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NPR1 is required for root colonization and the establishment of a mutualistic symbiosis between the beneficial bacterium *Rhizobium radiobacter* and barley

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Summary

Non-expressor of pathogenesis-related genes 1 (NPR1) is a key regulator of plant innate immunity and systemic disease resistance. The model for NPR1 function is based on experimental evidence obtained largely from dicots; however, this model does not fit all aspects of Poaceae family, which includes major crops such as wheat, rice and barley. In addition, there is little scientific data on NPR1's role in mutualistic symbioses. We assessed barley (*Hordeum vulgare*) HvNPR1 requirement during the establishment of mutualistic symbiosis between barley and beneficial Alphaproteobacterium *Rhizobium radiobacter* F4 (*Rr*F4). Upon *Rr*F4 root-inoculation, barley NPR1-knockdown (KD-*hvnpr1*) plants lost the typical spatiotemporal colonization pattern and supported less bacterial multiplication. Following *Rr*F4 colonization, expression of salicylic acid marker genes were strongly enhanced in wild-type roots; whereas in comparison, KD-*hvnpr1* roots exhibited little to no induction. Both basal and *Rr*F4-induced root-initiated systemic resistance against virulent *Blumeria graminis* were impaired in leaves of KD-*hvnpr1*. Besides these immune-related differences, KD-*hvnpr1* plants displayed higher root and shoot biomass than WT. However, *Rr*F4-mediated growth promotion was largely compromised in KD-*hvnpr1*. Our results demonstrate a critical role for HvNPR1 in establishing a mutualistic symbiosis between a beneficial bacterium and a cereal crop.

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Introduction

During co-evolution with pests and microbes, plants have evolved ingenious local and systemic immune pathways. Local immune responses are initiated when highly conserved microbe- or pathogen-associated molecular patterns are detected by cell surface-localized pattern recognition receptors. This recognition triggers pattern-triggered immunity (PTI), which often is sufficient to prevent further pathogen ingress. However, some pathogens are able to suppress PTI. In this situation, the host plant displays a low level of resistance, termed basal resistance. To combat these virulent pathogens, some plants can activate the second layer of local immunity, termed effector-triggered immunity (ETI). Both PTI and ETI are associated with increased synthesis of the phytohormone salicylic acid (SA) and the activation of various defence responses in the infected tissue (Jones and Dangl, 2006; Mishina and Zeiser, 2007; Choi and Klessig, 2016). The systemic immune pathways are broadly categorized into systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Fu and Dong, 2013; Pieterse *et al*., 2014; Klessig *et al*., 2018). SAR is induced in distal uninfected tissues by a prior inoculation of a pathogen (Ross, 1961); like PTI and ETI, it is dependent on the SA signalling pathway. By contrast, ISR is induced primarily by pests (insects) and root-colonizing non-pathogenic microbes. Activation of ISR is mediated by the jasmonic acid (JA) and ethylene (ET) signalling pathways (Van Loon *et al*., 2006.

The discovery that SA is a critical endogenous signal for SAR led to extensive efforts to identify downstream signalling components. Characterization of several Arabidopsis mutants that accumulated endogenous SA but failed to activate SAR after pathogen infection or exogenous SA treatment led to the identification of a single gene, *Non-expressor of pathogenesis-related (PR) genes 1* (*NPR1*)

(Cao *et al*., 1994; Delaney *et al*., 1995; Glazebrook *et al*., 1996; Shah *et al*., 1997). Subsequent studies revealed that NPR1 not only plays a critical role in the establishment of SAR but also during ISR (van Loon *et al*., 2006). Structural analyses indicated that NPR1 contains an N-terminal BTB/POZ (broad-complex, tamtrack, bric-a-brac/pox virus, and zinc finger) domain, an ankyrin repeat domain, a C-terminal transactivation domain and nuclear localization sequence (Klessig *et al*., 2018). NPR1 is a redox-sensitive protein that resides in the cytosol as an oligomeric complex formed by intermolecular disulfide bonds (Mou *et al*., 2003). Following microbial infection, SA induces a biphasic change in the cellular redox state. The initial oxidative burst is followed by a more reducing environment that causes the NPR1 oligomer to disassociate (Mou *et al*., 2003; Tada *et al*., 2008). In addition, direct binding of SA promotes NPR1 monomerization (Wu *et al*., 2012). NPR1 monomers are then transported to the nucleus, where they serve as transcriptional coregulators of defence-associated genes, such as *Pathogenesis-related 1*, via their direct interaction with basic leucine zipper transcription factors from the TGA family (Klessig *et al*., 2018).

In comparison to its role in the nucleus, cytosolic NPR1 does not promote SA-induced defence gene expression. Instead, it modulates crosstalk between the SA and JA signalling pathways (Spoel *et al*., 2003). These pathways are thought to form the backbone of plant immunity, with SA generally mediating resistance to biotrophic pathogens and JA regulating resistance to necrotrophic pathogens and herbivorous insects. ET frequently works synergistically with JA to activate resistance to necrotrophs. The relationship between the SA and JA/ET signalling pathways often is mutually antagonistic, although synergistic interactions have been noted (Pieterse *et al*., 2012; De Vleesschauwer *et al*., 2014; Caarls *et al*., 2015; Shigenaga *et al*., 2017). The balance between the SA and JA/ET pathways presumably enables deployment of defences best suited to combat pathogens with different lifestyles.

Although SA's and NPR1's roles in mediating defence signalling have been well documented in many dicot species, their function in monocots is less clear. Studies in rice, which constitutively accumulates elevated levels of SA, as well as other cereals, suggest that SA is involved in immune signalling triggered by at least some pathogens (Klessig *et al*., 2018). In addition, NPR1 is conserved in dicots and monocots (Kogel and Langen, 2005; Balmer *et al*., 2013; Sharma *et al*., 2013). Overexpression of *AtNPR1* either primes or enhances SA-associated disease resistance in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) against various pathogens, including *Xanthomonas oryzae*, *Magnaporthe oryzae* (*Mo*), *Fusarium verticillioides* and *Erwinia chrysanthemi* (Makandar *et al*., 2006; Chern *et al*., 2007; Quilis *et al*., 2008; Xu *et al*., 2017). Similarly, overexpression of wheat *TaNPR1* in barley (*Hordeum vulgare*) conferred enhanced resistance to *Mo*, whereas resistance to *Mo* was suppressed in a barley line with knocked-down (KD) expression of *HvNPR1* (Wang *et al*., 2018). Furthermore, protein interaction between NPR1 and TGAs is critical for NPR1 function in monocots and dicots (Després *et al*., 2003; Chern *et al*., 2007; Cantu *et al*., 2013), including expression of *PR* genes during resistance triggered by *P. syringae* DC3000 (Wang *et al*., 2016).

Despite these findings, the well-established model for NPR1's role in host–microbe interactions is not consistent with some aspects of the family of Poaceae, which includes major crops like wheat, rice and barley. These cereal crops do not develop a canonical SAR in which the activation of *PR* gene expression and broad-spectrum pathogen resistance in the systemic leaves is signalled by increased levels of endogenous SA (Kogel and Langen, 2005; Wang *et al*., 2018). In barley and wheat, inoculation with *Pseudomonas syringae* pv. *tomato* (*Pst*) induces enhanced resistance to secondary infection by other pathogens, a phenomenon termed acquired resistance (AR). Transcriptional profiling of barley tissue adjacent to the primary inoculation revealed similarities with the transcriptional profile of SAR in Arabidopsis, as well as transcripts previously associated with chemically induced AR in cereals (Beßer *et al*., 2000), suggesting that AR in barley and SAR in Arabidopsis may be mediated by analogous pathways. However, AR is not detected in systemic leaves, but rather in the region adjacent to the initial infection site (Colebrook *et al*., 2012; Gao *et al*., 2018). Alternatively, primary leaf infection of barley with *P. syringae* pv. *japonica* (*Psj*) induces systemic resistance in uninfected leaves against a subsequent challenge infection with *X. translucens* pv. *cerealis* (*Xtc*). Unlike SAR in Arabidopsis, however, systemic immunity in barley was not associated with HvNPR1, nor with the local or systemic accumulation of SA (Dey *et al*., 2014). Instead, it was associated with a moderate local, but not systemic, induction of abscisic acid (ABA). Local application of JA methyl ester or ABA, but not SA or BTH, triggered systemic immunity to *Xtc*. The systemic response correlated with the local and systemic induction of two WRKY and two ethylene-responsive factor-like transcription factors.

