Overexpression of the Apple *MpNPR1* Gene Confers Increased Disease Resistance in *Malus × domestica*

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The *NPR1* gene plays a pivotal role in systemic acquired resistance in plants. Its overexpression in *Arabidopsis* and rice results in increased disease resistance and elevated expression of pathogenesis-related (*PR*) genes. An *NPR1* homolog, *MpNPR1-1*, was cloned from apple (*Malus × domestica*) and overexpressed in two important apple cultivars, Galaxy and M26. Apple leaf pieces were transformed with the *MpNPR1* cDNA under the control of the inducible *Pin2* or constitutive *Cauliflower mosaic virus* (*CaMV*)35S promoter using *Agrobacterium tumefaciens*. Overexpression of *MpNPR1* mRNA was shown by reverse transcriptase-polymerase chain reaction. Activation of some *PR* genes (*PR2*, *PR5*, and *PR8*) was observed. Resistance to fire blight was evaluated in a growth chamber by inoculation of the shoot tips of our own rooted 30-cm-tall plants with virulent strain Ea273 of *Erwinia amylovora*. Transformed Galaxy lines overexpressing *MpNPR1* had 32 to 40% of shoot length infected, compared with 80% in control Galaxy plants. Transformed M26 lines overexpressing *MpNPR1* under the control of the *CaMV*35S promoter also showed a significant reduction of disease compared with control M26 plants. Some *MpNPR1*-overexpressing Galaxy lines also exhibited increased resistance to two important fungal pathogens of apple, *Venturia inaequalis* and *Gymnosporangium juniperi-virginianae*. Selected transformed lines have been propagated for field trials for disease resistance and fruit quality.

Plants have evolved sophisticated detection and response systems that decipher pathogen signals and induce appropriate defenses. Upon pathogen detection, plants activate a number of early responses that lead to the production of a broad spectrum of defense molecules (Chisholm et al. 2006; Takken and Joosten 2000). In many cases, products of host resistance (*R*) genes mediate pathogen recognition and activate pathways that are involved in local defenses, such as the hypersensitive response (HR) and the oxidative burst (Greengard and Yao 2004; Hammond-Kosack and Jones 1996). These induced defenses are often expressed not just locally but also systemically, thereby protecting the plant from subsequent pathogen attacks. One of the best-understood responses is systemic acquired resistance (SAR) (Durrant and Dong 2004; Ryals et al. 1996; Sticher et al. 1997). Salicylic acid (SA) is an essential signal in

the induction of SAR (Dempsey et al. 1999; Gaffney et al. 1993; Métraux et al. 1990). In addition, exogenous application of SA or its analogs, such as benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) and 2,6-dichlorosinonic acid (INA), triggers SAR (Friedrich et al. 2001; Gorchach et al. 1996; Métraux et al. 1990; Morris et al. 1998). Along with an increase of SA levels, increased expression of pathogenesis-related (*PR*) genes has also been tightly correlated with the onset of SAR. Only a few *PR* genes have been shown to have antimicrobial activity in vitro or to confer increased resistance when overexpressed in plants (Morrissey et al. 1999; Smith 1996), and no single *PR* gene has been shown to be essential for SAR, suggesting that the coordinated expression of these genes and other defense responses is needed to produce resistance.

Genetic analysis of plant mutants impaired in mounting a resistance response to invading pathogens have uncovered a number of distinct but interconnecting signal networks that are under both positive and negative control. In one such screen, the *Arabidopsis thaliana* gene *NPR1* (for nonexpressor of *PR*, also called *NIM1* for nonimmunity or *SAL1* for salicylic acid-insensitivity; hereinafter called *AtNPR1*) was identified as a key factor in transducing the signal leading to SAR (Cao et al. 1994; Delaney et al. 1995; Ryals et al. 1996; Shah et al. 1997). In addition, this gene also participates in induced systemic resistance (ISR) that is activated upon colonization of selected strains of nonpathogenic rhizobacteria around roots (Pieterse et al. 1998; Spoel et al. 2003). *AtNPR1* encodes a protein that contains a BTB/BOZ domain and an ankyrin repeat domain (Cao et al. 1997; Ryals et al. 1997). Both domains are known to mediate protein-protein interactions and are present in proteins with diverse functions (Aravind and Koonin 1999), including the transcriptional regulator I κ B, which mediates innate immune responses in animals (Baldwin 1996). Upon induction of SAR, *AtNPR1* is apparently translocated to the nucleus (Kinkeman et al. 2000; Somssich 2003), in which it interacts with the TGA family of basic leucine zipper (bZIP) transcription factors (Després et al. 2000; Fan and Dong 2002; Kim and Delaney 2002; Subramanian et al. 2001; Zhang et al. 1999; Zhou et al. 2000) that are involved in the SA-dependent activation of *PR* genes (Niggeweg et al. 2000).

Overexpression of *AtNPR1* in *A. thaliana*, rice, tomato, and wheat has been demonstrated to induce enhanced fungal and bacterial resistance (Cao et al. 1998; Chern et al. 2001, 2005; Fitzgerald et al. 2004; Friedrich et al. 2001; Lin et al. 2004; Makandar et al. 2006). In *A. thaliana*, overexpression of *AtNPR1* and enhanced resistance are correlated with both enhanced and quicker *PR* gene expression, suggesting that *NPR1* induces enhanced resistance through elevated expression of *PR* genes (Cao et al. 1998; Friedrich et al. 2001). Thus,

M. Malnoy and Q. Jin made equal contributions to this work.

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we decided to employ the *NPR1* overexpression strategy to generate apple plants with a broad-spectrum disease resistance, in particular to the devastating fire blight disease caused by *Erwinia amylovora* (Burr.).

