The Plant Growth-Promoting Fungus *Penicillium simplicissimum* GP17-2 Induces Resistance in *Arabidopsis thaliana* by Activation of Multiple Defense Signals

Md. Motaher Hossain 1, Farjana Sultana 1, Mayumi Kubota 2, Hiroyuki Koyama 2 and Mitsuro Hyakumachi 2,*

1 United Graduate School of Agricultural Sciences, Gifu University, Yanagido 1-1, Gifu, 501-1193 Japan
2 Faculty of Applied Biological Sciences, Gifu University, Yanagido 1-1, Gifu, 501-1193 Japan

*Corresponding author: E-mail, hyakumac@cc.gifu-u.ac.jp; Fax, 81-58-293-2847.

*Abbreviations: BGL, barley grain inoculum; BTH, benzothiadiazole; CF, culture filtrate; ET, ethylene; ISR, induced systemic resistance; JA, jasmonic acid; PDA, potato dextrose agar; PDB, potato dextrose broth; PGPF, plant growth-promoting fungi; PGPR, plant growth-promoting rhizobacteria; PR, pathogenesis related; Pst, *Pseudomonas syringae* pv. *tomato* DC3000; RT–PCR, reverse transcription–PCR; SA, salicylic acid; SAR, systemic acquired resistance.

**Introduction**

Plants utilize diverse and sophisticated signaling cascades for recognizing and responding to a wide range of biotic and abiotic stresses. Stress recognition and signaling is translated into biochemical reactions, metabolic adjustments and an altered physiological state. Thus, plants have evolved defense mechanisms by which they can increase their tolerance against such stresses. Consequently, a complex signaling network underlies plant adaptation to these adverse environmental conditions (Zhu 2001). There has been rapid progress in our understanding of these signaling pathways over recent years as a result of interdisciplinary studies. From these studies, it has become apparent that these pathways rely on endogenous regulators, such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA), to induce defense reactions (Glazebrook 2001). Depending on the initial stimulus, different signal transduction pathways are utilized. For instance, the signal transduction pathway for systemic acquired resistance (SAR) is independent of ET- and JA-mediated defense responses, but it requires accumulation of SA (van Loon 1985, Gaffney et al. 1993, Delaney et al. 1994, Thomma et al. 1998). Unlike SAR, induced systemic resistance (ISR) does not involve the accumulation of pathogenesis-related (PR) protein or SA (Pieterse et al. 1996), but instead relies on pathways regulated by JA and ET (Pieterse et al. 1996, Knoester et al. 1999, Yan et al. 2002). Other examples of ISR are linked to the production of SA by plant growth-promoting rhizobacteria (PGPR) strains and, therefore, have more in common with SAR (De Meyer and Höfte 1997, Ryu et al. 2004). Montesano et al. (2005) reported that multiple...
defense signals (JA, ET and cinnamic acid) are induced by the culture filtrate of 
Erwinia cartovora. An aqueous extract of the mycelium of \( Penicillium 
\) chrysogenum induces resistance independently of known signaling pathways
(Thuerig et al. 2006). Studies using active cellulose from 
Trichoderma longibrachiatum as elicitors have demonstrated the involvement of SA and JA/ET pathways in plant defense
(Martinez et al. 2001). It is likely that other forms of induced resistance exist that vary in their reliance on SA, JA, ET and
other as yet undiscovered plant regulators. In addition, these signaling pathways are not simple, linear, isolated cascades,
but can participate in cross-talk with one another (Reymond and Farmer 1998, Genoud and Métraux 1999).

Non-necrotizing mutualistic rhizosphere microorganisms can effectively trigger induced resistance (Tuzun and
Kloeper 1994, Pieterse et al. 1996, Tuzun and Bent 1999), the best studied of which are several species of PGPR.
Colonization of roots with plant growth-promoting fungi (PGPF) can also lead to systemic resistance in distal parts
of the plant (Meera et al. 1994, Meera et al. 1995). In the past few years, an increasing amount of research was devoted to
the study of ISR mechanisms. While most studies have focused on the interaction between rhizobacteria and plant
pathogens, little is known about the molecular mechanisms of response and resistance offered by PGPF. Only a few
studies of signaling pathways during PGPF-mediated ISR, all using 
Trichoderma spp., have been performed (Shoresh et al. 2005), while a mechanistic study with other PGPF has
not yet been conducted. An example of a PGPF is 
Penicillium simplicissimum GP17-2, which was collected from the rhizosphere of zoysiagrass (Zoysia tenuifolia) and
was found to enhance the growth of a variety of crop plants (Hyakumachi et al. 1994, Shivanne et al. 1994). This
PGPF isolate effectively controlled soil-borne diseases (Hyakumachi et al. 1994) and also has been shown to induce systemic defense responses in cucumber plants, when applied as barley grain inoculum (BGI) and culture filtrate
(CF), against several diseases (Shivanna et al. 1994, Koike et al. 2001). Both the 12,000 Da fraction and the lipid
fraction of CF of 
P. simplicissimum GP17-2 were highly effective in eliciting chemiluminescence activity and lignifi-
cation in cucumber plants (Koike et al. 2001). The potential of 
P. simplicissimum in elevating cucumber defense responses to pathogen infection has encouraged us to study the molecular mechanisms underlying this type of
ISR in the model plant 
Arabidopsis thaliana.