The role NPR1 and the SA signalling pathway play during establishment of mutualistic symbioses between plants and beneficial microbes also is poorly understood. Legumes are uniquely capable of forming symbiotic interactions with rhizobacteria belonging to the *Rhizobium* genus (Remigi *et al*., 2016). Inoculation of legume roots with symbiotic bacteria, such as *Sinorhizobium meliloti*,

or treatment with purified nodulation factors induces various early responses, such as root hair deformation and induction of early and late nodulin genes. Analyses of *Medicago truncatula* with altered levels of NPR1 expression revealed that *S. meliloti*-induced root hair deformation was suppressed in plants that overexpressed *AtNPR1*, while it was accelerated in plants silenced for *NPR1* expression (Peleg-Grossman *et al*., 2009). Interestingly, *S. meliloti*-induced root hair deformation and expression of early nodulin genes also were observed in the non-legume *Arabidopsis*, but only in the *npr1* mutant background rather than WT plants. Thus, NPR1 appears to suppress plant responses to *Rhizobia* (Peleg-Grossman *et al*., 2009). Further implicating the SA signalling pathway as a negative regulator of plant–*Rhizobium* symbiotic interactions, SA treatment of *M. truncatula* inhibits *S. meliloti*-induced root hair deformation, whereas this response is supported in SA-deficient *NahG* but not WT Arabidopsis. Additionally, SA levels are reduced in *M. truncatula* during the first days of *S. meliloti* infection, which may result in reduced NPR1-dependent gene expression (Martinez-Abarca *et al*., 1998). By contrast, the ectomycorrhiza (EM) fungus *Laccaria bicolor* promotes mutualism in *Populus* by expressing the effector protein MiSSP7 (Mycorrhiza-induced small secreted protein 7), which blocks the JA signalling network by binding and protecting the host protein PtJAZ6 (Jasmonate ZIM-domain [JAZ] protein 6) from degradation (Plett *et al*., 2014). Likewise, *Populus* roots colonized with the EM fungus *Paxillus involutus* accumulate elevated levels of the stress-related hormone ABA and SA compared with non-EM colonized roots, whereas JA and auxin levels are reduced (Luo *et al*., 2009). Together, these studies indicate that various plant immune signalling pathways can impact the establishment of mutualistic symbioses between different microbes and their hosts.

In the present work, we investigated whether NPR1 is involved in establishing a mutualistic symbiosis between the Alphaproteobacterium *Rhizobium radiobacter* (*Rr*F4) and the cereal plant barley. The beneficial bacterium *Rr*F4 was originally isolated from the sebacinoid basidiomycete fungus *Serendipita indica* (Weiß *et al*., 2016; syn. *Piriformospora indica*, Verma *et al*., 1998), a host-unspecific root endophyte that colonizes virtually all plants so far tested under greenhouse conditions (Sharma *et al*., 2008). *Rr*F4 shows a high degree of genomic similarity to the plant pathogen *R. radiobacter* (formerly: *Agrobacterium tumefaciens*) C58 (Glaeser *et al*., 2016). Similar to its fungal host *S. indica*, *Rr*F4 colonizes plant roots without host preference and forms aggregates of attached cells and dense biofilms at the root surface of maturation zones. *Rr*F4-colonized plants show increased biomass and systemically enhanced resistance against the powdery mildew fungus *B. graminis* f.sp. *hordei* (*Bgh*) in barley and bacterial leaf pathogens such as *Pst* DC3000 in Arabidopsis and *X. translucens* pv. *translucens* (*Xtt*) in wheat (Sharma *et al*., 2008; Glaeser *et al*., 2016; Alabid *et al.,* 2020).

Here, we examine the influence of *NPR1* on the beneficial bacterium *R. radiobacter* F4 to form mutualistic symbioses with roots of the cereal crop barley and further analyse the signalling pathways modulated during root colonization and induction of systemic resistance. Our results indicate that HvNPR1 plays a critical role in the establishment of a mutualistic symbiosis between a bacterium and a cereal crop. This study hence expands our understanding of the molecular nature of plant–microbe interaction in cereals.

Results and discussion

Phylogenetic analysis and in silico *identification of barley* NPR1-*like genes*

In Arabidopsis, *NPR1* belongs to a gene family that contains five additional members (*AtNPR2-6*). Previous analyses in barley identified *HvNPR1* (GenBank: AM050559.1), which encodes a protein containing the conserved domains identified in other NPR1 homologues such as the BTB/POZ domain, the DUF domain (Domain of Unknown Function), the ankyrin repeat domain and a NPR1/NIM1 like defence protein C terminal domain (Fig. Fig. S1a; Kogel and Langen, 2005). Two additional *HvNPR1*-like genes, *Cul4* (GenBank: AK360734.1) and *Lax-a* (GenBank: AK359086.1) have been published (Tavakol *et al*., 2015; Jost *et al*., 2016; Castelló *et al*., 2018). These genes share high similarity with Arabidopsis *Blade-On-Petiole 1* (*BOP1*; syn. *AtNPR5*) and *BOP2* (syn. *AtNPR6*) respectively (Fig. Fig. S1b). Phylogenetic analyses have divided NPR1-like proteins into three clades: clade I contains AtNPR1 and AtNPR2 homologues, clade II contains AtNPR3 and AtNPR4 homologues and clade III contains AtNPR5 and AtNPR6 homologues (Fig. 1; Backer *et al*., 2019). The clear separation of clade I and II is currently controversial (Toriba *et al*., 2019).

To mine additional barley *NPR1*-like genes, we conducted a genome-wide analysis across several species based on predicted protein data from the National Centre for Biotechnology Information. Using domain prediction analysis (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), we identified two additional genes that encode HvNPR1-like proteins, *HvNPR2* (BAJ86173.1) and *HvNPR3* (BAJ90272.1) (Fig. Fig. S1c,d). Protein sequences corresponding to *HvNPR2* (*HORVU3Hr1G074640.4*) and *HvNPR3* (*HORVU4Hr1G003040.1*) were also found in the barley cv. Morex sequencing database of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK,

