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

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ABSTRACT

In Arabidopsis, NPR1 is a key transcriptional co-regulator of systemic acquired resistance. Upon pathogen challenge, NPR1 translocates from the cytoplasm to the nucleus, where 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 *Puccinia NPR1 interactor* (PNPi). 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 over expressing *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.

Key words: wheat; stripe rust; NPR1; pathogen effector; yeast two-hybrid; bimolecular fluorescence complementation; transgenic barley.
INTRODUCTION

*Puccinia striiformis* Westend. f. sp. *tritici* Erikss. (*Pst*) is the causal pathogen of wheat stripe rust which is also known as yellow rust. New and more virulent *Pst* 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; Simons et al., 2011; Hovmøller et al., 2016). Many of the resistance genes that were effective against previous *Pst* races became ineffective against these new races (Chen et al., 2002) prompting the search for new sources of resistance (e.g. (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 *Pst* races revealed a large number of hypothetical effector proteins (Cantu et al., 2011; Cantu et al., 2013b; Zheng et al., 2013). In addition, sixteen *Pst* 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 leucine-rich receptor (NBS-LRR) 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 (Raffaele 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 salicylic acid (SA) hormone, and transcriptional activation of *Pathogenesis-Related (PR)* antimicrobial genes (reviewed in (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 where it interacts with TGA2 transcription factors to activate multiple *PR* genes (Cao et al., 1994; Delaney et al., 1995; Ryals et al., 1997; Shah et al., 1997; Mou et al., 2003).

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 (Chern 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 Liguless gene (Chern et al., 2001). The wNPR1 protein was also shown to interact with two wheat NRR proteins (Negative Regulator of Resistance) 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.,
At low SA levels, NPR1 is targeted for degradation in proteasomes via its binding to NPR4. As 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 wPR1 was induced by the fungal pathogen *Erysiphe graminis*, but did not respond to SA or its functional analogs 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) (Molina et al., 1999). In barley, HvPR1, HvPR3 (chitinase), HvPR5 (thaumatin-like), and HvPR9 (peroxidase) showed significant induction after infection with *E. graminis* or *Pseudomonas syringae* pv. *syringae*, but only infection with the latter resulted in higher SA accumulation (Vallelian-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 *P. 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 *Pst* protein that interacts with
wNPR1, and interferes with its binding to transcription factor wTGA2.2. We also show that overexpression of this Pst gene in barley results in the reduced induction of PR genes in the region adjacent to P. syringae infection sites. Based on these results, we hypothesize that this putative effector may have a role in manipulating wheat defense via its protein interaction with wNPR1.

RESULTS

Pst PNPi protein interacts with wNPR1 in a yeast two-hybrid (Y2H) screen.

The screening of a Y2H library of Pst 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 Pst, designated here as Puccinia NPR1 interactor (PNPi, 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} protein. Comparison of the full-length cDNA sequence of PNPi from Pst 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. Twenty-four amino acids after the end of the predicted signal peptide PNPi showed the sequence RSLL----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” (DPBB_1 domain, pfam 03330, Fig. 1). No trans-membrane domains were detected using the program TMHMM (Moller et al., 2001).

Sequence alignment of PNPi proteins from Pst races PST-78 (PSTG_16231, PRJNA123765), PST-21, PST-43, PST-87/7, PST-08/21 and PST-130 (Cantu et al., 2011; Cantu et al., 2013b) and two Puccinia striiformis f. sp. hordei (Psh) 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 Pst with its homologues from wheat stem rust Puccinia graminis f. sp. tritici (Pgt, XP_003325658) and wheat leaf rust Puccinia triticina (Pt, PTTG_03809) showed good conservation along the complete protein length (Fig. 1). The PNPi protein from Pst is 67.2% similar to the homologous protein in Pgt and 66.8% similar to homologous protein in Pt. 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*, etc.) 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 *Pst* infection.**

After inoculation of the susceptible common wheat variety “Fielder” with the virulent *Pst* race PST-130, we collected leaves at 5, 8, 15 and 22 days post inoculation (dpi). The first two collection points were done during haustoria formation and secondary hyphae expansion, whereas samples for the 15 dpi 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 qRT-PCR (primers in Supplementary Table S2) showed a clear up-regulation from 8 to 22 dpi (Supplementary Fig. S3). Analysis of published transcriptome data showed that *PNPi* is expressed in two datasets from isolated *Pst* 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 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-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(236-333) 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\textsubscript{(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\textsubscript{(23-333)} and wNPR4\textsubscript{(385-607)} in SD-Leu-Trp-His-Ade selection media, but no interaction was observed for wNPR3\textsubscript{(377-593)} (Fig. 2B). For all the negative Y2H assays, we confirmed by Western blots that the proteins were expressed (Supplementary Fig. S5).

