3 Bacterial Endophytes as Elicitors of Induced Systemic Resistance

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3.1 Introduction and Terminology

As indicated elsewhere in this book (e.g. Chap. 1 by Schulz and Boyle), the question of what are endophytes can be answered in different ways. For the purposes of this chapter, only those endophytes that could be isolated from surface-sterilized plant tissue or extracted from within the plant, as proposed by Hallmann et al. (1997), will be discussed. All of the rhizobacteria discussed here were isolated by grinding tissues of surface-sterilized plants, while maintaining sterility controls. It was subsequently discovered that some of these bacterial strains elicited systemic protection against pathogens when the bacteria were inoculated onto seeds or into the potting mix.

Application to crops of many plant-associated bacteria, including some endophytic bacteria, results in a reduction in the incidence or severity of diseases. This phenomenon is referred to as biological control. The most commonly reported mechanism of biological control is antagonism, where the bacterium causes a reduction in the pathogen population or its disease-producing potential. Antagonism includes the more specific mechanisms of predation, competition, and antibiosis. Antagonism is discussed in detail in Chap. 4 by Berg and Hallmann.

An alternative mechanism for biological control is that bacterial metabolites affect the plant in such a way as to increase the plant's resistance to pathogens, a process termed induced systemic resistance (ISR). Resistance can also be elicited in plants by the application of chemicals or necrosis-producing pathogens, and this process is termed systemic acquired resistance (SAR). Pieterse et al. (1998) proposed that ISR and SAR can be differentiated not only by the elicitor but also by the signal transduction pathways that are elicited within the plant. Accordingly, ISR is elicited by rhizobac-

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teria or other nonpathogenic microorganisms, while SAR is elicited by pathogens or chemical compounds. Further, the signal transduction pathway of ISR is independent of salicylic acid but dependent on jasmonate and ethylene, while the pathway of SAR is dependent on salicylic acid and shows variable dependency on jasmonate and ethylene. Recent discoveries show that some rhizobacteria, including some of the endophytic strains discussed in this chapter, elicit systemic protection that may be dependent on salicylic acid and independent of jasmonate or ethylene. Hence, ISR cannot be separated from SAR based on signal transduction pathways. In this chapter, ISR is used to describe the phenomenon whereby application of bacteria to one part of the plant results in a significant reduction in the severity or incidence of a disease following inoculation of a pathogen to another part of the plant.

3.2 Scope of Endophytes that Elicit Induced Resistance and Pathosystems Affected

The first indication that endophytic bacteria could elicit ISR dates to 1991 (Wei et al. 1991). *Pseudomonas fluorescens* strain G8-4, which was later designated 89B-61 and found to colonize plants internally, elicited systemic protection against cucumber anthracnose following application to cucumber seeds.

In efforts to find other strains of endophytic bacteria that elicited ISR, the research group at Auburn University performed isolations from cucumber plants in the field or from cucumber seeds. *Bacillus pumilus* strain INR7 was isolated from a surface-sterilized stem of a surviving cucumber plant in a field heavily infested with cucurbit wilt disease, caused by *Erwinia tracheiphila*. In two field trials, treatment with INR7 resulted in significant growth promotion relative to the nontreated control (Wei et al. 1996). In addition, the severity of angular leaf spot, following inoculation with *Pseudomonas syringae* pv. lachrymans, and the severity of naturally occurring anthracnose were significantly reduced by INR7. The cumulative yield of marketable cucumber fruit was also significantly enhanced by INR7 in both field trials. In the same study, strain 89B-61 also increased plant growth and yield and reduced the incidence of both angular leaf spot and anthracnose. In a subsequent field trial, INR7 reduced the severity of cucurbit wilt (Zehnder et al. 2001).

Erwinia tracheiphila is completely dependent on the striped cucumber beetle and the spotted cucumber beetles for survival and transmission. The finding that cucumber treated with strain INR7 exhibited reduced severity of cucurbit wilt in the field led to investigations aimed at determining if

ISR changed beetle feeding activity. In a 2-year field study, the number of beetles feeding on cucumber was significantly reduced following treatment with strain INR7 (Zehnder et al. 1997b). In both years of the study, the season-long average number of cucumber beetles per plant was significantly lower on plants treated with INR7 than on nonbacterized plants. Reduced beetle feeding on plants treated with strain INR7 was confirmed in subsequent greenhouse studies (Zehnder et al. 1997a). Beetle preference for nontreated plants was evident within the first 24 h of releasing the beetles into cages containing cucumber plants. After feeding for 17 days, beetle damage remained significantly lower on cotyledons and stems of plants treated with INR7 than on nontreated plants.

Elicitation of altered beetle behavior and feeding preferences in the field and greenhouse following seed treatment with INR7 was unexpected. As summarized by Zehnder et al. (1997a), cucumber beetle feeding behavior is influenced by a group of secondary plant metabolites called cucurbitacins, which are bitter compounds toxic to most insects. Cucumber beetles consume cucurbitacins without toxicity, apparently as an evolutionary adaptation that protects the cucumber beetles from predation. The beetles seek out cucurbitacins, and concentrations of 1 ng cause cucumber beetles to demonstrate arrested feeding behavior, whereby the beetles feed intensely on a single plant without moving from plant to plant. Hence, as an explanation for reduced beetle feeding on plants treated with INR7, Zehnder et al. (1997a) reasoned that elicitation of ISR by INR7 might be accompanied by reduced production of cucurbitacins by cucumber plants. Support for this hypothesis was found in a study (Zehnder et al. 1997a) in which treatment of cucumber with strain INR7 resulted in significantly reduced production of cucurbitacin C. Collectively, the results from studies on cucumber beetles demonstrate that specific endophytic bacteria can elicit unexpected yet important physiological changes in plants.