Most modern apple (*Malus × domestica*) cultivars are susceptible to *E. amylovora* and apple scab caused by the asco-

mycete fungus *Venturia inaequalis* (Cooke). Fire blight is the major bacterial disease of *Maloideae* (pear, apple, quince) and several other members of the *Rosaceae*. After entering the plants through natural openings or wounds, the bacteria spread quickly along the stems to the main branches, producing the characteristic symptom of this disease: a necrotic shoot blight

Fig. 1. Alignment of the deduced amino acid sequences of *MpNPR1-1*, *MpNPR1-2*, and *MpNPR1-3* to that of the *Arabidopsis thaliana* *AtNPR1* gene.

that can kill a tree in one season (Thomson 2000). Here, we report that overexpression of the *Malus NPR1* (*MpNPR1* hereinafter) gene in two apple cultivars, Galaxy and M26, confers increased resistance to a bacterial and two fungal pathogens.

RESULTS

Cloning and characterization of the apple *MpNPR1* gene.

To clone the ortholog of the *AtNPR1* gene from apple, we screened an apple cDNA library using *AtNPR1* as a probe. Three clones were obtained and designated *MpNPR1-1*, *MpNPR1-2*, and *MpNPR1-3*. *MpNPR1-1* shares 80% identity with *MpNPR1-2* and 85% identity with *MpNPR1-3* at the nucleotide level. The deduced amino-acid sequences of the three genes share about 35% identity with that of the *AtNPR1* protein (Fig. 1). The most conserved region corresponds to the ankyrin repeats, a protein-protein interaction domain that is a signature of the *AtNPR1* protein.

The *AtNPR1* gene was previously shown to be inducible by treatment with the SAR inducer BTH (Cao et al. 1998). To further examine the relatedness of the three putative apple *NPR1* genes to *Arabidopsis NPR1*, Northern blot analysis using gene-specific probes (5' noncoding regions) was conducted. Only *MpNPR1-1* was found to be induced by BTH (Fig. 2), suggesting that *MpNPR1-1* but not *MpNPR1-2* or *MpNPR1-3* is a likely ortholog of the *AtNPR1* gene. In support of this

Fig. 2. Northern blot analysis of expression of *AtNPR1* and *MpNPR1-1* genes by benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH). Left, Expression of *AtNPR1* in *Arabidopsis thaliana* treated with H₂O and BTH 3 days prior to the harvest of leaves. Right, Expression of *MpNPR1-1* in apple seedlings treated with H₂O and BTH. The 5' noncoding regions of *AtNPR1* and *MpNPR1-1* were used as probes.

notion, phylogenetic tree analysis was conducted, which showed that *MpNPR1-1* was most closely related to *AtNPR1* (Fig. 3). Therefore, *MpNPR1-1* was chosen for further experiments and referred to as *MpNPR1*.

Transformation efficiency and ploidy level.

We conducted apple transformation with *A. tumefaciens* EHA105 pCH32 containing binary vector pKYLX35S*AtNPR1*, pKYLX35SM*NPR1*, or pKYLXPIN*MpNPR1*. A total of 50 leaves taken from Galaxy and M.26 were transformed for each construct. We obtained 5, 12 and 9 transformed Galaxy lines, and 19, 5, and 20 transformed M.26 lines for pKYLX*AtNPR1*, pKYLX35SM*NPR1*, or pKYLXPIN*MpNPR1*, respectively. The efficiency of transformation (10 to 24% for Galaxy and 10 to 40% for M.26) was higher than that obtained with other *Malus × domestica* genotypes (Faize et al. 2003, 2004; Puite and Schaart 1996; Szankowski et al 2003) or other *Rosaceae* woody plants (Reynoird et al. 1999; Scorza et al. 1994). Presence of the *NPR1* genes was verified by polymerase chain reaction (PCR) analysis of transformed lines growing on selective medium. Ploidy level determined by flow cytometry showed that all of the transformed lines were diploid, like the nontransformed Galaxy and M.26.

Fig. 3. Phylogenetic tree generated by parsimony analysis based on shared amino acids of *MpNPR1-1*, *AtNPR1* (AT1G64280), *AtNPR2* (AT4G26120), *AtNPR3* (AT5G45110), and *AtNPR4* (AT4G19660). The scale is the expected number of substitutions per position. Numbers at the nodes are presented only for percentage bootstrap values above 50% of 1,000 resamplings.