In this article, we describe an Arabidopsis-based model system, in which the biocontrol strain 
P. simplicissimum
GP17-2 and its CF were used to induce an ISR in leaves to bacterial leaf speck pathogen 
Pseudomonas syringae pv.
tomato DC3000 (Pst). We have examined the resistance spectrum induced by GP17-2 and its CF, and have explored
the molecular basis for GP17-2-induced ISR.

**Fig. 1** Induced suppression of disease symptoms and number of 
P. s. tomato bacteria in Arabidopsis leaves expressing ISR and SAR in the soil experiment. ISR was induced by growing plants in soil containing 
P. simplicissimum GP17-2. SAR was induced by drenching the soil with 0.3 mM BTH 1 d prior to challenge inoculation. Pst was sprayed onto leaves until run-off. Disease severity was measured 5 d after challenge inoculation by recording the percentage of total leaf surface showing symptoms for each plant, where 0 = no symptoms; 100 = most severe necrotic symptoms (A). Corresponding bacterial proliferation data are given (B). Data are presented as numbers of c.f.u. g⁻¹ fresh weight, each from three sets of five whole shoots harvested 5 d after challenge inoculation with virulent pathogen Pst. Within each frame, different letters indicate a statistically significant difference between treatments (Fisher’s LSD, \( P < 0.05 \)). The data presented are from representative experiments that were repeated at least twice with similar results.

**Results**

*Induction of systemic resistance in Arabidopsis by barley grain inoculum of P. simplicissimum GP17-2*

To investigate whether 
P. simplicissimum GP17-2 is able to elicit ISR in Arabidopsis, GP17-2 was tested in
Arabidopsis Col-0 against the bacterial speck pathogen 
Pst. SAR was induced by soil drenching with benzothiadiazole
(BTH) (Lawton et al. 1996), while ISR was induced by growing the plants in soil containing 
P. simplicissimum GP17-2 for 3 weeks. Five days after challenge with Pst pathogen, the plants
developed typical bacterial speck disease symptoms, consisting of necrotic or water-soaked spots surrounded by
extensive chlorosis. Induced protection against the pathogen was quantified by determining the percentage area of
leaves with symptoms, as well as by assessing pathogen proliferation in the leaves. Results in Fig. 1A show that
Arabidopsis plants grown in soils amended with a BGI of
GP17-2 or drenched with BTH were more resistant to foliar diseases caused by Pst and exhibited a significant reduction
in disease symptoms compared with non-induced control plants. Fig. 2B shows typical differences in symptom
expression of Pst infection between untreated and GP17-2-treated Arabidopsis Col-0 plants. The reduction in disease
severity was recorded to be, on average, 51 and 46% in
BTH- and GP17-2-treated plants, respectively, and was consistent in three independent experiments. Determination of the number of colony-forming units (c.f.u.) of Pst in
challenged leaves revealed that proliferation of *Pst* was significantly inhibited in plants treated with either GP17-2 or BTH (Fig. 1B). Compared with control-treated plants, BTH- and GP17-2-treated plants showed a 6- and 4-fold decrease in growth of *Pst*, respectively, in challenged leaves. Clear differences in the inhibition of bacterial proliferation in leaves of GP17-2- or BTH-treated plants indicate that symptom reduction is associated with inhibition of bacterial proliferation.

**Plant growth promotion by GP17-2**

The influence of BGI amendment on shoot biomass and the number of rosette leaves per plant in Arabidopsis was also studied under the same experimental conditions. Arabidopsis grown in soil amended with GP17-2 exhibited an increase in shoot biomass and number of rosette leaves per plant compared with control plants (Fig. 2A). Four weeks after GP17-2 treatment, approximately one more leaflet and a 72 and 55% increase in shoot fresh and dry biomass was observed, respectively, in treated compared with control plants (Table 1). These results suggest that *P. simplicissimum* GP17-2 behaves as a PGPF for Arabidopsis.

**Root colonization**

Root colonization by GP17-2 was also examined in Arabidopsis Col-0 plants. Results reveal that GP17-2 was isolated at high frequencies from the roots. The re-isolation frequency was recorded to be, on average, 80% (data not shown). However, no *Penicillium* was detected in association with untreated roots.

**Induction of systemic resistance in Arabidopsis by a culture filtrate of *P. simplicissimum* GP17-2**

CF of GP17-2 was also tested for its ability to induce systemic resistance in Arabidopsis Col-0 plants against *Pst*. In order to select the optimum duration of root treatment at which considerable suppression of disease occurred without visible symptoms of stress, we performed a screening program of different durations of root treatment with diluted CF (50%). From these results, we selected 1 h root treatment for induction. Roots of 2-week-old Arabidopsis were dipped in either CF or potato dextrose broth

### Table 1 Effect of *Penicillium simplicissimum* GP17-2 colonization on growth of Arabidopsis plants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh weight (g/5 plants)</th>
<th>Dry weight (g/5 plants)</th>
<th>No. of rosette leaves/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.76</td>
<td>0.068</td>
<td>12.40</td>
</tr>
<tr>
<td>GP17-2</td>
<td>1.31*</td>
<td>0.105*</td>
<td>13.80*</td>
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</tbody>
</table>

*Significant difference by *t*-test (*P*<0.05).
(PDB) 1 d prior to pathogen inoculation. As a positive control, 5 mM BTH was applied in a similar manner. We observed that Arabidopsis plants pre-treated with CF of GP17-2 showed a clear reduction in disease symptoms (Figs. 2C, 3A) and an even more pronounced inhibition of bacterial growth in challenged leaves compared with untreated plants (Fig. 3B). CF-treated plants showed a 54% lower proportion of leaf surface with disease symptoms and a 4-fold decrease in growth of Pst in infected leaves, confirming that the CF of GP17-2 was equally effective as living Penicillium. No statistically significant difference was observed between the treatment effect of BTH and CF in this bioassay.

