A Conserved *Puccinia striiformis* Protein Interacts with Wheat NPR1 and Reduces Induction of Pathogenesis-Related Genes in Response to Pathogens

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In *Arabidopsis*, NPR1 is a key transcriptional coregulator of systemic acquired resistance. Upon pathogen challenge, NPR1 translocates from the cytoplasm to the nucleus, in which it interacts with TGA-bZIP transcription factors to activate the expression of several pathogenesis-related (PR) genes. In a screen of a yeast two-hybrid library from wheat leaves infected with *Puccinia striiformis* f. sp. *tritici*, we identified a conserved rust protein that interacts with wheat NPR1 and named it PNPI (for *Puccinia* NPR1 interactor). PNPI interacts with the NPR1/NIM1-like domain of NPR1 via its C-terminal DPBB_1 domain. Using bimolecular fluorescence complementation assays, we detected the interaction between PNPI and wheat NPR1 in the nucleus of *Nicotiana benthamiana* protoplasts. A yeast three-hybrid assay showed that PNPI interaction with NPR1 competes with the interaction between wheat NPR1 and TGA2.2. In barley transgenic lines overexpressing PNPI, we observed reduced induction of multiple PR genes in the region adjacent to *Pseudomonas syringae* pv. *tomato* DC3000 infection. Based on these results, we hypothesize that PNPI has a role in manipulating wheat defense response via its interactions with NPR1.

Puccinia striiformis Westend. f. sp. *tritici* Erikss. is the causal pathogen of wheat stripe rust, which is also known as yellow rust. New and more virulent *P. striiformis* f. sp. *tritici* races appeared at the beginning of this century and expanded rapidly into many of the wheat-growing regions of the world, where they are causing large yield losses (Chen et al. 2002; Hovmøller et al. 2010, 2016; Simons et al. 2011). Many of the resistance genes that were effective against previous *P. striiformis* f. sp. *tritici* races became ineffective against these new races (Chen et al. 2002), prompting the search for new sources of resistance (Maccaferri et al. 2015).

The successful biotrophic lifestyle of obligate parasitic fungi such as the rust pathogens depends upon their ability to deliver specialized effectors into the host cells to suppress or evade plant defenses. Uncovering how these effectors function is

critical to understand pathogenicity mechanisms and to develop new strategies to fight these pathogens. Recent whole-genome analyses of several *P. striiformis* f. sp. *tritici* races revealed a large number of hypothetical effector proteins (Cantu et al. 2011, 2013b; Zheng et al. 2013). In addition, 16 *P. striiformis* f. sp. *tritici* candidate effectors have been recently characterized in *Nicotiana benthamiana* and their target subcellular compartments have been identified (Petre et al. 2015).

Plants are under constant evolutionary pressure to recognize pathogen effectors or the modifications to their host targets (Jones and Dangl 2006). This is generally achieved by modifications in the recognition sites of intracellular receptors, which frequently belong to the nucleotide-binding site leucine-rich receptor class (Michelmore et al. 2013). Once an effector is recognized by the plant, the pathogen is under evolutionary pressure to modify or eliminate this effector to avoid recognition (Rafaele and Kamoun 2012). These recurrent evolutionary processes generate an arms race between pathogen and host that usually drives a rapid evolution of both resistance genes and effectors.

In addition to a local hypersensitive reaction, effector-triggered immunity can also result in systemic acquired resistance, an inducible form of plant defense that confers broad-spectrum immunity to secondary infections beyond the initial infection site. In *Arabidopsis*, this type of resistance involves the generation of mobile signals, accumulation of hormone salicylic acid (SA) and transcriptional activation of pathogenesis-related (PR) antimicrobial genes (Fu and Dong 2013). The *Arabidopsis* NPR1 protein (NONEXPRESSER OF PR GENES 1, also known as NIM1 and SAI1) is a master regulator required for transduction of the SA signal. Upon pathogen infection or artificial SA applications, NPR1 moves from the cytoplasm into the nucleus, in which it interacts with TGA2 transcription factors to activate multiple PR genes (Cao et al. 1994; Delaney et al. 1995; Mou et al. 2003; Ryals et al. 1997; Shah et al. 1997).

A previous analysis of the interactions between wheat NPR1 (wNPR1) and wheat homologs of known rice NPR1 interactors confirmed that wNPR1 interacts with four members of the basic-region leucine zipper (bZIP) transcription factor family (Cantu et al. 2013a). The interactions between wNPR1 and transcription factors wTGA2.1, wTGA2.2, and wTGA2.3 were also observed between the orthologous proteins in rice (Chen et al. 2001) and *Arabidopsis* (Després et al. 2003) and are critical to mediate NPR1 function. wLG2, the fourth bZIP transcription factor shown to interact with wNPR1, belongs to a separate subclass and is similar to the maize protein encoded by the *Liguleless* gene (Chen

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et al. 2001). The wNPR1 protein was also shown to interact with two wheat NRR (negative regulator of resistance) proteins and one NRR paralog designated as wNRRH1 (Cantu et al. 2013a). The rice homologs of the wheat NRR proteins were previously shown to downregulate NPR1 activity (Chern et al. 2005a).

NPR1 is a conserved protein that contains three different domains. The BTB/POZ (broad-complex, tramtrack, and bric-a-brac/poxvirus, zinc finger) domain, located at the N-terminal region, is a potential target for ubiquitin-dependent degradation by Cullin3-based E3 ligases (Petroski and Deshaies 2005). The central ankyrin-repeat domain is predicted to mediate protein-protein interactions with TGAs, and is essential for NPR1 function (Cao et al. 1997; Sedgwick and Smerdon 1999). The NPR1/NIM1-like domain in the C-terminal region, together with the BTB/POZ domain, is required for SA binding (Wu et al. 2012).

In *Arabidopsis*, NPR1 paralogs NPR3 and NPR4 are involved in the CUL3 E3 ligase-mediated degradation of NPR1 in a SA concentration-dependent manner (Fu et al. 2012). At low SA levels, NPR1 is targeted for degradation in proteasomes via its binding to NPR4. As the SA level increases after pathogen infection (basal resistance), SA binds to NPR4, releasing more NPR1, which activates the NPR1-mediated plant defense reactions; at very high SA levels (hypersensitive cell death), SA binds to NPR3 and promotes its interaction with NPR1, which finally leads to the turnover of NPR1 (Fu et al. 2012; Moreau et al. 2012).

In barley and wheat, the NPR1 resistance mechanism exhibits some differences from the mechanisms described above for *Arabidopsis*. In wheat, the NPR1-regulated gene *wNPR1* was induced by the fungal pathogen *Erysiphe graminis* but did not respond to SA or its functional analogs, 2,6-dichloroisonicotinic acid and benzothiadiazole (BTH) (Molina et al. 1999). In barley, *HvPR1*, *HvPR3* (chitinase), *HvPR5* (thaumatin-like), and *HvPR9* (peroxidase) showed significant induction after infection with *Erysiphe graminis* or *Pseudomonas syringae* pv. *syringae*, but only infection with the latter resulted in higher SA accumulation (Vallélian-Bindschedler et al. 1998). Wheat transgenic lines overexpressing *Arabidopsis NPR1* show a faster activation of defense response to *Fusarium* head blight and expression of *PR1* becomes BTH sensitive (Makandar et al. 2006). Injection of barley leaves with *Pseudomonas syringae* DC3000 results in acquired resistance in the area adjacent to the pathogen injection, but, in contrast to *Arabidopsis*, the resistance is not systemic (Colebrook et al. 2012).

