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**Resistant and Susceptible Responses in Tomato to Cyst Nematode are Differentially Regulated by Salicylic Acid**

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**Abbreviations:**

ADH, alcohol dehydrogenase; DK, Doctor-K; dpi, days postinoculation; ET, ethylene; HR, hypersensitive response; JA, jasmonic acid; J2, second-stage juvenile; KB, Kyouryoku-Beijyu; MC, Mini Carol; MF, Momotaro Fight; PAL, phenylalanine ammonia lyase; PCN, potato cyst nematode; PDC, pyruvate decarboxylase; PR protein, pathogen-related protein; RT-PCR, reverse transcription-PCR; SA, salicylic acid; SAG, salicylic acid glucoside; SAGE, serial analysis of gene expression; SAR, systemic acquired resistance; SL, Sugar Lamp; YC, Yellow Carol.

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**Abstract**

To understand the machinery underlying a tomato cultivar harboring the *Hero A* gene against cyst nematode using microarrays, we first analyzed tomato gene expression in response to potato cyst nematode (PCN; *Globodera rostochiensis*) during the early incompatible and compatible interactions at 3 and 7 days postinoculation (dpi). Transcript levels of the phenylalanine ammonia lyase (*PAL*) and *Myb*-related genes were upregulated at 3 dpi in the incompatible interaction. Transcription of the genes encoding pyruvate decarboxylase (*PDC*) and alcohol dehydrogenase (*ADH*) was also upregulated at 3 dpi in the incompatible interaction. On the other hand, the four genes (*PAL*, *Myb*, *PDC* and *ADH*) were downregulated in the compatible interaction at 3 dpi. When the expression levels of several pathogenesis-related (PR) protein genes in tomato roots were compared between the incompatible and compatible interactions, the SA-dependent *PR* genes were found to be induced in the incompatible interaction at 3 dpi. The *PR-1*(*P4*) transcript increased to an exceptionally high level at 3 dpi in the cyst nematode-infected resistant plants compared to the uninoculated controls. The free SA levels were elevated to similar levels in both incompatible and compatible interactions. We then confirmed that *PR-1*(*P4*) was not significantly induced in the *NahG* tomato harboring the *Hero A* gene, compared with the resistant cultivar. We thus found that *PR-1*(*P4*) was a hallmark for the cultivar resistance conferred by *Hero A* against PCN and that nematode parasitism resulted in the inhibition of the SA signaling pathway in the susceptible cultivars.

**Keywords:** Cyst nematode, *Globodera rostochiensis*, Plant pathogen, Resistance, Salicylic acid, Tomato

## Introduction

Plant parasitic cyst nematodes (*Globodera* and *Heterodera* spp.), which are sedentary and highly specialized endoparasites, are one of the most damaging agricultural pests in the world (Evans and Rowe 1998). Cyst nematodes spend most of their parasitic lifetime inside the root system of a host plant, feeding on a nematode-induced conglomerate of metabolically active root cells, known as the syncytium (Gheysen and Fenoll 2002). These specialized feeding cells are formed after the invading juveniles have become sedentary and injected secretions into plant cells, resulting in the enlargement of cells, breakdown of cell walls and the formation of the syncytium.

A number of studies have been conducted using large-scale host-gene-expression profiling to identify the plant genes that are differentially regulated by nematode infection during a compatible interaction (Alkharouf et al. 2006, Ithal et al. 2007a, b, Uehara et al. 2007, Szakasits et al. 2009). Many genes related to metabolic pathways, including phytohormone regulation, cell cycle alterations and cell wall architecture seem to be upregulated through the establishment of the syncytium. Moreover, phytohormones, such as auxin and ethylene (ET), are involved in the formation of syncytia and host susceptibility (Goverse et al. 2000, Wubben et al. 2001).

In agriculture practice, nematodes are controlled by crop rotation and use of nematocides and resistant cultivars. The exploitation of host resistance is the most effective and environmentally benign method for managing plant nematodes.

Resistant cultivars, having an incompatible interaction with pathogenic nematodes, are useful for controlling nematodes (Cook and Evans 1987, Trudgill 1991). Although

syncytia form during both resistant and susceptible reactions, the subsequent responses during the incompatible and compatible interactions differ. In susceptible roots, the syncytium continues to develop and expand, whereas a hypersensitive response (HR)-like cell death is initiated in resistant roots after the formation of the syncytium, which becomes surrounded by a layer of necrotic cells about 4 days after root invasion (Rice et al. 1985, Williamson and Hussey 1996, Sobczak et al. 2005). The syncytium then degenerates. This HR-like cell death is one of the various defense mechanisms that resistant cultivars have evolved to protect themselves against cyst nematodes. They also have a highly specialized resistance (*R*)-gene-mediated defense. The *R* gene in the host plant interacts specifically with the corresponding avirulence gene in the nematode. This gene-for-gene interaction results in the initiation of a cascade of defense responses.

Several *R* genes against cyst nematodes have recently been isolated and characterized. These include *Hs1*$^{pro-1}$ from sugar beet (Cai et al. 1997), *Cre3* from wheat (Lagudah et al. 1997), *Cpa2* and *Gro1* from potato (Van der Vossen et al. 2000, Paal et al. 2004), and *Hero A* from tomato (Ernst et al. 2002). All genes, except *Hs1*$^{pro-1}$, belong to the leucine zipper, nucleotide-binding site, leucine-rich repeat class of plant *R* genes (Martin et al. 2003). While knowledge about the nature of *R* genes against cyst nematodes has accumulated in recent years, any specific molecular mechanisms mediating incompatible plant–cyst nematode interactions are as yet unknown.

Salicylic acid (SA) and jasmonic acid (JA) are well known to regulate both basal and *R*-gene-mediated defense responses against pathogens and insects. (Glazebrook 2001,Hammond-Kosack and Parker 2003, Glazabrook 2005, Kim et al. 2008).

SA-dependent signaling seems to be crucial for resistance against biotrophic pathogens (Glazebrook 2005, Loake and Grant 2007). In contrast, JA-dependent resistance appears to be more effective against necrotrophic pathogens and insects (Keeler and Baldwin 2002, Glazebrook 2005). However, it is not yet clear whether the signal transduction pathways and defense strategies triggered by nematodes during root infection are the same as those observed in the resistance against biotrophic foliar pathogens (Williamson and Kumar 2006). Very little is known about the importance of SA- and JA-mediated signaling in root biotrophic interactions (Gutjahr and Paszkowski 2009).

In tomato, SA induces pathogenesis-related protein genes such as *PR-1*(*P4)*, *PR-1*(*P6*) and *PR-7*. PR-1 proteins belong to small, multigenic families. Both *PR-1*(*P4)* and *PR-1*(*P6*) inductions are used as a molecular marker for the activation of the SA signaling pathway (Jordá et al. 1999, Spletzer and Enyedi 1999, Sobzak et al. 2005, Hase et al. 2008). *PR-7* encodes a subtilisin-like protease (Zhao et al. 2003). JA induces the *PR-6* and *TPI-1* genes, which encode proteinase inhibitor II and I, respectively (Hondo et al. 2007, Hase et al. 2008). The *PR-5* gene encoding osmotin precursor has been reported as not inducible by SA in tomato (Van Kan et al. 1995, Sobzak et al. 2005).

To develop alternatives to environmentally damaging chemicals, we need to elucidate the molecular mechanisms underlying the plant defense responses against cyst nematodes. Recently, we found several tomato cultivars with strong resistance to potato cyst nematode (PCN; *Globodera rostochiensis*) (Uehara et al. 2008). In the present study, we confirmed that these resistant tomato cultivars contained the *Hero A* gene, while susceptible cultivars did not. Using these resistant and susceptible tomato cultivars, we performed microarray

analyses of incompatible and compatible host–PCN interactions. To understand the plant defense signaling pathways during infection, we analyzed the expression profiles of SA-, JA-dependent marker genes in response to PCN in roots of several resistant and susceptible tomato cultivars. To reduce endogenous SA, we introduced the *NahG* gene into the resistant tomato containing the *Hero A* gene and analyzed the expression of *PR1(P4)*. Based on these results, we found direct evidence for the involvement and importance of the SA pathway in the *Hero A*-mediated PCN defense.

## Results

### Reproduction of PCN on susceptible and resistant tomato

To investigate the resistance of tomato, we inoculated two commercial cultivars (resistant Doctor-K [DK] and susceptible Kyouryoku-Beijyu [KB]) with second-stage juveniles (J2) of PCN. The morphology and growth process of the seedlings of the two tomato cultivars are very similar. When cysts were isolated from the soil after 90 days, we observed significantly fewer cysts on DK than on KB ($P < 0.001$) (Fig. 1A and 1B). Because plants are defined as resistant to nematodes when they depress nematode reproduction (Trudgill 1991), DK is certainly a resistant cultivar. When segregated progenies from the selfing of DK were inoculated with PCN, the progenies segregated into 24 resistant and six susceptible plants, fitting the 3 : 1 ratio expected for single-gene inheritance ( $\chi^2 = 0.4$, $p = 0.53$). The mean numbers of cysts differed significantly between susceptible and resistant plants ($P < 0.001$) (Fig. 1C and D).

