Endophytic Actinobacteria Induce Defense Pathways in *Arabidopsis thaliana*

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Endophytic actinobacteria, isolated from healthy wheat tissue, which are capable of suppressing a number of wheat fungal pathogens both in vitro and in planta, were investigated for the ability to activate key genes in the systemic acquired resistance (SAR) or the jasmonate/ethylene (JA/ET) pathways in *Arabidopsis thaliana*. Inoculation of *A. thaliana* (Col-0) with selected endophytic strains induced a low level of SAR and JA/ET gene expression, measured using quantitative polymerase chain reaction. Upon pathogen challenge, endophyte-treated plants demonstrated a higher abundance of defense gene expression compared with the non-endophyte-treated controls. Resistance to the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* required the JA/ET pathway. On the other hand, resistance to the fungal pathogen *Fusarium oxysporum* involved primarily the SAR pathway. The endophytic actinobacteria appear to be able to “prime” both the SAR and JA/ET pathways, upregulating genes in either pathway depending on the infecting pathogen. Culture filtrates of the endophytic actinobacteria were investigated for the ability to also activate defense pathways. The culture filtrate of *Micromonospora* sp. strain EN43 grown in a minimal medium resulted in the induction of the SAR pathway; however, when grown in a complex medium, the JA/ET pathway was activated. Further analysis using *Streptomyces* sp. strain EN27 and defense-compromised mutants of *A. thaliana* indicated that resistance to *E. carotovora* subsp. *carotovora* occurred via an NPR1-independent pathway and required salicylic acid whereas the JA/ET signaling molecules were not essential. In contrast, resistance to *F. oxysporum* mediated by *Streptomyces* sp. strain EN27 occurred via an NPR1-dependent pathway but also required salicylic acid and was JA/ET independent.

Plants have developed a nonspecific mechanism of defense that can provide long-term protection against a broad number of pathogens. Systemic acquired resistance (SAR) is induced by prior inoculation with a necrotizing pathogen or the application of chemical agents such as salicylic acid (SA), 2,6-dichloro isonicotinic acid (INA), and benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Uknes et al. 1992). Once activated, the SAR pathway can confer resistance to a number of pathogens. SAR has been studied extensively in the dicots, tobacco (*Nicotiana tabacum*), and *Arabidopsis thaliana*

and is characterized by an early increase of endogenously synthesized SA and the enhanced production of pathogenesis-related (PR) proteins, specifically PR-1, PR-2, and PR-5 (Uknes et al. 1992). It has been suggested that a mobile signal is produced and thought to move from the site of attack via the phloem to the rest of the plant establishing systemic resistance (Maldonado et al. 2002).

In addition to the SAR pathway, the jasmonic acid/ethylene (JA/ET) pathway functions in an SA-independent manner and results in the production of antimicrobial compounds that confer resistance to a number of pathogens. JA are important plant-signaling molecules involved in a variety of critical functions, including fruit ripening, senescence, tuber formation, tendrils coiling, pollen formation, and defense response against pests and pathogens (Creelman and Mullet 1997). In *Arabidopsis*, JA inhibit root elongation and are required for pollen development and pathogen defense (Ellis and Turner 2001). Like JA, ET (C₂H₄) is an important plant-signaling molecule involved in many plant functions, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Wang et al. 2002). Application of ET, or its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), has been shown to trigger a pathway leading to the upregulation of the defense genes *PDF1.2* and *Hel*. ET production is increased upon exposure to abiotic and biotic stresses, including extreme temperatures, drought, anaerobic conditions, wounding, herbivory, and infection by viral, bacterial, and fungal pathogens (Abeles et al. 1992). JA and ET both are implicated in the activation of genes encoding plant defensins and enzymes involved in phytoalexin biosynthesis (Pieterse et al. 1998). The JA and ET signaling leads to the induction of the PR proteins: PR-3, a basic chitinase; PR-4, a chitin-binding protein; and PDF1.2. PDF1.2 is a member of the group of plant defensins, which is a family of peptides with antimicrobial activities and includes the thionins, lipid transfer proteins, hevein-type, knottin-type, and anti-microbial peptides (van Loon and van Stein 1999). In *Arabidopsis*, the JA/ET pathway induces resistance to the fungal pathogens *Alternaria brassicicola*, *Botrytis cinerea*, and *Fusarium oxysporum* f. sp. *matthiolae* (van Loon and van Stein 1999).

Nonpathogenic rhizobacteria have been shown to induce resistance in plants that is phenotypically similar to SAR. This has been termed induced systemic resistance (ISR) and it is believed that the pathway is distinct from the SAR pathway but is mediated by a JA/ET pathway (Hammerschmidt 1999; Pieterse et al. 2002; van Wees et al. 1999). Pieterse and associates (1998) investigated ISR in *Arabidopsis*, using as the inducing agent the nonpathogenic, root-colonizing *Pseudomonas fluorescens* WCS417r, which triggers ISR in carnation (van Peer et al. 1991), radish (Leeman et al. 1995), tomato (Duijff et al. 1996),

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and *Arabidopsis* (Pieterse et al. 1996). *P. fluorescens* WCS417r ISR is independent of SA accumulation and PR gene activation in *Arabidopsis* and, through investigation of mutants, it was concluded that the mechanisms involved in ISR could be different from those in the SAR and JA/ET pathways (Pieterse et al. 1998). *P. fluorescens* WCS417r confers resistance to *F. oxysporum* f. sp. *raphani*, the oomycetous leaf pathogen *Peronospora parasitica*, and the bacterial leaf pathogens *Xanthomonas campestris* pv. *campestris* and *Pseudomonas syringae* pv. *tomato*, indicating that ISR is effective against different types of pathogens (van Wees et al. 2000). Conrath and associates (2002) suggested that the nonpathogenic bacteria prime the plant for accelerated and enhanced response to a second stress stimulus, such as a pathogen. This theory was further enhanced by Verhagen and associates (2004), whose microarray analysis was used to identify ISR-related genes in *Arabidopsis*. More than 8,000 genes were surveyed and it was found, when using the ISR-inducing bacterium *P. fluorescens* WCS417r, that, although there was a substantial change in the expression of 97 genes in the roots, no changes in expression of any genes could be detected in the leaves. However, after subsequent challenge with *P. syringae* pv. *tomato* DC3000, there was a change in the expression of 81 genes in the leaves, indicating the role of rhizobacteria in priming the plant for ISR.