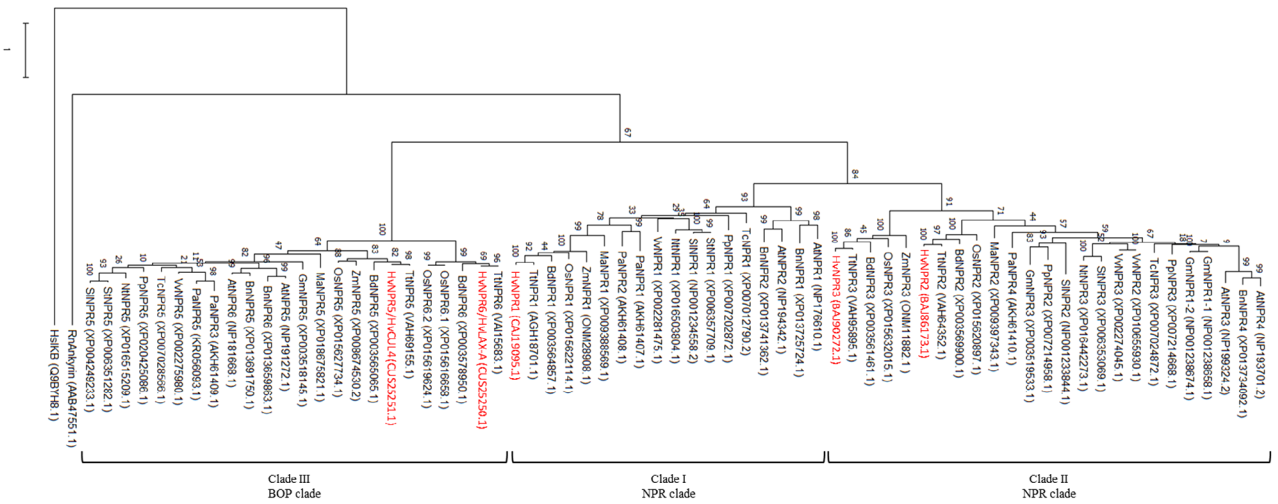

Fig 1. Phylogenetic relationship of barley NPRs with their homologues in other species. Amino acid (aa) sequences of HvNPR1 and its homologues were aligned using the MUSCLE algorithm and the maximum likelihood tree was generated using the MEGA software (MEGA X version 10.0.5, Kumar *et al*., 2018). Numbers in the tree nodes indicate confidence values based on 1000 bootstrap replicates. The following species were included in the analysis: the dicot model *Arabidopsis thaliana* (*At*), the grass model *Brachypodium distachyon* (*Bd*), *Brassica napus* (*Bn*), *Glycine max* (*Gm*), *Hordeum vulgare* (*Hv*, in red), *Musa acuminata* (*Ma*), *Nicotiana tabacum* (*Nt*), *Oryza sativa* (*Os*), *Persea americana* (*Pa*), *Prunum persica* (*Pp*), *Solanum lycopersicum* (*Sl*), *Solanum tuberosum* (*St*), *Theobroma cacao* (*Tc*), *Triticum turgidum* (*Tt*), *Vitis vinifera* (*Vv*) and *Zea mays* (*Zm*). The ankyrin-2 sequence from *Rattus norvegicus* (*Rn*) and human *NF-kappa-B inhibitor zeta* (*Hs*IkB) were used as outgroups. The scale bar at the bottom indicates the evolutionary distance corresponding to one aa substitution per site. [Color figure can be viewed at wileyonlinelibrary.com]

Gatersleben, Germany (https://webblast.ipk-gatersleben.de/barley_ibsc/viroblast.php)). Based on amino acid (aa) sequence, HvNPR2 shares the highest similarity with rice OsNPR2 and the grass model *Brachypodium distachyon* BdNPR2, and HvNPR3 shares the greatest level of similarity with OsNPR3 and BdNPR3, which all cluster in clade II (see Fig. 1). The domain structure of all barley *NPR1*-like genes was also tested by their exon-intron distribution frequency (http://gsds.cbi.pku.edu.cn), further confirming the placement of the barley NPR1-like proteins in the various phylogenetic clades (Fig. Fig. S1e).

HvNPR1 modulates colonization of barley roots by Rr*F4*

To assess the possibility that HvNPR1 plays a role in establishing a mutualistic symbiosis between the beneficial bacterium *Rr*F4 and barley, we monitored root colonization in *Rr*F4-inoculated WT plants and a barley line (cv. Golden Promise) that was partially silenced for *HvNPR1* expression (KD-*hvnpr1* E7L2 and E11L9 lines; Dey *et al*., 2014). As anticipated from a previous study, the relative level of *HvNPR1* transcript in homozygous KD-*hvnpr1* lines was 32% and 47% respectively, compared with wt plants (Fig. 2a), and KD-*hvnpr1* E7L2 plants lost sensitivity to the resistance inducer benzothiadiazole (BTH; Fig. 3; Görlach *et al*., 1996). To confirm that *HvNPR1* silencing was specific, we investigated possible off-target effects on other *HvNPRs*. As expected due to the lack of off-target detection with SiFi software, the KD-hvnpr1 lines E7L2 and E11L9 were silenced for *HvNPR1* expression, while *HvNPR2*, *HvNPR3*, *HvNPR5* and *HvNPR6* expression was not affected (Fig. 2b; Fig. S2).

The roots of 3-day-old WT and KD-*hvnpr1* seedlings were dip-inoculated with a *ß-glucuronidase* (*GUS*)-expressing *Rr*F4 strain (Glaeser *et al*., 2016). Subsequently, seedlings were cultivated in glass jars on half-strength Murashige and Skoog (1/2 MS) medium. Detached roots were treated with the GUS substrate 5-Bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexyl ammonium salt (X-gluc) for visualization of bacterial cells. Starting from 2 days post-inoculation (dpi), WT roots showed a dark blue colour that was spatially restricted to the root hair zone, whereas KD-*hvnpr1* roots showed a fainter colouring that was distributed across the root tips (Fig. 4a–d; Fig. S3). Based on the staining intensity and pattern, bacterial colonization of WT roots appears to be stronger than that of KD-*hvnpr1* roots. This finding raises the possibility that HvNPR1 positively regulates the spatiotemporal colonization pattern of *Rr*F4. To further investigate this possibility, WT and KD-*hvnpr1* plants were inoculated with *Rr*F4 and cultivated in the soil for 3 weeks; DNA was then extracted from roots and quantified by quantitative real-time PCR (qRT-PCR) using bacteria-specific internal transcribed spacer (ITS) primers (Glaeser *et al*., 2016). Based on the relative level of *Rr*F4 ITS, the roots of both KD-*hvnpr1* lines E7L2 and E11L9 supported substantially lower levels of *Rr*F4 than those of WT plants (Fig. 4e). We extended our analysis by transmission electron microscopy (TEM) to understand the

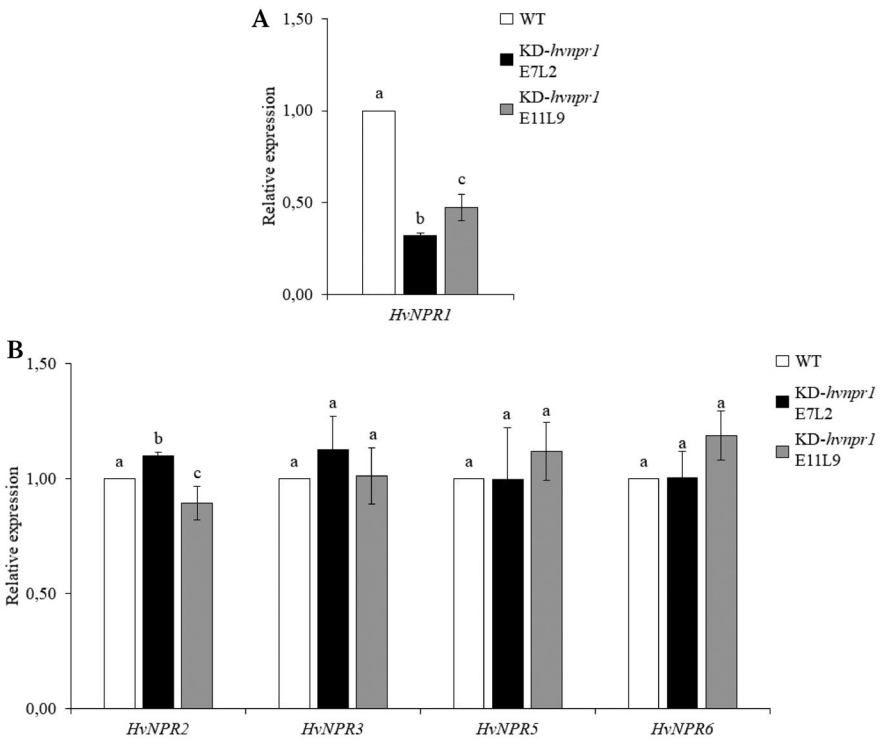

Fig 2. Relative expression of *HvNPR* genes determined by qRT-PCR in wild type (WT) barley cv. Golden Promise (GP) and in two KD-*hvnpr1* lines (Dey *et al*., 2014). The results were obtained using the T3 (E11L9) and T5 (E7L2) generation of transgenic plants. The transcript level of *HvNPR1* (A) and other *HvNPR* family members (B) was normalized to barley *Ubiquitin* (GenBank: M60176.1). Displayed is the mean of six technical repetitions (n = 10 plants). The experiment was conducted two times (n = 10 plants) with similar results. Error bars represent standard deviation (SD). Letters represent the statistical difference among each group means (Tukey's range test, α = 0.05).