Primer pair HERO_F2 and HERO_R3, based on the sequence of *Hero A* designed by

Cabrera Poch et al. (2006), was used. Cloning and subsequent sequencing of the RT-PCR fragment revealed that the sequence of the RT-PCR product from the resistant cultivars were identical to that of *Hero A.* When the genomic DNA was PCR-amplified for the *Hero A* gene, we detected two DNA fragments from the resistant cultivars and one fragment from the susceptible cultivars (Fig. 1E). These results were congruent with the results of Cabrera Poch et al. (2006).

**Expression profiling of tomato genes responsive to PCN**

The two cultivars, DK and KB, were also used to profile gene expression to understand the mechanisms of plant defense of DK against PCN. Four microarray analyses were performed to investigate the transcriptome profiles in the incompatible and compatible interactions at two times (3 and 7 days postinoculation [dpi]). At 3 dpi, the syncytium is induced, and at 7 dpi, the syncytium in resistant cultivars collapses (Rice et al. 1985, Sobczak et al. 2005). The selected genes were considered to be differentially regulated if the signal intensity ratios on the microarray had changed by more than 2.5-fold. In total, we obtained 164 genes after removal of redundant transcripts and statistically nonsignificant data. These genes were considered to be involved directly or indirectly in the defense responses to PCN. At 3 dpi, 34 genes were upregulated in the incompatible interaction, and 28 genes were upregulated in the compatible interaction. Among these upregulated genes, the two cultivars had three upregulated genes in common (Fig. 2A). At 7 dpi, 38 genes were upregulated in the incompatible interaction as opposed to 39 genes in the compatible interaction. Among these upregulated genes, 24 were common to the two

cultivars (Fig. 2B).

The results of cluster analysis for significantly (>2.5-fold) differentially induced genes in the compatible and incompatible interactions are shown in Fig. 3. Many transcripts of the genes involved in the defense against the cyst nematode were activated at 3 dpi in the incompatible interaction. For example, in the early incompatible interaction at 3 dpi, the transcript levels of phenylalanine ammonia lyase (*PAL*) and *Myb*-related genes were higher than those in the compatible interaction. In addition, transcription of the genes encoding pyruvate decarboxylase (*PDC*), alcohol dehydrogenase (*ADH*) and sucrose synthase was induced at 3 dpi in the incompatible interaction.

**Genes differentially expressed in compatible and incompatible interactions**

To confirm the results obtained from the microarray analysis, five genes were analyzed using a semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). First, we selected two genes (*PAL* and *Myb*-related genes) that were upregulated at 3 dpi in the incompatible root according to the expression profiles in the microarray analysis and evaluated the microarray data by semiquantitative RT-PCR (Fig. 4). The results confirmed that these two genes were induced at 3 dpi in the incompatible interaction. It is noteworthy that the two genes were downregulated in the compatible interaction at 3 dpi and that there was no difference between the transcripts levels in the compatible and incompatible interactions at 7 dpi. The genes encoding *PDC*, *ADH* and sucrose synthase were also confirmed to be induced at 3 dpi in the incompatible interaction by semiquantitative RT-PCR (Fig. 4). The *PDC* and *ADH* genes were also downregulated in the compatible

interaction at 3 dpi.

**Expression of *PR-1* (*P4*) is correlated with the resistance of the tomato cultivars to PCN**

The microarray approach is useful for a broad inventory of plant processes that are altered as a result of nematode infection, but because it is a hybridization-based method, it cannot necessarily accurately distinguish expression patterns of homologous members of individual gene families such as *PR-1*(*P4*) and *PR-1*(*P6*). In addition, not all the *PR* protein genes were on the microarray chip; we therefore further analyzed the expression profiles of SA- and JA-dependent *PR* protein genes in response to cyst nematode in roots of tomato cultivars DK and KB using a multiplex quantitative RT-PCR assay.

In the incompatible interaction of DK (*Hero A*) infected with PCN, transcripts of SA-dependent PR genes such as *PR-1(P4*) and *PR-1(P6*) accumulated faster (3 dpi) and more abundantly than in the compatible interaction of KB (Fig. 5). Conversely, transcripts of JA-dependent PR genes such as *PR-6* and *TPI-1* accumulated more slowly (7 dpi) and more abundantly in the compatible interaction of KB infected with PCN than in the incompatible interaction of DK (Fig. 5). In particular, the *PR-1*(*P4*) transcript levels increased markedly at 3 dpi in the cyst nematode-infected resistant plants compared to the uninoculated controls (Fig. 5). Furthermore, we also analyzed the expression of those PR protein genes in other susceptible cultivars (Momotaro-Fight [MF], Magnet [MA], Momotaro-8 [M8]) and *Hero A*-mediated resistant cultivars (Yellow-Carol [YC]), Mini-Carol [MC] and Sugar-Lamp [SL]). As a result, the *PR-1*(*P4*) transcript levels

increased markedly at 3 dpi in all three nematode-infected resistant cultivars but not in the susceptible cultivar (Fig. 6). In addition, we found that the expression levels of JA-dependent *PR-6* were higher in the susceptible cultivars than in the resistant cultivars (Fig. 6).

**Quantification of free SA in tomato roots and leaves after cyst nematode infection**

Because *PR-1*(*P4*) transcript levels were markedly elevated at 3 dpi in roots of cyst-nematode-infected resistant cultivars in comparison to susceptible cultivars, we examined free SA levels in roots and leaves of the resistant and susceptible cultivars at 3 dpi using the method of Matsuura et al. (2008). Total SA levels could be mainly considered to include inactive SA derivatives such as salicylic acid glucoside (SAG), and so it is important to measure active free SA levels (Seo et al. 1995, Huang et al. 2006). Free SA in root tissues of PCN-infected and control plants of resistant and susceptible cultivars were elevated 1.5-fold in both the resistant and susceptible cultivars after cyst nematode infection (Fig. 7A). Free SA in leaf tissues of PCN-infected and control plants were elevated 2-fold in both the resistant and susceptible cultivars after cyst nematode infection (Fig. 7B). Thus the SA levels were lower in roots than in leaves, but significantly ($P < 0.001$) higher levels of free SA were detected in roots and leaves of PCN-infected plants compared to the control plants. It is thus clear that the levels of free SA in the resistant and susceptible tomato cultivars increased after PCN infection.

***NahG* suppresses *PR-1*(*P4*) expression and resistance to PCN in the *Hero A* tomato**

**root during cyst nematode infection**

To test the interplay between elevation of the *Hero A*-dependent *PR-1*(*P4*) transcript and endogenous SA levels, we crossed tomato cv. SL (*Hero A*/*Hero A*) with cv. Moneymarker [MM] containing the *NahG* transgene (Brading et al. 2000). To confirm that the F1 progeny were hybrids, DNA was isolated and analyzed by PCR using the primer pair HERO_F2 and HERO_R3 to detect *Hero A* (Cabrera Poch et al. 2006) and primer pair NahG_F and NahG_R to detect *NahG*. All the F1 progeny tested had both the *Hero A* and *NahG* genes, indicating that they were true hybrids. In this study, the transcript of *PR-1*(*P4*), SA-inducible gene, increased markedly in the cyst nematode-infected *Hero A* plants (Figs. 5 and 6). In the *Hero A-NahG* plants, the levels of the *PR-1*(*P4*) transcripts after PCN infection were evaluated. As expected, the results showed that there was little difference between the *PR-1*(*P4*) transcripts levels in the susceptible and the *Hero A-NahG* plants (Fig. 8), indicating that the induction of the *PR-1(P4*) transcripts by PCN infection is at least partially mediated by SA.

As shown in Fig. 8B, we observed a number of cysts on the *Hero A-NahG* plants at 50 dpi. Very few cysts were observed on the SL plants (*Hero A*-resistant), suggesting that SA induces not only the level of *PR1(P4)* but also the resistance to PCN in the *Hero A* tomato.

**Discussion**

The resistant tomato cultivars used in this study were found to have the *Hero A* gene. The PCN resistance gene, called *Hero A*, was previously identified and cloned by map-based

cloning and a complementation test (Ernst et al. 2002). By studying the commercial cultivars that we found to be PCN-resistant (Uehara et al. 2008), we can begin to elucidate the role of SA and JA signaling in PCN infection. Although several studies to dissect signal transduction in plant–nematode interactions have focused on the roles of the SA and JA pathways (Branch et al. 2004, Bhattarai et al. 2008, Wubbenn et al. 2008), the detailed mechanisms underlying incompatible plant–nematode interactions are still not known. We thus used microarray analyses to identify the genes differentially regulated in a resistant tomato cultivar to PCN and gained insight into the dynamics of the defense response of tomato plants to PCN.