In this study, we report the identification of a conserved *P. striiformis* f. sp. *tritici* protein that interacts with wNPR1, and interferes with its binding to transcription factor wTGA2.2. We also show that overexpression of this *P. striiformis* f. sp. *tritici* gene in barley results in the reduced induction of *PR* genes in the region adjacent to *Pseudomonas syringae* infection sites. Based on these results, we hypothesize that this putative effector may have a role in manipulating wheat defenses via its protein interaction with wNPR1.

RESULTS

P. striiformis f. sp. *tritici* PNPI protein interacts with wNPR1 in a yeast two-hybrid (Y2H) screen.

The screening of a Y2H library of *P. striiformis* f. sp. *tritici*-infected wheat leaves using wNPR1 (JX424315) as bait (primers in Supplementary Table S1) yielded interactions with the wTGA2.2 (JX424317) protein (Cantu et al. 2013a) and with a protein from *P. striiformis* f. sp. *tritici*, designated here as PNPI (*Puccinia* NPR1 interactor) (GenBank accession number KT764125). The portion of PNPI included in the clone identified in the Y2H screen was 726 bp long and encoded an N-terminal truncated peptide, PNPI₍₉₃₋₃₃₃₎. Comparison of the full-length cDNA sequence of PNPI from *P. striiformis* f. sp. *tritici* race PST-08/21 (Cantu et al. 2013b) with the genomic

sequence of PST-130 (Cantu et al. 2011) showed that the *PNPI* gene has seven exons and encodes a predicted protein of 333 amino acids. The gene structure is annotated in KT764125.

The SignalP program predicted the presence of a secretory pathway signal peptide of 22 amino acids with high confidence. The 24 amino acids after the end of the predicted signal peptide PNPI showed the sequence RSL—DEEP, which is similar but not identical to the RxLR-dEER motif frequently found in oomycete effectors. Comparison with the conserved domains in the Pfam database indicated significant similarity of the C-terminal region of PNPI with a Rare lipoprotein A (RlpA)-like double-psi beta-barrel domain (Fig. 1, DPBB_1 domain, pfam 03330). No transmembrane domains were detected using the program TMHMM (Möller et al. 2001).

Sequence alignment of PNPI proteins from *P. striiformis* f. sp. *tritici* races PST-78 (PST_16231, PRJNA123765), PST-21, PST-43, PST1-87/7, PST-08/21, and PST-130 (Cantu et al. 2011, 2013b) and two *Puccinia striiformis* f. sp. *hordei* races, PSH-54 (GenBank accession KT764126) and PSH-72 (GenBank accession KT764127), showed 100% identity among all sequences. The coding regions of these genes were also 100% identical at the DNA level.

A sequence alignment of the PNPI protein from *P. striiformis* f. sp. *tritici* with its homologs from wheat stem rust *P. graminis* f. sp. *tritici* (XP_003325658) and wheat leaf rust *P. tritici* (PTTG_03809) showed good conservation along the complete protein length (Fig. 1). The PNPI protein from *P. striiformis* f. sp. *tritici* is 67.2% similar to the homologous protein in *P. graminis* f. sp. *tritici* and 66.8% similar to homologous protein in *P. tritici*. Similarity between PNPI proteins from the wheat rust pathogens and the closest homologs from more distantly related plant pathogens (e.g., *Melampsora larici*, *Ustilago maydis*, *Rhizoctonia solani*) were limited to the C-terminal region including the DPBB_1 domain (Supplementary Fig. S1). Only this conserved region was used to generate the phylogenetic tree presented in Supplementary Fig. S2.

PNPI is up-regulated during the late stages of *P. striiformis* f. sp. *tritici* infection.

After inoculation of the susceptible common wheat 'Fielder' with the virulent *P. striiformis* f. sp. *tritici* race PST-130, we collected leaves at 5, 8, 15 and 22 days postinoculation (dpi). The first two collection points were done during haustoria formation and secondary hyphae expansion, whereas samples at 15 and 22 dpi were collected during the initiation and full development of the sporulation phase, respectively.

Analysis of PNPI expression at the four time points, using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (primers in Supplementary Table S2), showed a clear upregulation from 8 to 22 dpi (Supplementary Fig. S3). Analysis of published transcriptome data found that PNPI was expressed in two datasets from isolated *P. striiformis* f. sp. *tritici* haustoria (Cantu et al. 2013b; Garnica et al. 2013) but not in the dataset from germinated urediniospores (Garnica et al. 2013). PNPI expression was detected in RNA extracted from haustoria (Cantu et al. 2013b) at 9 dpi (Garnica et al. 2013) and from a pool of haustoria collected at 6 and 14 dpi (Cantu et al. 2013b). These data suggest that PNPI is expressed in the mature haustoria.

The DPBB_1 domain in PNPI interacts with the NPR1/NIM1-like domain in wNPR1.

The full-length wNPR1 (JX424315) and a truncated PNPI₍₂₃₋₃₃₃₎ protein lacking the signal peptide (to avoid secretion) showed a strong interaction in the Y2H assays under SD (synthetic dropout) selection media lacking both histidine and adenine (SD–Leu–Trp–His–Ade) (Fig. 2B and negative controls for Y2H assays in Supplementary Fig. S4). To determine which portion of the PNPI and wNPR1 proteins were responsible for

their interaction, we tested two fragments of PNPI and three fragments of wNPR1 by Y2H assays (Fig. 2A). The N-terminal region of PNPI₍₂₃₋₂₃₅₎ failed to interact with the complete wNPR1. By contrast, the C-terminal region of PNPI₍₂₃₆₋₃₃₃₎ including the DPBB_1 domain showed a strong interaction with wNPR1 in SD media lacking leucine, tryptophan, histidine and adenine (SD–Leu–Trp–His–Ade) selection medium (Fig. 2B).

We then tested the interactions between the PNPI₍₂₃₋₃₃₃₎ protein lacking the signal peptide with each of the three wNPR1 fragments. Both the N-terminal wNPR1₍₁₋₁₇₀₎ and the central part wNPR1₍₁₉₆₋₃₆₃₎ including the DUF3420 and ANK domain showed no interaction with PNPI₍₂₃₋₃₃₃₎. By contrast, the C-terminal wNPR1₍₃₅₅₋₅₇₂₎ region including the NPR1/NIM1-like domain interacted with PNPI₍₂₃₋₃₃₃₎ in SD–Leu–Trp–His–Ade selection medium (Fig. 2B). Similar results were observed when PNPI₍₂₃₋₃₃₃₎ was replaced by the C-terminal region PNPI₍₂₃₆₋₃₃₃₎ (Fig. 2B). PNPI₍₂₃₋₃₃₃₎ also interacted in Y2H assays with the NPR1 homolog from *Arabidopsis*, suggesting that PNPI recognizes a conserved region in NPR1 (Fig. 2B).