Serratia marcescens strain 90-166 colonizes roots internally (Press et al. 2001) and has been shown to elicit ISR against various diseases of cucumber. Elicitation of ISR against Fusarium wilt was demonstrated using a split-root system (Liu et al. 1995a). Root systems of seedlings were mechanically separated into two halves, with each half then being placed into a separate pot. Strain 90-166 was applied to one pot and the pathogen to the other pot. Numbers of dead plants and severity of the disease were significantly reduced by strain 90-166 over a 6-week experimental period. In another study (Liu et al. 1995b), strain 90-166 was found to elicit ISR against angular leaf spot. Treatment of seeds or cotyledons with strain 90-166 resulted in significant reductions in numbers and size of angular leaf spot lesions when the pathogen was inoculated 3 weeks after planting. ISR against angular leaf spot was also elicited when 90-166 was injected into cotyledons 1 week before pathogen inoculation. When 90-166 was injected into cotyledons,

there was a reduction of 1.8 log units in the population of the pathogen inside leaves.

The capacity of strain 90-166 to elicit protection against cucumber anthracnose over a 5-week period was evaluated by Liu et al. (1995c). Strain 90-166 was applied to seeds at the time of planting, and *Colletotrichum orbiculare* was inoculated onto the first, second, third, fourth, or fifth leaf. There was approximately 1 week between each leaf stage. Treatment with strain 90-166 resulted in a significant reduction in the mean total lesion diameter when the pathogen was inoculated onto the fifth leaf, indicating that ISR elicited by the strain persists for at least 5 weeks on cucumber. Elicitation of ISR by strain 90-166 in tobacco against wildfire, caused by *P. syringae* pv. tabaci, was also demonstrated by Press et al. (1997). Stem injection of tobacco with strain 90-166 resulted in a significant decrease in disease severity when the pathogen was sprayed onto leaves 10 days after bacterial treatment. Raupach et al. (1996) reported that strain 90-166 also elicited ISR against *Cucumber mosaic virus* (CMV) on cucumber and tomato. On cucumber, seed treatment with 90-166 completely prevented development of CMV symptoms when the virus was inoculated onto cotyledons. On tomato, the effect of 90-166 was to delay symptom development over time. The area under the disease progress curve (AUDPC) was significantly reduced by strain 90-166.

Elicitation of ISR against viruses has also been reported for other strains of endophytic bacteria. Zehnder et al. (2000) conducted a greenhouse screen of PGPR (plant growth promoting rhizobacteria) for the potential to elicit ISR against CMV on tomato. PGPR were applied as seed treatments and as drenches upon transplanting 2 weeks after seeding. CMV was rub-inoculated with carborundum onto leaves 1 week after transplanting. From among 26 tested strains, three strains of endophytes were selected (*Bacillus subtilis* strain IN937b, *Bacillus pumilus* strain SE34, and *Bacillus amyloliquefaciens* strain IN937a). All of the selected strains significantly reduced disease incidence in each of five experiments. In the same study, Zehnder et al. (2000) conducted two field trials to evaluate the effects of strains IN937b, SE34, and IN937a on CMV. Treatment with all three endophytic bacterial strains resulted in significant reductions in the AUDPC compared to the nonbacterized control in both years of testing.

In another study with CMV in tomato, Murphy et al. (2003) used various two-strain combinations, where one strain was *B. subtilis* strain GB03, which is not reported to be an endophyte, and various endophytic bacteria, including strains IN937a, IN937b, SE34, and INR7. Spores of the bacteria were formulated on chitosan as a carrier and this preparation was mixed into potting mix. All of the bacterial treatments significantly reduced disease severity based on symptoms, decreased disease incidence based on

enzyme-linked immunosorbent assay (ELISA), and decreased virus accumulation, compared to controls.

Three field trials were conducted with various formulations of the endophytic strains IN937a, IN937b, and SE34 (Murphy et al. 2000) to determine their capacity to elicit ISR against *Tomato mottle virus* (ToMoV), which is vectored by the silver whitefly (*Bremisia argentifolii*). The plots were inoculated with ToMoV by natural movement of viruliferous whitefly adults from adjacent plantings of ToMoV-resistant tomato germplasm that was inoculated prior to transplanting into the field. The incidence and severity of ToMoV were significantly reduced by one or more of the formulations of each endophyte. The number of whitefly nymphs detected on plants was significantly reduced by strains IN937a and IN937b. Hence, as in the case with cucurbit wilt of cucumber, some endophytic bacteria can elicit ISR against both a plant disease and its insect vectors.