**Differential activation of defense-related genes in susceptible and resistant tomato cultivars infected with PCN**

The phenylpropanoid pathway is considered to be closely related to plant defense against nematode infection (Giebel 1982, Edens et al. 1995). The transcription level of the phenylalanine ammonia lyase (*PAL*) gene increased in the resistant tomato at 3 dpi but not in the susceptible cultivar. Our data are consistent with several reports that have suggested a key role for *PAL*-mediated metabolism in nematode resistance (Giebel 1973, Brueske 1980, Edens et al. 1995, Klink et al. 2007). We also found in this study that *Myb*-related genes were induced at 3 dpi in an incompatible interaction but not in a compatible interaction. Klink et al. (2007) also found that the *Myb*-related genes were upregulated at 3 dpi in an incompatible soybean–soybean cyst nematode (*Heterodera glycines*) interaction. Several studies have suggested that Myb transcription factor is involved in HR- and

SA-induced resistance (Yang and Klessig 1996, Vailleau et al. 2002, Raffaele et al. 2006). With further analysis, we found that the expression levels of *PAL* and *Myb*-related genes actually decreased in a susceptible tomato after PCN infection, suggesting that the cyst nematode can suppress the host defense response.

Furthermore, the genes encoding *PDC* and *ADH* were also confirmed to be induced at 3 dpi in the incompatible interaction. PDC catalyzes the decarboxylation of pyruvate to acetaldehyde and $CO_2$ during the first step of ethanolic fermentation. Acetaldehyde is then converted to ethanol by the action of ADH. The *PDC* in potato plant carrying resistance gene *R1* against *Phytophthora infestans* was induced by SA treatment or elicitor (Nakane et al 2003). Transgenic potato plants expressing bacterial *PDC* developed HR-like cell death, deposited callose, and acquired resistance to *Phytophthora infestans* (Tadege et al. 1998). Because *PDH* and *ADH* transcripts accumulated at 3 dpi in the incompatible interaction, acetaldehyde accumulation could also participate in defense signaling in tomato roots. These two genes were also downregulated in the compatible interaction at 3 dpi, suggesting that the cyst nematode can suppress the host defense response. Sucrose synthase was also induced in an incompatible interaction. Tagede et al. (1998) proposed that sugar metabolism played a crucial role in the execution of cell death programs in plants. Sugar metabolism might be involved in the resistance response.

**Defense signaling pathways**

The SA- and JA-regulated defense pathways are known to be important for both basal and *R*-gene-mediated resistance to foliar pathogens and insects (Glazebrook, 2005). However,

it is not clear whether these signaling molecules are generally effective for cyst nematode in the root. Prominent PR-1 proteins are often used as markers of the enhanced defensive state conferred by pathogen-induced systemic acquired resistance (SAR). Similarly, we analyzed the expression profiles of SA-, JA-inducible marker genes—two *PR-1*(*P4* and *P6*), *PR-5*, *PR-6*, *PR-7* and *TPI-1* in tomato.

In the incompatible interaction infected with PCN, SA-dependent PR gene transcripts accumulated faster (3 dpi) and more abundantly than in the compatible interaction. Common among several resistance cultivars at 3 dpi was the marked elevation of *PR-1*(*P4*). Sobczac et al. (2005) investigated the expression of *PR-1*(*P4*) in *Hero A* tomato in response to PCN and also found that the gene was upregulated in a resistant tomato cultivar at 3 dpi, in agreement with our observation. Until now, the role of *PR-1*(*P4*) in plant defense had been not well defined, but here we successfully demonstrated that the expression of SA-inducible *PR-1*(*P4*) is indeed associated with tomato resistance to PCN. We thus consider that *PR-1(P4)* is one of the hallmark genes associated with the SA-signaling pathway. When we measured the free SA levels in the resistant and susceptible cultivars, we found that in the two types of cultivars differed little in free SA levels in response to PCN infection. Therefore, we hypothesize that cyst nematode parasitism in the susceptible cultivars must somehow block the SA-inducible transcriptional induction of *PR-1*(*P4*), whereas *PR-1*(*P4*) was clearly upregulated by the SA elevation in the resistant cultivars.

To obtain direct evidence for the links between *Hero A*-dependent *PR-1*(*P4*) transcript and SA, we created *Hero A-NahG* tomato. As a result, the expression of *NahG*

actually suppressed the *PR-1*(*P4*) transcript accumulation after cyst nematode infection in the *Hero A-NahG* tomato. We thus conclude that SA can induce *PR-1*(*P4*) in the resistant cultivars. In addition, in the PCN inoculation experiments, we showed that PCN could reproduce more efficiently in the *Hero A-NahG* plants than in the *Hero A* plants, providing evidence that SA directly controls the *Hero A*-mediated PCN resistance.

Involvement of SA in the resistant response is also supported by the recent data on *Arabidopsis thaliana* mutants infected by beet cyst nematode, *Heterodera schachtii* (Wubben et al. 2008). They reported that a number of SA-deficient mutants were more susceptible than the wild type to the cyst nematode. In turn, resistance to the cyst nematode in the *npr1*-suppressor mutant *sni1* was elevated. Furthermore, an SA application indeed reduced parasitism by cyst nematodes (Kempster et al. 2001, Wubben et al. 2008), indicating that SA plays at least some role in the resistance to the nematode.

For JA, we observed that the expression level of *PR-6*, a JA-dependent PR, was higher in susceptible cultivars than in the resistant cultivars after PCN infection (Figs. 6 and 7). In addition, several protease inhibitors, senescence-associated protein and seed-specific protein, which are all associated with JA, increased in the compatible interaction (Uehara et al. 2007). We previously reported that several ET-induced genes, which are activated by ripening, were upregulated in the compatible interactions (Uehara et al. 2007). Wubben et al. (2001) reported that ET signal transduction positively influenced plant susceptibility to cyst nematodes. In cooperation with JA, ET antagonizes the SA-dependent resistance against biotrophic pathogens. Therefore, hormones such as JA and ET in the susceptible cultivars are likely to interfere with the tomato SA-inducible

PCN resistance pathway. In addition, we suggest that auxin also perturbs the SA-inducible pathway in the susceptible cultivars. Auxin and SA have actually been reported to be mutual antagonists in disease resistance (Wang et al. 2007, Zhang et al. 2007). Auxin itself has been found to be crucial for syncytium induction, morphogenesis and development (Goverse et al. 2000, Karczmarek et al. 2004). According to our microarray analysis, auxin response factor (AFR) was 2.4-fold induced in the compatible interaction.

Phytohormones thus participate in various stages of nematode parasitism such as invasion and induction of the syncytium, and they have complex networks of synergistic and antagonistic interactions. We thus believe that cultivar resistance to PCN is triggered essentially by SA, resulting in an elevated level of *PR-1*(*P4*), a hallmark gene for this type of resistance, but the SA-inducible pathway in the susceptible cultivars is somehow perturbed, perhaps by other hormones such as JA, auxin and ET (Fig. 9).

## Materials and methods

### Nematode and plant materials and infections

*Globodera rostochiensis* (PCN) pathotype Ro1 was propagated on greenhouse-grown potatoes. To isolate cyst nematode eggs, we crushed PCN cysts in a homogenizer, and the eggs were rinsed on a 38-μm sieve with sterile water. The eggs were soaked for 9 min in 1.3% commercial bleach, then washed four times by centrifugation for 5 min each at 500 × *g* with sterile water. Hatching of PCN eggs was stimulated over filter-sterilized tomato root diffusate at 22ºC for 6 days Second-stage juveniles (J2s) were allowed to crawl through eight layers of laboratory tissues into sterile water and washed with sterile water three

times by centrifugation for 5 min at 500 × *g* and suspended in sterile water and concentrated to around 2,000 J2/ml.

Four resistant tomato cultivars (Doctor K [DK], Sugar Lamp [SL], Mini Carol [MC] and Yellow Carol [YC]), five susceptible cultivars (Kyouryoku-Beijyu [KB], Momotaro Fight [MF], Magnet [MA], Momotaro 8 [M8], and Moneymaker [MM]) and the transgenic tomato expressing *NahG* in an MM background (Brading et al. 2000) were used in this study. Tomato plants were maintained in pots containing autoclaved commercial soil with a 16-h light cycle at 22ºC in a growth chamber (SANYO).

Plants were inoculated 28 days after germination with $10^4$ J2s of PCN per plant or mock-inoculated with sterile water, then kept for 90 days Three 50-g soil samples were taken from each pot, and cysts were isolated from the soil with the dry-flotation method. The cysts were counted using a dissecting microscope.

