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Salicylic Acid, Jasmonic Acid and Ethylene Involved in The Resistance Induced By The Rhizobacterium Pta-Ct2 In *Arabidopsis thaliana* Against *Botrytis cinerea*

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ABSTRACT

Salicylic acid (SA), Jasmonic acid (JA) and ethylene (ET) have the significant roles in the plants physiologically and in defense against pathogens. To elucidate the role of these three phytohormones in the development of Induced systemic resistance (ISR), it is a systemic immune response that occurs when the roots are colonized by beneficial microbes. The study model of SRI is the combination of specific beneficial strains of *Pseudomonas fluorescens* PTA-CT2 with *Arabidopsis thaliana*, the course of camalexin levels was monitored before and after infection with the fungus *Botrytis cinerea*. To conduct this, we use different mutants and transgenic plants that fail in the pathway of JA (*jar1*), ethylene (*ein2*) or NahG (transgenic line degrading salicylic acid [SA]). We are therefore monitoring the evolution of camalexin, a highly lipophilic phytoalexin, before and after bacterization and/or infection. As a consequence of the study, the bacterization induces potentiation of the defenses, which depends on the three signaling pathways. In addition, the recognition of the beneficial bacteria is reduced by JA and ET.

Keywords: Salicylic acid, Jasmonic acid, Ethylene, Induced Resistance, Rhizobacterium, *Botrytis cinerea*, *Arabidopsis thaliana*.

INTRODUCTION

The plant immune system is essential for the plants to perceive and defend against bacterial, fungal, insect pests, and pathogens. Following the attack of pathogens, the plants must defend themselves to ensure their survival. As a result, they will begin to accumulate secondary metabolites of defenses at the site of infection. However, in order to guard against another infection, the plant will establish a systemic resistance called Systemic Acquired Resistance (SAR) (Chen *et al.*, 2019; Iavicoli *et al.*, 2003). Although pathogens are not the only ones that can interact with plants. Indeed, in 1991, Van Peer *et al.*, has described Induced Systemic Resistance (ISR) which consists of the infection of microorganisms beneficial to the plant. The Induced Systemic Resistance results in the accumulation of phytoalexins, callose deposition, which increased activity of Pathogenesis-related proteins (PR) proteins, (Chen *et al.*, 2019; Magnin-Robert *et al.*, 2013; Verhagen *et al.*, 2010). These defenses are much less aggressive than when attacked by pathogens. However, ISR is different from SAR since it is induced by beneficial microorganisms, and allows the potentiation of defenses also called "priming" throughout early and effective establishment of defense responses (Chen *et al.*, 2019; Martinez-Medina *et al.*, 2016). The beneficial microorganisms inducing ISR are generally PGPRs (Plant Growth-Promoting Rhizobacteria) (Shine *et al.*, 2019). In addition, the bacteria are sometimes able to inhibit the pathogen directly by competing with it or by synthesizing antibiotics. (Van Loon, 2007; Son *et al.*, 2014). PGPRs are therefore a possible alternative to the use of

pesticides and the production of Genetically Modified Organisms (GMOs), which are poorly perceived by the general public due to their environmental impact and the lack of perspective on transgenic plants. For an application in agriculture, it is necessary to know effectively that the processes leading to ISR. The latter is induced remotely and therefore involves hormonal signaling pathways. During the ISR response, the genetic and hormonal signaling mechanisms deployed are partially understood (Pangesti *et al.*, 2016; Pieterse *et al.*, 2014). The three major defense hormones are jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) [Son *et al.*, 2014; Jalloul *et al.*, 2009].

Salicylic acid (SA) is known to be involved in resistance to biotrophic pathogens (Conrath *et al.*, 2002). Regarding JA and ET, they seem necessary for tolerance against biotrophs and necrotrophies even if the role of ethylene is sometimes ambivalent (Seilaniantz *et al.*, 2011).

The role of these three hormones in SAR is therefore well known, however, it is not known for the development of ISR. Given the absence of studies on the involvement of signaling pathways during the establishment of ISR, we are interested in them in the establishment of resistance induced by *Pseudomonas fluorescens* PTA-CT2, and to the potentiation of defenses against *Botrytis cinerea*. This bacterium, isolated from the Champagne vineyard in 2008, belongs to gram- bacteria and has a PGPR action on the vine (Compant *et al.*, 2013; Magnin-Robert *et al.*, 2007). The vine being too complex to study the signaling pathways, we are working on *Arabidopsis thaliana*. To do this, we use different mutants and transgenic plants that fail in the

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pathway of JA (*jar1*), ethylene (*ein2*) or NahG (transgenic line degrading salicylic acid [SA]). We are therefore monitoring the evolution of camalexin, a highly lipophilic phytoalexin (Rogers *et al.*, 1996), before and after bacterization and / or infection.