ISR elicited by the endophytes *B. pumilus* strain SE34, *S. marcescens* strain 90-166, and *Pseudomonas fluorescens* strain 89B-61 has been shown to reduce the severity of blue mold of tobacco, caused by *Peronospora tabacina* (Zhang et al. 2002a, 2002b, 2004). In one study (Zhang et al. 2002b), strains SE34, 90-166, and 89B-61 elicited ISR in detached leaf and microtiter plate bioassays as well as in pot trials in the greenhouse. In the pot assay, application of all three strains as a soil drench to 4-week-old plants of three tobacco cultivars resulted in significant reductions in the mean percentage of leaf area with lesions caused by *P. tabacina* inoculated onto leaves 1 week after bacterial treatment. Sporulation of the pathogen on lesions was significantly decreased by treatment with the three strains in pot trials. Strains SE34, 90-166, and 89B-61 also significantly reduced disease severity in the detached leaf (injection of a bacterial suspension into petioles) and microtiter plate bioassays (application of bacterial suspensions to roots). Sporulation of the pathogen was significantly reduced by both strains in the detached leaf bioassay.

In another study (Zhang et al. 2004), strains SE34 and 90-166 were used to explore the relationship between elicitation of plant growth promotion and ISR. Application of the endophytes as a seed treatment alone elicited significantly enhanced tobacco plant growth but not disease protection. When the strains were applied as seed treatments followed by a soil drench, both plant growth promotion and ISR were elicited. Overall, the results from this study indicated that while there was a relationship between growth promotion and ISR, elicitation of growth promotion can occur without elicitation of ISR; however, when ISR was elicited, growth promotion was also elicited with the bacterial strains used in the study.

Endophytic bacteria have also elicited ISR against tomato late blight, caused by *Phytophthora infestans* (Yan et al. 2002). Application of strains SE34 and 89B-61 by incorporation into the potting medium at the time of

planting elicited significant reductions in disease severity when *P. infestans* was inoculated onto leaves 5 weeks after planting.

Greenhouse screening of endophytic *Bacillus* spp. that have elicited ISR on some crops was conducted in Thailand (Jetiyanon and Kloepper 2002) as a first step toward employment of endophyte-elicited ISR in tropical agriculture. This study used four different host/pathogen systems: tomato and *Ralstonia solanacearum*, long cayenne pepper (*Capsicum annuum* var. *acuminatum*) and *Colletotrichum gloeosporioides*, green kuang futsoi (*Brassica chinensis* var. *parachinensis*) and *Rhizoctonia solani*, and cucumber and CMV. The goal of the study was to find mixtures of endophytic spore-forming bacteria that elicited ISR in all four host/pathogen systems. Seven individual strains and 11 combinations of 2 strains were tested. One strain (*B. amyloliquefaciens* IN937a) and four mixtures (IN937a + *B. subtilis* IN937b; IN937b + *B. pumilus* SE34; IN937b + *B. pumilus* SE49; and IN937b + *B. pumilus* INR7) significantly reduced incidence or severity of all four diseases. The results are noteworthy for two reasons. First, they indicate that ISR elicited by specific endophytic bacterial strains can protect hosts under tropical conditions. Second, the results show that mixtures of two bacterial strains are superior to individual strains for eliciting significant protection in multiple hosts against different pathogens.

Further evidence for the concept of using strain mixtures of *Bacillus* spp. to increase the repeatability of plant growth promotion or elicitation of ISR by bacteria was reported in a follow-up field investigation (Jetiyanon et al. 2003). Field tests were conducted in Thailand to find mixtures of bacteria that could protect several different hosts against the multiple diseases that are typical under the multi- or inter-cropping agricultural conditions predominant in Thailand. In tests conducted during the rainy and dry seasons, some two-strain mixtures of endophytes more consistently protected against disease than did a single strain. In each season, the mixture of strains IN937a and IN937b significantly protected against all the tested diseases (southern blight of tomato, CMV on cucumber, and anthracnose of long cayenne pepper). The same mixture of endophytes also resulted in significant yield increases of all crops during the rainy season.

Endophytic bacteria have also been shown to elicit ISR in conifers. Enebak and Carey (2000) tested the potential elicitation of ISR on loblolly pine (*Pinus taeda*) by strains *B. sphaericus* SE56 and *B. pumilus* strains INR7, SE34, SE49, and SE52 against *Cronartium quercuum* f. sp. *fusiforme*, which causes fusiform rust. Bacteria were applied at seeding, and suspensions of *C. quercuum* basidiospores from field-collected telia on water oak (*Quercus nigra*) were sprayed onto the pine seedlings at five different times. Six months after the final application of basidiospores, the incidence of fusiform rust was determined by noting the presence or absence of the typical symptoms of main-stem swellings or galls. The experiment was

conducted annually for 2 years. All strains except SE49 resulted in significant reductions in disease incidence in either one of the 2 years or in the pooled data from both years.

Before concluding this discussion of case studies of endophytic bacteria that have been shown to elicit ISR, a note should be made about *Azospirillum* spp. *Azospirillum brasilense* is a well characterized endophyte, and nearly all strains of this species have been shown to promote growth of many crop species (Bashan and de-Bashan 2002). Because many of the strains of *Bacillus* spp. cited above that elicit ISR also elicit plant growth promotion, one might expect that *A. brasilense* would also elicit ISR. However, this is not the case. Bashan and de-Bashan (2002) investigated the potential of *A. brasilense* to elicit ISR in tomato against bacterial speck, caused by *P. syringae* pv. Tomato, and concluded that this endophyte does not elicit ISR.

