Overexpression of a Rice NPR1 Homolog Leads to Constitutive Activation of Defense Response and Hypersensitivity to Light

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Arabidopsis NPR1/NIM1 **is a key regulator of systemic acquired resistance (SAR), which confers lasting broad-spectrum resistance. Previous reports indicate that rice has a disease-resistance pathway similar to the** *Arabidopsis* **SAR pathway. Here we report the isolation and characterization of a rice** *NPR1* **homologue (***NH1***). Transgenic rice plants overexpressing** *NH1* **(NH1ox) acquire high levels of resistance to** *Xanthomonas oryzae* **pv.** *oryzae***. The resistance phenotype is heritable and correlates with the presence of the transgene and reduced bacterial growth. Northern analysis shows that NH1ox rice spontaneously activates defense genes, contrasting with** *NPR1***-overexpressing** *Arabidopsis***, where defense genes are not activated until induction. Wild-type NH1, but not a point mutant corresponding to npr1-1, interacts strongly with the rice transcription factor rTGA2.2 in yeast two-hybrid. Greenhouse-grown NH1ox plants develop lesion-mimic spots on leaves at preflowering stage although no other developmental effects are observed. However, when grown in growth chambers (GCs) under low light, NH1ox plants are dwarfed, indicating elevated sensitivity to light. The GC-grown NH1ox plants show much higher salicylic acid (SA) levels than the wild type, whereas greenhouse-grown NH1ox plants contain lower SA. These results indicate that** *NH1* **may be involved in the regulation of SA in response to environmental changes.**

Systemic acquired resistance (SAR) is a long-lasting plant-defense response that confers broad-spectrum resistance to viral, bacterial, and fungal pathogens and induces expression of pathogenesis-related (*PR*) genes (Ryals et al. 1996). In dicots, such as *Arabidopsis* and tobacco, salicylic acid (SA) and the synthetic chemicals 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) are potent inducers of SAR (Friedrich et al. 1996). In monocots, SAR has been described for rice (Smith and Metraux 1991) and wheat (Gorlach et al. 1996) at least; and BTH has been shown to induce SAR in wheat (Gorlach et al. 1996) and disease resistance in rice (Rohilla et al. 2002; Schweizer et al. 1999) and maize (Morris et al. 1998).

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The *NPR1* (also known as *NIM1* and *SAI1*) gene is a key regulator of SA-mediated SAR in *Arabidopsis* (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Ryals et al. 1997; Shah et al. 1997). Upon induction by SA, INA, or BTH, *NPR1* expression levels are elevated (Cao et al. 1997; Ryals et al. 1997). *NPR1* affects the SAR pathway downstream of the SA signal. *Arabidopsis npr1/nim1* mutants are impaired in their ability to induce *PR* gene expression and mount a SAR response even after treatment with SA or INA. *NPR1* also is involved in the SA-promoted basal thermotolerance in *Arabidopsis* (Clarke et al. 2004). *NPR1* encodes a novel protein with a bipartite nuclear localization sequence and two potential protein–protein interaction domains: an ankyrin repeat domain and a BTB/POZ domain (Cao et al. 1997). Nuclear localization of NPR1 protein is essential for its function (Kinkema et al. 2000). Under uninduced states, NPR1 protein forms an oligomer and is excluded from the nucleus. Upon SAR induction, monomeric NPR1 emerges through redox changes, accumulates in the nucleus, and activates *PR* gene expression (Mou et al. 2003).

Overexpression of *NPR1* in *Arabidopsis* leads to enhanced disease resistance to both bacterial and oomycete pathogens in a dose-dependent manner (Cao et al. 1998). Similarly, overexpression of *Arabidopsis NPR1* in rice results in enhanced resistance to pathogen *Xanthomonas oryzae* pv. *oryzae* (Chern et al. 2001), indicating the presence of a similar defense pathway in rice. Although transgenic *Arabidopsis* plants overexpressing *NPR1* acquire enhanced sensitivity to SA and BTH (Freidrich et al. 2001), they display no obvious detrimental morphological changes and do not have elevated *PR* gene expression until activated by inducers or by infection of pathogens (Cao et al. 1998). However, in rice, overexpression of *Arabidopsis NPR1* potentiates a BTH- and low-light-environment-induced lesion mimic or cell death (LMD) phenotype (Fitzgerald et al. 2004).

In addition to SA, jasmonic acid (JA) and ethylene are well-studied signals that regulate distinct defense pathways (Turner et al. 2002). Cross talk between SA- and JA-mediated pathways has been well documented (Dong 1998; Glazebrook 2001; Kunkel and Brooks 2002). The function of *NPR1* also is essential for JA- and ethylene-regulated, SA-independent induced systemic resistance (ISR) (Pieterse et al. 1998). NPR1 also appears to modulate the cross talk between SA- and JA-dependent pathways; the antagonistic effect of SA on JA signaling requires NPR1, but not nuclear localization of the NPR1 protein (Spoel et al. 2003). The plant-specific transcription factor WRKY70 is identified as a common component in

SA- and JA-mediated signal pathways; overexpression of WRKY70 activates SA-induced *PR* genes, whereas antisense suppression results in activation of JA-responsive genes (Li et al. 2004). *WRKY70* expression is activated by SA and repressed by JA; functional *NPR1* is required for full-scale induction of *WRKY70* expression. Epistasis analysis suggests that WRKY70 is downstream of NPR1 in the SA signal pathway.

In *Arabidopsis*, NPR1 differentially interacts with the *Arabidopsis* TGA family members of basic-region leucine zipper (bZIP) transcription factors (Despres et al. 2000; Zhang et al. 1999; Zhou et al. 2000). Among the *Arabidopsis* TGA members, NPR1 preferentially interacts with TGA2 (also known as AHBP-1b), TGA3, TGA5, and TGA6 (Zhang et al. 1999; Zhou et al. 2000). The ankyrin repeats of NPR1 are necessary and sufficient for the interaction, although high-affinity interactions also require the N-terminal one third of NPR1 (Zhang et al. 1999). The interaction is abolished by *npr1-1* (carrying point mutation in the ankyrin repeats domain) and *npr1-2* (carrying point mutation in the N-terminal domain) mutants (Zhang et al. 1999). The interaction between NPR1 and TGA proteins facilitates binding of the TGA proteins to the SA-responsive *as-1* DNA element of the CaMV 35S promoter and the LS5 and LS7 elements of the *PR1* promoter (Despres et al. 2000). A GAL4:TGA2 fusion protein, consisting of GAL4 DNA binding domain and *Arabidopsis* TGA2, confers *NPR1*-dependent activation of a promoter containing GAL4 binding sites (Fan and Dong 2002), suggesting in vivo interaction between NPR1 and TGA2. The binding of NPR1 to *Arabidopsis* TGA transcription factors may facilitate TGA protein binding to SA-responsive elements (Despres et al. 2000; Johnson et al. 2003), activate the transcription factors, or recruit the TGA transcription factor to its functional location in the nucleus. The triple knockout mutant tga2tga5tga6, but not single or double knockouts, blocked induction of *PR* gene expression and pathogen resistance, showing an essential but redundant role of these transcription factors in SAR (Zhang et al. 2003). In rice, TGA2-like transcription factors interact with *Arabidopsis* NPR1 in a manner similar to *Arabidopsis* TGA2 (Chern et al. 2001). In summary, it has become clear that TGA proteins serve as a bridge between NPR1 and *PR* gene induction.