MATERIAL AND METHODS

Plant material:

We use *Arabidopsis thaliana* ecotype Columbia 0 (Col0), the inoculation of *Arabidopsis thaliana* roots with *Pseudomonas* as a model system for studying ISR against fungi pathogen (Pieterse *et al.*, 1996; Haney *et al.*, 2015), as well as two mutants in the signaling pathways: *jar1* (insensitive jasmonate), which no longer produces JA, *ein2* (insensitive ethylene), and no longer perceives ethylene. We also use the transgenic plant NahG which degrades SA through the production of SA hydroxylase. These plants are grown in pots containing around 80g of autoclaved soil, at a temperature of 22°C, a photoperiod of 12h day/12h night and a humidity level of 70%.

Microorganisms:

Beneficial bacteria:

A bacterial isolates used in this study (*Pseudomonas fluorescens* PTA-CT2) (Trotel-Aziz *et al.*, 2008), A preparation of bacterial is centrifuged at 5000g for 10 minutes. The pellet is taken up in 50 ml of 10 mM MgSO₄. Solutions of 5 ml containing 5.6×10^7 colony-forming units (CFU)/g of soil are inoculated at the base of the stems (without touching the leaves). For each plant genotype, 5 are inoculated with this PGPR and 6 others with 10 mM of MgSO₄. The bacteria batches are separated from the control batches in the culture chamber.

Pathogenic Fungus:

The fungus *Botrytis cinerea*, strain 630 isolated from the Champagne vineyard was cultured in Erlenmeyer flasks on potato dextrose agar medium (PDA) at 22 C for 14 days (Magnin-Robert *et al.*, 2012). The spores are collected by scraping: sterile distilled water is added to the spore-forming fungus so as to obtain a suspension of spores. The suspension is filtered to keep only the conidia. Inoculation is done by depositing 5 µl of solution, containing 106 conidia per ml, on each leaf. The plants tested are inoculated 2 weeks after infection with the beneficial bacteria. Three plants of each non-bacterial control phenotype and three plants of each bacterized phenotype are inoculated with the fungus. These plants are placed in a closed box containing 2cm of water and placed in the culture chamber.

Phytoalexins extraction:

The remainder of the plants not inoculated with *Botrytis* are used for the first extraction of camalexins (3 of each non-bacterized phenotype, 3 bacterized controls and 2 of each other bacterized phenotype). The second extraction is done a week later on plants previously infected with *Botrytis*. The extraction begins with the grinding in nitrogen of the leaves of the various plants. The powder is sampled in a brown Eppendorf tube at 250 mg. Eppendorf tubes are brown because phytoalexins are photodegradable. To the 250 mg of fresh material, extracted is added 1 ml of methanol (adapted according to the milligrams obtained). All samples are vortexed every 10 minutes for 1 hour. They are then centrifuged at 13000g for 10 minutes at room temperature. The supernatant is taken up to a minimum of 0.5 ml with a syringe, is filtered with Teflon filters and then

is deposited in vials fitted with stoppers having a Teflon membrane.

Dosage of camalexins:

The vials are then placed in a 48-place rack specially designed for dosing by UPLC (Ultra Performance Liquid Chromatography). The device takes a sample from each vials by piercing the membrane of the stopper to analyze it for 10 minutes. The peak that interests us, corresponding to camalexin, comes out at about 7.34 minutes. The concentration of camalexin in each sample is calculated using a standard curve provided by the Research Unit on Vine and Wine from Champagne (URVVC) of the University of Reims.

RESULTS AND DISCUSSION

Results

Effect of bacterization:

Figure 1 shows that the wild-type (WT) plants appear to have grown more when they underwent bacterization. Plants *ein2* and *jar1*, which from the start of the experiment were punier than WT, have the same phenotype whether or not they are bacteria. In contrast, the NahG plants were more developed at the start of the experiment. These non-bacterial plants developed a flower stalk earlier than when they were bacteria.

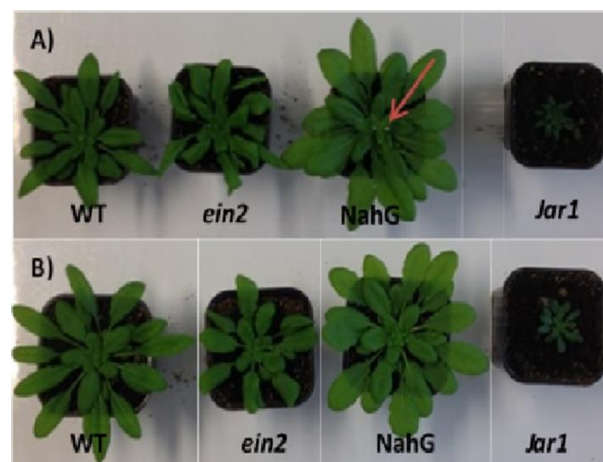


Figure 1. Effect of bacterization on 3 week old plants.