Endophytic actinobacteria isolated from healthy wheat plants have been shown to enhance disease resistance in wheat when applied as a seed coating. Actinobacteria are recognized as metabolically and morphologically more complex than sessile bacteria. Metabolically, they are prolific producers of an array of secondary metabolites, including antimicrobial agents, plant growth hormones, and siderophores. Morphologically, they produce mycelia and spore structures which will result in a different mode of plant colonization compared with sessile bacteria. These activities can be harnessed by employing endophytic actinobacteria for biocontrol and plant growth promotion. Inoculation of wheat with the endophytic actinobacterium, *Streptomyces* sp. strain EN27, has been shown to promote growth and enhance disease resistance both in vitro and in planta (Coombs 2002). Coombs and associates (2003) further demonstrated that, in steam-sterilized as well as field soils inoculated with *Gaeumannomyces graminis* var. *tritici*, the causative agent of "take-all," there was a significant disease reduction in *Streptomyces* sp. strain EN27-treated wheat plants compared with untreated controls. Actinobacteria are prolific producers of antibiotics which may account for the disease resistance, though it is possible the endophytes are capable of activating a systemic defense response similar to the ISR mediated by rhizobacteria.

The aim of this study was to determine the mechanism by which selected endophytic actinobacteria enhance disease resistance, using *Arabidopsis* as a model plant because its defense pathways have been studied extensively. Quantitative polymerase chain reaction (QPCR) was used to determine the level of gene induction in the SAR (*PR-1* and *PR-5*) and JA/ET (*PDF1.2* and *Hel*) pathways of *Arabidopsis* as a result of endophytic actinobacteria inoculation. The defense response that was recorded via both pathways was dissected further by employing the defense-compromised mutants *NahG* (SA de-

grading), *npr1-1* (nonexpression of PR proteins), *etr1-3* (ET insensitive), and *jar1-1* (JA insensitive).

RESULTS

Induction of gene expression in the SAR and JA/ET pathways using chemical elicitors.

The QPCR first was validated by quantifying the induction of the SAR genes, *PR-1* and *PR-5*, and the JA/ET genes, *PDF1.2* and *Hel*, by the application of the chemical elicitors SA, methyl jasmonate (MeJA), and ACC onto 6- to 8-week-old *Arabidopsis* plants. The application of SA to the *Arabidopsis* plant previously has been shown to trigger the SAR pathway and, as a result, the *PR-1* and *PR-5* transcripts are increased (Uknes et al. 1992) (Table 1). These genes were not induced by MeJA or ACC. As expected, the application JA and ET stimulated the upregulation of the PR genes *Hel* and *PDF1.2* (van Loon and van Stein 1999). The application of SA also induced the *Hel* transcript, but to a lower extent.

The effect of endophytic actinobacteria on key genes in the SAR and JA/ET pathways.

Inoculation of the *Arabidopsis* seed with actinobacteria endophytes was performed to determine whether induction of genes in the SAR and JA/ET pathways occurred in mature plants. Plants were harvested after approximately 7 weeks of growth and the changes in gene expression analyzed by QPCR (Fig. 1). The actinobacteria endophytes are nonpathogenic and all plants appeared healthy at the time of harvest. Though the endophytic actinobacteria used in this study have been isolated from wheat plants, they have a wide host range and have mediated growth promotion in *Arabidopsis*. Inoculation of *Arabidopsis* seed with *Streptomyces* sp. strain EN27 resulted in a

Fig. 1. Fold induction of the *PR-1*, *PR-5*, *PDF1.2*, and *Hel* transcripts in 7-week-old *Arabidopsis thaliana* (Col-0) plants preinoculated with the endophytic actinobacteria *Streptomyces* sp. strain EN27, *Streptomyces* sp. strain EN28, *Micromonospora* sp. strain EN43, and *Nocardioideus albus* EN46, compared with untreated plants.

Table 1. Fold induction of the *PR-1*, *PR-5*, *PDF1.2*, and *Hel* transcripts after treatment with salicylic acid (SA), methyl jasmonate (MeJA), and 1-aminocyclopropane-1-carboxylic acid (ACC) and normalized against actin

Treatment	Time after treatment (h)	<i>PR-1</i>	<i>PR-5</i>	<i>Hel</i>	<i>PDF1.2</i>
SA	6	1,682 ± 876	3.9 ± 1.4	1.6 ± 1.0	1.0 ± 0.2
SA	24	1,468 ± 508	1.1 ± 0.1	4.5 ± 0.2	1.2 ± 0.03
ACC	24	2.0 ± 0.57	0.5 ± 0.15	6.1 ± 2.26	11.1 ± 1.19
MeJA	24	1.4 ± 1.93	0.3 ± 0.07	8.3 ± 0.41	69.1 ± 3.5

19-fold induction of the *PR-1* transcript in 7-week-old plants, whereas the closely related strain, *Streptomyces* sp. strain EN28, was able to induce *PR-1*, *PR-5*, and *PDF1.2* by 4-, 3-, and 23-fold, respectively, compared with the uninoculated control. Inoculation with *Nocardioideis albus* EN46 resulted in induction of *PR-1* and *PR-5*. *Micromonospora* sp. strain EN43 had no effect on *PR-1* and *Hel*, but suppressed the expression of *PR-5* and *PDF1.2* compared with control plants.

The effect of *Erwinia carotovora* subsp. *carotovora* on the induction of SAR and JA/ET pathways genes following inoculation with endophytic actinobacteria.

Arabidopsis (Col-0) plants grown from endophytic actinobacteria-inoculated seed and untreated controls were challenged with the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* after approximately 7 weeks of growth. Three days after infection, the untreated plants showed severe disease symptoms of soft rot and, by day 5, were completely necrotic and had collapsed, whereas all the endophyte-treated plants showed a normal appearance (Fig. 2) and stayed healthy beyond 6 days after pathogen infection.