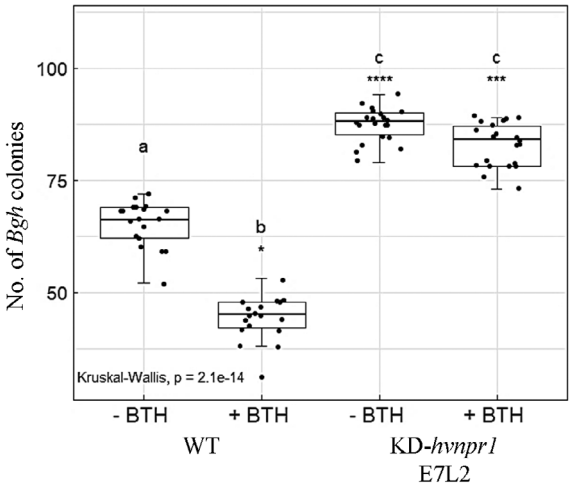

Fig 3. Sensitivity of barley to the resistance-inducing compound benzothiadiazole (BTH). Ten milliliters of 20 ppm BTH in wettable powder (WP) and WP alone as mock control were applied to a 5-day-old cv. Golden Promise WT and KD-*hvnpr1* seedlings as a soil drench. Two days later, detached first leaves were inoculated with *Bgh*A6 and at 6 dpi colonies were counted. *Bgh*A6 colony numbers on BTH-treated WT plants were lower than numbers on mock-treated plants. In contrast, BTH-treated KD-*hvnpr1* plants showed only minor reductions in *BghA6* colony number compared with mock-treated KD-*hvnpr1*, showing that BTH-induced resistance is dependent on HvNPR1. The experiment was conducted two times (n = 20 plants) with similar results. Comparisons between groups were performed via the Kruskal–Wallis test and Dunn's test of multiple comparisons. Asterisks represent the statistical differences of the groups against WT mock (*p < 0.05; ***p < 0.001; ****p < 0.0001). Letters represent statistical difference among all groups (α = 0.05).

rhizodermal colonization pattern of the bacteria. Due to the stronger *HvNPR1* silencing effect, these experiments were done with line KD-*hvnpr1* E7L2. At 5 dpi, *Rr*F4 cells had already penetrated into the WT roots. Significantly, and in accordance with Glaeser *et al*. (2016), *Rr*F4 predominantly colonize the extracellular spaces of the root cortex (Fig. 5A–C). In clear contrast, the roots of KD-*hvnpr1* plants showed a broad layer of bacteria that were located on the outside of the rhizodermis and bacteria could not be found in the extracellular spaces of the cortex (Fig. 5D–F). Thus, HvNPR1 appears to be required, at least in part, for effective colonization of barley roots by the beneficial microbe *Rr*F4.

Rr*F4-induced expression of SA but not JA marker genes is compromised in KD-*hvnpr1 *roots*

Whether *Rr*F4 inoculation impacts the local expression of plant defence genes was then assessed in WT and KD-*hvnpr1* roots over a 6-day time-course. Three-day-old barley seedlings were dip-inoculated with *Rr*F4 or dipped into buffer (mock) and axenically grown roots were harvested for qRT-PCR analysis at the indicated time points (Fig. 6). From 2 dpi onwards, expression of the SA marker genes *HvPR1b* and *HvPR2* was significantly higher in *Rr*F4-colonized WT roots compared with mock-treated roots (Fig. 6A and B). In contrast, *Rr*F4 colonization did not enhance the expression of either *PR* gene in KD-*hvnpr1* roots at 2 or 4 dpi, although a small induction

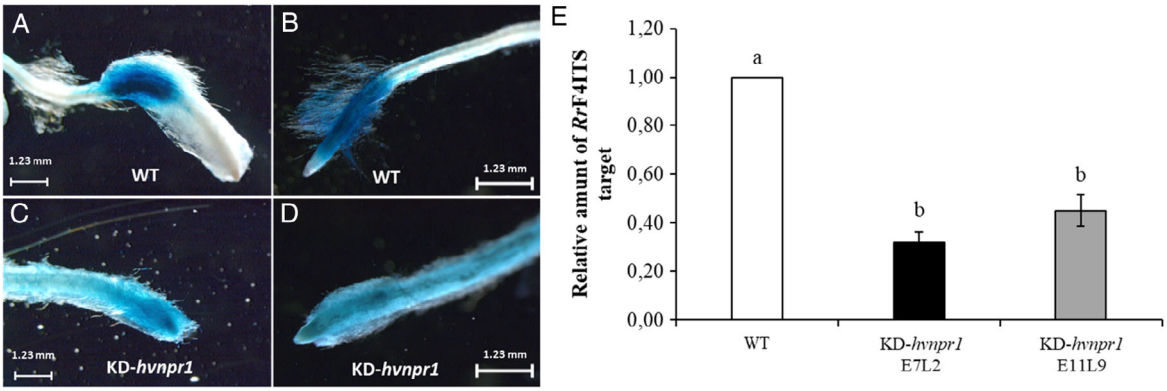

Fig 4. *Rr*F4 colonization pattern and strength in barley cv. Golden Promise WT and KD-*hvnpr1* roots. Root segments colonized by *GUS*-expressing *Rr*F4 at 5 dpi in WT (A, B) and KD-*hvnpr1* E7L2 (C, D) plants. (E) Relative qPCR analysis of the quantity of *Rr*F4 cells in roots of 3-day-old plants at 5 dpi using primers specific for barley *Ubiquitin* and *Rr*F4 *ITS*. The number of bacteria was significantly reduced in both roots of KD-*hvnpr1* mutant lines compared with WT plants. The experiment was conducted two times (n = 10 plants) with similar results. Displayed are means with standard errors of three independent biological experiments. Letters represent the statistical differences among the group means (Tukey's range test, α = 0.05). [Color figure can be viewed at wileyonlinelibrary.com]

was detected at 6 dpi, potentially due to residual NPR1 activity. The JA marker *S-adenosyl-l-methionine: jasmonic acid carboxyl methyltransferase* (*HvJMT*) also was induced by *Rr*F4 colonization of WT roots, although a dramatic increase was not detected until 4 dpi (Fig. 6C). In contrast to either *PR* gene, *HvJMT* expression was strongly enhanced in KD-*hvnpr1* roots after *Rr*F4 inoculation, with transcripts for this gene accumulating to even greater levels than in comparably treated WT plants at 2 and 6 dpi.

Taken together, these data suggest that root inoculation with the mutualistic microbe *Rr*F4 enhances local expression of *HvPR1b* and *HvPR2* via a pathway that is largely dependent on HvNPR1, whereas it induces *HvJMT* expression via a pathway that is largely HvNPR1-independent.