We then generated amino acid substitution mutations at the conserved sites of DPBB\_1 domain in PNPI based on the multi-sequences alignment (Supplementary Fig. S1). The point mutation C301W in PNPI\textsubscript{(23-333)} was sufficient to abolish the protein interaction between PNPI\textsubscript{(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 that 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 (BiFC) assays. Co-expression of YFP\textsuperscript{N}-PNPI\textsubscript{(23-333)} and YFP\textsuperscript{C}-wNPR1 in *N. benthamiana* protoplast resulted in strong YFP fluorescence in the
nucleus. We also observed clear YFP fluorescence in the positive control YFP\textsuperscript{N}-wHSP90.3 (ADF31760.1)/YFP\textsuperscript{C}-wRAR1 (EF202841.1), and no fluorescence in the negative controls using empty vector constructs YFP\textsuperscript{N}-EV and YFP\textsuperscript{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 NPR1. Protoplast co-transformed with YFP\textsuperscript{N}-wFDL2 and YFP\textsuperscript{C}-wFT1 showed strong YFP signal in the nucleus, whereas no fluorescence was detected in protoplasts co-transformed with YFP\textsuperscript{N}-PNPi\textsubscript{(23-333)}/YFP\textsuperscript{C}-wFDL2 or YFP\textsuperscript{N}-wFDL2/YFP\textsuperscript{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).

**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 (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, were included in the Y3H assays to visualize the strength of the protein-protein interactions. Expression of the *AUR1-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). The left panels of Fig. 4A excluding Aba selection are 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 (both in -Met, Fig. 4A). 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- galactosidase assay. In this assay, the α-
galactosidase 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 and Supplementary Fig. S4), 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). Yeast YTK12 strain transformed with the pSUC2 vector including the signal peptide of PNPi fused in frame to the invertase sequence were able to grow in both the SD-Trp and YPRAA medium (Supplementary Fig. S9). 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 Mg$^{87}_{(1-25)}$-pSUC2 and positive Ps$^{87}_{(1-25)}$-pSUC2 controls).

Attempts to test re-entry of PNPi into the plant cells using Agro-mediated transformation of *N. benthamiana* were not successful. We were unable to detect secretion of the predicted signal peptide (PNPi$_{1-22}$) fused with 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 pathogenesis-related (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 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 HvEF1a endogenous control (Supplementary Fig. S11).

PR genes were induced in the leaves of both transgenic and control untransformed plants by inoculation with P. syringae pv. tomato DC3000 infection (Fig. 5A) as described before 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 P. 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 Fig. 5B-F and those for events 2, 3, and 4 in Supplementary Fig. S12. The $P$ values presented below Fig. 5B-F panels indicate the significance of the differences between PNPi transgenic plants and their isogenic controls in combined ANOVAs using the four transgenic events as blocks. Comparison of the regions adjacent to the Pseudomonas inoculation showed
significant differences between the transgenic plants and the non-transgenic control for

HvPR1b ($P = 0.006$, Fig. 5B), HvPR2 ($P = 0.001$, Fig. 5C), HvPR4b ($P = 0.018$, Fig. 5D),

HvPR5 ($P = 0.032$, Fig. 5E), and HvChitinase 2a ($P = 0.004$, Fig. 5F). The differences
were consistent in all four transgenic events for all five genes: expression levels were
lower in the transgenic plants overexpressing PNPi than in the non-transgenic control. By
contrast, none of the five PR genes showed significant differences between transgenic
and control plants in water-inoculated control plants (Fig. 5 and Supplementary Fig. S12).

This experiment cannot be done using Psh instead of P. 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, Supplementary Fig. S13) (Dey et al., 2014).

After inoculation with \textit{P. syringae} pv. \textit{tomato} DC3000 we extracted RNA from the region adjacent to the infection area and evaluated \textit{PR} genes expression. In the RNAi transgenic plants with knocked-down HvNPR1 transcript levels, we observed a decrease in the relative expression of several barley \textit{PR} genes, which was significant for HvPR1b, HvPR4b, and HvChitinase 2a (Supplementary Fig. S14). In the transgenic barley plants overexpressing \textit{wNPR1} (\textit{Ubi::wNPR1}), we observed a significant increase in the transcript levels of all tested \textit{PR} genes relative to the control (Supplementary Fig. S15). The overexpression of the \textit{NPR1} gene was stronger in the transgenic event 7 than in event 8 and this was correlated with a stronger induction of the \textit{PR} genes in transgenic event 7 (Supplementary Fig. S13). In the control plants inoculated with water, we detected no significant differences, except for \textit{PR1b} in the \textit{Ubi::wNPR1} transgenic plants (Supplementary Fig. S14 and S15).