Table 1. Evaluation of fire blight resistance in our own rooted plants compared with grafted Galaxy plants containing PIN*MpNPR1*, 35S*AtNPR1*, and 35SM*NPR1*^a

Lines	Constructs	Rooted plants (%) ^b	Waller group	Grafted plants (%) ^c	Waller group
Galaxy		83.0	A	78.9	A
TGx-1508	35SM <i>NPR1</i>	65.9	AB	...	...
TGx-1517	35S <i>AtNPR1</i>	65.0	AB	...	...
TGx-1515	35SM <i>NPR1</i>	61.6	AB	...	...
TGx-1510	35SM <i>NPR1</i>	55.6	BC	...	...
TGx-1524	35SM <i>NPR1</i>	55.5	BC	...	...
TGx-1522	35SM <i>NPR1</i>	55.4	BC	...	...
TGx-1511	35SM <i>NPR1</i>	53.0	BCD	...	...
TGx-1320	PIN <i>MpNPR1</i>	52.9	BCD	...	...
TGx-1518	35S <i>AtNPR1</i>	48.0	BCDE	...	...
TGx-1491	PIN <i>MpNPR1</i>	47.3	BCDE	49.2	B
TGx-1321	PIN <i>MpNPR1</i>	46.9	BCDE	49.0	B
TGx-1521	35SM <i>NPR1</i>	45.4	BCDEFG	...	...
TGx-1322	PIN <i>MpNPR1</i>	40.3	CDEF	37.5	C
TGx-1323	PIN <i>MpNPR1</i>	35.0	CDEFG	35.0	C
TGx-1319	PIN <i>MpNPR1</i>	31.0	EFGH	34.0	C
TGx-1509	PIN <i>MpNPR1</i>	28.9	FGH	...	...
M.7	...	22.3	FGH	17.0	D
TGx-1520	35SM <i>NPR1</i>	17.2	GH	...	...
TGx-1523	35SM <i>NPR1</i>	10.6	H	...	...

^a By shoot inoculation with *Erwinia amylovora* Ea 273 at 5 × 10⁷ CFU ml⁻¹.

^b The percentage of shoot length infected in our own rooted plants in a growth chamber.

^c The percentage of shoot length infected in grafted plants in the greenhouse.

Southern blot analysis of seven selected transformed Galaxy lines containing the PINMpNPR1 insert showed that the tested transformed lines all contained a single copy of the transferred gene in their genome (data not shown).

Susceptibility of transformed lines to fire blight.

To characterize the response of the transformed lines to fire blight, 10 to 20 acclimated plants per line were inoculated with *E. amylovora* Ea 273 at 5×10^6 CFU ml⁻¹ for M.26 or 5×10^7 CFU ml⁻¹ for Galaxy in two to three independent experiments. The responses were similar in the replicated experiments, as indicated by the low confidence intervals of the means. Three weeks after inoculation, 83% of the shoot length infected in nontransformed Galaxy showed necrosis, whereas 22% of the shoot length in M.7 (resistant control) was necrotic (Table 1). By contrast, all of the transformed Galaxy lines expressing the *Malus × domestica* or *A. thaliana* NPR1 gene under the con-

trol of the CaMV35S or Pin2 promoter showed a reduction in susceptibility to *E. amylovora*. Two of these lines (TGx-1520 and TGx-1523) were numerically more resistant than the resistant control M.7. When we observed the responses of the transformed M.26 lines with the same expression constructs (Table 2), a different pattern appeared. Most of the PIN2MpNPR1 M.26 lines did not show a significant increase in resistance; only six of 20 lines tested showed an intermediate level of resistance compared with the nontransformed control M.26. In contrast, when the *Malus × domestica* or *A. thaliana* NPR1 gene was placed under the control of the strong CaMV35S promoter, all transformed M.26 lines showed significantly increased resistance (Table 2). However, only six of 20 PINMpNPR1 M.26 lines, in which the *MpNPR1* gene is expressed from the wound-inducible Pin2 promoter, showed an intermediate level of resistance compared with the nontransformed control M.26 (Table 2). Thus, the cultivar backgrounds have an effect on NPR1 overexpression-mediated resistance to fire blight (Table 3). In M.26, the *MpNPR1* gene gave the higher level of increased resistance to *E. amylovora* when it was under the control of the constitutive 35S promoter and a lower increase in resistance when it was under the control of the wound-inducible Pin2 promoter. Expression of the *AtNPR1* gene gave an intermediate level of resistance to fire blight. In the cultivar Galaxy, the difference in effect of the three constructs was not obvious. In contrast to what was observed in M.26, a higher level of resistance was observed in Galaxy when the *MpNPR1* was under the control of the Pin2 promoter.