**PCR for *Hero A* and *NahG* genes**

Genomic DNA was isolated from young seedling with Isoplant II (Nippon Gene), and total RNA was isolated from root after PCN infection with TRIzol reagent (Invitrogen). The primers used for PCR were as follows: for *Hero A*, HERO_F2 (5′-TTCCACCATTGTTTTACTGCCAAG-3′) and HERO_R3 (5′-GAATGCAGAAGGGATCTCCGTAAG-3′) (Cabrera Poch et al. 2006); for *NahG*, forward primer (5′-TCGTCGTAGGCTTCAAGCAG-3′) and reverse primer (5′-TGCCGACGGAATCAAGTCAG-3′). The cDNA fragments were amplified from 400 ng of total RNA using a gene-specific primer set and a TaKaRa RNA LA PCR kit (TaKaRa

Bio). The PCR was performed according to Cabrera Poch et al. (2006). The PCR products were then cloned and identified on the basis of sequence analysis and database searches.

**RNA preparation**

Root tissue samples were collected from PCN- and mock-inoculated plants at 3 and 7 dpi, we only collected roots only once from a single plant; at 7 dpi, we collected from another plants. The tissue samples were ground to fine powder in liquid N with a mortar and pestle. Total RNA was isolated from the resulting powder with TRIzol reagent.

**DNA microarray experiments**

FilgenArray Tomato (Filgen) was used in this experiment. This oligonucleotide microarray contains 12,404 tomato probe sets, which represent 12,404 UniGene clusters. Fluorescence-labeled antisense RNA was then synthesized by direct incorporation of Cy3-UTP or Cy5-UTP (GE Healthcare Bio-Science) using 20 μg of total RNA and an RNA Transcript SureLABEL Core Kit (TaKaRa Bio). The microarray was prehybridized for 1 h at 42 °C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS, 400 mg/mL bovine serum albumin, and 100 ng/μL salmon sperm DNA. The microarray was then washed three times in distilled water at room temperature for 2 min each time. Hybridization was performed for 16~20 h at 42 ºC in a solution containing 5× SSC, 0.1% SDS, 10% formamide, and heat-denatured, labeled cDNA. At room temperature, the microarray was then washed with 5× SSC and 0.05% SDS for 4 min, with 3× SSC and 0.05% SDS for 4 min, with 0.5× SSC for 1 min, and with 2× PBS for 4 min.

Fluorescence images of Cy3 and Cy5 dye channels were obtained using a GenePix 4000B scanner (Axon Instruments).

**Microarray data analysis**

Array-Pro Analyzer Ver4.5 (Media Cybernetics) was used to determine the signal intensity of each spot. We normalized the biases in net intensity between the two fluorescent dye channels in a microarray by local regression (loess) normalization using the software. Analyzed data were selected using the MicroArray Data Analysis Tool (Filgen). The normalized microarray data were clustered and visualized with CLUSTER and TREE-VIEW software, respectively (Eisen et al. 1998).

**Semiquantitative RT-PCR**

Total RNA was isolated from the roots of nematode-infected and mock-infected tomato plants at 3 and 7 dpi. The cDNA fragments were amplified from 400 ng of total RNA using a gene-specific primer set and a TaKaRa RNA LA PCR kit (Takara Bio). PCR amplification was performed in 18, 21 and 25 cycles. The primers used were as follows: for *PAL* gene (GenBank accession BI929434), forward primer (5′-GTCAAGGCAGCTCAGAAGCT-3′) and reverse primer (5′-GCAAGGAATTGAAGTTCTGAGC-3′); for *Myb*-related gene (BI422778), forward primer (5′-CAGTTATCTCCACAGGTGGAG-3′) and reverse primer (5′-ACTTGAGATTGGGACGACGAC-3′); for *PDC* gene (BF051095), forward primer (5′-CACCTTGACGTTGCTCGATC-3′) and reverse primer

(5′-TTGAATGCTGACCTTGGAGC-3′); for *ADH* gene (M86724), forward primer (5′-GTAACAGACCTTGCACCAGG-3′) and reverse primer (5′-GGGTTTCACAAACTCTGTCAC-3′); for sucrose synthase gene (L19762), forward primer (5′-GCTATTCGTTTGAGGCCTGG-3′) and reverse primer (5′-TCCGATTTCTTGGAACTTGTG-3′). PCR amplification was performed and the PCR products were electrophoresed on an agarose gel and visualized with ethidium bromide staining. The PCR products were then cloned and identified on the basis of sequence analysis and database searches.

**Multiplex quantitative RT-PCR assay**

We performed two separate multiplex RT-PCR assays to compare different expression patterns for genes, including the two closely related genes encoding *PR-1*(*P4*) and *PR-1*(*P6*). First, the expression patterns of *PR-1*(*P6*), *PR-5*, *PR-7*, *PR-6* and *TPI-1* were examined with an eXpress profiling multiplex RT-PCR assay, then the expression patterns for *PR-1*(*P4*) and *PR-6* were examined. *Actin* was used as an internal control (Correa-Aragunde et al. 2006). All primers used in this study for multiplex quantitative RT-PCR assay are listed in Supplementary Table S1. Each primer pair was designed to yield the PCR products ranging from 137 to 230 bp using Beckman Coulter eXpress Profiling Analysis Software (Beckman Coulter). Multiplex quantitative RT-PCR assays were carried out using Beckman CEQ 8800 (Beckman Coulter) as previously described by Kim et al. (2008).

**Measurement of free SA**

Using a method similar to that described by Schaff et al. (2007), tomato seedlings were grown in growth pouches in a growth chamber (SANYO) with a 16-h light cycle at 22ºC. Three-week-old seedlings were inoculated with 10,000 J2s of PCN per seedling. Four biological replicates were analyzed for free SA content after infected roots were harvested 3 dpi, weighed in plastic bags, and stored at –80°C. Each sample (1–2 g) was then crushed to a fine powder in liquid nitrogen. For each sample, the free SA concentrations were measured as described by Matsuura et al. (2009).

**Statistical analysis**

For statistical analysis of nematode bioassays, the statistical package JMP 8 (for Windows; SAS Institute) was used. Data having three or more treatments were analyzed using analyses variance.

**Supplementary data**

Supplementary data are available at PCP online.

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**Figure legends**

**Fig. 1.** Cyst production of susceptible (Kyouryoku-Beijyu [KB]) and resistant (Doctor-K [DK]) tomato cultivars at 90 dpi. Values are means of three biological replicates, and the error bars are standard errors. Bars with different letters denote a significant difference at $P < 0.001$. A: Mean number of cysts/50 g air-dried soil from three plants of either KB or DK. Experiments were repeated three times, and we obtained similar results. Results from one experiment are shown. B: Cysts from 100 g air-dried soil in a pot with KB (left) and DK (right). C: Mean number of cysts/50 g air-dried soil in progeny (segregating at 24 : 6 resistant : susceptible) from selfing of DK. D: Cysts from 50 g air-dried soil in a susceptible progeny (left) and a resistant progeny (right). E: PCR of genomic DNA from young seedling of susceptible cultivars (1, Kyouryoku-Beijyu [KB]; 2, Momotaro-Fight [MF]; 3, Magnet [MA]; 4, Momotaro-8 [M8]; and 5, Moneymarker [MM]) and resistant cultivars (6, Doctor-K [DK]; 7, Sugar-Lamp [SL]; 8, Mini-Carol [MC]; 9, Yellow-Carol [YC]) using the primer pair of HERO_F2 and HERO_R3. The size of the *Hero A*-specific PCR product is indicated by an arrow.

**Fig. 2.** Venn diagrams showing the numbers of probe sets for the microarray analysis depicting differential gene expression in tomato roots in the incompatible and compatible reactions after inoculation with potato cyst nematode. For each treatment, pooled RNA from three biological replicates was used. Experiments were done twice. The sizes of the circles do not represent the quantity of probe sets. Overlapping areas represent common probe sets. An absolute value >2.5 was used for the analysis. Induced or suppressed genes

were scored at 3 (A) and 7 dpi (B).

**Fig. 3.** Cluster analysis of differentially expressed genes at 3 and 7 dpi in the compatible and incompatible interactions. Tomato cultivars Kyouryoku-Beijyu (KB, susceptible) and Doctor-K (DK, resistant) were treated with potato cyst nematode. For each treatment, pooled RNA from three biological replicates was used. Experiments were done twice. Red indicates transcriptional activation, green represents suppression, and black indicates no change in expression. The expression ratio was converted to the color scale at the bottom.

**Fig. 4.** Semiquantitative RT-PCR analysis of the genes encoding the *Myb*, *PAL*, *PDC*, *ADH* and sucrose synthase differentially expressed in the compatible and incompatible interactions. Tomato cultivars Kyouryoku-Beijyu (KB, susceptible) and Doctor-K (DK, resistant) were inoculated with potato cyst nematode (PCN). M3: mock inoculation at 3 dpi, P3: PCN-inoculation at 3 dpi, M7: mock inoculation at 7 dpi, P7: PCN-inoculation at 7 dpi. The expression levels were compared by limiting the number of cycles (18, 21, 25) for RT-PCR as described in Materials and Methods. Primers for RT-PCR are also in Materials and Methods. The PCR products were cloned and sequenced. Ethidium bromide staining of rRNA is shown as an internal control. We obtained similar results in three biological replicates.