\section*{DISCUSSION}

\textbf{Discovery of a putative \textit{Pst} effector that directly targets \textit{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 *Pst* infected wheat leaves to identify *Pst* 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 (Després et al., 2003; Chern et al., 2005b), 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 SA-mediated plant defense pathway (reviewed in (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 salicylic acid (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 Cmu1 effector from Ustilago maydis, which affects both
pathogen virulence and SA levels in the Z. mays host plant (Djamei et al., 2011). Finally,
the HopM1 effector from P. 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
bimolecular fluorescence complementation 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
(Supplementary Fig. S6). 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 both
the function of wNPR1 on disease resistance and/or affect 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 over expressed 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 *Pst* races but also among different *formae specialis*. Not a single amino acid change was observed between the different *Pst* and *Psh* 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 constrains.

**Secretion and localization of PNPi.**

To interact with its target protein NPR1, PNPi needs to be secreted first from the *Pst*
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 (Supplementary Fig. S9). However, we were unable to detect secretion of the predicted signal peptide (PNPi\(_{(1-22)}\)) fused with GFP in \textit{N. benthamiana} plasmolyzed epidermal cells (Supplementary Fig. S10).

The RSLL-----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 \textit{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 \textit{N. benthamiana} failed to show re-entry into plant cells of effectors from \textit{Melampsora lini} and \textit{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 PNPi RSLL-----DEEP region in plant cell entry.

16  **Effect of PNPi on the induction of pathogenesis-related genes and the potential role of wNPR1**

18  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 up-regulation of a large set of \textit{PR} genes, and the establishment of systemic acquired
resistance (Zhang et al., 1999; Després et al., 2000; Kinkema et al., 2000; Fan and Dong, 2002). In rice, which has higher endogenous levels of SA, \textit{PR} genes are not effectively induced at SA concentrations that are effective in dicot species. However, at high SA concentrations some \textit{PR} gene induction is observed (Ganesan and Thomas, 2001). In spite of the limited effect of SA on the activation of \textit{PR} genes in rice, transgenic over-expression of \textit{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 \textit{PR} induction by \textit{P. syringae} in barley plants overexpressing the wheat \textit{NPR1} gene (Supplementary Fig. S15). In addition, down-regulation of \textit{NPR1} in rice leads to loss of resistance to the rice blast fungus \textit{Magnaporthe grisea} (Sugano et al., 2010; Feng et al., 2011) and in barley to enhanced susceptibility to \textit{Blumeria graminis} f. sp. \textit{hordei}, (Dey et al., 2014). This is consistent with the reduced induction of several barley \textit{PR} genes by \textit{P. syringae} in the transgenic RNAi plants with reduced expression of \textit{HvNPR1} (Supplementary Fig. S14). These results suggest that monocot and dicot plants share some parts of the signal transduction pathway controlling \textit{NPR1}-mediated resistance (Chern et al., 2001). When wheat and barley plants are exposed to various pathogens, \textit{PR} genes show a very similar induction as in Arabidopsis and rice (Colebrook et al., 2012; Dey et al., 2014). However, wheat and barley \textit{PR} genes are not induced by SA or BTH treatment as in the previous two model species (Kogel et al., 1994; Vallelian-Bindschedler et al., 1998; Colebrook et al., 2012).
This suggests that the enhanced resistance observed in wheat and barley leaves treated with BTH, is likely dependent on the up-regulation of a different set of resistance genes (Görlach et al., 1996; Besser et al., 2000).

In barley and wheat, the induction of PR genes in the region adjacent to the infiltration with *P. 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 referred as “acquired resistance” rather than as “systemic acquired resistance”. A recent research reported that the acquired resistance observed after infection of barley leaves with *P. 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 *P. 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 *P. syringae* is significantly reduced in barley plants overexpressing PNPi (Fig. 5 and Supplementary Fig. S12), 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 down-regulated levels of NPR1 (Supplementary Fig. S15 and S14) 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 Pst resistance.

MATERIALS AND METHODS

Screening of Y2H library using wheat wNPR1 as bait.