A disease resistance response often is accompanied by a hypersensitive response (HR), characterized by the rapid death of plant cells around the infection site (Mysore and Ryu 2004). In *Arabidopsis*, at least 37 spontaneous LMD mutants have been identified that exhibit misregulation in cell death and whose phenotypes resemble pathogen-inducible, HR cell death. Many of these LMD mutants display altered defense responses and, therefore, provide a direct link between programmed cell death and defense responses in plants (Lorrain et al. 2003). For example, *cpr* mutations, which result in constitutive expression of defense genes, cause spontaneous cell death. Similarly, mutations affecting salicylate-dependent signaling, such as *ssi* mutations (which suppress insensitivity to salicylate), also cause constitutive expression of defense genes and spontaneous lesions. In rice, lesion mimic mutants also have been identified that display activated expression of defense genes (Yin et al. 2000). The phenotypes of these mutants often resemble the HR, suggesting that the LMD results in activation of defense gene expression.

Here, we report the isolation and characterization of two rice cDNA clones encoding proteins similar to *Arabidopsis* NPR1. Transgenic rice plants overexpressing one of the cDNA clones display enhanced resistance to *X. oryzae* pv. *oryzae*. Growth stage-dependent lesion-mimic phenotype and constitutive activation of defense gene expression were observed in the transgenic plants, marking a difference in regulation of defense activation between rice and *Arabidopsis*.

RESULTS

Isolation of two NPR1 homologs from rice.

We reported (Chern et al. 2001) isolation of rice cDNA clones coding for four groups of proteins that interact with the *Arabidopsis* NPR1 in yeast-two hybrid screens. One of these, NRR (GenBank accession number AY846391), was used as bait to back-screen the rice cDNA library by the yeast two-hybrid method to search for rice NPR1. Characterization of NRR will be reported elsewhere.

We isolated two rice cDNA clones encoding NPR1 homologues (NH1 and NH2) after screening through approximately 16 million yeast clones with NRR as the bait. The NH1 cDNA appears to encode a full-length protein. The NH2 cDNA was apparently a truncated clone; the full-length NH2 protein sequence later was retrieved from the GenBank database (gi:34909872). A blast search (BLASTP) on the GenBank database with NH1 protein sequence gave a tobacco NPR1-like protein (gi:21552981) (Liu et al. 2002) as the highest hit and *Arabidopsis* NPR1/NIM1 in third. The rice NH1 (582 amino acids [aa]) and NH2 (635 aa) protein sequences were aligned with *Arabidopsis* NPR1 (593 aa) and the tobacco NPR1-like protein (588 aa) in Figure 1 by using the Pileup program of the Wisconsin GCG and SeqWeb package. Amino acids conserved among two or more of the proteins are highlighted in bold for recognition. Amino acids crucial for the NPR1 function as defined by genetic mutants, such as npr1-1, npr1-2, and nim1-4 (marked by arrowheads), are conserved.

By pairwise comparison using the Bestfit program, we found that rice NH1 shares with the tobacco NPR1-like protein 61% identity and 73% similarity; it shares 49% identity and 60% similarity with *Arabidopsis* NPR1. Rice NH2 is most similar to an *Arabidopsis* NPR1-like protein (gi:30694701), sharing 54% identity and 64% similarity. NH2 shares 48, 48, and 41% identity with NH1, the tobacco NPR1-like protein, and *Arabidopsis* NPR1, respectively. In addition to NH1 and NH2, two members (gi:50917587 and gi:50904471) in the NH1 family were identified using NH1 to BLASTP-search the GenBank database; the former shares 42% identity and the latter shares 31% identity with NH1. Using the *Arabidopsis* NPR1 protein to BLASTP-search the database, NH1 appears as the top hit in rice. Thus, the NH1 protein is most likely the rice ortholog of *Arabidopsis* NPR1.

Overexpression of rice NH1 confers enhanced resistance to *X. oryzae* pv. *oryzae*.

To test whether NH1 functions similarly to NPR1, we generated 32 independent transgenic lines overexpressing *NH1* (NH1ox) using a maize *ubiquitin* promoter in rice cv. LiaoGeng (LG). Six-week-old T0 transgenic plants and the LG control were challenged with *X. oryzae* pv. *oryzae* Korean race 1 (KR1), a highly virulent strain that overcomes *Xa21*-mediated resistance.

Inoculation data were collected from five independent experiments, each with its own LG control, because these plants were regenerated at different times (Fig. 2A). After inoculation with KR1, 10 lines (lines 11, 54, 58, 69, 85, 49, 63, 75, 56, and 57) of the 32 independently transformed NH1ox lines exhibited high levels of resistance to KR1 with lesion lengths 5.5 to 30% of the LG control. In all, 11 lines exhibited moderately enhanced resistance with leaf lesion lengths at least 40% shorter than the LG control. Lesion lengths of the remaining 11 lines (34%) were no different than the LG control, possibly because of chromosomal position effects.

Cosegregation of resistance with the NH1ox transgene.

To further characterize the NH1ox transgenic rice, we analyzed the progeny of four independent lines to see if the en-

hanced resistance phenotype correlated with the presence of the *Ubi-NH1* transgene. Leaf samples from individual progeny were collected and the polymerase chain reaction (PCR) performed using one primer specific to the *ubiquitin* promoter and the other specific to *NH1* to determine whether the individual progeny carried the transgene. These progeny were inoculated with KR1 at 6 weeks old. Inoculation and PCR results were compared. Typical results from progeny of two independent lines (lines 11 and 54) are presented in Figure 2B. Results from the other two lines were similar. These data demonstrate co-segregation of resistance and the NH1ox transgene, supporting the hypothesis that overexpression of *NH1* leads to the resistance phenotype.

Reduced bacterial populations correlate with shorter leaf lesion lengths.

We performed bacterial growth curves analysis to test whether reduced lesion lengths correlated with lower bacterial populations. Homozygous progeny of line 11 transgenic NH1ox plants and the LG control were inoculated with KR1. Four leaves from each were measured to obtain an average value for lesion length and each leaf ground up for *X. oryzae*

pv. *oryzae* population assessment at 0, 4, 8, 12, and 16 days after *X. oryzae* pv. *oryzae* inoculation.

Figure 3 presents results of the bacterial growth curve analysis (panel A) and leaf lesion development (panel B). No measurable leaf lesions developed till day 4, where bacterial populations had reached over 10^7 PFU/leaf in both NH1ox and LG control. Leaf lesions started to develop thereafter. The *X. oryzae* pv. *oryzae* populations in NH1ox plants stayed relatively constant and below 108 PFU/leaf from day 4 to day 16, whereas those in the LG control continued growing, reaching over 10^9 PFU/leaf. The *X. oryzae* pv. *oryzae* population in the LG control was 40 times larger than in the NH1ox line at day 16. Results (Fig. 3B) confirm the rapid and severe lesion development in LG and resistance in NH1ox plants described above. The reduced *X. oryzae* pv. *oryzae* growth in NH1ox plants was not due to smaller leaf size because these plants were no different from the LG control in their plant and leaf sizes when grown in the greenhouse, and only expanded leaves of nearly mature (6-week-old) plants were inoculated with *X. oryzae* pv. *oryzae*. Expanded leaves of NH1ox and LG control plants did not grow significantly differently during the incubation period after *X. oryzae* pv. *oryzae* inoculation. In summary, the shorter lesion

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             1
OsNH1                                      MEPPTSHVTNAF SDSDSAS.......VEEGDA D.........ADADVEALRR
NtNPR1                                       MDNSRTAF SDSNDISGSSSICCIGGGMT EFF...SPETSPAEITSLKR
AtNPR1                                       MDTTIDGF ADSYEISSTSFVATDNTDSS IVYLAAEQVLTGPDVSALQL
OsNH2    MPARSAVVVIAMEPSSSITI ASSSSYLSNGSSPCSVSLAP PGAGAVAAQAAPVAAGEGGG GGGGGGGGGGSSVEVVSLNR