Penicillium simplicissimum GP17-2 protects Arabidopsis mutants impaired in JA-/ET-dependent signaling and Arabidopsis transgenic plants defective in the SA-dependent signaling pathway

In order to learn more about the basis of resistance induced by *P. simplicissimum* GP17-2 and its CF, transgenic Arabidopsis or mutants impaired in signal transduction pathways were tested for induced responses towards infection with *Pst*. Analysis of the JA/ET signal transduction pathway was investigated with the ET-insensitive ein2 mutant (Guzman and Ecker 1990) and jar1, a mutant affected in the JA response pathway (Staswick et al. 1992). Both jar1 and ein2 plants developed systemic resistance after pre-treatment with BTH, confirming that BTH-activated resistance does not require the ability of the plant to respond to these signals (Figs. 4, 5). In the same way, although disease infection by *Pst* was more extensive in both mutants compared with wild-type plants, treatment with GP17-2 and its CF led to a similar reduction of disease severity and bacterial proliferation as measured 5 d after inoculation. Analysis of SA pathway involvement was investigated with NahG-expressing Arabidopsis, which are unable to accumulate SA (Lawton et al. 1995), and npr1, a mutant that is unresponsive to inducers of SAR (Cao et al. 1994). Results show that BTH elicited resistance in the NahG plant but not in the npr1 mutant, confirming the previous suggestion that
its action requires NPR1, but is independent of SA accumulation. In contrast, prevention of SA accumulation in NahG or mutation of NPR1 in npr1 plants had only a minor effect during systemic resistance induced by GP17-2 or its CF towards the Pst infection. Both NahG and npr1 plants were protected by GP17-2 or its CF (Figs. 4, 5), but to a lesser extent compared with similarly treated wild-type plants, and this difference in the protection values between wild-type and NahG or npr1 plants was statistically significant (Fig. 6). Thus, SA signaling and NPR1 appear to contribute to induced resistance, but neither appears to be a sole regulator of defense mechanisms.

**Induction of SA-, JA- and ET-responsive gene expression**

To determine whether *P. simplicissimum* GP17-2 treatment induced known plant defense-related genes, we studied the expression of SA-inducible genes encoding the pathogenesis-related protein *PR-1*, β-1,3-glucanase *PR-2* and the thaumatine-like protein *PR-5* (Uknes et al. 1992, Cao et al. 1994, Delaney et al. 1994). In addition, the JA-inducible gene encoding the vegetative storage protein *Vsp* (Berger et al. 1995), the ET-inducible gene encoding the hevein-like protein *Hel* that has been shown to exhibit antifungal activity (Potter et al. 1993) and the JA- and ET-inducible gene encoding the plant defensin, a small protein with antifungal activity, *PDF1.2* (Penninckx et al. 1996), were also studied. Expression of these genes was assessed locally and systemically 3 weeks after GP17-2 application (Fig. 7). Moreover, as a comparison, gene expression was assessed in the roots and leaves of plants that expressed SAR upon pre-treatment of roots with BTH (Fig. 7). We did not observe, by reverse transcription-PCR (RT–PCR) analysis, any local or systemic increase in the level of SA-inducible *PR-1* expression as a result of GP17-2 induction. However, SA-inducible *PR-2* and *PR-5* showed enhanced expression in leaves and roots of GP17-2-treated plants, while the level of *PR-5* expression was relatively weaker in roots than in leaves. Similarly increased expression of JA- or ET-inducible *PDF1.2* was seen in the leaves of
treated plants, although no expression was detected in roots. Expression of \textit{Hel} did not appear to be affected in the leaves of GP17-2-treated plants, although it was down-regulated in the roots of treated plants. In contrast, GP17-2 treatment led to no detectable expression changes of the JA-inducible \textit{Vsp} gene in both systemic and local tissues. Arabidopsis plants receiving a soil drench of BTH showed both local and systemic induction of SA-inducible PR genes in comparison with non-treated plants. The levels of PR genes were only slightly induced in roots following BTH application, although shoots of the same plants showed large increases. However, none of the JA- and ET-inducible genes was significantly activated by BTH in either roots or leaves, while \textit{PDF1.2} was apparently repressed in leaves.

Expression of these genes was analyzed locally, in roots, and systemically, in the leaves of 2-week-old Arabidopsis seedlings in response to GP17-2 culture filtrate. Expression was assessed at 1, 3, 6, 12 and 24 h after treatment (Fig. 8). Increased expression of \textit{PR-1}, \textit{PR-2} and \textit{PR-5} was seen in the leaves of CF-treated plants, starting at 1 h after CF application. In contrast, no significant expression of these genes was observed in the roots of treated plants. No transcript accumulation of \textit{PDF1.2} was evident in either leaves or roots, even 24 h after CF treatment. Increased expression of \textit{Vsp} was seen in the leaves as early as 1 h after CF treatment, while the expression level slightly declined between 12 and 24 h after treatment. In contrast, CF treatment showed no significant changes in the expression of \textit{Vsp} in the roots. Similarly, \textit{Hel} became elevated in the leaves between 3 and 24 h after CF treatment, while no induction was seen in the roots. These results show that both SA- and JA-/ET-inducible defense responses appear to play an important role in systemic resistance induced by CF.

