 causes the repression of high glucose affinity transporter. Cellular glucose is phosphorylated by hexokinase such as Hxk2. Phosphorylated glucose can then indirectly provoke the repression of genes subjects to CCR by the transcriptional repressor Mig1. To perform the repression, Mig1 is coupled with Tup1 and Ssn6 proteins in the nucleus and bind the promoter of genes. The repression is lifted during low glucose concentration by the activation of Snf1 complex, which leads to the phosphorylation of Mig1 and go back to the cytoplasm.

After entering the cell, glucose is phosphorylated by the hexokinases Glk2, Hxk1 and Hxk2, phosphorylated glucose causes indirect repression of the non-preferred C source (Vega et al., 2016). Under high glucose concentration conditions, the transcriptional repressor Mig1 is localized in the nucleus in complex with the co-repressor proteins Tup1 and Ssn6 where it exerts its repressing action on genes subject to CCR (Treitel and Carlson, 1995). Under low glucose conditions, Mig1 is exported out of the nucleus and phosphorylated by the Snf1 complex, and thus the repression is lifted (Welkenhuysen et al., 2017). Furthermore, according to Ahuatzí et al (2007), the hexokinase Hxk2 inhibits the action of Snf1 kinase on Mig1 under high glucose conditions by masking the serine 311 of Mig1. Another key element of the yeast CCR is the phosphatase Glc7, which dephosphorylates Mig1 when the glucose concentration starts to rise (Tabba et al., 2010) (Fig. 8).

Taking together, all these data present the fine-tuning regulation of CCR in yeast by a succession of phosphorylation/dephosphorylation in relation to the glucose concentration.

2. CCR in fungi

The CCR in filamentous fungi is quite similar to that previously described for yeast. Carbon sources sensing is under the control of numerous GPCR and the AC-activating G α subunit, which leads to increase of cAMP levels and activation of PKA (Fuller and Rhodes, 2012). The PKA system is also central to the regulation of fungal growth and CCR. In *Aspergillus fumigatus*, the presence of glucose leads to an increase of PKA activity that conducts to CCR (Oliver et al., 2002). In contrast to yeast, the concentration of other monosaccharides also affects the cellular localization of Mig1 homologous transcriptional regulator, CreA. Based on the same phosphorylation dependent state, unphosphorylated CreA is located within the nucleus, while phosphorylated CreA is moved to the cytoplasm (Ubersax and Ferrell, 2007; Ries et al., 2016). Phosphorylation of CreA is due to SnfA, homolog of Snf1, in the presence of cellulose or other saccharides while it is inhibited in the presence of glucose (Ubersax and Ferrell, 2007; Ries et al., 2016). In filamentous fungi, such as *A. nidulans*, ubiquitination also plays a role in CCR. Selective protein degradation by the ubiquitin-proteasome pathway is a powerful regulatory mechanism in a wide variety of cellular processes including CCR. Indeed, CreA activity and

stability depend on ubiquitination and deubiquitination promoted by other Cre proteins. CreD is an ubiquitin ligase protein, donating the Ub particle to CreA and responsible for its degradation by the proteasome (Boase and Kelly, 2004). The CreB-CreC complex is a deubiquitinase that removes the Ub particle from CreA to activate it (Lockington and Kelly, 2001; Lockington and Kelly, 2002). Activated CreA interacts with RcoA and SsnF, homologs to Tup1 and Ssn6 respectively, and binds to and represses the promoter region of genes subject to CCR (García et al., 2008) (Fig 8).

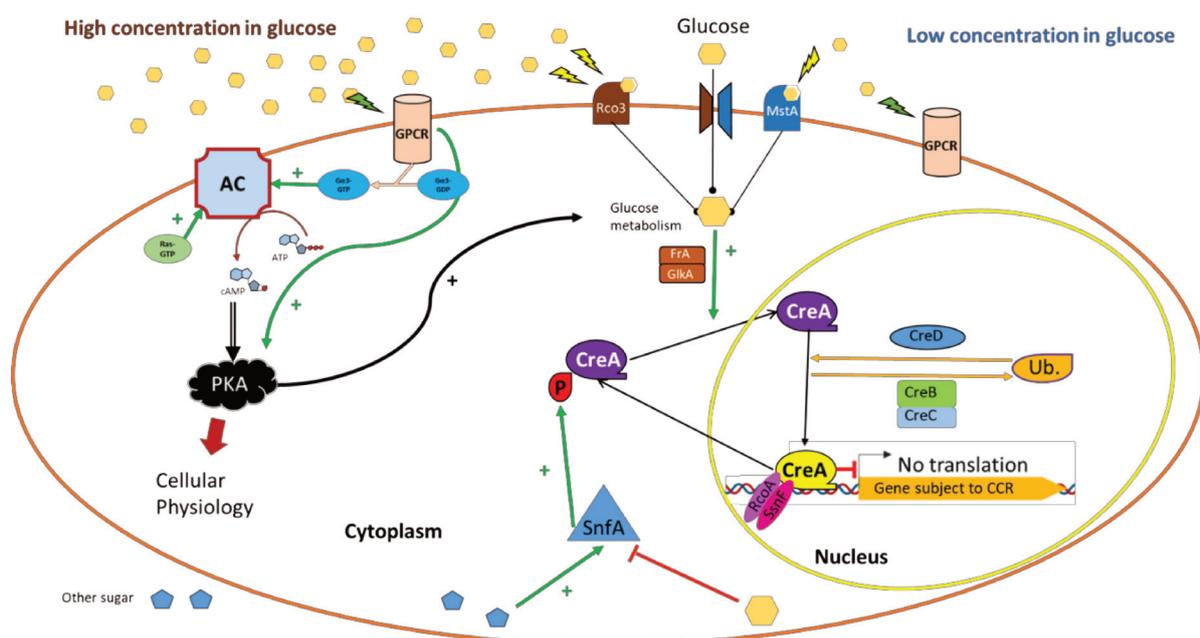


Figure 9: Schematic representation of the Glucose repression in Filamentous fungi (*A. nidulans*). Like in yeast, glucose is sensed and signaled by GPCR and Gα proteins, where some are homologue to what can be found in yeast. AC is stimulated by Ras and Gα activated proteins in presence of glucose. Which leads to an increase of PKA activity. Glucose is transported inside the cell by different glucose affinities transporters such as Rco3 and MstA. FrA and GlkA are hexokinase which participated in glucose metabolism and glucose mediated CCR. In filamentous fungi the repression is mediated by Mig1 homologue (Yeast transcriptional repressor) CreA. In presence of glucose, CreA is located in the nucleus. Inside the nucleus CreA is subsequently ubiquitinated by CreD protein complex and deubiquitinated by CreB-CreC. After the action of these two proteins complex, CreA interact with RcoA and Ssnf and bind to the promoter of genes subject to CCR and preformed the repression. On one hand, glucose inhibit the action of SnfA. On the other hand, in presence of other carbon sources, SnfA complex phosphorylate CreA and moves it to the cytoplasm which lift the repression.

CreA has been well identified in many filamentous fungi but in others filamentous fungi such as *Neurospora crassa* and *Trichoderma reesei*, the carbon catabolite repressor mentioned is Cre1 (Sun and Glass, 2011). CreA and Cre1 are homologous and have similar DNA bindings properties. However, it has been found that in *T. reesei* it must be phosphorylated in order to bind to DNA, furthermore, the Snf1 homolog does not appear to participate in the phosphorylation of Cre1 (Cziferszky et al., 2003). CreA binds to DNA by 5'-SYGGRG-3' motif (Tamayo et al., 2008). This DNA motif can be found in the promoter regions of genes such as xylanase encoding genes (*xlnA*, *xlnD*, *xlnR*) that are directly repressed by CreA during CCR (Tamayo et al., 2008). The repression exerted by CreA is also under the action of other regulators such as F box protein. Frp1, the F box protein required for *Fusarium oxysporium* pathogenesis, appears to negatively regulate CreA to activate transcription of pathogenic genes during CCR conditions (Jonkers and Rep, 2009) (Fig 9).

E. Objectifs de la thèse

De par leur métabolisme versatile, les microorganismes vont pouvoir mettre en place tout un système de régulation interne permettant l'expression de certaines fonctions à un instant bien défini. Comme nous venons de le voir lorsque les bactéries se retrouvent en présence de la plante, différentes fonctions clés permettant l'établissement de l'interaction plante-bactérie seront induites comme la capacité à former un biofilm bactérien pendant la colonisation racinaire, l'expression de facteurs de virulence lorsqu'il s'agit de microorganismes pathogènes ou la dénitrification. Ces fonctions bactériennes sont régulées par la répression catabolique du carbone (CCR) comme nous venons de le décrire.

La dénitrification est une fonction importante pour les microorganismes leur permettant de respirer en absence d'oxygène, et confère ainsi un avantage dans la colonisation de la rhizosphère. C'est pourquoi elle est considérée comme un trait de compétence rhizosphérique (Lecomte et al., 2018). Au sein de la rhizosphère, nous retrouvons toutes les conditions permettant l'induction de la dénitrification. En effet, dans des zones autour des racines il y a absence d'oxygène ce qui favorise la respiration anaérobie. La présence d'azote dans le sol, associée aux différents intrants azotés va servir de source d'N. En ce qui concerne la matière carbonée, la présence des exsudats racinaires va en être une des principales sources. Nous avons également vu que la dénitrification est fortement stimulée par la présence d'exsudats racinaires. Ainsi en prenant en compte les différents points abordés nous pouvons émettre **l'hypothèse que la dénitrification est régulée par la CCR via les exsudats racinaires.**

Les travaux de thèse présentés vont avoir pour objectif de démontrer que la nature des métabolites primaire exsudés par la plante régule la dénitrification au travers de la CCR et impacte aussi en retour la physiologie de la plante, en affectant probablement la composition de son microbiote. Pour se faire, elle se divise en deux parties expérimentales :

La première partie vise à mettre en avant **le rôle régulateur de la CCR sur la dénitrification. L'hypothèse étant qu'en fonction de la nature de la source de carbone en condition de dénitrification, la bactérie va plus ou moins stimuler la dénitrification via la CCR.**

Pour élucider cette régulation, nous avons fait le choix d'utiliser les deux organismes modèles suivants :

- ➔ La bactérie modèle *Pseudomonas fluorescens* F113 : une rhizobactérie capable de stimuler la croissance des plantes. Cette bactérie colonise de manière efficace les racines de différentes plantes (Vacheron et al., 2013) et est capable de réaliser la dénitrification (Haichar et al., 2012). La dénitrification est organisée en 4 opérons dont les gènes clefs sont *narG*, *nirS*, *norB*, *nosZ* (Figure 10).
- ➔ La plante modèle *Arabidopsis thaliana* : très utilisée en laboratoire pour sa petite taille, son cycle de vie court et par la disponibilité d'outils génétiques. Des lignées d'*A. thaliana* Col-0 mutées dans des ABC transporteurs ont également servi dans cette étude afin d'utiliser des profils d'exsudations différents en termes de métabolites primaires.

Durant ces travaux, nous nous sommes intéressés aux régulateurs clefs de la revCCR chez les *Pseudomonas* à savoir Crc et CbrB. L'expression des gènes de la dénitrification, ainsi que des ARNnc de la revCCR a été analysé *in planta* en interaction avec des mutants d'*A. thaliana* présentant différents profils d'exsudations.

La seconde partie a pour objectif **d'observer l'impact d'une communauté microbienne rhizosphérique sur la physiologie de la plante hôte en réponse à différents profils d'exsudations**. On s'est intéressé au suivi de deux phytohormones impliquées dans la régulation de la croissance et de la santé de la plante, à savoir l'auxine et le jasmonate. La communauté microbienne a été préalablement extraite de la rhizosphère de la plante hôte *A. thaliana* écotype Col-0 suite à sa culture dans un sol de type prairie provenant de La Côte-Saint-André. Le suivi de la signalisation des deux hormones, a été réalisé à l'aide de la construction de lignées de plantes portant des gènes rapporteurs de l'activation de ces deux hormones.

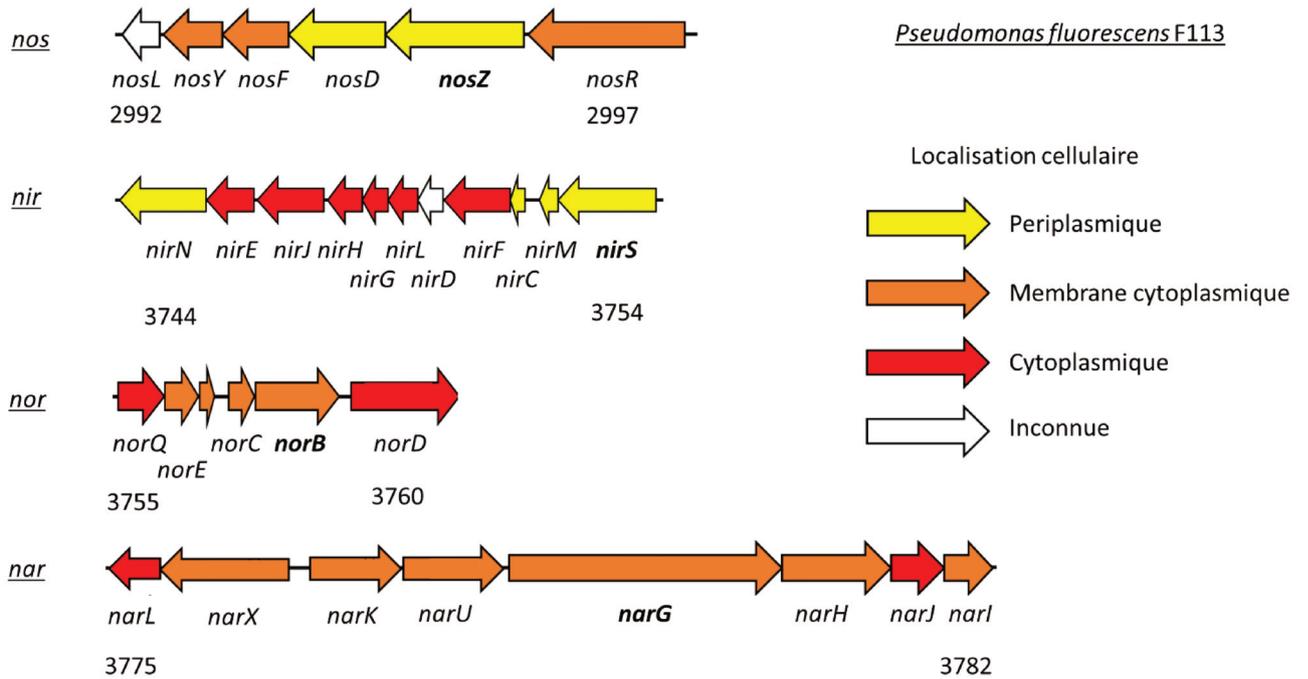


Figure 10 : Cluster des gènes de la dénitrification chez *Pseudomonas fluorescens* F113. Représentation schématique de l’environnement génique des gènes de la dénitrification de *Pseudomonas fluorescens* F113. Les nombre corresponde aux numéros des gènes d’après <https://www.pseudomonas.com>. Les gènes en gras sont les quatre gènes clefs de la dénitrification. La couleur des flèches représente la localisation cellulaire des produits des gènes représentés.

Chapitre 2 :

Lien entre les exsudats racinaires et la répression catabolique du carbone dans la régulation de la dénitrification chez *Pseudomonas fluorescens* F113

Ce chapitre rédigé sous forme d'article scientifique fera prochainement l'objet d'une soumission à publication dans le journal *New Phytologist*.

Résumé

Les exsudations racinaires, représentant jusqu'à 20% du carbone fixé lors de la photosynthèse, constituent une source d'énergie pour le développement et l'activité des communautés microbiennes du sol, comme le processus de dénitrification. En effet, ce dernier nécessite des conditions optimales pour sa réalisation : **disponibilité d'une source de carbone (C)** assimilable, absence d'oxygène et présence de nitrate. Chez les bactéries, il est connu que l'utilisation du carbone est régulée par **le mécanisme de répression catabolique du carbone (CCR pour Carbon Catabolite Repression)**. Ce mécanisme permet aux bactéries d'assimiler une source de C de façon préférentielle en présence d'un mélange de sources de C. Il est connu que de 5 à 10% des gènes bactériens sont régulés par la CCR.

Plusieurs études se sont intéressées à la régulation de la dénitrification par l'azote et l'oxygène, mais très peu sur l'effet régulateur du C. Cette étude vise à élucider comment la nature des métabolites primaires exsudés par les plantes régule le processus de dénitrification. Afin de démontrer le lien entre la source de carbone et la dénitrification, une première étude *in vitro* a été réalisée. Ainsi, nous avons utilisé la souche modèle *Pseudomonas fluorescens* F113 possédant (i) les gènes *crc* et *cbrB*, codant pour les régulateurs centraux de la CCR chez les *Pseudomonas* et (ii) les gènes impliqués dans la voie de dénitrification. Dans un premier temps, la régulation de la dénitrification par la CCR a été étudiée. **Nos résultats ont mis en évidence le type de CCR exercé par la souche modèle et son rôle dans le processus de dénitrification.** De manière intéressante, nous avons observé une préférence pour la source de carbone non préférée (glucose) dans les conditions d'anaérobies, ce qui suggère **le rôle répressif de la CCR sur les gènes de dénitrification.** Ensuite, nous avons mis en évidence **un rôle important de la CCR dans l'expression de traits bactériens impliqués dans l'interaction plante-bactérie.** Enfin, nous avons étudié l'impact des exsudats racinaires sur le processus de dénitrification. *Arabidopsis thaliana* et des mutants affectés dans le processus d'exsudation ont été inoculés avec des souches sauvages et mutantes (Δ *crc* et Δ *cbrB*) de *P.*

fluorescens F113 *in planta*. Les ARN de la suspension bactérienne et des bactéries associées aux racines ont été extraits pour quantifier l'expression des gènes clefs de la dénitrification (*narG*, *nirS*, *norB*, *nosZ*) ainsi que les ARN non codant impliqués dans la régulation de la répression catabolique. Nos résultats **démontrent une régulation positive sur l'expression des gènes de dénitrification en présence d'un profil d'exsudation enrichi en sucre.**

Linking root exudates and Carbon Catabolite Repression for the regulation of denitrification in *Pseudomonas fluorescens* F113.

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Abstract

Root exudations, representing up to 20% of the carbon fixed during photosynthesis, are a source of energy for the development and activity of soil microbial communities, such as the denitrification process. Indeed, the latter requires optimal conditions for its realization: availability of an assimilable of carbon (C) source, absence of oxygen and presence of nitrate. In bacteria, it is known that the use of carbon is regulated by the mechanism of carbon catabolic repression (CCR). This mechanism allows bacteria to assimilate a source of C in a preferential way in the presence of other sources of C. It is known that 5 to 10% of bacterial genes are regulated by CCR.

Several studies have focused on the regulation by nitrogen and oxygen, but very few on the regulatory effect of C on denitrification. This study aims to elucidate how the nature of primary metabolites exuded by plants regulates the denitrification process. In order to demonstrate the link between carbon source and denitrification, a first *in vitro* study was performed. Thus, we used the model strain *Pseudomonas fluorescens* F113 possessing (i) the *crc* and *cbrB* genes, encoding the central regulators of CCR in *Pseudomonas* and (ii) the genes involved in the denitrification pathway. In a first step, the regulation of denitrification by CCR was studied. Our results highlighted the type of CCR exercised by the model strain and its role in the denitrification process. Interestingly, we observed a preference for the non-preferred carbon source (glucose) under anaerobic conditions, suggesting the repressive role of CCR on denitrification genes. Second, we highlighted the important role of CCR in the expression of bacterial traits involved in plant-bacteria interaction. Finally, we studied the impact of root exudates on the denitrification process. *Arabidopsis thaliana* and mutants affected in the exudation process were inoculated with wild type and mutant strains (Δ *crc* and Δ *cbrB*) of *P. fluorescens* F113 *in planta*. RNAs from the bacterial suspension and root-associated bacteria were extracted to quantify the expression of key denitrification genes (*narG*, *nirS*, *norB*, *nosZ*) as well as non-coding RNAs involved in the regulation of catabolic repression. Our results demonstrate an up-regulation of denitrification gene expression in the presence of a sugar-enriched exudation profile.

Introduction

In the rhizosphere, the plant exudes up to 20% of the Carbon (C) fixed during photosynthesis (Bais et al., 2006; Haichar et al., 2008). Root exudation of carbonaceous molecules is a potential source of energy for the development and activity of soil microbial communities, including denitrifiers and participates greatly in the formation of plant-microorganism interactions (Haichar et al., 2012; Guyonnet et al., 2017; Guyonnet et al., 2018). Furthermore, differences in root exudation profiles can shape the denitrification activity in soil (Guyonnet et al., 2017).

Denitrification is an alternative respiration pathway used when there is absence of oxygen, consisting of the progressive reduction of nitrate to nitrogen gas (Zumft, 1997; Lecomte et al., 2018). It is an inducible function, which requires extremely low concentration or absence of oxygen, easily available organic compounds as energy sources and the presence of nitrate (Henry et al., 2008; Salles et al., 2009). Denitrification is also a major source of nitrogen (N) loss from soil (25-90%) (Radersma and Smit, 2011) that can affect plant productivity (van Der Heijden et al., 2008) and enhances N₂O atmospheric emission (Hu et al., 2015). Unfortunately, the N₂O reduction rate is lower than the reduction of nitrate and nitrite, leading to a higher N₂O/N₂ ratio in the atmosphere. Thus understanding how denitrification is regulated in the environment would allow reducing N₂O emission and limiting N loss in soils. If the quantity of available Carbon (C) appears as an important element in the regulation of denitrification, the nature of the C source is also very important.

Free-living bacteria frequently have a versatile metabolism that allows the use of many different compounds as carbon source and energy. This facilitates their colonization in very different niches and their adaptation to changing environmental conditions. Bacteria can either co-metabolize different carbon sources or preferentially assimilate a specific class of compounds that provide the most efficient growth. Simultaneously along the preferential assimilation, bacteria finely trigger inhibition of numerous functions including those involved in the catabolism of non-preferred compounds. This regulatory processes allowing for the selection of preferred carbon sources have been named Carbon Catabolite Repression (CCR) (Görke and Stülke, 2008). Genera like *Enterococcus* and *Firmicutes* demonstrate the classical CCR with a preference for sugar over organic acids, while *Pseudomonas* is characterized by the

reverse CCR (revCCR) and uses organic acids such as succinate as preferred carbon source (Deutscher, 2008; Franzino et al., 2022). It is known that 5-10 % of all bacterial genes are subject to CCR (Görke and Stülke, 2008).

The major transcriptional regulator of revCCR is the catabolite repression control protein “Crc”, well described in *Pseudomonas aeruginosa* PAO1 (Rojo, 2010; Sonnleitner et al., 2012). Crc controls different functions such as biofilm formation, virulence and antibiotics resistance in *P. aeruginosa* PAO1 (Linares et al., 2010). The activity of Crc is modulated by a small non-coding RNA (*CrcZ*) that binds the protein, thus competing with their target mRNAs (Sonnleitner et al., 2009; Sonnleitner et al., 2012). The expression of the ncRNA *CrcZ* which sequesters Crc in order to remove the repression is under the control of the two-component systems (TCS) CbrAB and the sigma factor RpoN (Sonnleitner et al., 2009). *CrcZ* is also involved in the regulation of bacterial functions like biofilm formation through the regulation of revCCR (Nishijyo et al., 2001; Sonnleitner et al., 2012; Pusic et al., 2016).

Several studies suggested a link between C metabolism and denitrification (Takaya et al., 2003; Otani et al., 2004; Salles et al., 2009). It is proposed that the quality of carbon sources provided by plant exudates and used by bacteria is an important factor controlling denitrifying genes regulation by catabolic regulation. However, the underlying molecular mechanism is still unknown. The aim of this study is to link the regulation of denitrification by primary metabolites exuded by plants to the carbon catabolism repression.

In this study, we explored the role of the revCCR in the regulation of denitrification and rhizosphere colonization traits in *Pseudomonas fluorescens* F113. F113 is a well know PGPR (Plante Growth Promoting Rhizobacteria) isolated from sugar beet and capable of colonizing a wide range of plant roots (Shanahan et al., 1992; Vacheron et al., 2018) and it is also a denitrifying bacterium (Redondo-nieto et al., 2013; Muriel et al., 2015). First, we confirmed the implication of revCCR and its regulators, Crc and CbrB, on the growth of F113 in aerobic and anaerobic conditions. We also show that Crc has a negative effect on biofilm formation and a positive effect in swarming ability. CbrB has also a positive effect on swarming ability and modulate auxin production in plant cells. And then, we demonstrated that the expression of denitrification genes is regulated by revCCR and root exudates from *Arabidopsis thaliana*.

Table 1. Strains and plasmids used in this study.

Strains	Genotype/relevant features	Source/reference
<i>P. fluorescens</i>		
F113	Wild type strain	Shanahan et al., 1992
F113 pBBR1-MCS5	F113 wt harboring pBBR1-MCS5 empty	This study
F113 Δcrc	F113 deleted of crc gene	This study
F113 Δcrc pBBR1-MCS5	F113 Δcrc harboring pBBR1-MCS5 empty	This study
F113 Δcrc-crc	F113 Δcrc harboring pBBR1-MCS5::crc F113	This study
F113 ΔcbrB	F113 deleted of cbrB gene	This study
F113 ΔcbrB pBBR1-MCS5	F113 ΔcbrB harboring pBBR1-MCS5 empty	This study
F113 ΔcbrB-cbrB	F113 ΔcbrB harboring pBBR1-MCS5::cbrB F113	This study
<i>E. coli</i>		
DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169. hsdR17(r _K ⁻ m _K ⁺), λ-	Grant et al., 1990
MDFpir	MG1655 RP4-2-Tc::[ΔMu1::aac(3)IV-ΔaphA-Δnic35-ΔMu2::zeo]ΔdapA::(erm-pir) ΔrecA	Ferrières et al., 2010
Plasmids		
pCM157	Vector containing Cre recombinase activity, Amp ^R , Tc ^R	Marx and Lidstrom 2002
pCM184	Cloning vector used for homologous recombination, Amp ^R , Km ^R , Tc ^R	Marx and Lidstrom 2002
pCM184::UpccrF113	pCM184 carrying Upstream sequence of F113 crc gene	This study
pCM184::UDcrrF113	pCM184::UpccrF113 carrying Downstream sequence of F113 crc gene	This study
pK18mobsacB	Cloning vector for allelic exchange; Km ^R	Schäfer et al., 1994
pK18mobsacB::cbrBF113	pK18mobsacB carrying F113 cbrB sequence cloned into pK18mobsacB,	This study
pBBR1-MCS5	pBBR1-MCS5 stable vector; Gm ^R	Kovach et al., 1995
pBBR1-MCS5::crc F113	pBBR1-MCS5 carrying F113 crc gene with promoting region sequence cloned into pBBR1-MCS5	This study
pBBR1-MCS5::cbrB F113	pBBR1-MCS5 carrying F113 cbrB gene with promoting region sequence cloned into pBBR1-MCS5	This study

Material and methods

Bacterial strains and growth conditions

The strains and the plasmids used in this study are listed in Table 1. Bacteria were grown in Lysogeny broth (LB) liquid or plate (1.5 % Agar) at 30 °C for *P. fluorescens* F113 or 37 °C for *Escherichia coli*. When required, antibiotics were added to the media at the following final concentration: 100 µg.ml⁻¹ for ampicillin, 10 µg.ml⁻¹ for tetracycline, 25 µg.ml⁻¹ for kanamycin and 15 µg.ml⁻¹ for gentamycin.

Construction of *P. fluorescens* F113 mutants

The primer used in this study are listed in Table 2.

F113 Δcrc

For this construction, Cre/Lox recombinase strategy was used (Marx and Lidstrom, 2002). Briefly, upstream and downstream region of *crc* were amplified by PCR (≈1kb) using pCM184-UPcrcF113 (fw and rv) and pCM184-DOWNcrcF113 (fw and rv) primers. Both purified products were then cloned into pCM184 vector using ABM[®] pro ligation free cloning kit (Applied Biological Materials Inc., Canada) between the restriction enzyme sites BglII/NotI and ApaI/SacI, respectively. The constructed plasmid was transferred into *P. fluorescens* F113 strain by biparental mating using *E. coli* MFDpir strain (Ferrières et al., 2010). Single crossing over between homologous regions present in this non-replicative plasmid allowed its integration in the targeted site of the genome (upstream or downstream region of *crc* gene). The second crossing event allowed to remove the plasmid (the part containing the tetracycline resistance) and the *crc* gene, leaving only the cloning region. Positives clones were then screened on agar plate supplemented with kanamycin (Km) or tetracycline (Tc), looking for a Km^R and Tc^S clones. After that, the pCM157 containing the *cre* recombinase gene was introduced by biparental mating to induce the recombination of the two LoxP sites, excising the kanamycin resistance gene, in order to finally obtain a mutant with a disruption of *crc* gene and without any resistance gene added. Kan^S and Tc^S clones were screened for mutated *crc* gene by PCR and sanger sequencing (Microsynth, France).

Table 2. Primers used in this study.

Name	SequenceA (5'→3')	Restriction site	Target	Type of utilization
pCM184-UPcbrF113fw	CCCGAAAAGTGCACCTGACGTAGCTAGATCTACCGACACTGGC	BglII	Upstream region of cbrC	
pCM184-UPcbrF113rv	tcgtataatgtagtctatacgaagtatgacggccgCATGATCCGCATAAATGGCCCC	NotI		Deletion of cbrC
pCM184-DOWNcbrF113fw	gaagttatggatccagcttctcgataccccgggCGTACCGCGACATCC	Apal	Downstream region of cbrC	
pCM184-DOWNcbrF113rv	tcggctggatcctctagtagctcGTTGATGGGATTGCTGACCG	SacI		
pK18-UPcbrBF113fw	aggaacacgctatgacatgattacgaattcaaggctgctgcaaggtttcatcg	EcoRI	Upstream region of cbrB	
pK18-UPcbrBF113rv	cgggtttacccccacacctcagctgatcaaaaatgfcggcattgattcg			Deletion of cbrB
pK18-DOWNcbrBF113fw	cgaatcaatgccacattttgatcagctgaggtgfgggggtaacaccg		Downstream region of cbrB	
pK18-DOWNcbrBF113rv	cctgaggtcgactctagaggatccccggggcgctggtggcagctcgaatcc	SmaI		
pBBR1-CompcbrF113fw	GGGCCCCGGCCCGgaaccggtgtgaacaggg	Apal	F113 cbrC gene and promoter	Complementation of cbrC
pBBR1-CompcbrF113rv	GAGCTCGAGCTCgcgatacgggcatcttcttggg	SacI		
pBBR1-CompcbrBF113fw	GGGCCCCGGGCCagtgaaagccttcgaacacacaggg	Apal	F113 cbrB gene and promoter	Complementation of cbrB
pBBR1-CompcbrBF113rv	GAGCTCGAGCTCgggtgtacccccacacctcag	SacI		
F113cbrBfw	ccgtcagagagaatcgaatcaatgc		F113 cbrB gene	Screen for F113 cbrB gene
F113cbrBrv	cgggtttacccccacacctcag			
M13fw	CGCCAGGGTTTTCCAGTCACGAC		MCS	Screen for plasmid construction
M13rv	TCCTGTGTGAAATTTGTTATCCGCT			
GABI_TDna_PDR2fw	CATATGCCGAGATTGGTCTAACCGCAAATG		Exon13 to Exon18 of AtPDR2 gene	Screen for T-DNA insertion
GABI_TDna_PDR2rv	CCCACCACGGAACTTTTGATGTCATCAAGC			
SALK_TDna_PDR6fw	GGTCAAACTGGAGATATCCTCAGTACAATGCC		Intron16 to Intron16 of AtPDR6 gene	Screen for T-DNA insertion
SALK_TDna_PDR6rv	GACTCATATCTCCAACACCAGTATGTGCACAAATCG			
LBb1,3 T-DNA	ATTTTGGCGATTTCGGAAAC		T-DNA SALK Line	Screen for T-DNA insertion

Table 2. Continued.