3.3 Internal Colonization of Endophytes that Elicit Induced Resistance

In most of the studies discussed in the previous section, extensive microbial ecology studies to determine the extent of internal colonization of plant tissues by the applied endophytes were not carried out. Typically, isolations are performed near the location where the pathogen was applied. Such isolation is done to test one of the suggested tenants of ISR: that there is physical separation of the pathogen and the inducing agent. According to this tenant, physical separation is required to differentiate ISR from antagonism as a mechanism for protection against pathogens. While testing for physical separation has validity, it also creates an inherent problem with endophytic bacteria that exhibit systemic colonization of plants. An endophyte that can move within the plant and colonize petioles could, theoretically, still elicit ISR at a level sufficient to reduce disease severity of a foliar pathogen. However, based on the tenant of physical separation, one could not state that ISR was the operable mechanism by which disease severity was reduced. Hence, some endophytic bacteria might actually elicit plant defense although they are not spatially separated from the pathogen.

An example illustrating the limited internal colonization of well characterized endophytes that elicit ISR is *P. fluorescens* strain 89B-61, which was initially designated as strain G8-4. Because this strain has reactions in biochemical tests that are intermediate between *P. putida* and *P. fluorescens*, some publications before 1997 refer to 89B-61 as *P. putida*. Later publications designate the strain as *P. fluorescens* based on repeated fatty acid analyses. Chen et al. (1995) found that strain 89B-61 significantly

reduced the severity of Fusarium wilt of cotton. In this system, 89B-61 was stab-inoculated into seedling stems 13 days prior to inoculation with *Fusarium oxysporum* f. sp. *vasinfectum* at a point 1.5 cm above the point of bacterial inoculation. Using a rifampicin-resistant mutant of 89B-61, no movement up the stem from the point of bacterial inoculation was detected. In a separate greenhouse study, Kloepper et al. (1992) reported that seed treatment of cucumber with strain G8-4 (89B-61) significantly reduced lesion numbers and size of anthracnose following challenge inoculation of leaves with *C. orbiculare*. This induced resistance was associated with colonization of internal root tissues at log 4.0 cfu/g at 14 days after emergence; however, the bacteria were not isolated from stems or leaves. Elicitation of induced resistance in cucumber by 89B-61 was confirmed in field studies (Wei et al. 1996), where application of the bacterium as a seed treatment resulted in significant reductions in severity of anthracnose and angular leaf spot.

Quadt-Hallmann et al. (1997) used isolation, ELISA, and immunogold labeling with *P. fluorescens* 89B-61-specific polyclonal antibodies to investigate the pattern of internal colonization of cotton by the ISR-eliciting strain 89B-61. Results from isolation studies indicated that, following treatment of seeds, 89B-61 colonized roots internally at a mean population of 1.1×10^3 cfu/g, while the bacterium was not recovered from stems, cotyledons, or leaves. With ELISA, strain 89B-61 was detected outside and inside roots but not inside stems, cotyledons, or leaves. Electron microscopy with immunogold labeling revealed that internal colonization of roots by 89B-61 was restricted mainly to intercellular spaces of the epidermis. Interestingly, the colonization pattern was quite distinct from that of another bacterium (*Enterobacter asburiae* strain JM22) that does not elicit ISR. JM22 colonized throughout the root cortex, including inside the vascular stele, in intercellular spaces close to the conducting elements, as was previously found in other plant species (Quadt-Hallmann and Kloepper 1996). It was suggested that the internal colonization of cotton by 89B-61 and JM22 could be considered as representative of two fundamental options for how endophytic bacteria colonize plants after application to seeds or soils. The first pattern is that of 89B-61 and consists of limited internal colonization of roots. The second pattern, demonstrated by JM22, consists of extensive internal root colonization and ultimately in some vascular colonization.

Internal colonization of roots by *S. marcescens* strain 90-166 was demonstrated by Press et al. (2001) in an investigation into the role of iron in elicitation of ISR. Cucumber root colonization by the wild-type strain was compared to that by a mutant deficient in siderophore production. While the total root population sizes (external and internal colonization) were statistically equivalent, the internal population size was significantly greater with the wild-type than with the siderophore-negative mutant. Because the mutant failed to elicit ISR against anthracnose, while ISR was elicited by the wild-type strain, it was concluded that capacity to elicit ISR was related to the population size of the bacterium inside roots.

3.4 Plant Responses to Endophytic Elicitors

Investigations aimed at determining how plants respond to inoculation with endophytes that elicit ISR is one approach to studying mechanisms of ISR by such bacteria. During the previously discussed study on cotton colonization by strain 89B-61 (Quadt-Hallmann et al. 1997), the epidermal cell walls of plant cells adjacent to cells of 89B-61 in the intercellular space developed electron-opaque appositions of an amorphous matrix.

Two cytological studies were conducted by Benhamou et al. (1996, 1998) with *B. pumilus* strain SE34. In the first study (Benhamou et al. 1996), colonization of pea roots by *Fusarium oxysporum* f. sp. *pisi* was restricted to the epidermis and outer cortex of roots treated with SE34, while in nonbacterized roots, the pathogen colonized the cortex, endodermis, and the paratracheal parenchyma cells. This reduction in fungal colonization by SE34 was associated with strengthening of the epidermal and cortical cell walls. In addition, roots treated with SE34 exhibited newly formed barriers beyond the site of fungal infection. These barriers were cell wall appositions that contained large amounts of callose and were infiltrated with phenolic compounds. Phenolic compounds were detected in transmission electron microscopy using gold-complexed laccase and were found to accumulate in host cell walls, in intercellular spaces, and on the surface of and inside the invading pathogen hyphae.