We then tested the ability of PNPI₍₂₃₋₃₃₃₎ to interact with the NPR1/NIM1-like domain from wNPR1 paralogs wNPR3 (Td-

k36_contig_20687) and wNPR4 (Td-k56_contig_528) from tetraploid wheat Kronos (Krasileva et al. 2013). A strong interaction was detected between PNPI₍₂₃₋₃₃₃₎ and wNPR4₍₃₈₅₋₆₀₇₎ in SD–Leu–Trp–His–Ade selection media, but no interaction was observed for wNPR3₍₃₇₇₋₅₉₃₎ (Fig. 2B). For all the negative Y2H assays, we confirmed by Western blots that the proteins were expressed (Supplementary Fig. S5).

Then, we generated amino acid substitution mutations at the conserved sites of DPBB_1 domain in PNPI, based on the multi-sequences alignment. The point mutation C301W in PNPI₍₂₃₋₃₃₃₎ was sufficient to abolish the protein interaction between PNPI₍₂₃₋₃₃₃₎ and wNPR1 in all three dilutions (in SD–Leu–Trp–His–Ade selection media). Point mutations at the other 14 conserved sites of the DPBB_1 domain showed interactions in all three dilutions in SD–Leu–Trp–His–Ade, with the exception of D257W, which was not detected only in the 1:1 and 1:10 dilutions (Supplementary Fig. S6).

PNPI-wNPR1 interaction was validated in *N. benthamiana* protoplasts.

To validate the Y2H interaction between wNPR1 and PNPI, we performed bimolecular fluorescence complementation

Fig. 1. Sequence alignment of PNPI proteins from different cereal rust pathogens. Multisequence alignment performed using MUSCLE showing conservation among PNPI homologs from *Puccinia striiformis* f. sp. *hordei* (Psh), *P. striiformis* f. sp. *tritici* (Pst), *P. graminis* f. sp. *tritici* (Pgt), and *P. trititica* (Pt). The predicted proteins include an N-terminal signal peptide followed by an RxLR-dEER-like motif, and a C-terminal region including a DPBB_1 domain.

(BiFC) assays. Coexpression of YFP^N-PNPi₍₂₃₋₃₃₃₎ and YFP^C-wNPR1 in *N. benthamiana* protoplast resulted in strong yellow fluorescent protein (YFP) fluorescence in the nucleus. We also observed clear YFP fluorescence in the positive control YFP^N-wHSP90.3 (ADF31760.1)/YFP^C-wRAR1 (EF202841.1), and no fluorescence in the negative controls using empty vector constructs YFP^N-EV and YFP^C-EV (Fig. 3). As an additional negative control, we used the nuclear localized protein wFDL2 (EU307112), which

interacts with wFT1 (Li et al. 2015) but not with PNPi or wNPR1. Protoplast cotransformed with YFP^N-wFDL2 and YFP^C-wFT1 showed strong YFP signal in the nucleus, whereas no fluorescence was detected in protoplasts cotransformed with YFP^N-PNPi₍₂₃₋₃₃₃₎/YFP^C-wFDL2 or YFP^N-wFDL2/YFP^C-wNPR1 (Supplementary Fig. S7). For the negative BiFC assays, we confirmed by Western blots that the proteins were expressed in the transformed *N. benthamiana* protoplast (Supplementary Fig. S8).

Fig. 2. wNPR1, wNPR3, wNPR4, and PNPi interactions in yeast two-hybrid (Y2H) assays. **A**, Domain predictions for wheat wNPR1, wNPR3, and wNPR4 and *Puccinia striiformis* f. sp. *tritici* PNPi, using Pfam. Segments indicated in black were cloned into Y2H vectors. **B**, Y2H assays to assess domain interaction between PNPi and wNPR1, wNPR3, and wNPR4. Yeast transformants coexpressing different bait and prey constructs were assayed on SD-Leu-Trp-His and SD-Leu-Trp-His-Ade. PNPi specifically interacted with NPR1/NIM1-like domain from wNPR1 via its DPBB_1 domain. PNPi also showed interaction with *Arabidopsis* NPR1 and NPR1/NIM1-like domain from wNPR4 but not from wNPR3.

PNPi competes with wTGA2.2 binding to wNPR1 in yeast three-hybrid (Y3H) assays.

A Y3H experiment based on the pBridge vector was performed to test if PNPi interferes with the interaction between wNPR1 and wTGA2.2 (Cantu et al. 2013a). The pBridge vector allows the expression of two proteins, a DNA-binding domain (BD) fusion and a second protein that positively or negatively affects the interaction between the BD and activation domain (AD) fusion, which is expressed in a separate vector. The second protein (designated Bridge protein) is conditionally expressed under the *MET25* (henceforth M25) promoter only in the absence of methionine (Met) and is repressed in its presence.

Two different reporters, aureobasidin A (Aba) and X- α -Gal (α -galactosidase), were included in the Y3H assays to visualize the strength of the protein-protein interactions. Expression of the *AURI-C* dominant mutant in response to protein-protein interactions in the Y2HGold yeast strain confers strong resistance to the otherwise highly toxic Aba drug (Clontech 2013). Panels (Fig. 4A, on the left) excluding Aba selection were used as transformation controls (only transformants containing both bait and prey vectors can grow on SD-Leu-Trp) and to confirm the correct normalization of the loaded samples to similar numbers of yeast cells. In the presence of Aba, a clear reduction in the strength of the wNPR1 and wTGA2.2 interaction was detected in the presence of PNPi₍₂₃₋₃₃₃₎ (–Met) compared with the absence of PNPi₍₂₃₋₃₃₃₎ (+Met) (Fig. 4A, indicated by arrows). This result suggests that PNPi₍₂₃₋₃₃₃₎ interferes with the wNPR1-wTGA2.2 interaction. This competitive effect of PNPi can be also observed by comparing the construct expressing both the PNPi₍₂₃₋₃₃₃₎ and wTGA2.2-BD protein with the construct

including only the wTGA2.2-BD protein (Fig. 4A, both in –Met). This effect was also observed in X- α -Gal reporter assays. The blue color of the reporter is less intense in the presence of PNPi₍₂₃₋₃₃₃₎ than in its absence (Fig. 4B). The PNPi₍₂₃₋₃₃₃₎-BD construct is included in both assays as a positive control of the interaction between PNPi₍₂₃₋₃₃₃₎ and wNPR1.

To quantify the extent of the interference of PNPi₍₂₃₋₃₃₃₎ on the wTGA2.2-wNPR1 interaction, we performed a quantitative α -Gal assay. In this assay, the α -Gal activity generated by the interaction between wTGA2.2 and wNPR1 was 40% lower ($P < 0.01$), in the presence of PNPi₍₂₃₋₃₃₃₎ (–Met) than in its absence (+Met) (Fig. 4C). Since we found no interaction between PNPi₍₂₃₋₃₃₃₎ and wTGA2.2 by Y2H (Fig. 2B), these results support the hypothesis that PNPi competes with wTGA2.2 for interaction with wNPR1 protein.

