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Enhanced disease resistance and hypersensitivity to BTH by introduction of an NH1/OsNPR1 paralog

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Summary

Non-expresser of pathogenesis-related genes 1 (NPR1) is the master regulator of salicylic acid-mediated systemic acquired resistance. Over-expression of Arabidopsis *NPR1* and rice *NH1 (NPR1 homolog1)/OsNPR1* in rice results in enhanced resistance. While there are four rice NPR1 paralogs in the rice genome, none have been demonstrated to function in disease resistance. To study rice NPR1 paralog 3, we introduced constructs into rice and tested for effects on resistance to infection by *Xanthomonas oryzae pv. oryzae* (*Xoo*), the causal agent of bacterial blight. While over-expression of *NH3* using the maize *ubiquitin-1* promoter failed to enhance resistance, introduction of an extra copy of *NH3* driven by its own promoter (nNT-NH3) resulted in clear, enhanced resistance. Progeny analysis confirms that the enhanced resistance phenotype, measured by *Xoo*-induced lesion length, is associated with the *NH3* transgene. Bacterial growth curve analysis indicates that bacterial population levels are reduced 10-fold in nNT-NH3 lines compared to control rice lines. The transgenic plants exhibit higher sensitivity to benzothiadiazole (BTH) and 2,6-dichloroisonicotinic acid (INA) treatment as measured by increased cell death. Expression analysis of *pathogenesis-related* (*PR*) genes showed that nNT-NH3 plants display greatly enhanced induction of *PR* genes only after treatment with BTH. Our study demonstrates an alternative method to employ a regulatory protein to enhance plant defence. This approach avoids using undesirable constitutive, high-level expression and may prove to be more practical for engineering resistance.

Introduction

Plants survive pathogen attack by employing various defence strategies, including strengthening of cell walls, the accumulation of phytoalexins, synthesis of salicylic acid (SA) and induction of pathogenesis-related (*PR*) genes. A hypersensitive response (HR) is often associated with the defence response and limits pathogen growth to the infected site. After an initial local infection, systemic acquired resistance (SAR) often occurs, which coordinately induces expression of a set of *PR* genes, leading to a long-lasting enhanced resistance against a broad spectrum of pathogens (Durrant and Dong, 2004). In dicots, like Arabidopsis and tobacco, SA and its synthetic analogues, such as 2,6-dichloroisonicotinic acid (INA), benzothiadiazole (BTH) and probenazole are potent inducers of SAR (Ward *et al.*, 1991; Friedrich *et al.*, 1996; Yoshioka *et al.*, 2001). In monocots, SAR can be induced by BTH in wheat (Gorlach *et al.*, 1996) and by *Pseudomonas syringae* in rice (Smith and Metraux, 1991). BTH can also induce disease resistance in rice (Schweizer *et al.*, 1999; Rohilla *et al.*, 2002; Shimono *et al.*, 2007) and maize (Morris *et al.*, 1998).

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). *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* encodes a protein with a bipartite nuclear localization sequence and two 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). The ankyrin domain is required for interaction with TGA transcription factors (Zhang *et al.*, 1999; Despres *et al.*, 2000), and the BTB/POZ domain interacts with the repression domain of TGA2 to negate its function (Boyle *et al.*, 2009). During non-induced 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). NPR1 also appears to modulate the crosstalk between SA- and jasmonic acid (JA)-dependent pathways; the antagonistic effect of SA on JA signalling requires NPR1, but not nuclear localization of the NPR1 protein (Spoel *et al.*, 2003).

Over-expression 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, over-expression of Arabidopsis *NPR1* or the rice NPR1 ortholog, NH1, in rice results in enhanced resistance to rice bacterial blight pathogen *Xanthomonasoryzae* pv. *oryzae* (*Xoo*) and blast pathogen *Magnaporthe grisea* (Chern *et al.*, 2001; Yuan *et al.*, 2007; Quilis *et al.*, 2008), indicating the presence of a similar defence pathway in rice. Although transgenic Arabidopsis plants over-expressing *NPR1* acquire enhanced sensitivity to SA and BTH (Friedrich *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, over-expression of rice NH1 results in a development- and environment-dependent lesion-mimic phenotype, which can be further enhanced by application of BTH (Chern *et al.*, 2005a). These results suggest that over-expression of NH1 in rice activates the defence response in the absence of inducer treatment or pathogen challenge, an undesirable consequence in terms of practical application. Thus, although rice possesses a pathway similar to the NPR1-mediated one in Arabidopsis, there may be significant differences in their regulation.

There are six NPR1-like genes in Arabidopsis (Liu *et al.*, 2005; Zhang *et al.*, 2006) and five NPR1-like genes in rice (Yuan *et al.*, 2007). Despite extensive investigations carried out on NPR1, very little is known concerning the NPR1-like genes with regard to their possible involvement in plant defence. Arabidopsis *NPR5* and *NPR6* have recently been named *BOP2* (*Blade-On-Petiole2*) and *BOP1* (*Blade-On-Petiole1*), respectively. BOP1 and BOP2 regulate Arabidopsis leaf formation. Like NPR1, these proteins function as transcriptional coactivators targeting the *AS2* (*Asymmetric Leaves2*) gene (Jun *et al.*, 2010). Thus, NPR5 (BOP2) and NPR6 (BOP1) are mainly involved in regulating plant development rather than defence. Contradictory results concerning the function of Arabidopsis NPR4 have been reported. Liu *et al.* (2005) reported that Arabidopsis *NPR4* is required for basal resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and *Erysiphe cichoracearum* because the *npr4-1* mutant is more susceptible to these two pathogens. This group suggested that *NPR4* may be also involved in the crosstalk between SA- and JA-dependent signalling pathways because expression of the JA-dependent marker gene *PDF1.2* is compromised in *npr4-1* leaves following application of methyl-JA. However, Zhang *et al.* (2006) reported that Arabidopsis *NPR3* and *NPR4* are the negative regulators of *PR* gene expression and disease resistance. They showed that *npr3* mutants have slightly increased basal *PR-1* expression and the *npr3npr4* double mutant shows even higher *PR-1*, *PR-2* and *PR-5* expression. The double-mutant plants display enhanced resistance against virulent bacterial (including *Pst* DC3000) and oomycete pathogens (Zhang *et al.*, 2006). Thus, the roles of *NPR4* in disease resistance from these two reports contradict each other. In rice, Yuan *et al.* (2007) have over-expressed OsNPR1/NH1, OsNPR2 and OsNPR3 in rice and tested for enhanced resistance to *Xoo* and rice blast. These authors found that only OsNPR1 (but not OsNPR2 or OsNPR3) over-expression conferred enhanced resistance, leaving in doubt whether any rice NPR1 paralogs are involved in defence against pathogens.