Name	SequenceA (5'->3')	Restriction site	Target	Type of utilization
qPCR F113 rpoD fw	GTCGCGGAAGAAAGAGATG			
qPCR F113 rpoD rv	AGATCGAGGAATTGCAGGCC		<i>rpoD</i>	qPCR rpoD
qPCR F113 narG fw	AAGAACGGCCTGATCACCTG			
qPCR F113 narG rv	GCTTGCGGATTTTCGGGTAC		<i>narG</i>	qPCR narG
qPCR F113 nirS fw	TTGTCGTCGATCACACCAG			
qPCR F113 nirS rv	GTATCAAGAAAGGCGCGCTG		<i>nirS</i>	qPCR nirS
qPCR F113 norB1 fw	CITTTGGGTGGAAGGCGTTTG			
qPCR F113 norB1 rv	AGCCACTTCTCGACCACCTC		<i>norB</i>	qPCR norB
qPCR F113 nosZ1 fw	CTGGGGAGCTGGACGAATAC			
qPCR F113 nosZ1 rv	CITTGCTTCGTTGGTCAGGC		<i>nosZ</i>	qPCR nosZ
qPCR F113 crcZ fw	AGAGGCGTTGTATTTGGGGC			
qPCR F113 crcZ rv	TGCTTTGATCGCTGTGTTGC		<i>crcZ</i>	qPCR crcZ
qPCR F113 crcY fw	TTGCTCTTGATCCCGGATGG			
qPCR F113 crcY rv	AAITTTGTCGTCGCGTGCTC		<i>crcY</i>	qPCR crcY

F113 ΔcbrB

For this construction, suicide vector strategy by pK18mobsacB vector was used (Schäfer et al., 1994). Two regions, upstream and downstream of *cbrB*, were amplified by PCR (≈1kB) using pK18-UPcbrBF113 (fw and rv) and pK18-DOWNcbrBF113 (fw and rv) primers respectively and then assembled by PCR overlap to form a 2 kb product containing a deleted version of *cbrB*. The newly purified product was then cloned by recombination into linearized pK18mobsacB with the ABM pro-ligation free cloning kit (Applied Biological Materials Inc., Canada). The cloned region was verified by sanger sequencing (Microsynth, France). Then, the plasmid construct was introduced into *P. fluorescens* F113 strain by biparental mating using *E. coli* MFDpir strain. Simple crossing over between homologous regions present in this non-replicative plasmid allowed its integration into the targeted site of the genome (upstream or downstream region of *cbrB* gene). Thus, transconjugants were screened by using one primer outside the region (F113cbrBfv or F113cbrBrv) and one primer inside the plasmid (M13fw or M13rv). The second crossing event will remove the plasmid (the part containing *sacB* and kanamycin resistance) with or without the *cbrB* gene. Positives clones were then screened on agar plate supplemented with sucrose 15 % or kanamycin, looking for a Km^S and Suc^R clones. Clones were then validated to get a correct excision of *cbrB* gene by PCR and sanger sequencing (Microsynth, France).

Complementation

To perform complementation of the two mutant strains F113 Δ*crc* and F113 Δ*cbrB*, *crc* and *cbrB* genes with their promoting region (200 nucleotides before starting codon) were amplified by PCR (pBBR1-Comp*crc*F113 and pBBR1-Comp*cbr*BF113) and then cloned by ligation into pBBR1-MCS5 vector using *Apal*/*SacI* sites. The two cloned regions were sequenced. Then the constructed vectors were transferred in F113 mutants strain using electroporation. Good conjugants were selected on LB plate supplemented with gentamycin.

Bacterial growth conditions

P. fluorescens F113 and its derivatives mutants were grown at 30 °C on 5 ml of M9 minimal salt (Difco, USA) supplemented with 40 mM of carbon from either glucose or succinate and with 20 mM of KNO₃. Under denitrification conditions, helium was injected into sterile tubes in order to generate anoxic conditions and cells were grown without shaking. Overnight cultures were harvested and washed with a 0.9 % NaCl solution and then each strain was inoculated in each medium condition at a final OD_{600nm} about 0.075. Growth was then monitored each 2 h for aerobic condition and each 24 h for anaerobic condition using an Ultrospec 10 Cell density meter (Amersham Biosciences, UK). For aerobic condition growth rate (μ) was calculated during the exponential phase; $\mu = (\log_2 N_2 - \log_2 N_1) / (t_2 - t_1)$ where N_2 and N_1 are the OD measured at time t_2 and t_1 respectively. Each experiment was performed in triplicates and in three biological replicates.

Carbon (C) and nitrogen (N) quantification

One mL of bacterial culture samples was collected from each tube during growth under aerobic or anaerobic conditions. Samples were then filtered using a 0.2 μ m filter to remove bacterial cells from medium. Samples were analyzed by HPLC for succinate and glucose quantification. Briefly, succinate was separated using the elution protocol described by Rasclé et al (2018) whereas glucose was monitored by derivatization at its reducing end with *p*-aminobenzoic ethyl ester (Gomis et al., 2001; Wang and Yan, 2004). Under our conditions, succinate was eluted at a retention time of 7 min and glucose at 8.5 min. Quantification was performed by measuring peak areas using the Mass-Link 4.1 software (Waters, France). Peak areas were then compared with a standard curve of succinate or glucose to measure the amount in each sample.

For N source quantification, N-NO₃⁻ concentrations in samples were determined using standard colorimetric methods (Grasshoff et al., 1999) using a sequential analyzer (SmartChem200, AMS Alliance, Frépillon, France).

Biofilm formation

Biofilm forming ability of *P. fluorescens* F113 wild-type and mutant strains on abiotic surface was assayed according to Sivakumar et al (2020). Briefly, overnight cultures were washed with NaCl 0.9 % and resuspended in K10T-1 medium (Monds et al., 2007) at OD_{600nm} of 0.05. Two ml samples were dispensed into glass tubes in 4 replicas. The sterile medium served as a negative control. Tubes were incubated at 30 °C without shaking. After 24 h, the medium with planktonic cells was discarded, tubes were washed with NaCl 0.9 % and the biofilm was stained with 2.5 ml of crystal violet (0.1 % w/v) for 20 min. The dye was discarded, the tubes were washed with sterile water, air-dried and photographs were taken. In order to measure biofilm formation, produced biofilm was eluted with 95 % ethanol by shaking tubes for 20 min to ensure the crystal violet dissolution. Then, 200 µl was transferred into a 96-well flat-bottom plate and the absorbance was measured at 590 nm on a TECAN Spark.

Motility assays

Overnight cultures were washed with sterile saline buffer (0,9 % NaCl) and diluted to OD_{600nm} = 0,1. Five µl were stabbed at the center of minimal medium M9 plate containing 0.4% agar and supplemented with glucose or succinate in 4 replicates for each strain. The swarming zone was measured after 120 h at 30°C. *Dickeya dadantii* was used as classic CCR swarming control strain (Jahn et al., 2008).

Plant culture and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) was used as WT for all experiments. *Atpdr2* (GK-041D08-015219) and *Atpdr6* (SALK_082843) ABC transporters mutant lines were obtained from Nottingham Arabidopsis Stock Centre (NASC). These mutants were confirmed by PCR-based genotyping using primers listed in Table 2.

For plant growth, seeds were sterilized by vapor-phase according to (Clough and Bent, 1998) sown on Murashige and Skoog (MS) (Duchefa, Nederland) medium plates containing 0.8 % plant agar and then grown under a 16 h light / 8 h dark condition at 22 °C in a growth chamber.

Root exudates characterization

Seven days old seedlings were transferred individually into sterile magenta pot (Dutscher, France) containing 20 mL of liquid MS medium supplemented with 1 % sucrose and grown in a growth chamber under a 16 h light at 25 °C / 8 h dark at 21 °C condition. When plants were 18 days old, they were washed with sterile water to remove the surface-adhering exudates and transferred to 6-well plates containing 6 mL of fresh MS liquid medium supplemented with 1 % sucrose and were grown for 3 days after in a growth chamber under the same conditions. Five ml of exudates were collected from each plant line in triplicate, centrifuged at 8000 rpm during 20 min and then filtered using a 0.45 µm filter to remove root cells. Exudates were then lyophilized, weighed, and stored at -20°C.

Trimethylsilyl (TMS) derivatives were obtained by manual derivatization based on the methodology described by (Ribeiro et al., 2014). Before the derivatization, 1.1 mg of exudates were placed for 30 min in a CentriVap® concentrator to remove residual humidity. First, 40 µL of methoxylaminehydrochloride (20 mg.mL⁻¹ in pyridine) was added. Samples were vortexed, sonicated for 5 min at room temperature and then incubated 1 h 30 min at 30 °C. Then, 70 µL of Bistrimethylsilyltrifluoroacetamine (BSTFA) was added, vortexed and incubated for 30 min at 37 °C. Derivatized extracts were transferred into vials with insert prior to analysis. Sample aliquots of 1 µL were injected (Agilent 7683 Series Injector) at 290 °C (splitless mode) in a Hewlett Packard 6890A gas chromatograph (Agilent Technologies) coupled to a Hewlett Packard 5973 mass spectrometer (Agilent Technologies), using a DB-5MS capillary column (Agilent; 60 m × 0.25 mm × 0.25 µm) with helium as carrier gas at a column flow rate of 2.3 mL.min⁻¹. The oven temperature program was 5 min at 70 °C, followed by a 4 °C.min⁻¹ ramp up to 306 °C. The transfer line temperature was set at 300 °C. The column effluent was ionised by electron impact at 70 eV. Mass spectra were recorded at 1.29 scans.s⁻¹ within a mass-to-charge ratio range of 50–600 m/z at a source temperature of 230 °C. A solvent delay of 7min was set. Using retention times and peak areas obtained from GC-MS chromatograms of studied extracts, data matrices were built in order to carry Partial Least Squares Discriminant Analysis (PLS-DA). Peak areas were normalized by adding 9.4 µL of 0.5 mM norvaline in each sample prior to derivatization. Identification of compounds was done by comparing mass spectra with public databases (NIST/CNRS/WILEY275/FIEHN).

Gene expression during root colonization

Three biological repetitions of five 7 days old seedlings from Col-0, *Atpdr2* and *Atpdr6* were transferred into sterile magenta pot containing 30 mL of liquid MS supplemented with 1 % sucrose and incubated in growth chamber under a 16h light at 25 °C / 8h dark at 21 °C conditions. When plants were 14 days old, they were washed in sterile water to remove traces of sucrose and transferred into new sterile pot containing fresh MS liquid media without sucrose. At 18 days old, suspension of 5.10^6 cells of *P. fluorescens* F113 wild type, F113 Δ *crc* or F113 Δ *cbrB* mutants were inoculated in each pot containing Col-0 plants and only F113 WT for *Atpdr2* and *Atpdr6*. Three days after the inoculation, 3 mL of RNA protect (QIAGEN, Germany) were added to each pot. Root associated bacteria were collected by vortexing the root system with 1 mL of medium. Culture medium was collected and bacteria were pelleted by centrifugation at 7300 rpm at 4 °C. Bacteria were freeze-dried in liquid nitrogen and conserved at -80 °C until RNA extraction.

Total RNA from pellets were extracted using RNeasy Plus Mini Kit (QIAGEN, Germany). DNA traces were eliminated with DNase I treatments (NEB, USA). Isolated RNA was quantified using Nanodrop ND-1000 spectrophotometer (Labtech, Italy), and 500 ng of total DNA-free RNA was reverse transcribed using PrimeScript™ RT Reagent Kit (Perfect Real Time) (TAKARA, Japan). QRT-PCR was performed using the TB Green Premix Ex Taq (Tli RNase H Plus) kit (TAKARA, Japan). CFX Connect Real-Time PCR detection system from BIO-RAD was used for thermal cycling reactions according to the following protocol: an initial step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and a final step at 72 °C for 3 min in order to amplify, *crcZ*, *crcY* and denitrification (*narG*, *nirS*, *norB1*, *nosZ1*) genes. Primers in Table 2 Normalization of genes expression was performed using *rpoD* housekeeping gene. Fold change was calculated with the $\Delta\Delta$ Ct method according to Livak and Schmittgen (2023). Each condition was performed in triplicate.

Bacterial effect on auxin accumulation

Ten days old *A. thaliana* seedlings harboring the *DR5::GFP* auxin reporter (Friml et al., 2003) were inoculated with 1.10^6 cells of *P. fluorescens* F113 wild type, Δ *crc* or Δ *cbrB* mutants in a

100 μL of 0.9 % NaCl suspensions. Those suspensions were spread along the root systems, and incubated in growth chamber under a 25 °C, 16h light/ 21 °C 8h dark condition for 24 h prior to observations. At least, seven root apexes by plant conditions were imaged by using a Zeiss LSM700 laser confocal scanning microscope with same parameters than those described by Brunoud *et al* (2020). Integrated fluorescens density was quantified by ImageJ software (<http://imagej.net/Welcome>).

Statistical analysis

All experiments were performed in at least three biological replicates. The standard deviation was calculated and statistical significance was determined using Welch's t-test with a P-value < 0.05 for significant result.

All metabolomics analyses were done using R software version 4.1.1 (<https://www.r-project.org/>). Each compound concentration was normalized for each sample by calculating logarithm of the compound concentration divided by the mean concentration of all compounds in the sample. To determine if there were differences between conditions, a PLSDA was performed as a multivariate analysis with normalized concentrations using `plsda` function from `mixOmics` package (Lê Cao and Le Gall, 2011; Hervé et al., 2018). `plsda.VIP` function from `RVAideMemoire` package was used to assign which compound was most discriminant to construct PLSDA model (Galindo-prieto et al., 2014). To determine if a compound concentration was significantly different for a condition, a Kruskal-Wallis test was applied to each compound followed by a Dunn test using `Kruskal.test` and `kwAllPairsDunnTest` functions from `stats` and `PMCMRplus` packages respectively (<https://CRAN.R-project.org/package=PMCMRplus>). PLSDA and boxplot representation was performed using `ggplot2` package (Gómez-Rubio, 2017). Heatmap was done using `pheatmap` package (<https://CRAN.R-project.org/package=pheatmap>) after centering concentration with `scale` function from `base` package.

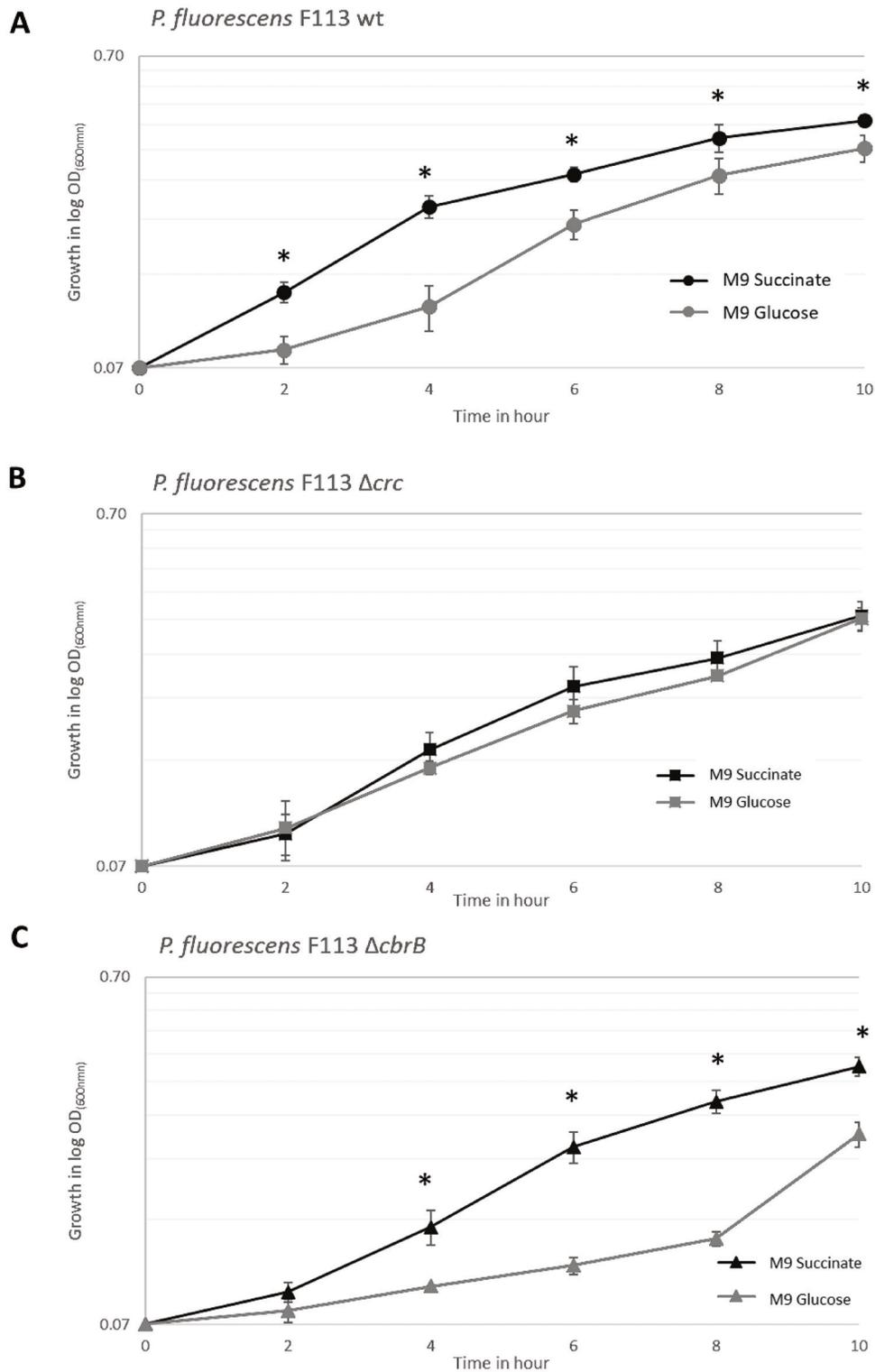


Figure 1. Kinetic growth in aerobic conditions of *Pseudomonas fluorescens* F113 WT and its mutants deleted in *crc* and *cbrB* genes. Growth curve of (A) F113 wild type (circles), (B) F113 Δ *crc* (squares) and (C) F113 Δ *cbrB* (triangles). Cells were grown in M9 minimal medium supplemented with 40 mM of carbon either from succinate (black line) or glucose (grey line) as sole carbon sources in aerobic conditions and OD 600nm were measured during time course in hours. Error bars correspond to standard deviation of three biological replicates. Stars indicate a p-value < 0.05.

Results

***Pseudomonas fluorescens* F113 presents revCCR mechanism where Crc and CbrB act as regulators under aerobic conditions**

To study in detail, the CCR expressed by *P. fluorescens* F113, first we tested its growth in M9 minimum medium supplemented with 40 mM of carbon either from succinate or glucose as sole carbon source under aerobic conditions. The growth start of the wild type strain is delayed by 2 h in the presence of glucose as C source compared to succinate condition (Fig 1A). F113 WT growth rate μ at exponential phase, between 2 and 4 h, in the presence of succinate was about $\mu=0.45 \text{ h}^{-1}$ (± 0.02) whereas it was about 0.22 h^{-1} (± 0.09) in presence of glucose demonstrating that *P. fluorescens* F113 prefer succinate over glucose as C source and hence present revCCR mechanism. Then, to assess the role of Crc and CbrB in the regulation of *P. fluorescens* F113 CCR, we constructed a *crc* and a *cbrB* deletion mutant (F113 Δ *crc* and F113 Δ *cbrB*, respectively) and tested their growth in the same conditions as the WT strain. No difference in growth was observed between the two C sources for the Δ *crc* mutant (Fig 1B) indicating the perturbation of revCCR resulting in an indifference towards carbon sources and demonstrating the importance of Crc in substrate ranking.

The *cbrB* mutant grow similar to WT under succinate condition while its growth was impaired under glucose condition as we observed a delay during the first 8h (Fig 1C) compared to WT. In addition, between 4 and 6 h, growth rate of F113 Δ *cbrB* in the presence of succinate was about $\mu=0.38 \text{ h}^{-1}$ (± 0.04), whereas, it was about 0.09 h^{-1} (± 0.04) in the presence of glucose indicating that F113 Δ *cbrB* has a longer adaptation time to accept sugar as carbon source, compared to WT which indicates the important role of CbrB in the relief of the repression. The complementation of the corresponding two mutants by the expression of either *Crc* or *CbrB* under the control of their native promoter, successfully restored the preference of succinate over glucose as sole carbon source as observed with the WT strain (data not shown).

Taking together, these data confirm the revCCR mechanism of *P. fluorescens* F113 with preference for succinate (organic acids) over glucose (sugar) as sole carbon source for growth in aerobic condition and demonstrate the role of Crc and CbrB as regulators of the repression.

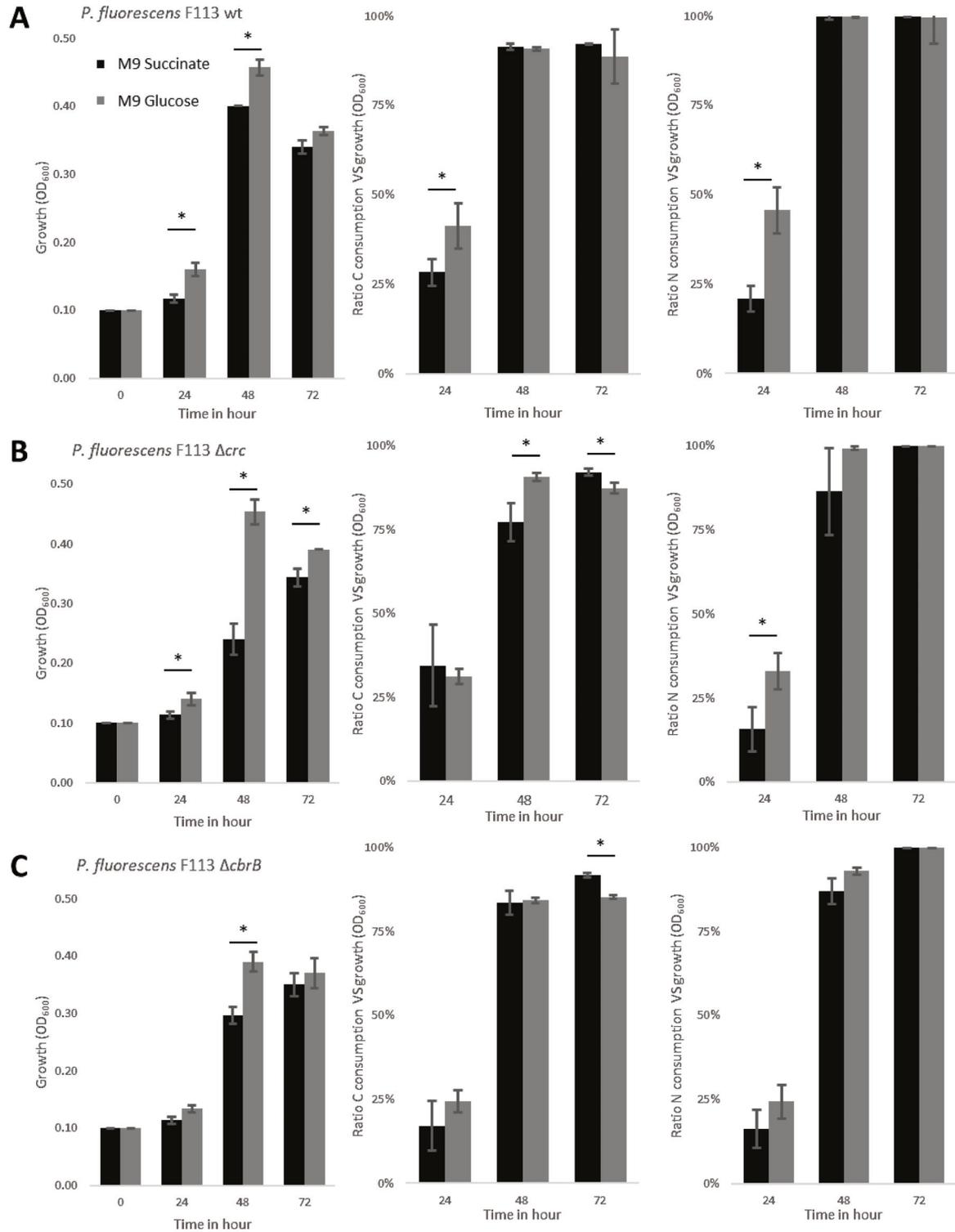


Figure 2. Kinetic growth in anaerobic conditions of *Pseudomonas fluorescens* F113 WT and its mutants deleted in *crc* and *cbrB* genes. Growth curve and ratio of carbon or nitrogen sources consumption reported by the growth for F113 (A), F113 Δ *crc* (B) and F113 Δ *cbrB* (C) under anaerobic conditions. Cells were grown in M9 minimal medium supplemented with 20 mM of KNO_3 and with 40 mM of carbon either from succinate (black bar) or glucose (grey bar) as the sole carbon source under anaerobic conditions. Error bars correspond to standard deviation of three biological replicates. Stars indicate a p-value < 0.05.

Effect of the nature of carbon source on denitrification

To determine the impact of the nature of C source on denitrification process, *P. fluorescens* F113 WT and F113 Δcrc and F113 $\Delta cbrB$ mutant strains were grown under anaerobic conditions in M9 minimal medium supplemented with 20 mM of KNO_3 as N source and with 40 mM of C either from succinate or glucose as sole C source. Under these conditions, the WT strain grew better using glucose as C source and we observed a better C and Nitrate consumption by a factor of 1.5 and 2, respectively at 24 h in comparison with succinate condition (Fig 2A). In presence of glucose as sole carbon source the repression is relieved. And thus having a better growth and utilization of nitrate in presence of sugar suggests that revCCR exerts a negative effect on denitrification and by extension on growth in *P. fluorescens* F113.

Similarly, to WT, F113 Δcrc grew better in the presence of glucose than succinate (Fig. 2B). Indeed, at 48 h significant differences between those growth conditions were observed for C and Nitrate consumption (Fig 2B). Interestingly, at 48 h, the growth of F113 Δcrc in glucose is almost 2-fold higher than the growth observed for succinate indicating a growth defect in the presence of succinate as sole C source compared to the WT strain (Fig 2A-B).

Regarding F113 $\Delta cbrB$, preferential use of glucose is delayed compared to WT strain and only seen at 48h (Fig 2C). However, compared to the WT strain, growth of F113 $\Delta cbrB$ seemed to be negatively affected under both conditions suggesting a key role of CbrB for the optimal growth of F113 under denitrification condition. It is interesting to note that under our conditions, the N source was totally consumed after 48 h of growth (Fig. 2). These data taken together indicate that under anaerobic conditions sugar is preferentially used over organic acids for optimal growth and nitrate utilization. These data confirm our hypothesis that the nature of the carbon source regulates denitrification and so revCCR and its regulators are linked to this regulation.

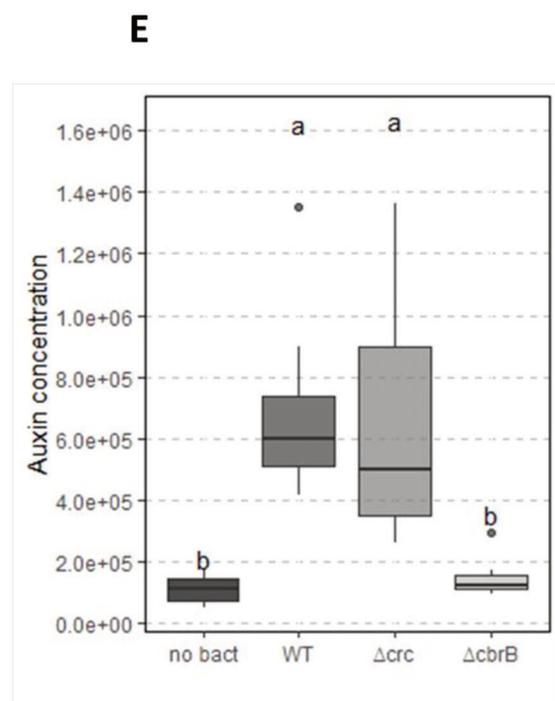
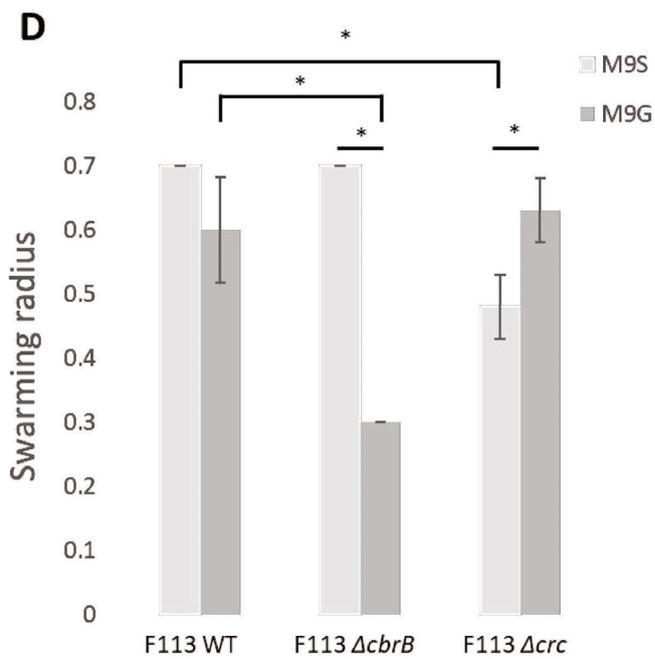
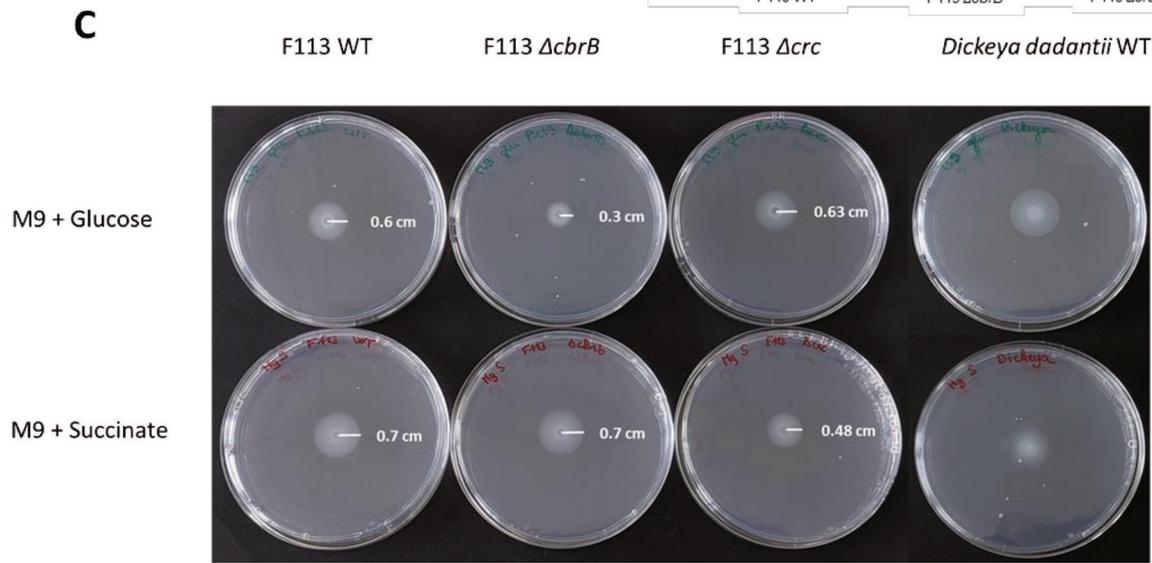
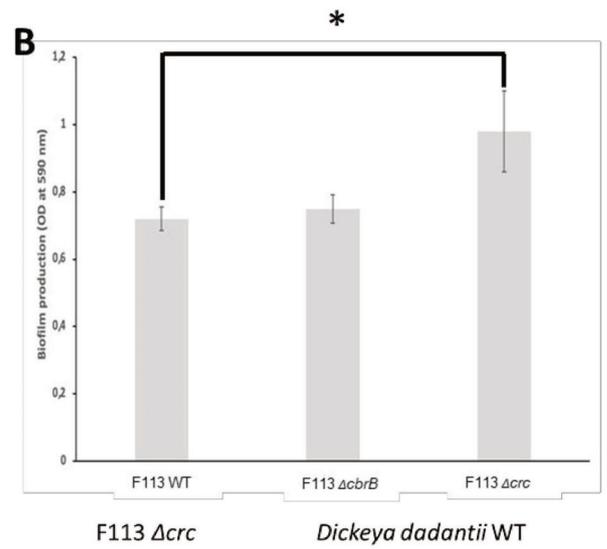
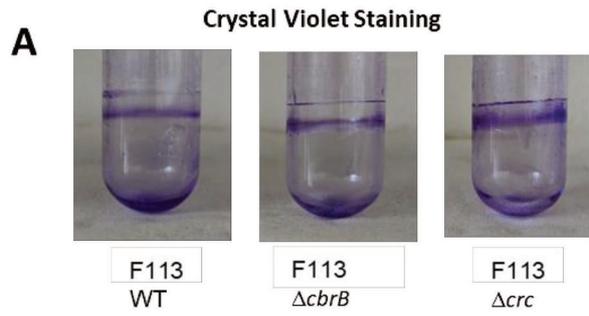


Figure 3. Impact of revCCR on different rhizospheric functions.