PNPi signal peptide was sufficient to induce invertase secretion.

A yeast invertase secretion assay (Gu et al. 2011) was used for the functional validation of PNPi predicted signal peptide (22 amino acids). The yeast YTK12 strain, transformed with the pSUC2 vector including the signal peptide of PNPi fused in frame to the invertase sequence, was able to grow in both the SD-Trp and YPRAA (yeast extract peptone raffinose agar medium) medium. By contrast, the YTK12 control strain that is unable to secrete invertase could not grow on the YPRAA medium (Supplementary Fig. S9, includes additional negative Mg87₍₁₋₂₅₎-pSUC2 and positive Ps87₍₁₋₂₅₎-pSUC2 controls).

Attempts to test re-entry of PNPi into the plant cells using agromediated transformation of *N. benthamiana* were not

Fig. 3. Bimolecular fluorescence complementation assays showed interaction between YFP^N-PNPi₍₂₃₋₃₃₃₎ and YFP^C-wNPR1 in *Nicotiana benthamiana* protoplast. YFP^N-wHSP90.3 and YFP^C-wRAR1 were used as positive controls. Coexpression of each recombinant vector with its corresponding non-fused YFP^N and YFP^C empty vectors served as negative controls. BF = bright field; EV = empty vector; YFP = yellow fluorescent protein. Scale bars = 100 μ m.

successful. We were unable to detect secretion of the predicted signal peptide (PNPi₍₁₋₂₂₎) fused with green fluorescent protein (GFP) in *N. benthamiana* plasmolyzed epidermal cells (Supplementary Fig. S10). PNPi₍₁₋₂₂₎-GFP fusion showed a similar cytoplasmic localization as the fusions including a larger N-terminal region (PNPi₍₁₋₆₄₎-GFP), the complete PNPi protein (PNPi₍₁₋₃₃₃₎-GFP), or the GFP control.

Overexpression of PNPi reduces induction of PR genes.

Based on the previous experiment, we hypothesized that the interference of PNPi on the wTGA2.2-wNPR1 interaction could also interfere with the wNPR1 regulation of downstream PR genes. To test this hypothesis, we generated transgenic barley plants overexpressing PNPi₍₂₃₋₃₃₃₎ (without the signal peptide) under the maize *Ubiquitin* promoter. Four independent transgenic events were obtained and were confirmed both by

PCR of genomic DNA and qRT-PCR. Expression levels of the PNPi transgene were between 4 and 17% of the levels of *HvEF1α* endogenous control (Supplementary Fig. S11).

PR genes were induced in the leaves of control untransformed plants by inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 infection (Fig. 5A), as previously described in similar experiments performed with the same barley variety used here (Colebrook et al. 2012). All five PR genes showed induction in the adjacent region to the *Pseudomonas syringae* inoculation (48 h after inoculation), relative to the regions adjacent to the water-infiltrated control. We present the results for transgenic event 1 in Figure 5B to F and those for events 2, 3, and 4 in Supplementary Fig. S12. The *P* values presented in Figure 5B to F indicate the significance of the differences between PNPi transgenic plants and their isogenic controls in combined analyses of variance using the four transgenic events

Fig. 4. Yeast three-hybrid assay to determine the effect of competing PNPi₍₂₃₋₃₃₃₎ protein on the interactions between wTGA2.2 and wNPR1. **A**, Yeast transformants coexpressing EV^{M25}/wTGA2.2-BD, EV^{M25}/PNPi₍₂₃₋₃₃₃₎-BD, or PNPi₍₂₃₋₃₃₃₎^{M25}/wTGA2.2-BD with wNPR1-pGADT7. Panels on the left without Aba (with and without Met) were used to normalize yeast cell number. Yeast transformants were assayed on SD-Leu-Trp +Aba medium with and without Met. The interaction between wTGA2.2 and wNPR1 was weaker in the presence of PNPi₍₂₃₋₃₃₃₎ (-Met) than in its absence (+Met). EV = empty vector site; Met = methionine; Aba = aureobasidin. **B**, Yeast transformants were then assayed on SD-Leu-Trp-Met+X-α-Gal₄₀ selection medium. The blue color intensity of the wTGA2.2-BD interaction with wNPR1-AD in the presence of PNPi₍₂₃₋₃₃₃₎^{M25} was weaker than in the absence of the putative effector (EV^{M25}/wTGA2.2-BD). **C**, The quantitative α-galactosidase (α-Gal) assay showed that the interaction between wTGA and wNPR1 was significantly reduced in the presence of PNPi₍₂₃₋₃₃₃₎ (**, *P* < 0.01). Relative α-Gal activity values for each interaction were the average of six replicates (error bars = standard error). EV = empty vector.

as blocks. Comparison of the regions adjacent to the *Pseudomonas* inoculation site showed significant differences between the transgenic plants and the nontransgenic control for *HvPR1b* (Fig. 5B, $P = 0.006$), *HvPR2* (Fig. 5C, $P = 0.001$), *HvPR4b* (Fig. 5D, $P = 0.018$), *HvPR5* (Fig. 5E, $P = 0.032$), and *HvChitinase 2a* (Fig. 5F, $P = 0.004$). The differences were consistent in all four transgenic events for all five genes, i.e., expression levels were lower in the transgenic plants overexpressing PNPI than in the nontransgenic control. By contrast, none of the five *PR* genes showed significant differences between transgenic plants and water-inoculated control plants (Fig. 5). This experiment cannot be done using *P. striiformis* f. sp. *hordei* instead of *Pseudomonas syringae*, because the rust pathogen would introduce to the control plants the same PNPI protein expressed in the transgenic barley plants.

Previous studies in *Arabidopsis* have shown that NPR1 interactions with TGA transcription factors play an important role in the regulation of several *PR* genes (Després et al. 2000; Kinkema et al. 2000). Therefore, we hypothesized that the observed downregulation of the *PR* genes in the PNPI transgenic plants could be associated with the ability of PNPI to interfere

with the NPR1 and TGA protein interactions (Fig. 4). To test the connection between NPR1 and the *PR* genes in Triticeae species, we overexpressed the wheat *NPR1* gene under the maize *Ubiquitin* promoter (*Ubi::wNPR1*) in barley and obtained two independent transgenic events with 1.8- and 6.3-fold higher *NPR1* transcript levels than the wild type (Supplementary Fig. S13). We also obtained previously published RNA interference (RNAi) barley transgenic plants with reduced transcript levels of *HvNPR1* (32 and 46% of the wild-type levels) (Dey et al. 2014).