In another study (Benhamou et al. 1998), the effect of SE34 alone or in combination with chitin on structural and cytochemical changes of tomato infected with *F. oxysporum* f. sp. *radicis-lycopersici* was investigated. Treatment with SE34 reduced the severity of typical symptoms, including wilting of seedlings and numbers of brown lesions on lateral roots. This disease protection by strain SE34 was associated with more limited fungal colonization of roots and with marked changes in host physiology. Physiological changes elicited by strain SE34 included an increase in host cell wall density, the accumulation of polymorphic deposits at sites of potential pathogen penetration, and the occlusion of epidermal cells and intercellular spaces with an osmophilic, amorphous material that appeared to trap the invading fungal hyphae. The extent and magnitude of the physiological changes in the host elicited by SE34 were enhanced by the addition of chitosan. Interestingly, the overall chitin component of the pathogen was structurally preserved in roots treated with SE34 with or without chitosan at the time

when hyphal degradation was apparent. This suggests that synthesis of chitinase in bacteria-treated roots is not an early event in the cascade of physiological steps in signal transduction that lead to induced resistance. Benhamou et al. (1998) concluded: "According to our cytological observations, the induction of resistance triggered by *B. pumilus* strain SE34 involves a sequence of events including first the elaboration of structural barriers and the production of toxic substances such as phenolics and phytoalexins, and second the synthesis and accumulation of other molecules including chitinases and other hydrolytic enzymes such as β-1,3-glucanases which probably contribute to the release of oligosaccharides that, in turn, can stimulate other defense reactions."

Jeun et al. (2004) conducted a cytological comparison of cucumber plants in which systemic resistance had been elicited by bacteria or by chemicals. In this study, the endophytes 89B-61 and 90-166 were used to elicit ISR against *C. orbiculare*. Significantly fewer numbers of anthracnose lesions developed on plants treated with 89B-61 and 90-166 than on the control (chemical treatment). Cytological studies using fluorescent microscopy revealed a higher frequency of autofluorescent epidermal cells, which are related to accumulation of phenolic compounds, at the sites of fungal penetration in plants treated with either strain and inoculated with *C. orbiculare*. In addition, callose-like structures (β-1,3-glucan polymers) were frequently deposited at the site of fungal penetration of the leaves of plants treated with either strain.

Investigations on plant responses to elicitation of ISR can also examine the signal transduction pathway of plants to determine general biochemical pathways in plants during ISR. As previously discussed, according to the model pathway for signal transduction (Pieterse et al. 1998), ISR pathways are independent of salicylic acid, but dependent on ethylene, jasmonic acid, and the regulatory gene *npr-1*. Further, according to the model, ISR elicited by bacteria does not result in the accumulation of pathogenesis-related (PR) proteins. PR proteins are accumulated during SAR elicited by pathogens and chemicals, and SAR is dependent upon salicylic acid.

A few studies on signal pathways have been reported with endophytic bacteria that elicit ISR. In the tomato late blight system, ISR was elicited by *B. pumilus* strain SE34 on NahG lines, which breakdown endogenous salicylic acid, but not in the ethylene-insensitive *NR/NR* line or in the jasmonic acid-insensitive *dfl/dfl* line (Yan et al. 2002). These results are consistent with the model of Pieterse et al. (1998). Similar results were reported by Zhang et al. (2002a). In the tobacco blue mold system, *B. pumilus* strain SE34, as well as two strains of Gram-negative bacteria, elicited ISR on both wild-type and NahG transgenic tobacco lines, as evidenced by significant reductions in the severity of blue mold on bacterized plants compared to nonbacterized plants.

Different results were found with strain 89B-61 (Park and Kloepper 2000), which elicits ISR in tobacco against wildfire caused by *P. syringae* pv. tabaci. In this system, a transgenic line of tobacco with a β-glucuronidase (GUS) reporter gene fused to the *PR-1a* promoter had significantly reduced severity of wildfire compared to nonbacterized controls. Elicitation of ISR by strain 89B-61 was associated with a significant increase in GUS activity in microtiter plate and whole plant bioassays. Hence, with strain 89B-61, elicitation of ISR results in activation of the *PR-1a* gene, which is activated during SAR but not during bacterial-induced ISR according to the model of Pieterse et al. (1998).

Signal pathways in ISR elicited by *P. fluorescens* strain CHA0 in *Arabidopsis* against *Peronospora parasitica* were investigated by Iavicoli et al. (2003) using various transgenic and mutant plant lines: NahG (for degradation of salicylic acid), *sid2-1* (lacks production of salicylic acid), *npr1-1* (nonexpressor of *PR* genes), *jar1-1* (insensitive to jasmonic acid), *ein2-1* (insensitive to ethylene), *eir1-1* (insensitive to ethylene/auxin), and *pad2-1* (phytoalexin-deficient). ISR was elicited by strain CHA0 in all lines except *jar1-1*, *eir1-1*, and *npr1-1*.