(A) Visualization and (B) quantification of biofilm formation in *P. fluorescens* F113 WT, $\Delta cbrB$ and Δcrc mutant strains. Biofilm adhered were stained with crystal violet. Quantification of the formed biofilm using a spectrophotometer at OD_{590nm}. Error bars correspond to standard deviation of three biological replicates. Stars indicate a p-value < 0.05. (C) Visualization and (D) quantification of swarming motility in *P. fluorescens* F113 WT and mutant strains. Strains were grown on soft M9 medium supplemented succinate (M9S) or glucose (M9G) as sole carbon sources. The experiment was performed in quadruplicates and image of representative plates are shown. Error bars correspond to standard deviation of four biological replicates. Stars indicate a p-value < 0.05. (E) Changes in auxin concentration (arbitrary unit) during root colonization in *Arabidopsis thaliana* Col-0 by F113 WT and its $\Delta cbrB$ and Δcrc mutant strains. A condition without bacteria inoculation (No bact) was used as negative control. Box plot from integrated fluorescens density. Root apex from *A. thaliana* DR5::GFP were imaged using confocal scanning microscopy Zeiss LSM700. Integrated fluorescent density was determined using ImageJ software. Differences indicated by different letters are statistically significant (p-values < 0.01), and those by the same letter are not statistically significant (n=7).

RevCCR mediated control of bacterial rhizospheric traits

To evaluate the involvement of revCCR in root colonization traits we evaluated biofilm formation, swarming motility and auxin modulation during root colonization. We first tested the ability of F113 WT, F113 Δcrc and F113 $\Delta cbrB$ strains in biofilm formation on abiotic surface (Fig. 3A-B). Significant increase, by a factor 1.5 in biofilm formation was observed and quantified for the Δcrc mutant compared to the WT strain at 24 h indicating that Crc negatively regulates biofilm formation in *P. fluorescens* F113. No difference was observed for the $\Delta cbrB$ mutant.

Next, we tested the swarming ability of these strains on M9 medium supplemented in glucose or succinate as sole carbon source (Fig. 3C-D). The swarming ability of F113 WT strains was slightly but not significantly reduced in glucose condition (swarming radius (sr) = 0.6 ± 0.08 cm) compared to succinate condition (sr = 0.7 ± 0 cm). For the Δcrc mutant, the ability to swarm was impacted in succinate conditions where the swarming radius was lower (sr = 0.48 ± 0.05 cm) compared to WT strain (sr = 0.7 ± 0 cm). This sustains the role of Crc in maintaining the optimal swarming in presence of the preferred carbon source. The swarming ability of $\Delta cbrB$ mutant was significantly impaired in glucose condition (sr = 0.3 ± 0 cm) compared to succinate

condition ($sr = 0.7 \pm 0$ cm) or WT strain in glucose condition ($sr = 0.6 \pm 0.08$ cm) indicating the role of CbrB as a revCCR reliever regulator in presence of sugar (Fig 1C).

PGPR are known to stimulate the plant growth by auxin production (Spaepen and Vanderleyden, 2011). To determine whether the revCCR of F113 affects auxin homeostasis in *A. thaliana* during root colonization, F113 WT and mutant strains were inoculated on root surface of Col-0 containing the DR5::GFP auxin sensor (Friml et al., 2003). After 24 h of root colonization, in presence of F113 WT auxin maxima in the roots was five times higher than in absence of bacteria (Fig. 3E) demonstrating a positive effect of F113 WT on auxin accumulation. Inoculation by F113 Δcrc strain resulted in similarly high level of auxin accumulation than WT, indicating that Crc is not implicated in this processes. On the other hand, in presence of F113 $\Delta cbrB$ no difference was observed compared to the control roots without bacterial inoculation (Fig. 3E). This result suggests an important role of CbrB for plant-bacteria interaction during root colonization. Taking together, these results confirmed the importance of revCCR mechanism in rhizospheric functions such as biofilm formation, motility and auxin modulation in plant cells.

Impact of root exudates on the expression of denitrifying genes in *Pseudomonas fluorescens* F113

To determine the impact of primary metabolites exuded by plants on denitrification, we followed changes in denitrification genes expression (*narG*, *nirS*, *norB*, *nosZ*) and revCCR ncRNA expression (*crcZ*, *crcY*) after root colonization of *Arabidopsis thaliana* Col-0 as well as two ABC transporters plant mutants by *Pseudomonas fluorescens* F113. ABC transporter mutants are known to influence plant exudate composition (Badri et al., 2008).

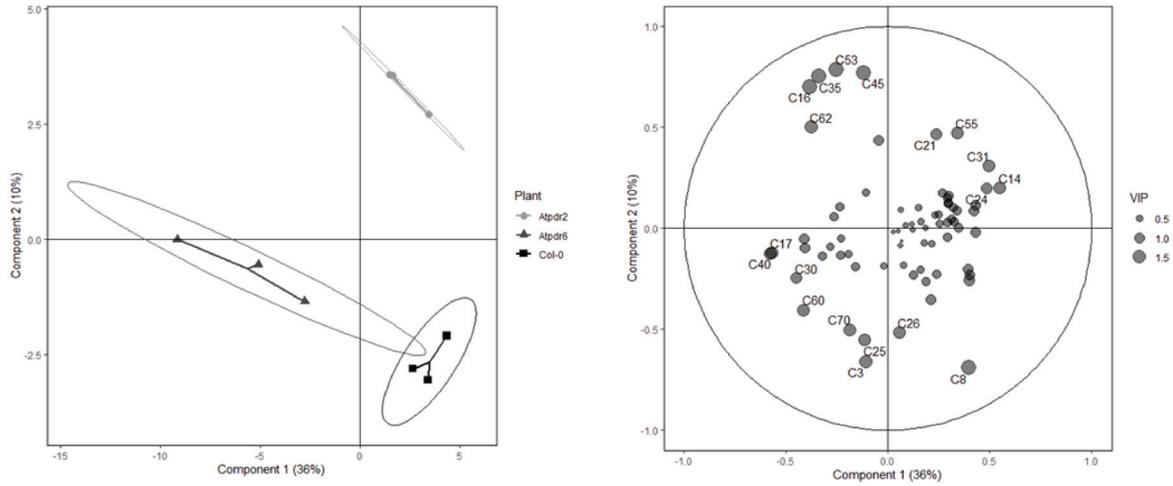
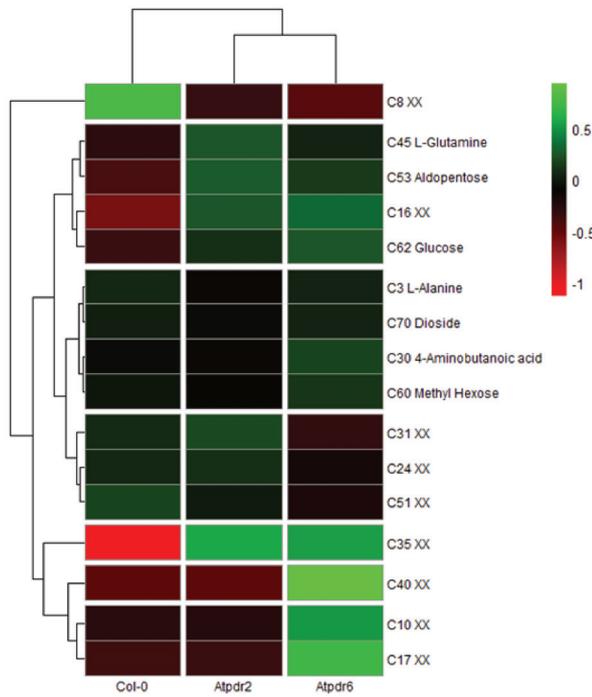
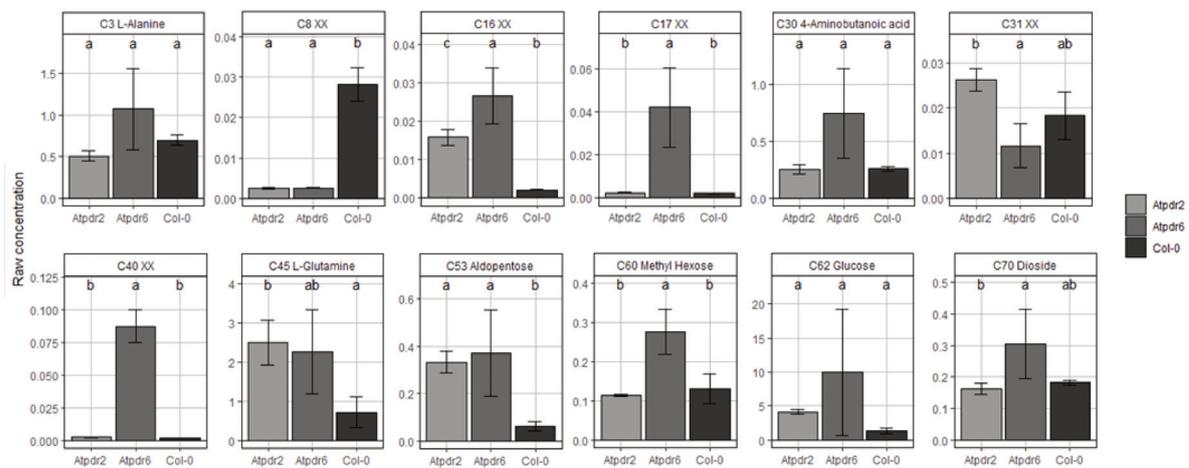
A**B****C**

Figure 4. *Arabidopsis thaliana* ABC transporter mutants show differences in root exudates profiles. (A) Partial Least Square-Discriminant Analysis (PLS-DA) and most discriminating compounds (Variable Importance in the Projection =VIP), performed from GC-MS data obtained from root exudates collected from *Arabidopsis thaliana* Col-0 (black squares), *Atpdr2* (light grey circles) and *Atpdr6* (grey triangles) mutant lines. **(B)** Hierarchical Cluster Analysis in heat map representing the abundance of most discriminating compounds with a VIP score greater than 1. Red represents a lower relative abundance and Green represents a higher relative abundance. **(C)** Bar plot of raw concentration of annotated most discriminating compounds. Statistical differences between plant lines are indicated using letters a–c (Tukey’s HSD test, $P < 0.05$). Means and standard error are represented ($n = 3$).

First, we compared the primary metabolites exuded by *A. thaliana* Col-0 and *Atpdr2*, *Atpdr6* ABC-transporter mutant lines. Our results demonstrated important differences between the three lines (Fig. 4A). Most of the variability in the data could be explained by component 1 with 36% of the total variability (Fig. 4A). Among 70 peaks detected on chromatographic profiles, 19 discriminate compounds with a VIP (Variable Importance in the Projection) score greater than one are highlighted (Fig. 4A). Those compounds were used to perform a Hierarchical Cluster Analysis (Fig 4B). Among them, seven were identified: L-Alanine (C3), 4-Aminobutanoic acid (C30), L-Glutamine (C45), Aldopentose (C53), Methyl Hexose (C60), Glucose (C62) and Dioside (C70). Briefly, according to HCA (Hierarchical cluster analysis) (Fig 4B), both ABC transporter mutant lines seem to exude more L-Glutamine, Glucose, Aldopentose and an unidentified C35 compound but less of the unidentified C8 compound. Interestingly, *Atpdr6* exudes two unidentified compounds, C17 and C40, that were absent in Col-0 and *Atpdr2* (Fig. 4C). In fact, mutant lines seem to be enriched in the exudation of sugar compounds, and particularly *Atpdr6*, which exude more of a Methyl Hexose (C60) and a Dioside (C70) compared to Col-0 and *Atpdr2* (Fig. 4C). Taking together our results showed differences in primary metabolites exuded by the different *A. thaliana* lines.

To study the impact of different root exudates profiles on bacterial gene expression during root colonization, we performed quantitative RT-PCR and analyzed the expression profile of bacterial genes implicated in revCCR and denitrification (*crcZ*, *crcY*, *narG*, *nirS*, *norB*, *nosZ*) during root colonization of *A. thaliana* Col-0 and ABC transporter plant mutants. We observed a more than 5-fold increase for *narG*, *crcZ* and *crcY* expression when Col-0 WT was inoculated by Δ *crc* and Δ *cbrB* mutant strains compared to the inoculation with F113 WT (Fig. 5A). The *nirS*

expression was also up regulated (5-fold) but only after inoculation with Δcrc mutant indicating that Crc negatively affects *nirS* (Fig. 5A). These results suggest an increase in nitrate and nitrite reduction during denitrification process in *P. fluorescens* F113 Δcrc and $\Delta cbrB$ mutant strains suggesting that denitrification genes expression are regulated by revCCR mechanism. Surprisingly, *crcY* and *crcZ* were overexpressed when F113 Δcrc and $\Delta cbrB$ mutants were inoculated comparing to F113 WT strains suggesting a strong implication of the two sRNA during the interaction of the two mutant strains with the plant. Upon exposure to different root exudates profiles, the expression of denitrification and revCCR genes of F113 WT was also affected. In the *Atpdr2* and *Atpdr6* mutants, *nosZ* and *crcZ* were up-regulated (Fig. 5B) compared to inoculated Col-0 WT. Moreover, in the presence of *Atpdr6* exudation, *narG*, *norB*, and *crcZ* were also up regulated (Fig. 5B), suggesting a better denitrification activity with *Atpdr6* compared to *Atpdr2*. These results support our hypothesis that the regulation of denitrification is regulated by the quality of root exudates *via* revCCR mechanism.

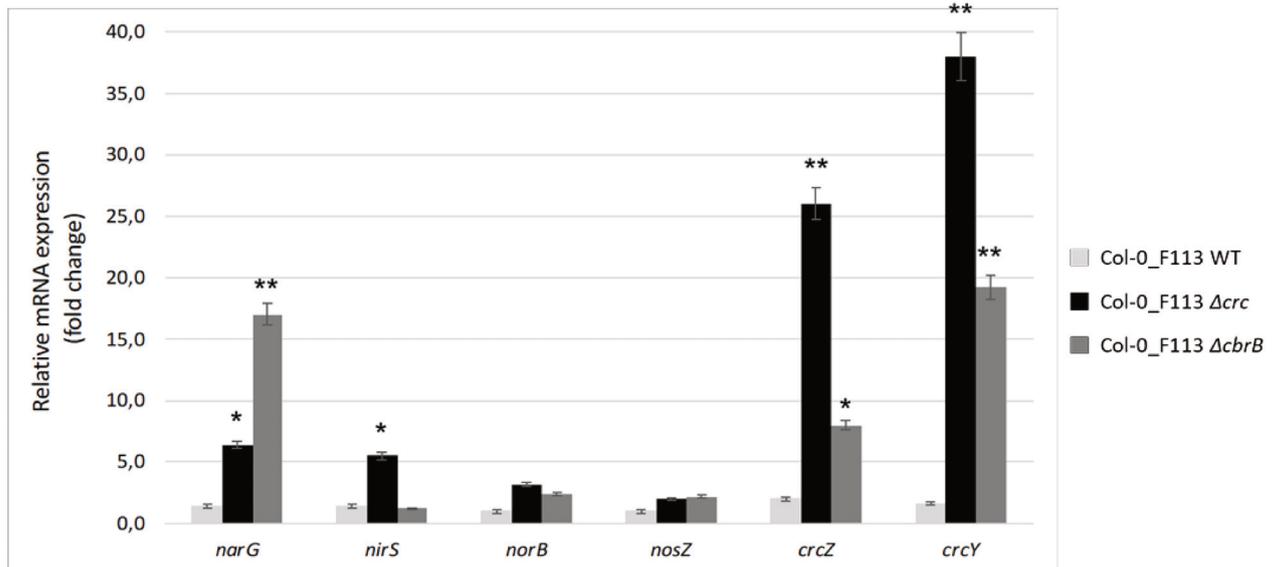
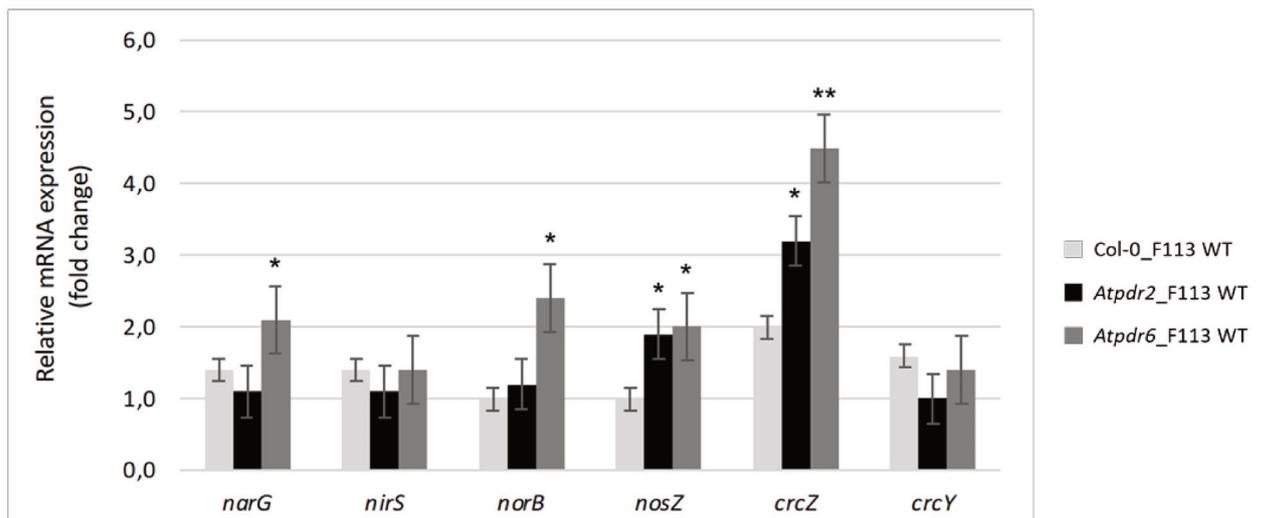
A**B**

Figure 5. Expression profile of genes involved in revCCR and denitrification during root colonization of *Arabidopsis thaliana* WT and ABC transporter mutants. Gene expression profile were analyzed after 3 days post inoculation. **(A)** Effect of Δ *crc* and Δ *cbrB* F113 mutants on expression of *narG*, *nirS*, *norB*, *nosZ*, *crcZ* and *crcY* after their inoculation in *A. thaliana* WT line. **(B)** Effect of *Atpdr2* and *Atpdr6* plant mutants on expression of *narG*, *nirS*, *norB*, *nosZ*, *crcZ* and *crcY* of WT F113. Fold change in gene expression was normalized using *rpoD* as housekeeping bacterial gene and using the $\Delta\Delta$ Ct method. Quantitative real-time PCR was performed in triplicate, and data presented are mean of relative change, and the error bar represents standard deviation. *, significant difference $p < 0.05$; **, significant difference $p < 0.01$.

Discussion

RevCCR regulates aerobic and anaerobic growth in F113

The revCCR mechanism is well described in *P. aeruginosa* PAO1, where Crc and CbrB act as main regulators (Rojo, 2010; Franzino et al., 2022). Metabolic pathways of non-preferred carbon source, such as sugar compounds, are under the repression of revCCR and thus PAO1 will have a reduced growth in presence of glucose compared to that in presence of succinate, which is the preferred carbon source (Sonnleitner et al., 2009; Rojo, 2010; Sonnleitner et al., 2012). Here, we observed the same growth profile for *P. fluorescens* F113 WT than PAO1 under aerobic condition. In addition, our data show that in the absence of Crc, *P. fluorescens* F113 was able to grow in the presence of glucose as non-preferred carbon source without any delay as shown with the WT strain demonstrating the role of Crc in catabolic repression. This result is also in accord with Molina and colleagues (2019) where *Pseudomonas putida* KT2440 Δ crc mutant presents a higher glucose consumption rate during the early stages of growth concomitant with a high growth level. Moreover, in the presence of glucose as sole carbon source the F113 Δ cbrB mutant was still able to grow but with a greater delay than the wild type strain, which is in agreement with Nishijyo et al (2001) where PAO1 Δ cbrB mutant express a growth defect in presence of glucose as sole carbon source. This result demonstrates the regulator role of CbrB in the relief of the repression. CbrB is implicated in the expression of the sRNA CrcZ which sequester Crc in order to remove the repression (Sonnleitner et al., 2009). In PAO1 only one sRNA participating in the removal of revCCR repression was found, but in other *Pseudomonas* species such as *P. putida* KT2440 and *Pseudomonas syringae* pv. *tomato* DC3000, other sRNA are present: *crcY* and *crcX*; respectively (Moreno et al., 2012; Filiatrault et al., 2013). BLAST analysis revealed the presence of *crcY* in addition to *crcZ* in *P. fluorescens* F113 (Franzino et al., 2022). CrcY is likely to be redundant with CrcZ (Liu et al., 2017) and in *P. putida* KT2440, the transcription of *crcY* seems to be partially due to CbrB unlike for *crcZ* (Moreno et al., 2012). As even in the absence of CbrB, *P. fluorescens* F113 ends of growing in the presence of non-preferred C source suggest that CrcY might take the lead over CrcZ in the relieved of the revCCR mechanism in our conditions and therefore other regulatory systems independent of CbrB might interplay in the transcription of *crcY* in order to relieved the repression.

Many bacteria can use nitrate (NO_3^-) as terminal electron acceptor during denitrification process, allowing them to grow under anoxic condition (Lecomte et al., 2018). In our study, we confirm the role of revCCR on *P. fluorescens* F113 growth under aerobic condition, but to our knowledge, no study explored its role during anaerobic growth. Under anoxic condition, our results show a better growth associated with a better C and NO_3^- consumption in presence of glucose as C source rather than succinate for F113 WT strain. This result suggests that Crc inhibits the use of NO_3^- as terminal acceptor electron under anaerobic conditions and hence its reduction suggesting the role of revCCR in denitrification regulation. Other regulating systems can also participate in the regulation of denitrification. Indeed, it has been shown that the expression of denitrification genes in PAO1 is under the control of many regulators such as the nitrate sensing system NarXL, or the oxygen sensors ANR and DNR (FNR-like proteins) (Schreiber et al., 2007). Pusic and colleagues (2016) suggested that *crcZ* might be regulated by ANR, since a putative motif was found in the promoter region of *crcZ* in *P. aeruginosa* PAO1. In *P. fluorescens* F113, we found the same putative ANR site in the promoter region of *crcY* but surprisingly not in the promoter region of *crcZ* (data not shown). This indicates that CrcY might be more effective to relieve the repression during anaerobic growth than CrcZ. Thus, it seems important to consider these other denitrification regulatory systems in order to deeply understand denitrification regulation.

RevCCR mechanism is involved in *P. fluorescens* F113 rhizospheric traits

Franzino and colleagues (2022) underlined that CCR is an important trait involved in plant-microbe interactions. In order to colonize roots efficiently, bacteria possess a set of rhizospheric traits such as motility or the ability to form biofilm. Mutation in genes implicated in biofilm formation or motility in *P. putida* resulted in a decrease in its ability to colonize seeds and roots (Yousef-Coronado et al., 2008). To explore the role of revCCR in regulating rhizospheric functions we investigated *P. fluorescens* F113 WT, Δ *crc* and Δ *cbrB* mutant strains in their capacity to form biofilm on abiotic surface as well as their swarming ability. In our conditions, F113 Δ *crc* mutant produced more biofilm on abiotic surface than WT strain. This result is similar to Chakravarthy et al (2017) where *P. syringae* pv *tomato* DC3000 *crc* mutant showed an increase in biofilm formation. This can suggest that F113 Δ *crc* mutant might be

able to colonize more efficiently plant roots. Then we observed a significant reduced swarming ability in the presence of glucose for $\Delta cbrB$ mutant strain. Similarly, Sivakumar et al., (2021) showed that *P. aeruginosa* PGPR2 $\Delta cbrB$ mutant was impaired in swarming ability in presence of sugar as C source in agreement with our findings. During this study they demonstrated that PGPR2 $\Delta cbrB$ mutant was depleted in flagella production which is crucial for root colonization which explained the observed decrease in corn root colonization (Sivakumar et al., 2021). From this we can suggest that F113 $\Delta cbrB$ is also impaired in root colonization, therefore demonstrating the important role of the revCCR during plant - bacteria interaction.

Pseudomonas fluorescens F113 is known to be a PGPR as it possesses many plants beneficial function that promotes the plant growth (Vacheron et al., 2018; Ortiz-Castro et al., 2019; Alani et al., 2020) Castro and colleagues (2019) demonstrated that *P. putida* and *P. fluorescens* stimulate the growth of *Arabidopsis thaliana* with an induction of auxin response element in root tips. In our study, F113 $\Delta cbrB$ was unable to stimulate the auxin signaling in Col-0 roots compared to F113 WT and Δcrc strains probably due to its inability to colonize plants roots. In fact, at ten days of growth, *A. thaliana* root exudates are mostly composed of sugar compounds (Chaparro et al., 2013). F113 $\Delta cbrB$ mutant is impaired in swarming ability in presence of glucose suggesting that F113 $\Delta cbrB$ mutant might be impaired in root colonization and hence in auxin induction in *A. thaliana* root tips. Taking together, our results demonstrate that CbrB may plays a crucial role in root colonization.

Rev CCR regulators control denitrification genes and non-coding RNA expression *in planta*

The upregulation of *narG* and *nirS*, *in planta*, in the absence of Crc confirm that Crc negatively affect nitrate reduction indicating that revCCR inhibits denitrification process as shown in our previous results. The overexpression of *narG* in the absence of CbrB could be explained by the disequilibrium of C/N balance held by CbrB and NtrBC systems (Li and Lu, 2007). For small RNA, the strong induction of *crcZ* and *crcY* in Δcrc mutant compared to $\Delta cbrB$ might be due to the fact that CbrB control the expression of these ncRNA (Moreno et al., 2012). In addition, our observation that the expression is not totally repressed in the absence of CbrB suggest the involvement of another regulator in *crcZ* and *crcY* expression. We also

show that *crcY* is more expressed than *crcZ* which can be explained by the potential activation of *crcY* by ANR regulator under anaerobic conditions as suggested by Pusic et al (2016).

The expression of denitrifying genes and non-coding RNA is also modulated according to plant root exudates composition. For example, *Atpdr6* mutant, enriched in sugar exudation, induces *narG*, *norB*, *nirS* and *crcZ*. The increase in *crcZ* expression seems to be responsible of the upregulation of denitrifying genes as CrcZ participates in the relieved of the repression mediated by revCCR (Rojo 2010). We previously reported a negative control of denitrification gene expression by revCCR mechanism. Therefore, we suggest that as we have more sugar exuded, CrcZ is more transcribed and thus the repression of the denitrification is more relieved. These results confirm our main hypothesis that the nature of primary metabolites exuded by plants controls the regulation of denitrification by revCCR.

Conclusion

So far, little was known about the link between the C metabolism and denitrification process. In this study, we demonstrated the regulatory role of the revCCR mechanism on the (i) growth of *P. fluorescens* F113 under aerobic and anaerobic conditions and (2) on the expression of certain rhizospheric traits of F113. We also revealed the impact of primary metabolites on the regulation of denitrification gene expression mediated by the revCCR mechanism. The present work evidenced the importance of revCCR mechanism during plant-bacteria interactions.

Chapitre 3 :

Influence du microbiote sur la
physiologie de la plante *via* les
exsudats racinaires

Préambule

L'objectif de ce chapitre va être de comprendre comment la nature des exsudats racinaires influence le microbiote de la plante et quel va être le retour du microbiote recruté sur la physiologie de la plante.

Pour cela, nous avons utilisé les lignées d'*A. thaliana* WT et les mutants *Atpdr2* et *Atpdr6*, qui ont des compositions d'exsudat différentes (voir chapitre 2). Dans un premier temps, *A. thaliana* WT a été cultivé sur un sol de type prairie provenant de La Côte-Saint-André (coordonnées GPS : 45°22'29.1"N 5°16'37.4"E ; 38130, France) afin de récupérer le microbiote au niveau des racines et du sol adhérent aux racines (SAR). Le microbiote extrait a ensuite été inoculé de façon *in vitro* sur les racines des différentes lignées de l'étude. L'effet de ce microbiote sur la signalisation hormonale des différentes lignées de plante a été étudié. Ce chapitre sera inclus dans une future publication où certains résultats sont en cours d'analyse.