After inoculation with *Pseudomonas syringae* pv. *tomato* DC3000, we extracted RNA from the region adjacent to the infection area and evaluated *PR* gene expression. In the RNAi transgenic plants with knocked-down *HvNPR1* transcript levels, we observed a decrease in the relative expression of several barley *PR* genes, which was significant for *HvPR1b*, *HvPR4b*, and *HvChitinase 2a* (Supplementary Fig. S14). In the transgenic barley plants overexpressing *wNPR1* (*Ubi::wNPR1*), we observed a significant increase in the transcript levels of all tested *PR* genes relative to the control (Supplementary Fig. S15). The overexpression of the *NPR1* gene was stronger in transgenic event 7 than in event 8, and this was correlated with a stronger

Fig. 5. Functional characterization of PNPI₍₂₃₋₃₃₃₎. **A**, Infiltration of young barley leaves with either *Pseudomonas syringae* pv. *tomato* DC3000 or sterile water as control. The borders of the infiltrated region were marked in black. Samples for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were collected from the leaf region adjacent to the infection 48 h after inoculation. **B** to **F**, Relative expression of antimicrobial pathogenesis-related (*PR*) genes *HvPR1b*, *HvPR2*, *HvPR4b*, *HvPR5*, and *HvChitinase 2a* genes was measured by qRT-PCR in the region adjacent to the inoculation. Data for event 1 are presented here. The y axis indicates transcript levels relative to barley endogenous control *HvEF1α*. *P* values indicate significance of the differences between transgenic and control plants in combined analyses of variance, using transgenic events as blocks. Error bars indicate standard error of the means calculated from eight independent biological replicates.

induction of the *PR* genes in transgenic event 7. In the control plants inoculated with water, we detected no significant differences, except for *PR1b* in the *Ubi::wNPR1* transgenic plants.

DISCUSSION

Discovery of a putative *P. striiformis* f. sp. *tritici* effector that directly targets wNPR1.

The Y2H system has been used for both the discovery and validation of protein interaction between pathogen effectors and plant defense-related proteins. Good examples of this strategy include the interactions between the CSEP0055 effector from *Blumeria graminis* f. sp. *hordei* and barley defense protein PR17c (Zhang et al. 2012), between the *Parastagonospora nodorum* effector SnTox3 and wheat TaPR1 (Breen et al. 2016), and between the AvrL567 effector from *Melampsora lini* and L5/L6 R protein from flax (Ravensdale et al. 2012). In this study, we screened a Y2H library from *P. striiformis* f. sp. *tritici*-infected wheat leaves to identify *P. striiformis* f. sp. *tritici* proteins that interact with wNPR1, a master regulator of systemic acquired resistance.

The conservation of NPR1 protein interactions between wheat and rice (Cantu et al. 2013a) and between rice and *Arabidopsis* (Chern et al. 2005b; Després et al. 2003) suggests that this is an ancient component of the plant immune system. The conservation of NPR1 protein sequence across the monocot-dicot divide (Supplementary Fig. S16) is also supported in this study by the ability of both wheat and *Arabidopsis* NPR1 proteins to interact with PNPI (Fig. 2B). The discovery of this interaction was an exciting result because no pathogen effector has been reported, so far, to target NPR1 directly. There are, however, multiple effectors from different pathogens that have been reported to target NPR1 indirectly by targeting the SA-mediated plant defense pathway (Kazan and Lyons 2014). For example, the type III effector XopJ from *Xanthomonas campestris* interacts with the plant proteasomal subunit RPT6 and is involved in the reduction of SA (Üstün et al. 2013). Two additional examples are the downy mildew effector HaRxL44, which interacts with Mediator subunit 19a in a proteasome-dependent manner, suppressing SA-triggered immunity in *Arabidopsis* (Caillaud et al. 2013), and the *Cm1* effector from *Ustilago maydis*, which affects both pathogen virulence and SA levels in the *Zea mays* host plant (Djamei et al. 2011). Finally, the HopM1 effector from *Pseudomonas syringae* pv. *tomato* DC3000 suppresses expression of *PR1* by targeting AtMIN7 (Gangadharan et al. 2013).

The direct PNPI-wNPR1 interaction detected in the Y2H screen was validated by BiFC in *N. benthamiana* protoplasts (Fig. 3) and was characterized in more detail by testing interactions between different regions of both proteins and different PNPI mutants by Y2H. Strong interactions were observed between DPBB_1 and NPR1/NIM1-like domains, located in the C-terminal regions of PNPI and wNPR1, respectively. We also showed that the amino acid substitution C301W in the DPBB_1 domain of PNPI is sufficient to abolish its interaction with NPR1. The DPBB_1 domain of PNPI was also shown to interact in the Y2H assays with the C-terminal region of the wNPR1 homolog wNPR4, which encodes a proteasomal adaptor protein that regulates proteasome-mediated turnover of NPR1 in a SA-dependent manner (Fu et al. 2012). These results suggest that PNPI may affect either or both the function of wNPR1 on disease resistance and its stability through its interactions with wNPR4.

Characterization of the PNPI putative effector.

Several lines of evidence suggest that the protein encoded by *PNPI* is an effector. This is a small protein (333 amino acids) with a secretory signal peptide that is encoded by a gene expressed in the haustoria. In addition, it interacts with at least

two host proteins (wNPR1 and wNPR4) and, when overexpressed in barley cells, it downregulates the induction of *PR* genes after pathogen infection. However, the evolutionary conservation of the PNPI protein sequence among a relatively wide range of plant pathogens is an unusual characteristic for an effector. The continuous arms race between resistance genes and effectors usually results in a rapid evolution of both gene classes. Signatures of positive selection are often found when comparing strain-specific variants of protein effectors, suggesting that effectors play a key role in the arms race with the host immune system (Guttman et al. 2014). By contrast, PNPI seems to be conserved, not only among different *P. striiformis* f. sp. *tritici* races but, also, among different *formae speciales*. Not a single amino acid change was observed between the different *P. striiformis* f. sp. *tritici* and *P. striiformis* f. sp. *hordei* races sequenced in this study. A relatively high level of conservation was also observed among PNPI proteins from wheat stripe, leaf, and stem rust pathogens (Fig. 1). These results suggest that PNPI likely plays an important role in the evolutionary success of this group of pathogens and that changes in the structure of this protein are under evolutionary constraints.

Secretion and localization of PNPI.

To interact with its target protein NPR1, PNPI needs to be secreted first from the *P. striiformis* f. sp. *tritici* cells into the extra-haustorial matrix and, then, translocated into the host cells. The predicted signal peptide of PNPI was sufficient to induce invertase secretion from transformed yeast cells. However, we were unable to detect secretion of the predicted signal peptide (PNPI₍₁₋₂₂₎) fused with GFP in *N. benthamiana* plasmolyzed epidermal cells.

The RSL—DEEP sequence in the N-terminal region of PNPI is similar but not identical to the RxLR-dEER amino acid motif observed in many oomycete effectors (Kale and Tyler 2011; Wang et al. 2011). In *Phytophthora sojae* effectors, the RxLR-dEER motif has been proposed to be sufficient for re-entry into plant cells, even in the absence of the pathogen (Dou et al. 2008; Wang et al. 2011). However, a recent study in *N. benthamiana* failed to show re-entry into plant cells of effectors from *Melampsora lini* and *Phytophthora infestans* fused to a signal peptide and fluorescent proteins (Petre et al. 2016). Therefore, other methods may be required to test the role of the PNPI RSL—DEEP region in plant cell entry.