We have taken a similar approach to investigate possible functions for rice NPR1-like genes. We found that transgenic rice plants carrying an NH3 transgene driven by its native promoter show enhanced resistance to the *Xoo* pathogen, whereas those carrying an NH3 transgene driven by the commonly employed maize *Ubi-1* promoter fail to yield resistant plants. These transgenic plants also exhibit elevated sensitivity to BTH treatment and enhanced induction of defence-related genes upon treatment with plant defence activators. These results not only differ from previous reports but also point out an alternative way for engineering plants for resistance to pathogens.

Results

The five rice NPR1-like proteins form three subgroups

In rice, there are five NPR1-like proteins, namely NH1, NH2, NH3, NH4 and NH5 (NPR1 homologs 1-5), encoded by six genes (TIGR gene ID Os01g09800, Os01g56200, Os03g46440, Os01g72020, Os11g04600 and Os12g04410) in the genome. Protein NH5 is encoded by two duplicate genes, Os11g04600 and Os12g04410, which are recently duplicated genes because of chromosomal segmental duplication (Jacquemin *et al.*, 2009). In Arabidopsis, the six NPR1-like proteins are divided into three groups with each group containing two proteins (Liu *et al.*, 2005). As depicted in Figure 1, the five rice NPR1-like proteins can also be divided into three groups based on Blast search results: NH1 in group 1, NH2 and NH3 in group 2 and NH4 and NH5 in group 3. Thus, unlike in Arabidopsis, rice NH1 is most unique in sequence among rice NPR1-like proteins.

The identity levels across different groups of rice NPR1-like proteins are higher than those in Arabidopsis. For example, in Arabidopsis, NPR1 shares about 35% identity with group 2 and 20% identity with group 3, while in rice NH1 shares 43% identity with group 2 and 31% identity with group 3. These data indicate that the five rice NPR1-like proteins are closer to each other than the six Arabidopsis NPR1-like proteins to each other. Within groups, rice NH2 shares 54% identity with NH3, while NH4 shares 62% identity with NH5. Between rice and Arabidopsis, rice NH1 shares the highest homology with Arabidopsis NPR1 and NPR2, carrying 46% identity and 66% similarity with NPR1 and 46% identity and 64% similarity with NPR2. NH2 has the highest homology to Arabidopsis NPR3 and NPR4, sharing 51% identity and 69% similarity with NPR3 and 51% identity and 70% similarity with NPR4; NH3 also shares the highest homology with Arabidopsis NPR3 and NPR4, carrying 48% identity and 65% similarity with NPR3 and 46% identity and 65% similarity with NPR4. NH4 is most homologous to Arabidopsis BOP2 (NPR5), sharing 70% identity and 78% similarity; NH5 is most homologous to Arabidopsis BOP1 (NPR6), sharing 65% identity and 78% similarity.

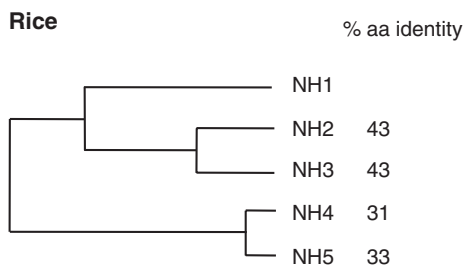

Figure 1 A phylogenic tree of rice NPR1-like proteins. Sequences of the five rice NPR1-like proteins are based on TIGR annotations and confirmed by isolated cDNA clones. Sequences are aligned using BlastP. The percentage of amino acid (aa) identity was calculated by comparing individual protein to NH1, respectively.

Construct using the Ubi-1 promoter to over-express NH3 yielded no transgenic plants with enhanced resistance

Over-expression is a useful tool to study the possible function of a gene of interest. The *Ubi-1* promoter is commonly used for such purpose because of its ability to express a gene to very high levels, especially in monocots, including rice. In an attempt to study possible involvement of rice NH1 paralogs in disease resistance, we tried to over-express the rice NH3 (also called OsNPR3) gene using the maize *Ubi-1* promoter in the Kitaake (Kit) rice variety. A 1.8-kb rice NH3 cDNA was introduced into an Ubi-C1300-based binary vector. We were able to produce many (approximately 20) transgenic green calli and generated more than 10 transgenic rice seedlings. Upon transfer to greenhouse, most of these transgenic rice plants developed lesion mimics and dwarfism and eventually died. Only four plants eventually survived. These plants were challenged with *Xoo* pathogen PXO99 to test for possible effects on disease resistance. No obvious enhanced disease resistance was observed, as shown in Figure 2a, in these transgenic plants.

Enhanced disease resistance is accomplished by introduction of a modified copy of NH3

In the same attempt to study the function of NH3, we also created a binary construct based on the C1300 vector in which expression of the NH3 cDNA is directed by its own native promoter, contained in a 1-kb DNA fragment. An N-terminal tag [N-terminus tandem affinity purification (NTAP)] was added to the NH3 protein to potentially facilitate detection of the protein. This construct is designated nNT-NH3. When this construct was introduced into the Kit recipient, 12 transgenic plants were obtained and eventually survived. These plants were subject to challenge by *Xoo*. Our results (Figure 2a) showed that about half (7 of 12) of the T0 transgenic plants exhibit obvious enhanced resistance to *Xoo*.