A. Introduction

Dans le sol, les interactions plantes - microorganismes sont médiés par les exsudats racinaires. Ces molécules carbonées vont constituer une ressource nutritive de choix pour les microorganismes ou bien servir comme signal annonçant la présence de la plante (Haichar et al., 2014). L'exsudation racinaire est influencée par de nombreux facteurs biotiques comme abiotiques qui vont modifier la quantité et la composition des molécules sécrétées (Carvalhais et al., 2011; Haichar et al., 2014). Ainsi, les voies de biosynthèse et de transport des exsudats de la plante vont jouer un rôle central dans cette exsudation. Parmi ces transporteurs, on retrouve des ABC transporteurs, qui sont des complexes protéiques transmembranaires utilisant de l'énergie sous forme d'ATP pour leur fonctionnement (Kang et al., 2011; Hwang et al., 2016). Il a ainsi été confirmé que la mutation de certains de ces ABC transporteurs provoque une altération de la composition de l'exsudat de la plante (Badri et al., 2007; Badri et al., 2008; Chapitre 2). La mutation dans le gène *Atpdr2* codant un ABC transporteur entraîne une exsudation plus importante en acide organique, tandis que la mutation dans *Atpdr6*

résulte en une exsudation plus importante des sucres (Badri et al., 2008; Oa et al., 2009; chapitre 2). Or la nature de l'exsudat est déterminante pour la composition du microbiote qui est recrutée par la plante (Chaparro et al., 2013; Vannier et al., 2019; Feng et al., 2021). Ainsi, on pose comme hypothèse que des lignées de plantes mutées sur les gènes codant pour des ABC transporteurs recrutent des microbiotes différents.

Une fois établies, les interactions plantes - microorganismes peuvent être bénéfiques ou néfastes pour la plante. Notamment, durant ces interactions, la balance hormonale de la plante va être modifiée. Au sein du microbiote, il existe de nombreuses espèces capables de promouvoir la croissance de la plante et/ou de stimuler les défenses contre différents pathogènes (Acosta and Farmer, 2010; Spaepen and Vanderleyden, 2011; Pieterse et al., 2014). Certaines bactéries telles que les *Azospirillum*, *Bacillus* ou *Pseudomonas* sont des bactéries dites PGPR (Plant Growth Promoting Rhizobacteria) qui sont capables de stimuler la croissance des plantes par la production d'auxines exogènes (Ortiz-castro et al., 2009; Spaepen and Vanderleyden, 2011; Ortiz-Castro et al., 2019). En effet l'auxine est une des principales phytohormones produites par les plantes qui jouent un rôle majeur dans la coordination de la croissance et l'adaptation aux changements environnementaux (Gallei et al., 2020; Yu et al., 2022). L'auxine la plus répandue dans la nature est l'acide indole-3-acétique (IAA : Indole-3-acetic acid) dont le tryptophane est le précurseur (Won et al., 2011; Napier, 2016). Une fois produite, l'auxine va être transportée et redistribuée dans toute la plante afin de promouvoir différents processus tels que le développement ou la maturité des différents organes végétaux (Napier, 2016). Ainsi, la production de l'auxine est plus importante dans les tissus en croissance active. Par exemple dans les racines, l'auxine est principalement retrouvée dans le méristème et la columelle, zones d'élongation de la pointe apicale (Petrášek, 2009; Smolko et al., 2021).

L'autre phytohormone majeure des plantes est le jasmonate (JA) qui fait partie de la famille de molécules dérivées des oxylipines (Acosta and Farmer, 2010; Wasternack and Kombrink, 2010). Le JA est également impliqué dans le développement de la plante mais il contribue aussi à l'établissement de la réponse défensive de la plante face à divers agresseurs (Zhu and Napier, 2017). La stimulation des défenses de la plante implique l'induction de la réponse systémique (ISR pour Induced Systemic Resistance), résultant de l'activation de la voie de réponse aux jasmonates (Pieterse et al., 2014). De nombreux microorganismes sont

capables de stimuler la réponse ISR afin de rendre la plante plus robuste face aux nombreuses agressions dont elle va faire face tout au long de son développement (Pieterse et al., 2014). D'autre part, il a été démontré que de nombreuses bactéries pathogènes sont capables de produire une molécule semblable au JA pour contourner la réponse défensive de la plante à leur présence (Zhang et al., 2017). Par exemple, la bactérie phytopatogène *P. syringae* est productrice de la toxine coronatine (COR) ayant des propriétés structurales et fonctionnelles mimant celles du jasmonoyl-isoleucine (JA-Ile) (Bender et al., 1999). Ainsi par la production de COR, *P. syringae* détourne la réponse de la plante par l'activation de la voie du jasmonate au lieu de déclencher la voie de signalisation de l'acide salicylique, réponse critique en cas d'attaque par un pathogène biotrophe ou hemibiotrophe (Zhang et al., 2015).

L'interaction entre la plante et son microbiote est donc complexe et doit être finement coordonnée pour assurer la croissance optimale de la plante, mais également de la bactérie. Si la plante régule les interactions qu'elle va établir avec son microbiote par le biais de son exsudation, quel va être l'impact de ce microbiote sélectionné via les exsudats racinaires sur la physiologie de la plante ? Pour répondre à cette question, une communauté microbienne issue de la rhizosphère d'*A. thaliana* WT (racines + SAR) a été inoculée sur différentes lignées d'*A. thaliana* mutées pour des gènes codant des ABC transporteurs et présentant un profil d'exsudation différent. Afin d'analyser l'impact sur la physiologie de la plante, nous avons suivi l'accumulation d'auxine et la présence de JA dans les cellules à l'aide de plantes portant des gènes rapporteurs de la signalisation hormonale (Thines et al., 2007; Jedličková et al., 2022).

B. Matériel et méthodes

1. Condition de culture et croissance des plantes

Arabidopsis thaliana Columbia-0 (Col-0) a été utilisé en tant que plante de lignée sauvage (WT). *Atpdr2* (GK-041D08-015219) et *Atpdr6* (SALK_082843) sont des lignées mutées dans un transporteur ABC. Ces lignées ont été obtenues depuis Nottingham Arabidopsis Stock Centre (NASC). Ces lignées mutantes ont été vérifiées par génotypage par PCR en utilisant les amorces suivantes : GABI_TDNA_PDR2fw (CATATGCCGAGATTGGTCTAACCGCAAATG) et

GABI_TDNA_PDR2rv (CCCACCACGGAATCTTTGATGTCATCAAGC) pour le criblage de l'insertion du T-DNA dans le gène *Atpdr2* ; SALK_TDNA_PDR6fw (GGTCAAACCTGGAGATATCCTCAGTACAATGCC) et SALK_TDNA_PDR6rv (GACTCATATCTCCAACACCAGTGATGTGCACAATCG) pour le criblage de l'insertion du T-DNA dans le gène *Atpdr6* ; LBb1,3 T-DNA (ATTTTGCCGATTTCCGGAAC) ciblant spécifiquement le T-DNA.

Pour suivre la signalisation, nous avons utilisé des lignées rapporteur d'auxine *DR5::GFP* (Friml, 2003) ou rapporteur de l'acide jasmonique (JA) *JAZ1-GUS* (Thines et al., 2007). Ces gènes rapporteurs, s'ils sont activés, reflètent l'induction d'une réponse de la plante via l'activation des voies de biosynthèse de l'hormone dans la cellule (activation de la transcription ou dégradation protéique) plutôt qu'une concentration de ladite hormone. Afin de générer les lignées *Atpdr2-DR5::GFP*, *Atpdr2-JAZ1-GUS*, *Atpdr6-DR5::GFP* et *Atpdr6-JAZ1-GUS* nous avons croisé les lignées mutantes *Atpdr2* et *Atpdr6* avec les plantes contenant les gènes rapporteurs (Fig 1 - croisement). Les plantes de la génération F2 ont été génotypées à l'aide du kit PCR Phire Plant Direct PCR Master Mix (Thermo Scientific™) selon les recommandations du fournisseur et les lignées doubles homozygotes mutation/rapporteur ont été sélectionnées.

Les graines d'*Arabidopsis thaliana* ont été stérilisées par vapeur de gaz selon Clough et Bent (1998) et semées sur milieu Murashige et Skoog (MS) (Duchefa, Pays-bas) contenant 0,8 % d'agar puis incubées en chambre de culture sous un cycle de 16 h jour / 8 h nuit à 22 °C.

2. Extraction et culture de la communauté microbienne

Arabidopsis thaliana Col-0 a été cultivé sur un sol de prairie provenant de La Côte-Saint-André (coordonnées GPS : 45°22'29.1"N 5°16'37.4"E ; 38130, France) pendant 9 semaines de croissance. La communauté microbienne (CM) représentative de Col-0 a été ensuite extraite à partir des racines et du SAR de la plante. Brièvement, 5 g de sol et les racines ont été broyées à l'aide d'un mortier stérile et de 45 mL d'eau stérile. Après décantation, le surnageant a été ensuite récupéré et dilué au dixième avec du NaCl 0,9 %, puis étalé sur gélose de Lysogeny Broth dilué au 10^{ème} (LB 1/10) ainsi qu'en LB 1/10 liquide, puis mis à incuber pendant 48 h à 30 °C. La CM a ensuite été récupérée depuis la boîte et mélangée à la culture liquide et mise

en conservation dans du glycérol 40 % à -80 °C. L'identification de cette communauté microbienne cultivable est en cours.

3. Effet de la communauté microbienne sur l'accumulation d'auxine

Des plantules âgées de dix jours de *A. thalina* WT-DR5::GFP, *Atpdr2*-DR5::GFP, *Atpdr6*-DR5::GFP ont été inoculées avec la CM dans une suspension de 100 µL à DO = 0,1 de 0,9 % NaCl étalé le long de la racine, puis incubées en chambre de culture sous un cycle de 16h de jour à 25 °C et 8 h de nuit à 21 °C pendant 24 h avant observation. Dans chaque condition, 7 pointes apicales ont été observés par microscopie confocale à balayage laser ZEISS LSM800 en utilisant un laser de 488 nm pour l'excitation. La densité de fluorescence intégrées a été quantifiée à l'aide du logiciel ImageJ (<http://imagej.net/Welcome>).

4. Effet de la communauté microbienne sur l'accumulation de jasmonate

Des plantules âgées de dix jours de *A. thalina* WT_JAZ1-GUS, *Atpdr2*_JAZ1-GUS, *Atpdr6*_JAZ1-GUS ont été inoculées et incubées de la même façon que précédemment. Dans chaque condition, le protocole de coloration GUS a été réalisé sur huit plantules. Pour cela, les plantules ont été immergées dans un volume d'acétone 80 % glacial puis incubées pendant 30 min à température ambiante. L'acétone a été ensuite retiré et suivi par deux lavages des plantules avec un tampon phosphate (composition : 0,05 M NaH₂PO₄ + 0,05 M Na₂HPO₄). Les plantules ont ensuite été immergées dans un volume de solution de coloration GUS (composition : 0,2 g d'acide 5-bromo-4-chloro-3-indolyl-β-D-glucuronique (X-Gluc) + 0,1% Triton + 0,002 M K₃[Fe(CN)₆] + 0,002 M K₄[Fe(CN)₆]) puis incubées dans le noir à 37 °C sur la nuit. Après incubation les plantules sont rincées avec un tampon phosphate (composition : 0,05 M NaH₂PO₄ + 0,05 M Na₂HPO₄) et sont conservées dans de l'éthanol à 70 % à 4°C jusqu'à l'observation. Les plantules ont été observées puis photographiées par une loupe binoculaire.

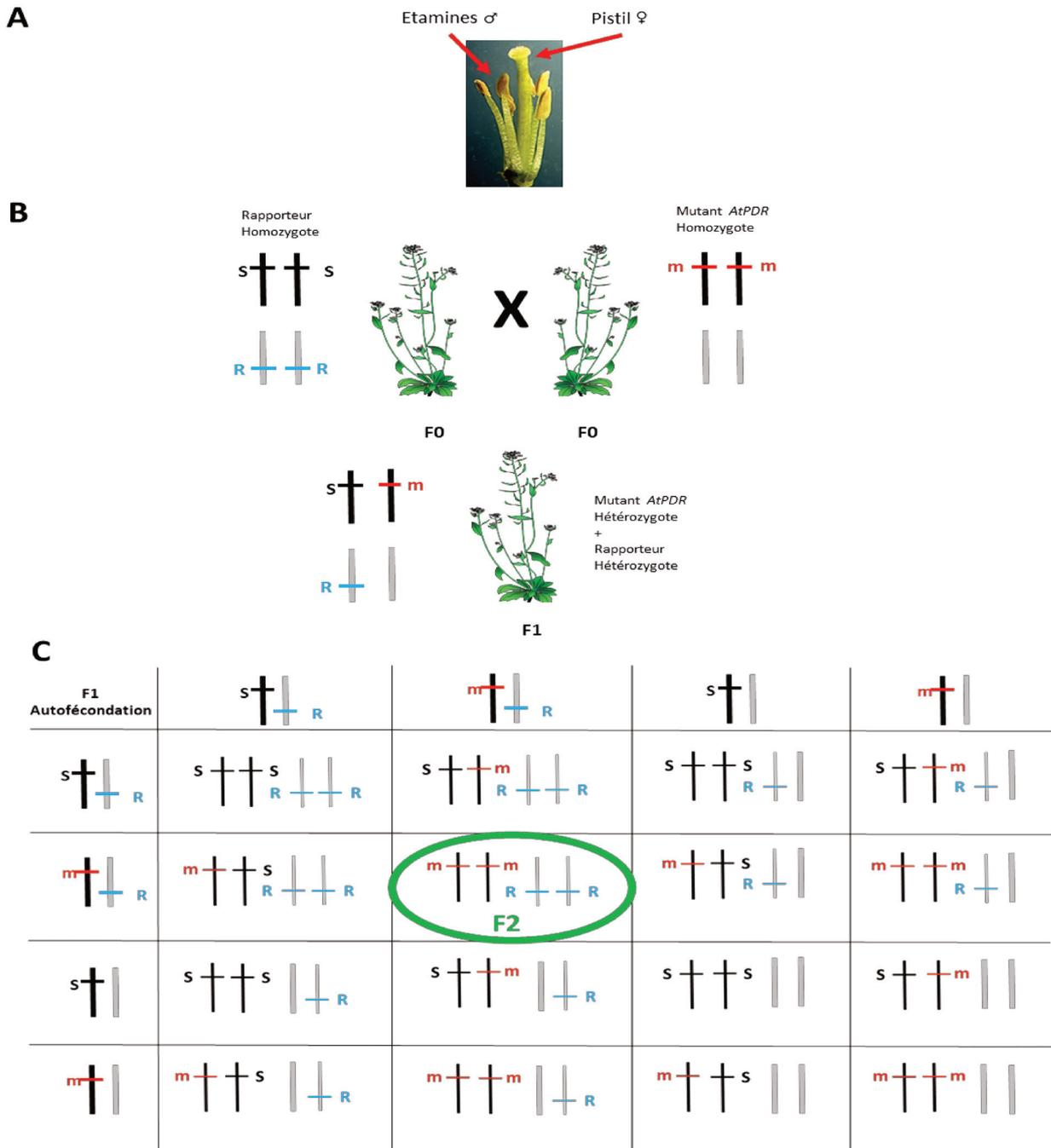


Figure 1 : Croisement entre deux lignées d'*A. thaliana*. (A) Photographie des étamines et du pistil d'*A. thaliana* Col-0. (B) Représentation schématique du croisement entre une plante homozygote pour le rapporteur et une plante homozygote pour la mutation *Atpdr* (plantes F0). Toutes les plantes issues de ce croisement (génération F1) sont hétérozygotes pour la mutation *Atpdr* et pour le rapporteur de la signalisation hormonale. Les différents gènes sauvages, mutés et rapporteurs sont représentés, avec en noir « S » le gène codant pour l'ABC transporteur sauvage ; en rouge « m » le gène codant pour l'ABC transporteur muté ; en bleu « R » le rapporteur de la signalisation hormonale. (C) Schéma d'autocroisement de la génération F1. La plante F2 double homozygote pour la mutation et pour le marqueur de la signalisation hormonale se retrouve d'une fréquence 1:16 dans cette population (entouré en vert). Ces plantes ont été sélectionnées pour la suite des expérimentations.

C. Résultat et discussion

Comme nous avons vu précédemment, il existe une communication entre la plante et son microbiote *via* la production des hormones pour assurer une interaction optimale. La plante régule ces interactions par le biais de son exsudation et en retour son microbiote impacte sa physiologie. Afin de déterminer comment la communauté microbienne affecte la balance hormonale de l'auxine et du jasmonate chez les lignées *Atpdr2* et *Atpdr6* d'*A. thaliana* présentant un profil d'exsudation différent (Badri et al., 2008; Franzino et al en préparation), la communauté a été inoculée sur leurs racines. Nous avons le suivi de l'accumulation d'auxine ou la présence de JA dans les cellules en suivant la fluorescence des lignées portant le gène rapporteur *DR5::GFP* (Friml, 2003) ou regardant l'accumulation de bleu dans les lignées portant le gène rapporteur *JAZ1-GUS* (Thines et al., 2007), respectivement. Les observations ont été réalisées après 24 h de colonisation racinaire.

I. Effet de la communauté microbienne sur l'homéostasie du jasmonate

Ces expériences ont été conduites en utilisant le reporteur *JAZ1-GUS* qui nous a permis de suivre l'accumulation des JAs *in planta*. Après coloration de X-Gluc, l'expression de la β -glucuronidase (GUS) si le gène rapporteur est activé produit un précipité bleu responsable de la coloration. Dans la construction *JAZ1-GUS* l'enzyme est fusionnée à la protéine *JAZ1*, répresseur de la voie de réponse du Jasmonate (Memelink, 2009). En effet, *JAZ1* se lie à des facteurs de transcription, intervenant dans la voie de défense et de réponse aux blessures et attaques d'herbivores tel que *MYC2*, et inhibe leur activité (Memelink, 2009). En présence de JA, la protéine *JAZ1* est ubiquitinée et va être dégradée par le protéasome et par conséquent l'activité GUS sera absente. Ainsi la dégradation de la protéine *JAZ1-GUS*, en présence de JAs, va conduire à une absence de coloration indiquant que les voies de réponse au jasmonate ont été activées (Thines et al., 2007). Après coloration GUS, sans inoculation de la CM, nous

observons une forte coloration GUS aussi bien dans la racine que dans la partie aérienne des lignées Col-0_JAZ1-GUS et *Atpdr2*_JAZ1-GUS, indiquant un niveau bas ou une absence de JA dans les plantes sans inoculation de la communauté microbienne (Fig. 2).

En absence de communauté, une plus faible coloration bleue a été observée dans la lignée *Atpdr6*_JAZ1-GUS indiquant un niveau d'accumulation basale du JA plus importante comparée à la lignée sauvage Col-0 ou *Atpdr2* (Fig. 2). Nos observations nous laissent supposer que la voie du JA est perturbée par la mutation *Atpdr6*, et plus particulièrement dans la partie aérienne de la plante. La lignée *Atpdr6* est une lignée mutée d'un gène codant un ABC transporteur. Or, ce transporteurs pourraient intervenir dans le transport et l'accumulation du jasmonate dans les différents compartiments cellulaires (Li et al., 2017). Ainsi, la mutation du gène *atpdr6* entrainerait une perturbation dans ces processus de transport de jasmonate et aboutirait à un niveau de base plus élevé de JA dans ces plantes.

En présence de la CM, la coloration des lignées Col-0_JAZ1-GUS et *Atpdr2*_JAZ1-GUS est moins intense comparée aux conditions sans inoculum (Fig. 2) alors qu'aucune coloration bleue n'est observée pour la lignée *Atpdr6*_JAZ1-GUS (Fig. 2). Ce résultat nous indique qu'en présence de la CM, il y a une production massive de jasmonate pour les trois lignées et plus particulièrement pour la lignée *Atpdr6*_JAZ1-GUS. De nombreux microorganisme, notamment les *Pseudomonas* et *Bacillus*, sont capables d'induire la réponse systémique (ISR) de la plante (Pieterse et al., 2014). Nous supposons que l'inoculation de la CM sur les racines des plantes a déclenché l'ISR de la plante via la production de jasmonate puis son transport vers les parties aériennes (absence ou faible coloration GUS). Cela est supporté par l'observation de l'accumulation des JA non seulement dans les racines qui ont été mises en contact avec les CMs, mais également dans la partie aérienne de la plante (absence de la coloration GUS dans les feuilles).

Un lien entre la voie du jasmonate, l'exsudation racinaire et le microbiote associé a déjà été démontré auparavant (Carvalhais et al., 2015). En effet, chez les mutants *myc2* et *med25*, participant dans la réponse au jasmonate, une baisse d'exsudation en asparagine, ornithine et en tryptophane a été observée. Cela a conduit à un recrutement plus faible en bactéries du genre *Clostridium* et cela pour les deux mutants comparés à la lignée sauvage. Cette altération d'exsudation a été également corrélée à un changement au niveau du microbiote associé aux

racines de la plante avec un enrichissement de bactéries participant à la défense de la plante telles que certains *Bacillus* comme *Bacillus gibsonii* et *Bacillus nealsonii*, au détriment de bactéries participant à la stimulation de la croissance de la plante comme *Bacillus niacini* et *Pseudomonas putida* (Carvalhais et al., 2013). Nous pouvons ainsi supposer qu'une modification dans le profil d'exsudation va modifier une partie de la CM en favorisant certaines espèces qui vont avoir un impact plus important dans la stimulation de la production de jasmonate chez la plante. Cependant, nous ne pouvons pas encore conclure si l'accumulation de jasmonate observée va contribuer au développement ou à la défense de la plante. Des expériences complémentaires sont en cours de réalisation pour avoir plus de précision (Voir "Perspectives").

Nos observations indiquent un rôle important pour *Atpdr6* dans la communication entre la plante et son microbiote (Fig. 2), car la lignée *Atpdr6_JAZ1-GUS* est marquée par une accumulation basale du JA plus importante comparée à la lignée sauvage Col-0 et une accumulation de JA suivant l'inoculation avec les CM. La lignée *Atpdr6* mutante d'un ABC transporteur possède un profil d'exsudation enrichi en sucre comparé aux plantes Col-0 (Chapitre 2; Franzino en préparation). Comme suggéré par Chaparro et ses collègues (2013), une exsudation plus importante en sucre permet l'attraction d'un grand nombre de microorganismes. Les sucres sont les substrats préférés de nombreuses bactéries exprimant la répression catabolique du carbone (CCR) (Franzino et al., 2022). Des fonctions clés dans l'interaction plante-bactéries sont régulées par la CCR notamment pour la colonisation racinaire et la stimulation de la plante (Franzino et al., 2022). Ainsi nous pouvons supposer que la lignée *Atpdr6* est capable de recruter un plus grand nombre de microbes ayant pour conséquence une stimulation plus importante de la voie de réponse au jasmonate.

Il est démontré que *Atedr6* joue un rôle dans la défense d'*A. thaliana* face à la maladie, par exemple, chez le pathogène *Alternaria brassicicola*, par la sécrétion de camalexine (Khare et al., 2017). La camalexine est un métabolite secondaire de type alcaloïde indole, elle possède des propriétés phytoalexines et permet à la plante de se défendre en cas d'agression. De plus l'expression de *Atpdr6* est stimulée par la présence de méthyl-jasmonate (MeJA) indiquant son rôle dans la voie de réponse au jasmonate (Khare et al., 2017). Ainsi, nous pouvons supposer que le mutant *Atpdr6* possède une sensibilité accrue face à la CM qui se traduit par une plus forte montée de JA (absence de coloration GUS) dans les cellules de la plante

comparée à Col-0. Nous ne pouvons aussi exclure le fait que la mutation de *Atpdr6* entraîne un défaut de sécrétion de camalexine ce qui entraîne son accumulation au sein des tissus. De par sa nature, la camalexine peut également être toxique pour les cellules de la plante (Glawischnig, 2007). Ainsi, nous pouvons supposer qu'une accumulation de camalexine dans les cellules de la plante peut entraîner une réponse liée au jasmonate. Cependant, comme indiqué plus haut, la signalisation du jasmonate peut intervenir dans soit le développement de la plante, soit son système de défense face aux stress. Au vu des différents résultats que nous avons obtenus, nous ne pouvons statuer sur la direction prise par la stimulation de la voie de réponse aux jasmonate.

De plus, Carvalhais et ses collègues (2013) ont montré qu'en stimulant la voie du jasmonate par exposition au MeJA, il en résultait une modification du profil d'exsudation chez *A. thaliana*. Donc nous pouvons imaginer une communication bilatérale entre la plante et son microbiote, où l'exsudat racinaire influence le microbiote et *vice versa*, jusqu'à un équilibre qui est optimale aussi bien pour la plante que pour la bactérie.

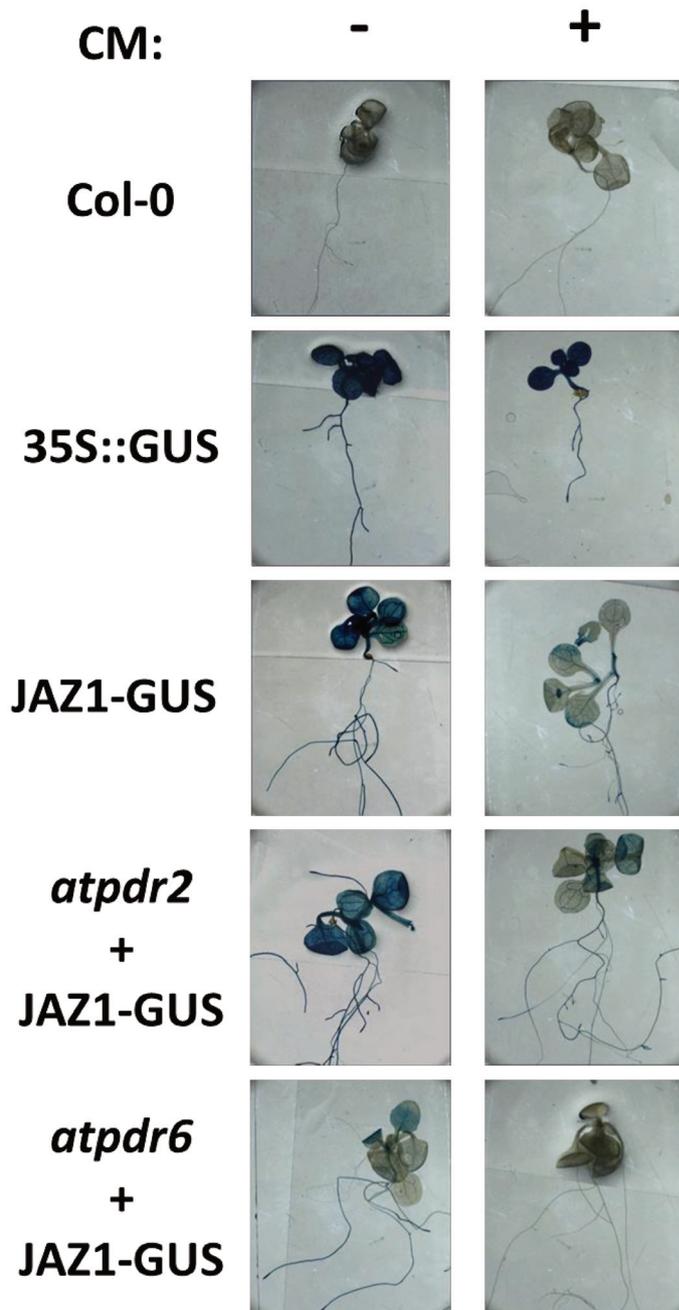


Figure 2 : Changement dans la réponse au jasmonate des différentes lignées d'*A. thaliana* après colonisation racinaire par la communauté microbienne synthétique. Les racines de différents génotypes ont été mises en contact avec la communauté microbienne pendant 24h, puis l'accumulation de JAs a été visualisé à l'aide de reporteur JAZ1-GUS. Images obtenues à partir d'une loupe binoculaire couplée à une caméra numérique. Les lignées Col-0 sans rapporteur et 35S::GUS ont servies comme contrôle négatif et positif de coloration respectivement. La colonne de droite (+) correspond à la condition où la communauté microbienne a été inoculée le long des racines, la colonne de gauche (-) correspond à la condition sans inoculation. Images représentatives de 4 échantillons.

II. Effet de la communauté microbienne sur l'homéostasie de l'auxine

Ces expériences ont été conduites en utilisant le rapporteur DR5::GFP qui nous a permis de suivre l'accumulation des auxines *in planta*. L'étude des promoteurs des gènes répondant à l'auxine a révélé un site spécifique nommé AuxRE permettant leur induction en présence des auxines. DR5 possède en son promoteur le motif AuxRE, ce qui lui permet d'être rapporteur indirect de la transcription de gènes en réponse à l'auxine (Jedličková et al., 2022). Des études mené par Benkova et ses collègues (2003) ont démontré que l'activité de DR5 était en adéquation avec leur observation d'accumulation d'auxine et donc que DR5 est un système fiable pour suivre l'accumulation de l'auxine dans les cellules. Ce système de suivi de la concentration d'auxine est notamment utilisé notamment dans le cadre des interactions plant-microorganisme (Xiao et al., 2020). Sans CM, DR5::GFP se localise au niveau du méristème de l'apex racinaire (Fig. 3A). Ceci indique que les auxines sont concentrées au niveau du méristème indiquant la présence de cellules en cours de division pour assurer la croissance et l'élongation de la racine. Ceci est en accord avec les observations faites par Petersson et al (2009).

Après 24h de colonisation racinaire suite à l'inoculation de la CM, nous pouvons observer une diminution significative de l'accumulation d'auxine chez le mutant *Atpdr2* (Fig. 3). Au contraire, pour le mutant *Atpdr6* on observe une augmentation significative de l'accumulation d'auxine dans la racine (Fig. 3). Ces deux lignées mutantes présentent un profil d'exsudation modifié par rapport à la lignée sauvage Col-0 (Chapitre 2 ; Franzino en préparation). L'exsudation de certains composés permet le recrutement spécifique de certaines espèces plutôt que d'autres. Nous savons que le microbiote de la rhizosphère, notamment les bactéries du genre *Pseudomonas*, sont capables de produire des auxines ou de stimuler la production d'auxines chez la plante (Ortiz-Castro et al., 2019). Ainsi nous pouvons supposer que de part de la modification du profil d'exsudation, il va y avoir un changement dans la composition du microbiote qui va avoir une réponse différente dans l'interaction plante-microbiote et dans notre cas sur la modulation de l'auxine.

Dans le cas du mutant *Atpdr2* nous avons une communauté qui semble inhiber la présence d'auxine au niveau du méristème des racines de la plante ce qui suggère un ralentissement dans sa croissance. L'activité inhibitrice de l'auxine sur la racine des plantes est bien documentée (Gallei et al., 2020), mais cet effet reste non élucidé. En effet, l'auxine est aussi bien capable d'inhiber que de stimuler la croissance racinaire (Yu et al., 2022). De plus, la balance hormonale de l'auxine orchestre de nombreux autres phénomènes en lien avec la croissance de la plante, tels que le phototropisme de la partie aérienne, le gravitropisme des racines en quête de ressources ou pour éviter certains stress (Yu et al., 2022).