A yeast two-hybrid (Y2H) cDNA library was previously developed from Pst infected and non-infected leaves of T. turgidum ssp. 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, Mountain View, CA, USA). 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 (MATα) following the Clontech protocol.
The cDNA library was screened using the full-length \textit{wNPR1} sequence as bait. \textit{wNPR1} was cloned into the Y2H bait vectors pLAW10 (Cantu et al., 2013a) and introduced into the yeast strain “Y2H Gold” (Clontech, Mountain View, CA, USA) using the lithium acetate method (Gietz and Woods, 2002; Cantu et al., 2013a). \textit{wNPR1} does not show auto-activation when tested against an empty vector on SD-Leu-Trp-His-Ade (Cantu et al., 2013a). The bait colonies of pLAW10-\textit{wNPR1} were grown to approximately $10^8$ cfu/ml in 50 ml liquid medium of SD-Trp. Yeast cells were pelleted, washed once with sterile H$_2$O and resuspended in 50 ml liquid media of 2×YPAD. One aliquot of the Y187 target yeast ($>2\times10^7$ cells) was combined with the bait. Yeast strains were allowed to mate for 20-24 hours at 30°C with slight shaking. Yeast cells were then isolated and washed twice with sterile water and plated on SD media lacking Leucine, Tryptophan, Histidine and Adenine (SD-Leu-Trp-His-Ade). Yeast putative positive diploids from the primary screens were isolated and plasmids extracted using Zymoprep I™ Yeast Plasmid Minipreparation Kit (Zymo Research, CA, USA). The Matchmaker AD-LD primers were used to amplify the inserted gene fragments (Supplementary Table S1). Sequence annotation were carried out with Blastx homology searches against the NCBI GenBank nr database.

\textbf{Cloning and characterization of \textit{PNPi}.}

The primers designed to amplify the coding region of the \textit{wNPR1} interactor \textit{PNPi}
identified in the Y2H screen are described in Supplementary Table S1. The complete coding region of \textit{PNPi} was amplified from cDNA synthesized using the RNA isolated from seedling leaves of \textit{Triticum turgidum} ssp. \textit{durum} cv. Langdon line RSL65 infected with \textit{Puccinia striiformis} f. sp. \textit{tritici} race PST-113 and harvested at 24 hours post-inoculation.

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 trans-membrane domains (Moller et al., 2001). Multiple sequence alignments and Neighbor Joining trees were generated using MUSCLE as implemented in 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 cultivar Fielder were inoculated with \textit{P. striiformis} f. sp. \textit{tritici} race PST-130 (virulent) in a CONVIRON growth chamber as described before (Cantu et al., 2013b). Leaves were harvested at 0, 5, 8, 15 and 22 days post-inoculation (dpi) for RNA isolation. Sporulation was observed at 15 dpi. All samples were rapidly frozen in liquid nitrogen and 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). Quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR Green® (Life Technologies) and a 7500 Fast Real-Time PCR system (Applied Biosystems). Stripe rust elongation factor (PstEF, Supplementary Table S2) was used as internal reference. Transcript levels were expressed as linearized fold-PstEF levels calculated by the formula $2^{(\Delta\Delta C_T)}$. Primer sequences and amplification efficiencies are listed in Supplementary Table S2. 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 (DNA-binding domain, BD) and pLAW11 (activation domain, AD). These vectors were provided by Richard Michelmore (University of California, Davis) and were described before (Cantu et al., 2013a). Two non-overlapping 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 generated also fifteen amino acid substitutions at conserved sites of the

DPBB_1 domain in PNPi by overlap-PCR, and incorporated them into Y2H BD vectors

(primers in Supplementary Table 1). The co-transformed 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

a 0.1 optical density at 600 nm (\( OD_{600} \)). Samples were then incubated at 30 °C for ~5h

with shaking at 230 nm 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 additional 100

µl 0.2M NaOH. After a brief centrifugation at 13,000 rpm briefly, the supernatant was

removed and 50 µl of 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-HA-HRP antibody (1:2000 dilution, Sigma Catalog#12013819001), and in those transformed with Y2H BD using the anti-cMyc-HRP antibody (1:500 dilution, Santa Cruz Biotechnology Catalog#9E10).

Validation of PNPi-wNPR1 interactions using bimolecular fluorescence complementation.

Bimolecular fluorescence complementation (BiFC) assays were conducted using a split yellow fluorescent protein (YFP) system (Bracha-Drori et al., 2004) in N. benthamiana protoplasts as described before (Schütze et al., 2009; Cantu et al., 2013a; Wang et al., 2014). The complete coding region of wNPR1 and of a truncated PNPi excluding the signal peptide were recombined with the N-terminal and C-terminal regions of YFP in Gateway destination vectors pSY736 (YFP\textsuperscript{N}-PNPi\textsubscript{(23-333)} fusion) and pSY735 (YFP\textsuperscript{C}-wNPR1 fusion), respectively. The fusion proteins were co-expressed 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).