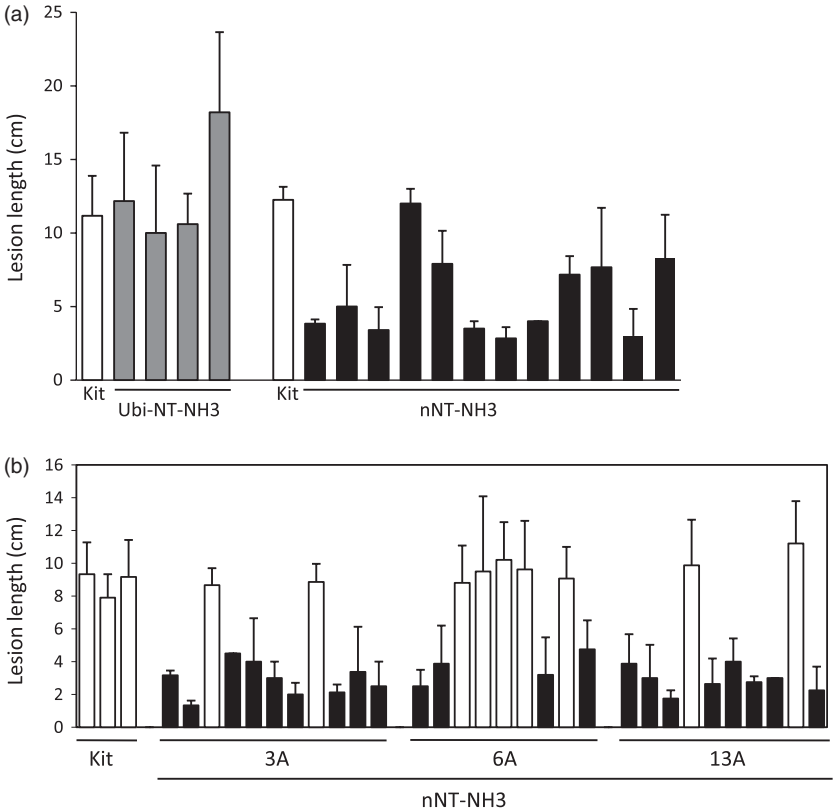

Figure 2 Lesion length measurements of NH3 transgenic plants. (a) Resistance analysis of T0 plants. Four Ubi-NT-NH3 (grey bars) and 12 nNT-NH3 (filled bars) T0 transgenic plants were inoculated with PXO99, and lesion lengths were measured 2 weeks after inoculation. Wild-type Kitaake (Kit) plants were included as control (open bars). Each bar represents the average and standard deviation of two to five leaves. (b) Enhanced resistance and transgene co-segregation analysis. T1 progeny from three T0 lines (#3A, #6A and #13A) of nNT-NH3 transgenic plants were genotyped by PCR for the presence of the nNT-NH3 transgene. The progeny containing the transgene are presented as filled bars and null segregants, which no longer contain the gene, are as open bars. Kit plants were included as control (open bars). Six-week-old plants were inoculated with PXO99 and lesion lengths measured in 2 weeks. On average, three to four leaves were inoculated. The error bars represent standard deviations.

Progeny segregation analysis was performed subsequently to test whether the observed enhanced resistance phenotype is associated with the nNT-NH3 transgene. The presence of the nNT-NH3 transgene was detected by conducting polymerase chain reactions (PCR) targeting the NH3 mini-gene, which differs from the endogenous NH3 gene in lacking introns. Shown in Figure 2b are progeny segregation analysis results of such three (lines #3A, 6A, and 13A) transgenic lines. The results clearly show that all progeny plants containing the nNT-NH3 gene (closed bars in Figure 2b) are resistant to PXO99. On the contrary, null segregants (open bars), which no longer contain the transgene, are as susceptible to PXO99 as the wild-type control Kit plants.

A more detailed analysis of the progeny of lines #6A and 13A was conducted to study the resistance phenotype. Progeny plants were genotyped for the presence of the nNT-NH3 gene. Those that contained the gene (both homozygous and heterozygous) were pooled together to represent lines #6A (labelled NH3-6) and #13A (labelled NH3-13), respectively. Figure 3a shows the lesion development and 3b the growth curves of *Xoo*. Consistent with previous results, Figure 3a shows that NH3-6 and NH3-13 plants clearly developed much shorter lesions compared with the Kit control at day 8 and day 12. Bacterial growth curves in Figure 3b also show clear differences between the NH3-6 and NH3-13 transgenic plants and the Kit control at day 8 and day 12. The bacterial populations in Kit are more than 10-fold higher than those in the transgenic plants. *T*-tests on NH3-6 vs. Kit and NH3-13 vs. Kit at day 12 yield *P* values = 0.0036 and 0.0042, respectively, indicating that the differences are highly statistically significant. Two typical leaves (12 days after *Xoo* inoculation) from each line and the Kit control are shown in Figure 3c. An RT-PCR was conducted to confirm that transgenic lines NH3-6 and NH3-13 express the nNT-NH3 gene. RT-PCR results shown in Figure 3d reveal that the NH3 transcript level is higher in both NH3-6 and NH3-13 (including both native NH3 and NTAP-NH3 transcripts) than in Kit (by approximately threefold) and that the NTAP-tagged NH3 is expressed only in the transgenic plants. These results clearly demonstrate that introduction of the nNT-NH3 transgene into wild-type rice enhances resistance to the *Xoo* pathogen.

NH3 transgenic plants show strong responses to plant defence activators BTH and INA

We have previously reported that application of BTH to the Ubi-NH1 transgenic plants induces/enhances