             81
OsNH1    LSDNLAAAF.RSPEDFAFLA DARIAVPGGGGGGGDLRVHR CVLSARSPFLRGVFARR... ...AAAAAGGGGEDGSERL.
NtNPR1   LSETLESIFDASLPEFDYFA DAKLVV...SGPCKEIPVHR CILSARSPFFKNLFC..... ..........GKKEKNSSKV.
AtNPR1   LSNSFESVFD.SPDDF..YS DAKLVLSG.....REVSFHR CVLSARSFFKSALA..... ...AAKKEKDSNNTAAVKL.
OsNH2    LSANLERLLLDSDLD...CS DADVDVADGGPP...VPVHR CILAARSTFFYNLFAARGRG GDGAAGGGGGGGGGGGERTG

             161                                          ▽ npr1-2
OsNH1    .....ELRELLGGGGEEVEV GYEALRLVLDYLYSGRVGDL PKAACLCVDEDCAHVGCHPA VAFMAQVLFAASTFQVAELT
NtNPR1   .....ELKEVM....KEHEV SYDAVMSVLAYLYSGKVRPS PKDVCVCVDNDCSHVACRPA VAFLVEVLYTSFTFQISELV
AtNPR1   .....ELKEI....AKDYEV GFDSVVTVLAYVYSSRVRPP PKGVSECADENCCHVACRPA VDFMLEVLYLAFIFKIPELI
OsNH2    GRPRYKMEELVPGG....RV GRDAFLSLLGYLYTGKLRPA PDDVVSCADPMCPHDSCPPA IRFNVEQMYAAWAFKITELI

             241
OsNH1    NLFQRRLLDVLDKVEVDNLL LILSVANLCNKSCMKLLERC LDMVVRSNLDMITLEKSLPP DVIKQIIDARLSLGLISPE.
NtNPR1   DKFQRHLLDILDKTAADDVM MVLSVANICGKACERLLSSC IEIIVKSNVDIITLDKALPH DIVKQITDSRAELGLQGPE.
AtNPR1   TLYQRHLLDVVDKVVIEDTL VILKLANICGKACMKLLDRC KEIIVKSNVDMVSLEKSLPE ELVKEIIDRRKELGLEVPK.
OsNH2    SLFQRRLLNFVDKTLVEDVL PILQVA..FHSELTPVLEKC IRRIARSNLDNVSLDKELPP EVAVQIKEIRQKSQPNEGDT

             321                                                       npr1-1 ▽
OsNH1    ..NKGFPNKHVRRIHRALDS DDVELVRMLLTEGQTNLDDA FALHYAVEHCDSKITTELLD LALADVNHRNPRGYTVLHIA
NtNPR1   ..SNGFPDKHVKRIHRALDS DDVELLQMLLREGHTTLDDA YALHYAVAYCDAKTTAELLD LALADINHQNSRGYTVLHVA
AtNPR1   ..VK....KHVSNVHKALDS DDIELVKLLLKEDHTNLDDA CALHFAVAYCNVKTATDLLK LDLADVNHRNPRGYTVLHVA
OsNH2    VISDPVHEKRVRRIHRALDS DDVELVKLLLNESEITLDDA NALHYAAAYCDSKVVSELLD LRLANLNLKNSRGYTALHLA

             401
OsNH1    ARRREPKIIVSLLTKGARPA DVTFDGRKAVQISKRLTKQG DYFGVTEEGKPSPKDRLCIE ILEQAERRDPQLGEASVSLA
NtNPR1   AMRKEPKIVVSLLTKGARPS DLTSDGRKALQIAKRLTRLV DFSKSPEEGKSASNDRLCIE ILEQAERRDPLLGEASVSLA
AtNPR1   AMRKEPQLILSLLEKGASAS E.TLEGRTALMIAKQATMAV ECNNIPEQCKHSLKGRLCVE ILEQEDKREQIPRDVPPSFA
OsNH2    AMRREPAIIMCLLNKGAAVS QLTADGQSAMSICRRLTRMK DYNTKMEQGQESNKDRLCID ILDREMIRKPMAVEDSVTSP

             481       nim1-4 ▽
OsNH1    MAGESLRGRLLYLENRVALA RIMFPMEARVAMDIAQVDGT LEFNLGSG.ANPPPERQRTT VDLNESPFIMKEEHLARMTA
NtNPR1   MAGDDLRMKLLYLENRVGLA KLLFPMEAKVAMDIAQVDGT SEFPLASI.GKKMANAQRTT VDLNEAPFKIKEEHLNRLRA
AtNPR1   VAADELKMTLLDLENRVALA QRLFPTEAQAAMEIAEMKGT CEFIVTSLEPDRLTGTKRTS PGVKIAPFRILEEHQSRLKA
OsNH2    LLADDLHMKLLYLENRVAFA RLFFPAEAKVAMQIAQADTT PEF...GIVPAASTSGKLKE VDLNETPVTQNKRLRSRVDA

             561
OsNH1    LSKTVELGKRFFPRCSNVLD KIMD.D..ETDPVS.LGRDT SAE...KRKRFHDLQDVLQK AFHEDKEENDR.SGLSSSSS
NtNPR1   LSRTVELGKRFFPRCSEVLN KIMDAD..DLSEIAYMGNDT AEERQLKKQRYMELQEILTK AFTEDKEEYDKTNNISSSCS
AtNPR1   LSKTVELGKRFFPRCSAVLD QIMNCE..DLTQLACGEDDT AEKRLQKKQRYMEIQETLKK AFSEDNLELG.NSSLTDSTS
OsNH2    LMKTVELGRRYFPNCSQVLD KFLEDDLPDSPDALDLQNGT SDEQNVKRMRFCELKEDVRK AFSKDRADNSMFSILSSSSS