Une diminution de l'expression de DR5::GFP au niveau de l'apex principal chez *A. thaliana* suite à l'inoculation de *B. megaterium* a été observé (López-bucio et al., 2007) associée à une redistribution de l'auxine au niveau de l'émergence de racines secondaire. Ainsi, nous pouvons supposer que la modification du profils d'exsudation d'*Atpdr2* va favoriser le recrutement d'espèces microbiennes, notamment des PGPRs, pour favoriser la relocalisation de l'auxine. Ainsi l'élongation des racines secondaires sera stimulée au détriment de la racine principale.

De l'autre côté, nous observons une accumulation d'auxine au niveau de l'apex racinaire de la lignée *Atpdr6* après inoculation de la CM ce qui suggère une stimulation de la croissance de la racine (Figure 3). Comme dit plus haut, de nombreux microorganismes sont capables de stimuler la production d'auxines, mais également capable d'en produire. Les bactéries du genre *Pseudomonas* tels que *P. putida*, *P. fluorescens* et *P. aeruginosa* sont capables de stimuler la croissance de leur plante hôte par production d'IAA (Ortiz-Castro et al., 2019). Nous pouvons donc supposer que le profil d'exsudation d'*Atpdr6* va favoriser le recrutement et la prolifération d'espèces capable de stimuler la production d'auxine dans la racine.

Une complexité supplémentaire est due au fait que l'auxine n'agit pas seul, mais la croissance des organes dont les racines sont régulées par une balance entre différentes phytohormones. De multiples hormones sont responsable de l'élongation de la racine principale, de son arrêt de développement ainsi que la prolifération de racines secondaires. On retrouve à côté des auxines les cytokines (CK), l'éthylène (ET) ou encore l'acide abscisique (ABA). L'ABA est également un régulateur de croissance des plantes pouvant être synthétisé par des rhizobactéries tels que *Azospirillum brasillense* sp. ou encore *B. cereus* et *B. megaterium*

(Karadeniz et al., 2006; Cohen et al., 2008). Il a été récemment observé que l'ABA modifie le gravitropisme de l'apex racinaire en inhibant certains transporteurs d'auxines comme PIN2 (Xie et al., 2021). Dans leur étude, Xie et ses collègues (2021) ont également observé une diminution de l'accumulation d'auxines à l'apex chez *A. thaliana* via le gène rapporteur DR5 suite à l'ajout d'ABA exogènes. Donc, il est fort probable que les changements observés en accumulation de l'auxine dans les mutants avec différents profils d'exsudation reflètent un changement dans la balance hormonale en générale de la plante.

Dans le cas du mutant *Atpdr6*, nous avons observé une augmentation aussi bien de l'auxine que des JAs (Fig. 2-3). Lors de l'ISR il y a également intervention de l'éthylène. L'éthylène va avoir pour effet d'inhiber l'expansion cellulaire et l'élongation de la racine principale favorisant le développement des racines secondaire. De plus, l'éthylène stimule également la production d'auxine ainsi que son transport jusqu'aux apex nouvellement formés (Swarup et al., 2007; Lewis et al., 2011). Ainsi nous pouvons croiser nos résultats et supposer que *Atpdr6* semble être plus sensible à la CM, ce qui en retour provoque une plus forte induction de l'ISR par montée de JA, mais aussi une hausse dans la production d'auxine potentiellement due à la montée également d'éthylène.

D'autre part, il existe une communication croisée entre l'auxine et le JA (Xu et al., 2020). En effet le JA participe également à l'inhibition de l'élongation de la racine en diminuant le nombre et la taille des cellules de la pointe apicale (Chen et al., 2011). Lors de l'activation de MYC2 par la voie de réponse aux jasmonates, MYC2 réprime l'expression des gènes codant PLT1 et PLT2 qui sont des facteurs de transcription clefs pour la croissance de la racine (Chen et al., 2011). Ces facteurs de transcriptions PLTs répondent positivement à la présence d'auxine dans la cellule (Chen et al., 2011). Ainsi l'interaction entre le JA et l'auxine est un élément important dans le développement et la croissance de la plante qui reste un processus compliqué faisant intervenir de nombreux acteurs finement régulés (Xu et al., 2020).

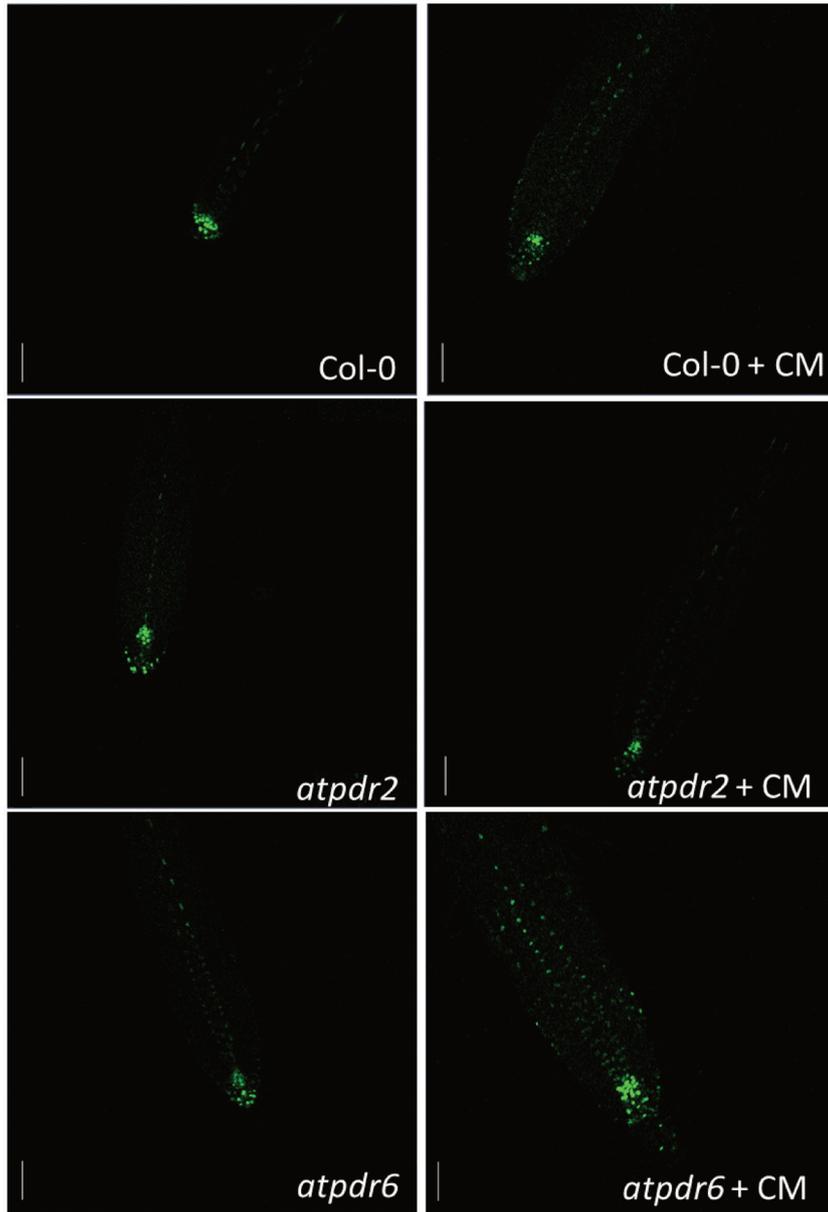
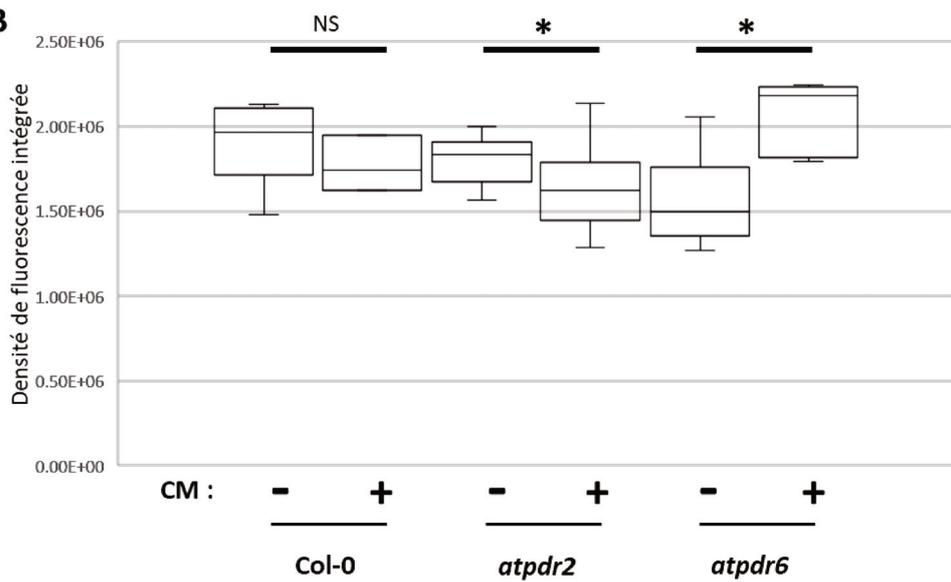
A**B**

Figure 3 : Changements dans l'accumulation de l'auxines des différentes lignées d'*A. thaliana* après colonisation racinaire par la communauté microbienne synthétique. Les racines de différents génotypes ont été mises en contact avec la communauté microbienne synthétique pendant 24h, et l'accumulation de l'auxine a été visualisée à l'aide de reporteur DR5::GFP. **(A)** Observation d'apex racinaire en présence ou non de la CM après 24h de colonisation racinaire par microscopie confocale à balayage laser ZEISS LSM800 objectif x20. Image représentative d'au moins 4 échantillons. En blanc, la barre d'échelle de 50 μm . **(B)** Diagramme en boîte à moustache de la densité de fluorescence intégrée en présence ou non de la CM et sur les différentes lignées de plante. La densité de fluorescence intégrée a été déterminée en utilisant le logiciel ImageJ. NS : non significatif, * indique une différence significative ($p\text{-value} < 0.05$) après un test t de Student ($n=4$).

D. Conclusion

L'objectif de cette étude était de comprendre comment la nature des exsudats racinaires influence le microbiote de la plante et quel est le retour de ce microbiote sur la physiologie de la plante. L'inoculation d'une communauté microbienne rhizosphérique sur différentes lignées d'*A. thaliana* présentant des profils d'exsudation différents nous a permis de mettre en avant des différences dans l'impact du microbiote sur la physiologie de la plante. Nous avons observé qu'à partir de la même communauté il pouvait y avoir soit une inhibition, soit une stimulation de l'accumulation de l'auxines dans la racine. Cela peut se traduire par des différences au niveau du développement et de la structuration du système racinaire pour une croissance optimale. Nous avons également observé que la plante pouvait être plus sensible à la communauté dans sa réponse aux jasmonates suivant son profil d'exsudation. Ceci peut indiquer de différences de niveau de l'ISR. En conclusion, nous avons montré qu'il y a une communication active entre la plante et son microbiote. A partir d'une même communauté microbienne, l'influence qu'elle va avoir sur la physiologie de son hôte est médiée par son hôte lui-même via l'exsudation racinaire.

Chapitre 4 :

Discussion et Perspectives

A. Contexte et rappel des objectifs.

L'objectif de ces travaux de thèses était de comprendre comment la nature des métabolites primaires exsudés par la plantes régule le processus de dénitrification microbienne au travers de la répression catabolique du carbone (CCR), mais aussi d'étudier son impact sur la physiologie de la plante *via* la communauté microbienne sélectionnées. Dans un premier temps nous nous sommes intéressés à la régulation de la dénitrification par la nature de la source de C *via* la CCR en présence de plantes (type sauvage et mutants) présentant différents profils d'exsudation, puis nous avons observé l'impact de l'exsudation racinaire sur la sélection de la communauté microbienne rhizosphérique et son impact en retour sur la physiologie de la plante. Pour ce faire, différentes approches *in vitro* et *in planta* ont été réalisées allant de l'étude de la régulation de la dénitrification à l'échelle de la souche *P. fluorescens* F113 et à l'échelle d'une communauté microbienne complexe en interaction avec la plante *Arabidopsis thaliana*.

Dans ce chapitre final, nous discuterons les différents résultats obtenus tout au long de cette thèse et ensuite nous proposerons de nouvelles perspectives afin d'ouvrir sur des nouveaux travaux visant à approfondir notre compréhension sur la régulation de la dénitrification dans la rhizosphère.

B. Discussion

I. Lien entre répression catabolique du carbone et dénitrification

L'étude de la littérature nous a permis de mettre en avant le rôle régulateur de la CCR lors des interactions plantes-bactéries (Franzino et al., 2022). La dénitrification est une voie de respiration alternative réalisée par les microorganismes en absence d'oxygène et induite par la présence d'une source d'azote comme le nitrate, mais également par la présence d'une source de carbone (Zumft, 1997; Philippot, 2002). La dénitrification est fortement stimulée par les exsudats racinaires dans la rhizosphère (Haichar et al., 2012). Bien que la régulation de la dénitrification par l'oxygène et l'azote ait longtemps été décrite, peu d'études se sont intéressées sur le lien entre la régulation catabolique du C (CCR) et la dénitrification. **Ainsi une des hypothèses de cette thèse était que la dénitrification est régulée par la nature de la source de C via la CCR.** Dans un premier temps nous avons montré l'impact de la revCCR (CCR de type inverse pour une préférence en acide organique chez les *Pseudomonas*) sur la croissance en aérobie et en anaérobie (condition de dénitrification) chez *P. fluorescens* F113, une rhizobactérie dénitrifiante largement retrouvés dans la rhizosphère d'*A. thaliana* (Haichar et al., 2012). Nous avons confirmé la préférence du succinate (acide organique) par rapport au glucose (sucre) en culture en aérobiose. En anaérobiose, et donc en condition de dénitrification, cette tendance est inversée. En effet, on observe une meilleure croissance associée à une meilleure consommation de la source de C et de la source de N en présence de glucose par rapport à la présence de succinate. Nous en avons conclu que la revCCR réprime la dénitrification chez *P. fluorescens* F113. Cependant d'autres systèmes de régulation peuvent être impliqués. En effet, il a été démontré chez *P. aeruginosa* PAO1, que l'expression des gènes de la dénitrification est régulée par le système senseur du nitrate NarXL, le senseur d'oxygène ANR ainsi que par la protéine régulatrice DNR (Schreiber et al., 2007). Chez PAO1, la levée de la répression de la revCCR fait intervenir un ARN non codant (ARNnc) CrcZ (Sonnleitner et al., 2012). De plus, Pusic et ses collègues (2016) ont mis en avant la présence

d'un site consensus d'ANR dans la région promotrice de *crcZ* suggérant ainsi le contrôle de *crcZ* par ANR en condition d'anaérobiose. Durant notre étude, nous avons trouvé le même site consensus d'ANR dans la région promotrice de *crcY*. *CrcY* est un deuxième ARNnc participant également à la levée de la revCCR chez certaines *Pseudomonas* comme *P. fluorescens* F113 (Franzino et al., 2022). Ainsi nous pouvons suggérer une régulation croisée entre les différents régulateurs de la dénitrification et la revCCR. Cette première approche nous a permis d'observer le rôle régulateur de la revCCR dans la dénitrification. L'utilisation du mutant F113 Δ *crc*, après délétion du gène codant pour répresseur de la revCCR, nous a permis de confirmer le rôle inhibiteur de la revCCR sur le processus de la dénitrification. De plus, L'utilisation du mutant F113 Δ *cbrB*, gène codant pour le régulateur de réponse du système à deux composante CbrAB, nous a permis de confirmer son rôle dans la levée de la répression lors de la dénitrification. Ainsi *Crc* et *CbrB* semblent réguler la dénitrification *in vitro*.

Dans un second temps, nous avons analysé l'expression des gènes de la dénitrification en présence de la plante modèle *Arabidopsis thaliana*. En absence de *CbrB*, protéine permettant de contrôler l'expression de *crcZ* et donc la levée de la répression, nous avons observé la surexpression du gène *narG* codant pour la nitrate réductase. Cette observation peut être expliquée par le déséquilibre de la balance C/N. En effet, *CbrB* participe en commun avec *NtrBC* pour le maintien de la balance C/N de la cellule. Il serait donc intéressant d'étudier le rôle de *NtrBC* dans la régulation de la dénitrification. Ainsi *Crc* et *CbrB* semblent réguler la dénitrification *in planta*. Au terme de cette étude, nous avons également démontré que l'expression des gènes de la dénitrification et des ARNnc (*CrcZ* et *CrcY*) était modulée par la nature des exsudats racinaires de la plante en utilisant les mutants d'*A. thaliana*. Nous avons observé qu'en présence d'un profil d'exsudation enrichi en sucre, il en résulte une surexpression de *crcZ* et des gènes de la dénitrification. Nous en avons déduit que la surexpression des gènes de la dénitrification était due à l'accentuation de la levée de la répression de la revCCR par *CrcZ* en présence de la source non préférée de *P. fluorescens* F113. Confirmant ainsi notre hypothèse de départ à savoir que la nature des métabolites primaires exsudés par les plantes régule le mécanisme de la dénitrification *via* la revCCR.

II. Impact des exsudats racinaires sur le microbiote de la plante et son retour sur la physiologie de la plante

Dans le chapitre 3, nous avons observé la réponse sur la physiologie de la plante d'une communauté microbienne rhizosphérique inoculée au niveau des racines de plantes d'*A. thaliana* présentant des profils d'exsudation racinaires différents. La communauté microbienne provenant d'*A. thaliana* Col-0 a été extraite après 9 semaines de croissance sur un sol de type prairie provenant de La Côte-Saint-André. Cette communauté microbienne (CM) va représenter l'ensemble des microorganismes cultivables en milieu liquide et solide qui sont présents au niveau du sol adhérent aux racines et sur les racines de la plante. Étant donné que la CM a été extraite après 9 semaines de culture, nous pouvons considérer qu'elle est bien établie dans la rhizosphère d'*A. thaliana*. Afin d'observer l'impact de cette CM sur la physiologie de la plante et le rôle des exsudats racinaires dans cet effet, nous avons inoculé la CM le long des racines de plantules d'*A. thaliana* Col-0 WT et des deux lignées mutantes *Atpdr2* et *Atpdr6*, présentant des profils d'exsudations différents (Franzino et al en préparation). Nous nous sommes intéressés de suivre des changements d'accumulation de deux phytohormones : le jasmonate et l'auxine. Le jasmonate est impliqué dans la réponse défensive de la plante et contribue également à son développement (Zhu and Napier, 2017). L'auxine est également une hormone impliquée dans la croissance et le développement de la plante (Napier, 2016).

Nos résultats indiquent qu'en fonction de la nature des exsudats, une même communauté pouvait soit inhiber (*Atpdr2*) soit stimuler (*Atpdr6*) l'accumulation de l'auxine au niveau de la pointe apicale. Nous pouvons supposer que le changement d'exsudation influence la composition du microbiote, mais aussi son activité ce qui en découle une réponse différente dans la modulation de l'auxine. Une diminution dans la concentration d'auxine au niveau de la pointe apicale, est souvent synonyme de sa relocalisation pour l'émergence de racines secondaires. Nous pouvons, (i) d'une part, supposer que le profil d'exsudation de *Atpdr2* va favoriser le recrutement de microorganismes qui vont influencer de manière positive cette relocalisation. (ii) D'autre part, supposer que la lignée *Atpdr6* a recruté des microorganismes qui vont stimuler la production d'auxine.

Nos résultats ont également montré qu'en présence de la CM, il y a une production de jasmonate. Cela indique, comme attendu, que la présence de microorganismes non pathogènes permet l'induction de la résistance systémique de la plante (ISR pour Induced Systemic Resistance). Cependant nous avons observé une plus forte production de jasmonate chez la lignée *Atpdr6* que pour les autres lignées. De la même façon que pour l'auxine, nous pouvons supposer que le profil d'exsudation de *Atpdr6* a favorisé le recrutement de communauté microbienne stimulant plus fortement l'ISR. Par ailleurs, l'exsudation de *Atpdr6* a montré un enrichissement en sucre (Chapitre 2, Franzino en préparation). Cet enrichissement en sucre pourrait conduire à un recrutement plus important de microorganismes. En effet, le sucre est le substrat préféré d'un grand nombre de microorganismes au travers du mécanisme de la répression catabolique du carbone (Franzino et al., 2022). Il est donc possible que *Atpdr6* va favoriser le recrutement des organismes exprimant la CCR. De plus, nous avons vu dans les chapitres précédents que la CCR est un système important dans l'établissement des interactions plante-bactéries. Il est donc possible qu'en modulant la CCR par l'exsudation racinaire, nous pouvons moduler les interactions entre la plante et son environnement.

C. Perspectives

Elucider la régulation de la dénitrification dans la rhizosphère

Au cours du chapitre 2, nous avons montré le lien entre la régulation de la dénitrification et la répression catabolique du carbone. De façon générale, nous proposons d'analyser les autres systèmes de régulation de la dénitrification en présence des différents profils d'exsudation. Afin de lever la répression en présence de la source de carbone non préférée, CbrB régule la transcription de l'ARNnc CrcZ (Sonnleitner et al., 2012). CbrB participe avec NtrBC, système à deux composantes intervenant dans l'assimilation du nitrate, dans le maintien de la balance C/N (Li and Lu, 2007). Etant donné l'implication de NtrBC dans le métabolisme du N, nous proposons d'étendre ces analyses en construisant le mutant F113 $\Delta ntrBC$. La possible régulation croisée entre le senseur d'oxygène ANR et l'ARNnc CrcY est une piste intéressante, c'est pourquoi nous proposons d'analyser la transcription de *crcY* chez un

mutant F113 *Δanr* afin de confirmer le lien entre ANR et CrcY. ANR et NarXL contrôlent également la transcription de *narG* (Schreiber et al., 2007), il serait donc également intéressant d'analyser leur transcription en fonction de la source de carbone utilisée afin de voir si il existe un lien entre la CCR et ces systèmes de régulation.

Afin de comprendre comment la nature des métabolites primaires exsudés par les plantes régule la dénitrification au sein de la rhizosphère, notre étude peut être complétement par des analyses en sol sur communauté microbienne rhizosphérique. Le traçage par isotope stable (SIP pour Stable Isotope Probing) pourrait être réalisé sur les lignées d'*A. thaliana* Col-0 WT, *Atpdr2* et *Atpdr6* cultivées sur le sol de La Côte-Saint-André, utilisé dans le chapitre 3. Cette technique est utilisée pour tracer les communautés microbiennes impliquées dans l'assimilation des exsudats racinaires (Haichar et al., 2008; Haichar et al., 2016). Les plantes sont incubées en présence du $^{13}\text{CO}_2$ qui sera assimilé par la plante pendant la photosynthèse afin de produire des métabolites primaires et secondaires marqués qui seront exsudés dans le sol. Les microorganismes qui vont assimiler ces exsudats marqués vont être à leur tour marqués par le C^{13} par anabolisme au niveau de leur constituant cellulaire. Le C^{13} étant plus dense que le C^{12} , il sera par la suite possible après extraction ADN/ARN de séparer les fractions marquées des non marquées par gradient de densité. Il sera donc ensuite possible de différencier les communautés microbiennes actives ayant assimilées les exsudats marqués de celles assimilant la matière organique du sol (non marqué). Après couplage d'analyses métabarcoding et métatranscriptomiques sur les différentes fractions, nous serons en mesure, en fonction des profils d'exsudations racinaire, d'identifier (i) les communautés présentes et actives dans la rhizosphère de chaque plante et (ii) les gènes exprimés par cette communauté dans la rhizosphère. Nous serons également en mesure de statuer sur la régulation de l'expression des gènes de la dénitrification dans la rhizosphère ainsi que des différentes CCR au sein de communautés actives et complexes. Comme annoncé au début de ce manuscrit (introduction générale et chapitre 1), la dénitrification a une incidence sur l'effet de serre au travers des émissions de N_2O produit durant la dénitrification (Galloway et al., 2008; Hu et al., 2015). La dénitrification est également responsable d'une importante perte de N dans les sols affectant la productivité végétale (van Der Heijden et al., 2008; Radersma and Smit, 2011). **Ainsi comprendre comment la nature des exsudats racinaires régule la dénitrification au sein de la rhizosphère nous permettra d'établir une stratégie applicable**

en agronomie où par la connaissance des profils d'exsudation des différents cultivars et de la composition microbienne des sols agricoles, nous pouvons sélectionner les cultivars limitant le processus de dénitrification et donc à la fois le rejet de N₂O dans l'atmosphère et la perte de N dans le sol.

Nous avons vu dans le chapitre 3, que microbiote pouvait impacter la plante différemment suivant l'exsudation de la plante. Il serait intéressant de caractériser le microbiote qui a été sélectionné dans chaque rhizosphère suivant les différents profils d'exsudation. En corrélant les observations effectuées sur la modulation hormonale de la plante et les communautés microbiennes identifiées, nous pourrions ainsi établir de nouvelles stratégies de manipulation du microbiote de la plante afin d'en stimuler sa croissance et sa résistance face à divers stress.

D. Conclusion de la thèse

Les travaux réalisés tout au long de cette thèse nous ont donc permis de démontrer (i) l'influence de la répression catabolique du carbone (CCR) sur la régulation de différents traits rhizosphériques bactériens en utilisant différentes natures de source de carbone. (ii) De mettre en avant que la régulation de l'expression des gènes de la dénitrification par la nature des métabolites primaires exsudés par la plante est en lien avec la CCR. (iii) Que les exsudats racinaires influencent le comportement de la communauté microbienne qui elle-même impact en retour la physiologie de la plante associée à cette communauté. En résumé, ces travaux démontrent donc le rôle des exsudats racinaires dans la médiation des interactions plante-microorganismes et mettent en avant le rôle important de la répression catabolique du carbone dans ces interactions et notamment dans la régulation de la dénitrification.

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ANNEXES

Conférences et séminaires

Franzino Théophile (2019). “How the nature of primary metabolites exuded by plants controls the denitrification process in the rhizosphere?” Poster presentation at the IX seminar of Association Francophone d’Ecologie Microbienne Conference at Bussang, France.

Franzino Théophile (2021). “How the nature of primary metabolites exuded by plants controls the denitrification process in the rhizosphere?” Poster flash-presentation at the X webinar of Association Francophone d’Ecologie Microbienne.

Franzino Théophile (2022). “How the nature of primary metabolites exuded by plants controls the denitrification process in the rhizosphere?” Talk presentation at 15th Plants-Bacteria Meetings (webinar version).

Franzino Théophile (2022). “How the nature of primary metabolites exuded by plants regulates nitrate and NO reduction by rhizobacteria ?” Talk presentation at 10th Congress of the International Society of Symbiosis-3rd International Conference on Holobionts at Lyon, France.

Productions Scientifiques

Laith Khalil Tawfeeq Al-Ani, **Théophile Franzino**, Liliana Aguilar-Marcelino, Feth el Zahar Haichar, Edson Luiz Furtado, Waqas Raza, Ghulam Hussain Jatoi, Mubashar Raza, “Chapter 14 - The role of microbial signals in plant growth and development: Current status and future prospects”, Editor(s): Ali Asghar Rastegari, Ajar Nath Yadav, Neelam Yadav, New and Future Developments in Microbial Biotechnology and Bioengineering, Elsevier, 2020, Pages 225-242, ISBN 9780128205266, <https://doi.org/10.1016/B978-0-12-820526-6.00015-4>.

Solène Lecomte, Xavier Nesme, **Théophile Franzino**, Camille Villard, Mariane Pivard, Ludovic Vial, Jeanne Doré, Florence Hommais, Feth el Zahar Haichar, “Agrobacterium fabrum C58 involved nitrate reductase NapA and antisense RNA NorR to denitrify”, FEMS Microbiology Ecology, Volume 97, Issue 1, January 2021, fiae233, <https://doi.org/10.1093/femsec/fiae233>

Théophile Franzino, Hasna Boubakri, Tomislav Cernava, Danis Abrouk, Wafa Achouak, Sylvie Reverchon, William Nasser, Feth el Zahar Haichar. Implications of carbon catabolite repression for plant–microbe interactions. *Plant Communications*, Cell Press, 2021, 3, pp.100272. <https://dx.doi.org/10.1016/j.xplc.2021.100272>

Formations

Module A10 : Ethique de la recherche (cours en ligne) - 25h

Module A36 : Histoire des sciences et épistémologie - 10h

Module B03b : S'affirmer face à des situations et à des personnalités difficiles – 20h

Module B33 : L'intelligence Artificielle... avec intelligence ! (cours en ligne) – 10h

Module Statistique : Rappel et approfondissement des méthodes statistiques de base et mise en pratique avec le logiciel R – 13h

Module Statistique : Module Analyses multivariées, principes des analyses multivariées classiques et mise en pratique avec le logiciel R - 13h

Module Statistique : Réalisation de représentations graphique avec le logiciel R – 6,5h

Module Statistique : Modèle linéaire – 13h

The role of microbial signals in plant growth and development: Current status and future prospects

Laith Khalil Tawfeeq Al-Ani^{a,b}, Théophile Franzino^c, Liliana Aguilar-Marcelino^d, Feth el Zahar Haichar^c, Edson Luiz Furtado^e, Waqas Raza^f, Ghulam Hussain Jatoi^g and Mubashar Raza^h

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14.1 Introduction

Microbes are present in the surroundings, on the surface, in the rhizosphere, and inside the plant all day. The continuous connection between microbes and plants leads to several interactions between them. The microbes play an important role for the improvement in plant growth and development. Microbes live in the environment of plants and exist on the surface, in the rhizosphere, and inside the plants. Microbes have different relationship with plants. These relationships affect plant growth and development. The microbes coexist with the plants by utilizing the various secretions by plants. The plants secrete several materials that are useful for the growth and proliferation of microbes that live on the surface of the plant and in the rhizosphere, while other microbes existing inside the plant are called endophytes and utilize water and nutrients of the plant tissue. The primary metabolites such as sugars, amino acids, and organic acids are root exudates of plants that are helpful for microbes (Canarini et al., 2019). On the other side, microbes can affect the growth and development of plants through colonization and growth in the rhizosphere, on the surface, and in the tissues of the plant. The colonization and the growth of microbes are possible because of the secretion of several secondary compounds and enzymes that potentially useful for plant.

However, more microbes such as bacteria and fungi have a relationship with a plant. Plant microbiota include both pathogenic and beneficial such as nonsymbiotic (microbes as endophytic and rhizospheric) and symbiotic. Many species of fungi live around and are endophytic in the plant such as *Trichoderma*, *Fusarium*, *Colletotrichum*, entomopathogenic fungi such as *Beauveria* sp., and nematophagous fungi such as *Pochonia* sp. The well-known species of bacteria that are very useful for plants include *Pseudomonas*, *Bacillus*, and *Serratia marcescens*.