\textbf{Potentiation of defense-related gene expression after pathogen inoculation}

In order to investigate the possibility that GP17-2 treatment potentiates defense-related genes resulting in faster or greater activation after pathogen inoculation, we checked the expression of defense-related genes 0, 1, 2, 4 and 6 d after challenge with \textit{Pst}. For comparison, gene expression was also studied in plants treated with BTH and inoculated

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig7}
\caption{Local and systemic expression of defense-related genes in Arabidopsis plants in response to \textit{P. simplicissimum} GP17-2 and BTH treatment. Induction treatment with GP17-2 was performed by growing the plants in soil containing GP17-2. BTH (0.3 mM) was applied as a soil drench 1 d before challenge. For each treatment, total RNA was extracted from the pool of leaves and roots collected from three different plants. Transcript levels of SA-responsive (\textit{PR-1}, \textit{PR-2} and \textit{PR-5}), JA-responsive (\textit{Vsp}), ET-responsive (\textit{Hel}) and JA-/ET-responsive (\textit{PDF1.2}) marker genes were analyzed by RT–PCR using primers specific for each gene. A constitutively expressed \textit{\beta}-tubulin (\textit{Tub}) (Snustad et al. 1992) was used as a control in RT–PCR analysis.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{fig8}
\caption{Local and systemic expression of defense-related genes in Arabidopsis plants in response to treatment with PDB (control) or CF of \textit{P. simplicissimum} GP17-2. RNA was extracted from leaves and roots of 2-week-old Arabidopsis plants harvested at the indicated time points after treatment with either PDB or CF of GP17-2. Transcript levels of SA-responsive (\textit{PR-1}, \textit{PR-2} and \textit{PR-5}), JA-responsive (\textit{Vsp}), ET-responsive (\textit{Hel}) and JA-/ET-responsive (\textit{PDF1.2}) marker genes were analyzed by RT–PCR using primers specific for each gene. A constitutively expressed \textit{\beta}-tubulin (\textit{Tub}) (Snustad et al. 1992) was used as a control in RT–PCR analysis.}
\end{figure}
with *Pst*. Even though there was strong PR gene activation in leaves of BTH-pre-treated plants (Fig. 7), the response was not further enhanced by subsequent stimulation by *Pst* attack (Fig. 9), nor were any of the JA-/ET-inducible genes. Similarly, in GP17-2-treated ISR-expressing plants, the SA-responsive marker genes *PR-2* and *PR-5* did not show additional stimulation after pathogen inoculation, but remained elevated due to the pre-treatment effect up to 2 d after pathogen inoculation compared with plants inoculated with pathogen only. In contrast, previously induced JA-/ET-responsive *PDF1.2* declined 1 d after inoculation and showed a dramatic elevation 4 d after infection in both treated and untreated plants. Additionally, although JA-responsive *Vsp* was not induced in GP17-2-treated plants, *Pst* infection resulted in greater expression of the *Vsp* gene in ISR-expressing plants. However, the enhanced transcript accumulation of the *Vsp* gene was only observed at later stages of infection beginning at 4 d post-infection. These results show that the expression pattern of SA-responsive genes is clearly different from that of JA-responsive genes.

In a hydroponic experiment, the leaves of CF-treated plants were also analyzed for defense-related genes after pathogen inoculation. Fig. 10 shows that pre-treatment with CF strongly induced SA-inducible PR genes as well as the ET-inducible *Hel* gene in leaves of Arabidopsis plants. Expression was considerably enhanced within 1 day after challenge. In comparison with PR genes, the JA- or ET-inducible *PDF1.2* gene was not induced in treated plants, and increased expression was observed only at 4 d post-infection. On the other hand, the transcript levels of the JA-inducible *Vsp* gene, which was induced in treated plants before *Pst* infection, were radically diminished within 1 d after challenge inoculation, followed by re-elevation 4 d after infection. This specific pattern of *PDF1.2* and *Vsp* gene expression was repeatedly observed in independent experiments, and is similar to results of our study with BGI in the soil system.

**Analysis of marker gene expression induced by GP17-2 and its CF in different defense mutants**

To explicate the role of SA, JA and ET signal transduction pathways in the regulation of genes inducible by GP17-2 and its CF, we characterized the induction pattern of marker genes in plants affected in these signaling pathways. GP17-2-induced defense responses in mutant plants were examined by studying the expression of *PR-2*, *PR-5* and *PDF1.2*, while CF-induced defense responses were investigated by analyzing the expression of *PR-1*, *PR-2*, *PR-5*, *Hel* and *Vsp* genes, since these genes were
found to be activated in Col-0 plants in response to treatment with GP17-2 and its CF.