Analysis of gene expression (Fig. 3) in the untreated (diseased) plants infected with *E. carotovora* subsp. *carotovora* showed that the JA/ET pathway was strongly upregulated, because *PDF1.2* demonstrated the largest induction of gene expression. In all the endophytic actinobacteria-treated (healthy) plants, the *PDF1.2* transcript was induced after the challenge with the *Erwinia* sp., with highest induction present in the *Micromonospora* sp. strain EN43- and *N. albus* EN46-treated plants. Of the endophyte-treated plants, only the *Micromonospora* EN43-treated plants demonstrated an induction of the *Hel* gene at levels above those shown in the untreated control plants.

The *PR-1* transcript was not induced above the level of the non-endophyte-treated infected plant in the *Streptomyces* sp. strain EN27-, EN28-, and *N. albus* EN46-treated infected plants; whereas, in the *Micromonospora* sp. strain EN43-treated plants, *PR-1* was induced at levels 17 times higher than the untreated control plants. The *PR-5* gene was induced at a low level in both untreated and endophyte-treated infected plants.

The effect of *F. oxysporum* on the induction of genes in the SAR and JA/ET pathways after pre-inoculation with endophytic actinobacteria.

Seven-week-old *Arabidopsis* (Col-0) plants grown from endophyte-inoculated seed were challenged with the fungal

pathogen *F. oxysporum*. Three days after this challenge, the non-endophyte-treated plants showed symptoms of disease (yellowing leaves) which could not be seen in any of the endophyte-treated plants (data not shown). Infection of *Arabidopsis* with *F. oxysporum* resulted in the induction of genes (Fig. 3) in both the SAR and JA/ET pathways to some degree. The *PR-1* and *PR-5* transcripts were further induced in the endophyte-treated infected plants, with the highest level of gene expression change detected in the *Streptomyces* sp. strain EN28-treated plants, with an increase of 47.5 times and 12.7 times for the *PR-1* and *PR-5* genes, respectively, compared with the untreated infected plants. The *PDF1.2* transcript was induced in the *Streptomyces* sp. strain EN27-treated infected plants 2.5 times and the *Hel* transcript 2.1 times higher in the *Streptomyces* sp. strain EN28-treated infected plants compared with expression in the untreated infected plants.

The roots and leaves of *Arabidopsis* plants treated with *Streptomyces* sp. strain EN27 and infected with *F. oxysporum* were separated and the gene expression analyzed independently in the roots and leaves (data not shown). The *PR-1* and *PDF1.2* transcripts could be detected only in the leaves, with an 850- and 84-fold induction, respectively, compared with the unprimed, uninoculated control plants. The *PR-5* and *Hel* transcripts were induced in both the roots and leaves, four- and ninefold, respectively. This indicated that the changes in gene expression by the endophytes were indeed systemic, and appear to correlate with the resistant phenotype.

The effect of endophytic actinobacterial culture filtrates on defense gene expression.

Actinobacterial culture filtrates were used to treat *Arabidopsis* (Col-0) plants and the changes in gene expression were analyzed by QPCR (Fig. 4). Two different media were used to culture the actinobacteria: a minimal (FL026) and a complex (FL031) medium. The application of the culture filtrates from the *Streptomyces* sp. strains EN27 and EN28 cultures did not result in any significant gene induction compared with untreated control plants. When the FL026 culture filtrate of *Micromonospora* sp. strain EN43 was applied, the *PR-1* and *PR-5* transcripts were induced 9- and 16-fold, respectively, indicating activation of the SAR pathway. However, when the culture filtrate of the same strain grown in the complex medium FL031 was applied, the JA/ET pathway was more strongly induced: the *PDF1.2* transcript 24-fold and the *Hel* transcript 8-fold. The metabolites of *N. albus*

Fig. 2. Wild-type *Arabidopsis thaliana* Col-0 infected with *Erwinia carotovora* subsp. *carotovora*. **A**, Wild-type *Arabidopsis* 3 days after inoculation with *E. carotovora* subsp. *carotovora* at 3×10^8 CFU ml⁻¹. **B**, Wild-type *Arabidopsis* preinoculated with *Streptomyces* sp. strain EN27 at 1×10^8 CFU ml⁻¹ 3 days after inoculation with *E. carotovora* subsp. *carotovora* at 3×10^8 CFU ml⁻¹.

EN46 induced the *PDF1.2* transcript eightfold only when cultured in the complex medium.

Expression of defense genes in *Streptomyces* sp. strain EN27-inoculated defense-compromised mutants after challenge with *E. carotovora* subsp. *carotovora*.

Defense-compromised mutants of *Arabidopsis* were employed to determine the role of the specific defense pathways involved in actinobacterial endophyte-mediated systemic defense. Inoculation of the SA-degrading lines of *Arabidopsis*, *NahG*, with *E. carotovora* subsp. *carotovora* resulted in severe disease symptoms within the first 24 h and, by day three, the plants had started to collapse (Fig. 5A); all plants collapsed completely after 5 days. There was no enhanced disease resistance in the *Streptomyces* sp. strain EN27-inoculated *NahG* plants, whereas the Col-0 plants treated with *Streptomyces* sp. strain EN27 showed enhanced resistance (Fig. 2), with endophyte-treated plants remaining disease free and healthy after 6 days of pathogen treatment. No significant level of defense gene induction could be detected in the *Streptomyces* sp. strain EN27-inoculated infected *NahG* plant (data not shown). In the untreated infected *NahG*, there was a low level of *PR-1* induction but no *PR-5* transcript could be detected. However, there was a significant induction of *PDF1.2* and *Hel* (data not shown).