             641
OsNH1    STSI..GAIRPRR
NtNPR1   STSK..GVDKPNKLPFRK
AtNPR1   STSKSTGGKRSNRKLSHRRR
OsNH2    SSPPPKVAKK
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Fig. 1. Sequence alignment of rice NPR1 homologues 1 (osNH1) and 2 (osNH2), *Arabidopsis* NPR1, and a tobacco NPR1-like protein. OsNH1, osNH2, *Arabidopsis* NPR1, and the tobacco NPR1-like protein (gi:21552981) were aligned using the Pileup program of the Wisconsin GCG and WebSeq package (version 2). Amino acids conserved among two or more of the proteins are highlighted in boldface. Gaps are represented by dots. The amino acids changed in npr1-1, npr1-2, and nim1-4 mutants are marked by arrowheads.
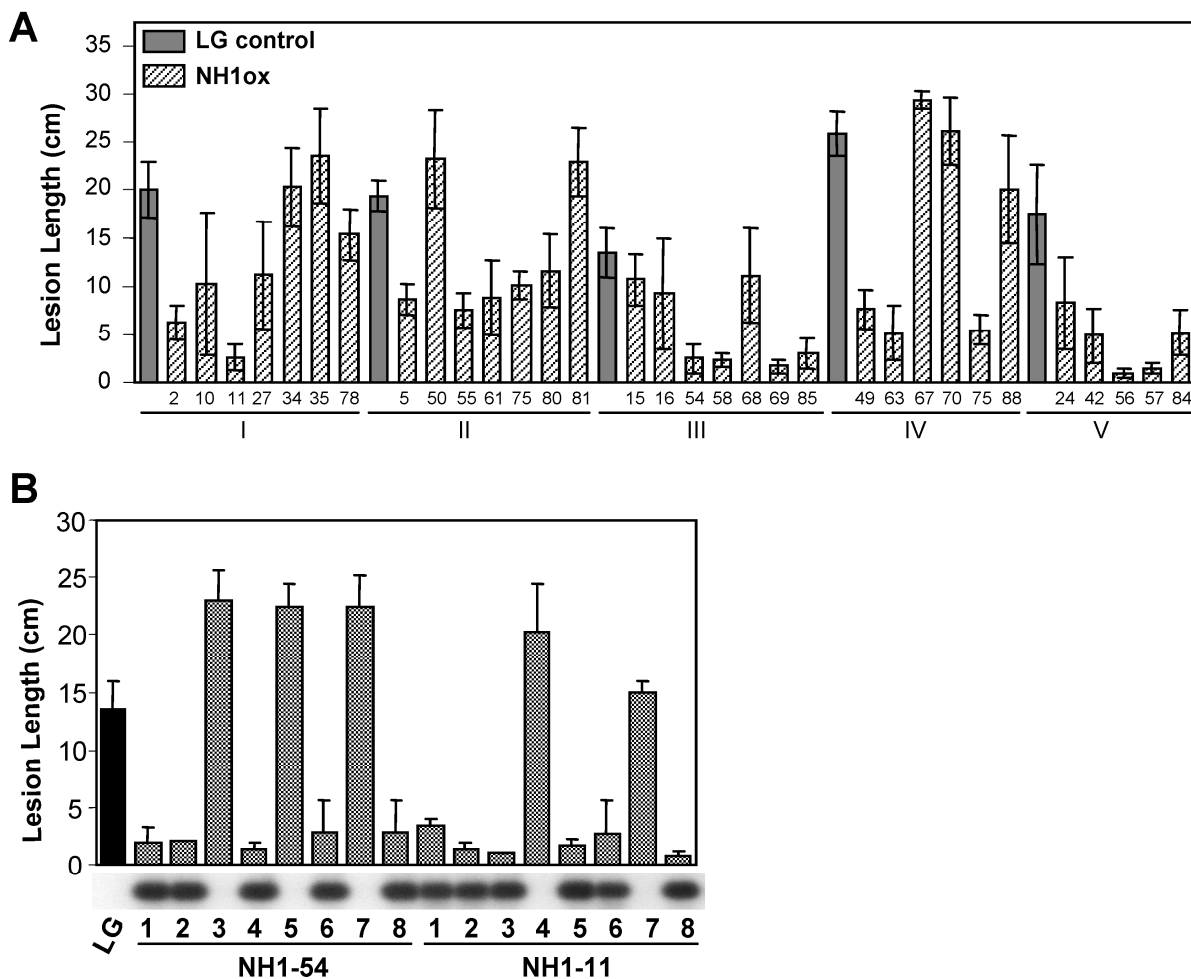
Fig. 2. Leaf lesion length of transgenic rice over-expressing *NH1* (NH1ox) inoculated with *Xanthomonas oryzae* pv. *oryzae* Korean race 1 (KR1). **A,** Thirty-two independently transformed 6-week-old T0 NH1ox transgenic plants and the LiaoGeng (LG) control were challenged with *X. oryzae* pv. *oryzae* in five separate inoculations (labeled I to V). **B,** Co-segregation of the *Ubi-NH1* transgene and the enhanced resistance phenotype. Lesion lengths of eight (labeled 1 to 8) segregating progeny from each of two lines (lines 11 and 54) are presented. Polymerase chain reaction (PCR) was performed using one primer specific to the *ubiquitin* promoter and the other specific to the *NH1* cDNA. PCR results hybridized with a *NH1* probe are shown below the bar graph.
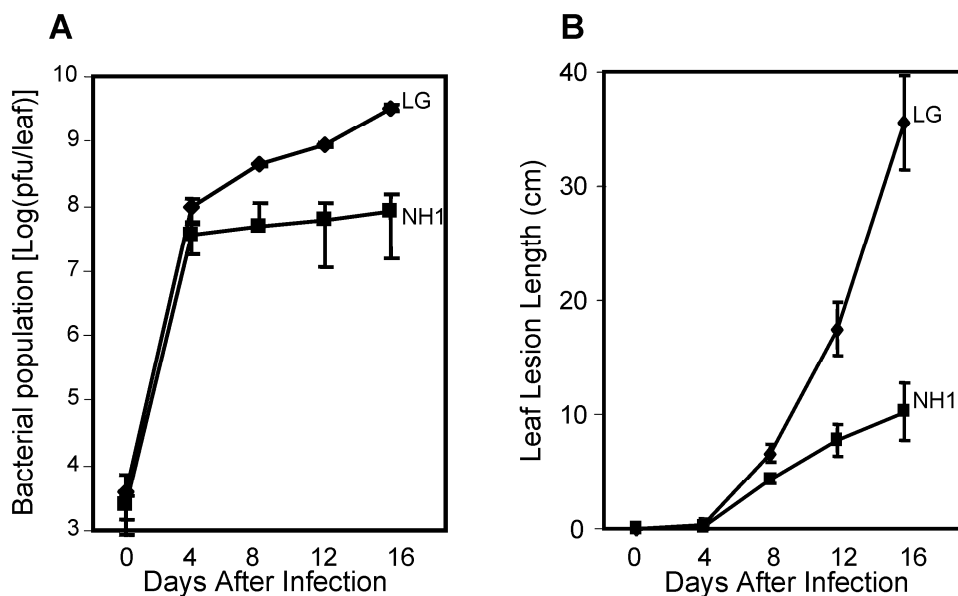
Fig. 3. Correlation of *Xanthomonas oryzae* pv. *oryzae* population growth and leaf lesion development. **A,** Bacterial growth curves. **B,** Leaf lesion development. Homozygous NH1ox transgenic plants (T2 progeny from line #11) and LiaoGeng (LG) control plants were inoculated with *X. oryzae* pv. *oryzae* KR1. Four leaves each were collected from NH1ox or LG at day 0, 4, 8, 12, and 16 post inoculation. Lesion length for each individual leaf was measured; each leaf then was ground individually in water and plated out to assess *X. oryzae* pv. *oryzae* population. Each data point represents the average and standard deviation of four leaves.

development phenotype in NH1ox plants is accompanied by a reduction in *X. oryzae* pv. *oryzae* growth.

Activation of defense genes.

In *Arabidopsis*, *NPR1* regulates expression of many *PR* genes, such as *PR1*, *PR2*, and *PR5*. Not only do *npr1/nim1* mutants abolish *PR* gene expression after induction, but also overexpression of *NPR1* results in stronger induction of *PR* genes by chemical inducers and pathogens. Although overexpression of *Arabidopsis NPR1* in rice also leads to enhanced resistance, it was not known whether defense genes are activated. We carried out Northern hybridizations to test whether defense genes are activated in the NH1ox rice plants. Total RNA samples were extracted from 6-week-old, greenhouse-grown, untreated plants of two homozygous NH1ox lines

Fig. 4. RNA expression levels of *NH1* and defense-related genes of NH1ox lines 11 and 54 and the LiaoGeng (LG) control. Total RNA samples were sequentially probed with *PBZ1*, *PAL*, *NH1*, and 25S rDNA, or with *PR1*, *POX*, *NH1*, and 25S rDNA. Total RNA (10 μg) was loaded in each lane.

(lines 11 and 54) and the LG control and hybridized with four defense-related genes: rice *PR1b*, *PBZ1/PR10* (Qi and Yang 1999), *PAL* (phenylalanine ammonia lyase), and *POX* (peroxidase).