As shown in Figs. 11 and 12, BTH treatment induced the expression of PR-1, PR-2 and PR-5 in the ET response mutant ein2, the JA-insensitive mutant jar1 and the SA-defective NahG plants, in both soil and hydroponic experiments. However, BTH treatment failed to induce full expression of PR genes in the npr1 plant. Although CF-induced expression of PR-1 was moderately blocked in SA-defective NahG plants in comparison with wild-type plants, it was completely eradicated in npr1 plants. In contrast, the induction of PR-2 and PR-5 by GP17-2 and its CF was not fully impaired in NahG and npr1 plants. The jar1 mutation had little effect on expression of PR genes, while expression was up-regulated in ein2 mutants, indicating that defective ET signaling provoked increased sensitivity to SA signaling. GP17-2-induced expression of PDF1.2 was abolished in jar1 and ein2 plants as well as npr1 plants (Fig. 11). Both CF- and BTH-induced expression of Hel remained unaffected in NahG, jar1 and ein2 plants (Fig. 12). However, expression was partially blocked in npr1 plants. Although CF-treated NahG plants showed reduced expression of Vsp in comparison with wild-type plants, the gene was still inducible in jar1 plants. In contrast, expression was considerably enhanced in ein2 plants, while it was eradicated in npr1 plants. These results suggest that full CF induction of the Vsp gene requires SA, acting in an NPR1-dependent system.

Discussion

Systemic resistance in plants is induced by PGPR via several different mechanisms. PGPF may also induce systemic resistance by a number of different mechanisms, although, due to limited research (Bent 2006), this remains unclear. In the present study, we have analyzed induced defense responses in the model plant Arabidopsis using a PGPF, P. simplicissimum GP17-2, and challenging with Pst. One of the experimental advantages of this study was the use of both living Penicillium and its CF as inducers for systemic resistance, making it possible to gain insight into the molecular mechanisms of ISR.

When Arabidopsis Col-0 plants were grown in soil containing GP17-2 or treated with BTH, bacterial speck disease caused by Pst was suppressed at a distance above the treatment site. Protection of the plant was manifested by both a reduction in disease severity and a decrease in pathogen proliferation in the leaves. GP17-2 suppressed Pst infection via induced systemic resistance, since disease reduction took place without direct contact between the two microorganisms. This was confirmed by evidence that GP17-2 used to treat the roots did not colonize the above-ground parts of Arabidopsis plants. This suggestion is further supported by the observation that treatment of the roots of hydroponically grown Arabidopsis with a cell-free filtrate also protected leaves against Pst. Since the CF did not affect pathogen proliferation (data not shown), the observed disease suppression seemed to be neither a direct
parasitic or antibiotic activity of GP17-2, nor an inhibitory effect of the filtrate. Apparently, signals originating in the roots reached the upper part of plant and activated defense mechanisms. This is the first report of typical PGPF-mediated ISR in the model plant Arabidopsis.

We made use of well-characterized mutants and transgenic plants to clarify the signaling pathways involved in *P. simplicissimum* GP17-2-mediated ISR. Mutants (*jar1* and *ein2*) defective in JA- and ET-dependent defense responses are more sensitive than wild-type plants to *Pst* infection. This, however, had no effect on the plants that were already induced with either GP17-2 or its CF. Our results with Arabidopsis expressing the NahG transgene or containing disruption in NPR1 (*npr1*) show that both plants underwent ISR after treatment with GP17-2 or CF, albeit that the level of protection was lower than that observed for wild-type plants. From these results, it appears that the SA-dependent pathway is partially required for the establishment of GP17-2-mediated resistance in plants. These observations may suggest the possible contribution of additional signaling pathway(s) in the regulation of GP17-2-mediated ISR, although they do not show what the additional pathway(s) is. On the other hand, consistent with previous observations (Lawton et al. 1996, Ryu et al. 2003), Arabidopsis NahG, *jar1* and *ein2* plants were protected by BTH, while *npr1* was not. Thus, the GP17-2-mediated ISR and BTH-induced SAR signaling pathways differ in their requirement for NPR1.

Our results also show that BTH-induced SAR was accompanied by strong systemic and relatively weak local induction of PR genes responsive to the SA pathway. Similarly, SA-inducible PR-2 and PR-5 genes have been found to be continuously expressed at a higher level in GP17-2-treated Arabidopsis leaves and roots, indicating that root colonization by GP17-2 promotes the up-regulation of defense genes involved in the SA-responsive pathway. Accumulation of PR protein or SAR gene expression in response to PGPR inoculation has been described in several plant-PGPR systems (Cartieux et al. 2003, Tjamos et al. 2005, Wang et al. 2005). In contrast, *Pseudomonas fluorescens* WCS417r, one of the most frequently studied PGPR in Arabidopsis, elicited ISR without activation of PR genes (van Wees et al. 1999). Thus, the systemic resistance induced by GP17-2 root colonization seems to be distinct from that reported in WCS417r-induced ISR, but overlaps to some extent with that observed in SAR. However, the results also show that GP17-2 triggers an additional defense response pathway, as evidenced by induction of the JA-/ET-dependent *PDF1.2* gene. Conversely, root colonization by GP17-2 was associated with a parallel down-regulation of the ET-inducible *Hel* gene in the roots. Interestingly, some positive transcription factors of ET-responsive genes were down-regulated in Arabidopsis roots colonized by WCS417r (Verhagen et al. 2004). Verhagen et al. (2004) postulated that the onset of ISR is associated with a reduction in ET signaling in roots. If this is correct, GP17-2-induced systemic resistance may partially share signaling pathways with WCS417r-induced ISR. Therefore, plant defense gene changes induced by GP17-2 overlap to some extent with those reported in typical SAR- or ISR-expressing plants, but appear to be distinct from either of them.

Treatment with CF resulted in enhanced expression of SA-dependent PR genes as well as the ET-inducible *Hel* and JA-inducible *Vsp* gene in the leaves, but not the roots, of Arabidopsis plants, suggesting that the systemic effect activating plant defense responses is more evident than the local effect. However, CF treatment revealed a slight deviation in the amount of co-expressed genes compared with GP17-2 treatment. This may be due to differences between these two experiments with regard to elicitor characters, plant age and treatment duration.