In another study of signaling pathways, Ryu et al. (2003b) used endophytic bacterial strains in *Arabidopsis* to elicit ISR against two different pathovars of *P. syringae* (pvs. tomato and maculicola). Strains SE34, 90-166, and 89B-61 elicited ISR against both pathogens. Strain SE34 elicited a salicylic acid-independent pathway against one pathovar and salicylic acid-dependent pathway against a different pathovar. Additional tests of strains 89B-61 and SE34 on various mutant lines of *Arabidopsis* (Ryu et al. 2003b) revealed that, in agreement with the model of Pieterse et al. (1998), ISR elicited by both strains was dependent on NPR1 and ISR elicited by SE34 was dependent on jasmonic acid and ethylene. However, in contrast to the model, ISR elicited by strain 89B-61 was independent of ethylene and jasmonic acid, and ISR by strain 90-166 was dependent on jasmonic acid but independent of ethylene.

Endophyte strains 90-166 and SE34 also elicited ISR against CMV in *Arabidopsis* (Ryu et al. 2004b). Strains 90-166 and SE34 reduced disease severity in NahG plants, indicating that ISR elicited by strains 90-166 and SE34 was independent of salicylic acid. Further investigation on the signal pathway of ISR against CMV elicited by strain 90-166 with lines NahG, *npr1*, and *fad3-2 fad7-2 fad8* (insensitive to jasmonic acid) indicated that ISR against CMV by strain 90-166 is independent of salicylic acid and NPR1, but is dependent on jasmonic acid (Ryu et al. 2004b).

Collectively, the results on signaling pathways of ISR elicited by endophytic bacteria indicate that different pathways are elicited by various strains. Further, the specific signal transduction pathway that is activated during ISR depends on the host plant and, at least in one case, on the pathogen used on a given host.

A new approach to investigations on plant responses to endophytic bacteria was recently opened by the finding that volatile organic compounds produced by the endophyte *B. amyloliquefaciens* IN937a elicit plant growth promotion (Ryu et al. 2003a) and ISR (Ryu et al. 2004a). Significant growth promotion of *Arabidopsis* by IN937a was observed in I-plates, which have a raised plastic divider separating agar on each half of the dish, thus preventing movement of soluble compounds. When IN937a was placed on one side of an I-plate, *Arabidopsis* plants growing on the other side exhibited enhanced growth, presumably as a result of volatiles produced by the bacteria. Characterization of the volatile organic compounds (VOCs) produced by IN937a, coupled with bioassays of fractions of VOCs, revealed that 2,3-butanediol and acetoin elicited plant growth promotion. In a separate study (Ryu et al. 2004a), exposure of *Arabidopsis* to VOCs from strain IN937a resulted in significantly less disease caused by *Erwinia carotovora* subsp. carotovora. Tests with various mutant lines of *Arabidopsis* revealed that elicitation of ISR by VOCs of IN937a is independent of jasmonic acid, ethylene, salicylic acid, and *npr1*. Such a pattern of signal pathway has not been reported with ISR elicited by bacteria and, therefore, it is likely that VOCs of IN937a elicit a distinct, and as yet uncharacterized, pathway in *Arabidopsis*.

3.5 Implementation in Production Agriculture: Two Case Studies

The principle that endophytic bacteria can elicit ISR or plant growth promotion has been extended to use in production agriculture and horticulture through the development of two products. These two products are discussed, not as endorsements of the products, but as case studies indicating that our growing scientific knowledge of endophytic bacteria can be put to practical use.

In the first case study, an agricultural product has been developed using the capacity of a single endophytic strain of *Bacillus* spp. to elicit both ISR and plant growth promotion. The product is Yield Shield, which is produced by Gustafson, LLC. Yield Shield consists of a spore preparation of the *B. pumilus* strain listed in this review as INR7 (Table 3.1) and designated by Gustafson as GB34 (http://www.gustafson.com/Labels/yield_shield_label.pdf). The product received registration from the United States Enivironmental Protection Agency (EPA) in 2003 for use on soybeans to protect against *Rhizoctonia solani* and *Fusarium* spp. Seed treatment of soybean with Yield Shield and strain INR7 results in significant seedling growth promotion and in ISR, which is apparent both by a significant de-

Table 3.1. Endophytic bacterial strains that have been reported to elicit induced systemic resistance (ISR) in at least two publications