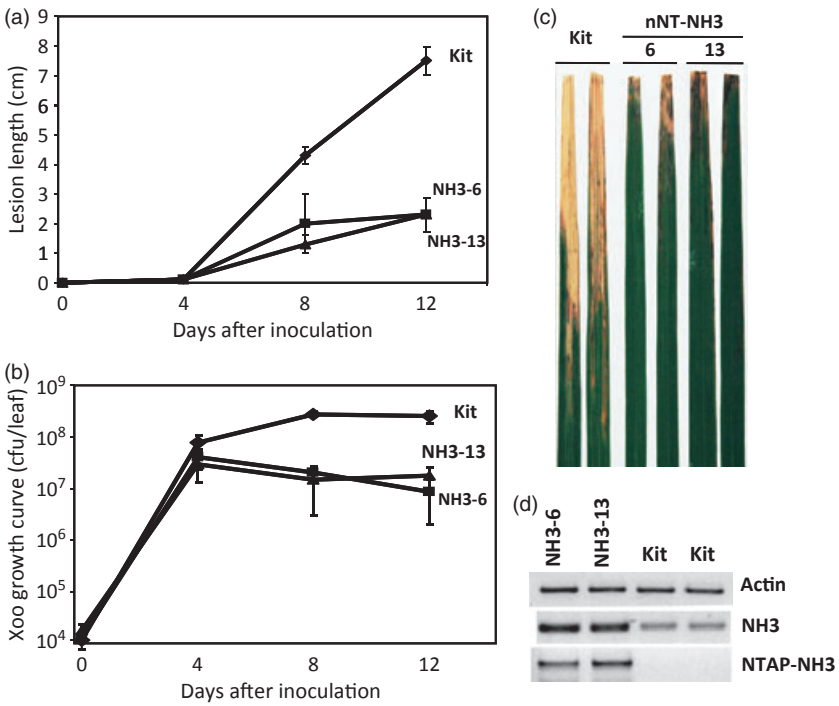

Figure 3 Analysis of nNT-NH3 lines #6A and #13A. (a) Lesion development. Progeny of nNT-NH3 lines #6A and #13A (labelled NH3-6 and NH3-13) were first genotyped. Those containing the nNT-NH3 transgene were pooled to represent the line and inoculated with PXO99. Lesion lengths were measured at days 0, 4, 8 and 12, after inoculation. Each data point represents the average and standard deviation of three leaf samples. (b) Bacterial growth curves. Inoculated leaf samples described in (a) were used to extract *Xoo* for growth curve analysis. (c) Leaf lesions caused by *Xoo*. Two representative leaves, each from Kit control, NH3-6 and NH3-13, 2 weeks after PXO99 inoculation are shown. (d) RT-PCR results. Total RNA samples were extracted from NH3-6, NH3-13 and Kit. An equal amount of RNA was reverse-transcribed, and the cDNA was used for RT-PCR. The amounts of input cDNA were further equalized based on expression of actin. The expression of the NH3 genes (including endogenous and the nNT-NH3 transgene) was measured by targeting the NH3 transcripts. The expression of the nNT-NH3 transgene (labelled NTAP-NH3) was measured using one primer targeting the NTAP tag and the other targeting NH3. NTAP, N-terminus tandem affinity purification.

lesion-mimic development (Chern *et al.*, 2005a). We tested whether the nNT-NH3 transgenic plants would have a similar response to BTH treatment. To our surprise, when sprayed with 10 mM BTH, the nNT-NH3 plants responded to BTH very strongly, leading to extremely severe cell death. As shown in Figure 4a, the severity of the cell death induced by BTH treatment is extremely high, as evidenced by the dry-out of whole leaves within 3–4 days. On the contrary, plants that carry an NTAP control driven by the *Ubi-1* promoter (Park *et al.*, 2008) are completely clear of such cell death phenotype. These results suggest that the development of the severe cell death phenotype after BTH application is because of the NH3 transgene.

We then treated the nNT-NH3 plants as well as the Kit control with 1 mM BTH or 1 mM INA to test for effects. When treated with 1 mM BTH, the nNT-NH3 plants developed lesion-mimic spots in general together with some leaves showing bleached areas, representing severe cell death, as shown by the two representative leaves in Figure 4b (labelled NH3, BTH). In contrast, when treated with 1 mM BTH, Kit developed no lesion-mimic spots at all and was free of bleached areas (see Kit, BTH). Mock nNT-NH3 plants treated with no BTH only occasionally carry some small lesion-mimic spots (NH3, mock). When treated with 1 mM INA, the nNT-NH3 plants developed typical lesion-mimic spots, but no bleached areas on leaves (NH3, INA). Treatment with 1 mM INA had little effects on Kit plants (Kit, INA). These results suggest that the nNT-NH3 plants respond to plant defence activators, such as BTH and INA, with clearly elevated sensitivity.

Defence-related genes are induced more strongly in the NH3 transgenic plants

To investigate the BTH- and INA-induced responses at the molecular level, we looked at the transcript expression levels of several rice defence-related genes, including *PR1a*, *PR3*, *PR10*, *POX* (peroxidase) and *NH1*. RT-PCR results of these genes for response to BTH treatment are shown in

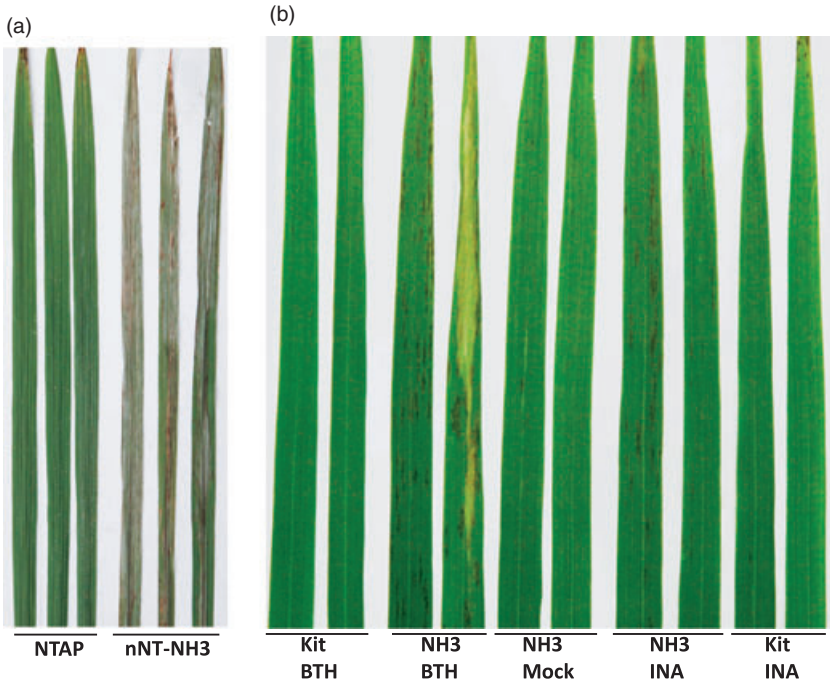

Figure 4 Responses of nNT-NH3 plants to plant defence activators. (a) Responses to 10 mM benzothiadiazole (BTH). nNT-NH3 and N-terminus tandem affinity purification (NTAP) control plants were foliar-sprayed with 10 mM BTH. Three representative leaves each from the NTAP control and nNT-NH3 plants 1 week after the treatment. (b) Responses to 1 mM BTH and 2,6-dichloroisonicotinic acid (INA). Plants were foliar-sprayed with either 1 mM BTH, 1 mM INA or mock solution. Two typical leaves from each combination of plant and treatment are shown. Symptoms on leaves were allowed to develop for 1 week following the treatment.