The increase in defense-related gene expression in response to GP17-2 and its CF treatment raised questions concerning to what extent these genes are activated following challenge infection with pathogen. Challenge inoculation of BTH-treated plants did not result in further stimulation of SA-inducible PR genes, indicating that BTH activates these genes by itself (Kohler et al. 2002). Similarly, GP17-2-treated plants also showed direct activation of SA-inducible PR-2 and PR-5 genes in comparison with challenged control over a period of 2 d after infection. In contrast, the expression of JA-/ET-inducible *PDF1.2*, interestingly, declined over the next few days after *Pst* infection and was re-elevated 4 d after infection without showing a distinct difference from the control plant. Moreover, even induction of the *Vsp* gene was not observed in ISR-expressing plants before pathogen inoculation, although transcript levels accumulated to greater levels in GP17-2-treated plants at later stages of *Pst* infection (4 and 6 d post-infection). Thus, systemically resistant Arabidopsis plants are primed for potentiated activation of the *Vsp* gene, which is subsequently triggered by phytopathogenic *Pst*, indicating that ISR is associated with potentiated expression of defense-related genes (priming) (reviewed in Conrath et al. 2006). Potentiation of SA-inducible PR genes, the ET-inducible *Hel* gene and JA-/ET-inducible *PDF1.2* was also observed in CF-treated plants infected with *Pst*, since these genes were further enhanced upon *Pst* infection. In contrast, the expression pattern of the JA-inducible *Vsp* gene in CF-treated plants was similar to that of the *PDF1.2* gene in GP17-2-treated plants after *Pst* infection. The fact that *PDF1.2* or *Vsp* genes initially declined after *Pst* infection but resurfaced at later stages of infection suggests that the expression pattern of JA-inducible genes was different from that of SA-inducible
genes and occurred in an apparently two-step sequential manner. The first step is elevation of SA responses with a parallel repression of JA responses, and the second step is a subsequent activation of JA responses. Transient accumulation of the JA-inducible transcript Vsp has been noted in WCS417r-treated Arabidopsis 1 d after infection with Pst (van Wees et al. 1999). This argues against our observation, since the expression of Vsp was exclusively detected at later stages of infection in repeated experiments. It is also noteworthy that Vsp genes are needed as a nitrogen source to synthesize various substances for plant defense and/or for healing damaged tissues (Utsugi et al. 1998). Thus, the delayed enhancement of Vsp transcripts may be significant for healing diseased tissues faster in ISR-expressing plants compared with untreated plants. This reason for PDF1.2 and Vsp mRNA levels increasing at later time points of Pst infection is most probably due to coronatine from Pst, which is believed to be a JA mimic (Weiler et al. 1994, Block et al. 2005). Therefore, it is likely that GP17-2 induces expression of these genes by weakly activating the JA pathway; next, the SA induced by Pst infection shuts down the JA pathway; and then coronatine from Pst overcomes SA suppression and turns on the JA pathway again.

Our results have shown that although SA is required for full CF induction of the PR-1 gene, SA did not appear to explain fully the induction of PR-2 and PR-5, since induction of these genes by GP17-2 or its CF was only partially eradicated in NahG plants. Other signals triggered by GP17-2 or its CF that involve ET may also interfere with the SA response, as evidenced by the elevated induction of PR genes in the ein2 mutant after treatment with GP17-2 or its CF. In contrast, CF induction of JA-dependent Vsp was reduced in NahG plants, while it was enhanced in the ein2 mutant. This reveals that SA is required for full CF induction of the Vsp gene, while ET inhibits induction of this gene by interfering with the SA pathway. Induced expression of Vsp in jar1 mutants resembled that seen in wild-type plants after CF treatment, which has been blocked in another JA signaling mutant, coil, during bacterial CF induction (Norman-Setterblad et al. 2000), indicating that multiple JA signaling pathways operate during the plant defense response (Oñate-Sánchez and Singh 2002). Induction of PDF1.2 by GP17-2 was eliminated in jar1 and ein2 mutants, confirming that both pathways are required for this response. Treatment with CF and BTH stimulated the expression of Hel in wild-type plants as well as in mutants in all three signaling pathways, indicating that Hel is activated in a manner independent of SA, ET and JA production/perception (Alméras et al. 2003). Furthermore, although Hel expression was observed in the leaves of 2-week-old BTH-treated plants, the induction was absent in 3-week-old treated plants. This indicates that regulation of the Hel gene may be controlled during different developmental stages, as observed for SA induction of the ET-responsive factor ERF13 and ERF1 (Oñate-Sánchez and Singh 2002).

The npr1 mutant plant showed complete inhibition of SA-inducible PR-1, JA-inducible Vsp and JA-/ET-inducible PDF1.2 gene expression. This indicates that npr1 can differentially regulate the downstream effects of both SA- and JA-/ET-inducible signaling pathways (Pieterse et al. 1998). However, the npr1 plant was not fully impaired in induction of GP17-2- or its CF-mediated ISR as well as PR-2, PR-5 and Hel genes. Zhang and Shapiro (2002) have shown that PR-5 induction differs from SAR or PR-1 induction, and that mutant npr1 is not fully impaired in SAR induction or PR-5 gene expression in response to P. syringae carrying avrB. It is noteworthy that none of the characterized npr1 mutants has a large deletion (Cao et al. 1997, Ryals et al. 1997). Therefore, the induction of resistance in npr1 plants by GP17-2 or its CF could be due to residual function of any of these gene products, which individually or in combination contribute to resistance.