Co-transformation of wHSP90.3-pSY736 and wRAR1-pSY735 vectors was used as positive control and co-transformations of YFP\textsuperscript{N}-PNPi\textsubscript{(23-333)} and YFP\textsuperscript{C}-wNPR1 with empty vectors YFP\textsuperscript{C}-EV and YFP\textsuperscript{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). Co-transformation of wFDL2-pSY736 and wFT1-pSY735 vectors was used to confirm the previously published interaction (Li et al., 2015), whereas co-transformations of YFP<sup>N</sup>-PNPi<sub>(23-333)</sub> and YFP<sup>C</sup>-wNPR1 with YFP<sup>C</sup>-wFDL2 and YFP<sup>N</sup>-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 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 12000 rpm for 10 min, and loaded 50 µl in the SDS-PAGE gel. To detect protein expression, we used anti-HA-HRP antibodies (1:2000 dilution, Sigma Catalog#12013819001) for the protoplasts transformed with the BiFC pSY736 vector, and anti-cMyc-HRP antibodies (1:500 dilution, Santa Cruz Biotechnology Catalog#9E10) for the protoplasts transformed with the BiFC pSY735 vector.

**Subcellular localization.**

To study the function of PNPi signal peptide and N-terminal region on subcellular localization, we generated four constructs using GFP fusions in vector pGWB5. Construct 35S::PNPi<sub>(1-22)</sub>-GFP included only the signal peptide of PNPi fused to GFP. Construct 35S::PNPi<sub>(1-64)</sub>-GFP included both the signal peptide and the N-terminal region
of PNPi fused to GFP. Finally, constructs 35S::PNPi_{(1-333)}-GFP included the complete PNPi coding region. These constructs were transformed into *Agrobacterium* strain GV3101 (Hofgen and Willmitzer, 1988). Infiltration experiments were performed on four- to six-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 six minutes.

**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.

**Yeast three-hybrid assays for PNPi, wTGA2.2 and wNPR1.**

We used the pBridge vector-based yeast three-hybrid 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 activation domain (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 the 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 co-transformed separately with each of the three pBridge constructs described above into the yeast strain AH109 (Clontech). Clones were first grown on SD-Trp-Leu medium, isolated and diluted equally after counting yeast cell number under the microscope. Aureobasidin A (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-galactosidase 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 2–5×10^6 cells ml^{-1} in SD-Leu-Trp +Met and SD-Leu-Trp -Met medium at 30°C. Cells were pelleted using a micro-centrifuge, 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-a-D-galactopyranoside), and was incubated at 30°C for 12–24 h. Reactions were stopped by adding 200 µl of 2 M Na₂CO₃, and activity was measured as the optical density at 410 nm (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 variety Golden Promise using Agrobacterium at the UC Davis transformation facility (http://ucdptf.ucdavis.edu/). 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). The primers used to generate the binary vector are described in Supplementary Table S1. We selected three independent transgenic lines overexpressing PNPᵢ and two overexpressing wNPR1 by PCR using primers described in Supplementary Table S1. We used both T₁ and T₂ plants for qRT-PCR assays. RNA interference (RNAi) transgenic barley plants with knockdown expression of HvNPR1 (HvNPR1-RNAi, T₅ homozygous lines) were generously provided by Corina 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 the PR gene expression was induced by inoculation with *P. syringae pv. tomato* DC3000 (Colebrook et al., 2012). Briefly, *P. syringae* DC3000 was grown on King’s B medium with Rif antibiotics and then diluted to OD$_{600} = 0.2$ in sterile water.

Third leaves were inoculated with a 1 ml needless 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 (~1 cm from the border of the infiltrated region, 48 h post-inoculation).

The number of biological replicates used for each transformation event is described in Supplementary Table S3.

RNAs were extracted using Sigma Plant total RNA Kit following the manufacturer’s instruction and first-strand cDNA was synthesized using the Reverse Transcription kit (Applied Biosystems®). Primers for qRT-PCR are described in Supplementary Table S2.

Gene expression was quantified as described before using the barley Elongation Factor 1-alfa (*HvEF1a*, Supplementary Table S2) as an internal reference. The PR genes induced by *P. 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). The GenBank accessions numbers
for the sequences used to design the qRT-PCR primers are listed in Supplementary Table
S2.

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 wildtype and transgenic plants. This is a stringent analysis
because the interaction between Event and Genotype is included in the error term. The
figures for PNPi-OE transgenic event 1 are presented in the text as Fig. 5, and those for
transgenic events 2, 3 and 4 are presented in the Supplementary Fig. S12. Figures for
*HvNPR1-RNAi* transgenic event 5 and 6 are presented in Supplementary Fig. S14 and
those for wNPR1-OE transgenic event 7 and 8 are presented in Supplementary Fig. S15.

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FIGURE LEGENDS

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

Fig. 2. wNPR1, wNPR3, wNPR4 and PNPi interactions in yeast two-hybrid assays. A, Domain predictions for wheat wNPR1, wNPR3, and wNPR4 and *Pst* PNPi using Pfam. Segments indicated in black were cloned into Y2H vectors. B, Yeast two-hybrid assays to assess domain interaction between PNPi and wNPR1, wNPR3 and wNPR4. Yeast transformants co-expressing 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 wNPR3.