Northern analysis results are presented in Figure 4. First of all, in both NH1ox lines, the NH1 RNA levels were much higher than the endogenous level represented by the LG control. In fact, we have tested many of the NH1ox lines that display the resistance phenotype and all of them express high levels of *NH1* mRNA. In LG, the four defense genes examined, especially *PBZ1* and *PR1*, are expressed at very low levels. In contrast, all four defense-related genes obviously are constitutively expressed at elevated levels in the two NH1ox lines. These results demonstrate that overexpression of the *NH1* gene in rice leads to constitutive expression of defense genes in the absence of induction by chemical or pathogen treatment.

NH1ox plants develop lesion-mimic spots in the greenhouse and LMD spots in the growth chamber.

In a previous study, we observed that LMD spots develop on leaves of transgenic rice overexpressing *Arabidopsis NPR1* (NPR1ox) (Fitzgerald et al. 2004) when the plants were transferred from the greenhouse (high light conditions) to the growth chamber (approximately one-sixth light intensity, high humidity; details discussed below). Similarly, NH1ox plants develop LMD spots 3 weeks after transfer from the greenhouse to the growth chamber (Fig. 5A). These LMD spots normally start near the leaf tips and slowly move down the leaves. In severe cases, the entire leaf will senesce and die.

In addition to the LMD phenotype, we have observed a second lesion-mimic phenotype on the NH1ox but not the NPR1ox plants. Older leaves of the NH1ox plants spontaneously develop lesion-mimic spots in the greenhouse when the plants are 8 to 9 weeks old, at the preflowering (booting) stage. Two leaves from NH1ox plants displaying such typical lesion-mimic spots are shown in Figure 5B (indicated by the arrowhead). Leaves from LG plants are free of such spots. In contrast to the LMD phenotype, development of the lesion
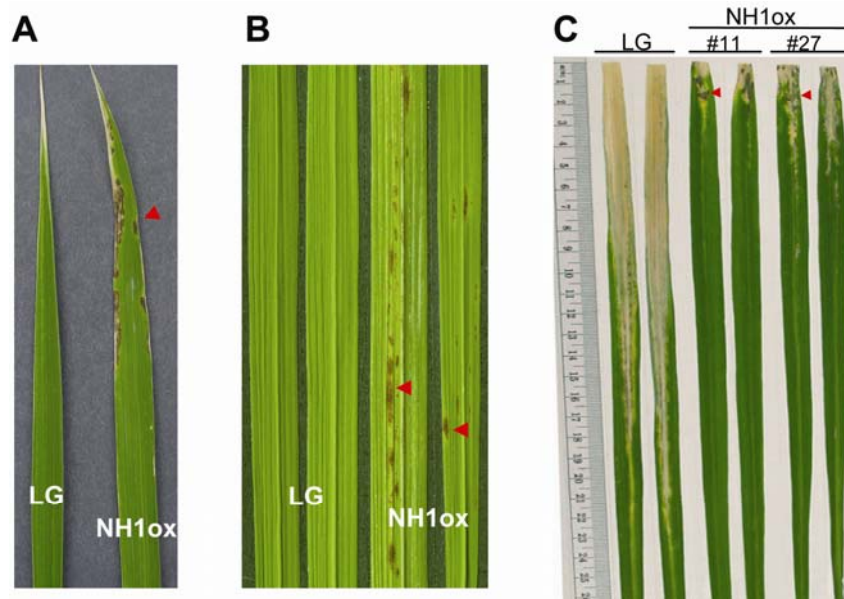
Fig. 5. Environment-induced lesion mimic or cell death (LMD), spontaneous lesion-mimic spots, and *Xanthomonas oryzae* pv. *oryzae*-induced hypersensitive response (HR)-like necrosis on NH1ox plants. **A,** Environment-induced LMD (marked by the arrowhead); 3-week-old plants grown in the greenhouse were transferred to the growth chamber and grown for another 3 weeks. **B,** Spontaneous, stage-dependent lesion-mimic spots. Leaves of 9-week-old NH1ox transgenic plants and the LiaoGeng (LG) control grown in the greenhouse. The arrowheads mark typical lesion-mimic spots. **C,** *X. oryzae* pv. *oryzae*-induced HR-like necrosis of NH1ox plants. Leaf tips were infected with *X. oryzae* pv. *oryzae* when plants were 6 weeks old. Pictures were taken 2 weeks after inoculation. The HR-like necrosis normally becomes visible 3 days after inoculation with *X. oryzae* pv. *oryzae*.

mimic spots on the NH1ox rice in the greenhouse is growth-stage dependent and its initiation is not restricted to the leaf tips. It occurs only on mature leaves of older plants and appears throughout the leaf. Nevertheless, NH1ox transgenic plants grow as well as the LG control in the greenhouse and show a seed set comparable to the control (data not shown).

Older leaves of NH1ox rice plants show a tendency to senesce precociously compared with wild-type plants. This tendency becomes more obvious after pathogen infection; although *X. oryzae* pv. *oryzae*-inoculated leaves of NH1ox plants develop shorter lesions, older leaves (below the inoculated ones) of the same plant display accelerated senescence (not shown).

In addition, NH1ox plants show more obvious HR-like response a few days after challenge with the *X. oryzae* pv. *oryzae* pathogen. Leaves of the NH1ox plants develop *X. oryzae* pv. *oryzae*-induced, HR-like necrotic spots (Fig. 5C, arrowheads) near the *X. oryzae* pv. *oryzae* inoculation sites, whereas fully susceptible leaves of the LG control show few

spots. These results further support the conclusion that the NH1ox transgenic plants possess an altered defense response to *X. oryzae* pv. *oryzae* infection.

Growth of NH1ox plants is more sensitive to light.

When grown in the greenhouse, NH1ox plants show no obvious difference from the wild-type LG control in appearance and seed setting. However, when grown in the growth chamber, the NH1ox plants display retarded growth relative to the LG control. The difference in growth chamber-grown plants and the similarity in the greenhouse-grown plants at 3 weeks old is shown in Figure 6A. In addition, under the growth chamber conditions, older leaves of NH1ox plants tend to senesce precociously.

To quantify the results, plant height, fresh weight of aerial parts, number of tillers per plant, and leaf width were measured at 8 weeks old. The results summarized in Figure 6B show that, although greenhouse-grown NH1ox plants were essentially the