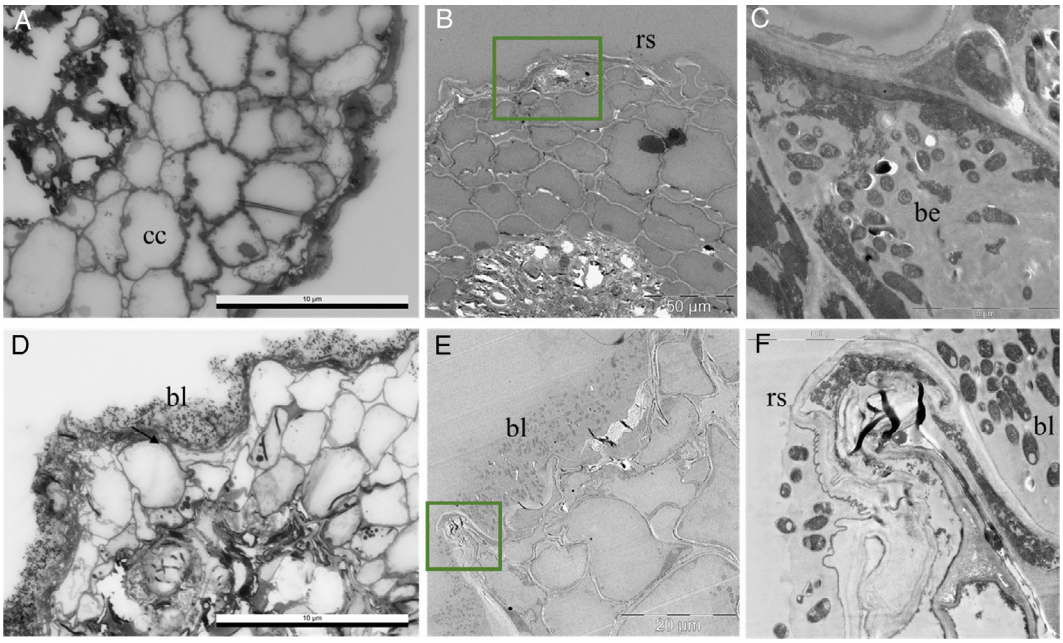

Fig 5. TEM analysis of the colonization pattern of *Rr*F4 in WT vs. KD-*hvnpr1* barley roots. Three-day-old seedlings were dip-inoculated for 30 min into bacterial suspensions (OD_{600} = 1.4–2), the colonization pattern was analysed at 5 dpi. In WT plants, bacterial colonization was located mainly in the root cortex (A, B, C), whereas in KD-*hvnpr1* roots it was located on the outside of the rhizodermis (D, E, F). Cc, root cortex cells; bl, bacterial layer outside on the root surface; be, bacteria in the extracellular space of cortex cells; rs, root surface. [Color figure can be viewed at wileyonlinelibrary.com]

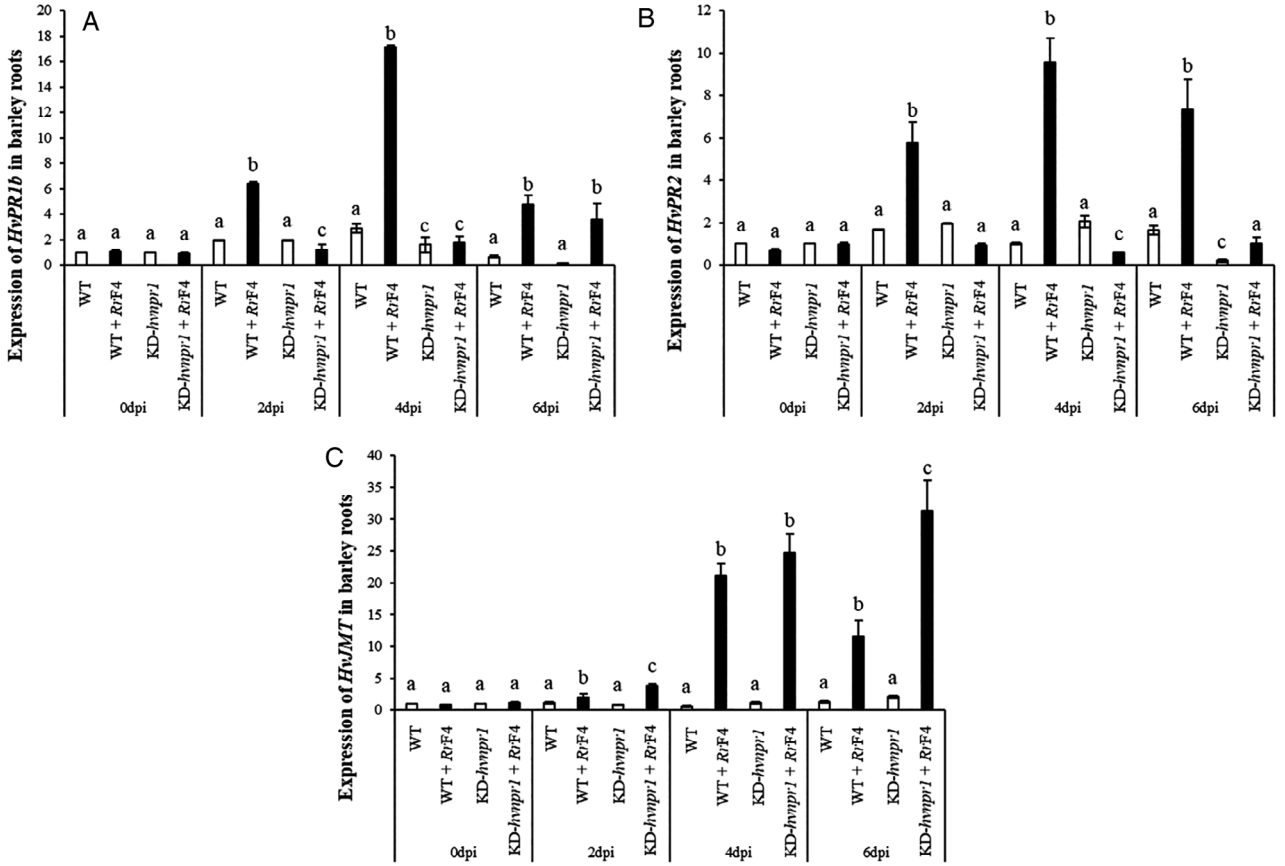

Fig 6. Relative expression of immune-related genes in the roots of KD-*hvnpr1* vs. WT barley in the presence or absence of *Rr*F4. Transcripts of *HvPR1b* (A), *HvPR2* (B), or *HvJMT* (C) were assessed by qRT-PCR and normalized to barley *ubiquitin*. Roots of 3-day-old seedlings were dip-inoculated with *Rr*F4 (OD_{600} = 1.4–2) and harvested at 0, 2, 4 and 6 dpi. The experiment was conducted three times (n = 7 plants) with similar results. Error bars indicate standard deviation. For each gene, the different letters above the bars indicate significant differences in the means determined by one-way ANOVA with post-hoc Tukey HSD test (α = 0.05).

Our data are consistent with a report showing that elevated levels of JA prevent endophytic colonization of rice roots by the nitrogen-fixing *Azoarcus* sp. strain BH72 (Miché *et al*., 2006). Furthermore, our results confirm that an intact immune status of the roots is important for the establishment of a mutualistic interaction, as has been shown for fungal sebacinoid endophytes, such as *S. indica* in Arabidopsis (Lahrmann *et al*., 2015).