Effect of PNPI on the induction of *PR* genes and the potential role of wNPR1.

In *Arabidopsis*, pathogen infection or SA treatment results in the translocation of NPR1 from the cytoplasm to the nucleus, its interaction with TGA transcription factors, the upregulation of a large set of *PR* genes, and the establishment of systemic acquired resistance (Després et al. 2000; Fan and Dong 2002; Kinkema et al. 2000; Zhang et al. 1999). In rice, which has higher endogenous levels of SA, *PR* genes are not effectively induced at SA concentrations that are effective in dicot species. However, at high SA concentrations, some *PR* gene induction is observed (Ganesan and Thomas 2001). In spite of the limited effect of SA on the activation of *PR* genes in rice, transgenic overexpression of *NPR1* in this species results in constitutive activation of defense responses and improved resistance to bacterial blight (Chern et al. 2005b; Yuan et al. 2007). We also observed, in this study, a higher level of *PR* induction by *Pseudomonas syringae* in barley plants overexpressing the wheat *NPR1* gene. In addition, downregulation of *NPR1* in rice leads to loss of resistance to the rice blast fungus *Magnaporthe grisea* (Sugano et al. 2010; Feng et al. 2011) and, in barley, to enhanced susceptibility to *Blumeria graminis* f. sp. *hordei* (Dey et al. 2014). This is consistent with the reduced induction of several barley *PR* genes by *Pseudomonas syringae* in the transgenic

RNAi plants with reduced expression of *HvNPR1*. These results suggest that monocot and dicot plants share some parts of the signal transduction pathway controlling NPR1-mediated resistance (Chern et al. 2001). When wheat and barley plants are exposed to various pathogens, *PR* genes show a very similar induction as in *Arabidopsis* and rice (Colebrook et al. 2012; Dey et al. 2014). However, wheat and barley *PR* genes are not induced by SA or BTH treatment as in the previous two model species (Colebrook et al. 2012; Kogel et al. 1994; Vallélian-Bindschedler et al. 1998). This suggests that the enhanced resistance observed in wheat and barley leaves treated with BTH is likely dependent on the upregulation of a different set of resistance genes (Beßer et al. 2000; Görlach et al. 1996).

In barley and wheat, the induction of *PR* genes in the region adjacent to the infiltration with *Pseudomonas syringae* pv. *tomato* DC3000 does not expand beyond the infected leaf (Colebrook et al. 2012). This indicates that the response is not systemic, as in *Arabidopsis*, and therefore, should be called “acquired resistance” rather than “systemic acquired resistance.” A recent research reported that the acquired resistance observed after infection of barley leaves with *Pseudomonas syringae* pv. *japonica* is associated with a moderate local but not systemic induction of abscisic acid (Dey et al. 2014). The significant induction of five different barley *PR* genes (including *HvPR1b*, *HvPR2*, *HvPR4b*, *HvPR5*, and *HvChitinase 2a*) in the leaf region adjacent to a *Pseudomonas syringae* infiltration was not observed in plants infiltrated with water, demonstrating a specific response to the pathogen.

In this study, we show that the induction of these five *PR* genes by *Pseudomonas syringae* is significantly reduced in barley plants overexpressing *PNPi* (Fig. 5) and hypothesize that NPR1 is involved in this reduction. This hypothesis is based on the connection observed between NPR1 and *PR* genes in barley plants with up- or downregulated levels of *NPR1* and on the reduced interactions between wNPR1 and wTGA2.2 proteins observed in the presence of *PNPi* in Y3H assays (Fig. 4). Previous studies in rice and *Arabidopsis* have demonstrated that the interactions between NPR1 and different TGA2 transcription factors are critical to mediate the upregulation of multiple *PR* genes (Chern et al. 2001; Després et al. 2003; Johnson et al. 2003). Therefore, the *PNPi* disruption of this interaction provides a simple hypothesis to explain the reduced induction of *PR* genes observed in the barley plants overexpressing *PNPi*. This reduction also suggests that *PNPi* plays a role in the manipulation of the wheat defense response and that it may contribute to the virulence of the rust pathogens. We are currently developing a null *NPR1* mutant in tetraploid wheat to test its effect on *P. striiformis* f. sp. *tritici* resistance.

MATERIALS AND METHODS

Screening of a Y2H library using wheat wNPR1 as bait.

A Y2H cDNA library was previously developed from *P. striiformis* f. sp. *tritici*-infected and noninfected leaves of *T. turgidum* subsp. *durum* cv. Langdon (Yang et al. 2013). Briefly, RNAs were reverse-transcribed into cDNA, using the Make Your Own Mate & Plate library system, following the company's protocol (Clontech). The cDNA was then recombined into the library prey vector (pGADT7Rec) using Clontech's SMART technology. The final library was transformed into the yeast strain Y187 (MAT α) following the Clontech protocol.

The cDNA library was screened using the full-length wNPR1 sequence as bait. wNPR1 was cloned into the Y2H bait vector pLAW10 (Cantu et al. 2013a) and was introduced into the yeast strain Y2HGold (Clontech), using the lithium acetate method (Cantu et al. 2013a; Gietz and Woods 2002). wNPR1 does not show autoactivation when tested against an empty vector on SD–Leu–Trp–His–Ade (Cantu et al. 2013a). The bait colonies of pLAW10–wNPR1 were grown to approximately 10⁸ CFU per

milliliter in 50 ml of liquid medium of SD–Trp. Yeast cells were pelleted, were washed once with sterile H₂O, and were resuspended in 50 ml of liquid media of 2× YPDA (yeast extract peptone dextrose agar). One aliquot of the Y187 target yeast (>2 × 10⁷ cells) was combined with the bait. Yeast strains were allowed to mate for 20 to 24 h at 30°C with slight shaking. Yeast cells were then isolated and were washed twice with sterile water and were plated on SD–Leu–Trp–His–Ade. Yeast putative positive diploids from the primary screens were isolated and plasmids were extracted using Zymoprep yeast plasmid miniprep kit (Zymo Research). The Match-maker AD–LD primers were used to amplify the inserted gene fragments. Sequence annotations were carried out with Blastx homology searches against the National Center for Biotechnology Information GenBank nonredundant database.

Cloning and characterization of *PNPi*.

The primers were designed to amplify the coding region of the wNPR1 interactor *PNPi* identified in the Y2H screen. The complete coding region of *PNPi* was amplified from cDNA synthesized using the RNA isolated from seedling leaves of *Triticum turgidum* subsp. *durum* cv. Langdon line RSL65 infected with *Puccinia striiformis* f. sp. *tritici* race PST-113 and harvested at 24 h postinoculation.