Table 2. Fire blight resistance in our own rooted M.26 plants^a

Lines	Constructs	Shoot length infected (%)	Waller group
T26-1304	PINMpNPR1	100	A
T26-1309	PINMpNPR1	97.6	AB
T26-1306	PINMpNPR1	97.1	ABC
T26-1297	PINMpNPR1	95.8	ABCD
T26-1307	PINMpNPR1	95.4	ABCD
M.26		92.7	ABCDE
T26-1292	PINMpNPR1	91.8	ABCDE
T26-1466	35SAtnPR1	90.4	ABCDEF
T26-1299	PINMpNPR1	90.2	ABCDEF
T26-1302	PINMpNPR1	88.8	ABCDEFG
T26-1287	PINMpNPR1	86.8	ABCDEFHG
T26-1305	PINMpNPR1	84.4	ABCDEFHGI
T26-1301	PINMpNPR1	81.8	BCDEFGHIJ
T26-1308	PINMpNPR1	81.4	CDEFGHIJ
T26-1290	PINMpNPR1	79.4	CDEFGHIJK
T26-1291	PINMpNPR1	78.7	DEFGHIJKL
T26-1288	PINMpNPR1	78.3	DEFGHIJKL
T26-1501	35SMpNPR1	75.0	EFGHIJKLM
T26-1505	35SAtnPR1	73.6	FGHIJKLMN
T26-1325	PINMpNPR1	71.5	GHIJKLMN
T26-1303	PINMpNPR1	70.9	GHIJKLMNO
T26-1479	35SAtnPR1	70.8	GHIJKLMNO
T26-1300	PINMpNPR1	69.7	HIJKLMNO
T26-1457	35SAtnPR1	67.4	IJKLMNOP
T26-1324	PINMpNPR1	64.2	JKLMNOPQ
T26-1470	35SAtnPR1	62.0	KLMNOPQ
T26-1480	35SAtnPR1	61.8	KLMNOPQ
T26-1462	35SAtnPR1	60.9	LMNOPQR
T26-1528	35SMpNPR1	60.9	LMNOPQR
T26-1460	35SAtnPR1	60.8	LMNOPQR
T26-1468	35SAtnPR1	59.4	MNOPQRS
T26-1456	35SAtnPR1	57.2	MNOPQRS
T26-1298	PINMpNPR1	57.0	MNOPQRS
T26-1461	35SAtnPR1	54.8	NOPQRS
T26-1459	35SAtnPR1	54.1	NOPQRS
T26-1506	35SAtnPR1	53.5	NOPQRS
T26-1326	PINMpNPR1	53.0	OPQRS
T26-1467	35SAtnPR1	51.0	PQRS
T26-1463	35SAtnPR1	50.4	PQRS
T26-1478	35SAtnPR1	49.2	QRST
T26-1481	35SAtnPR1	48.8	QRST
T26-1458	35SAtnPR1	47.9	QRST
T26-1528	35SMpNPR1	47.8	QRST
T26-1502	35SMpNPR1	43.4	RST
T26-1464	35SAtnPR1	41.9	STU
T26-1471	35SMpNPR1	31.2	TUV
T26-1469	35SAtnPR1	25.1	UV
M.7	19.2	V	

^a Evaluation of fire blight resistance in our own rooted plants containing PINMpNPR1, 35SAtnPR1, and 35SMpNPR1 by shoot inoculation with *Erwinia amylovora* Ea 273 at 5×10^6 CFU ml⁻¹ in a growth chamber.

Table 3. The overall effectiveness of three expression constructs on fire blight resistance^a

Constructs or controls	Shoot length infected (%)	Waller group
M.26		
M.26 (susceptible)	92.7	A
PINMpNPR1	78.9	B
35SAtnPR1	56.7	C
35SMpNPR1	47.4	D
M.7 (resistant)	19.2	E
Galaxy		
M.26 (susceptible)	83.0	A
35SAtnPR1	53.3	B
35SMpNPR1	50.2	B
PINMpNPR1	39.9	C
M.7 (resistant)	22.3	D

^a Effectiveness of PINMpNPR1, 35SAtnPR1, and 35SMpNPR1 on fire blight resistance of our own rooted Galaxy plants and M.26-transformed plants following shoot inoculation with *Erwinia amylovora* in a growth chamber. The data from all the transformed lines expressing a given construct were used to calculate the overall effectiveness of the construct.

Table 4. Resistance to *Venturia inaequalis* (apple scab)^a

Transformed lines	Infection rating ^b	No. of leaves with sporulation
Galaxy	2.48	4.86 ± 0.5
TGx-1319	1.12	2.95 ± 0.3
TGx-1320	1.35	3.02 ± 0.4
TGx-1323	1.01	2.62 ± 0.2
TGx-1509	1.09	2.83 ± 0.3
TGx-1520	0.91	2.63 ± 0.3
TGx-1521	0.71	2.45 ± 0.2
TGx-1523	0.77	2.50 ± 0.5
TGx-1524	0.85	3.00 ± 0.2

^a Resistance to *Venturia inaequalis* (apple scab) of our own rooted transformed Galaxy lines containing PINMpNPR1 and 35SMpNPR1 in a growth chamber. Three leaves were inoculated.

^b Symptom and infection severity scale: 0 = <5% leaf surface with sporulation, 1 = 5 to 25% leaf surface with sporulation, 2 = 25 to 50% leaf surface with sporulation, and 3 = >50% leaf surface with sporulation.

We further analyzed the transformed apple plants in the greenhouse. Selected transformed Galaxy lines were grafted onto seedling rootstocks and were inoculated with *E. amylovora* Ea 273 at 5×10^7 CFU ml⁻¹. Three weeks after inoculation, 78.9% of the shoot length of nontransformed Galaxy showed necrosis, whereas 17% of the shoot lengths infected in M.7 (resistant control) were necrotic (Table 1). By contrast, all the transformed Galaxy lines expressing the *Malus* × *domestica* or *A. thaliana* *NPR1* gene, under the control of the

CaMV35S or Pin2 promoter, showed a reduction in susceptibility to *E. amylovora*, which was similar to the reduction of susceptibility observed in the growth chamber.

Resistance to *Venturia inaequalis* and *Gymnosporangium juniperi-virginianae*.

In order to evaluate the range of protection offered by overexpression of the *NPR1* genes, we examined selected transformed Galaxy lines for resistance to two major fungal

Fig. 4. Microscopic analysis of *MpNPR1* transformed Galaxy apple line TGX-1319 and nontransformed control Galaxy 3 weeks after inoculation with *Gymnosporangium juniperi-virginianae*.

pathogens (*V. inaequalis* and *G. juniperi-virginianae*) of apple.

After *V. inaequalis* inoculation on plants growing in the growth chamber, macroscopic symptoms were evaluated 21 days later and the percentage of leaves with sporulation was determined. Interestingly, there was a reduction of symptoms on leaf surfaces as well as a reduction in the number of leaves infected compared with the Galaxy control (Table 4).