Figure 5a and real-time quantitative PCR results in Figure 5b. The input cDNA amounts were equalized first by using actin as the reference in Figure 5a. Quantitative expression data in Figure 5b are expressed as fold of actin gene expression. As expected, the NH3 levels in the nNT-NH3 plants (filled bar) are elevated by about three- to fourfold compared to Kit (open bar) control before treatment. Interestingly, in Kit control plants (open bars), the NH3 transcript levels appear slightly reduced after BTH application, especially at day 4 and day 8. In nNT-NH3 plants, the NH3 levels appear further modestly induced at day 1 but otherwise remain constantly elevated compared to Kit after BTH treatment. Mock treatment also appears to have effects on the NH3 levels. In Kit controls, NH1 levels remain relatively constant, except at day 4 and day 8 where the NH1 levels appear slightly reduced. Interestingly, in untreated nNT-NH3 plants, the NH1 level is reduced by half compared to Kit and is induced by about 3.5-fold (twofold compared to untreated Kit) 1 day after BTH treatment; the NH1 levels then remain at levels compatible to that of untreated Kit control, except at day 8, where it is significantly higher.

Results in Figure 5a,b show that expression of defence gene *PR1a* is at comparable levels in Kit and in nNT-NH3 plants before treatment, both at very low levels. Upon 1 mM BTH treatment, *PR1a* expression is induced up to 11-fold in Kit. *PR1a* is induced dramatically more by BTH (up to 244-fold) in the nNT-NH3 plants than in Kit. The *PR3* expression level in nNT-NH3 plants is about twofold that in Kit. *PR3* expression is slightly (up to twofold) induced by BTH in Kit but strongly induced (up to 10-fold) by BTH in nNT-NH3 plants. The *PR10* level in nNT-NH3 plants is lower than in Kit. Similarly, expression of *PR10* is slightly induced (up to twofold) in Kit but strongly induced (up to 51-fold over untreated nNT-NH3 or 12-fold over untreated Kit) in nNT-NH3 plants. Expression levels of peroxidase genes *POX8.1* and *POX22.3* in nNT-NH3 are comparable to those in Kit. BTH treatment significantly induces *POX8.1* and *POX22.3* expression in both Kit and nNT-NH3 plants, but more strongly for *POX22.3* in nNT-NH3 plants. However, BTH treatment results in a faster drop in the *POX8.1* expression level, leading to a lower level of *POX8.1* in nNTNH3 than in Kit at day 4.

Results in Figure 5c show that 1 mM INA treatment also slightly reduces the levels of NH3 expression. However, the NH3 levels in the nNT-NH3 plants remain constantly elevated. Treatment with INA slightly induces expression of *PR1a*, *PR3* and *PR10* in Kit plants. The elevated levels of NH3 have no obvious effects on induction of these genes by INA. INA also induces expression of POX in Kit and nNT-NH3 plants. The elevated levels of NH3 appear to have similar but mild effects on peroxidase gene expression.

These results suggest that elevated NH3 levels, in general, enhance induction of defence-related genes but may have the opposite effect on some other genes, such as