Interestingly, microbes produce different secondary compounds and enzymes through interaction with plants. The microbes secrete two types of plant hormones: enzymes and secondary compounds; one type is useful and the other is harmful to plants. The phytohormones produced by microbes include gibberellin (GA) (French and Iyer-Pascuzzi, 2018) and indole-3-acetic acid (IAA) (Foo et al., 2013). Secondary compounds include phytotoxins (Durbin, 1988; Panaccione et al., 2001), volatile (Al-Ani, 2019a,b,c,d,e,f) and nonvolatile compounds (Li et al., 2019), siderophores (Al-Ani, 2019b), auxins (Nnamdi and Onyeka, 2016), and phytoalexin (Claverie et al., 2018). Microbes produce several enzymes such as chitinase (Naumann et al., 2011), cutinases (Chen et al., 2007), pectinolytic (Reignault et al., 2008). Plant's responses to several biotic and abiotic stresses are always signaled as the production of proteins called pathogenesis-related proteins or PR proteins. The accumulation of these proteins produced as a response to stress is in general related to pathological infections (Van Loon, 1999). The defense reactions comprise the activation of genes

of the host that are not active or expressed in fundamental levels. For instance, leaves not inoculated with tobacco accumulate 5%–10% less PR proteins compared to leaves inoculated with TMV (tobacco mosaic virus) presenting hypersensitivity symptoms (Sticher et al., 1997).

Interestingly, microbial has the potential to activate secondary metabolites in plants (Al-Ani, 2019a). Therefore, the endophyte microbial enhances the secondary metabolites and utilizes these as natural products for producing biopesticides and plant activators (Al-Ani et al., 2012; Mohammed et al., 2012; Egbuna et al., 2019).

Therefore, the relationship between microbes and plant are more attractive and is divided into two categories: one is useful for plant and the other is harmful and devastating for plants. It shows the role microbes in the two categories in the growth and development of plants.

14.2 Signals beneficial to plant microbiota

The rhizosphere is the area of the soil under the influence of the plant root system, where the plant exudes up to 20% of the fixed carbon during photosynthesis (Haichar et al., 2014). Root exudates are considered a rich banquet of nutrients for soil microorganisms. Because of this, the rhizosphere can be considered as a hotspot for the complex interaction between microorganisms and the plant (Bais et al., 2006; Haichar et al., 2008; Badri and Vivanco, 2009; Drogue et al., 2013; Vacheron et al., 2013). The microbial community in interaction with the plant roots is called the rhizomicrobiome (Chaparro et al., 2013). Inside this rhizomicrobiome, some microorganisms are well known to promote plant growth and/or stimulate the plant defenses against numerous pathogens (Couillerot et al., 2009; Richardson et al., 2009). Bacteria known to promote plant growth and defenses are referred to as plant growth-promoting rhizobacteria (PGPR) and the fungi as plant growth promoting-fungi (PGPF) (Bent, 2006; Al-Ani, 2017a). In the following sections, the plant growth and defense stimulation are discussed.

14.2.1 Enhancement of the microbial signals for the plant growth and development

Beneficial microbials such as bacteria and fungi secrete several enzymes and phytohormones that promote plant growth and development. These signals of bacteria and fungi improve several characteristics for the growth and development of plant such as (A) the biomass of root and shoot, (B) enlargement of the cell, (C) cell divisions, (D) increase in the number of hairs and lateral root, and (E) decrease in cellular aging (Fiorilli et al., 2015; Dash et al., 2018; Sansinenea, 2019). Bacteria and fungi can send signals for the plant during attaching the plant tissue that leads to regulate the morphogenesis and plant growth (Guether et al., 2009; Bordiec et al., 2011; Trdá et al., 2013). The signals for enhancing the plant growth are produced by phytohormones, secondary metabolites, and secreting enzymes (Berg, 2009; Yuan et al., 2010; Mabood et al., 2014). Beneficial bacteria include several genera such as *Azospirillum*, *Azotobacter*, *Burkholderia*, *Paraburkholderia*, *Aneurinibacillus*, *Enterobacter*, *Serratia*, *Aeromonas*, *Bacillus*, and *Pseudomonas* called plant growth-promoting bacteria (PGPB) (Caballero-Mellado et al., 2007; Nabti et al., 2010; Mohammed et al., 2011, 2013, 2014; Lee et al., 2016; Hammami et al., 2013; Esmaeel et al., 2018; Singha et al., 2018; Kumar et al., 2018), also include the bacteria group in rhizosphere called PGPR.

For producing phytohormones, is producing by most of PGPB. Absolutely, PGPB secretes many phytohormones like GA, CK, and IAA (Bottini et al., 2004; Farrar et al., 2014). *Rhodospseudomonas* produce important plant hormones such as IAA and 5-aminolevulinic acid (ALA) (Koh and Song, 2007). Two species of the genus *Bacillus* such as *Bacillus licheniformis* and *Bacillus pumilus* produce four gibberellins (Gutierrez-Manero et al., 2001). These hormones of PGPB affect lateral roots of the plant by increasing their number and length, as well as, enhance the biomass of root and shoot (Sansinenea, 2019). In additional, PGPB can secrete secondary metabolites affecting hormonal pathways (Vejan et al., 2016). *Pseudomonas*, *Aneurinibacillus aneurinilyticus*, and *Aeromonas* sp. produce siderophores (Kumar et al., 2018). However, several enzymes are produced by PGRB. Glick (2014) noted the ability of PGPB to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase that breaks down the plant ethylene into two materials that promote the plant growth such as α -ketobutyrate and ammonia.

Surprisingly, many fungi can be more beneficial for the plant by producing signals called plant growth-promoting fungi (PGPF) including the fungal group symbiont, nonsymbiont, endophyte, phyllosphere, and rhizosphere, as well as, other fungal group comprising entomopathogen, and nematophagous. PGPF includes many fungi such as *Trichoderma*, *Taxus wallichiana*, *Paecilomyces (Isaria)*, *Fusarium*, *Pochonia*, *Metarhizium*, *Lecanicillium*, *Beauveria*, *Metarhizium*, *Mycorrhizae*, *Aspergillus*, and *Penicillium* (Al-Ani and Salleh, 2010; Al-Ani et al., 2013a,b, 2018; Jaber and Enkerli, 2016; Al-Ani, 2018a,b, 2019a; Al-Ani and Albaayit, 2018a,b; Dash et al., 2018; Todeschini et al., 2018; Adhikari and Pandey, 2019; Sharma et al., 2020; Aguilar-Marcelino et al., 2020; Al-Ani and Furtado, 2020;

Al-Ani and Mohammed, 2020). But some genus depend on the characteristics of the strains (pathogenic or beneficial for the plant) such as *Trichoderma*, *Aspergillus*, *Fusarium*, and *Penicillium* (Attitalla et al., 2010a,b; Al-Ani, 2017b). A study conducted on the action of Brazilian semiarid saprobe fungi observed that *Curvularia eragrostidis* and *Memnoniella levispora* induce rooting in *Eucalyptus* cuttings (Furtado, not published).

Interestingly, PGPF produces several important phytohormones such as IAA and GA (Khan et al., 2012). *Fusarium konzum*, *Fusarium sacchari*, *Fusarium fujikuroi*, *Fusarium subglutinans*, *Aspergillus fumigatus*, *Penicillium resedanum*, *Paecilomyces formosus*, and *Penicillium janthinellum* produce fascinating phytohormones gibberellins (Tudzynski and Holter, 1998; Khan et al., 2011, 2015a; Bilal et al., 2017). *Trichoderma virens*, *Trichoderma asperellum*, and *Trichoderma atroviride* produce IAA (Nieto-Jacobo et al., 2017). Also, *Trichoderma* affects plant physiology and production of phytohormones that enhances the growth of the plant (Martínez-Medina et al., 2014).

Many enzymes helpful for the plant are secreted by PGPF such as phytase and ACC deaminase (ACCD) (Viterbo et al., 2010; Gaind and Nain, 2015; Khan et al., 2016). PGPF such as the genus *Trichoderma* produces secondary metabolites such as siderophores which is helpful for plant growth and development (Al-Ani, 2017b). Nieto-Jacobo et al. (2017) noted that the several species of *Trichoderma* such as *T. virens*, *T. asperellum*, and *T. atroviride* produce volatile organic compounds beneficial to plant growth. In addition, *Trichoderma hamatum* stimulate secondary metabolite-producing gene clusters with genes unique, and transcripts encoding small-secreted cysteine-rich proteins that improve the plant growth in the lettuce (Shaw et al., 2016). The plant growth stimulation by the microbiota can be another effect of direct stimulation through the production of phytohormones like auxins and gibberellins (Drogué et al., 2012) or by the improvement in nutrient uptakes like phosphate solubilization and nitrogen fixation (Richardson et al., 2009).

14.2.1.1 Auxins

Auxins are the principal plant growth hormones present in plant cells, which regulate the plant root development in cross talk with other hormones (Napier, 2016). The rhizomicrobiome can affect the hormonal balance by producing exogenous auxins like IAA that can interfere with auxin signaling pathway and then modifying the root system architecture (Spaepen et al., 2007). Many PGPRs are known to produce IAA. *Azospirillum brasilense* is a good example of IAA producer as mutant altered in the production of IAA showed a decrease in their plant growth stimulation effect (Dobbelaere et al., 1999). Other species like *Pseudomonas fragi* and *Bacillus cereus* are good potential IAA producers and improve the growth quality of rice (Susilowati et al., 2018).

14.2.1.2 Gibberellins

Naturally present in plants, gibberellins regulate many key processes during the plant growth from seed germination to leaf expansion and root growth. *Exogenous gibberellins*, produced by a wide range of microorganism such as bacteria, for example, *Azotobacter*, *Pseudomonas*, *Azospirillum*, and fungi, for example, *Sphaeloma* sp., *Neurospora* sp., *Phaeosphaeria* sp. (Rademacher, 1994; Santos et al., 2003; Parmar et al., 2018), can be used to improve the growth and the yield of rice under saline stress and to reduce metal stress during growing (Prakash and Prathapasenan, 1990; Moya et al., 1995).

14.2.1.3 Phosphate solubilization

The bioavailability of phosphorus (P) in the soil can be a limiting factor for plant growth because of its nonsoluble form. The plant can count on phosphorus solubilizing microorganisms (PSM) from its microbiota in order to gain access to this key nutrient. The presence of PSM increases the availability of P in soil and promotes plant growth by increasing the uptake of P by the plant. PSM has been reported to strengthen the growth of several cereals (Chabot et al., 1996; Murty and Ladha, 1988; Rodríguez and Fraga, 1999; Kalayu, 2019).

14.2.1.4 Nitrogen fixation

For nitrogen nutrition, plants benefits from nitrogen-fixing bacteria commonly found in the rhizomicrobiome (Richardson et al., 2009; Lugtenberg and Kamilova, 2009). It has been reported that approximately 70% of the total N uptake in field crops come from nitrogen-fixing PGPR (Montañez et al., 2009; Zakry et al., 2012; Kuan et al., 2016). For example, nitrogen-fixing bacteria, the symbiont *Rhizobium*, forming root nodules in association with its host to fix the atmospheric N, is a good choice. The symbiosis process is well reviewed by Garg and Geetanjali (2007). Free-living bacteria such as *Azospirillum* are also good examples of microorganisms capable of fixing nitrogen and capable of promoting plant growth (Fukami et al., 2018) in combination with all the other characteristics mentioned previously.

14.2.2 Enhancement of the microbial signals for induced of plant defenses

It is well known that enhancement in the plant growth is associated with an increase in the plant defense system. Inside the plant microbiota, many PGPR and PGPF can trigger the induced systemic resistance (ISR) promoting the vigor and the health of their plant host (Al-Ani and Al-Ani, 2011; Pieterse et al., 2014) for direct interaction. In addition, the microbiota can prevent plant infection by pathogens by controlling the pathogen presence by either competing or producing antimicrobial compounds.

14.2.2.1 Resources competition

The uptake of iron is important for the good growth of microorganisms; to gain access to this resource, they produce iron chelators called siderophores. After chelating ferric ion, the complex so formed is absorbed (Andrew et al., 2003; Boukhalfa and Crumbliss, 2002). The ability to produce numerous siderophore confers some competitive advantages to the beneficial microorganism against pathogens, for example, the suppression of fungal pathogens by fluorescent pseudomonads (Dwivedi and Johri, 2003).

14.2.2.2 Antagonist effect

Pseudomonads are also good specimen for the production of other compounds such as 2,4-diacetylphloroglucinol (DAPG) antibiotics that can damage the membrane of *Pythium* spp. (De Souza et al., 2003). Other antibiotics produced from *Bacillus* spp. like colistin, polymyxin have a broad spectrum of action (Maksimov et al., 2011).

14.2.2.3 Induced systemic resistance

The ISR is the result of the interaction between the plant and a beneficial microbe triggering the defense mechanism. The regulation of the plant immune system is mediated by different hormones such as salicylic acid (SA) or jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 2014). But in most cases, beneficial microbes induce ISR by modulating salicylic acid (Van Loon and Bakker, 2006).

14.3 The form of relationship between plant defense and plant growth

The beneficial microbial is present in the plant environment. The beneficial microbial occur due to the interaction between the plant and phyllosphere, rhizosphere, and endophyte. Two main microbial such as bacteria and fungi are beneficial for the plant. There are two points very interesting that need for elucidation the effect of the signals of beneficial microbes on a plant by enhancing the plant growth connecting with succeeding in induced of the plant defense.

Generally, the interaction between microbes and plants induce signals responsible for the stimulation of the plant growth as well as the plant immunity (PI) and plant defense (PD). Microbial can induce several chemical signals such as plant hormones useful for enhancing the plant growth, PI, and PD. Plant hormones can be categorized into two groups: first group comprises SA (salicylic acid), JA (jasmonate), and ET (ethylene), and the second group comprises CK (cytokinin), ABA (abscisic acid), BR (brassinosteroid), auxin (AUX), SLs (strigolactones), and GA (gibberellic acid) (Li et al., 2019). The first group plays the role of the main hormones in the stimulation of PI compared with the second group is a helper for the main hormones. There is a close relationship between the main and helper signals. Walters (2011) mentioned the following six hypotheses to explain the form of the relationship between the plant defense and the growth of a plant:

1. The growth-differentiation balance hypothesis.
2. Optimal defense hypothesis.
3. Plant apparency hypothesis.
4. The carbon-nutrient balance hypothesis.
5. The growth rate hypothesis.
6. Hypothesis of plant defense—where next?

Interestingly, to the increase in the capability of plant defense against plant enemies (plant pathogen, pests, and weeds), beneficial microbes need to use the stored energy from the plant. The plant after getting the defense signals from beneficial microbes induce the defense protein genes to makes a lot of protein copies to get a defense power against plant enemies. The ability of a plant to produce high number of copies connects with the nutrient storage to support production of high number of protein defenses. In addition to beneficial microbes, the defense genes and also PI stimulate the cells to utilize the defense

protein copies against plant enemies. Beneficial microbial play a major role to save the activity of the defense in plants for the production of high protein copies by inducing important plant hormones, as well as can affect the nutrient elements important for plant growth and development. Beneficial microbes produce many secondary metabolites, and enzymes affect the nutrient elements in soils that lead to modify the complex elements to the utilization unit being easier for absorption from the plant roots. The essential element such as iron is necessary for the growth of the plant and production of proteins such as defense copies. Many beneficial microbes such as PGPR (e.g., *Chryseobacterium* spp., *Pseudomonas*, *Burkholderia* sp., *A. aneurinilyticus*, *Aeromonas* sp., and *Bacillus*) and PGPF (e.g., *Trichoderma* spp.) produce the most of siderophores (chemical chelates) forming the more stable iron complex for plant (Radzki et al., 2013; Al-Ani, 2017b; Kumar et al., 2018; Gowtham et al., 2018). PGPR can make nutrient available to plant by solubilizing elements such as phosphate and potassium, as well as by fixing nitrogen (Vejan et al., 2016).

14.4 The effect of signals of plant-microbial pathogen on the growth and development of plant compared with beneficial microbes

The interaction of pathogenic microbes causes a huge economic loss compared to beneficial microbes. This interaction impacts the signaling pathway of plant defense and phytohormones. This influence kills or reduces the growth and development of the plant. Pathogenic microbes such as viroids, viruses, phytoplasma, bacteria, and fungi affect the host plant in two ways: direct and indirect. They directly affect the host plant by producing secondary compounds, enzymes, and proteins. While the indirect effect is that microbes cannot produce secondary compounds and enzymes but utilize the gene tools of cells to transcriptome and to create proteins and structure of microbes that cause a different disease to a host plant.

Indeed, the role of the plant-microbial pathogen through interaction with plant is to secrete several effectors such as proteins and nonproteins (called pathogen-associated molecular patterns [PAMPs]) on the plant surface through contacting to start the invasion (Camejo et al., 2016). The success of the microbial pathogen to invade the plant is related to the ability of PAMPs to suppress plant immunity. Plant-microbial pathogen regulates the host cell by controlling the nutrient and preventing response of the host plant to infection (Kuhn and Panstruga, 2014). The plant has a surveillance system that components from two types of immune receptors: (1) receptors in intracellular and (2) receptors numerous localized on the cell surface (Qi et al., 2017). The surveillance system is a sensing system to detect molecules of the microbial pathogen during the interaction with the plant (Qi et al., 2017). The plant-pathogen interaction stimulates the plant defenses from the plants to recognize microbe-associated molecular pattern (MAMP) receptors or PAMP (de Torres Zabala et al., 2015). MAMPs provide plants protection against microbial pathogens.

However, the perception of PAMPs is that it activates the pattern-recognition receptors (PRRs) with other immune responses (Sang and Macho, 2017). The plant through interaction with microbial pathogens produces three signals such as reactive oxygen species (ROS), calcium (Ca^{2+}) wave, and electric signals (Gilroy et al., 2016). ROS acts as signal molecules to arrange the cellular activities by using the stomatal movement (Qi et al., 2017). The apoplast is a major site for the production ROS like hydrogen peroxide (H_2O_2), the hydroxyl radical (OH), the superoxide anion (O_2^-), and singlet oxygen ($^1\text{O}_2$) (Camejo et al., 2016) and can transport ROS to the cytoplasm by regulating redox molecules (Qi et al., 2017). ROS possibly downstream from different signals then accumulate in cells and possibly acts at a low level (useful signal transduction molecules) or at a high level (toxic molecules) (Foyer and Noctor, 2005; Miller et al., 2010). ROS blocks the pathogen entry and acts as the local and systematic secondary messengers to elicit the immune responses like stomatal closure and gene expression (Suzuki et al., 2011). The inducing and accumulation of ROS through interacting with plant pathogens is causing inhibition for the plant pathogen growth (Camejo et al., 2016). Several signaling pathways are integrated with ROS-dependent systemic response during induced SAR by plant pathogens (Návarová et al., 2012). The systems of ROS interact like cellular thiol in a complex network in the redox regulation of pathways for the phytohormone signaling which are crucial for both defense and development in the plant against threats of plant pathogens and other stresses (Noctor et al., 2018).

Surprisingly, the generation of ROS through interaction between the plant pathogens and host plant cells occurs at three sites: (I) chloroplasts, (II) mitochondria, and (III) peroxisomes (Camejo et al., 2016). ROS is produced during photosynthesis in chloroplasts, through apoplastic NADPH oxidases, photorespiratory reactions in peroxisomes-localized, mitochondrial respiration, and other oxidases (Mhamdi and Van Breusegem, 2018). Camejo et al. (2016) explained their role in the cells: (I) Chloroplasts are metabolically active organelles and are major producer of ROS. (II) Mitochondria are considered to be very dynamic and metabolically activate organelles, as well as, major ROS producer. (III) Peroxisomes are very interesting and ubiquitous organelles which participate in metabolic pathways and have association with other cellular metabolism. Chloroplasts are major generators of nitric oxide, calcium signaling, and ROS (Lu and Yao, 2018).

Chloroplasts are responsible for photosynthesis process. Photosynthesis produced NADPH, carbohydrates, and ATP that are utilized as resources for the synthesis of several important compounds for plants like defense-related hormones (including ET, JA, SA, and ABA), antimicrobial compounds, and primary metabolites (Lu and Yao, 2018). Chloroplasts generating signals produce substrates for peroxisomal pathways and the signals interchange between chloroplast and mitochondria, and also regulate intercellular communication via plasmodesmata (Bobik and Burch-Smith, 2015). The chloroplast is able to communicate with their neighbors by volatile compounds produced in response to attack by plant enemies (Bobik and Burch-Smith, 2015).

However, plant-microbial pathogens can be classified depending on the trophic method and strategic infection as biotrophs, necrotrophs, and hemibiotrophs (Fatima and Senthil-Kumar, 2015). Plant pathogens repress the immunogenic signals and alter the immune response with the inappropriate gene expression of the host plant by producing the pathogen effectors (Sowden et al., 2017). The plant pathogens cause defects in host plant leading to a decrease in or unresponsive signals from the microbial intruders. The fault in the activity of chloroplast, abnormality in organelles of the cell both in size and in number, and metabolic pathways cause a reduction in the nutrients, gene expression, and production of compounds and enzymes. Various components of plant cell machinery are exposed due to degrading of several effector molecules produced by plant pathogens (Fatima and Senthil-Kumar, 2015). Plant fungal pathogens target various organelles with metabolite pathway through invading the plant cell. Fungal pathogens target several organelles of plant cells such as chloroplast, mitochondria, nucleus, nucleoli, and discrete cellular bodies (Petre et al., 2015). The microbial pathogen attacks chloroplast and alters the function leading to degradation of the capability of plant defenses and enhances virulence directly or indirectly (Serrano et al., 2016).

On the other hand, beneficial microbial can increase the chlorophyll content leading to the enhancement of the growth and development of the plant. The increase in chlorophyll content means an increase in the area of photosynthesis that reflects on the ability of a plant to benefit from signals for the growth and development of beneficial microbes as well increase in levels of plant defense. *Trichoderma* increases the chlorophyll content leading to an improvement in plant vigor as well as control of *Fusarium* wilt in banana (Al-Ani, 2017b). PGPR enhances the chlorophyll content in a plant causing an increase in protein content and plant growth with inducible plant defenses (Chitra, 2014; Habib et al., 2016).

Interestingly, plant-microbial pathogens cause defect in plant physiology through interaction several ways: (A) by secreting enzymes and secondary compounds affecting or degrading the plant tissue, (B) by preventing the host plant from producing enzymes and secondary compounds useful for the growth and development of the plant, (C) by consuming the nutrient and helpful materials of the host plant that exist intercellular and intracellular of the plant tissue, (D) by the induction of the plant tissue to produce important enzymes and secondary compounds for the growth and development of the plant tissue at very high levels, that is, abnormal levels, (E) blocking plant vessels from transferring the nutrients from root to leaf and vice versa, (F) degrading plant tissue indirectly by producing important enzymes and secondary compounds, and (G) degrading plant tissue directly by direct pressure exerted by the plant pathogen.

On the other hand, the influence of plant-microbial pathogens through interacting with plants is possibly again summarized theirs using three important tools that are helpful in a reduction both of growth and development and then destroy the plant. First, the ability of microbial pathogens to produce several extracellular enzymes including pectinase (Ramos et al., 2016), hemicellulases (Ramos et al., 2016), arabanase (Karimi and Ward, 1989), xylanase (Ramos et al., 2016), cutinase (Choi et al., 2013s), β -Glucosidase (Xue et al., 2018a,b), cellulase (Ramos et al., 2016), phospholipase (Barman et al., 2018), polygalacturonase (Xue et al., 2018a,b), ligninase (Wu et al., 2006), and protease (Moreira et al., 2005). Second, the possibility of plant-microbial pathogen producing several secondary compounds causing a disorder in plant physiology and damage to plant tissues. Phytotoxin is an important secondary compound that affects the plant growth and development. Phytotoxins lead to disorder in plant physiology and the appearance of symptoms such as wilting, necrosis, and chlorosis (Yamane et al., 2010). Third, the plant-microbial pathogen possibly affects the plants by influencing the production of phytohormones. Strange (2003) mentioned to the role the plant pathogens in causing an imbalance in plant hormones that lead to the appearance of several symptoms such as abnormal growth, crinkled leaves, witches' broom, induce a redirection of nutrients, necrosis, epinasty, abscission, and chlorosis. Indeed, the plant-microbial pathogen interaction causes a defect in meiosis (hyperplasia) and normal mitosis, which lead to an increase in size the infectious part and appearance of marked abnormalities.

14.4.1 Viruses and viroids

These two microbes affect the growth and development of plants by replicating inside the plant cell but do not affecting the metabolism directly. This step causes a defect in the genome of plant cell and lead to the loss of some useful gene expressions for growth and development. Viruses and viroids show special symptoms after the occurrence the compatibility

with the plant host. These pathogens do not affect the host plant until they invade the plant cell and replicate and then transmit the infection to other cells of the host plant. These pathogens do not have genes that are responsible for the production of toxins, enzymes, active compounds, and growth regulators (Agrios, 2005).

However, the interaction of plant viruses and viroids with a plant affects indirectly. The effect of these pathogens on the host plant is different from other plant-microbial pathogens because they not produce any toxins, growth regulators, and bioactive compounds. They affect the plant host through utilizing the cell organelles to produce one or more proteins by induced synthesis from virus and viroid. The protein is responsible for causing defect in growth and development of plants. For plants, the virus causes high damage but rarely kill plants. The viral protein modifies the cellular functions of the host plant after infection (Zhu et al., 2005). Cucumber mosaic virus (CMV) is a very interesting plant virus that attacks many plants, about 1000 plants, and causes different symptoms on plants without any killing symptoms for the plants (Schneider and Roossinck, 2001; Roossinck, 2001). Al-Ani (2006) mentioned about different symptoms through the development of cucumber mosaic disease on the plant leaves of cucumber after infection with cucumber mosaic virus (CMV) (Fig. 14.1). The pathogen of plant viruses and viroids affects the function and the cell organelles of the host plant through interacting indirectly, such as chloroplast, phytohormones, and metabolite activity.

Interestingly, the chloroplast is targeted by the plant virus and viroid. This leads to a defect in the photosynthesis reflecting symptoms. Therefore, the symptoms appear as mosaic, vein, chlorosis, and mottle. These symptoms are shown in many plant viruses such as CMV, pepper mild mottle virus (PMMV), strawberry vein banding virus (SVBV), grapevine rupestris vein feathering virus (GRVfV), petunia vein clearing virus (PVCV), clover yellow vein virus (CYVV), and potato yellow vein virus (PYVV). The symptoms of plant viroids are peach latent mosaic viroid (PLMVd) and chrysanthemum chlorotic mottle viroid (CChMVd). A destruction of the chloroplast causes weakening of the plant and the inability to confront the plant viruses and viroids. Chloroplasts are responsible for responding to the plant host to infection with plant pathogen. Zhao et al. (2016) demonstrated that genes/proteins related to chloroplast that conduct photosynthesis in plant

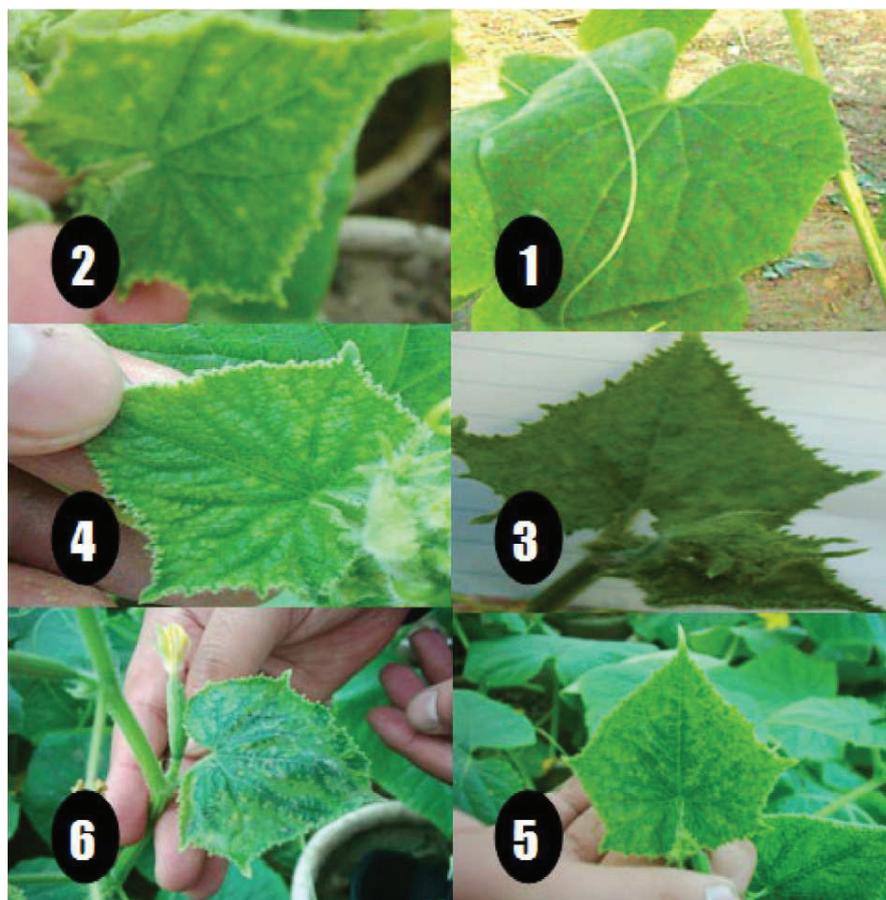


FIG. 14.1 Development of the symptoms the cucumber mosaic disease on the cucumber plant leaf causing by cucumber mosaic virus (CMV): (1) intact (no infection), (2) local circular spots, (3) mottle, (4) the beginning of mosaic (mild symptoms), (5) mosaic (50%), (6) green- to dark-green mosaic with vein banding (severe symptoms) Al-Ani (2006).

host play a role in defense against virus and boosts plant defenses. Citrus bark cracking viroid (CBCVd) causes modification in chloroplast after infection of the plant host (Štajner et al., 2019). A chloroplast mediate signaling pathway of plant immunity (Nomura et al., 2012). Chloroplasts transport innate immunity to nucleus and subcellular compartments by a microscopic structure called stromules (dynamic tubular extensions) (Caplan et al., 2015).

However, the plant viruses and viroids cause dwarf and stunting in a plant by affecting the balance of phytohormones. The plant virus inhibits the growth of the host plant (Fraser, 1987). The interactions between plant virus and host plant affect the plant growth causing quick alternations in the signaling pathways and phytohormones (Islam et al., 2019). CMV decreases the accumulation of gibberellic acid and appearance of dwarf symptoms in the host plant (Ben-Tal and Marco, 1980). CMV affects the accumulation of gibberellic acid and inhibits the growth of Arabidopsis (Bueso et al., 2017). Apple stem grooving virus (ASGV) affects the root development of plant *Pyrus pyrifolia* cv. “Jinshui no. 2” and causes a defect in the phytohormone balance such as CK (cytokinin)/IAA and IAA/ABA ratios (Chen et al., 2017). Citrus bark cracking viroid (CBCVd) affects hormones of the plant host (Štajner et al., 2019). The plant hormones such as ABA, JA, SA, and ET play a role in regulating the plant defense as an antiviral against plant virus (Alazem and Lin, 2014).

On the other hand, the proteins of plant virus and viroid are responsible for modifying the metabolite pathways leading to a weak activity or inactivity in the host plant. CMV affects metabolic pathways of gibberellins through infection of cucumber and leads to the reduction in the activities of gibberellins (Ben-Tal and Marco, 1980). The interaction between the capsid P2 proteins of plant virus rice dwarf virus (RDV) and the rice plant causes a reduction in the biosynthesis of gibberellins and phytoalexin that affect the plant growth and development causing a dwarf symptom for rice (Zhu et al., 2005). The protein (2b) of CMV interacts with catalase such as “2b-catalase” that decreases the activity of catalase causing a necrosis symptom on Arabidopsis (Masuta et al., 2012). Therefore, the targeting of the chloroplast, plant hormones, and metabolite activities is a strategic step for the successful infections by the plant virus and viroid.