Inoculation with *G. juniperi-virginianae* caused the formation of lesions on the leaf surface. One month after inoculation, a mean of more than 66 lesions could be counted on leaf surfaces of the nontransformed control. The transformed lines tested (TGx-1319, 1320, 1322, 1323, and 1509) showed a reduction of 40% in the number of necrotic lesions on the adaxial surface of the leaves and a reduction in formation of aecia on the abaxial surfaces (data not shown). After staining of 3-week-old leaves infected with *G. juniperi-virginianae*, microscopic analysis revealed that most of the lesions observed on the transformed lines had necrosis or mycelia without sporulation

(Fig. 4), compared with the nontransformed lines of Galaxy, in which all lesions had mycelial growth with abundant sporulation (Fig. 4).

Molecular characterization of NPR1 gene expression in transformed lines.

NPR1 mRNA and protein levels were determined in transformed lines grown in the greenhouse compared with those in nontransformed Galaxy and M.26 plants under the same conditions. As expected, different transformed lines displayed different levels of *NPR1* mRNA, as revealed by reverse transcriptase (RT)-PCR (Fig. 5), while a faint *NPR1* mRNA signal was detected in the nontransformed control Galaxy (Fig. 5) and M.26 (data not shown). Overexpression of the *NPR1* protein in the transformed lines was also confirmed in all the transformed lines by Western blot for the Galaxy transformed lines expressing the *MpMPR1* gene under the control of the Pin2 promoter (Fig. 6). Similar results were obtained for the transformed M.26 and Galaxy lines expressing the *MpNPR1* or

Fig. 5. Comparative reverse transcriptase-polymerase chain reaction (PCR) analysis of *NPR1*, *PR1*, *TPR1*, *PR2*, *PR5*, *PR8*, *PR10*, and *EF-1α* in leaves of acclimated plants from nontransformed Galaxy and transformed lines expressing the *MpNPR1* gene under the control of the Pin2 promoter. Differences among transcription levels of transformed plants were estimated after 20 cycles of PCR. The *EF-1α* transcript was used as an internal control. Experiments were repeated at least twice with similar results. Results are from one of these experiments.

AtNPR1 gene under the control of the CaMV35S or Pin2 promoter (data not shown).

Abundance of *NPR1* transcripts, evaluated by the ratio of RT-PCR signal intensity between *NPR1* and the control *EF1- α* , was correlated in Galaxy with the level of resistance in the growth chamber ($R = 0.60$, $P < 0.01$ and $R = 0.75$, $P < 0.01$ for the PINMpNPR1 and 35SMpNPR1 constructs, respectively). In M.26, a similar correlation was observed.

We used the Pin2 promoter, in addition to the CaMV 35S promoter, because it was shown to be inducible by *E. amylovora* infection. We were, therefore, interested in examining the effect of *E. amylovora* infection on the expression of the *MpNPR1* gene under the control of the Pin2 promoter. Interestingly, before inoculation, the *MpNPR1* transcript levels were already higher in all transformed lines, as compared with the Galaxy control (Fig. 7). After inoculation with *E. amylovora*, the abundance of *NPR1* transcripts increased rapidly in the transformed lines compared with that in the Galaxy control. This activation of transcription was specific to *E. amylovora* inoculation, because after inoculation with water the abundance of *NPR1* transcripts increased only slowly (Fig. 7).

Molecular characterization of *PR* gene expression in the *NPR1*-transformed lines.

We examined whether overexpression of the *NPR1* gene in apple enhanced the expression of *PR* genes. Specifically, we checked the expression over time of *PR1*, *PR2*, *PR5*, *PR8*, and *PR10* in selected transformed Galaxy Pin2MpNPR1 lines after inoculation with *E. amylovora* or water. We found that, after inoculation with *E. amylovora*, the expression of these *PR* genes, except *PR10*, was greater in transformed plants than in the Galaxy control inoculated with *E. amylovora* or in the same transformed lines inoculated with water. The transcript levels of these different *PR* genes reached maximum levels 48 h after inoculation (Fig. 8), which was 24 h after the *NPR1* gene had reached its maximum expression (Fig. 7).

DISCUSSION

Genetic engineering of disease resistance through transfer of plant defense-related genes or pathogen-originated genes into crops is valuable in terms of cost, efficacy, and reduction of

pesticide usage (Rommens and Kishore 2000; Salmeron and Vernooij 1998; Shah 1997; Stuiver and Custers 2001). Among the strategies used for the genetic engineering of disease resistance, the employment of SAR is of special interest. SAR is long-lasting and appears to be effective against a broad spectrum of pathogens (Durrant and Dong 2004; Ryals et al. 1996). By screening an apple cDNA library with an *AtNPR1* probe, we cloned three apple *NPR1*-like genes, which showed 35% identity with *AtNPR1* at the amino-acid level. Only one of these genes (*MpNPR1-1*) was found to be induced by the SAR inducer BTH. *MpNPR1-1* is, therefore, the most likely candidate for being the ortholog of the *Arabidopsis NPR1* gene. *MpNPR1-1* has no obvious biochemical functions, except for the presence of two protein-interacting domains (the BTB/BOZ and ankyrin repeat domains), suggesting that its function is through interaction with other proteins, as has been previously described in *A. thaliana* (Després et al. 2000; Kim and Delaney 2002; Subramaniam et al. 2001; Zhang et al. 1999, 2006; Zhou et al. 2000). *AtNPR1* is located in the cytoplasm as an oligomer under noninducing conditions, and Cys216 is one of the cysteines that are required for keeping *AtNPR1* in the cytoplasm (Mou et al. 2003). Sequence comparisons between the three apple *NPR1*-like proteins indicate that residues near this cysteine are conserved in *MpNPR1-1* but not conserved in the *MpNPR1-2* and *MpNPR1-3* proteins, suggesting that *MpNPR1-2* and *MpNPR1-3* may have distinct functions.