Growth condition	Cultivar/Line	Height (cm)	FW (Aerial) (g)	No. Tillers	Leaf width (mm)	Sample size
Green house	LG	80.8 ± 5.8	18.57 ± 7.67	5 ± 2	10.88 ± 0.68	20
	NH1ox-54	79.7 ± 6.4	16.05 ± 4.18	5 ± 1	11.09 ± 0.69	21
Growth chamber	LG	91.0 ± 8.9	5.97 ± 2.45	1 ± 0	10.01 ± 1.69	20
	NH1ox-54	51.0 ± 7.7	0.75 ± 0.32	1 ± 0	4.54 ± 1.28	22
	NH1ox-11	48.9 ± 9.4	0.9 ± 0.6	1 ± 0	4.88 ± 1.28	20
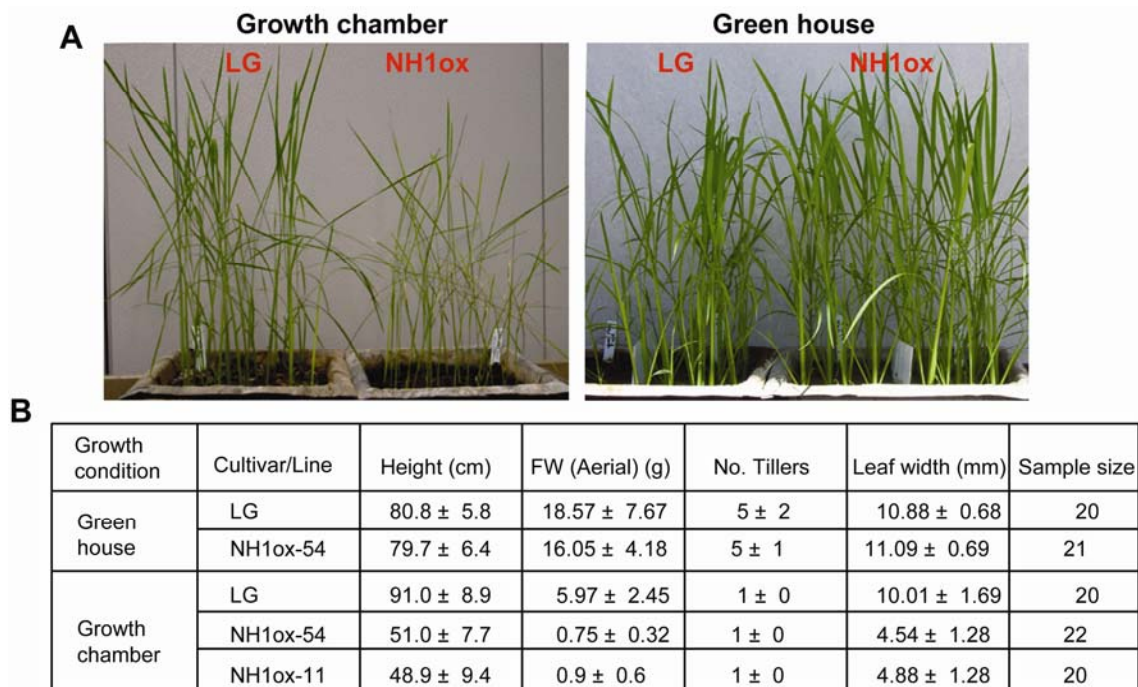
Fig. 6. Growth of NH1ox rice plants under greenhouse and growth chamber conditions. **A,** Approximately 30 homozygous T2 progeny from NH1ox transgenic lines 54 and 11 and the LiaoGeng (LG) control were grown either in the growth chamber or greenhouse. Pictures were taken when plants were 3 weeks old. **B,** At 8 weeks old, NH1ox and LG plants were harvested and measured for total height and fresh weight (FW) of the aerial portions of the plant, as well as tiller number and leaf width.
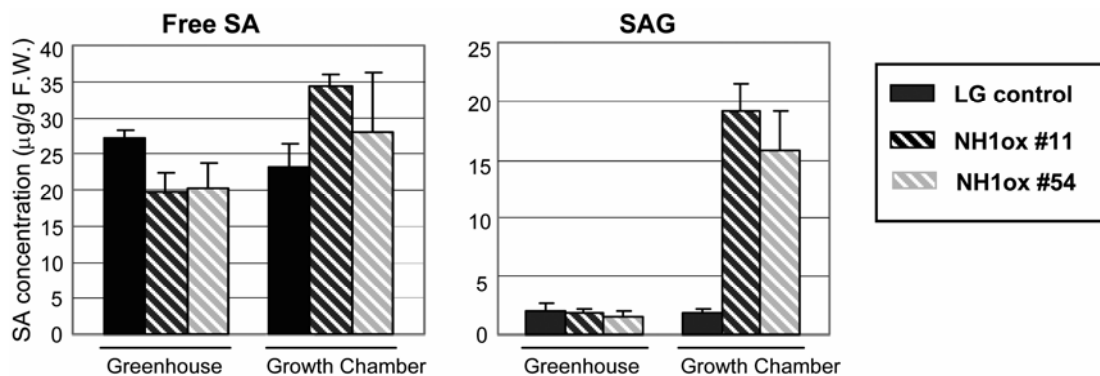
Fig. 7. Free and conjugated salicylic acid (SA) contents. The third leaf of each plant was harvested from 3-week-old LiaoGeng (LG) or NH1ox (lines 11 and 54) rice grown in the greenhouse or growth chamber. Two to three replicate samples per cultivar or line (approximately 15 plants per cultivar or line for each growth environment) were analyzed by high-performance liquid chromatography for both free-SA and glucose-conjugated SA (SAG) contents. Each bar represents the mean and standard deviation for the sample.

same as the LG wild-type control, growth chamber-grown NH1ox plants (both lines 11 and 54) have much smaller statures (2×), lower fresh weights (7×), and narrower (2×) or smaller leaves. Under the growth chamber condition, both LG and NH1ox plants failed to develop additional tillers. These results indicate that the NH1ox rice plants are more sensitive to the growth chamber environment, which has a different light spectrum and lower light intensity than the greenhouse environment (Fitzgerald et al. 2004).

NH1ox plants have altered levels of SA contents.

We previously reported that overexpression of *Arabidopsis NPR1* in rice correlated with reduced SA accumulation in leaves (Fitzgerald et al. 2004). To assess possible effects of overexpressing *NH1* on SA accumulation in rice, we measured SA levels. Both free SA and SA-glucoside (SAG) contents were determined for the third leaves of NH1ox (lines 11 and 54) and LG plants grown under greenhouse and growth chamber conditions. Free SA levels are lower in NH1ox lines than in the LG wild type under greenhouse conditions, similar to the observation for NPR1ox rice plants (Fig. 7). However, free SA levels are higher in NH1ox lines under the growth chamber condition. Moreover, under the growth chamber conditions, the SAG levels in both NH1ox lines are strikingly (more than 8×) higher than the LG wild type. Although all the rice plants grown in the greenhouse contain low levels of SAG, the SAG levels of the two growth-chamber-grown NH1ox lines reached levels similar to free SA, suggesting that free SA might be converted to SAG when it reached a certain threshold level. In short, the NH1ox plants accumulate much higher total SA (free SA + SAG) than the wild type in the growth chamber, whereas they contain slightly lower total SA when grown in the greenhouse. Thus, SA accumulation appears more sensitive to environmental changes in the NH1ox plants compared with the LG controls.

**Interaction of rice NPR1
with TGA transcription factors is conserved.**

The rice *NH1* was identified in two-hybrid screening by using an NPR1-interacting protein NRR rather than an rTGA2 protein. Therefore, we tested directly whether rice NH1 interacted with rTGA2.2 in the yeast two-hybrid assay. The rTGA2.2 protein was fused to the B42 activation domain, whereas each of NH1 and its mutants was fused to the LexA DNA binding domain. The wild-type NH1 interacted strongly with rTGA2.2, showing intense blue color, and the vector-only control displayed little blue color (Fig. 8A).

We also generated two NH1 point mutations at amino acids critical for NPR1 function and conserved with rice NH1 and tested their effects on interaction with rTGA2.2. Mutation $H_{338}Y$ (HY) corresponded to npr1-1 and mutation $C_{150}Y$ (CY) to npr1-2. Each of the mutations was capable of completely abolishing the interaction with rTGA2.2 (Fig. 8A). The Western results of yeast protein (extracted from yeast cell carrying these constructs) (Fig. 8B) show a slightly reduced protein level for the HY mutant. However, the slightly lower protein expression cannot account for the complete abolishment of interaction by the HY mutation. The CY mutant had a significantly reduced protein level. Thus, protein instability of CY may have contributed to the lack of interaction with rTGA2.2. Overall, the HY mutation had the same effect on NH1 as on NPR1 with regards to the interaction with rTGA2.2.