The *npr1-1* mutant is compromised in *PR* gene expression and application of 5 mM SA was not able to induce *PR-1* or *PR-5* gene expression (data not shown). Inoculation of the *npr1-1* with *E. carotovora* subsp. *carotovora* resulted in disease symptoms comparable with infection in the Col-0 plant, whereas the *Streptomyces* sp. strain EN27-inoculated mutant plants demonstrated an enhanced resistance, though not to the extent seen in the wild-type plant (Fig. 5B). Comparative QPCR analysis revealed that the *Streptomyces* sp. strain EN27-treated *npr1-1* plants had a decreased level of *PR-1* transcript compared with the untreated infected *npr1-1* plant (Fig. 6A), and that the JA/ET pathway genes were induced more strongly.

Application of the chemical elicitors MeJA and ACC to the *jar1-1* and *etr1-3* mutants confirmed that they were defective in their perception to JA and ET, respectively (data not shown). Inoculation of the *jar1-1* and *etr1-3* plants with *E. carotovora* subsp. *carotovora* resulted in disease symptoms comparable with infection in the wild-type plants. However, when seed were treated with *Streptomyces* EN27, the *jar1-1* and *etr1-3* plants showed enhanced resistance (Fig. 5C and D). The JA/ET pathway genes were significantly induced in the *jar1-1* and *etr1-3* mutants after challenge with the *Erwinia* sp., with a stronger induction of the *PDF1.2* transcript than the *Hel* transcript (Fig. 6A).

Even though the *Streptomyces* sp. strain EN27-treated JA and ET mutant plants were resistant to *Erwinia* infection, the level of gene expression of *PDF1.2* and *Hel* was lower than in the primed *jar1-1* and *etr1-3* mutants, though *PR1* generally was higher.

Expression of defense genes in *Streptomyces* sp. strain EN27-inoculated defense-compromised mutants after challenge with *F. oxysporum*.

F. oxysporum infection of *NahG* and the *npr1-1* mutant resulted in little expression of defense genes in both the SAR and JA/ET pathways and disease symptoms appeared earlier than in the infected wild type (Col-0). The same result was observed in EN27-treated mutants infected with *F. oxysporum*. QPCR revealed minimal expression of the *PR-1* and *PR-5* transcripts in both the uninfected untreated and endophyte-treated *NahG* and *npr1-1* mutants compared with infected Col-0 (Fig. 6B).

F. oxysporum infection of the *jar1-1* and *etr1-3* plants resulted in a considerably different gene expression profile compared with the SAR mutants (Fig. 6B). Most noticeably, the *PR-1* transcript was significantly induced in the *jar1-1* and *etr1-3* mutants (380- and 468-fold, respectively) and was further enhanced in the *Streptomyces* sp. strain EN27-treated plants (1,188- and 663-fold, respectively). The *PDF1.2* and *Hel* transcripts were significantly induced in the untreated *jar1-1* and *etr1-3* plants; however, this level of induction was reduced when the plants were inoculated with *Streptomyces* sp. strain EN27.

DISCUSSION

Nonpathogenic rhizobacteria have been shown to enhance disease resistance by stimulating the systemic defense pathways (Hammerschmidt 1999). In this study, inoculation of *Arabidopsis* with selected endophytic actinobacteria also was found to stimulate the plant defense pathways, though the elicitation profile of the genes involved was different from that previously

Fig. 3. Fold induction of the *PR-1*, *PR-5*, *PDF1.2*, and *Hel* transcripts in 7-week-old *Arabidopsis thaliana* (Col-0) plants preinoculated with the endophytic actinobacteria *Streptomyces* sp. strains EN27 and EN28, *Micromonospora* sp. strain EN43, and *Nocardioideus albus* EN46 and 3 days postchallenge with **A**, *Erwinia carotovora* subsp. *carotovora* and **B**, *Fusarium oxysporum*, compared with untreated plants; * represents a fold induction of 2,138.

reported using other plant-growth-promoting bacteria or bio-control bacterial strains. The SAR pathway in *Arabidopsis* normally is activated by biotrophic pathogens either as a part of the hypersensitive response or as a symptom of disease and the JA/ET pathway is triggered by infection with necrotrophic pathogens (Dong and Durrant 2004; Glazebrook 2005; Thaler et al. 2004). Inoculation with *Streptomyces* sp. strain EN27 resulted in activation of the SAR pathway via induction of the *PR-1* gene, whereas *Streptomyces* sp. strain EN28 primarily triggered the JA/ET pathway because the *PDF1.2* gene was expressed to a higher degree than the *PR-1* and *PR-5* transcripts. The *Streptomyces* sp. strains EN27 and EN28 are highly similar to each other morphologically and by 16S rRNA gene sequence homology; however, it is well known that different strains of the same actinobacterial species can produce a different range of secondary metabolites (Hopwood 2003). The *N. albus* EN46 strain appeared to activate the SAR pathway, whereas the *Micromonospora* sp. strain EN43 was unable to upregulate the plant defense genes examined. In contrast to the *Streptomyces* and *Nocardioides* strains, the well-studied nonpathogenic rhizobacteria *P. fluorescens* WCS417r (van Wees et al. 1999) and *P. fluorescens* LSW17S (Ahn et al. 2007) do not induce SA- or JA-responsive genes by inoculation with the rhizobacterium alone. The selected endophytic actinobacteria used in this study activated the plant defense genes in the absence of a pathogen, suggesting that the actinobacteria are detected as “minor” pathogens which do not trigger a full resistance response on their own, because they do not have pathogenic determinants, and this may result in more effective priming of the defense response, as was seen in this study.

Systemic induction of the SAR and JA/ET genes is thought to occur via a lipid-derived signal that can be transported through the phloem (Maldonado et al. 2002). Because actinobacteria are prolific producers of secondary metabolites, including antifungal agents (Nolan and Cross 1988), it was necessary to determine whether the elicitation of the defense pathways could be mediated by a secreted secondary metabolite or excreted cell factor. Induction of SAR and JA/ET genes was detected only with the *Micromonospora* sp. strain EN43 and *N.*

albus EN46 culture filtrates. Interestingly, the culture filtrate of EN43 grown in a minimal medium resulted in the induction of the SAR pathway but, when grown in a complex medium, the JA/ET pathway was activated. This suggests that a different suite of (secondary) metabolites produced in each medium is responsible for the activation of the defense pathways.