Strain no. and identification	Effects reported and systems used[a]	Reference
IN937a *Bacillus amyloliquefaciens*	Reduced incidence or severity of vegetable diseases (caused by *Cucumber mosaic virus* (CMV), *Sclerotium rolfsii*, *Ralstonia solanacearum*, *Colletotrichum gloeosporioides*, and *Rhizoctonia solani*) in greenhouse and field trials in Thailand	Jetiyanon et al. 2002; Jetiyanon and Kloepper 2003
	Reduced incidence of CMV on tomato in the greenhouse	Zehnder et al. 2000
	When applied to tomato with the non-endophyte *Bacillus subtilis* GB03, reduced severity of CMV in the greenhouse	Murphy et al. 2003
	Reduced incidence and severity of tomato mottle virus in the field. Also reduced numbers of the white fly vector feeding on plants	Murphy et al. 2000
	Volatile organic compounds of the strain elicit growth promotion of *Arabidopsis* and ISR against *Erwinia carotovora* subsp. carotovora	Ryu et al. 2003a, 2004
	Component of the product, BioYield (Gustafson LLC, http://www.gustafson.com)	Kloepper et al. 2004
IN937b *Bacillus subtilis*	Reduced incidence of CMV on tomato in the greenhouse	Zehnder et al. 2000
	When applied to tomato with the non-endophyte *B. subtilis* GB03, reduced severity of CMV in the greenhouse	Murphy et al. 2003
	Reduced incidence and severity of tomato mottle virus in the field. Also reduced numbers of the white fly vector feeding on plants	Murphy et al. 2000
90-166 *Serratia marcescens*	Reduced incidence and delayed development of symptoms of Fusarium wilt of cucumber, caused by *Fusarium oxysporum* f. sp. *cucumerinum*	Liu et al. 1995a
	Reduced severity of bacterial angular leaf spot of cucumber, caused by *Pseudomonas syringae* pv. lachrymans	Liu et al. 1995b
	Reduced severity of anthracnose, caused by *Colletotrichum orbiculare*, in two cucumber cultivars over a 5-week period after bacterial treatment	Liu et al. 1995c
	Reduced incidence of CMV on cucumber and tomato and reduced the area under the disease progress curve (AUDPC)	Raupach et al. 1996
	Decreased severity of tobacco wildfire, caused by *Pseudomonas syringae* pv. tabaci	Press et al. 1997

Table 3.1. (continued)

Strain no. and identification	Effects reported and systems used[a]	Reference
	Wild-type strain reduced severity of cucumber anthracnose. A siderophore-negative mutant did not elicit ISR and colonized roots internally at lower populations than the wild-type	Press et al. 2001
	Decreased severity of tobacco blue mold, caused by *Peronospora tabacina*, in NahG transgenic tobacco lines that degrade salicylic acid	Zhang et al. 2002a
	Decreased severity of tobacco blue mold in microtiter plate assays and detached leaf assays. Reduced pathogen sporulation	Zhang et al. 2002b
	Reduced symptoms of *Pseudomonas syringae* pvs. tomato and maculicola on *Arabidopsis*	Ryu et al. 2003b
	Decreased severity of tobacco blue mold, caused by *Peronospora tabacina*	Zhang et al. 2004
SE34 *Bacillus pumilus*	Reduced incidence or severity of vegetable diseases (caused by cucumber mosaic virus, *Sclerotium rolfsii*, *Ralstonia solanacearum*, *Colletotrichum gloeosporioides*, and *Rhizoctonia solani*) in greenhouse and field trials in Thailand	Jetiyanon et al. 2002; Jetiyanon and Kloepper 2003
	Decreased severity of tobacco blue mold, caused by *Peronospora tabacina*, in NahG transgenic tobacco lines that degrade salicylic acid	Zhang et al. 2002a
	Decreased severity of tobacco blue mold in microtiter plate assays and detached leaf assays. Reduced pathogen sporulation	Zhang et al. 2002b
	Decreased severity of tobacco blue mold when applied as both seed treatment and drench in the greenhouse. Elicitation of ISR was associated with growth promotion	Zhang et al. 2004
	Decreased severity of tomato late blight, caused by *Phytophthora infestans*, and decreased germination of sporangia and zoospores of the pathogen	Yan et al. 2002
	Reduced incidence of CMV on tomato in the greenhouse	Zehnder et al. 2000
	When applied to tomato with the non-endophyte *B. subtilis* GB03, reduced severity of CMV in the greenhouse	Murphy et al. 2003
	Reduced incidence and severity of tomato mottle virus in the field	Murphy et al. 2000
	Reduced incidence of Fusiform rust, caused by *Cronartium quercuum* f. sp. *fusiforme*, on loblolly pine	Enebak and Carey 2000

Table 3.1. (continued)

Strain no. and identification	Effects reported and systems used[a]	Reference
	Restricted colonization of pea roots by *F. oxysporum* f. sp. *pisi* and induced formation of structural barriers in the plant	Benhamou et al. 1996
	Reduced damage of tomato roots to *F. oxysporum* f. sp. *radicis-lycopersici* and induced structural and cytochemical barriers in the plant	Benhamou et al. 1998
	Reduced symptoms of *Pseudomonas syringae* pvs. tomato and maculicola on *Arabidopsis*	Ryu et al. 2003b
INR7 *Bacillus pumilus* (available as the product Yield Shield; Gustafson LLC)	Reduced incidence or severity of vegetable diseases (caused by CMV, *Sclerotium rolfsii*, *Ralstonia solanacearum*, *Colletotrichum gloeosporioides*, and *Rhizoctonia solani*) in greenhouse and field trials in Thailand	Jetiyanon et al. 2002; Jetiyanon and Kloepper 2003
	Decreased severity of anthracnose (caused by *Colletotrichum orbiculare*) and angular leaf spot (caused by *Pseudomonas syringae* pv. lachrymans) on cucumber in field trials	Wei et al. 1996
	Decreased the incidence of cucurbit wilt disease, caused by *Erwinia tracheiphila*, in field trials	Zehnder et al. 2001
	Decreased numbers of cucumber beetles on plants in the field	Zehnder et al. 1997b
	Decreased beetle feeding activity and transmission of *E. tracheiphila* on cucumber in cages where beetles had a choice between bacterial-treated and nontreated plants	Zehnder et al. 1997a
	When applied to tomato with the non-endophyte *B. subtilis* GB03, reduced severity of CMV in the greenhouse	Murphy et al. 2003
	Reduced incidence of Fusiform rust, caused by *Cronartium quercuum* f. sp. *fusiforme*, on loblolly pine	Enebak and Carey 2000
	Decreased severity of anthracnose (caused by *Colletotrichum orbiculare*) and angular leaf spot (caused by *Pseudomonas syringae* pv. lachrymans) on cucumber in field trials	Wei et al. 1996
89B-61 *Pseudomonas fluorescens* (earlier referred to as G8-4)	Decreased severity of tobacco blue mold, caused by *Peronospora tabacina*	Zhang et al. 2004
	Decreased severity of Fusarium wilt of cotton, caused by *Fusarium oxysporum* f. sp. *vasinfectum*	Chen et al. 1995
	Following seed treatment of cotton, 89B-61 reached an internal root population of 1.1×10^3 cfu/g. Bacteria were not detected inside cotyledons, stems, or leaves	Quadt-Hallmann et al. 1997