The predicted amino acid sequence of *PNPi* protein was used to search the Pfam database (Finn et al. 2014) to identify conserved domains or motifs. SignalP v 4.0 was used to identify signal peptides (Petersen et al. 2011) and TMHMM v2 to detect the presence of transmembrane domains (Möller et al. 2001). MUSCLE multiple sequence alignments and neighbor-joining trees were generated using MEGA6 (Tamura et al. 2013). Confidence of nodes in the neighbor-joining trees were calculated using 1,000 bootstrap cycles.

Expression profile of *PNPi* by qRT-PCR assay.

Seedlings of the susceptible common wheat ‘Felder’ were inoculated with *P. striiformis* f. sp. *tritici* race PST-130 (virulent) in a CONVIRON growth chamber as described previously (Cantu et al. 2013b). Leaves were harvested at 0, 5, 8, 15, and 22 dpi for RNA isolation. Sporulation was observed at 15 dpi. All samples were rapidly frozen in liquid nitrogen and were stored at –80°C. Four independent biological replications were included for each time point.

The mRNAs were isolated using the MagMAX express magnetic particle processors (Thermo Fisher Scientific), according to the manufacturer's instructions. First-strand cDNA was synthesized using the reverse transcription kit (Applied Biosystem). qRT-PCR was performed using SYBR Green (Life Technologies) and a 7500 fast real-time PCR system (Applied Biosystems). Stripe rust elongation factor (*PSTEF*) was used as internal reference. Transcript levels were expressed as linearized fold *PSTEF* levels calculated by the formula $2^{(PSTEF-CT - TARGET-CT)}$. Dissociation curves were generated for each primer to confirm primer specificity.

Dissection of protein regions involved in *PNPi* and wNPR1 Y2H interactions.

Different regions of the *PNPi* and wNPR1 genes were cloned into Y2H vectors pLAW10 (BD) and pLAW11 (AD). These vectors were provided by R. Michelmore (University of California, Davis) and were described previously (Cantu et al. 2013a). Two nonoverlapping regions of *PNPi* were cloned into pLAW10. The first one included *PNPi*_(23–235), which started immediately after the end of the 22-amino acid long predicted signal peptide and included 213 amino acids from the N-terminal region of the *PNPi* protein. The second one, designated as *PNPi*_(236–333), included the DPBB_1 domain located in the C-terminal region of the protein.

Three regions of *wNPR1* were cloned into the pLAW11 vector. Clone *wNPR1*₍₁₋₁₇₀₎, included the BTB/POZ domain, clone *wNPR1*₍₁₉₆₋₃₆₃₎ the DUF3420 and ANK domains, and clone *wNPR1*₍₃₅₅₋₅₇₂₎ the NPR1/NIM1-like domain. This last domain was also cloned into the bait vector from *wNPR1* paralogs *wNPR3* (*wNPR3*₍₃₇₃₋₅₉₃₎) and *wNPR4* (*wNPR4*₍₃₈₅₋₆₀₇₎). A bait vector with the full-length *Arabidopsis NPR1* homolog and a prey vector with a full-length *wTGA2.2* gene were obtained from a previous study (Cantu et al. 2013a). We also generated 15 amino acid substitutions at conserved sites of the DPBB_1 domain in PNPI by overlap PCR and incorporated them into Y2H BD vectors. The cotransformed yeast strains were assayed on plates with SD–Leu–Trp–His and SD–Leu–Trp–His–Ade selection media.

For Y2H assays showing negative results, we confirmed the presence of the proteins using Western blots. Transformed yeast strains were shaken in SD medium overnight, and 1 ml of the overnight culture was transferred into fresh YPDA medium until they reached an optical density at 600 nm (OD_{600}) = 0.1. Samples were then incubated at 30°C for approximately 5 h, with shaking at 230 rpm, until they reached an OD_{600} = 0.4 to 0.6. Yeast cells were harvested by centrifugation. The pellet was washed with ice-cold water, was resuspended in 100 µl of water, and was incubated for 10 min at room temperature, with an additional 100 µl of 0.2 M NaOH. After a brief centrifugation at $11,000 \times g$, the supernatant was removed and 50 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer was added. From each sample, 50 µl was loaded in an SDS-PAGE gel. Protein expression in cells transformed with the Y2H AD vector was detected using the anti-hemagglutinin horseradish peroxidase (anti-HA-HRP) antibody (1:2,000 dilution) (Sigma) and, in those transformed with Y2H BD, using the anti-cMyc-HRP antibody (1:500 dilution) (Santa Cruz Biotechnology).

Validation of PNPI-*wNPR1* interactions using BiFC.

BiFC assays were conducted using a split YFP system (Bracha-Drori et al. 2004) in *N. benthamiana* protoplasts, as described before (Cantu et al. 2013a; Schütze et al. 2009; Wang et al. 2014). The complete coding region of *wNPR1* and of a truncated PNPI excluding the signal peptide were recombined with the N- and C-terminal regions of YFP in Gateway destination vectors pSY736 (YFP^N-PNPI₍₂₃₋₃₃₃₎ fusion) and pSY735 (YFP^C-*wNPR1* fusion), respectively. The fusion proteins were coexpressed in *N. benthamiana* protoplasts using the polyethylene glycol method. Fluorescence was monitored between 24 and 48 h after transformation, using a Zeiss Axiovert 25 fluorescence microscope with the Zeiss YFP filter cube 46HE (excitation, BP500/25; beam splitter, FT515; emission, BP535/30).

Cotransformation of *wHSP90.3*-pSY736 and *wRAR1*-pSY735 vectors was used as positive control and cotransformations of YFP^N-PNPI₍₂₃₋₃₃₃₎ and YFP^C-*wNPR1* with empty vectors YFP^C-EV and YFP^N-EV, respectively, were used as negative controls. As an additional control for false positive nucleic signals, we used the nuclear wheat protein wFDL2 from previous research (Li et al. 2015). Cotransformation of wFDL2-pSY736 and wFT1-pSY735 vectors was used to confirm the previously published interaction (Li et al. 2015), whereas cotransformations of YFP^N-PNPI₍₂₃₋₃₃₃₎ and YFP^C-*wNPR1* with YFP^C-wFDL2 and YFP^N-wFDL2, respectively, were used as negative controls.

In the BiFC assays showing negative results, we confirmed protein expression by Western blots. Transformed protoplasts were collected by centrifugation at $100 \times g$ for 4 min. After removing half of the supernatant, we added 50 µl of SDS-PAGE sample buffer, boiled the samples for 10 min, centrifuged them at $10,000 \times g$ for 10 min, and loaded 50 µl in the SDS-PAGE gel. To detect protein expression, we used anti-HA-HRP antibodies (1:2,000 dilution) (Sigma) for the protoplasts

transformed with the BiFC pSY736 vector and antiMyc-HRP antibodies (1:500 dilution) (Santa Cruz Biotechnology) for the protoplasts transformed with the BiFC pSY735 vector.

Subcellular localization.