HvNPR1 is required for both Rr*F4-induced root-initiated systemic resistance and basal resistance to powdery mildew*

In Arabidopsis, root colonization with *Rr*F4 results in enhanced systemic resistance against *Pst* DC3000 (Glaeser *et al*., 2016). Mutational analysis showed that this systemic resistance does not require NPR1 or SA, but instead is dependent on the JA-induced ISR pathway. The unavailability of similar mutants in cereals has hampered such analyses in these important crops. To assess the requirement of HvNPR1 in root-initiated systemic resistance of a monocotyledonous plant, roots of 3-day-old KD-*hvnpr1* and WT barley seedlings were either dip-inoculated in an *Rr*F4 suspension or mock treated with buffer. The seedlings were grown for 3 weeks in the soil; leaves were then harvested and inoculated with the virulent isolate A6 of *Bgh* (*Bgh*A6). At 6 dpi with *BghA6*, the detached leaves from *Rr*F4-colonized WT plants displayed fewer fungal colonies than the leaves from mock-treated plants (Fig. 7). Thus, root colonization with *Rr*F4 initiated systemic resistance to this virulent fungal pathogen. By contrast, as after BTH treatment (see Fig. 3) comparable numbers of *Bgh*A6 colonies were observed on the detached leaves of KD-*hvnpr1* plants regardless of whether their roots were treated with *Rr*F4 or buffer. It should be noted that the leaves of buffer-treated (as well as *Rr*F4-colonized) KD-*hvnpr1* plants supported a greater number of fungal colonies than the leaves of buffer-treated WT plants.

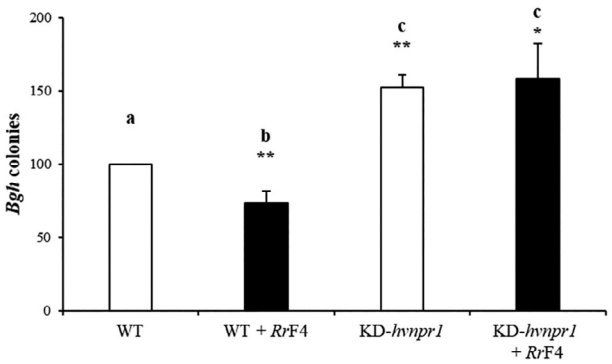

Fig 7. Knock-down (KD) of barley *HvNPR1* results in altered basal and root-initiated systemic disease resistance to the powdery mildew fungus *B. graminis* f.sp. *hordei* (*Bgh*). Number of *Bgh* colonies on detached leaves of WT and KD-hvnpr1 plants whose roots were or were not colonized by *Rr*F4. After dip-inoculating the roots of 3-day-old seedlings in an *Rr*F4 suspension (OD_{600} = 1.4) or 10 mM $MgSO_4$ $7H_2O$ buffer, plants were grown in soil for 3 weeks. Twenty-four-day-old detached third leaves were inoculated with 3–5 *Bgh* conidia/mm^{-2} and fungal colonies were counted 6 days later. The graph shows the percentage in pustules count of three independent experiments (n = 15 plants). Comparisons between groups were performed via Kruskal–Wallis test and Dunn's test of multiple comparisons. Error bars represent standard deviation. Asterisks indicate statistical difference of the group means against WT mock (*p < 0.05; **p < 0.01; ***p < 0.001). Letters represent the statistical differences among all group means (α = 0.05).

Together, these results both confirm our prior demonstration that HvNPR1 is required in the inoculated leaf for basal resistance to *Bgh*A6 (Dey *et al*., 2014), and they reveal a critical role for HvNPR1 in root-initiated systemic resistance. This latter finding extends an earlier report investigating the role of NPR1 in barley during AR (Gao *et al*., 2018). Previously, foliar inoculation of WT barley with *Pst* DC3000 was shown to induce heightened resistance in the adjacent tissue (outside of the initial infection zone) to a secondary infection by *Mo*. This *Pst* DC3000-induced AR was suppressed in the *HvNPR1* knock-down line E7L2 line but enhanced in a barley line overexpressing wheat *wNPR1* (Gao *et al*., 2018). By contrast, a different study indicated that HvNPR1 is not required for systemic immunity triggered by inoculating a lower leaf of barley plants with either *Xtc* or *Psj*. In comparison to plants that received a primary mock inoculation, the systemic leaves of KD-*hvnpr1* (line E7L2) and WT plants that received a primary inoculation with *Xtc* or *Psi* displayed a similar reduction in bacterial growth following challenge inoculation with *Xtc* (Dey *et al*., 2014). Further studies will be required to determine how the location of the primary infection (root vs. leaf) and/or the identity of the pathogen influence activation of systemic resistance via NPR1-dependent or -independent signalling pathways.

Rr*F4-induced systemic defence gene expression is compromised in KD-*hvnpr1 *plants*

Next, we investigated whether the HvNPR1-dependent systemic resistance triggered by *Rr*F4 root colonization is associated with increased defence gene expression in barley leaves. To this end, the roots of WT and KD-*hvnpr1* seedlings were dip-inoculated with *Rr*F4 or dipped into the buffer (mock). After growing the seedlings on soil for 3 weeks, leaves were detached and inoculated with *BghA6* conidia. Relative levels of *HvPR1b*, *HvPR2* and *HvPR5* expression were then determined by qRT-PCR analysis at 0, 18, 36, 48 and 72 h post-inoculation (hpi) (Fig. 8; Fig. S4). At all time points after *Bgh*A6 inoculation, expression levels of *HvPR1b*, *HvPR2* and *HvPR5* were substantially lower in the leaves of *Rr*F4-colonized KD-*hvnpr1* plants compared with comparably treated WT plants. Thus, the ability of *Rr*F4 root colonization to effectively induce systemic *PR* gene expression appears to be largely dependent on HvNPR1.

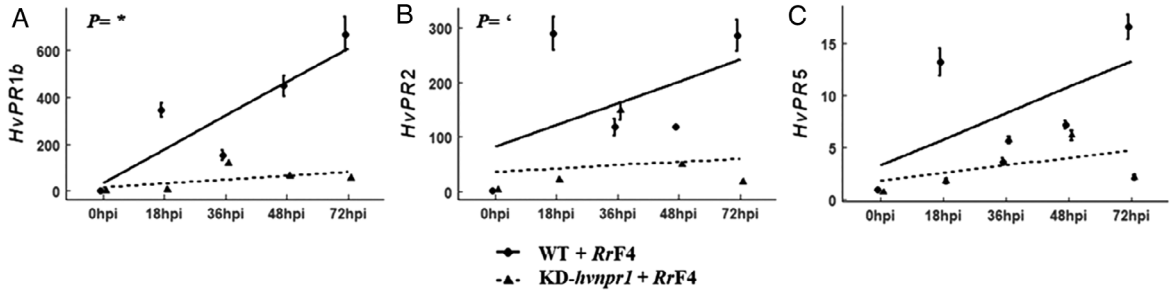

Fig 8. Scatterplots with trendlines of the relative systemic expression of defence-related genes upon *Bgh*A6 challenge inoculation of *Rr*F4-colonized WT or KD-*hvnpr1* plants. Transcripts of *HvPR1b* (A), *HvPR2* (B) and *HvPR5* (C) were assessed by qRT-PCR and normalized to barley *ubiquitin*. Roots of WT and KD-*hvnpr1* seedlings were dip-inoculated with *Rr*F4 (OD_{600} = 2). After growing the seedlings in the soil for 3 weeks, the detached youngest leaves were inoculated with 10–15 *Bgh*A6 conidia/mm^{-2} and harvested 0, 18, 36, 48 and 72 hpi. Displayed are the means of three biological repetitions (n = 4 plants). Error bars indicate standard deviation. Significant differences between the linear regression analyses were determined by one-way ANOVA ('p < 0.1, *p < 0.05).