In conclusion, our results show that blockage of SA accumulation or the SA signaling pathway in Arabidopsis only partially attenuates, but does not abolish, GP17-2-mediated ISR to Pst. The observation that the ISR effect is only partially dependent on SA is a reasonable basis to argue that multiple pathways are involved, although it does not specify what the other pathway(s) might be. Activation of SA-, JA- and ET-inducible genes in plants treated with GP17-2 or its CF also implies that not only SA, but other signals as well, may play an important role in inducing resistance. Induction of multiple defense signals has been found for several PGPR (Ahn et al. 2001) as well. This may be explained by the fact that these microorganisms produce multiple pathogen-associated molecular patterns (PAMPs), each of which is recognized by different receptors and activates different pathways.

Materials and Methods

Origin of seeds, pathogen and inoculator

Seeds of A. thaliana ecotype Columbia (Col-0) were provided by K. S. Park (NIAST, Suwon, Korea). The mutants ein2, jar1 and npr1 were obtained from the Nottingham Arabidopsis Stock Center. The transgenic line NahG was a personal gift. All the mutants and transgenic Arabidopsis were generated in the Col-0 background. The rifampicin-resistant virulent pathogen Pst was provided by Y. Ichinose (Okayama University, Okayama, Japan). The PGPF P. simplicissimum GP17-2 was isolated from the rhizosphere of zoysia grass (Z. tenusifolia).

Cultivation of plants in soil for the barley grain inoculum experiment

Arabidopsis plants were grown in commercial Star bed potting medium (soil-less, peat-based potting medium, Kyohohiro Co. Ltd, Aichi, Japan), which contains humus, peat, rock phosphate and composted plant materials. After autoclaving twice at 24 h intervals at 121 °C for 1 h, about 40 g of the potting
medium was placed in each sterilized paper pot. Five seeds of Arabidopsis plants were sown in each pot. Only one plant was allowed to grow in each pot and the rest of the plants were thinned out after 5 d of germination. Plants were cultivated in a growth chamber with a 9 h day and 15 h night cycle at 23.5°C and watered on alternate days.

Hydroponic culture of plants for the culture filtrate experiment
Hydroponic culture was carried out using a culture apparatus, which was developed by Toda et al. (1999). This apparatus contains a plastic photo slide mount (Fuji film, Japan) and a mesh (50 holes per inch) made of thick nylon wire. The basic culture solution consisted of 1/10 strength of MGRL nutrients (Fujiwara et al. 1992). Seeds of Arabidopsis were soaked in 0.5 ml of distilled water in Eppendorf tubes, and kept in a refrigerator for 3 d at 4°C to synchronize germination. Then 24 seeds, each in one line, were placed on the apparatus. All apparatus were floated on 5 liters of nutrient solution and kept in the growth chamber at 20°C with a 9 h day and 15 h night cycle at 23.5°C. After 5 d of germination, plants were cultivated in a growth chamber with a 9 h day and 15 h night cycle at 23.5°C and watered on alternate days.

Preparation of inducer isolates
Arabidopsis roots were treated with the following two forms of PGPF: BGI and CF.

Barley grain inoculum (BGI).
Autoclaved barley grains (100 g in 100 ml of water in a 500 ml Erlenmeyer flask) were inoculated with 10–15 disks (5 mm) obtained from the actively growing margin of 7-day-old potato dextrose agar (PDA) (2% agar) cultures of *P. simplicissimum* GP17-2. After 10–12 d of incubation at 25°C in the dark, the completely colonized barley grains were air-dried at laboratory temperature (23–25°C). The dried BGI was ground to a 1–2 mm particle size and stored at 4°C until use.

Culture filtrate (CF).
*Penicillium simplicissimum* GP17-2 was grown in PDA medium for 7 d. Twenty mycelial disks of GP17-2 culture were obtained from the growing margin of a colony on PDA and transferred to a 500 ml Erlenmeyer flask containing 200 ml of PDB. The fungus was cultured without shaking at room temperature (25°C) for 10 d. The culture filtrate was separated from mycelia and filtered through two layers of Whatman No. 2 filter paper. The CF was then filtered sterilized (0.22 µm Millipore filters, Millipore Products Division, Bedford, MA, USA).

Induction treatments with BGI and CF
Arabidopsis plants grown in soil were treated with ISR-inducing *P. simplicissimum* GP17-2 by mixing BGI of GP17-2 with potting medium to a concentration of 0.5% (w/w) just before the seeds were sown. Potting medium supplemented with an equal volume of autoclaved barley grain was used as a control.

In the case of hydroponically grown plants, induction treatment was performed 1 d before challenge inoculation by dipping the roots of 2-week-old seedlings in 50% diluted CF of GP17-2 for 1 h. Control plants were treated with 50% diluted PDB.

Root colonization
Root colonization was determined after 3 weeks of treatment application. Roots were harvested from nine randomly selected plants, washed free of soil with running tap water, rinsed three times in sterilized distilled water and blotted dry. Roots were then cut into about 1 cm long segments, plated on PDA (20 segments per plate) amended with chloramphenicol (200 mg·l⁻¹) and incubated for 2 d at 25°C. After incubation, colonies of GP17-2 growing from root segments were counted and the isolation frequency was determined as described by Meera et al. (1994). Root colonization ability was measured based on isolation frequency.