DISCUSSION

We have isolated two rice cDNA clones encoding NPR1 homologs by yeast two-hybrid screening. Our results showed

that NH1 strongly interacted with rTGA2.2, a rice bZIP transcription factor similar to *Arabidopsis* TGA2; the interaction was abolished by HY and CY mutations corresponding to npr1-1 and npr1-2, respectively, suggesting that the NH1-rTGA2.2 interaction is similar to that of NPR1-TGA2. To study *NH1* function, we overexpressed *NH1* in the rice cv. LG, which is highly susceptible to *X. oryzae* pv. *oryzae* KR1. Transgenic NH1ox plants show high levels of resistance when challenged with KR1. The resistance phenotype is heritable and correlates with the presence of the *Ubi-NH1* transgene. Bacterial growth curve analysis showed a correlation between the resistance phenotype and the reduction in *X. oryzae* pv. *oryzae* population in the NH1ox rice. These results are consistent with our previous report, which showed that overexpression of the *Arabidopsis NPR1* in rice enhances resistance to *X. oryzae* pv. *oryzae* and suggested that rice shares a resistance pathway similar to the *NPR1*-mediated pathway (Chern et al. 2001). Overexpression of rice *NH1* seems to be more effective in conferring resistance to *X. oryzae* pv. *oryzae* than overexpression of *Arabidopsis NPR1* because the NH1ox plants exhibit a strong enhancement in resistance when challenged with KR1. NPR1ox rice plants exhibited only very moderate resistance to this strain (data not shown). However, because the transgenic lines are in different genetic backgrounds, these results should be interpreted with caution.

RNA blot analysis indicated that the NH1ox rice plants constitutively express defense genes. This defense gene expression was independent of the presence of visible lesion mimic spots which normally occur at 8 weeks or older, because RNA samples taken from 6-week-old and younger (data not shown) greenhouse-grown plants showed elevated defense gene expression. In contrast, PR1 and PBZ1/PR10 were significantly expressed in NPR1ox plants only when LMD spots were visible after the plants were transferred to growth chambers. Thus, NH1ox plants appeared to activate the defense gene more readily than NPR1ox rice. The NH1ox results also contrasted with findings from *Arabidopsis* over-
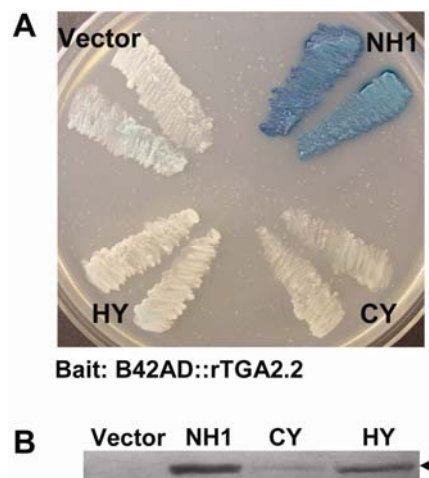

Fig. 8. Yeast two-hybrid assay for interaction of rTGA2.2 and NH1 wild-type and mutants. **A,** In-plate β-galactosidase activity assay. The rTGA2.2 protein was fused to the B42 activation domain while each of NH1 and its mutants was fused to the LexA DNA binding domain. NH1 mutant $C_{150}Y$ (CY) corresponds to the npr1-2 mutation and $H_{338}Y$ (HY) to the npr1-1 mutation. X-gal was included in the medium. The picture was taken 2 days after yeast cells were streaked on the plate. **B,** Western analysis of yeast protein. Protein samples were extracted from yeast cells expressing either wild-type NH1, mutant protein CY or HY fused to the LexA DNA binding domain, or the LexA domain alone. Protein samples were probed with an antibody (Clontech) against LexA. The arrowhead indicates the location of the fusion proteins. Approximately 150 μg of protein were loaded in each lane.

expressing NPR1, in which defense gene expression was not observed until induction by chemical or pathogen treatment (Cao et al. 1998). In short, our data showed a difference between rice and *Arabidopsis* in the regulation of defense gene induction, possibly due to the fact that rice contains much higher levels of endogenous SA than *Arabidopsis* (Silverman et al. 1995). Although the high levels of SA in rice may cause the overexpressed NH1 protein to be constitutively activated, the low levels of SA in *Arabidopsis* would keep overexpressed NPR1 protein inactive until SAR induction and SA synthesis to induce defense.

The development of lesion-mimic spots on leaves of NH1ox plants at preflowering stage in the greenhouse indicated that the defense pathway or pathways was activated, consistent with the Northern results. However, we did not observe any obvious detrimental developmental effects in the NH1ox plants, probably because the rice plants already were at a productive stage when the lesion mimic spots occurred. We also did not find significant reduction in seed set of the NH1ox plants, although large-scale experiments would be needed to draw a more definitive conclusion.

The spontaneous and age-dependent development of the lesion-mimic spots on NH1ox plants was distinct from the LMD spots that we observed for both NPR1ox and NH1ox rice plants. Transgenic NPR1ox and NH1ox rice lines did not exhibit LMD in the greenhouse; the LMD spots developed only when the plants were transferred from the greenhouse to growth chambers (low light). The LMD spots normally start near the leaf tips and slowly move down the leaves in severe cases (Fitzgerald et al. 2004). In contrast, initiation of the lesion mimic spots on the NH1ox rice was not restricted to the leaf tips but occurred throughout the leaves. Although the LMD spots can develop on young plants, development of the lesion-mimic spots on NH1ox rice in the greenhouse was limited strictly to plants approximately 8 weeks old and older. These results also suggest a difference in the rice NH1 and *Arabidopsis* NPR1 proteins. Like the spontaneous induction of defense genes, development of the lesion-mimic spots may be due, in part, to the high endogenous SA levels in rice.

SA is involved in leaf senescence in *Arabidopsis*. SA levels are increased fourfold in senescent leaves compared with mature green ones. Senescence-enhanced genes, such as LSC94 and LSC460, were induced by SA application. Upregulation of these genes at senescence was inhibited in *npr1* and *pad4* mutants and *NahG* transgenic *Arabidopsis*; changes in gene expression were accompanied by a delayed yellowing and reduced necrosis (Morris et al. 2000). Thus, SA levels and NPR1 both are involved in senescence-associated gene expression in *Arabidopsis*.

Older leaves of NH1ox rice plants show a tendency to senesce early when compared with wild-type plants. This tendency becomes more obvious after pathogen infection. Although *X. oryzae* pv. *oryzae*-inoculated leaves of NH1ox plants develop shorter lesions, older leaves (below the inoculated ones) of the same plant experience accelerated senescence. This phenomenon is reminiscent of INA-treated *Arabidopsis* plants, where wild-type *Arabidopsis* experience early senescence in the lower leaves approximately a week after the treatment, whereas *npr1* mutants remain green and unchanged (*unpublished data*). This suggests that *NH1* in rice most likely is involved in a SAR-like response and elevated levels of NH1 may augment the response.

Interestingly, NH1ox plants display growth retardation when grown in growth chambers, indicating that they are more sensitive to the growth-chamber light conditions. We compared the wavelength spectrum and intensity of light in our growth chamber and greenhouse. Growth chambers evidently had much lower light intensity (approximately one-sixth of the greenhouse) and very different light quality or wavelength spectrum from the greenhouse (Fitzgerald et al. 2004). Thus, it is likely that suboptimal light quality and low light intensity may cause the growth retardation effects of NH1ox plants. In *Arabidopsis*, it has been reported that phytochrome signaling modulates a SA-perceptive pathway (Genoud et al. 2002). The induction of *PR1* by SA is dependent on *phyA*- and *phyB*-controlled light signaling pathways; in darkness as well as dim light, SA-induced *PR* gene expression and the HR to pathogens are strongly reduced.