A) non-bacterial plants (5 mL of water at the base of the stems).

B) Bacterized plants with 5mL containing 5.6×10^7 CFU/g of soil inoculated at the base of the stems. The arrow indicates the flower stalk developed earlier in the non-bacterial NahG than in the bacterium. These results reflect three replicates.

Regarding camalexin (Fig 2.), it seems that there is less of it in the leaves of the bacterized WT than in those of the non-bacterial WT. However, in view of the standard deviations, the difference does not seem significant. The *jar1* and *ein2* mutants have a constitutively higher level unlike the NahG plants. In fact, the level of camalexin in NahG plants, whether or not they are bacterized, is extremely low (close to 0 µg of camalexin / g of FW [fresh weight]). Concerning the two mutants *jar1* and *ein2*, the bacterized plants have a much higher level of camalexin than the non-bacterized with a however a non-significance in the mutant *jar1*.

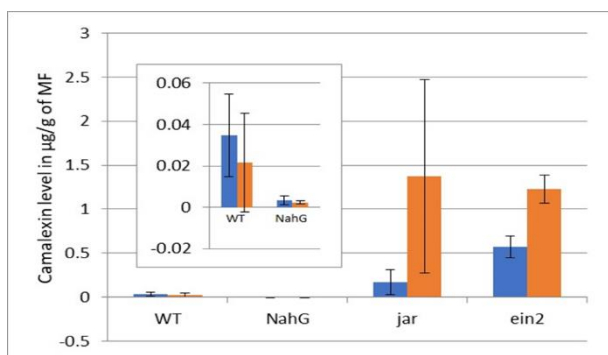


Figure 2. Camalexin level in plants bacteria or not and not infected with *B. cinerea*. In blue, the non-bacterial plants (5 mL of water at the base of the stems) and in red the bacterized plants (5mL containing 5.6×10^7 CFU/g of soil inoculated at the base of the stems). These results reflect three replicates.

Potential of plants:

Bacterized WT plants show less necrosis due to *B. cinerea*. Mutant and transgenic plants show a similar frequency of necrosis whether or not they are bacteria. When WT is infected, the level of camalexin is once lower than when they are bacteria. For non-bacterial plants, the jar mutant contains as many camalexins as WT. In contrast, NahG at a consistently very low rate (1.5 times lower than WT) and ein2 plants, about twice as high as WT. For each of the mutant and transgenic plants, no difference between bacteria and non-bacteria can be observed (Figure 3B).

Discussion

Effect of bacterization:

Our data indicate that accessions of *Arabidopsis* actively inhibit growth of some species within the Pseudomonadaceae while leaving the majority of the microbiome intact. Given that the NahG transgenic plants do not accumulate SA and that they do not seem to produce camalexin, the hypothesis of a positive control of the biosynthesis of camalexin by SA can be made (Seilaniantz *et al.*, 2013). The strong accumulation of camalexin in the jar1 and ein2 mutants suggests a downregulation of the synthesis of this phytoalexin by JA and ET. Following the bacterization of plants, WT like NahG do not seem to recognize the bacterium PTA-CT2 as a pathogen because their level of camalexin does not increase. On the other hand, the mutants recognize PTA-CT2, inducing a strong increase in the level of camalexin. Recognition of the beneficial bacteria therefore appears to be inhibited by JA and ET. Regarding SA, it is difficult to conclude since it is involved in the biosynthesis of camalexin, which is therefore almost absent in all cases (Nawrath *et al.*, 1999; Roetschi, 2001).