**Fig. 5.** Comparison of the transcript levels of the pathogenesis-related genes by quantitative RT-PCR in roots between susceptible and resistant tomato cultivars

Kyouryoku-Beijyu (KB, susceptible) and Doctor-K (DK, *Hero A*-resistant) were inoculated with potato cyst nematode (PCN) and harvested at 3 and 7 dpi. Data were normalized for variation using *actin* expression values (expression value set at 1). Values are mean fold-change in three biological replicates, and error bars represent standard errors.

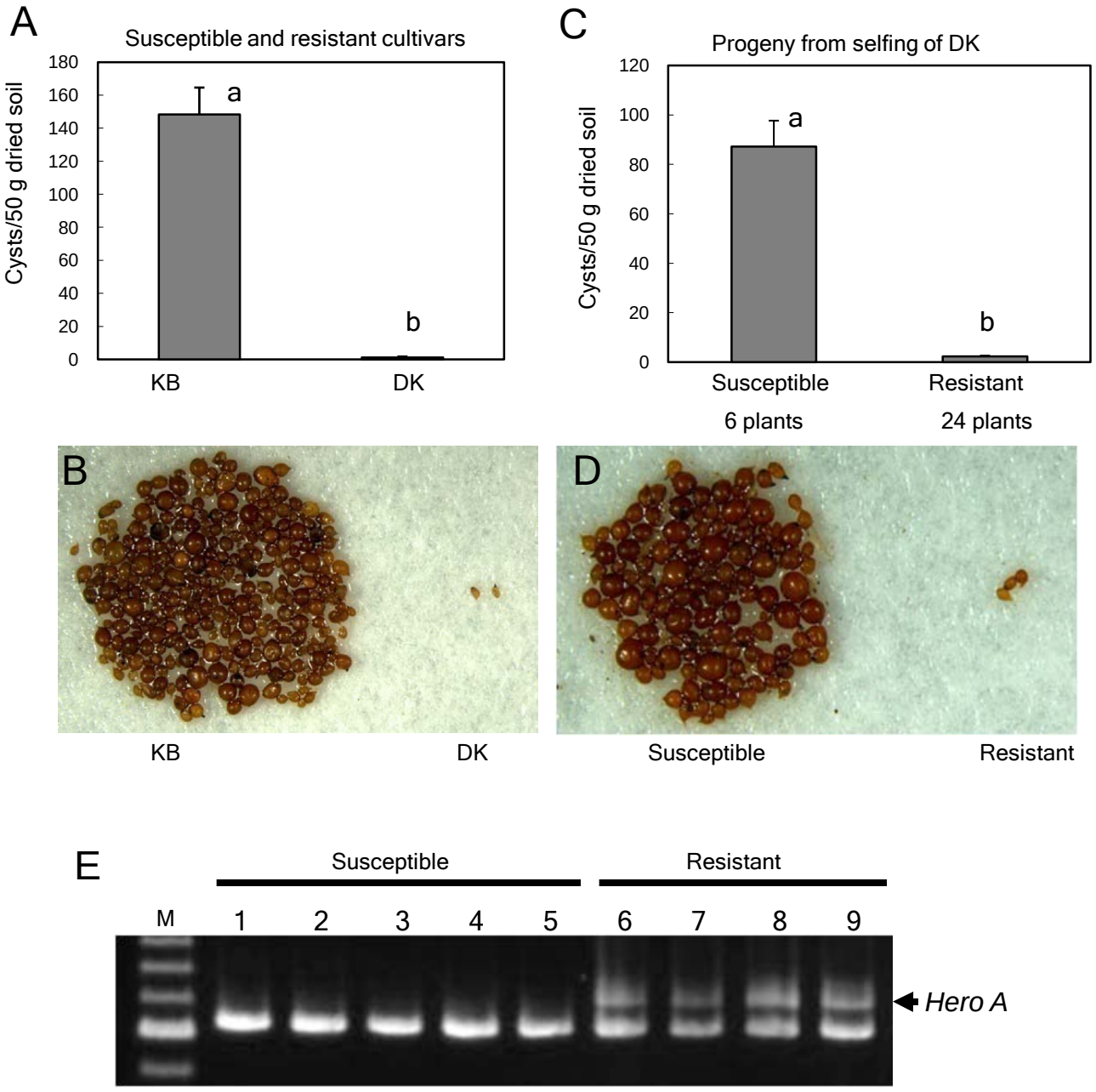
**Fig. 6.** Analysis of the transcript levels of the pathogenesis-related genes by quantitative RT-PCR in roots of several resistant tomato cultivars. Susceptible cultivars (Momotaro-Fight [MF], Magnet [MA], Momotaro-8 [M8]) were used as a control. The resistant cultivars (Sugar-Lamp [SL], Mini-Carol [MC] and Yellow-Carol [YC]) all contained the *Hero A* gene. Cultivars were inoculated with potato cyst nematode (PCN) and harvested at 3 and 7 dpi. Data were normalized for variation using *actin* expression values (expression value set at 1). Values are mean fold-change in three biological replicates and error bars represent standard errors.

**Fig. 7**. Free salicylic acid (SA) in root (A) and leaf (B) tissues of susceptible Kyouryoku-Beijyu (KB) and resistant Doctor-K (DK) cultivars of tomato at 3 dpi with either mock-inoculation or potato cyst nematode (+PCN). Values are means of four biological replicates, and error bars are standard errors. Bars with different letters denote a significant difference at $P < 0.001$.

**Fig. 8.** Experiments using *Hero A-NahG* tomato plants. (A) Analysis of the transcript levels of the *PR(P4*) gene by quantitative RT-PCR in roots of *Hero A* tomato expressing

*NahG*. MM (susceptible), SL (*Hero A*-resistant) and *NahG*×SL were inoculated with potato cyst nematode (PCN) and harvested at 3 dpi. Data were normalized for variation using *actin* expression values (expression value set at 1). Values are mean fold-change in three biological replicates, and error bars represent standard errors. (B) A number of cysts were observed on the *Hero A-NahG* tomato plants at 50 dpi (left). No cysts were observed on tomato containing *Hero A* (SL) at 50 dpi (right).

**Fig. 9.** Model to explain resistance and susceptibility of tomato cultivars to potato cyst nematode. *R*-gene-mediated resistance is induced through the SA-mediated signaling pathway. Signaling pathways mediated by other hormones such as auxin, ethylene (ET) and jasmonic acid (JA), which perhaps interfere with the SA pathway, may be activated by syncytium formation because some hormones are involved in the formation of syncytia. Arrows indicate positive regulation; the blunt end indicates negative regulation.


A
Susceptible and resistant cultivars
Cysts/50 g dried soil
180
160
140
120
100
80
60
40
20
0
a
b
KB
DK
C
Progeny from selfing of DK
Cysts/50 g dried soil
120
100
80
60
40
20
0
a
b
Susceptible
Resistant
6 plants
24 plants
B
KB
DK
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Susceptible
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Susceptible
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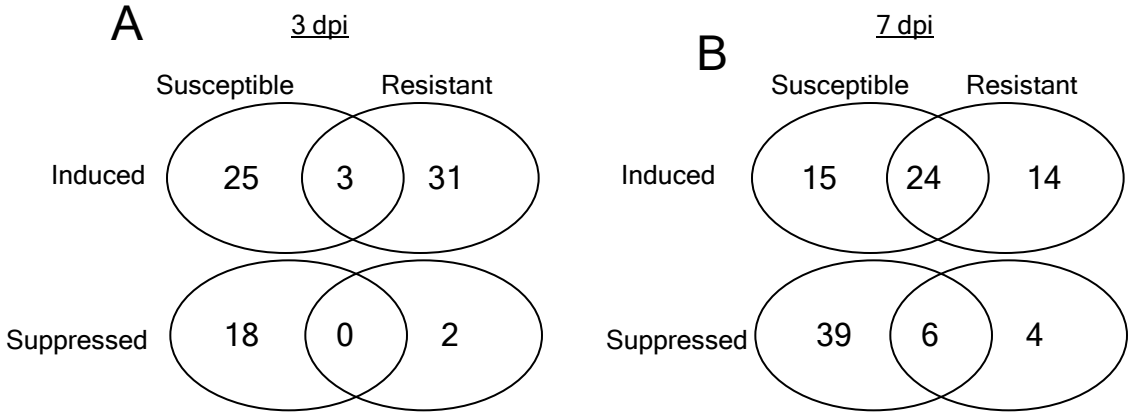
9
Hero A