Our results showed that overexpression of the *MpNPR1* gene increased the resistance of apple to three important pathogens, fire blight bacterium, apple scab fungus, and cedar apple rust fungus. The broad-spectrum resistance to both bacterial and fungal diseases observed is consistent with previous reports in *Arabidopsis*, rice, and wheat (Cao et al. 1998; Chern et al. 2001, 2005; Fitzgerald et al. 2004; Friedrich et al. 2001; Lin et al. 2004; Makandar et al. 2006). The fact that overexpression of *NPR1* proteins causes similar effects in different plants suggests the presence of similar defense pathways in apple, *Arabidopsis*, rice, and wheat. It is important to note, however, that there are differences between these different species with regard to their response to the elevated levels of *NPR1* gene expression. Although transformed *Arabidopsis* plants overexpressing *AtNPR1* acquire enhanced sensitivity to SA and BTH (Friedrich et al. 2001), they display no obvious

Fig. 6. Western blot analysis of *MpNPR1* expression in greenhouse leaves of nontransformed Galaxy plants and *MpNPR1*-transformed Galaxy lines. Leaf crude extract (10 μ g) was used for each sample. M = molecular marker (BenchMark prestained protein ladder, Invitrogen, Carlsbad, CA, U.S.A.).

detrimental morphological changes and, mostly, do not have elevated *PR* gene expression until activated by an inducer or by infection (Cao et al. 1998; Friedrich et al. 2001). However, overexpression of NH1 (rice homolog of AtNPR1) in rice leads to constitutive activation of defense genes and causes a developmentally controlled lesion-mimic phenotype (Chern et al. 2005). In addition, overexpression of either AtNPR1 or NH1 in rice potentiates a lesion mimic and cell death phenotype that can be triggered by BTH treatment or low light intensity (Fitzgerald et al. 2004). In apple, the overexpression of AtNPR1 or MpNPR1 does not create detrimental morphological changes in our experimental conditions.

Overexpression of the *NPR1* genes in *Arabidopsis*, tomato, and rice leads to enhanced nonspecific disease resistance in a dosage-dependent fashion (Cao et al. 1998; Chern et al. 2001; Lin et al. 2004). Consistent with these observations, transformed Galaxy lines that accumulated higher levels of NPR1 mRNA and protein exhibited broad-spectrum resistance to several important and different pathogens tested (fire blight, apple scab, and cedar apple rust). It will be of interest to determine

in the future whether these transformed plants have enhanced resistance to all major apple diseases. The expression pattern of the *PR* genes in our Galaxy transformed lines was generally consistent with some of the studies previously reported. In transformed *Arabidopsis* plants overexpressing *AtNPR1*, *PR* gene expression was induced in an AtNPR1-dosage dependent manner (Cao et al. 1998). In this study, expression of some *PR* genes (e.g., *PR2*, *PR5*, and *PR8*) was enhanced in some of our transformed apple lines; however, the degree of induced *PR* gene expression did not always correlate with the levels of NPR1 expression, except for the *PR2* gene. Our results are similar to what Lin and associates (2004) observed in tomato. Previously, it had been shown that *PR* gene expression could be either NPR1-dependent or -independent (Zhang and Shapiro 2002) and be uncoupled from bacterial and viral resistance (Clarke et al. 1998). Therefore, the enhanced broad-range disease resistance observed in transformed apple lines may involve the activation of *PR2* and some other defenses that were not analyzed in this study. In fact, a major challenge in the field is to elucidate what exact end compounds or cell

Fig. 7. *NPR1* transcript levels in MpNPR1. Top, Galaxy-transformed lines TGx-1319 and bottom, TGx-1322 compared with those in the control Galaxy after inoculation with water (W) or *Erwinia amylovora* (E.a) at 10^8 CFU.mL⁻¹.

Fig. 8. Pathogenesis-related (PR) transcript levels in *MpnPR1*-transformed Galaxy lines TGx-1319 and TGx-1322 compared with nontransformed Galaxy after inoculation with water (W) or *Erwinia amylovora* (E.a) at 10^8 CFU.ml⁻¹.

structural modifications, or both, plants use to achieve the resistance state.

MATERIALS AND METHODS

Cloning of the *MpNPR1* gene.

Green Jonathan apple leaves, sprayed with 0.3% BTH (wt/vol, commercially available BTH formula dissolved in distilled water) 5 days prior to collection for two consecutive days, were collected from the Michigan State University (East Lansing, MI, U.S.A.) educational apple orchard in the summer of 1998. Total RNA was extracted from these leaves and was used to construct the apple cDNA library in ZAP express vector (Stratagene, La Jolla, CA, U.S.A.). The cDNA library was screened by using the *AtNPR1* gene as a probe, following the manual provided by Stratagene. cDNA inserts present in all positive isolates were characterized by sequencing and were blasted against *AtNPR1* gene.