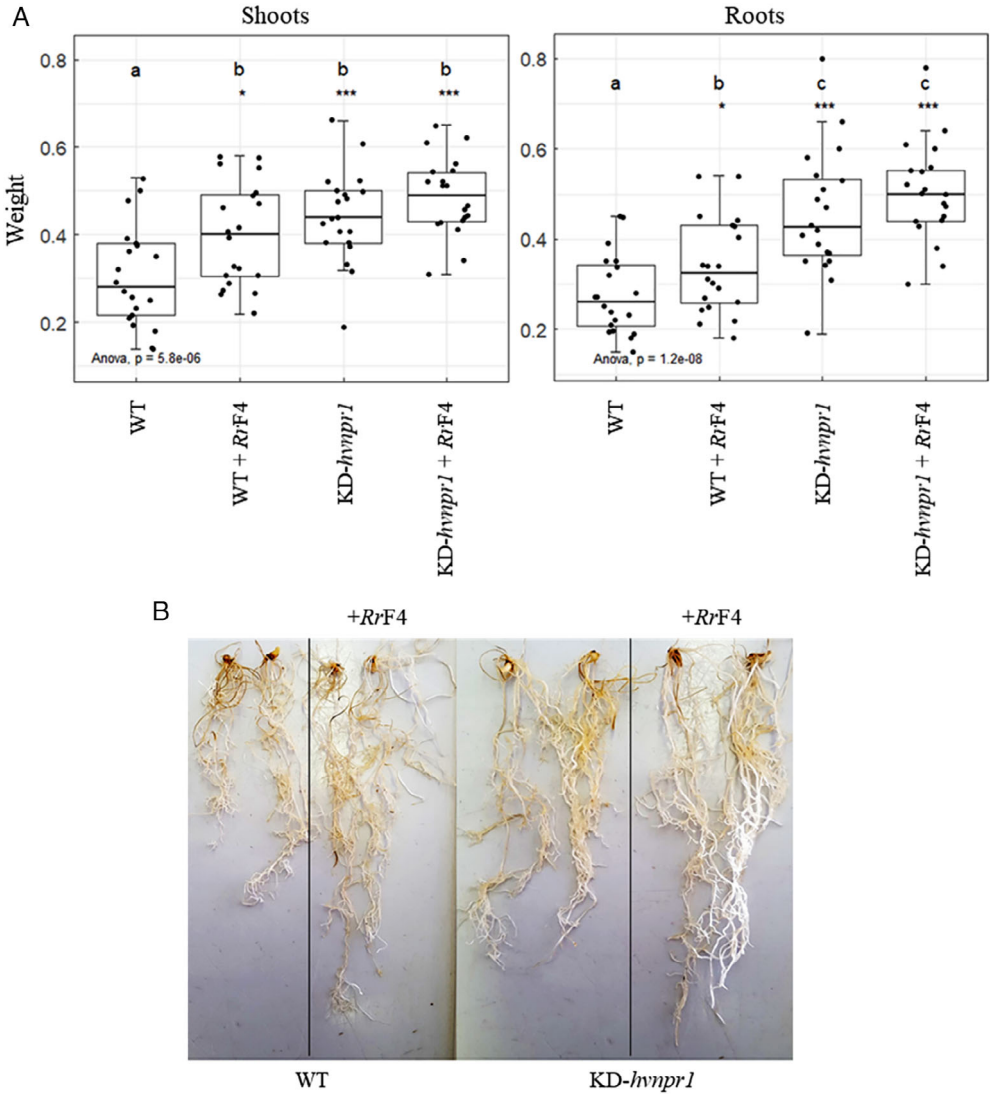

Fig 9. Root and shoot biomass of 3-week-old WT and KD-*hvnpr1* plants after colonization with *Rr*F4 was compared with non-colonized plants. Plants were cultivated in artificial soil containing 2:1 mixture of expanded clay (Seramis) and Oil-Dri in a growth chamber at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density) (A) Root and shoot fresh weight (FW) and (B) root morphology. The experiment was conducted two times ($n = 20$ plants) with similar results. Comparisons between groups were performed via one-way ANOVA and Tukey's range test. Asterisks represent statistical difference of the group means against WT mock (*$p < 0.05$; ***$p < 0.001$). Letters represent the statistical differences among all group means ($\alpha = 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

KD-hvnpr1 *plants have a higher biomass but are compromised for* Rr*F4-induced growth promotion*

From an agronomic viewpoint, it is critical to determine whether NPR1's function as a key regulator of *PR* gene expression and pathogen defence also has an impact on plant yield (Xu *et al*., 2017). Previous studies have shown that the biomass of Arabidopsis and barley plants is enhanced after root inoculation with *Rr*F4 (Sharma *et al*., 2008; Glaeser *et al*., 2016). To assess whether this response is dependent on HvNPR1, we recorded the biomasses of WT and KD-*hvnpr1* plants whose roots were inoculated with either buffer or *Rr*F4 over a growth period of 3 weeks. *Rr*F4-colonized WT plants showed a strong increase in root and shoot fresh weight (FW) compared with mock-treated WT plants, corroborating the findings of Sharma *et al*. (2008). Strikingly, the root and shoot FWs of mock-inoculated KD-*hvnpr1* plants were significantly higher (Tukey's range test $p < 0.001$) than those of either mock- or *Rr*F4-inoculated WT plants (Fig. 9; Fig. S5). In comparison to WT plants, however, the FW of *Rr*F4-colonized KD-*hvnpr1* plants showed only a slight, statistically insignificant increase over that of mock-treated KD-*hvnpr1* plants. To further substantiate the hypothesis that NPR1 is required for plant fitness and

growth, we also recorded the root and shoot biomasses of KD-*hvnpr1* E11L9, which shows a weaker (53%) silencing effect. Both root and shoot FWs were significantly higher compared with WT plants (Fig. S6), suggesting a negative correlation between *HvNPR1* transcript levels and growth promotion. That plants with reduced *HvNPR1* expression display better fitness in terms of root and shoot growth is consistent with the hypothesis that a weakened immune system results in a stronger growth phenotype (Heil and Baldwin, 2002; Abreu and Munné-Bosch, 2009; Yang *et al*., 2012; Huot *et al*., 2014).

Conclusion

The results presented here suggest that *HvNPR1* plays a vital role in the establishment of a mutualistic symbiosis. Following *Rr*F4 inoculation, the roots of KD-*hvnpr1* plants displayed a different spatiotemporal colonization pattern than the roots of WT plants, and they supported substantially fewer bacterial cells. The reduced multiplication of *Rr*F4 in KD-*hvnpr1* roots was associated with reduced local and systemic expression of several SA marker genes, including *HvPR1b*, *HvPR2* and/or *HvPR5*, while local expression of the JA marker *HvJMT* was either comparable to or higher than that detected in *Rr*F4-inoculated WT plants. Based on these findings, we hypothesize that *Rr*F4-mediated activation of the SA signalling pathway may help to downregulate the JA pathway, thereby enhancing the colonization of barley roots. In addition, *KD-hvnpr1* plants were compromised for *Rr*F4-induced root-initiated systemic resistance to *Bgh*A6. Together, these findings suggest that HvNPR1 plays important roles in both modulating the tissue-specific capacity for successful *Rr*F4 colonization, as well as transducing the signal for *Rr*F4-induced immune responses in barley. Finally, HvNPR1 function negatively interferes with the growth of barley roots and shoots, however, reinforces *Rr*F4-induced growth responses.

Experimental procedures

Plant material and inoculation with Bgh

Seeds of spring barley (*H. vulgare*) cv. Golden Promise (GP) and GP-derived KD-*hvnpr1*-E7L2 plants were surface sterilized and grown under sterile conditions for 3 days (Glaeser *et al*., 2016). The generation of KD-*hvnpr1*-E7L2 plants is described in Dey *et al*. (2014). A conserved domain of HvNPR1 (aa 204–333) was used to generate hairpin RNA constructs for RNAi-mediated silencing of *HvNPR1*. Seeds were germinated on sterile filter paper for 3 days at 22°C/18°C (day/night cycle) and roots were dipped in *Rr*F4 suspension buffer (OD_{600} = 1.4–2) or just in suspension buffer (10 mM $MgSO_4$ $7H_2O$) for 2–3 h. Subsequently, the seedlings were transferred, depending on the experiment, to pots (⌀12 cm) containing soil (Fruhstorfer Erde Typ T) or alternatively in 2.5-L glass jars on 1/2 MS medium (150 ml tot. vol.). Plants were cultivated then in a growth chamber at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). Plants in soil were fertilized weekly with 0.1% WUXAL top N solution (N/P/K: 12/4/6; Aglukon, Düsseldorf, Germany). The detached leaf assay was done with the third leaves of 3-week-old plants. Leaf segments were laid on 1% (wt./vol.) water agar and inoculated with fresh conidia of *Blumeria graminis* f.sp. *hordei* (*Bgh*) race A6 as described in Dey *et al*. (2014). For the root defence-gene analysis, after plants were moved in ½ MS medium, at 0, 2, 4 and 6 dpi roots were harvested, crushed in liquid nitrogen with the help of a mortar and pestle and extracted DNA/RNA analysed via qPCR.