Previously, it was shown by Ryu and associates (2004) that volatile organic compounds from *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a were able to induce ISR in *Arabidopsis* and reduce the disease severity of *E. carotovora* subsp. *carotovora*. Isolating the actinobacteria endophyte compound or compounds responsible for the activation of the defense genes could lead to the identification of a biochemical biological control agent. However, it may be possible that the physical interaction of the endophyte and host plant is needed for the full range of defense responses to be activated.

The priming response was displayed in *Arabidopsis* plants inoculated with endophytic actinobacteria at planting when challenged after 7 weeks with pathogens. Infection of *Arabidopsis* with the nonspecific bacterial plant pathogen *E. carotovora* subsp. *carotovora*, which causes soft-rot disease in a wide range of hosts (Aguilar et al. 2002; Norman et al. 1999), has been shown to induce the local and systemic expression of genes that are mainly responsive to ET or JA (e.g., *Hel*, *ChiB*, *PDF1.2*, and *Atvsp*) (Norman-Setterblad et al. 2000). It was confirmed in this study by QPCR that strong induction of the *PDF1.2* and *Hel* transcripts occurred in the Col-0 plants infected with this pathogen. Inoculation with all four actinobacteria endophytes resulted in an enhanced level of resistance because the plants appeared healthy 3 days after challenge compared with the untreated plants, which exhibited disease symptoms. This resistance correlated with induction of *PDF1.2* transcript above the level detected in the untreated infected control. *Micromonospora* sp. strain EN43 was the only endophyte that did not induce the SAR or JA/ET pathway genes in the absence of a pathogen; however, it also carried the capacity to prime the SAR and JA/ET pathways, enabling a stronger defense response upon pathogen invasion, which correlated with a resistant phenotype. In this regard, *Micromonospora* sp. strain EN43 appears

Fig. 4. Effect of culture filtrates of actinobacteria endophytes *Streptomyces* sp. strain EN27 and EN28, *Micromonospora* sp. strain EN43, and *Nocardioides albus* EN46, grown in two different media, FL026 and FL031, on genes in the systemic acquired resistance and jasmonic acid/ethylene pathways in 7-week-old *Arabidopsis thaliana* (Col-0), 24 h posttreatment.

to be able to induce resistance in a manner similar to *P. fluorescens* WC417r or LSW17S, where the treatment with these nonpathogenic root-colonizing bacteria alone do not elicit a detectable defense response. However, upon challenge with a pathogen such as *P. syringae* pv. *tomato* DC3000, the *P. fluorescens* WC417r-treated plants displayed a potentiated defense response compared with that detected by pathogen alone. It was concluded that *P. fluorescens* WC417r was priming the plant defense pathways—where inoculation with this bacterium alone does not induce the plant defense response but, when attacked by a pathogen, the defense response is mounted quickly, inducing systemic resistance (Conrath et al. 2002; Verhagen et al. 2004).

Resistance to *E. carotovora* subsp. *carotovora*, in terms of both plant survival and defense gene expression, mediated by *Streptomyces* spp. EN27 and EN28 and *N. albus* EN46, appears to occur via the JA/ET pathway, whereas *Micromonospora* EN43-mediated resistance correlates with increased expression of genes in both the JA/ET and SAR pathways. The SAR and ISR pathways have been shown to be additive by van Wees and associates (2000), who demonstrated, in *Arabidopsis*, that

the SAR and ISR pathways singly resulted in 40 to 60% reduction, respectively, in disease symptoms of *P. syringae* pv. *tomato*. When ISR and SAR genes were expressed simultaneously, disease reduction was enhanced up to 80%. Therefore, because *Micromonospora* sp. strain EN43 has the ability to activate both the SAR and JA/ET pathways, it may prove to be a broad-ranging biocontrol agent.

Induced resistance to the soilborne fungus *F. oxysporum*, mediated by the endophytic actinobacteria, occurred primarily by the SAR pathway. The *PR-1* and *PR-5* genes both were expressed at a higher level in the plants inoculated with the endophytic actinobacteria. Previously, it has been shown that infection of *Arabidopsis* with *F. oxysporum* induces the defense-related gene, thionin (*Thi2.1*), and overexpression of this gene results in resistance (Eppe et al. 1997). Berrocal-Lobo and Molina (2004) found induction of the *PR-1* transcript 4 days after infection and, by 8 days, both *PR-1* and *PDF1.2* were induced, demonstrating activation of both the SAR and JA/ET pathways by *F. oxysporum*. The endophytic actinobacteria, though, can upregulate both the SAR pathway and JA/ET pathway defense genes and, when both pathways are in opera-

Fig. 5. Defense-compromised *Arabidopsis thaliana* plants. **A**, *NahG* mutants; **B**, *npr1* mutants; **C**, *etr1-3* mutants; **D**, *jar1-1* mutants; column 1, 7-week-old untreated plants; column 2, 7-week-old plants 3 days after inoculation with *Erwinia carotovora* subsp. *carotovora* at 3×10^8 CFU ml⁻¹; column 3, 7-week-old plants grown from seed inoculated with *Streptomyces* sp. strain EN27 at 1×10^8 CFU ml⁻¹; column 4, 7-week-old plants grown from seed inoculated with *Streptomyces* sp. strain EN27 at 1×10^8 CFU ml⁻¹ and 3 days after inoculation with *E. carotovora* subsp. *carotovora* at 3×10^8 CFU ml⁻¹.

tion, a stronger protection may be manifested. In contrast, defense responses by *P. fluorescens* WC417r or LSW17S and *Bacillus* spp. is SA independent and associated with JA and ET (Kloepper et al. 2004; Pieterse et al. 1996, 1998).