BTH treatment on apple seedlings for Northern blot analysis.

Apple seedlings were grown to the four-true-leaf stage in a controlled-environment chamber. Leaves were liberally sprayed with BTH (0.3%) until completely wet. Five days after treatment, leaves were harvested for isolation of RNA, which was subjected to Northern analysis as indicated elsewhere (Sambrook and Russell 2001).

Bacterial inocula.

Erwinia amylovora (fire blight). The wild-type strain of *E. amylovora* (Ea 273) was grown overnight at 28°C on Kado medium. Inoculum was prepared by suspension of strain Ea273 in distilled water at a concentration of 5×10^6 or 5×10^7 CFU ml⁻¹.

Venturia inaequalis (apple scab). A mixed inoculum of *V. inaequalis* isolates 1805-2, 1777-8, 1771-2, 1778-6, and 1810-1, representing the five races, race 1 through race 5, was used. Inocula were prepared by suspension of the conidia in distilled water at a concentration of 2.7×10^7 conidia ml⁻¹.

Gymnosporangium juniperi-virginianae (cedar apple rust). Inocula were prepared by suspension of the *G. juniperi-virginianae* basidiospores in distilled water at a concentration of 2.7×10^7 spores ml⁻¹.

Plasmid constructions.

Both *AtNPR1* and *MpNPR1-1* genes were amplified and inserted in-frame into pKYLX71 under the control of the CaMV35S promoter or a modified pKYLX71 under the control of the wound-inducible promoter Pin2. Three binary expression vectors, pKYLX35SAtnPR1, pKYLX35SMpNPR1, and pKYLXPINMpNPR1, were finally chosen to be electroporated into super-virulent *Agrobacterium tumefaciens* EHA105 PCH32 (Hood et al. 1993) for apple transformation.

The primers used for amplifying AtNPR1 and MpNPR1-1 were as follows.

AtNPR1 forward:

5'SalI-TCTTCAGTCGACATGGACACCACCA
TTGATGGATTTCGCC

AtNPR1 reverse:

5'SacI-GACCATTAGAGCTCACCGACGACGA
TGAGAGAGTTTACG

MpNPR1-1 forward:

5'SalI-TCTTCAGTCGACATGGCTCATTCA
GCCGAACCATCATCC

MpNPR1-1 reverse:

5'SpeI-CCACTAGTCAGTAAACCTCCGAA
GGCTTATTAGATGC

Plant material and transformation.

The apple cultivar Galaxy and the rootstock M.26 were chosen for this study because they can be genetically transformed at high efficiency (Borejsza-Wysocka et al. 1999; Norelli et al. 1999). Leaf segments were excised from in vitro-grown shoots of these two cultivars 3 weeks after subculturing. Transformation experiments were carried out as previously reported (Borejsza-Wysocka et al. 1999; Norelli et al. 1999), using *A. tumefaciens* EHA105 containing one of the binary vectors pKYLXAtNPR1, pKYLX35SMpNPR1, or pKYLXPINMpNPR1. DNA was isolated from the youngest leaf of putative transformed plants and nontransformed control plants as described by Cheung and associates (1993), and PCR was performed as described by Bolar and associates (1999). In order to check for the presence of promoter-*NPR1* fusions, specific primers were designed to amplify a fragment of the Pin2 or 35S promoter region and of the *MpNPR1* or *AtNPR1* sequence (Table 1). Nontransformed Galaxy, M.26, and transformed clones were propagated in vitro (Norelli et al. 1988). Acclimation of the plants was performed as described by Bolar and associates (1998).

Determination of ploidy level.

Ploidy level in the transformed and nontransformed clones was estimated by flow cytometry. Nuclei were isolated from in vitro leaves by manually chopping them with a razor blade directly into the buffer described by the manufacturer (Partec, Münster, Germany). After addition of 4,6 diamino-2-phenylindole dihydrochloride (DAPI; 2% vol/vol) and filtration through a 20-µm nylon mesh, the mixture was analyzed with a flow cytometer (Partec II, Partec).

Inoculation procedures.

Erwinia amylovora. The youngest expanded leaves from our own rooted plants in a growth chamber were inoculated by cutting the leaves transversely with scissors dipped in a suspension (5×10^6 CFU ml⁻¹ for M.26 or 5×10^7 CFU ml⁻¹ for Galaxy) of the virulent *E. amylovora* Ea273.

Venturia inaequalis. The youngest expanded leaf was tagged and the plants were inoculated with a suspension of *V. inaequalis* (2.7×10^6 conidia per milliliter), using an atomizer connected to a compressed air supply (Yepes and Aldwinckle 1993). The plants were incubated in a mist chamber (16-h photoperiod of 40 µmol m⁻² s⁻¹, 18 ± 1°C, and 100% relative humidity) for 48 h and, later, were moved to a growth chamber. Macroscopic symptoms and infection severity were evaluated