Fig. 3. Bimolecular fluorescence complementation assays. Bimolecular fluorescence complementation assays showed interaction between YFP_N-PNPi_{23-333} and YFP_C-wNPR1 in *N. benthamiana* protoplast. YFP_N-wHSP90.3 and YFP_C-wRAR1 were used as positive control. Co-expression 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 μm.
Fig. 4. Yeast three-hybrid assay to determine the effect of competing PNPi\textsubscript{(23-333)} protein on the interactions between wTGA2.2 and wNPR1. A, Yeast transformants co-expressing EV\textsuperscript{M25}/wTGA2.2-BD, EV\textsuperscript{M25}/PNPi\textsubscript{(23-333)}-BD or PNPi\textsubscript{(23-333)}\textsuperscript{M25}/wTGA2.2-BD with wNPR1-pGADT7. Left panels 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\textsubscript{(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-\(\alpha\)-Gal\textsubscript{40} selection medium. The blue color intensity of the wTGA2.2-BD interaction with wNPR1-AD in the presence of PNPi\textsubscript{(23-333)}\textsuperscript{M25} was weaker than in the absence of the putative effector (EV\textsuperscript{M25}/wTGA2.2-BD). C, Quantitative \(\alpha\)-gal assay showed that the interaction between wTGA and wNPR1 was significantly reduced in the presence of PNPi\textsubscript{(23-333)} (** = \(P < 0.01\)). Relative \(\alpha\)-galactosidase activity values for each interaction were the average of six replicates (error bars = Standard Error). EV = empty vector.

Fig. 5. Functional characterization of PNPi\textsubscript{(23-333)}. A, Infiltration of young barley leaves with either \textit{Pseudomonas syringae} pv. \textit{tomato} DC3000 or sterile water as control. The borders of the infiltrated region were marked in black. Samples for qRT-PCR assays were collected from the leaf region adjacent to the infection 48 hours after inoculation. B-F,
Relative expression of antimicrobial 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 is presented in this figure and events 2, 3 and 4 in Supplementary Fig. S5. The Y scale indicates transcript levels relative to barley endogenous control *HvEF1a*. *P* values indicated below the water and DC3000 treatments indicate significance of the differences between transgenic and control plants in combined ANOVAs using transgenic events as blocks. Error bars indicate standard error of the means calculated from eight independent biological replicates.

**SUPPLEMENTARY MATERIALS**

**Supplementary Tables**

**Supplementary Table S1.** Primers for cloning, yeast two- and three-hybrid assays, subcellular localization, and constructs for PNPi transgenic plants.

**Supplementary Table S2.** Primers used for qRT-PCR expression studies.

**Supplementary Table S3.** Transgenic lines used in qRT-PCR assays.

**Supplementary Figures**

**Supplementary Fig. S1.** Alignments of DPBB_1 domains from PNPi homologs.

**Supplementary Fig. S2.** Neighbor-joining tree for PNPi and closest homologs from
other plant pathogens.

Supplementary Fig. S3. Expression of PNPi during PST130 infection.

Supplementary Fig. S4. Negative controls for Y2H assay.

Supplementary Fig. S5. Western blot validation of protein expression in yeast two-hybrid assays with negative results.

Supplementary Fig. S6. Amino acid substitutions in PNPi and their effect on the interactions with wNPR1 in yeast two-hybrid assays.

Supplementary Fig. S7. Negative controls for BiFC assays.

Supplementary Fig. S8. Western blot validation of protein expression in BiFC assays.

Supplementary Fig. S9. Functional validation of PNPi predicted signal peptides using a yeast invertase secretion assay.

Supplementary Fig. S10. Subcellular localization of PNPi in N. benthamiana epidermal cells.

Supplementary Fig. S11. Transcript levels of PNPi in different Ubi::PNPi transgenic events in barley.

Supplementary Fig. S12. Functional characterization of transgenic barley lines overexpressing PNPi (PNPi-OE).

Supplementary Fig. S13. Transcript levels of NPR1 in Ubi::wNPR1 (overexpression) and HvNPR1-RNAi (downregulation) in barley transgenic plants.

Supplementary Fig. S14. Transcript levels of PR genes in HvNPR1-RNAi transgenic
1 barley plants.

2 **Supplementary Fig. S15.** Transcript levels of PR genes in *Ubi::wNPR1* transgenic barley plants.