Fig.1


A
3 dpi
Susceptible
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25
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39
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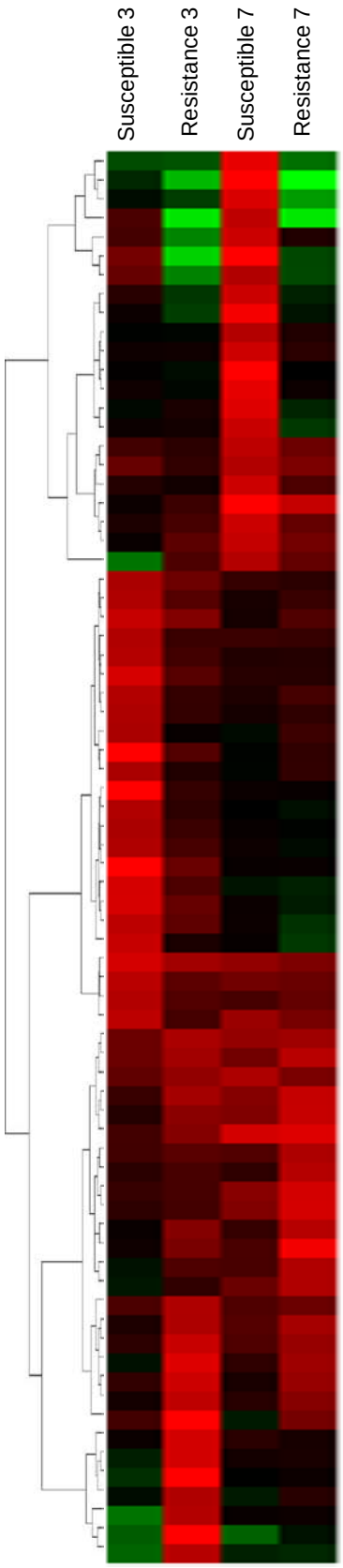
Fig. 2




| Accession | Gene Information<br>Unigene Description |
|---|---|
| BE449812 | Hypothetical protein SPAPB1E7.07 [*Schizosaccharomyces pombe*] |
| M86724 | Alcohol dehydrogenase |
| BF051095 | Pyruvate decarboxylase [*Arabidopsis thaliana*] |
| BW689974 | Non-symbiotic hemoglobin class 1 (Glb1) |
| BT012973 | pi1: Putative pathogen-induced protein (pi1 gene) |
| M80608 | Beta-1,3-glucanase |
| AY257487 | PR5-like protein |
| BI204707 | DNA binding / transcription factor [*Arabidopsis thaliana*] |
| Z15140 | Chitinase |
| BT012707 | LeEF-1 mRNA for elongation factor 1 alpha |
| BW692175 | Transcribed locus |
| BW688144 | Transcribed locus |
| BI935086 | Transcribed locus |
| L19762 | Sucrose synthase |
| BM408837 | Pyruvate decarboxylase [*Arabidopsis thaliana*] |
| BT013168 | Clone 134231R, mRNA sequence |
| BT013336 | Clone 135126R, mRNA sequence |
| Y08804 | PR1b1: PR (pathogenesis related) protein (P6) |
| BI422778 | Myb-related protein A (A-Myb) [*Rattus norvegicus*] |
| BI203380 | Transcribed locus |
| BG643345 | Unknown protein [*Arabidopsis thaliana*] |
| BI929434 | PAL:; Phenylalanine ammonia-lyase [*Arabidopsis thaliana*] |
| L08255 | Abscisic stress ripening protein 1 |
| AY140893 | Ovate protein |
| Y15846 | TSI-1: TSI-1 protein |
| BE458634 | Transcribed locus |
| BI931264 | Putative ferredoxin-thioredoxin reductase [*Oryza sativa*] |
| BG734703 | Transcribed locus |
| BG128541 | PDE166: Magnesium chelatase [*Arabidopsis thaliana*] |
| AW735879 | NEF1 (NO EXINE FORMATION1) [*Arabidopsis thaliana*] |
| BT013038 | Clone 114288R, mRNA sequence |
| BG627683 | Transcribed locus |
| AW944773 | Transcribed locus |
| CK715748 | ATP:ADP antiporter [*Arabidopsis thaliana*] |
| AB036338 | ETAG-A3 |
| BG628658 | Transcribed locus |
| BI204103 | ATP binding / kinase/ protein kinase [*Arabidopsis thaliana*] |
| BI206819 | LePK1: Protein kinase (LePK1) gene |
| BW686663 | cDNA, clone: FC06DA12, HTC in fruit |
| BG129721 | Ripening regulated protein (DDTFR14) mRNA, partial sequence |
| BG627157 | Transcribed locus |
| CK715778 | DNA binding zinc finger protein-like [*Oryza sativa*] |
| AY281152 | TPx1: Thioredoxin peroxidase 1 (TPx1) |
| BI204145 | Transcribed locus |
| BT014085 | Clone 133186R, mRNA sequence |
| X72731 | (ERT 13) ripening-related mRNA |
| BT013281 | Clone 134862F, mRNA sequence |
| BI206752 | Glycerophosphodiesterphosphodiesterase [*Arabidopsis thaliana*] |
| BG127137 | Protein binding /ubiquitin-protein ligase [*Arabidopsis thaliana*] |
| BI935985 | WRKY transcription factor [*Oryza sativa*] |
| BI422044 | BAP2 (BON ASSOCIATION PROTEIN2) [*Arabidopsis thaliana*] |
| BG123295 | Hydrolase [*Arabidopsis thaliana*] |
| AW626298 | Unknown protein [*Arabidopsis thaliana*] |
| BI422101 | OSJNBa0039C07.10 [*Oryza sativa*] |
| AI486815 | Transcribed locus |
| AI489841 | OSJNBa0011F23.20 [*Oryza sativa*] |
| BP887560 | ATSEB1 [*Arabidopsis thaliana*] |
| AW031368 | ATP binding / transmembranereceptor [*Arabidopsis thaliana*] |
| BI422554 | Putative arm repeat containing protein [*Oryza sativa*] |
| BW691354 | *Lycopersicon esculentum* GRAS9 (GRAS9) |
| BT013863 | Clone 132843R, mRNA sequence |
| BI422134 | Putative trehalose-6-phosphatephosphatase [*Oryza sativa*] |
| BI421885 | Unknown protein [*Oryza sativa* (japonica cultivar-group)] |
| BI926142 | Putative pollen-specificprotein NTP303 precursor [*Oryza sativa*] |
| AI487447 | Anion exchanger [*Arabidopsis thaliana*] |
| AW621399 | Transcribed locus |
| BI211052 | Transcribed locus |
| BI921870 | Hypothetical protein [*Plasmodium falciparum* 3D7] |
| BI208885 | ARPN (PLANTACYANIN); copper ion binding [*Arabidopsis thaliana*] |
| BI925360 | Lipid binding [*Arabidopsis thaliana*] |
| BI208939 | ATHVA22C [*Arabidopsis thaliana*] |
| AW093123 | Unknown protein [*Oryza sativa*] |
| BF113820 | Unknown protein [*Arabidopsis thaliana*] |
| AW220367 | Unknown protein [*Arabidopsis thaliana*] |

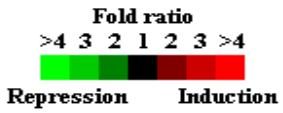



Fig.3

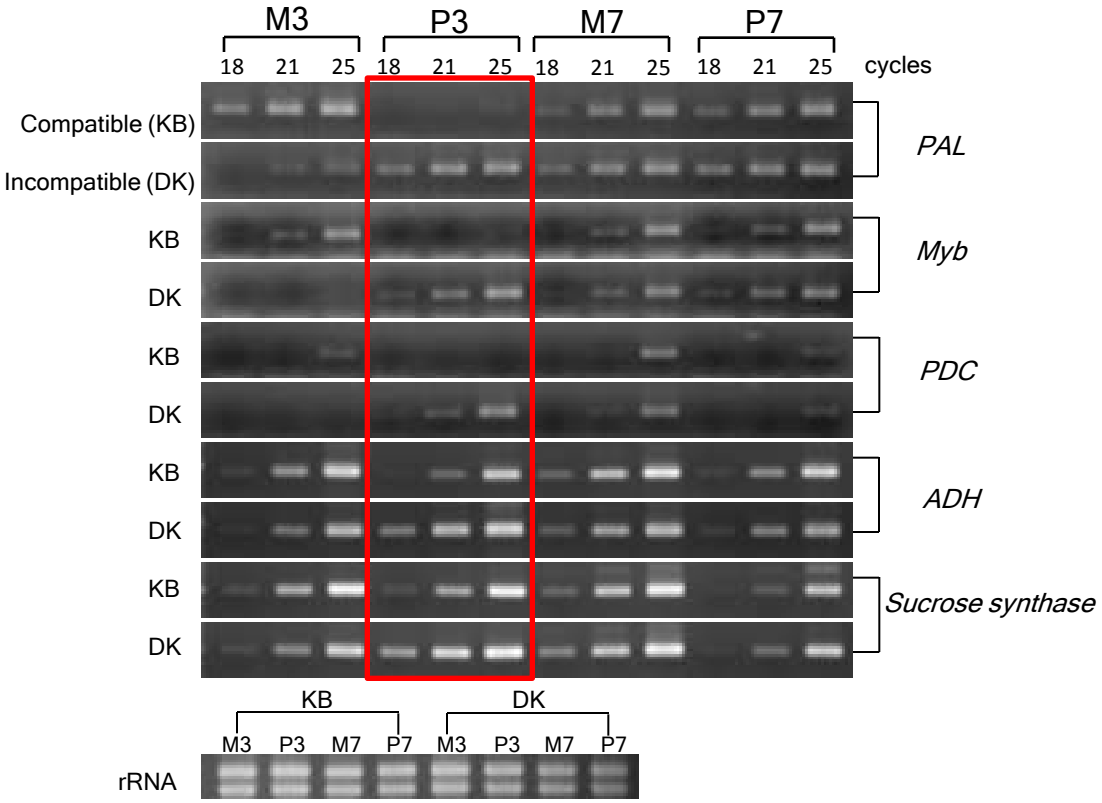

M3
P3
M7
P7
18 21 25 18 21 25 18 21 25 18 21 25
cycles
Compatible (KB)
Incompatible (DK)
KB
DK
KB
DK
KB
DK
KB
DK
PAL
Myb
PDC
ADH
Sucrose synthase
KB
DK
M3 P3 M7 P7 M3 P3 M7 P7
rRNA