To study the function of the PNPI signal peptide and N-terminal region on subcellular localization, we generated four constructs, using GFP fusions in vector pGWB5. Construct 35S::GFP including only GFP was used as positive control. Construct 35S::PNPI₍₁₋₂₂₎-GFP included only the signal peptide of PNPI fused to GFP. Construct 35S::PNPI₍₁₋₆₄₎-GFP included both the signal peptide and the N-terminal region of PNPI fused to GFP. Finally, construct 35S::PNPI₍₁₋₃₃₃₎-GFP included the complete PNPI coding region. These constructs were transformed into *Agrobacterium* sp. strain GV3101 (Höfgen and Willmitzer 1988). Infiltration experiments were performed on 4- to 6-week-old *N. benthamiana* plants as described before (Wang et al. 2011). An empty pGWB5 vector expressing only GFP was used as control. Green fluorescence was detected 48 h after infiltration by fluorescence microscopy. Epidermal peels from *N. benthamiana* leaves were plasmolyzed in 800 mM mannitol for 6 min.

Yeast secretion assays for the validation of signal peptide of PNPI.

The signal peptide of PNPI₍₁₋₂₂₎ was fused in frame to the invertase sequence in the pSUC2 vector and were transformed into yeast strain YTK12. As controls, we used untransformed YTK12 and YTK12 carrying either Ps87₍₁₋₂₅₎-pSUC2 (positive control) or Mg87₍₁₋₂₅₎-pSUC2 (negative control). Yeast strains unable to secrete invertase can grow on SD–Trp medium but not on YPRAA medium.

Y3H assays for PNPI, *wTGA2.2* and *wNPR1*.

We used the pBridge vector-based Y3H system to test if the presence of PNPI can disrupt the interactions between *wTGA2.2* and *wNPR1*. For these experiments, the full-length *wNPR1* was fused with the AD in vector pLAW11 (*wNPR1*-AD). The full-length coding region of *wTGA2.2* was fused to the BD in the pBridge vector, whereas a truncated PNPI lacking the signal peptide was expressed under the *M25* promoter as the bridge protein in the same vector (PNPI₍₂₃₋₃₃₃₎^{M25}/*wTGA2.2*-BD). In this pBridge construct PNPI₍₂₃₋₃₃₃₎ is not expressed in the presence of Met and is expressed in its absence. As controls, both the full-length *wTGA2.2* and the truncated PNPI were expressed as BD fusions in separate pBridge constructs with an empty *M25* promoter (EV^{M25}/*wTGA2.2*-BD and EV^{M25}/PNPI₍₂₃₋₃₃₃₎-BD, respectively).

The resulting *wNPR1*-AD was cotransformed separately with each of the three pBridge constructs described above into yeast strain AH109 (Clontech). Clones were first grown on SD–Trp–Leu medium, were isolated, and were diluted equally, after counting yeast cell number under the microscope. Aba at a concentration of 62.5 ng/ml was used as reporter for BD-AD interactions (Clontech) in the Y3H assays. Protein interactions were tested on SD–Leu–Trp+Met+Aba (bridge protein repressed by Met) or SD–Leu–Trp–Met+Aba (bridge protein expressed).

The quantitative α -Gal assay was used to compare the strength of the interaction between *wTGA2.2* and *wNPR1* in the presence or absence of the PNPI₍₂₃₋₃₃₃₎ bridge protein. Cell populations from PNPI₍₂₃₋₃₃₃₎^{M25}/*wTGA2.2*-BD and *wNPR1*-AD were grown to a density of 2×10^6 to 5×10^6 cells per milliliter in SD–Leu–Trp+Met and SD–Leu–Trp–Met medium at 30°C. Cells were pelleted using a microcentrifuge, and an aliquot of 200 µl from the supernatant was mixed with 600 µl of the assay buffer (0.33 M sodium acetate, pH 4.5, 33 mM p-nitrophenyl- α -D-galactopyranoside) and was incubated at 30°C for 12 to 24 h.

Reactions were stopped by adding 200 µl of 2 M Na₂CO₃, and activity was measured as OD₄₁₀. We also tested the interaction between wTGA2.2 and PNPI₍₂₃₋₃₃₃₎ in Y2H assays, using a wTGA2.2-AD construct from previous research (Cantu et al. 2013a).

Evaluation of PNPI-OE, wNPR1-OE, and HvNPR1-RNAi barley transgenic lines.

We cloned a truncated *PNPI* gene encoding a protein lacking the signal peptide (PNPI₍₂₃₋₃₃₃₎) under the regulation of the maize *Ubiquitin* promoter in a modified Gateway binary vector, pGWB17. We transformed this construct into the barley 'Golden Promise', using *Agrobacterium* spp. at the University of California Davis transformation facility. We used a similar approach to generate barley transgenic plants expressing the full-length wheat *wNPR1* transcript under the regulation of the maize *Ubiquitin* promoter (*Ubi::wNPR1*). We selected three independent transgenic lines overexpressing PNPI and two overexpressing wNPR1, by PCR. We used both T₁ and T₂ plants for qRT-PCR assays. RNAi transgenic barley plants with knockdown expression of *HvNPR1* (*HvNPR1-RNAi*, T₅ homozygous lines) were provided by C. A. Volt (Helmholtz Zentrum Muenchen, Germany) (Dey et al. 2014). Supplementary Table S3 summarizes the transgenic lines used in the qRT-PCR assays.

Upregulation of *PR* gene expression was induced by inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (Colebrook et al. 2012). Briefly, *Pseudomonas syringae* DC3000 was grown on King's B medium with *Rif* antibiotics and was then diluted to OD₆₀₀ = 0.2 in sterile water. Third leaves were inoculated with a 1-ml needleless syringe by pressure infiltration of bacterial suspensions through the leaf abaxial surface. The borders of the infiltrated region were marked using a marker pen. Control seedlings were infiltrated in the same way with sterile water. After bacterial inoculation, seedlings were transferred to a constant 23°C condition to facilitate bacterial growth. Samples for qRT-PCR assay were collected from both wild type and transgenic lines from regions adjacent to the infiltration region (approximately 1 cm from the border of the infiltrated region, 48 h postinoculation).

RNAs were extracted using a Sigma plant total RNA kit, following the manufacturer's instruction, and first-strand cDNA was synthesized using the reverse transcription kit (Applied Biosystems). Gene expression was quantified as described before, using the barley elongation factor 1-α (*HvEF1α*) as an internal reference. The *PR* genes induced by *Pseudomonas syringae* pv. *tomato* DC3000 and characterized by qRT-PCR include *HvPR1b* (Colebrook et al. 2012), *HvPR2* (encoding a β-1-3-glucanase), *HvPR4b* (encoding a chitin-binding protein), *HvPR5* (encoding a thaumatin-like protein TLP6), and *HvChitinase 2a* (X78671.1, encoding a Chitinase).

Transcript levels were quantified separately for the different transgenic events and, therefore, comparisons were restricted to treatments within the same gene and event. The significance of the differences in expression levels between transgenic and control plants for the different *PR* genes were calculated using SAS program version 9.4. The water-inoculated and *Pseudomonas*-inoculated plants were analyzed separately because the responses were very different. In these statistical analyses, the independent transgenic events were used as blocks, separating the variability among events from the analysis of the differences between the wild type and transgenic plants. This is a stringent analysis, because the interaction between event and genotype is included in the error term.

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