In contrast to many other plants, rice normally contains more free-SA than SAG (Fitzgerald et al. 2004; Silverman et al. 1995). The growth-chamber-grown NH1ox plants accumulated much higher SAG and slightly higher free SA than the wild type, whereas greenhouse-grown NH1ox plants contained slightly lower free SA and SAG levels. These results suggest that NH1 may be involved in SA signaling and in the regulation of SA content in response to environmental changes (Fig. 9). The light quality and intensity may be the environmental factor that NH1 responds to, leading to changes in SA levels. The strikingly higher levels of SAG in the growth-chamber-grown NH1ox plants indicated that, under low light conditions, NH1 may activate SA-conjugating enzymes, leading to much higher SAG levels. A possible reason that leaves of NH1ox plants have such high free SA contents in the growth chamber is that NH1ox plants might be undergoing senescence. Support for this hypothesis is the observation that leaves of growth-chamber-grown NH1ox plants displayed LMD symptoms resembling senescence. The LMD symptoms
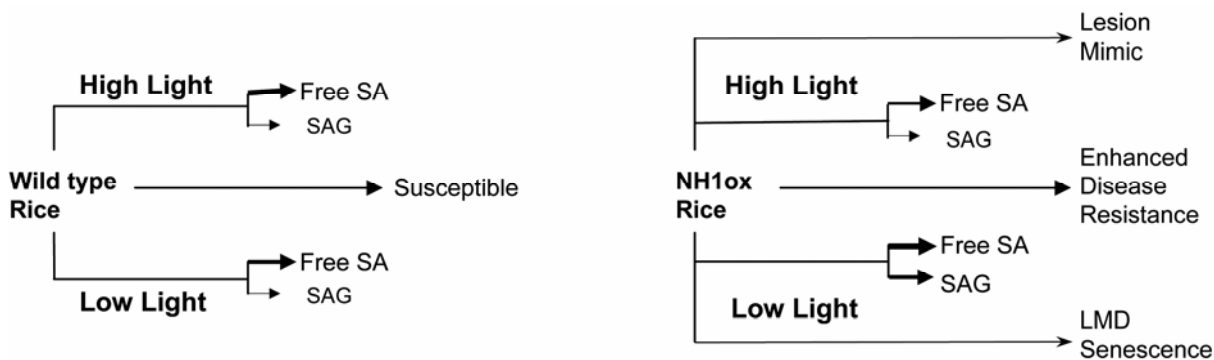

Fig. 9. Schematic representation of the modulation of free salicylic acid (SA) and glucose-conjugated SA (SAG) accumulation by the light intensity in wild-type (left panel) and NH1ox (right panel) rice plants and effects on lesion mimic and disease resistance. High light is represented by greenhouse conditions and low light by growth chamber conditions. NH1ox represents high levels of NH1 expression. Levels of free SA and SAG accumulation are depicted by the different sizes of the arrows; thicker arrows indicate higher levels.

on NH1ox plants induced by the growth chamber environment were similar to the LMD observed for NPR1ox plants under the same environment. However, NPR1ox plants did not accumulate high SA under this environment.

In summary, the study of rice *NH1* has revealed similarities as well as differences between rice and *Arabidopsis* with regard to the defense responses and plant growth. It appears that, although rice and *Arabidopsis* share conserved defense pathways, the regulation of these pathways and the links to other plant pathways may be quite divergent. Therefore, it is important to directly study *NH1* in rice in order to understand its involvement in rice defense and other biological pathways.

MATERIALS AND METHODS

Plant materials and growth conditions.

Rice (*Oryzae sativa* L.) plants were maintained in the greenhouse, which had a photosynthetically active radiation (PAR) value equal to 674 μmol m^{-2} s^{-1} in spring. The growth chamber had a light intensity PAR equivalent to 103 μmol m^{-2} s^{-1}, set on a 14-h daytime period and a temperature cycle of 28 and 26°C, 90% humidity. For *X. oryzae* pv. *oryzae* inoculation, rice plants were grown in the greenhouse until they were 6 weeks old and transferred to a growth chamber. The *X. oryzae* pv. *oryzae* strain KR1 was used to inoculate rice by the scissors-dip method (Kauffman et al. 1973).

Rice transformation.

Rice cv. LG was used as the recipient for transformation. *Agrobacterium* EHA105 was used to infect rice callus for transformation. Rice transformation protocol was as described before (Chern et al. 2001).

Plasmid construction.

For rice transformation, a 1.95-kb full-length *NH1* cDNA fragment was PCR amplified from the original yeast two-hybrid pAD-Gal4 clone using primers prNH13 (AAATCTAGAGGAT CCCAATGGAGCCGCCGACCA) and prNH6 (CCTCGAGTA CAAGCACTA). The *NH1* PCR product was cloned into pBluescript II SK– and its sequence confirmed by sequencing. The full-length *NH1* cDNA insert was excised by cutting with *Bam*HI and *Spe*I and cloned into binary vector Ubi-C1300, digested with the same enzymes, creating plasmid Ubi-NH1/C1300. The Ubi-C1300 binary vector was generated by cloning a maize *ubiquitin* promoter (Christensen and Quail 1996) and a nos3′ fragment into the Cambia 1300 vector by the same way as described before (Chern et al. 2001) for Ubi-C1301.

To create the LexA fusion protein for the yeast two-hybrid test, a 2-kb, full-length *NH1* cDNA was excised with *Eco*RI and *Xho*I and cloned into plasmid pNLex digested by *Eco*RI and *Sal*I enzymes. The C$_{150}$Y mutant was generated by using primers prNH8 (GTCCTCGTCGACGTAGAGGCACGCCGC CT) and SS020 (AGGGATGTTTAATACCACTAC) to amplify a 0.5-kb 5′ end of *NH1* cDNA; the 0.5-kb CY fragment was cut with *Eco*RI and *Sal*I and ligated to a 1.5-kb *Sal*I-*Xho*I fragment using the unique *Sal*I site to assemble the full-length cDNA. To create mutant H$_{338}$Y, a 3′ end was amplified using primers prNH9 (ACTGTTCTTTACATTGCTGCGAGGCG AA) and prNH6. A middle piece of *NH1* was amplified with prNH10 (GCAGCAATGTAAAGAACAGTATAACCTCTTG) and prNH16 (AACTCGAGATCTACGAGGCGCTGCGGCT GGT). The two pieces were annealed together and the 1.5-kb fragment amplified with prNH16 and prNH6. The 1.5-kb HY fragment was joined with a 0.5-kb *NH1* 5′ end fragment at the unique NotI site to assemble the full-length cDNA. All PCR products were confirmed by DNA sequencing. The full-length

CY and HY mutants were cloned into the pNLex vector using the *Eco*RI and *Xho*I sites the same way as with the wild-type *NH1* cDNA.

PCR, DNA, and RNA blot hybridization.

Rice genomic DNA extraction was done according to a protocol described previously (Dellaporta et al. 1984). PCR of the *Ubi-NH1* transgene was carried out with the maize *ubiquitin* promoter-specific primer Ubi-1 (TGATATACTTGGATGATG GCA) and *NH1*-specific primer prNH22 (GGACGGCGATG CGCGCGTC). DNA and RNA blotting and hybridization was performed as described before (Chern et al. 2001). For the rice *PR1b* probe, a 1.3-kb fragment of *PR1b* was amplified from IR24 genomic DNA using primers osPR1-1 (AAGAATTCAA GTCCTGCGTACAAATC) and osPR1-2 (AAGAATTCTAGA GAAGTGCGGCGATG). The PCR product was cut with *Eco*RI and cloned into pBluescript II SK–; the sequence of the insert was verified by DNA sequencing. The rice *PBZ/PR10* probe was the same as described before (Fitzgerald et al. 2004).

SA measurement.

The measurement of SA contents of rice leaf tissues was done as described previously (Fitzgerald et al. 2004).

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