Chemical induction treatment
Arabidopsis plants grown in soil were treated with 0.3 mM of the chemical inducer, BTH (Novartis Agro, Tokyo, Japan) by soil drenching 1 d prior to challenge inoculation. However, BTH treatment of Arabidopsis plants grown in a hydroponic system was performed by dipping the roots in 5 mM BTH solution 1 d prior to pathogen inoculation.

Challenge inoculation and disease assessment
Plants were challenge inoculated at 3 weeks of age in a soil system. In contrast, in the hydroponic system, pathogen inoculation was done 1 d after induction treatment. One day before challenge, the plants were placed at 100% relative humidity. The virulent bacterial pathogen *Pst* was cultured in liquid King's medium B (KB) at 28°C. After overnight incubation, cells were collected by centrifugation, washed twice and resuspended in 10 mM MgSO₄. Inoculation was carried out by spraying the *Pst* suspension containing 2.5 × 10⁶ c.f.u. ml⁻¹ bacteria, and 0.01% (v/v) Silwet L-77 (Nihon Unika, Tokyo, Japan) as detergent on to the rosette leaves until run-off. The inoculated plants were kept at 100% relative humidity in darkness for 2 d in order for disease to develop. Plants were then transferred to a growth chamber. Five days after pathogen challenge, disease severity was measured for each plant by recording the percentage of total plant leaf surface showing symptoms from 0 = no symptoms to 100 = most severe with necrotic symptoms. The number of *Pst* in inoculated leaves was assessed by collecting all leaves separately from three replications. Leaves were then weighed, rinsed thoroughly in sterile water and homogenized in sterilized distilled water. Subsequently, appropriate dilutions were plated onto KB medium supplemented with 50 mg·l⁻¹ rifampicin. After 48 h of incubation at 28°C, the number of rifampicin-resistant c.f.u. per gram of infected leaf tissues was determined.

RT–PCR analysis
RNA was isolated from Arabidopsis tissues using the modified technique of Suzuki et al. (2004). Leaves of randomly selected plants were placed together in 1.5 ml Eppendorf tubes and frozen in liquid nitrogen. Using a drill driver with a pellet mixer at the tip, the tissues were ground and homogenized with extraction buffers: 100 mM Tris–HCl (pH 9.5), 10 mM EDTA (pH 8.0), 2% (w/v) lithium dodecyl sulfate, 0.6 M NaCl, 0.4 M trisodium citrate and 5% (v/v) 2-mercaptoethanol. The aqueous phase resulting from centrifugation (≥12,000 × g) at room temperature was re-extracted with a chloroform:isoamylalcohol (24:1) mixture. Water-saturated phenol containing 35% (w/v) of guanidium thiocyanate and 0.1 vol. of 2 M sodium acetate (pH 4.0) was added to the collected supernatant. After centrifugation at 4°C for 5 min, the upper phase was transferred to a new tube, mixed with 0.6 vol. of isopropanol and incubated for 10 min at room temperature. The precipitated RNA was collected by centrifugation at 4°C for 15 min, washed with 75% (v/v) ethanol, air-dried briefly and dissolved in RNAse-free water. After treatment with RNAse-free DNase and inactivation of the DNase according to the supplier’s instructions (TAKARA BIO INC., Shiga, Japan), approximately 1 µg of total RNA was reverse transcribed in the presence of oligo(dT)₁₂ primer and 25% (v/v) TE buffer was added to a final volume of 100 µl. An aliquot of the
obtained cDNA (1 μl) was used as a template in a 10 μl PCR performed with 0.25 U of rTaq DNA polymerase (TAKARA BIO INC., Shiga, Japan), as described by Suzuki et al. (2004). For each pair of specific primers, the number of cycles needed to perform the amplification within the exponential phase was determined. The PCR products were resolved on an agarose gel stained with ethidium bromide to visualize the gene expression level. The gene-specific primer pairs used were PR-1 (5’-GTAGGTCGCTTGTTCTTCC-3’; 5’-TTACGATAATTCGCCAGGAG-3’) (Pieterse et al. 1998), PR-2 (5’-TCAGGAAGGTCCAGGATG-3’; 5’-TCCGTGTAT CCATTCCTCA-3’), Hel (5’-GTACACCGCGCAGACTCTG T-3’; 5’-CAATGAGATGCGCTTGTGA), PR-5 (5’-ATGGC AAATATCTCCGATATCCAC-3’; 5’-ATGGCTGGGCGAAG CGCGTTAGG-3’) (Ohate-Sánchez and Singh 2002), Vsp (5’-TTTACGCCAAAGGACTGTGC-3’; 5’-ATCCGAGGTCCACAGGTT-3’), PDF1.2 (5’-AATAGCTCTCCTAGCTAAGITT GTTCAC-3’; 5’-AATCCATGGAAATACACGATTTGACAC C-3’) (Penninckx et al. 1996) and b-ub crystallin (5’-CTGGGATCTCAGCAATACAGAGCC-3’; 5’-CCTCTTCGACTTTCACCTGC TCTTC-3’).

Statistical analysis

The experimental design was completely randomized, consisting of three replications for each treatment. The experiment was repeated at least twice, and treatment means obtained were separated using a Fisher’s least significant difference (LSD) test. However, when comparing two or more means, the values were separated using Dunnet’s multiple comparison test (when appropriate). All analyses were done at P<0.05.

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References


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