Fig.4

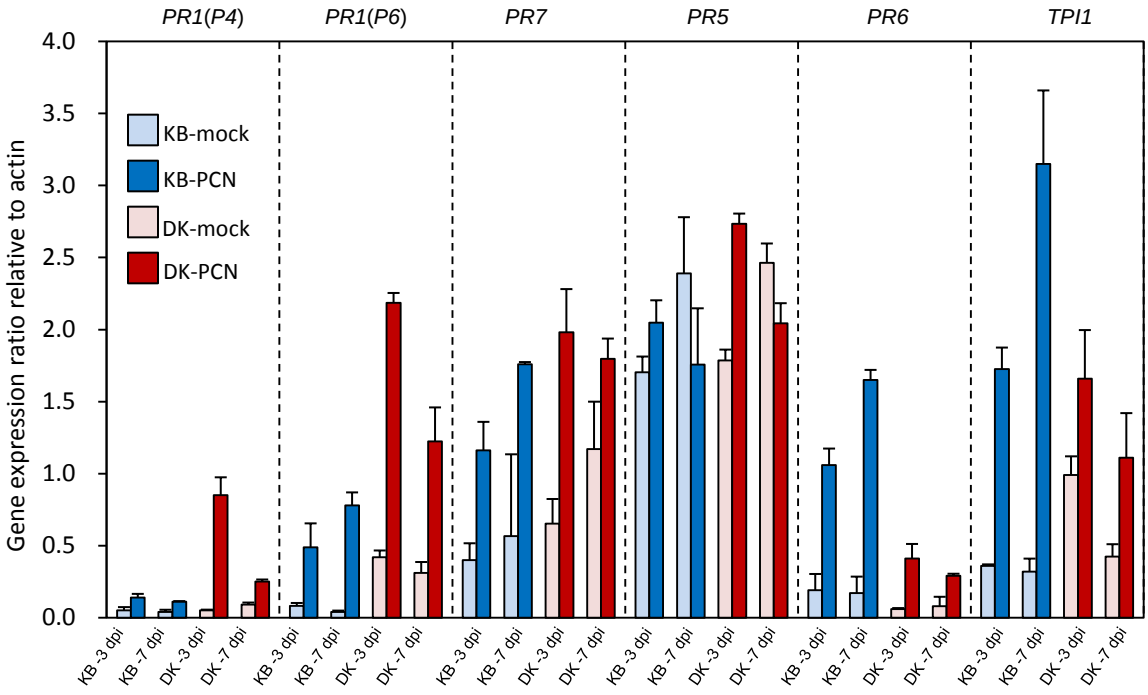

PR1(P4)
PR1(P6)
PR7
PR5
PR6
TPI1
Gene expression ratio relative to actin
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
KB-mock
KB-PCN
DK-mock
DK-PCN
KB-3 dpi
KB-7 dpi
DK-3 dpi
DK-7 dpi

Fig.5

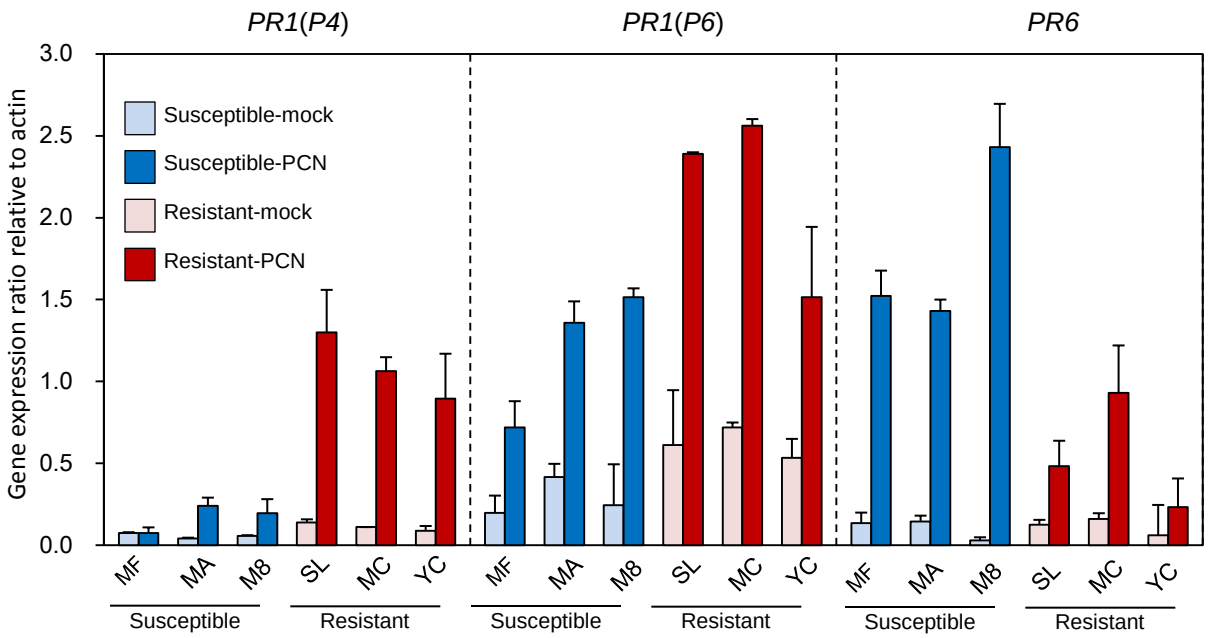

PR1(P4)
PR1(P6)
PR6
Gene expression ratio relative to actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Susceptible-mock
Susceptible-PCN
Resistant-mock
Resistant-PCN
MF
MA
M8
SL
MC
YC
Susceptible
Resistant