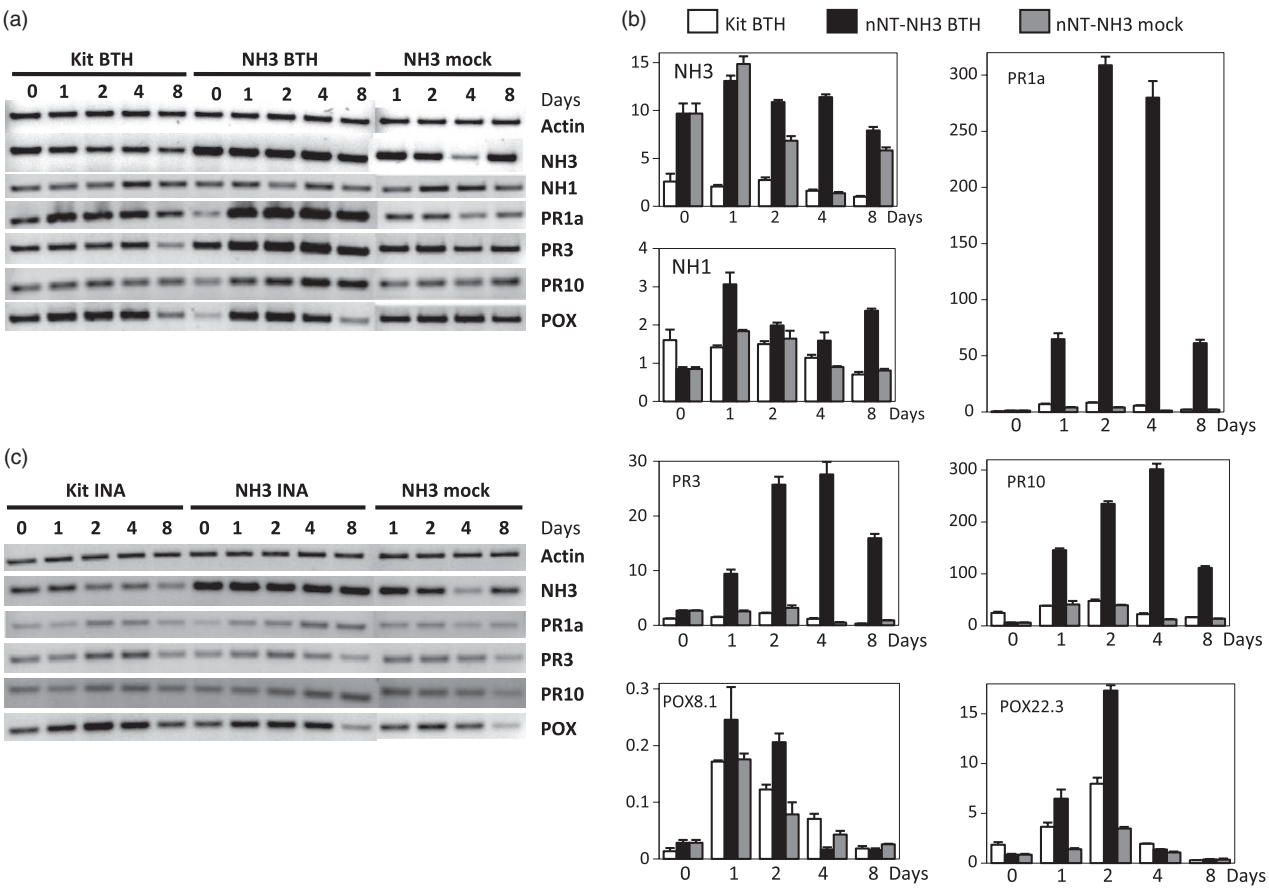

Figure 5 Effects of introduction of *nNT-NH3*on expression of defence-related genes. (a) Effects of nNT-NH3 on induction by benzothiadiazole (BTH) treatment. Kit control and nNT-NH3 plants were foliar-sprayed with 1 mM BTH or with mock solution. Leaf samples were collected at days 0, 1, 2, 4 and 8. Total RNA was extracted, and equal amount of RNA was used to synthesize cDNA for RT-PCR. The amounts of cDNA input were further equalized using actin as the reference. The same amount of cDNA was then used in the RT-PCR for NH3 and defence-related genes: NH1, PR1a, PR3, PR10 and peroxidase (POX22.3). (b) Quantitative real-time RT-PCR. Real-time qPCR experiments were performed using the cDNA prepared above (a). Open bars represent kit treated with 1 mM BTH, Filled bars for nNT-NH3 treated with 1 mM BTH and grey bars for nNT-NH3 with mock treatment. Days after BTH application are indicated under each panel. The targeted gene is indicated in each panel. The numbers on the *X*-axis in each graph represent the expression levels normalized to actin expression. Each bar represents three technical replications and its standard deviation. (c) Effects of nNT-NH3 on induction by 2,6-dichloroisonicotinic acid (INA). Kit control and nNT-NH3 plants were treated with 1 mM INA or mock solution. Leaf samples were collected at days 0, 1, 2, 4 and 8. Total RNA samples were extracted. The amounts of cDNA input were equalized using actin as the reference. RT-PCR were performed for genes NH3, PR1a, PR3, PR10 and peroxidase (POX22.3).

peroxidases, at certain stages. Nevertheless, these results support the observation that higher levels of *NH3* transcript enhance responses to BTH treatment, which induces *PR* gene expression and disease resistance. More importantly, even at these higher levels of *NH3* transcript, the defence-related genes are not highly induced without inducer treatment.

The enhanced disease resistance phenotype is because of the NH3 protein itself

There are no reports so far describing possible involvement of rice NH3 in plant defence or any function of NH3. The enhanced disease resistance effects and elevated responses to BTH and INA, conferred by the nNT-NH3 plants, are most likely due to the function of the NH3 protein itself because control transgenic plants expressing the NTAP tag alone show no such effects (see Figure 4a above; Park *et al.*, 2008). However, the possibility remains that introducing the NTAP tag to the N-terminus of the NH3 protein may cause the protein to function differently, leading to the observed phenotypes. To rule out this possibility, we created another modified NH3 construct, NH3Myc, which would produce an NH3 protein fused to the c-Myc tag at the C-terminus and its expression driven by the same 1-kb NH3 promoter. We transformed Kit with this construct and generated more than 20 T0 transgenic plants. These plants were challenged with PXO99 along

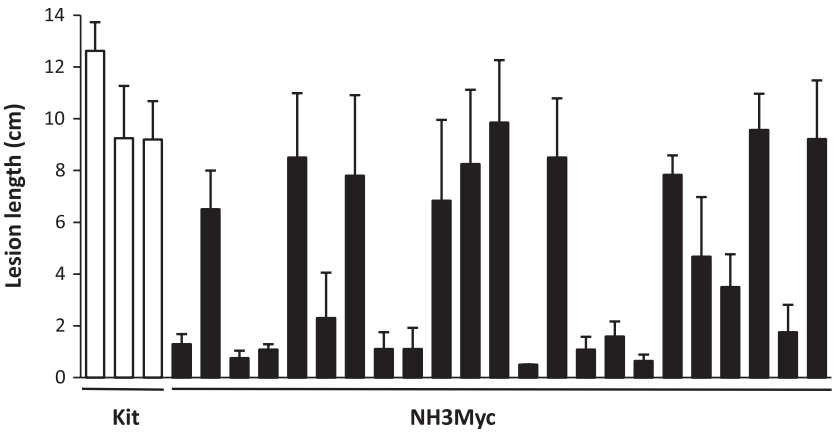

Figure 6 Lesion length measurement of NH3Myc transgenic plants. Three Kit control (open bars) and 23 independent NH3Myc (filled bars) T0 transgenic plants were inoculated with PXO99 and lesion length measured 2 weeks postinoculation. On average, four leaves were measured for each plant. The error bars represent standard deviations.

with Kit as control. Approximately half of the NH3Myc transgenic plants show high levels of enhanced resistance to *Xoo* challenge, as shown in Figure 6, similar to the case of nNT-NH3 transgenic plants. These results show that the NH3Myc construct has the same function as the nNT-NH3 construct. Because the NH3Myc protein carries a tag different from the TAP tag at a totally different location, these results suggest that the observed effects are from the function of the NH3 protein itself.