3 Supplementary Fig. S16. Comparison of NPR1 proteins.
Figure 1
Figure 2

A

B

SD-Leu-Trp-His

SD-Leu-Trp-His-Ade

AD / BD

1:1 1:10 1:100

1:1 1:10 1:100

wNPR1/PNPi$_{(23-333)}$

AtNPR1/PNPi$_{(23-333)}$

wNPR1/PNPi$_{(23-355)}$

wNPR1/PNPi$_{(236-333)}$

wNPR1$_{(1-170)}$/PNPi$_{(23-333)}$

wNPR1$_{(196-363)}$/PNPi$_{(23-333)}$

wNPR1$_{(355-572)}$/PNPi$_{(23-333)}$

wNPR1$_{(1-170)}/PNPi_{(236-333)}$

wNPR1$_{(196-363)}/PNPi_{(236-333)}$

wNPR1$_{(355-572)}/PNPi_{(236-333)}$

wNPR3$_{(373-593)}/PNPi_{(23-333)}$

wNPR4$_{(385-607)}/PNPi_{(23-333)}$

wTGA2.2 /PNPi$_{(23-333)}$

Positive

Negative
Figure 3
Figure 4
Figure 5

Pseudomonas syringae pv. tomato DC3000

A

Water
adjacent region

HvPR1b

B

Water
DC3000

P = 0.9157
P = 0.0058

HvPR2

C

Water
DC3000

P = 0.2517
P = 0.0012

HvPr4b

D

Water
DC3000

P = 0.2332
P = 0.0183

HvPR5

E

Water
DC3000

P = 0.963
P = 0.032

HvChitinase 2a

F

Water
DC3000

P = 0.8725
P = 0.0044
## Supplementary Tables

### Supplementary Table S1. Primers for cloning, yeast two- and three-hybrid assays, subcellular localization and constructs for PNPi transgenic plants.

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**Supplementary Table S2.** Primers used for qRT-PCR expression studies.

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Supplementary Table S3. Transgenic lines used in qRT-PCR assays.

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Supplementary Fig. S1. Alignments of DPBB_1 domains from PNPi homologs.

Alignment of DPBB_1 domains from PNPi and its homologs in more distantly related plant pathogens using the multiple alignment program Muscle as implemented in MEGA 6.0.

Fifteen point mutations at the DPBB_1 domain (*) were generated to test their interactions with wNPR1.
Supplementary Fig. S2. Neighbor-joining tree for PNPi and closest homologs from other plant pathogens. The Neighbor-joining tree was generated using the software MEGA v6. Values in the tree nodes indicate confidence values based on 1000 bootstrap replications. Alignments were based on the most conserved C-terminal region including the PPDB1 domain (last 99 amino acids of PNPi from *Puccinia striiformis* f. sp. *tritici*).
Supplementary Fig. S3. Expression of PNPi during PST-130 infection. Transcript levels of PNPi were determined by qRT-PCR in wheat leaves (cv. “Fielder”) infected with Pst virulent race PST-130. Leaf samples were collected at 5, 8, 15, 22 days post-inoculation (dpi) with Puccinia striiformis f. sp. tritici. The Y scale indicates transcript levels of PNPi relative to the endogenous control PstEF. The mean and standard error were calculated from four independent biological replications.
Supplementary Fig. S4. Negative controls for Y2H assay. All the bait and prey constructs were co-transformed with the corresponding empty AD or BD vectors to test auto-activation. Yeast transformants were assayed on plates with SD-Leu-Trp-His and SD-Leu-Trp-His-Ade selection media.
Supplementary Fig. S5. Western blot validation of protein expression in yeast two-hybrid assays with negative results. Western blot assays using anti-HA-tag and anti-cMYC-tag antibodies were applied to validate the protein expressed by either AD or BD vectors, respectively. “−” indicates empty vector.
**Supplementary Fig. S6.** Amino acid substitutions in PNPi and their effect on the interactions with wNPR1 in yeast two-hybrid assays. Fifteen amino acid substitutions of PNPi\(_{(23-333)}\) were generated by overlap-PCR and cloned into Y2H BD vectors. Yeast transformants co-expressing different bait and prey constructs were assayed on SD-Leu-Trp-His and SD-Leu-Trp-His-Ade. Point mutation C301W in PNPi was sufficient to abolish the protein interaction between these two proteins in SD-Leu-Trp-His-Ade (there is some auto-activation in SD-Leu-Trp-His).
Supplementary Fig. S7. Negative controls for BiFC assays. In addition to the empty vector control, wheat protein wFDL2 was used as a nuclear localization control. Bimolecular fluorescence complementation assays showed interaction between YFPN-wFDL2 and YFPF-wFT in the nuclei of N. benthamiana protoplast. Co-expression of YFPF-wNPR1 and YFPN-PNPi(23-333) with YFPN-wFDL2 and YFPF-wFDL2, respectively, served as negative controls. BF = bright field; YFP = yellow fluorescent protein. Scale bars = 200 μm.
Supplementary Fig. S8. Western blot validation of protein expression in BiFC assays.