Table 3.1. (continued)

Strain no. and identification	Effects reported and systems used[a]	Reference
	Decreased severity of anthracnose (caused by *Colletotrichum orbiculare*) and angular leaf spot (caused by *Pseudomonas syringae* pv. lachrymans) on cucumber	Wei et al. 1996
	Decreased severity of tomato late blight, caused by *Phytophthora infestans*, and decreased germination of sporangia and zoospores of the pathogen when applied to seeds	Yan et al. 2002
	Decreased severity of tobacco blue mold, caused by *Peronospora tabacina*, in NahG transgenic tobacco lines that degrade salicylic acid	Zhang et al. 2002a
	Decreased severity of tobacco blue mold in microtiter plate assays and detached leaf assays. Reduced pathogen sporulation	Zhang et al. 2002b
	Decreased severity of tobacco wildfire, caused by *Pseudomonas syringae* pv. tabaci. Activated the promoter for PR1a [a pathogenesis-related (PR) protein]	Park and Kloepper 2000
	Reduced symptoms of *Pseudomonas syringae* pvs. tomato and maculicola on *Arabidopsis*	Ryu et al. 2003b
	Reduced number of lesions of *Colletotrichum orbiculare* on cucumber and increased deposition of callose-like polymers on leaf cells at the site of pathogen penetration	Jeun et al. 2004
	Reduced mean numbers and size of anthracnose lesions on cucumber	Wei et al. 1991
	Colonized roots internally at log 4.0 cfu/g at 2 weeks after emergence when applied as seed treatments. Bacteria were not detected in leaves or stems	Kloepper et al. 1992
CHA0 *Pseudomonas fluorescens*	Reduced severity of *Tobacco necrosis virus* (TNV) on tobacco.	Maurhofer et al. 1994
	Severity of TNV on tobacco was reduced equivalently by the wild-type strain and a transgenic strain carrying *pchAB* genes for synthesis of salicylic acid.	Maurhofer et al. 1998
	Reduced sporulation of *Peronospora parasitica* on *Arabidopsis*	Iavicoli et al. 2003

[a] In all cases, the stated reductions in disease incidence or severity and the effects on insects are statistically significant at $P \leq 0.05$

crease in incidence and severity of *R. solani* inoculated onto stems at a point where INR7 does not colonize, and by a systemic increase in lignification of plant cell walls (C.-M. Ryu and C.-H. Hu, unpublished). It should be emphasized that Yield Shield is a unique case for a rhizobacterium that elicits ISR in that economically significant efficacy sufficient to warrant the costs of product development and EPA registration was shown for a single bacterial strain.

In the second case study, the product consists of a two-strain mixture of *Bacillus* spp., where one strain (IN937a) is an endophyte that elicits ISR (Table 3.1) and the other strain (GB03) is a non-endophyte. The product is BioYield and is also produced by Gustafson. The development of BioYield was recently reviewed (Kloepper et al. 2004). The underlying concept was to develop a biological formulation consisting of components known to exert different mechanisms for control of diseases. The selected components and their mechanisms were chitosan (as a carrier) for nematode control via promotion of indigenous soil predators and antagonists to root-knot nematodes, *B. subtilis* strain GB03 for control of soil-borne pathogens via production of the antibiotic iturin, and one of several tested endophytic *Bacillus* spp. that elicit ISR. The most unexpected finding was that the three-component combination (chitosan plus two bacterial strains) exhibited more consistent, and a greater magnitude of, growth promotion and systemic protection against pathogens than did any of the individual components (Kloepper et al. 2004). Based on the results, the two-strain combination of *B. amyloliquefaciens* strain IN937a and *B. subtilis* strain GB03 was selected for product development.

3.6 Conclusions

As discussed in this review, selected strains of nonpathogenic endophytic bacteria can elicit ISR in plants, leading to reductions in severity of various diseases. Research on such endophytes has concentrated both on delineating the pathosystems where protection results and in understanding plant responses that occur during the signal transduction pathways that culminate in disease protection. In many cases, elicitation of ISR by endophytic bacilli is associated with increased plant growth, and the relationship between ISR and growth promotion should be further investigated. Elucidation of specific bacterial determinants that account for elicitation of ISR is just beginning, and further work is needed to understand why one strain of a given bacterial species can elicit ISR while another strain of the same species cannot. It is encouraging that implementation of ISR by endophytic bacilli is beginning, even while some basic questions remain to be answered.

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