Table 5. Primer sequences used for polymerase chain reaction

Target gene	Primer sequence	Tm ^a °C
<i>AtNPR1</i>	F: ATTGCCGGAAGAGCTTGTTA R: AAGACGTTGAGCAAGTGCAA	58
<i>MpNPR1</i>	F: TCTTCAGTCGACATGGCTCATTACAGC CGAACCATCATCC R: CCACTAGTCAGTAAACCTCCGAAGG CTTATTAGATGC	58
<i>TPR1</i>	F: GTAGGCGTTGGTCCCCTTGAC R: GATTGCAGTCGCCAACATGT	55
<i>PR2</i>	F: GGAATTCAGTAGTATTCTCGAAG R: CAAATAGGTAAGTCTCTGTAGC	58
<i>PR3</i>	F: GCCGCGAATTCAGTAGTG R: CCAAACGTAGTCAAATAAGC	54
<i>PR5</i>	F: GAACCAGATGCTCCACGGAC R: GGCCGCGAATTCAGTAGTGAT	54
<i>PR8</i>	F: GTTAACATAGCCTTCCTC R: TGGACATTGAGGGGCTGC	54
<i>PR10</i>	F: TGGGTGTCTACACATTG R: TTAGTTGTATGCGTCGG	54

^aTm = temperature of hybridization.

on all leaves 21 days postinoculation according to the following scale: 0 = <5% of leaf surface with sporulation; 1 = 5 to 25%, 2 = 25 to 50%, and 3 = >50% leaf surface with sporulation. The number of leaves with symptoms was also determined. The median and confidence limits of the three leaves with most infection were calculated to describe the overall response of each line.

Gymnosporangium juniperi-virginianae. The youngest expanded leaf was tagged and the plants were inoculated with a suspension of *G. juniperi-virginianae* (2.7×10^6 conidia ml⁻¹), using an atomizer connected to a compressed air supply (Yepes and Aldwinckle 1993). The plants were incubated in a mist chamber (16-h photoperiod of 40 μ mol m⁻² s⁻¹, 18 $\pm$ 1°C, and 100% relative humidity) for 48 h and, later, were moved to a growth chamber. Macroscopic symptoms and infection severity were evaluated on all leaves 21 days postinoculation, by counting the number of lesions.

The presence of *V. inaequalis* conidia and *G. juniperi-virginianae* lesions on the leaf surface of inoculated plants was assessed 3 weeks postinoculation, by optical microscopy. At 21 days postinoculation, leaves were harvested and bleached for 24 h in a solution of acetic acid/ethanol 100% (vol/vol). The leaves were transferred into a solution of lactophenol (20 g of phenol, 6.6 ml of acetic acid, 32.1 ml of glycerol, 20 ml of water) for 24 h, followed by staining for 15 min with a solution of 0.5% methylene blue diluted in lactophenol. After washing the leaves with distilled water, the number of conidia and lesions was determined by microscopy.

DNA extraction and detection of T-DNA integration.

DNA from the transformed lines and nontransformed control were isolated following the procedure of the Nucleon phytopure plant and fungal DNA extraction kits (Amersham, Piscataway, NJ, U.S.A.). HindIII- or EcoRI-digested genomic DNA from a greenhouse-acclimated plant was transferred to Hybond-N nylon membrane. This membrane was hybridized with a fluorescein-labeled probe following the standard procedure of the DIG DNA labeling and detection kit (Roche, Penzberg, Germany).

Semiquantitative reverse transcription-PCR analysis.

Total RNA (50 to 100 μ g) was extracted from 0.5 g of young leaves excised from in vitro shoots as described by Venisse and associates (2002). Reverse transcription was performed with 2 μ g of total RNA. In order to evaluate relative differences in cDNAs between transformed clones, comparative kinetic analysis was conducted by PCR, as suggested by Horikoshi and associates (1992). Initial amounts of PCR substrates were adjusted for each clone on the basis of an equivalent amplification of a cDNA encoding the α subunit of translation elongation factor 1 (EF1- α), a member of a constitutively expressed gene family (Mahe et al. 1992). In order to evaluate differences among clones proportional to differences in initial amounts, we limited the amplification to 20 cycles. PCR of EF1- α was carried out with the specific primers of apple (forward, 5'-ATTGTGGTCATTGGTCATGT-3' and reverse, 5'-CCAATCTTGTAGACATCCTG-3'). In a second step, comparative amplification was carried out with *AtNPR1*-, *MpNPR1*-, *PR2*-, *PR5*-, *PR8*-, *PR10*-, and *TPR1*-specific primers (Table 5) on equivalent total cDNA. The PCR reaction was run with products from Promega (Madison, WI, U.S.A.). The thermocycler program was 94°C, 5 min, (20 cycles of 94°C, 30 s; temperature of hybridization (Table 5), 1 min; 72°C, 1 min) and 72°C, 15 min for each amplification. After electrophoresis on 1.2% agarose gel, amplified products were blotted onto a Hybond-N nylon membrane. This membrane was hybridized with a fluorescein-labeled probe following the standard

procedure of Random prime labeling and detection systems (Amersham, Piscataway, NJ, U.S.A.).

Protein extraction and Western blot.

Protein extraction was carried out from 200 mg of leaves from in vitro shoots for each transformed clone, according to Ko and associates (2002). For Western analysis, 50 μ g aliquots of protein extracted from control and transformed clones in Laemmli buffer were separated on 16% sodium dodecyl sulfate-tricine polyacrylamide (wt/vol) gel. After electrophoresis, proteins were blotted onto Hybond C nitrocellulose membrane (Amersham) by passive transfer. Polyclonal rabbit anti-MpNPR1 antiserum or anti-AtNPR1 was used. NPR1 proteins were detected with the enhanced chemiluminescence Western blotting detection system (ECL, Amersham) using horseradish peroxidase-labeled secondary antibody, according to the manufacturer's instructions.

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