14.4.2 Phytoplasma

Interestingly, according to Bertamini et al. (2002), Xue et al. (2018a,b), and Pradit et al. (2019), the effective role played by phytoplasma in the growth and development of a host plant after interaction with it are as follows: (1) it degrades the stroma lamellae and grana of chloroplasts causing the inhibition of the photosystem II activity in photosynthesis. (2) It increases the level of carbohydrates by overexpressing genes for starch, glucose, total saccharides, and sucrose synthesis. (3) It decreases the soluble proteins, contents of chlorophyll, and the biomass of leaf. (4) It stimulates energy metabolisms and amino acids. (5) It accumulates phytoplasmas in the sieve elements of phloem leading to a defect in the transformation of the nutrients. (6) It reduces nitrate reductase, 14CO_2 fixation, and ribulose-1,5-bisphosphate carboxylase activities. (7) It represses the defensive pathway of the host plant. (8) It changes the size, regions, and number in the cell structure in the host plant (Rudzińska-Langwald and Kamińska, 2001).

14.4.3 Bacteria

The signals of plant-bacterial pathogens cause a reduction in the growth and development of the plant host directly. The signals of bacterial pathogens include secondary compounds (phytotoxins and other compounds), enzymes, and proteins. Armijo et al. (2016) produced several CWD (cell wall-degrading enzymes) enzymes comprising endoxylanases, endo-1,4- β -glucanases, cellobiohydrolase, a polygalacturonase (PG), and β -xylosidases that cause degradation of xylem pit membranes. In addition, the accumulation of cell bacteria inside the vascular plant causes defect in plant physiology such as growth and development. *Pseudomonas syringae* pv. *actinidiae* caused by bacterial cankers on *Actinidia deliciosa* and *Actinidia chinensis* secrete phytotoxic metabolites and other toxins such as antimetabolite phaseolotoxin (Andolfi et al., 2014). Hogenhout and Loria (2008) mentioned that the strategy of bacteria to destroy the host plant is to produce phytotoxins, proteins, and cytokinins. The plant-bacterial pathogen reduces photosynthesis then degeneration due to the toxins. Photosynthesis provides important energy for plant defense and to fight against plant-bacterial pathogens (Göhre, 2015).

However, bacteria pathogen for colonizing host plant needs strategies such as modification of the plant habitat, regression, ingress, and aggregation (Beattie and Lindow, 2007). Plant-bacterial pathogens cause many symptoms for the host plant including soft rot, wilt, leaf spotting, cankers, translucence, water soaking, greasy aspect, yellowing, collapse, necrosis, scab, blackleg, and maceration. A J-domain protein of phyto-bacterial *P. syringae* virulence effector leads to an increase in the invasion of the host plant because bacterial pathogen alters the function and structure of the chloroplast (Jelenska et al., 2007). Several effector molecules act a tool for plant-bacterial pathogens to attack different components of the host plant to obtain the available nutrients (Fatima and Senthil-Kumar, 2015). The effector protein of *P. syringae* such as effector HopK1 impacts the chloroplast by ingressing the effector protein into the chloroplast that allows the effector access

to deactivate within the organelle the photosynthetic machinery and proteins (Brown, 2016). The abilities of HopK1 in stopping the responses of the host plant were elucidated by repression of the photosynthetic response including callose deposition and ROS production (Brown, 2016).

Similarly, de Torres Zabala et al. (2015) determined virulence strategies of phyto-bacterial pathogen *P. syringae* by large-scale repression of nuclear-encoded chloroplast-targeted genes (NECGs). NECG expression in the host plant modifies chloroplast activity and suppresses photosynthetic CO₂ assimilation leading to the inhibition of photosystem II, as well as prevents the activity of ROS burst (de Torres Zabala et al., 2015). The primary virulence of *P. syringae* targets the type-III effector proteins existed in chloroplasts by importing a transit peptide to chloroplasts (Li et al., 2014).

Interestingly, plant-bacterial pathogen targets plant mitochondrial and β -barrel proteins of outer bacteria membrane recognized by mitochondrial, and the ability to import bacterial proteins into mitochondria transporters (Kozjak-Pavlovic et al., 2008). The type III effector HopG1 of *P. syringae* enhances the disease by causing a defect in mitochondrial functions (Block et al., 2010). *Staphylococcus aureus* secretes α -toxin that causes cytotoxicity such as mitochondria (Bantel et al., 2001). The bacterial pathogen toxin causes a calcium-dependent mitochondrial swelling (Matarrese et al., 2007). The bacterial pathogen affects the signal and metabolites of the host plant. The modification in the host auxin IAA signaling enhances the bacterial pathogen growth (Kunkel and Harper, 2018). *Pantoea agglomerans* and *Pseudomonas savastanoi* pv. *savastanoi* produced by IAA inside the plant cause hyperplastic symptoms that interfere with development directly (Barash and Manulis-Sasson, 2007; Aragon et al., 2014).

However, many effector proteins of plant-bacterial pathogen have various functions such as colonization of bacterial pathogen in the host plant, repression the plant host defense, defect in the gene expression and hormone homeostasis (e.g., synthesis, activity, and metabolism) and signaling that cause a defect in the plant cell growth by stimulating gall, canker, knot, or pustule formation leading to the disease symptom development (Kunkel and Harper, 2018). The phytotoxin coronatine (JA isoleucine analog) produced by plant-bacterial pathogen as mimicry for functional JA causes repression of SA that ruins the signal of the plant defense (Brooks et al., 2005; Laurie-Berry et al., 2006). Toxin and effectors produced by bacterial pathogens suppress the signaling pathway of plant defense (such as SA, ET, and JA), and regulate the plant hormones such as auxin, brassinosteroid (BR), cytokinin (CK), and gibberellin (GA) (Ma and Ma, 2016).

14.4.4 Fungi

Plant fungal pathogen is a prominent pathogen causing several diseases in plants that lead to a high loss in the economy. Fungal pathogens produce mycotoxins and effectors targeting the capability of the host plant for growth and development. The mycotoxins and effectors show decrease in the accumulation of photosynthetic, activity of mitochondria, and metabolite pathway, as well as, other cell organelles. Fungal pathogen effectors modify plant physiology and repress plant defense responses to get the nutrients that help fungal pathogens to colonize the plant host with functional interaction between the fungal hyphae and host or by transferring to the plant host (Lo Presti et al., 2015). Plant-fungal pathogens produce phytotoxins and plant cell wall-degrading enzymes (PCWDEs) as tools to absorb the nutrient after degrading the cell wall of the host plant (Choi et al., 2013). The CWDEs produced by fungal pathogen utilizes carbon sources for the successful infection such as spore germination, penetration, and invasion of the plant tissues (Feng et al., 2014). CWDEs typically include several enzymes such as hemicellulases, cellulases, cutinases, xylanases, pectinases, and cellulases, as well as phytotoxins and oxalic acid kills the plant host (Prins et al., 2000; Kubicek et al., 2014; Rai et al., 2015).

Interestingly, effectors of fungal pathogens can transport into the host plant by forming the haustorium-like structure for suppressing the signals of plant defense, for example, *Magnaporthe oryzae* (Khang et al., 2010) and *Phytophthora* (Wang et al., 2017). While *Fusarium oxysporum* and *Verticillium dahliae* secrete effector proteins through colonizing the xylem vessels of the host plant without the haustorium-like structure (Zhao et al., 2014). The enzyme activity of fungal pathogens such as *endo*- β -1,4-xylanase inhibits plant defense (Brito et al., 2006).

However, fungal pathogen *Oidium heveae* cause degradation of the structure and function of chloroplasts through decrease in chlorophyll contents and mitochondria (Wang et al., 2014). Magyarosy et al. (1976) demonstrated the ability of *Erysiphe polygoni* to cause alterations in the ultrastructure of chloroplast and activity of enzymes. Rust fungus *Melampsora larici-populina* alters the host plant response through colonizing that target the chloroplast and mitochondria by the effector proteins, as well as, other organelles of the cell such as nucleoli, discrete cellular bodies, and nucleus (Petre et al., 2015). Fungal pathogen *V. dahliae* produces effector proteins repressing the plant immunity by targeting the plant nucleus (Zhang et al., 2017). Zhu et al. (2017) detected the ability of fungal pathogen *Botrytis cinerea* to affect chloroplast and become disorganization. The fungal pathogen of rust *M. larici-populina* secretes effector proteins such as chloroplast-targeted protein 1 that accumulates in the stroma of chloroplasts and mitochondria and cause damage to plant-specific signals (Petre et al., 2016).

Surprisingly, toxins ruin mitochondrial function (Singh and Sharma, 2018). Mycotoxin of plant fungal pathogen ruins the activity of mitochondria. T-toxin affects the activity of mitochondria by impacting the URF13 protein (Scharf et al., 2014). Fungal pathogens such as *Alternaria brassicicola* possibly causes alteration in oxidative phosphorylation of mitochondrial and also inhibits glycolysis or the tricarboxylic acid cycle (Calmes et al., 2015).

On the other hand, the fungal pathogen causes a defect in both signal and metabolite pathways of the host plant. Fungal pathogens cause a defect or alter in signal pathways and produce secondary metabolites of the host plant. Fungal pathogen secretes many secondary metabolisms such as hormonal pathways through plant-fungal interaction that can mimic the hormonal pathways of plant and subvert the response of plant defense (Chanclud et al., 2016). Pusztahelyi et al. (2015) mentioned the capability of secondary metabolisms of a fungal pathogen to subvert the plant growth and defense. The strategy of a fungal pathogen is to invade and cause infection in a plant by secreting secondary metabolites and sRNA, as well as cysteine-rich proteins for altering plant physiology and ruin the growth and development for completing a life cycle (Rodriguez-Moreno et al., 2018). *Fusarium*-secreted mycotoxin deoxynivalenol causes the inhibition of signaling of plant defense such as apoptosis-like programmed cell death (Diamond et al., 2013). Plant fungal pathogens can modulate in producing the plant hormones that possibly play a useful role in the growth and development of a plant. The acidification and loosening of the cell wall by IAA are helpful in invading a plant by a plant pathogen (Vrabka et al., 2019).

Interestingly, the plant hormone such as indole acetic acid (IAA) utilizes fungal pathogen to invade the plant by inducing adhesion (Prusty et al., 2004). The production of plant hormones by plant fungal pathogen suppresses the production of plant hormones by plant tissues. The *Fusarium* genus as a plant fungal pathogen causes a decrease in the growth and development of plant by producing five important plant hormones such as gibberellins, IAA, ethylene, abscisic acid, and cytokinins (Chanclud and Morel, 2016; Vrabka et al., 2019). Endophytic *F. proliferatum* ET1 showed more IAA synthesis from other *Fusarium* (Tsavkelova et al., 2012). Low concentration of IAA enhances the growth of plant fungal pathogen *Fusarium delphinoides* that causes infection to chickpea (Chanclud and Morel, 2016). *F. fujikuroi* secretes gibberellin leading to an elongation in the stalks of a plant such as rice plant and leading to the repression of the biosynthesis of gibberellin in plant tissues (Tudzynski and Holter, 1998; Hedden and Sponsel, 2015; Vrabka et al., 2019). In addition, Vrabka et al. (2019) mentioned that *F. mangiferae* induces increase in the cytokinin levels through invading and infecting a plant by changing the regulation of plant genes relevant for their biosynthesis and aggregation of cytokinin in the mango tree causing damage the reproductive organs. The fungus *Claviceps purpurea* utilizes cytokinin through interaction with a plant to complete infection (Hinsch et al., 2015). *M. oryzae* as rice blast fungus modulates the function of cytokinin hormones to ruin the plant defense and alters distribution of sugar and amino acids in and around the site of infection (Chanclud et al., 2016). *Magnaporthe grisea* can repress the defense response like SA-dependent responses by producing abscisic acid (Jiang et al., 2010).

In the Brazilian semiarid saprobe, fungus *Phialomyces macrosporus* showed signs of being a great biotic inducer for rust inhibiting the germination of urediniospores of *P. psidii*, and increased the activity of the enzyme phenylalanine ammonia-lyase, peroxidase, and β -1,3-glucanase. Other fungi also showed promising results, such as *C. eragrostidis* that decreases the severity of rust and induced rooting; *Stachybotrys chartarum* that reduces the disease severity and increases the activity of β -1,3-glucanase, and *Curvularia inaequalis* that increases the activity of the enzyme phenylalanine ammonia-lyase (Furtado, not published)

14.5 Conclusion and future prospect

In nature around the plant, many microbes impact the growth and development of a plant that can be divided into two kinds: useful and harmful microbes. These two kinds of microbes send many signals that play a role in the control of plant physiology, growth, and development. The microbial signals can be molecules, enzymes, and secondary metabolites. These signals can be more useful in different fields such as agriculture and environment. The signals of microbes can be utilized in the manufacture of biopesticides, bioherbicides, plant activators, and biofertilizers by, first, utilizing the microbes (beneficial and plant pathogens), and, second, as natural compounds. The role of microbial signals is to induce plant defenses against the plant pathogen and pests, while the role of signals from pathogenic microbes is to control weeds by causing a defect in plant physiology, and growth. Phytotoxins of plant pathogens affect secondary metabolite on the cell structure and plant physiology, which can cause devastation as possible for several species of weeds by increasing in concentration. Phytotoxins can be utilized as alternative methods of chemical herbicides. Indeed, the determinant of signals is more important to separate among microbes either plant pathogens or beneficial microbes due to diversity in the microbial community around economic plants such crops, fruit trees, and others that need to be discovered. This discovery is a chance for a

new method for controlling plant enemies such as pests, plant pathogens, and weeds. The new biotechnologies such as nanotechnology and OMICS analysis are interesting and can be more useful in the determination of signals. Finally, the utilization of signals as an alternative to synthetic chemicals in the agriculture fields to clean the residues of chemical pesticides from the ecosystem.

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RESEARCH ARTICLE

Agrobacterium fabrum C58 involved nitrate reductase NapA and antisense RNA NorR to denitrify

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One sentence summary: The nitrate reductase NapA is involved in *Agrobacterium fabrum* C58 fitness and the non coding RNA NorR is involved in denitrification regulation.

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ABSTRACT

Agrobacterium fabrum C58 is a plant-associated bacterium that is able to denitrify under anoxic conditions. The cluster of denitrification genes harbored by this strain has been well characterized. It includes *nir* and *nor* operons encoding nitrite and nitric oxide reductases, respectively. However, the reductase involved in nitrate reduction has not yet been studied and little information is available on denitrification regulators in *A. fabrum* C58. In this study, we aimed to (i) characterize the nitrate reductase, (ii) determine its role in *A. fabrum* C58 fitness and root colonization and (iii) reveal the contribution of small RNA on denitrification regulation. By constructing a mutant strain defective for *napA*, we demonstrated that the reduction of nitrate to nitrite was catalyzed by the periplasmic nitrate reductase, NapA. We evidenced a positive role of NapA in *A. fabrum* C58 fitness and suggested that *A. fabrum* C58 is able to use components exuded by plant roots to respire anaerobically. Here, we showed that NorR small RNA increased the level of *norCBQ* mRNA and a decrease of NorR is correlated with a decrease in N₂O emission. Together, our results underscore the importance of understanding the denitrification pathway at the strain level in order to develop strategies to mitigate N₂O production at the microbial community level.

Keywords: denitrification; NapA; nitrate reductase; fitness; root colonization; small RNA

INTRODUCTION

The diversification of respiration in prokaryotes has been a major contributor to the ability of these microbes to colonize a wide range of environments from the oceans to the Earth's crust (Richardson 2000) and to adapt to changing environments, such

as the rhizosphere (Haichar *et al.* 2014). Among the mechanisms of anaerobic respiration, the ability to denitrify has been shown to be advantageous to bacteria in the rhizosphere and was suggested to be a rhizospheric competence trait (Lecomte *et al.* 2018). Denitrification is a key function for the biochemical

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cycling of nitrogen. This process is a major source of atmospheric nitrous oxide (N₂O) and contributes to the emission of nitric oxide (NO). Emission of NO is undesirable because NO is toxic, and N₂O is a potent greenhouse gas (Houghton et al. 2001) and a dominant ozone-depleting substance (Ravishankara, Daniel and Portmann 2009). The denitrification process sustains respiratory growth under oxygen limitation via the dissimilatory reduction of nitrate to gaseous end products (Zumft 1997). Denitrification consists of four successive steps: the first step (NO₃⁻ → NO₂⁻) is catalyzed by the NarG or NapA nitrate reductases; the second step (NO₂⁻ → NO) is catalyzed by the NirK or NirS nitrite reductases; the third step, leading to N₂O formation (NO → N₂O), is mediated by the NorB nitric oxide reductase and the final step, reduction of N₂O (N₂O → N₂) is mediated by the nosZ nitrous oxide reductase (this step constitutes the only known microbial process that can reduce N₂O to N₂ in the biosphere; Zumft 1997; Jones et al. 2013; Hallin et al. 2018). Nearly one-third of nirS- or nirK-containing denitrifiers lack the nosZ gene (Philipot et al. 2011; Bakken et al. 2012), and therefore, these nosZ-lacking microbes do not have the genetic capacity to reduce N₂O and hence participate in N₂O emission and N loss from soils. An understanding of the physiology and regulatory biology of key N₂O-producing microorganisms may lead to the development of strategies to eliminate NO/N₂O production and/or promote NO/N₂O consumption at the microbial community level.

To date, different regulators have been reported for their involvement in the regulation of denitrification (Gaimster et al. 2018), for example, the Crp-Fnr superfamily regulators, such as Fnr (fumarate and nitrate reductase) and NnrR (response to NO in denitrification) (Zumft 2002; Körner, Sofia and Zumft 2003). Fnr is a transcription regulator that controls the expression of the genes required for anaerobic metabolism in response to O₂ availability (Zumft 2002). NnrR is a protein that controls the free NO concentration by regulating the expression of NO-producing (NIR) and NO-consuming (NOR) enzymes (Tosques et al. 1996; Bartnikas et al. 1997). Regarding NO toxicity, regulation of NO production and consumption is a critical step in the denitrification process, especially for rhizospheric bacteria that are subjected to different environmental changes, such as rapid transition from oxic to anoxic conditions. Small regulatory RNA (sRNA; Harfouche, Haichar and Achouak 2015) are also appropriate for this fine-tuned regulation. Indeed, Tata et al. (2017) evidenced the induction of Pail sRNA after shift to anaerobiosis conditions in the presence of nitrate and glucose in *Pseudomonas aeruginosa* PA14 strain. Recently, Gaimster et al. (Gaimster et al. 2016, 2019) demonstrated the role of denR sRNA in the inhibition of nitrite reductase and the reduction of NO and N₂O levels in *Parococcus denitrificans*.

Among nosZ-lacking bacteria, *Agrobacterium fabrum* C58 is a facultative denitrifying bacterium that belongs to the Alphaproteobacteria and is found in soils in free living form as well as in association with plants in the rhizosphere (Chèneby et al. 2004; Haichar et al. 2008). The *A. fabrum* C58 genome carries on its linear chromosome a gene cluster of approximately 60 kb that is involved in denitrification (Goodner et al. 2001; Wood et al. 2001). The accurate re-annotation performed by Baek, Hartsock and Shapleigh (2008) revealed the presence of the copper-containing Nir protein (encoded by the nirK gene) that is involved in nitrite reduction and the nor gene cluster, encoded by norCBQD, that is involved in nitric oxide reduction. Annotation of the *A. fabrum* C58 genome revealed the presence of a putative nitrate reductase named NapA which was demonstrated to be involved in nitrate reduction in different strains, such as *Bradyrhizobium*

japonicum (Bedmar, Robles and Delgado 2005). However, no evidence is available regarding its role in nitrate reduction in *A. fabrum* C58. In this strain, NnrR and ActR are transcriptional activator members of the Crp-Fnr regulator superfamily, control the expression of the genes encoding the Nir and Nor proteins (Baek, Hartsock and Shapleigh 2008). Evidence suggests that NnrR is activated by Fnr and nitric oxide (NO; Baek, Hartsock and Shapleigh 2008; Fig. 1A). Indeed, the presence of nitrate or nitrite leads to low-level production of NO, which activates NnrR, in turn increasing the transcription of nirK. NnrR also activates nor expression, mitigating NO accumulation and toxicity. RNA sequencing analysis performed by Dequivre et al. (2015) revealed the presence of putative sRNA. RNA829 between the norD and norQ genes on the antisense strand of the norCBQD operon. However, no study has looked at the role of sRNA in denitrification control in this strain.

The aim of this study was to (i) demonstrate the role of NapA in nitrate reduction, (ii) determine its role in the fitness and root colonization behavior of *A. fabrum* C58 and (iii) reveal the contribution of RNA829 in denitrification regulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli and *Agrobacterium* strains used in this study are summarized in Table 1. *A. fabrum* C58 wild-type and its derivatives were grown aerobically at 28°C with shaking (180 rpm) on YPG rich medium (per liter: 5 g of yeast extract, 5 g of peptone, 10 g of glucose and pH 7.2). Media were supplemented, when needed, with appropriate antibiotics (100 µg/mL of ampicillin, 30 µg/mL of gentamycin, 25 µg/mL of kanamycin, 25 µg/mL of neomycin and 10 µg/mL of tetracycline). In denitrification conditions, *A. fabrum* C58 and its derivatives were grown on 20 mL of YPG supplemented with 20 mM of KNO₃ at 28°C without shaking in 160 mL flasks closed hermetically with corks. A total of 140 mL of helium were injected into flasks to be under anoxic conditions since less than 0.5% of O₂ was measured.

Construction of markerless deletion mutant and fluorescent strain

C58ΔnapA

The linear chromosome DNA fragments corresponding to napA (atu4408) were deleted by double-recombination from C58 WT as described by Lassalle et al. (2011), resulting in C58ΔnapA strain (Table 1). Briefly, mutagenic PCR fragments were created by joining two fragments corresponding to the two regions flanking the sequence to be deleted (average 1 kb each) using the In-Fusion cloning kit (Clontech lab, USA; Table S1, Supporting Information). This fragment was cloned into the pre-open pJQ200SK, a plasmid carrying the gentamycin resistance cassette and the sacB gene conferring sucrose sensitivity (Quandt and Hynes 1993; Table 1). The resulting plasmid was introduced in C58 WT by electroporation (Lassalle et al. 2011). Single recombinants were selected on YPG media supplemented with gentamycin. Double recombinants were selected on YPG media supplemented with 5% sucrose. Deletion mutants were verified by PCR with appropriate primers and Sanger sequenced.

Complementation of C58ΔnapA with napA gene was performed using a pLAFR3 cosmid containing napA gene from *A. fabrum* C58 (Staskawicz et al. 1987). Briefly, a cosmidic bank constructed from *A. fabrum* C58 DNA was screened by PCR with

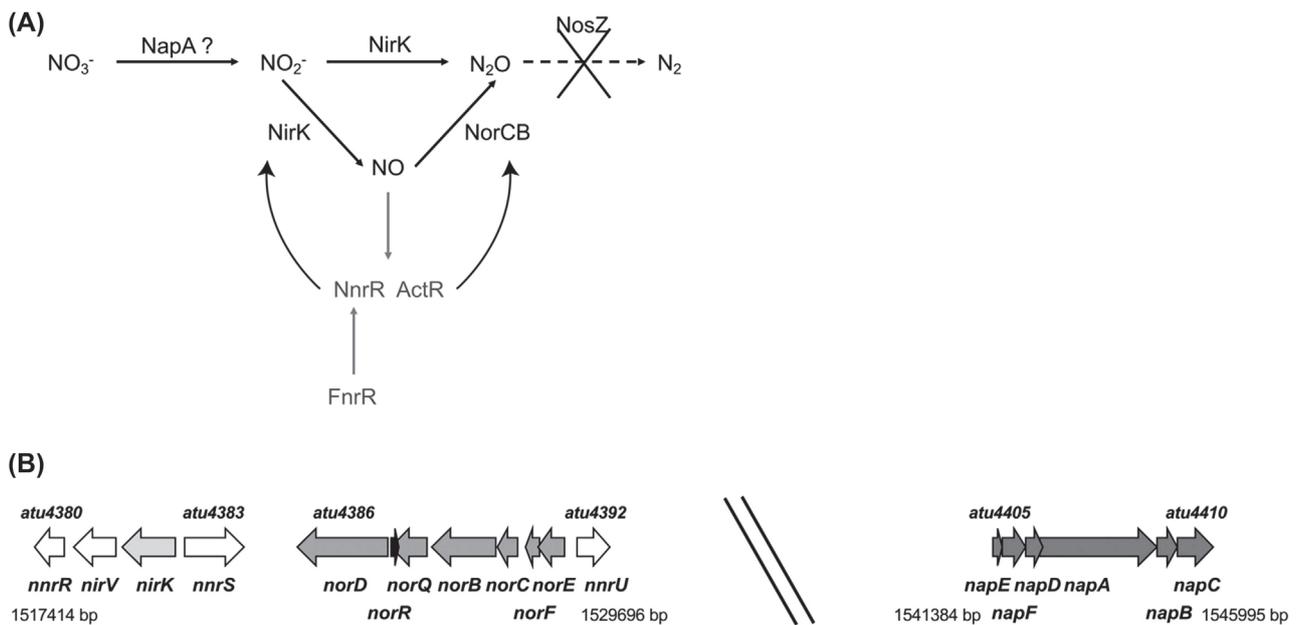


Figure 1. Denitrification pathway and its genetic organization in *Agrobacterium fabrum* C58 (A) Denitrification pathway and its regulation in *A. fabrum* C58. The reduction of soluble nitrate to nitrite is thought to be catalyzed by NapA, the periplasmic nitrate reductase. Reduction of soluble nitrite to nitric oxide gas is catalyzed by a copper (Cu-Nir) nitrite reductase. Lastly, the reduction of NO to N₂O is catalyzed by (Nor) of nitric oxide reductase. NnrR, FnrR and ActR, transcriptional regulator members of the Crp-Fnr superfamily regulators are involved in denitrification control. NnrR and ActR control the expression of the genes encoding Nir and Nor proteins (Baek, Hartsock and Shapleigh 2008). Whereas, FnrR activate NnrR expression (Baek, Hartsock and Shapleigh 2008). (B) Genes involved in denitrification in *A. fabrum* C58 are localized on the linear chromosome. This genomic region from *atu4380* to *atu4410* includes *nnrR*, *nirK*, *norCBQD* operon and *napEFDABC* operon. The arrows represent the gene orientation. The length of the arrows is related to the length of the genes. The genes are grouped by function figured by specific filling colors.

primers targeting *napA* gene. Cosmids were introduced by conjugation into *A. fabrum* C58Δ*napA*.

Overexpression and reduced expression of NorR

The linear chromosomal DNA fragment corresponding to *norR* (from 1 524 962 to 1 525 071 bp) was amplified by PCR using two different couples of primers (Table S1, Supporting Information). The fragments were digested using the HindIII and BamHI enzymes and were then ligated into the pBBR1MCS-2 and pBBR1MCS-5 plasmids, respectively, pre-linearized with the same enzymes, resulting in the *pnorR*⁺ and *pnorR*⁻ plasmids. The total sequence of *norR* was constitutively expressed under plasmid promoter control from the plasmid *pnorR*⁺, leading to overexpression of NorR. In contrast, a fragment of the *norR* sequence was constitutively expressed under plasmid promoter control from the plasmid *pnorR*⁻ leading to hybridization of genomic NorR with plasmid NorR and decreased NorR levels. Electroporation of the *pnorR*⁺ and *pnorR*⁻ stable plasmids into *A. fabrum* C58 yielded C58 NorR⁺ and C58 NorR⁻, respectively. The pBBR1MCS-2 and pBBR1MCS-5 empty plasmids were also introduced by electroporation into the *A. fabrum* C58 strain as a control.

Construction of *nirK* translational fusion

To construct translation eGFP fusion to *nirK*, a fragment containing the *nirK* promoter region and the translation initiation sites was amplified by PCR using the appropriate primers (Table S1, Supporting Information) and chromosomal DNA of strain C58 as the template. The fragment was digested with *Apal*-*SpeI* and cloned into the corresponding sites of pOT1eM plasmid in-frame of *egfp* generating pOT1eM-*PnirK*::GFP. pOT1eM plasmid

also contains the *m-cherry* gene. The constitutively expressed *m-cherry* gene (red color) indicates the bacteria presence whereas the inducible expressed *egfp* gene (green color) reports expression of *nirK*.

Denitrification activity

A. fabrum strains were grown under denitrification conditions (anaerobically on YPG media supplemented with 20 mM KNO₃). Flasks were inoculated at 4.2 × 10⁷ bacterial cells/mL from a 48 h anoxic culture on YPG supplemented with 20 mM of NO₃⁻ or with 2 mM of NO₂⁻. Denitrification activities were performed by measuring N₂O accumulation over the time (every 4 h) using gas chromatography coupled to a micro-catharometer detector (μGC-R3000, SRA instruments, Marcy l'Etoile, France) as previously describe by Guyonnet et al. (2017). Experiments were running for 24 h to capture the stationary phase of N₂O emission. Each experiment was performed in triplicate.

RNA characterization

RNA isolation

Strains were grown under denitrification conditions for 24 h (corresponding to the beginning of the stationary phase of denitrification activity). Cells were harvested and total RNA was extracted by a phenol chloroform method as previously described by Hommais et al. (2008). DNA traces were eliminated with two DNase I treatments (DNA-free kit, Ambion, Lithuanie). Isolated RNA was quantified using Nanodrop spectrophotometry (NP80, Implen, Germany), visualized on an agarose gel to check quality and stored at -80°C. Each experiment was performed in triplicate.

Table 1. Bacterial strains and plasmids used in this study.