The defense pathways activated by the endophytic actinobacterium *Streptomyces* sp. strain EN27 were further dissected using *Arabidopsis* defense-compromised mutants. In this study, it was found that the *Streptomyces* sp. strain EN27-mediated resistance to *E. carotovora* subsp. *carotovora* required SA because the *NahG* mutants were unable to mount a defense response upon infection. This response is opposite to that obtained with *P. fluorescens* LSW17S, which was able to reduce pathogen proliferation with this *NahG* mutant (Ahn et al. 2007). *NahG* mutants are unable to accumulate SA, because the *NahG* transgene salicylate hydroxylase converts SA to the inactive form catechol and, therefore, is unable to induce *PR-1* (Lawton et al. 1995). However, because *NahG* mutants have manifested pleiotropic effects, these results need to be con-

firmed using SA-deficient *sid2* mutants (Heck et al. 2003; van Wees and Glazebrook 2003).

Although SA is essential for *Streptomyces* sp. strain EN27-mediated resistance to the pathogen, it occurred via an NPR1-independent pathway. *Streptomyces* sp. strain EN27-treated *npr1-1* mutants infected with *E. carotovora* subsp. *carotovora* demonstrated *PR-1*, *PDF1.2*, and *Hel* induction correlating with resistance because the plants showed little symptom of disease, indicating that an NPR1-independent pathway must have been functioning. Shah and associates (1999) identified the dominant mutation *ssi1* (*suppressor of SA immunity*), where *PR-1*, *PR-2*, and *PR-5* were expressed constitutively and the requirement for NPR1 was bypassed. SSI1 is thought to function as a switch, modulating cross-talk between the SA- and JA/ET-mediated defense pathways. Evidence for SA-dependent but NPR1-independent pathways for regulation of *PR-1* gene expression and resistance to bacterial pathogens has been reported previously (Bowling et al. 1997; Clarke et al.

Fig. 6. Induction of systemic acquired resistance (*PR-1* and *PR-5*) and jasmonic acid/ethylene pathway genes (*PDF1.2* and *Hel*) 3 days after challenge with **A**, *Erwinia carotovora* subsp. *carotovora* or **B**, *Fusarium oxysporum*. Wild-type (Col-0), and defense-compromised plants *NahG*, *npr1-1*, *etr1-3*, and *jar1* of *Arabidopsis* were either untreated or treated at sowing with *Streptomyces* sp. strain EN27 (+EN27).

1998; Devadas et al. 2002; Rairdan and Delaney 2002; Rate et al. 1999) and we conclude that a similar mode of resistance is operational with the endophytic actinobacteria.

Norman-Setterblad and associates (2000) found that resistance to *E. carotovora* subsp. *carotovora* occurred via two types of defense pathways, one JA-dependent and inhibited by SA and the other ET- and JA-dependent but potentiated by SA. Infection with *E. carotovora* subsp. *carotovora* was shown to trigger the JA/ET pathway in wild-type (Col-0) plants and the *PDF1.2* transcript was further upregulated upon *Streptomyces* sp. strain EN27 treatment. Therefore, it was anticipated that resistance mediated by *Streptomyces* sp. strain EN27 would be abolished in plants defective in JA and ET signaling. However, analysis of the gene expression in *E. carotovora* subsp. *carotovora*-infected *jar1-1* and *etr1-3* plants revealed that *PDF1.2* still could be expressed, similar to the findings of Penninckx and associates (1996) with the *etr1-3* mutant upon infection with *Alternaria brassicicola*. When the JA and ET mutants were treated with *Streptomyces* sp. strain EN27, resistance to *E. carotovora* subsp. *carotovora* was observed but the expression of *PDF1.2* and *Hel* were significantly reduced. Instead, the SAR pathway gene *PR-1* expression was increased compared with the untreated plants. It is possible that JA/ET mutants inoculated with *Streptomyces* sp. strain EN27 are able to detect that the JA/ET pathway is defective and develop resistance by triggering the SAR pathway. Our findings suggest that resistance to *E. carotovora* subsp. *carotovora* mediated by *Streptomyces* sp. strain EN27 occurs via an NPR1-independent pathway that requires SA, but not JA and ET.

Infection of the two SAR mutants, *NahG* and *npr1*, with the hemibiotroph *F. oxysporum* revealed minimal SAR and JA/ET gene expression, indicating that SA and the NPR1 protein are essential for the development of a resistant phenotype. Analysis of the JA- and ET-insensitive mutants infected with *F. oxysporum* revealed that JA/ET signaling is not correlated with the ability to mount a defense response. In fact, when the JA/ET mutants were inoculated with *Streptomyces* sp. strain EN27 and infected with either pathogen, there was a clear shift to the SAR pathway and *PR-1* expression was significantly enhanced. It appears that treatment with *Streptomyces* sp. strain EN27 enabled detection of the defective JA/ET pathway and switching to the functioning SAR pathway. We conclude that, in contrast to the bacterial pathogen, resistance to the fungus *F. oxysporum*, mediated by *Streptomyces* sp. strain EN27, occurred via an NPR1-dependent pathway but also required SA and was JA/ET independent.

It is recognized that there is not always a strong correlation between expression of defense-related genes and disease resistance in ISR (Ahn et al. 2007), and that other defense-related mechanisms such as callose deposition, accumulation of active oxygen species, and papillae formation also may be activated (Verhagen et al. 2004; Wang et al. 2005). Of interest, the endophytic streptomycete used in this study is able to offer protection to the *npr1*, *etr1-3*, and *jar1-1* plants but not *NahG*,

whereas *P. fluorescens* LSW17S was able to protect only *NahG* plants (Ahn et al. 2007). Subsequently, priming with *P. fluorescens* LSW17S was able to elicit expression of *PDF1.2* in *NahG* plants only, whereas this gene was expressed in all the other mutants primed with *Streptomyces* sp. strain EN27.

Inoculation of wild-type *Arabidopsis thaliana* with selected strains of endophytic actinobacteria was able to prime the defense pathways by inducing low-level expression of SAR and JA/ET genes. Upon pathogen infection, the defense genes are strongly upregulated and the endophyte-treated plants were demonstrated to have a higher abundance of the defense gene transcripts. This ability to prime the plant for an augmented response to pathogen attack is expected to confer a fitness advantage (van Hulten et al. 2006) to the endophyte-treated plants. This capacity to induce disease resistance in a versatile manner is expected to work in concert with their proven antibiotic effects and their colonization efficiency, making these endophytes excellent candidates for effective biocontrol agents.