Potential of plants:

We conclude that whether of microbiome community provides benefit or harm by plant genotype when essential determinant for the host. Random encounters with beneficial microbes are not sufficient to explain the health benefits of the plant microbiome; the plant will not benefit from the plant genotype because incompatible with growth of the beneficial microbe, when its presence in the environment. The PTA-CT2 bacterium effectively induces a priming of WT but none of the mutant or transgenic plants appears to be potentiated (FIGS. 2 and 3). The level of camalexin in bacterialized WT is lower because there is

less necrosis. The potentiation of *A. thaliana* by PTA-CT2 not only affects camalexin levels but also other defenses (Iavicoli *et al.*, 2003; Verhagen *et al.*, 2010). In fact, camalexin is cytotoxic in high doses, so it is healthier for the plant not to accumulate in excess (Rogers *et al.*, 1996). Transgenic plants are infected with *B. cinerea* in a similar fashion whether or not they are bacteria (Figure 2.). This eliminates the camalexin level (Figure 3.) to conclude that AS seems to lead to the priming of *Arabidopsis thaliana* by PTA-CT2 against *B. cinerea* since the potentiation disappears in the absence of SA. The same is true for mutant plants, they show no change in either the infection rate (significantly similar) or the camalexin level (Figure 2. and 3.). The JA and the ET, like the SA, therefore intervene in this process of potentiation.

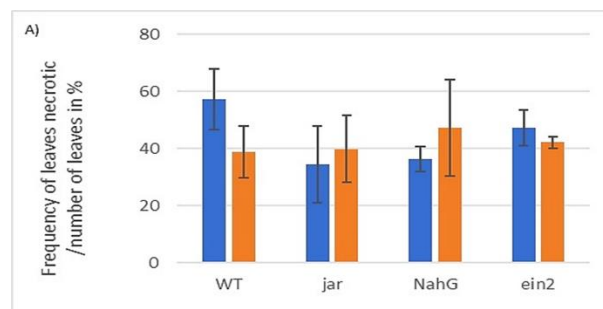


Figure 3A. Potential of plants by PTA-CT2.

Frequency of contamination of plants contaminated with *B. cinerea*.

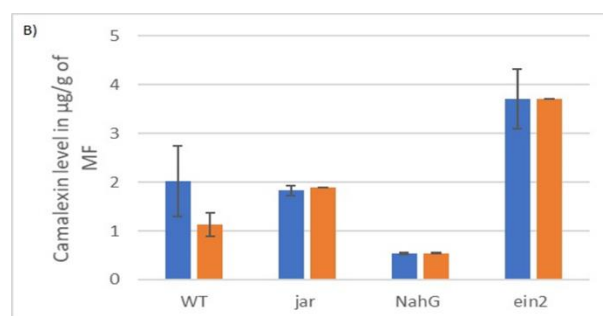


Figure 3B. Potential of plants by PTA-CT2.

Camalexin level in plants infected with *B. cinerea*. In blue, the non-bacterial plants (5 mL of water at the base of the stems) and in red the bacterized plants (5mL containing 5.6×10^7 CFU/g of soil inoculated at the base of the stems). These results reflect three replicates.

CONCLUSION

The role of JA and ET is to decrease the recognition of the bacteria by *Arabidopsis thaliana* during bacterization. These hormones, in association with SA, would induce potentiation. However, there are other untested phytohormones such as abscisic acid (ABA), which interacts with the three signaling pathways studied in this study (Jalloul *et al.*, 2009).

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مشاركة حمض الساليسيليك وحمض الياسمونيك والإيثيلين في المقاومة التي تسببها البكتيريا الجذرية PTA-CT2 في نبات (*Arabidopsis thaliana*) ضد العفن الرمادي (*Botrytis cinerea*)

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يلعب حمض الساليسيليك (SA) وحمض الياسمونيك (JA) والإيثيلين (ET) دوراً مهماً في النباتات من الناحية الفسيولوجية وفي الدفاع ضد مسببات الأمراض. لتوضيح دور هذه الهرمونات النباتية الثلاثة في تطوير المقاومة المناعية المستحثة (ISR) ، تحدث استجابة مناعية جهازية عندما يتم استعمار الجذور بواسطة الميكروبات المفيدة. نموذج دراسة المقاومة المناعية المستحثة هو مزيج من سلالات مفيدة محددة من PTA-CT2 مع *Pseudomonas fluorescens* مع *Arabidopsis thaliana* ، تم تقدير مستويات camalexin قبل وبعد الإصابة بالفطر *Botrytis cinerea* ، لإجراء ذلك ، تم استخدام طفرات مختلفة ونباتات معدلة وراثياً تقفل في مسارات حامض الياسمونيك والإيثيلين وحمض الساليسيليك ، لذلك نقوم بدراسة تطور مركب camalexin ، وهو المركب النباتي الطبيعي الغني بالدهون ، قبل وبعد العدوى البكتيرية. أظهرت نتائج الدراسة ، تحفيز البكتيريا تقوية الدفاعات المناعية ، والتي تعتمد على مسارات الإشارات الثلاثة تحت الدراسة. بالإضافة إلى ذلك ، يتم تقليل التعرف على البكتيريا المفيدة بواسطة حامض الياسمونيك والإيثيلين.