Western blot assays using anti-HA-tag and anti-cMYC-tag antibodies were applied to validate the protein expressed by either pSY735 or pSY736 vectors, respectively.
Supplementary Fig. S9. Functional validation of PNPi predicted signal peptides using a yeast invertase secretion assay. The signal peptide of PNPi was fused in frame to the invertase sequence in the pSUC2 vector and were transformed into yeast YTK12 strain. Untransformed YTK12 strain, YTK12 carrying the Ps87(1-25)-pSUC2 (positive) and Mg87(1-25)-pSUC2 (negative) were used as control. Strains that are unable to secrete invertase can grow on SD-Trp medium but not on YPRAA medium.
**Supplementary Fig. S10.** Subcellular localization of PNPi in *N. benthamiana* epidermal cells.

Transient expression of GFP fused PNPi segments in *N. benthamiana* leaves by Agrobacterium infiltration. Expression in all constructs was driven by the 35S promoter. The PNPi(1-22)-GFP fusion included only the putative signal peptide fused to GFP. The PNPi(1-64)-GFP fusion included both the putative signal peptide and the N-terminal region including the RxLR-dEER-like motif fused to GFP. Finally, the PNPi(1-333)-GFP fusion included the complete PNPi protein. GFP alone was used as control. Leaf epidermal peels were plasmolyzed in 800 mM mannitol for six minutes. Yellow arrows indicate examples of plasmolyzed positions, where the GFP fluorescence remains associated to the plasma membrane.
Supplementary Fig. S11. Transcript levels of PNPi in different Ubi::PNPi transgenic events in barley. Four independent barley transgenic events expressing Ubi::PNPi were tested. The Y scale indicates transcript levels of PNPi relative to the barley endogenous control HvEF1a. The mean and standard errors were calculated from 16 (event 1), 28 (event 2), 15 (event 3) and 6 (event 4) independent biological replicates (more information in Supplementary Table S3).
**Supplementary Fig. S12.** Functional characterization of transgenic barley lines overexpressing *PNPi* (*PNPi-OE*). Infiltration of young barley leaves with water (control) or *Pseudomonas syringae* pv. *tomato* DC3000. Samples for qRT-PCR assays were collected from the leaf region adjacent to the infection 48 hours after inoculation, when a weak chlorosis or yellowing occurs. Transgenic event 1 is presented in Fig. 5. Transcript levels are expressed relative to endogenous control *HvEF1a* using the 2^{-ΔCT} method. Scales are not comparable between different genes or events because different optimum thresholds were used in the qRT-PCR analyses. Error bars indicate standard error of the means calculated from independent biological replicates.
Supplementary Fig. S13. Transcript levels of NPR1 in Ubi::wNPR1 (overexpression) and HvNPR1-RNAi (downregulation) in barley transgenic plants. Expression of NPR1 in different transgenic lines were measured by qRT-PCR. The Y scale indicates transcript levels of NPR1 relative to the barley endogenous control HvEF1a. The mean and standard errors were calculated from independent biological replicates of each experiment (detail information for each transgenic event see Supplementary Table S3).
Supplementary Fig. S14. Transcript levels of PR genes in HvNPR1-RNAi transgenic barley plants. Infiltration of young barley leaves with water (control) or P. syringae pv. tomato DC3000. Samples for qRT-PCR assays were collected from the leaf region adjacent to the infection 48 h after inoculation, when a weak chlorosis or yellowing occurs. Transcript levels are expressed relative to endogenous control EF1a using the 2^{-ΔΔCT} method. P values indicated indicate significance of the differences between transgenic and control plants in combined ANOVAs using transgenic events as blocks.
Supplementary Fig. S15. Transcript levels of PR genes in Ubi::wNPR1 transgenic barley plants.

Infiltration of young barley leaves with water (control) or Pseudomonas syringae pv. tomato DC3000.

Samples for qRT-PCR assays were collected from the leaf region adjacent to the infection 48 hours after inoculation, when a weak chlorosis or yellowing occurs. Transcript levels are expressed relative to endogenous control HvEF1a using the 2^{-ΔΔCT} method. P values indicate significance of the differences between transgenic and control plants in combined ANOVAs using transgenic events as blocks.
Supplementary Fig. S16. Comparison of NPR1 proteins. Alignment of NPR1 and NPR1-like proteins from Arabidopsis (GenBank AAM65726.1), rice (GenBank NP_001042286.1) and wheat (GenBank AGH18701) using the multiple alignment program Muscle as implemented in MEGA 6.0. BTB = Broad-Complex, Tramtrack and Bric a brac (smart00225), DUF3420 = Domain of unknown function (pfam11900), ANK = ankyrin repeats that mediate protein-protein interactions (cd00204), NPR1/NIM1 = NPR1/NIM1-like defence protein C terminal (pfam12313).