BTH treatment

Barley plants were grown in 200 g capacity pots in soil (Fruhstorfer Erde, Vechta, Germany) under controlled condition 16 h light (240 μmol m^{-2} s^{-1} photon flux density) and 60% relative humidity (22/18°C day/night cycle). Ten milliliters of 20 ppm BTH (CGA245704, Bion®, Novartis, Basel, Switzerland) formulated as 50% active ingredient with wettable powder (WP) in water was applied to 5-day-old seedlings as a soil drench. Control plants were treated with WP. Two days after BTH treatment, first leaf segments were placed on 0.5% (wt./vol.) water agar containing 20 mg L^{-1} benzimidazole (Merck-Schuchardt, Munich, Germany) and inoculated with *Bgh*A6 (5 conidia/mm^2 density) by air current dispersion in an inoculation tower and saved in the same climate chamber for 7 days. *Bgh* colonies were counted using a binocular on a 2.5 cm^2 segment. Comparisons between groups were performed via ANOVA + Tukey with a 95% family-wise confidence level.

Inoculation of roots with Rr*F4, genomic DNA isolation and qRT-PCR*

Bacteria culturing, root inoculation and DNA extraction were performed as described in Glaeser *et al*. (2016). Briefly, the Alphaproteobacterium *R. radiobacter* F4 (*Rr*F4; syn. *Agrobacterium fabrum*, syn. *Agrobacterium tumefaciens*) originally isolated from the beneficial fungus *P. indica* DSM 11827 (Sharma *et al*., 2008; Glaeser *et al*., 2016) was grown overnight in modified LB broth (1% casamino hydrolysate, 0.5% yeast extract and 5% NaCl, pH 7.0, supplemented with 100 μg ml^{-1}

gentamicin) at 28°C and 150 r.p.m. GUS-expressing *Rr*F4 was cultured in the presence of 100 μg ml^{-1} spectinomycin. Bacterial cells were collected by centrifugation (3202*g*, 10 min), washed and resuspended in a 10 mM $MgSO_4$ $7H_2O$ solution. Roots of 3-day-old barley seedlings were dip-inoculated for 2–3 h in *Rr*F4 suspensions (OD_{600} = 1.4–2). Control seedlings were dipped into 10 mM $MgSO_4$ $7H_2O$. RNA extraction, qRT-PCR with specific oligonucleotides (Supplemental Table S1) was performed as described (Imani *et al*., 2011). Relative DNA or transcript levels were determined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Protein structure comparison and phylogenetic analysis

Protein sequences of NPRs from selected crop species were used for the protein structure and phylogenetic analysis. Visualization and comparison of the different NPRs domains were done via the online-tool CDD/SPARCLE (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, Marchler-Bauer *et al*., 2016). Multiple sequence alignments were carried out using the MUSCLE algorithm (Edgar, 2004). The phylogenetic tree was built using the maximum likelihood statistical method based on the WAG protein substitution model (Whelan and Goldman, 2001). Tree nodes accuracy was tested via the bootstrap method with 1000 bootstrap replicates. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software (MEGA X version 10.0.5, Kumar *et al*., 2018). Exon-intron distribution analysis was carried out via the online-tool gene extraction display server ($GSDS_{2.0}$, http://gsds.cbi.pku.edu.cn, Hu *et al*., 2015). Corresponding coding and genomic sequences were obtained from the JGI Phytozome 12.1.6 Plant Comparative Genomics portal (https://phytozome.jgi.doe.gov/pz/portal.html).

Microscopy

Visualization of root colonization by Rr*F4.* The colonization of plant roots was visualized using GUS-expressing *Rr*F4 strains combined with light- and epifluorescence microscopy. Root cross-sections also were analysed by TEM according to methods described in Glaeser *et al*. (2016) (see also Supplementary Materials and Methods).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

Fig. S1. Domain and genomic analysis of the various HvNPR1-like family members with their homologues in *Oryza sativa*, *Brachypodium distachyon* and *Arabidopsis thaliana*. (a-d) Domain structure comparison via the online-tool CDD/SPARCLE (Marchler-Bauer *et al*., 2016). (e) Comparison of the predicted exon-intron frequency in the genomic sequences. Exons are displayed as yellow boxes while introns as straight black lines.

Fig. S2. Blast alignment of *Hvnpr1*_RNAi (Dey *et al*., 2014) against *HvNPR1* and RNAi off-targets prediction analysis. (a) Blast analysis of the RNAi construct was conducted by EMBOSS Needle (Madeira *et al*., 2019; https://www.ebi.ac.uk/Tools/psa/emboss_needle). (b) Off-targets simulations were run using SiFi software (v1.2.3), program designed for RNAi off-target analysis and silencing efficiency predictions (Lueck, 2017; http://labtools.ipk-gatersleben.de). siRNA hits were found only against *HvNPR1* sequence, while no off-targets hits were found in the other *HvNPR* genes.

Fig. S3. *Rr*F4 colonization pattern and strength in WT and KD-*hvnpr1* roots. Primary root segments colonized by *GUS*-expressing *Rr*F4 at 2 dpi, 4 dpi and 10 dpi. The number of bacteria was reduced in roots of KD-*hvnpr1* as compared to WT plants (methods see Fig. 3).

Fig. S4. Scatterplots with trendlines of the relative systemic expression of defence-related genes upon *Bgh* inoculation in non-colonized WT vs. KD-*hvnpr1* barley. Transcripts of *HvPR1b* (a), *HvPR2* (b), and *HvPR5* (c) were assessed by qRT-PCR and normalized to barley *ubiquitin*. After growing the seedlings in soil for three weeks, the detached youngest leaves were inoculated with 10 to 15 *Bgh* conidia per mm^{-2} and harvested 0, 18, 36, 48, and 72 hpi. Displayed are means of three biological repetitions ($n = 4$ plants). Error bars indicate standard deviation. Significant differences between the linear regression analyses were determined by one-way ANOVA.

Fig. S5. Phenotypic analysis of WT and KD-*hvnpr1* barley cv. Golden Promise seedlings grown for 10 days in artificial soil containing 2:1 mixture of expanded clay and Oil-Dri® in a growth chamber at 22°C/18°C (day/night cycle) with 60%

relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). Plants were fertilized one time with 0.1% WUXAL top N solution (N/P/K: 12/4/6; Aglukon, Düsseldorf, Germany).

Fig. S6. Plant root and shoot biomass of three-week-old barley cv. Golden Promise WT and two KD-*hvnpr1* mutant lines (Dey *et al*., 2014). The results were obtained using the T3 (E11L9) and T5 (E7L2) generation of transgenic plants. Plants were cultivated in artificial soil containing 2:1 mixture of expanded clay (Seramis®, Masterfoods, Verden, Germany) and Oil-Dri® (Damolin, Mettmann, Germany) in a growth chamber at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). The experiment was conducted two times (n = 15 plants) with similar results. Comparisons between groups was performed via One-way Anova and Tukey's Range Test. Asterisks represent statistical difference of the group means against WT mock (**p < 0.01). Letters represent statistical difference among all group means (α = 0.05).

Table S1. List of primers used in the study.