MATERIALS AND METHODS

Endophytic actinobacteria cultures.

The endophytic actinobacteria cultures EN27 (*Streptomyces* sp. strain AY148075), EN28 (*Streptomyces* sp. strain AY148076), EN43 (*Micromonospora* sp. strain AY291589), and EN46 (*N. albus* AY148081) were maintained on half-strength potato dextrose ($1/2$ PDA) agar and EN2 (*Microbispora* sp. strain AY148073) on oatmeal agar. All the actinobacteria used in this study were isolated previously from surface-sterilized healthy wheat roots, as described previously (Coombs and Franco 2003b).

Plant pathogens.

E. carotovora subsp. *carotovora*, originally isolated from leek, was grown on $1/2$ PDA at 27°C for 3 to 4 days. A suspension of the bacterial cells was prepared in sterile saline (0.9% NaCl) to a final concentration of 1×10^8 CFU ml⁻¹. *F. oxysporum*, originally isolated from cucumber, was maintained on $1/2$ PDA. A plug of the culture was used to inoculate 50 ml of Sabouraud broth and incubated at 27°C for 3 days at 150 rpm. The *F. oxysporum* culture was adjusted to 1×10^8 CFU ml⁻¹. The *E. carotovora* subsp. *carotovora* and *F. oxysporum* cultures were kindly provided by the Horticultural Pathology and Nematology Group at the South Australian Research and Development Institute, Adelaide, Australia.

Plant cultivation.

Arabidopsis thaliana (ecotype Columbia-0 and mutants *npr1-1*, *jar1-1*, *etr1-3*, and *NahG*) plants were grown on half-strength Murashige and Skoog ($1/2$ MS) salt medium (Sigma-Aldrich, Sydney, Australia), with 0.8% Bacto-agar (Bacto Laboratories, Liverpool, Australia) as the gelling agent when the plants were grown on plates with a horizontal orientation or 0.8% phytagel agar (Sigma-Aldrich) if grown on plates with a

Table 2. Amplification efficiency and sequence of primers used for quantitative polymerase chain reaction

Target gene	Accession no.	Primer sequence	Amplification efficiency ^a
<i>Hel</i>	NM_111344	Forward: 5' CAA GTG TTT AAG GGT GAA GA 3' Reverse: 5' CGG TGT CTA TTT GAT TGA AC 3'	1.89
<i>PDF1.2</i>	AY133787	Forward: 5' CTG CTC TTG TTC TCT TTG CT 3' Reverse: 5' GTG TGC TGG GAA GAC ATA 3'	1.78
<i>PR-1</i>	AY117187	Forward: 5' GCC TTA CGG GGA AAA CTT A 3' Reverse: 5' CTT TGG CAC ATC CGA GTC T 3'	1.64
<i>PR-5</i>	AY059114	Forward: 5' CGG AAA CGG TAG ATG TGT AAC 3' Reverse: 5' GTT GAG GTC AGA GAC ACA GCC 3'	1.69

^a The amplification efficiency for *Actin* is 1.61.

vertical orientation. Growth on agar was selected because sterile conditions can be maintained which enabled the effects from only the experimental microorganism, in this case actinobacteria, to be studied and interference from contamination microorganisms to be ruled out. This is especially critical for the study of ISR. The plants appeared healthy on agar and did not represent a stressed condition. In all cases, untreated plants were used as controls to provide a direct comparison of expression levels. Seed were surfaced sterilized by immersing in 70% (vol/vol) ethanol for 1 min followed by 1 ml of 12% sodium hypochlorite (as fresh Domestos bleach) for 10 min. The seed then were rinsed twice in sterile 2% (wt/vol) sodium thiosulfate to remove the residual chlorine (Miche and Balandreau 2001) and a minimum of 5 washes with sterile H₂O to remove all traces of sodium hypochlorite. Seed were placed onto 1/2 MS salt medium and the plates were sealed with micropore tape and placed at 4°C overnight to achieve even germination. Plants then were transferred to a light box with a 9-h light cycle, 2,000 lux, with the temperature of 20 ± 4°C. *Arabidopsis* mutant seed *npr1-1* (CS3726) (Cao et al. 1994), *jar1-1* (Salk_011510) (Alonso et al. 2003), and *etr1-3* (CS3070) (Guzman and Ecker 1990) were obtained from the *Arabidopsis* Biological Resource Centre. The *NahG* (Lawton et al. 1995) B15 seed were kindly provided by Novartis, Research Triangle Park, North Carolina, U.S.A.

Chemical treatment and endophytic actinobacterial inoculation of plants.

Six- to eight-week-old Col-0, *npr1-1*, *jar1-1*, *etr1-3*, and *NahG* plants were sprayed with 5 mM SA, 50 µM MeJA, or 0.01% (wt/vol) ACC and whole plants were harvested at 6 and 24 h. Surface-sterilized Col-0 seed were inoculated with endophytic actinobacterial suspensions containing 10⁸ spores, or H₂O for control plants, and left to dry in the laminar flow cabinet. The seed was inoculated to ensure that the endophyte had maximum opportunity to colonize the plant as the seed germinated, as demonstrated in previous studies by Coombs and Franco (2003a).

Inoculation of *Arabidopsis* (Col-0) with endophytic actinobacteria culture filtrates.