Strains	Relevant genotype and description	Reference or source
Escherichia coli		
DH5 α	<i>E. coli</i> strain used for over and reduce expression of NopR	NEB catalog
Stellar	<i>E. coli</i> strain used for <i>napA</i> and <i>nasA</i> mutant construction	Takara catalog
Agrobacterium fabrum		
C58 WT	<i>Agrobacterium fabrum</i> C58 Wild-type	CFBP 1903
C5801	C58 deleted of <i>napA</i> (<i>atu4408</i>) gene	This study
C5803	C58 harboring pME6010::GFP plasmid	This study
C5804	C58 harboring pME6010::RED plasmid	This study
C5805	C58 Δ <i>napA</i> harboring pME6010::GFP plasmid	This study
C5806	C58 Δ <i>napA</i> harboring pME6010::RED plasmid	This study
C5807	C58 harboring pOT1eM-PnirK::GFP plasmid	This study
C5808	C58 harboring pBBR1-MCS2 empty plasmid	This study
C5809	C58 harboring pBBR1-MCS2:: <i>norR</i> ⁺ plasmid	This study
C5810	C58 harboring pBBR1-MCS5 empty plasmid	This study
C5811	C58 harboring pBBR1-MCS5:: <i>norR</i> ⁻ plasmid	This study
Plasmid	Description	Reference or source
pJQ200SK	Suicide vector; P15a <i>sacB</i> (used for mutant construction); Gm ^R	Quandt and Hynes (1993)
pME6010	Stable vector (used for reporter plasmid construction); Tet ^R	Heeb et al. (2000)
pOT1eM	pOT1e derivative harboring <i>Ptac-m-cherry</i> inserted in <i>Clal-Sall</i> site (used for fluorescent reporter protein); Gm ^R	Meyer et al. (2018)
pJQ200SK:: <i>napA</i>	pJQ200SK:: <i>napA</i> ; 600 pb upstream and downstream regions of <i>napA</i> gene inserted in <i>SmaI</i> site in pJQ200SK; Gm ^R	This study
pBBR1-MCS2	pBBR1-MCS2 stable vector; Neo ^R (used for RNA overexpression construction)	Kovach et al. (1994)
pBBR1-MCS5	pBBR1-MCS5 stable vector; Gm ^R (used for RNA silencing construction)	Kovach et al. (1994)
pBBR1-MCS2:: <i>norR</i> ⁺	pBBR1-MCS2 carrying <i>norR</i> region inserted in <i>BamHI-HindIII</i> site; Neo ^R	This study
pBBR1-MCS5:: <i>norR</i> ⁻	pBBR1-MCS5 carrying <i>norR</i> region inserted in <i>BamHI-HindIII</i> site; Gm ^R	This study
pME6010::GFP	pME6010 carrying gene encoding GFP fluorochrome inserted in <i>XhoI</i> site; Tet ^R	Meyer et al. Unpublished
pME6010::RED	pME6010 carrying gene encoding RED fluorochrome inserted in <i>XhoI</i> site; Tet ^R	Meyer et al. Unpublished
pOT1eM-PnirK::GFP	pOT1eM-PnirK::GFP; promoting <i>nirK</i> region inserted in <i>ApaI-SpeI</i> site in pOT1eM in front of <i>gfp</i> gene; Gm ^R	This study

Quantitative real-time reverse-transcription PCR (qRT-PCR)

A total of 2.5 μ g of total DNA-free RNA was reverse transcribed using random hexamer primer according to manufacturer's recommendations (ABM). qRT-PCR were performed using the SYBR Green PCR Master mix kit (Thermo Scientific, Lithuania) as previously described by Dequivre et al. (2015). LC480 Lightcycler from Roche was used for thermal cycling reactions according to the following protocol: an initial step at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 58°C for 30 s and 72°C for 20 s in order to amplify *norR* (Table S1, Supporting Information). Normalization of genes expression was performed using *rpoD* and *atpD* house-keeping genes. Melting curves were checked in order to verify PCR primer specificity.

Northern blot

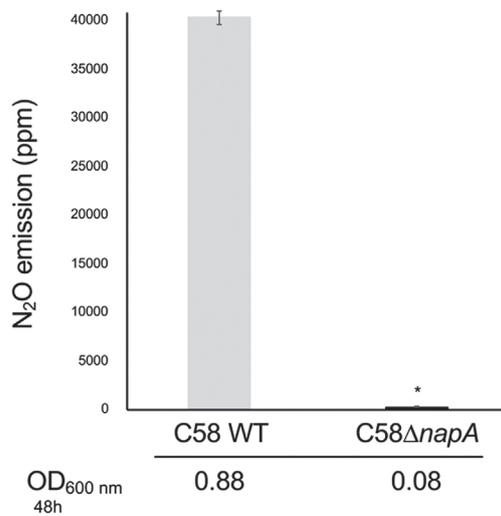
Ten μ g of total RNA extracted from C58 NorR⁺, C58 NorR⁻, C5808 and C5810 and 2 μ g of DynaMarker RNA High ladder (BioDynamics Laboratory Inc, Japan) were used for electrophoresis performed on 1% agarose gel containing 25 mM guanidium thiocyanate and ethidium bromide. After migration, RNA was transferred by capillary action on nitrocellulose membrane. Hybridization was

achieved with specific digoxigenin (DIG)-labelled probes complementary to the genomic regions of *norC*. Anti-digoxigenin-AP antibody (ROCHE) was used conjugated with CSPD substrate (ROCHE), and chemiluminescent detection was carried out with the FUSION-FX7 Spectra (Vilber, France). The northern blot analysis was performed in two replicates for each condition.

Determination of 5' and 3' ends by RACE PCR

RACE-PCR was performed as previously described by Dequivre et al. (2015). Briefly, 12 μ g of total DNA-free RNA were treated successively with different enzymes as follows: the XRN-I exoribonuclease (Biolabs, New England) were used to eliminate cleaved monophosphate RNA, then tobacco acid pyrophosphatase (Epicentre, UK) was used to treat the resulting triphosphate RNAs and finally T4 RNA ligase (Biolabs) was used to circularize RNA. An EasyScript Reverse Transcriptase kit was used to reverse transcribe 2.5 μ g of total circular DNA-free RNA using primers specific to the gene *norR* (Table S1, Supporting Information). The 5' and 3' end junction was specifically amplified by PCR. The products were cloned into pGEMT-Easy (Promega, USA)

(A) Nitrate as terminal electron acceptor



(B) Nitrite as terminal electron acceptor

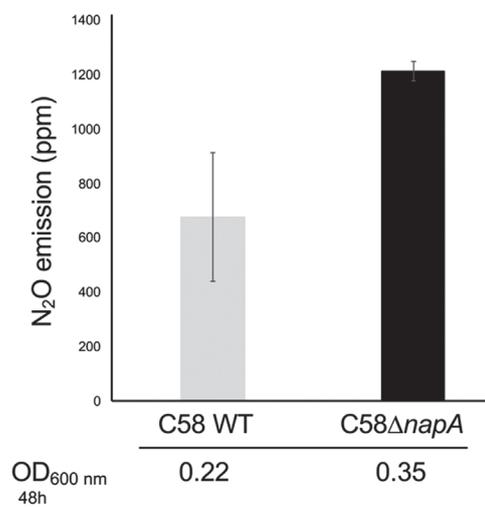


Figure 2. N₂O emission and bacterial growth after 48 h of incubation of C58 WT and C58Δ*napA* strains growing under denitrification conditions. (A) On 20 mM of nitrate. (B) On 2 mM of nitrite. For both, N₂O emissions were measured in ppm on strains incubated for 48 h under denitrification conditions and in presence of nitrogen oxide. Optical density (OD_{600 nm}) indicated growth of strains. WT and strains defective for the *napA* (C58Δ*napA*) gene were analyzed. Error bars correspond to standard deviation. Means that differed from the WT strains are indicated by a star (Wilcoxon's test, $\alpha < 0.05$). Experiments were performed in triplicates.

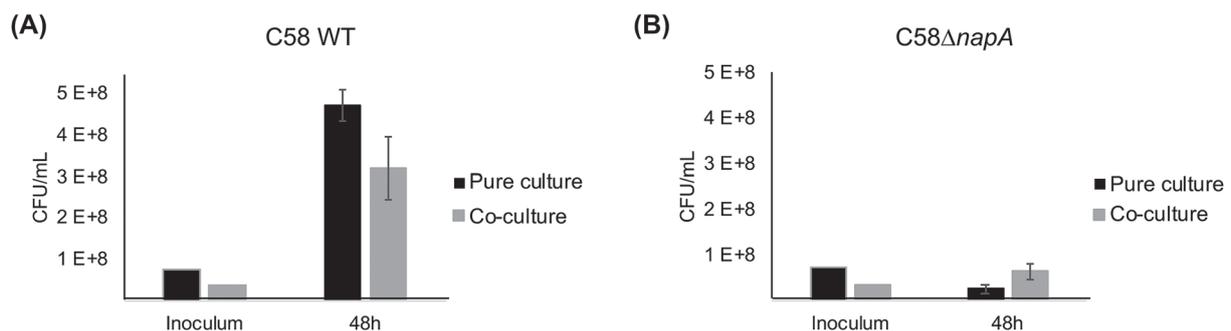


Figure 3. Competition assays between C58 WT and C58Δ*napA* strains under anoxic conditions *in vitro* and *in planta*. The numerations of cells were performed using strains colored with fluorescent proteins (RED and GFP proteins). The two combinations of colored strains were tested, and no difference was observed. (A) Growth of C58 WT strain in pure and co-culture during *in vitro* assays. The cell concentrations were determined for the initial inoculum and 48 h after inoculation under anoxic conditions. (B) Growth of C58Δ*napA* strain in pure and co-culture during *in vitro* assays. The cell concentrations were determined for the initial inoculum and 48 h after inoculation under anoxic conditions. Error bars correspond to standard deviation ($n = 3$). (C) Validation of anoxic conditions during *in planta* assays by observing *nirK* expression using GFP as reporter gene after 24 h of bacterial inoculation of maize roots using ZOE microscopy under oxic and anoxic conditions. Red fluorescence from m-cherry constitutive expression indicates bacterial presence (arrows as examples) whereas green fluorescence indicates active bacteria expressing *nirK* (arrows as examples). Plant auto fluorescence is represented by red and green vegetal cells. eGFP expression was intense under anoxic conditions whereas it was switched off under oxic conditions. (D) Growth of C58 WT and C58Δ*napA* strains in pure culture during *in planta* assays. The cell concentrations of C58 WT or C58Δ*napA* recovered from the maize roots were determined after 9 days of inoculation under anoxic conditions. (E) Proportion of C58 WT and C58Δ*napA* strains in co-culture during *in planta* assays. The proportion of each strain recovered from the maize roots was determined after 9 days of inoculation under anoxic conditions. Error bars correspond to standard deviation ($n = 4$).

and were Sanger sequenced using M13fwd primer. A total of five independent clones were analyzed.

Secondary structure

Secondary structure was predicted from *norR* gene sequence obtained by RACE-PCR using basic options of RNAfold algorithm (<http://ma.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) as previously describe by Dequivre et al. (2015).

Similarity sequence analysis

Comparative genome analysis was performed on whole genomes of *Agrobacterium* genus available on Genoscope

website (<http://www.genoscope.cns.fr/agc/microscope/home/index.php>) to study the synteny of denitrification genes and the conservation of NorR non-coding RNA in this genus.

Competition assays

In vitro

A culture of only one strain (C58 WT or C58Δ*napA*) or a co-culture of both strains (C58 WT and C58Δ*napA*) containing a plasmid with constitutively expressed fluorescent protein (pRED or pGFP) at a 1:1 ratio was grown under denitrification conditions (Table S2, Supporting Information). After 48 h of growth, the absolute number of colony forming units (CFUs) of WT and

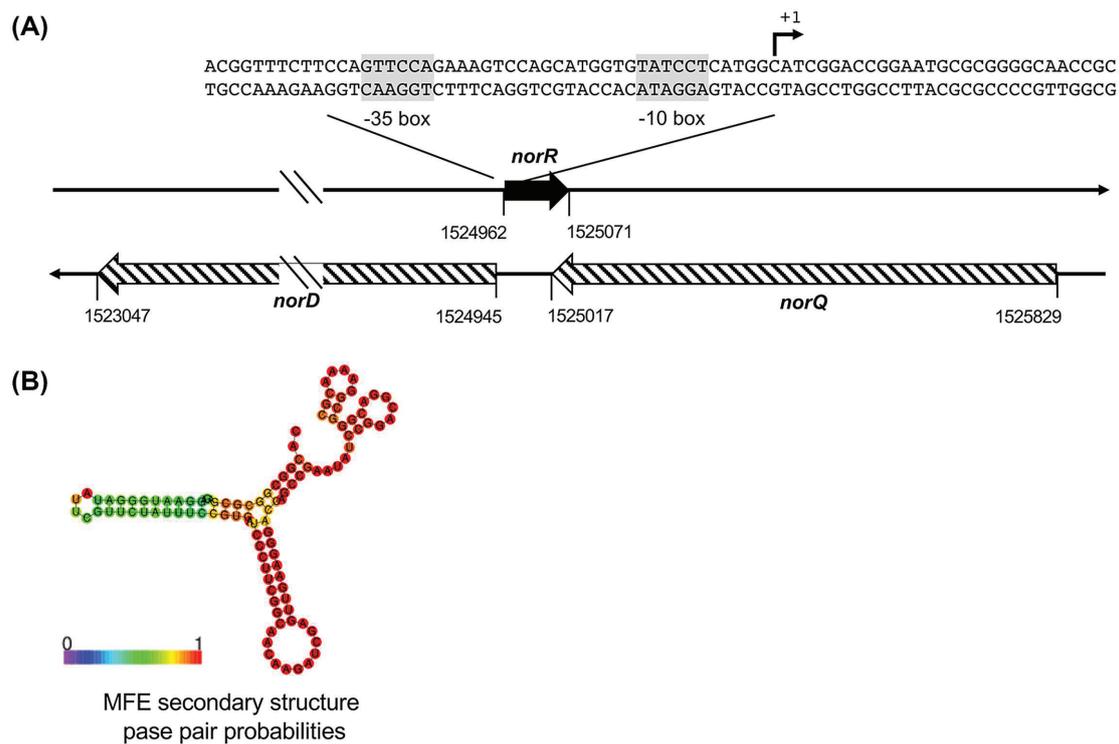


Figure 4. NorR characterization by RACE PCR and secondary structure. (A) The 3' and 5' end of *norR* sequence was identified by RACE PCR. The -10 box (TATCCT) and -35 box (GTTCCA) were identified using consensus sequence of these boxes known for *Agrobacterium tumefaciens* (Wilms et al. 2012). The *norR* sequence begins at 1 524 962 bp and finishes at 1 525 071 bp. (B) The minimum free energy (MFE) structure is obtained from RNAfold algorithm. The free energy of the thermodynamic ensemble is -39.32 kcal/mol. The frequency of the MFE structure in the ensemble is 26.44%. The colors represent base-pair probabilities with high value in red and low value in blue.

mutant strains were counted by plating these bacterial suspensions on YPG supplemented with tetracycline. The colonies, colored in red or green according to the harbored plasmid, were differentiated by the trained eye. The two combinations of reporter plasmids were performed to verify that no effect was noticeable on bacterial fitness.

In planta

The surfaces of maize seeds were disinfected by immersion in bleach and washing in ultra-pure water. For pre-germination, seeds were plated on agar for plant culture at 8 g/L (Sigma Aldrich, Germany) and incubated in the dark for 48 h at 28°C. The germinated seeds were then transferred in glass tubes, tightened approximately 5 cm from the bottom, containing 5 mL of plant nutrient solution supplemented with 5 mM nitrate. Then, these seeds were grown in a climatic chamber for 5 days at 24°C and 18°C with a 16/8-hour day/night cycle and 70% humidity. A total of five-day seedlings were inoculated with bacterial cell suspensions containing either only one strain (C58 or C58 Δ *napA*) or a mixture of both strains (C58 and C58 Δ *napA*) harboring the pRED or pGFP reporter plasmid at a 1:1 ratio. Then, these inoculated 5-day seedlings were grown for 9 days under anoxic conditions by hermetically sealing the tubes with corks. No carbon sources were added. Anoxic conditions were tested by inoculating C58 pOT1eM-PnirK::GFP in *in planta* supplemented with 20 mM of nitrate and visualizing fluorescence expression by microscopy (ZOE, fluorescent cell imager). Red fluorescence from constitutively expressed m-Cherry indicates bacterial presence, whereas green fluorescence indicates active bacteria expressing *nirK* only under anoxic conditions. After 9 days, the colonizing bacteria were recovered. Roots were crushed for 5 min with metallic balls

and 5 mL of water to recover bacterial cells, which were plated on YPG supplemented with tetracycline. Bacterial enumeration was performed as previously described. A total of two combinations of reporter plasmids were used to verify that there was no noticeable effect on bacterial fitness.

RESULTS

napA is responsible for the reduction of nitrate to nitrite

The gene *napA* is located on the linear chromosome in the cluster of denitrification genes begins at *atu4380*, corresponding to *nnrR*, and ends at *atu4410*, corresponding to *napC* (Fig. 1B). This cluster is composed of two parts: part 1 ranges from *atu4380* to *atu4392* and part 2 ranges from *atu4405* to *atu4410* (Fig. 1B). This cluster harbors genes encoding known denitrifying reductases: as the *nirK*-encoded nitrite reductase and *norC*-encoded nitrous oxide reductase. Among 11 representative strains of 10 genomic species of the *Agrobacterium tumefaciens* complex, the cluster of denitrification genes is present in *Agrobacterium* sp. G2 (= *A. pusense*), *Agrobacterium* sp. G3, *Agrobacterium* sp. G4 (= *A. radiobacter*), *Agrobacterium* sp. G7 (= *A. deltaense*) and *Agrobacterium* sp. G8 (= *A. fabrum*; Figure S1, Supporting Information). Moreover, the synteny of this cluster is conserved among these strains (Figure S1, Supporting Information). Accordingly, these strains were able to denitrify and to grow under anoxic conditions, whereas, those without (*Agrobacterium* sp. G1, *Agrobacterium* sp. G5, *Agrobacterium* sp. G9, *Agrobacterium* sp. G13 and *Agrobacterium* sp. G15 (= *A. viscosum*)) the cluster were unable to denitrify (Table S1, Supporting Information).

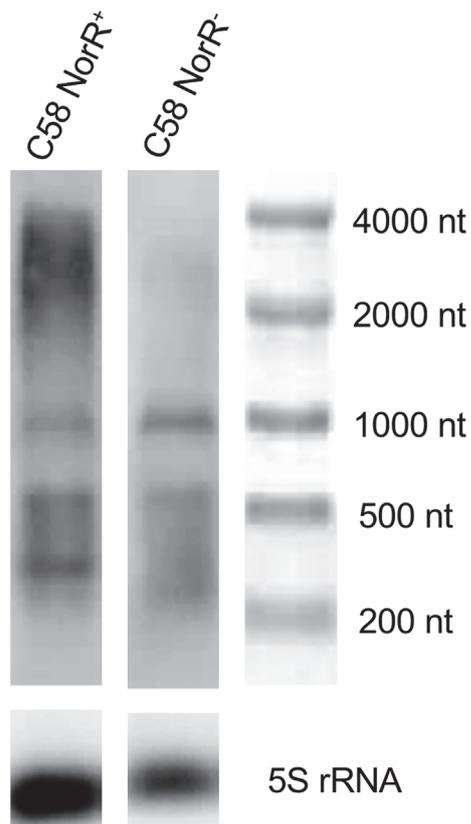


Figure 5. *norCBQD* mRNA expression in C58 NorR⁺ and C58 NorR⁻ strains. The expression of *norCBQD* mRNA transcripts was analyzed by northern blot using strand-specific probes for *norC* mRNA. No transcript corresponding to the isoform of *norCBQD* (around 4000 bases) could be detected. A transcript of 3000 bases corresponding to the isoform *norCBQ* of the mRNA is only detected in C58 NorR⁺ strain. A transcript of around 400 bases corresponding to *norC* alone was detected in both strains. The northern blot was performed in two replicates for each condition.

A mutant strain defective for *napA* was constructed (C58 Δ *napA*) and its capacity to denitrify was tested by measuring N₂O emission on nitrate or nitrite as terminal electron acceptors (TEA). C58 Δ *napA* strain was not able to denitrify and grow on nitrate, whereas, C58 WT strain reached 40 000 ppm of N₂O emission and its growth was 10-fold higher than C58 Δ *napA* strain (Fig. 2A). In contrast, in the presence of nitrite as TEA, C58 Δ *napA* and C58 WT were able to denitrify and to grow with no significant difference between both strains ($P > 0.05$; Fig. 2B). C58 Δ *napA* complemented with cosmid containing *nap* operon grown under denitrification condition as C58 WT strain. These results demonstrated that NapA is a nitrate reductase responsible for the reduction of nitrate to nitrite under denitrification conditions.

Is NapA involved in the fitness and rhizosphere colonization behavior of *A. fabrum* C58?

To test the involvement of NapA in the fitness and rhizosphere colonization behavior of *A. fabrum* C58 under denitrification conditions, we evaluated the growth of C58 WT and C58 Δ *napA* during *in vitro* and *in planta* competition assays.

In vitro assays

C58 WT and C58 Δ *napA* were both inoculated at 7×10^7 CFU/mL in pure culture and at 3.5×10^7 CFU/mL in co-culture (Fig. 3A).

After 48 h of growth, C58 WT reached 4.7×10^8 CFU/mL in pure culture and 3.2×10^8 CFU/mL in co-culture. After 48 h, cell numbers of C58 Δ *napA* decreased in pure culture (2.6×10^7 CFU/mL) and increased in co-culture (6.4×10^7 CFU/mL) (Fig. 3B). The growth of C58 Δ *napA* was 2 times higher in the presence of C58 WT than in pure culture, whereas the growth of C58 WT was lower in the presence of C58 Δ *napA* than in pure culture (Fig. 3A and B).

In planta assays

Anoxic conditions were verified *in planta* by analyzing the expression of the *nirK* gene using the pOT1eM-P*nirK*::GFP reporter plasmid after 24 h, 48 h, 72 h and 9 days of bacterial inoculation of maize roots. *A. fabrum* C58 root colonization was shown by constitutive red fluorescence. Anoxic condition establishment was verified by the induction of *nirK* expression reported by green fluorescence. *nirK* expression was induced as early as 24 h after bacterial inoculation (Fig. 3C) attesting the establishment of denitrification conditions.

C58 Δ *napA* and C58 WT strains were inoculated on maize roots at 3×10^7 CFU/mL in pure culture and at 1.5×10^7 CFU/mL in co-culture. After 9 days of growth, both strains grew and colonized maize roots at approximately 3×10^8 CFU/mL and 1.7×10^8 CFU/mL in pure culture and co-culture, respectively (Fig. 3D and E). Maize root colonization was identical regardless of the strain used in pure or co-culture.

A small transcript NorR is located in the denitrification cluster of *A. fabrum* C58

The determination of NorR (former RNA829) transcriptional start and stop by RACE-PCR revealed a small transcript of 109 bases in length beginning at base 15 224 962 and ending at base 15 225 071 (Fig. 4A). The RTqPCR analysis reveals that the expression level of this small transcript is low in oxic conditions but increased by a factor of 5 in anoxic conditions. The prediction of its secondary structure comprises several stem loops with long stems and single-stranded loops containing several A- and G-rich regions (Fig. 4B). Thus, this small transcript is in antisense of the intergenic region between *norD* and *norQ* and overlaps the coding sequence of *norQ* by about 50 bases. We renamed it NorR sRNA and evaluated its impact on the *norCBQD* mRNA. We constructed strains with altered levels of NorR (a strain overexpressing this small transcript (C58 NorR⁺) and a strain silencing NorR (C58 NorR⁻) and we performed a northern blot with these strains cultivated in anoxic conditions (Fig. 5). We used a probe targeting *norC*, the first gene of the operon. No transcript corresponding to the isoform of *norCBQD* (around 4000 bases) could be detected whatever the strain analyzed but a transcript of around 400 bases corresponding to *norC* alone was detected. Interestingly, we also observed a transcript of about 3000 bases in length in the strain overexpressing NorR but this transcript is absent in the strain silencing NorR. This transcript could correspond to the isoform *norCBQ* of the mRNA since *norC* is about 453 bases, *norB* about 1347 bases and *norQ* about 813 bases I.E. *norCBQ* transcript is about 2700 bases (Fig. 5).

NorR is involved in the regulation of denitrification

NorD and NorQ are chaperone proteins involved in the activation of the nitric oxide reductase NorC (Boer et al. 1996). Taken together this allows us to test the involvement of NorR in the regulation of denitrification. We thus evaluated the denitrifying enzyme activities (DEA) of these strains and compared them to

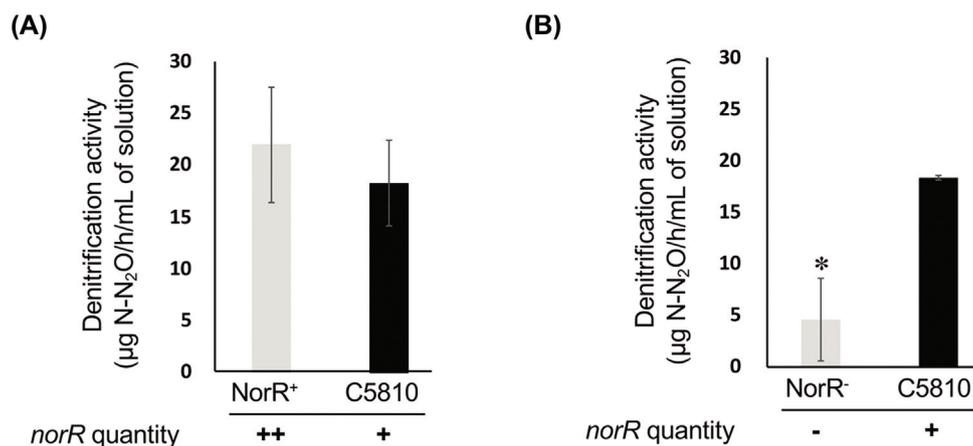


Figure 6. Denitrification activity of C5808, C58 NorR⁺, C5810 and C58 NorR⁻ strains. The graphic shows the denitrification activity (DEA, $\mu\text{g N-N}_2\text{O/h/mL}$ of solution) of C5808 (WT strain harboring empty plasmid for overexpression construction), C58 NorR⁺ (a strain overexpressing NorR sRNA), C5810 (WT strain harboring empty plasmid for silencing construction) and C58 NorR⁻ (a strain silencing NorR sRNA). Quantity of NorR is indicated by (++) for C58 NorR⁺, (+) for WT strains and (-) for C58 NorR⁻. Error bars correspond to standard deviation ($n = 6$). Significant difference is indicated by a star (Wilcoxon's test, $\alpha < 0.05$).

those of the wild type strain by measuring N_2O emission for 24 h under denitrification conditions. Every strain was able to grow under anoxic conditions (Fig. 6). While DEA of 18 $\mu\text{g N-N}_2\text{O/h/mL}$ was measured for the wild-type strains a slight increase to 22 $\mu\text{g N-N}_2\text{O/h/mL}$ was observed for the strain overexpressing NorR. In contrast, the DEA measured for the strain silencing NorR was only 5 $\mu\text{g N-N}_2\text{O/h/mL}$ showing a significant decrease of 4-fold compared to strain overexpressing NorR. Taken together, the results demonstrated a reduction of denitrification activity when NorR was silenced (Fig. 6).

DISCUSSION

Nitrate reduction is catalyzed by NapA

The cluster of denitrification genes harboring the *nir* and *nor* operons is well known (Baek and Shapleigh 2005; Baek, Hartsock and Shapleigh 2008; Bergaust et al. 2008) and is conserved among denitrifier species of *A. tumefaciens* complex. This cluster was probably lost by the other species (Lassalle et al. 2017) suggesting that denitrification is a species-specific pathway in the *A. tumefaciens* complex. NapA was suggested as the reductase involved in nitrate reduction in different studies, such as Kampschreur et al. (2012) and Kuypers, Marchant and Kartal (2018), but was not yet characterized. Thus, we demonstrated that under anoxic conditions, the growth and denitrification ability of the *napA* null mutant were affected with nitrate as TEA and presented the same level as C58 WT with nitrite as TEA. These results demonstrated that NapA is the nitrate reductase that catalyzes the conversion of nitrate to nitrite in *A. fabrum* C58 and probably in the whole *Agrobacterium* genus harboring the cluster of denitrification genes.

NapA plays a role in *A. fabrum* C58 fitness

In soil and rhizospheric environments, bacteria need the ability to explore host resources and compete with the resident microbiota (Gonzalez-Mula et al. 2019). The denitrification pathway is one of the traits known to provide competitive advantage for rhizosphere colonization (Philippot, Clays-Josserand and Lensi 1995; Lecomte et al. 2018). In our study, using *in vitro* assays under anoxic conditions, the mutant C58 Δ *napA* was unable to grow on nitrate demonstrating that NapA is involved in C58 fitness. During competition assays, we observed poor growth of

C58 Δ *napA* simultaneously with decreased growth of C58 WT, which can be explained by the NO_2^- flux. Indeed, C58 WT is responsible for the reduction of NO_3^- to NO_2^- , and NO_2^- can diffuse from cells to media. However, we could not detect it NO_2^- in the media. As NO_2^- is toxic for cells, it is probably rapidly diverted by C58 Δ *napA* to aid its own growth instead of C58 WT's, as demonstrated in the soil environment by Robertson and Groffman (2007). In the same ecological niches, species of *Agrobacterium* can coexist (Lassalle et al. 2011), notably species that encode the denitrification function with those lacking it. This finding suggests a Black Queen Hypothesis (BQH) context in which some *Agrobacterium* species called 'helpers', as these species provide the denitrification function, build obligate associations with species that avoid the denitrification function, called 'beneficiaries' (Morris, Lenski and Zinser 2012; Mas et al. 2016; Zomorodi and Segrè 2016). In addition, because helpers provide nitrite for the growth of beneficiaries under certain conditions, these species also produce NO, which may play a role in efficient plant colonization (Ghiglione et al. 2000).

NapA is not involved in *A. fabrum* C58 root colonization

For *in planta* competition assays, we chose *Zea mays* plants that exude different carbon sources, such as rhamnose, maltose, succinate and citric acid (Krafczyk, Trolldenier and Beringer 1984), and are efficiently colonized by *Agrobacterium* species (Chèneby et al. 2004). Surprisingly, no differences in growth were observed between C58 WT and C58 Δ *napA* in pure and in co-culture during *in planta* experiments under anoxic conditions. This finding suggests that (i) NapA is not involved in C58 fitness during *in planta* growth, (ii) C58 Δ *napA* could alternatively use exudates as TEA, allowing the growth of this strain and favoring root colonization under anoxic conditions. Indeed, certain plants root exudates, such as fumaric acid could be used as TEA by microorganisms under anoxic conditions (Guyonnet et al. 2017; Lecomte et al. 2018).

NorR contributes to denitrification regulation

To adapt to environmental changes, such as rapid transition from oxic to anoxic conditions, coordination of the regulation and activity of the different reductases involved in denitrification is necessary and may be mediated by non-coding RNA. The

role of non-coding RNA in the repression of denitrification activity was already demonstrated in *Paracoccus denitrificans* (Gaimster et al. 2019). However, nothing is known regarding the activation of the denitrification process by non-coding RNA. In this study, we discovered a novel sRNA named NorR (nor regulation sRNA) that is involved in the activation of denitrification in *A. fabrum* C58. NorR is a cis-encoded antisense sRNA that is located on the opposite strand of the *norQD* genes, expressed under anoxic conditions and conserved among genomic species harboring the denitrification cluster. We evidenced that *norCBQ* mRNA level is increased concomitantly with N₂O emissions level in C58 NorR⁺ strain. Therefore, we suggest that during anoxic condition, NorR forms base pairs with the 3' end of the *norQ* mRNA and confers an increase in *norCBQ* mRNA levels allowing *norQBC* mRNA accumulation and hence N₂O emission elevation.

CONCLUSION

Our results have shown that NapA catalyzes nitrate reduction under denitrification conditions. As expected, a positive effect of NapA on *A. fabrum* C58 fitness was observed, while NapA didn't appear to play a role in *A. fabrum* C58 root colonization. In addition, we evidenced the contribution of a sRNA NorR in denitrification regulation. NorR increases the level of *norCBQ* mRNA and a decrease of NorR is correlated with a decrease in N₂O emission.

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SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://academic.oup.com/femsec) online.

Conflicts of interest. None declared.

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