Discussion

Despite numerous studies using NPR1 or its orthologs to achieve enhanced disease resistance in many plant species (Cao *et al.*, 1998; Friedrich *et al.*, 2001; Chern *et al.*, 2001; Lin *et al.*, 2004; Chern *et al.*, 2005a; Meur *et al.*, 2008; Le Henanff *et al.*, 2009) for application purposes, no NPR1 paralogous genes have been shown to be able to enhance disease resistance so far. In Arabidopsis, BOP1 (NPR6) and BOP2 (NPR5) were shown to regulate development of leaf. Among the three groups of NPR1-like proteins, BOP1 and BOP2 are the most distant members from NPR1. Thus, this group of NPR1-like proteins may function to regulate leaf development rather than defence. In support of this hypothesis, we have shown that increased expression of rice NH4 or NH5, using either their native promoters or the maize *Ubi-1* promoter, does not confer enhanced resistance in transgenic plants (M.S. Chern and P. Ronald, unpubl. data).

Liu *et al.* (2005) reported that Arabidopsis *NPR4* is required for basal resistance to *P. syringae* pv. *tomato* (*Pst*) DC3000 and *Erysiphe cichoracearum* because they observed that the *npr4-1* mutant is more susceptible to these two pathogens. However, Zhang *et al.* (2006) reported that Arabidopsis *NPR3* and *NPR4* are negative regulators of *PR* gene expression and disease resistance. Zhang *et al.* showed that *npr3* mutants have slightly increased basal *PR-1* expression and the *npr3npr4* double mutant shows higher *PR-1*, *PR-2* and *PR-5* expression. The double-mutant plants display enhanced resistance against virulent bacteria, such as *Pst* DC3000, and oomycete pathogens (Zhang *et al.*, 2006). The conclusions of these two reports contradict each other. Moreover, other than NPR1 itself, no Arabidopsis NPR1-like genes have been shown to enhance disease resistance when expressed at higher levels.

Yuan *et al.* (2007) showed that over-expression of OsNPR2 and OsNPR3 in rice had no significant effects on resistance to *Xoo*. Thus, whether any of the rice NPR1 paralogs is similarly involved in regulating defence responses was in serious doubt. We tried to over-express NH3 in the Kit rice variety using the maize *Ubi-1* promoter, which has been successfully used in many cases to over-express a gene in rice (Chern *et al.*, 2001, 2005a,b). We failed to obtain plants carrying enhanced resistance. Instead, most of the transgenic plants died before or after transfer to greenhouse. Presumably, ultra-high levels of NH3 expression, as driven by the *Ubi-1* promoter (whose expression often reaches 10-fold or higher than the endogenous gene), cause toxicity leading to lethality. A similar situation may have occurred in Yuan's over-expression experiment with the *OsNPR3* gene when they used the *Ubi-1* promoter.

When we used the NH3 native promoter to drive the expression, many of the transgenic plants exhibited enhanced resistance. These native promoter NH3 transgenic plants survived, most likely because they do not express extremely high levels of NH3, but only moderate levels (about three- to fourfold of the endogenous level) sufficient to enhance resistance. In addition, the native

promoter NH3 constructs may express NH3 only in relevant tissues at the right developmental stages and the right timing, minimizing possible detrimental effects. This is supported by the observation that expression of *PR* genes in nNT-NH3 plants is not highly elevated until induction by BTH (Figure 5a,b). However, once it is triggered by BTH, *PR1a* induction reaches as high as 244-fold in the nNT-NH3 plants whereas induction by BTH reaches 11-fold in Kit plants. Thus, the NH3 transgene has little effects before BTH induction but magnifies the BTH effect by 20-fold. Practically, this likely means that less BTH is needed to induce same levels of defence response. In contrast, rice transgenic plants carrying the *Ubi-NH1* transgene, which exhibit normal developmental phenotypes, show elevated levels of *PR* gene expression without induction (Chern *et al.*, 2005a). Thus, the use of native promoter to express an extra copy of NH3 clearly carries advantages. In *Arabidopsis*, the NPR1 transcript level is elevated only two- to threefold after induction (Cao *et al.*, 1997). These levels of NPR1 transcript are high enough to enhance disease resistance significantly. In the results presented here, introduction of an extra copy of NH3 is able to achieve similar levels (three- to fourfold) of NH3 transcript, leading to enhanced disease resistance. Similarly, we have also used the same strategy to introduce an extra copy of NH1 into Kit rice and observed enhanced resistance (MS Chern and PC Ronald. unpubl. data). Therefore, this strategy may be widely applicable when using regulatory genes, such as *NH3* and *NH1*, to engineer plants with enhanced disease resistance and other desirable traits.

The dramatic cell death responses of the nNT-NH3 plants to BTH are striking. At 1 mM of BTH, these plants start to show bleached areas on leaves, a symptom of severe cell death and strongly activated defence response. At 10 mM of BTH, treated leaves are bleached and dried out completely. These symptoms are more severe than those observed on NH1 over-expression plants treated with the same concentrations of BTH. Thus, NH3 may respond to BTH more dramatically than NH1 in certain ways. Additional molecular characterization is needed to reveal the mechanism leading to the difference in response to BTH.

It is interesting to notice that while the NH1 transcript level is lower in nNT-NH3 than in Kit plants without induction, it is induced to a level twice as high as that in Kit plants 1 day after BTH treatment (Figure 5b). In contrast, in Kit plants after application of 1 mM BTH, NH1 expression levels are relatively unchanged. These data suggest that the levels of NH1 and NH3 expression may be highly coordinated to maintain a cellular homeostatic state. In support of this notion, our microarray results show that in the NH1 over-expression plants, NH3 expression is up-regulated by 1.6-fold (unpubl. data). This data is consistent with the idea that NH3 is involved in plant defence responses. Also in support of a positive role of NH3 in plant defence response is that our microarray data on negative regulator of resistance (NRR) over-expressing (NRRox) plants show a 2.4-fold down-regulation of NH3 expression (unpubl. data). NRRox plants exhibit super-susceptibility, evidenced by long lesions and few HR-like spots following *Xoo* challenge (Chern *et al.*, 2005b). Down-regulation of NH3 expression in NRRox plants may contribute to the super-susceptible phenotype.

Experimental procedures

Plant materials, growth conditions and pathogen challenge

The Kitaake (Kit) japonica rice (*Oryza sativa* L) cultivar was used for this study. Kit rice is susceptible to the Philippine *Xoo* strain PXO99AZ. Rice plants were grown in greenhouses at UC Davis at 27–32 °C under sunlight. For *Xoo* inoculation, 5–6-week-old plants were transferred to a growth chamber and inoculated with PXO99AZ by the scissor-dip method (Kauffman *et al.*, 1973). *Xoo* growth curve measurements were taken as described before (Chern *et al.*, 2005b). Growth chambers were set at the same temperatures with a day/night time cycle of 14 h/10 h.