Endophytic actinobacterial culture filtrates were prepared by inoculating 50 ml of inoculum medium (glucose at 15 g liter⁻¹, soybean meal at 15 g liter⁻¹, corn steep liquor at 5 g liter⁻¹, NaCl at 5 g liter⁻¹, and CaCO₃ at 2 g liter⁻¹; pH 7.2) with a loop of the actinobacteria spores. The inoculum medium was grown at 27°C for 3 days with shaking at 150 rpm. The well-grown inoculum culture (5%, vol/vol) then was used to inoculate 250-ml Erlenmeyer flasks containing 50 ml of FLO26 medium (glucose at 20 g liter⁻¹, soybean meal at 10 g liter⁻¹, CaCO₃ at 4 g liter⁻¹, and CoCl₂ · 6H₂O at 1 mg liter⁻¹; pH 7.2) or FLO31 medium (glycerol at 15 g liter⁻¹, glucose at 5 g liter⁻¹, Pharmamedia at 20 g liter⁻¹, yeast extract at 5 g liter⁻¹, KH₂PO₄ at 1 g liter⁻¹, MgSO₄ · 7H₂O at 0.5 g liter⁻¹, CaCO₃ at 2 g liter⁻¹, CuSO₄ · 5H₂O at 7 mg liter⁻¹, FeSO₄ · 7H₂O at 1 mg liter⁻¹, MnCl₂ · 4H₂O at 8 mg liter⁻¹, and ZnSO₄ · 7H₂O at 2 mg liter⁻¹; pH 7.2). After 8 days at 27°C with shaking at 150 rpm, the cultures were transferred to sterile 50-ml falcon tubes and centrifuged at 4,000 × g for 5 min. The culture broth was removed and filtered through a 0.8-µm Millipore filter to remove bacterial cells. The filtrate was used to treat the roots of 6- to 8-week-old Col-0 plants.

Arabidopsis pathogen challenge.

Six- to eight-week-old endophyte-inoculated and untreated Col-0, *npr1-1*, *jar1-1*, *etr1-3*, and *NahG* plants were challenged separately with *F. oxysporum* and *E. carotovora* subsp. *caroto-*

vora. *F. oxysporum*, as a 1 × 10⁸ CFU ml⁻¹ suspension, was applied directly to the roots of the plants. *E. carotovora* subsp. *carotovora*, as a 1 × 10⁸ CFU ml⁻¹ suspension, was applied by pressure infiltration to all of the leaves using a needleless syringe. Sterile saline was applied to control plants. The whole plants were harvested 3 days postchallenge, which was chosen on the basis of a time course of the disease progression and expression of defense genes for *E. carotovora* subsp. *carotovora* (Aguilar et al. 2002) and *F. oxysporum* (Epple et al. 1995). Plant material was harvested by carefully removing the plants from the medium and immediately freezing in liquid nitrogen.

Analysis of gene expression by QPCR.

Total RNA was extracted from 100 to 200 mg of snap-frozen *Arabidopsis* tissue with Trizol (Invitrogen, Sydney, Australia). Contaminating DNA was removed from RNA preparations using RQ-1 RNase-free DNase-1 (Promega, Annandale, Australia) or Turbo DNA-free (Ambion, Cambridge, U.K.). RNA was converted to cDNA using Omniscript reverse transcriptase (Qiagen, Valencia, CA, U.S.A.) with 1 µg of RNA pooled from nine plants and Oligo dT primers (50 ng µl⁻¹). The cDNA was diluted 1:4 with sterile H₂O and 2 µl of cDNA used per 10-µl QPCR reaction. The housekeeping gene Actin was used to normalize the experimental genes. Primers designed by An and associates (1996) and Charrier and associates (2002) were used to amplify *Actin2* and *Actin8* genes simultaneously. QPCR primers for the *Hel*, *PDF1.2*, *PR-1*, and *PR-5* genes were prepared by Geneworks (Adelaide, Australia) and the primer sequences designed in this study are presented in Table 2. PCR products using the primers were sequenced to confirm correct amplification of the target gene. For all samples, duplicate reactions with a final volume of 10 µl consisted of: 10 ng of each primer, 200 µM dNTPs, 1.5 mM MgCl₂, 0.4× SYBR Green-1 (Molecular Probes, Eugene, OR, U.S.A.), 1× Qiagen PCR Buffer, 0.25 units of Hot Star Taq (Qiagen), and 2 µl of cDNA per reaction. Amplification of *Actin*, *Hel*, *PDF1.2*, and *PR-5* products were performed in the Rotorgene 2000 (Corbett Research, Sydney, Australia) with the following thermal cycle profile: 95°C for 15 min followed by 35 cycles of 94°C for 20 s, 54°C for 20 s, and 72°C for 20 s. For amplification of *PR-1*, the following thermal cycle profile was used: 95°C for 15 min followed by 40 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 20 s. Products then were melted by heating from 65 to 92°C over 5 min. Data was acquired on the SYBR-green channel with the gain set at 7 and, for both quantitation and melt analysis, a "light" digital filter was utilized to smooth raw fluorescence readings. Melt-curve analysis was carried out with the dF/dT threshold set above the fluorescence background at a value of 1 for detection of single melt products. The amplification efficiency of each primer set was determined using serial dilutions of purified PCR products. PCR products for each gene were purified with the UltraClean PCR Purification kit (MoBio, Carlsbad, CA, U.S.A.). Serial 10-fold dilutions of the purified PCR products were made and 2 µl used as the template in the QPCR. A standard curve was generated using the Rotorgene 2000 software based on the least-squares linear regression method with the cycle threshold (Ct) values plotted against the log₁₀ (copy number). The slope of this standard curve was used to determine amplification efficiency (*E*) using the calculation $E = 10(-1/\text{slope})$. Standard deviations and coefficient of variance were determined for each run and found to be less than 2% intra-run and 1 to 10% inter-run.

Relative quantification of gene expression (QPCR).

Ct values were obtained from raw fluorescence data using quantitation analysis options in the Rotorgene 2000 software

(Corbett Research, Sydney, Australia). The fluorescence threshold was set at 0.03, dynamic tube normalization was applied to correct for background fluorescence in raw data, and the first five cycles were ignored. The Ct values were used to calculate the fold induction of the target gene in each sample. The comparative Ct or $\Delta\Delta$ -Ct equation developed by Pfaffl (2001) was used to calculate relative gene expression, normalized against the expression of the housekeeping gene transcript *Actin* (Livak and Schmittgen 2001). The reverse-transcription PCR results are presented as means of duplicate QPCR where the RNA was pooled from nine plants. Two independent experiments were carried out to validate the results.

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