Fig. 6

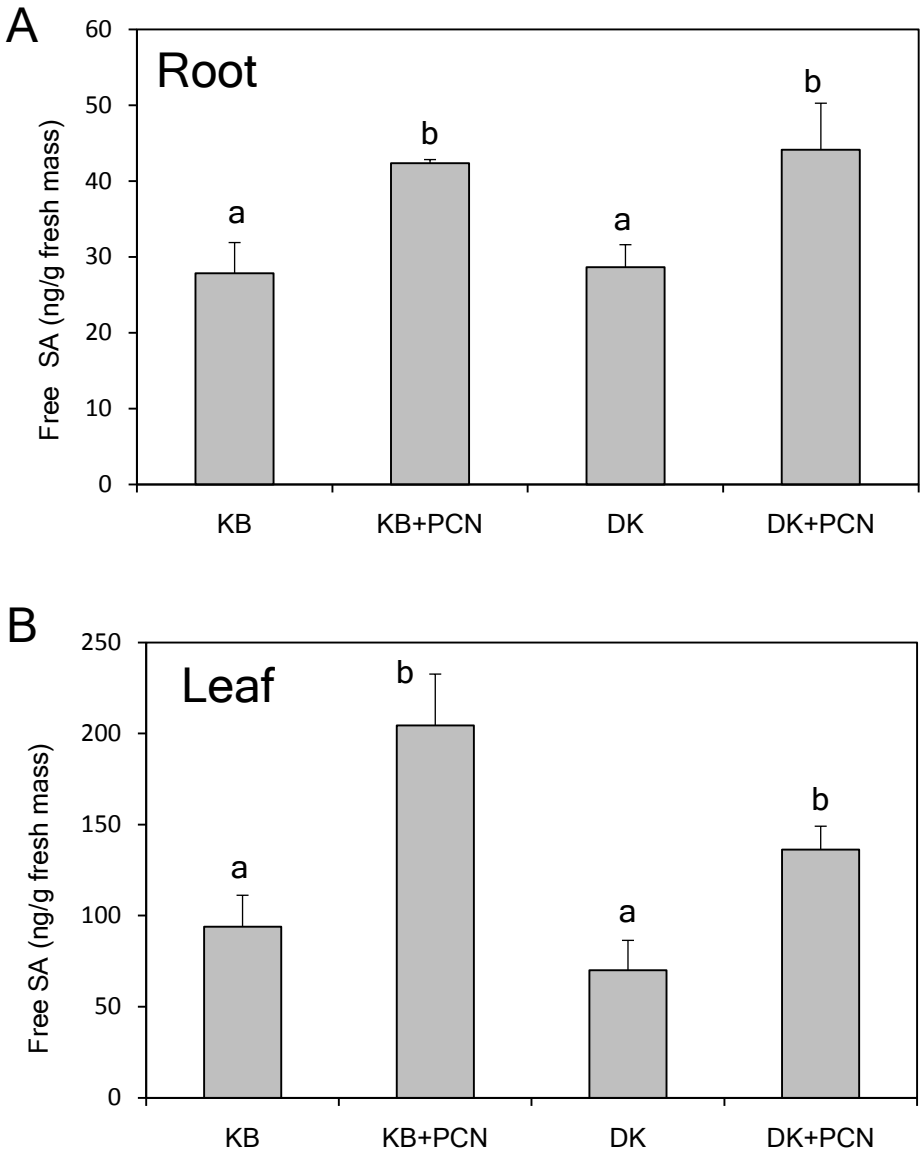

A
Root
Free SA (ng/g fresh mass)
60
50
40
30
20
10
0
a
b
a
b
KB
KB+PCN
DK
DK+PCN
B
Leaf
Free SA (ng/g fresh mass)
250
200
150
100
50
0
a
b
a
b
KB
KB+PCN
DK
DK+PCN

Fig.7

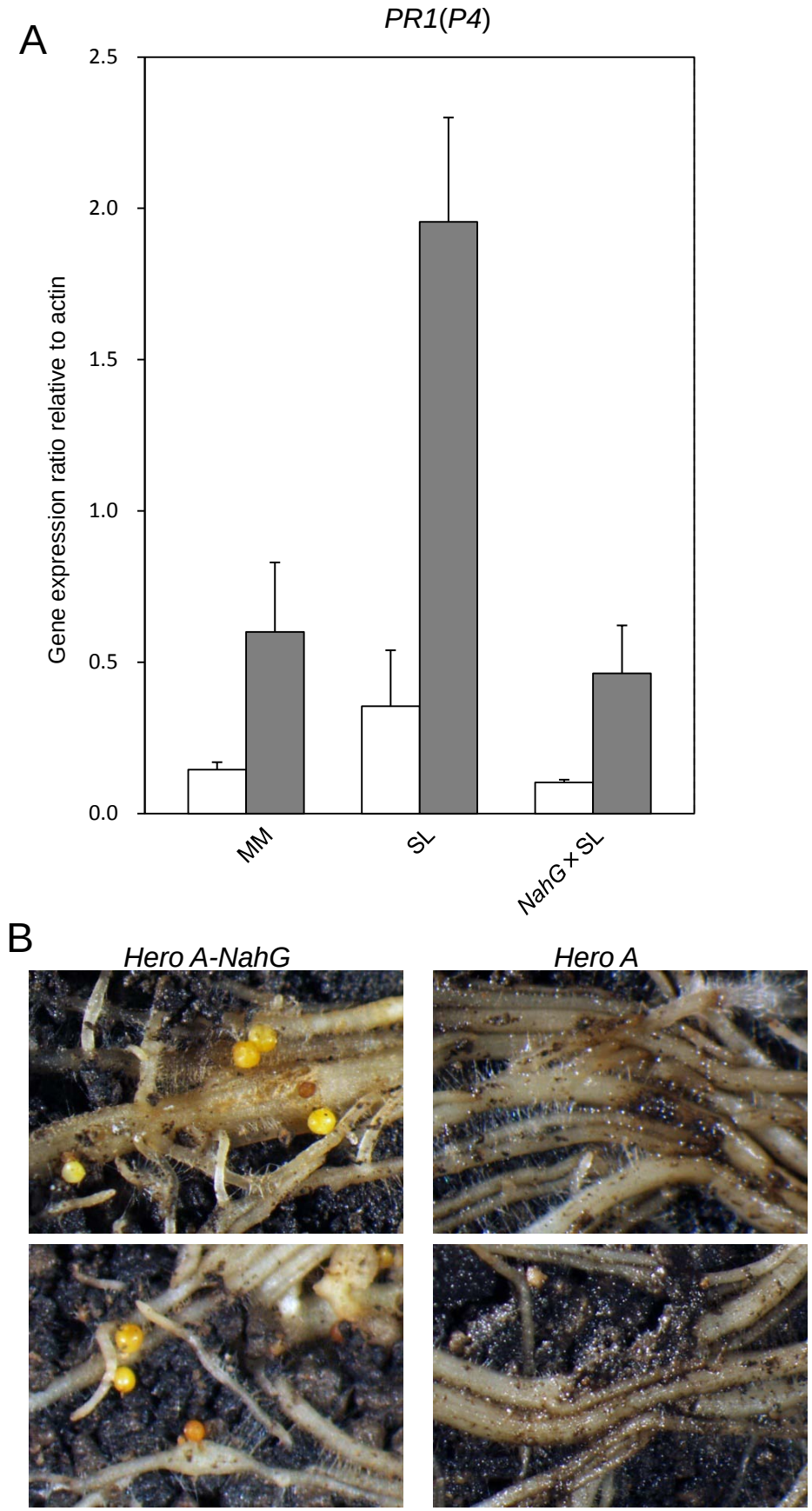

A
PR1(P4)
Gene expression ratio relative to actin
2.5
2.0
1.5
1.0
0.5
0.0
MM
SL
NahG×SL
B
Hero A-NahG
Hero A

Fig.8

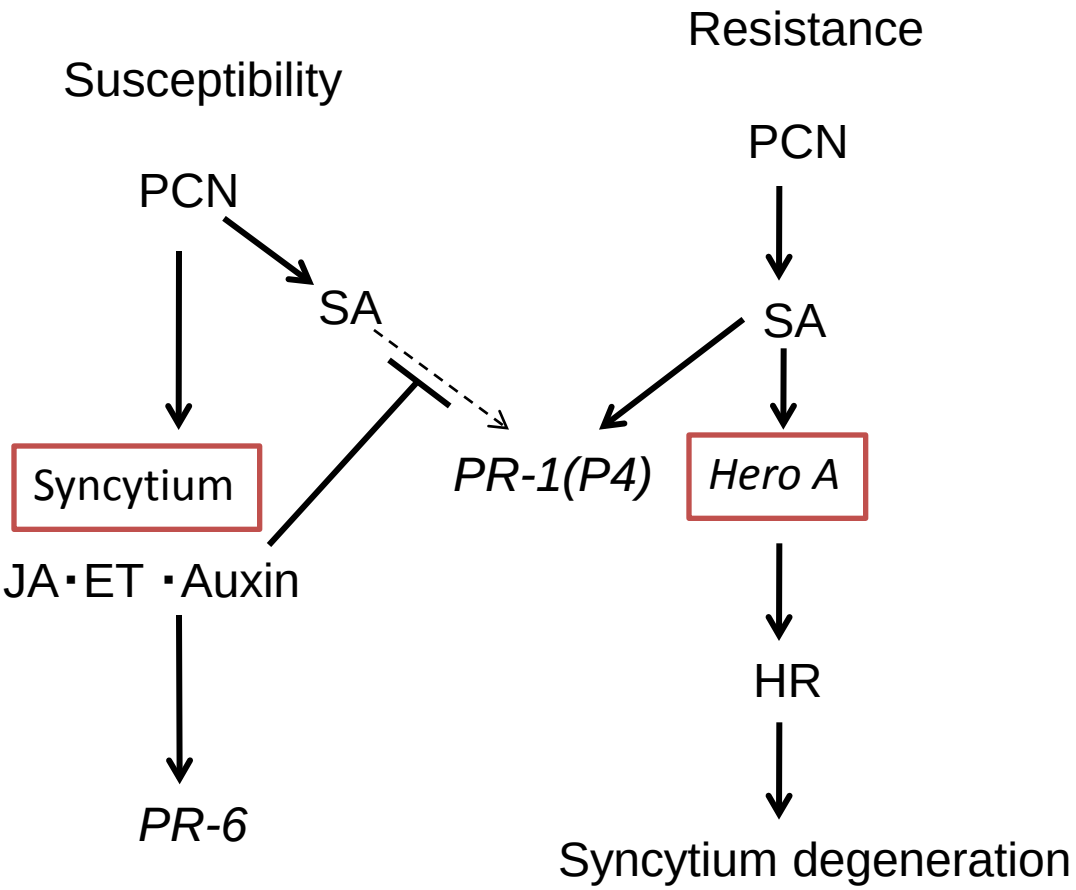

Susceptibility
PCN
SA
Syncytium
JA・ET・Auxin
PR-6
PR-1(P4)
Resistance
PCN
SA
Hero A
HR
Syncytium degeneration

Fig. 9