Gene isolation and plasmid construction

NH3 cDNA was amplified from a Nipponbare rice cDNA pool with primers NH3TAP1 (5′CACCGAGACGTCCACCATAAGCTTCTC3′) and NH3TAP3 (5′ACTGCAGATTAGACTTAACTGCTG3′). The NH3 cDNA PCR product was cloned into the pENTR-D vector and confirmed by sequencing. The 1-kb NH3 promoter was amplified with primers NH3P-1 (5′TTTTAAGCTTCGTTGGATGAACTACATTG-CTGAT3′) and NH3P-2 (5′TTGGATCCAGATCTTATCCGGAAATTT-CGCGCGTGT3′) and cloned into pBluescript II SK- using HindIII + BamHI. The insert was sequence-confirmed. The NH3 cDNA was cloned into the Gateway-compatible Ubi-NTAP-1300 vector (Rohila *et al.*, 2006) by recombination to generate the Ubi-NT-NH3 over-expression construct. To create a native promoter NH3 construct, the NH3 promoter was first cloned into the Ubi-NTAP-1300 vector using HindIII + BglII to replace the Ubi-1 promoter. The resultant plasmid was used to accommodate the NH3 cDNA by recombination, yielding the nNT-NH3 construct.

For the NH3-Myc construction, the same 1-kb NH3 promoter fragment (NH3P1.0) was amplified with primers NH3P-3 (5′CAC-CTCGTTGGATGAACTACATTGCTGAT3′) and NH3P-NcoI (5′TCC-ATGGCTCTTATCCGGAAATTTCGCGCGTGT3′) and cloned into the pENTR-D vector. The NH3 cDNA was re-amplified sequentially, first with primers NH3ATG (5′CACCATGGAGACGTCCACCATAA-

G3′) and NH3-cMyc (5′GGAGATGAGCTTCTGCTCCCGTGATAGCTTCCCTTTCTTG3′), then with primers NH3ATG and cMyc-SpeI (5′ACTAGTTATTTCTCCAACAGGTCTTCCTCGGAGATGAGCTTCTGCTC3′). The NH3Myc PCR product was cloned into the pENTR-D vector and confirmed by sequencing. The NH3P1.0 fragment was excised with NotI + NcoI and cloned into the NH3Myc/pENTR plasmid, predigested with NcoI + NotI, generating the P1-NH3Myc/pENTR construct. This construct was used to transfer the P1-NH3Myc fragment into a Gateway-C1300 vector by recombination, creating P1-NH3Myc/C1300. The P1-NH3Myc construct was used to transform Kit rice, generating NH3Myc transgenic plants.

Treatment with plant defence activators

For 10 mM BTH treatment, the commercial product Actigard (Syngenta) was first used for foliar spray on the rice plants. Pure BTH chemical Acibenzolar-S-methyl (Wako) was subsequently used in place of Actigard. One millimolar of BTH and INA solutions were prepared in 0.05% Tween 20 for foliar spray.

RNA extraction and RT-PCR

Leaf samples were collected and frozen immediately in liquid nitrogen. Leaf samples were stored at −80 °C until use. Total RNA was extracted using the Trizol reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instruction. RNA was reverse-transcribed using reverse transcriptase Superscript (Invitrogen, Carlsbad, CA, USA). Approximately 10 μg of RNA was used for each reverse transcription. RT-PCR was first performed with primers targeting an actin gene as the reference. An equal amount of cDNA input was then used for RT-PCR for NH3 or defence-related genes. RT-PCR for NH3 used primers NH3-RT1 (5′GTGCATTGGCGTCTTACAGCA3′) and NH3-RT2 (5′GGGAAGTATCGTCGTCCGAGT3′). RT-PCR for NTAP-NH3 used primers NH3-2 (5′GTGGCTGCAGCCGTCGTCCA3′) and NTAP-5 (5′ATGCCCAAGCCCCAAAGGACTACG3′). RT-PCR for NH1 used primers NH1-RT1 (5′ACTTAGCTCGGATGACGGCAC3′) and NH1-TAP2 (5′AGCAATGGTGTTCATCTCCTTGGT3′). Genes *PR1a* (Os07g03710) and *PR10* (Os12g36830) as markers for plants defence have been described earlier (Park *et al.*, 2008).

Quantitative real-time PCRs were performed on a Bio-Rad CFX96 Real-Time System coupled to a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). For qPCRs, the Bio-Rad SsoFast-EvaGreenSupermix was used. QPCR primers used are as follows: Actin-Q1 (TCGGCTCTGAATGTACCTCCTA) and Actin-Q2 (CACTTGAGTAAAGACTGTCACTTG) for actingene. NH1-RT3 (CTGATCCGTTTCCCTCGGA) and NH1-RT4 (GACCTGTCATTCTCCTCCTTG) for the NH1 gene. NH3-RT3 (TGCTACACCTCTGCTGGTTGA) and NH3-RT4 (GACCAGCAAACTCTTGAGTTGAG) for the NH3 gene. PR1a-C1 (CGTCTTCATCACCTGCAACT) and PR1a-C2 (TGTCCATACATGCATAAACACG) for the PR1a gene. PR3-1 (CTTGGACTGCTACAACCAGA) and PR3-2 (CATTGTGGGCATTACTGATG) for the PR3 gene. PR10-C1 (CTCATCCTCGACGGCTACTT) and PR10-C2 (ATCAGGAAGCAGCAATACGG) for the PR10 gene. POX8.1-1 (CAAACTGGATACAAAAGCAAACAC) and POX8.1-2 (CATGGGCTTCCTGATCTG) for the POX8.1 gene. POX22.3-1 (ATCGTGTCGACGACGACAT) and POX22.3-2 (CTCTGCTCCATACACTTGATG) for the POX22.3 gene. QPCRs were run at 56 °C annealing for 12 s and 95 °C for 8 s for 40 cycles.

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