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 symbiosis. 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.

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 effectortriggered 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 signaling 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)
CAARLS 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, tramtrack, 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 VLEESCHAUWER 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 (DESPRS 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 (BESER 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 uninected 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* (RrF4) and the cereal plant barley. The beneficial bacterium RrF4 was originally isolated from the sebacoinid basidiomycete fungus *Serendipita indica* (Weiβ 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). RrF4 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*, RrF4 colonizes plant roots without host preference and forms aggregates of attached cells and dense biofilms at the root surface of maturation zones. RrF4-colonized plants show increased biomass and systemically enhanced resistance against the powdery mildew fungus *B. graminis* t.s. *hordei* (Bgh) in barley and bacterial leaf pathogens such as *Pst* DC3000 in *Arabidopsis* and *X. translucens* pv. *translucens* (Xtf) 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. 1A; 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. 1B). 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. 1C; 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, Zimbabwe), which contains the sequence for the barley cDNA HvNPR2 (BAJ86173.1) and HvNPR3 (BAJ90272.1). The sequence similarity among all three HvNPR2 and HvNPR3 homologues is high, as well as the sequence similarity between HvNPR2 and HvNPR3 and those of *Arabidopsis*, *Medicago*, and *Rhizobium* (Fig. 1B). This analysis indicates that HvNPR2 and HvNPR3 are highly conserved among various plant species and are close homologues of each other.
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://gds.cbi.pku.edu.cn), further confirming the placement of the barley NPR1-like proteins in the various phylogenetic clades (Fig. S1e).

**HvNPR1 modulates colonization of barley roots by RrF4**

To assess the possibility that HvNPR1 plays a role in establishing a mutualistic symbiosis between the beneficial bacterium RrF4 and barley, we monitored root colonization in RrF4-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 β-glucuronidase (GUS)-expressing RrF4 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 β-glucuronidase 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 RrF4. To further investigate this possibility, WT and KD-hvnpr1 plants were inoculated with RrF4 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 RrF4 ITS, the roots of both KD-hvnpr1 lines E7L2 and E11L9 supported substantially lower levels of RrF4 than those of WT plants (Fig. 4e). We extended our analysis by transmission electron microscopy (TEM) to understand the
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, RrF4 cells had already penetrated into the WT roots. Significantly, and in accordance with Glaeser et al. (2016), RrF4 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 RrF4.

RrF4-induced expression of SA but not JA marker genes is compromised in KD-hvnpr1 roots

Whether RrF4 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 RrF4 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 RrF4-colonized WT roots compared with mock-treated roots (Fig. 6A and B). In contrast, RrF4 colonization did not enhance the expression of either PR gene in KD-hvnpr1 roots at 2 or 4 dpi, although a small induction

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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 BghA6 and at 6 dpi colonies were counted. BghA6 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).
NPR1 contributes to mutualism

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 RrF4 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-hvnp1 roots after RrF4 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 RrF4 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 4. RrF4 colonization pattern and strength in barley cv. Golden Promise WT and KD-hvnp1 roots. Root segments colonized by GUS-expressing RrF4 at 5 dpi in WT (A, B) and KD-hvnp1 E7L2 (C, D) plants. (E) Relative qPCR analysis of the quantity of RrF4 cells in roots of 3-day-old plants at 5 dpi using primers specific for barley Ubiquitin and RrF4 ITS. The number of bacteria was significantly reduced in both roots of KD-hvnp1 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]

Fig 5. TEM analysis of the colonization pattern of RrF4 in WT vs. KD-hvnp1 barley roots. Three-day-old seedlings were dip-inoculated for 30 min into bacterial suspensions (OD600 = 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-hvnp1 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]

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Our data are consistent with a report showing that elevated levels of JA prevent endophytic colonization of rice roots by the nitrogen-fixing Azotobacter 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 sebacinoïd endophytes, such as S. indica in Arabidopsis (Lahrmann et al., 2015).

HvNPR1 is required for both RrF4-induced root-initiated systemic resistance and basal resistance to powdery mildew

In Arabidopsis, root colonization with RrF4 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 seedlings were dip-inoculated with RrF4 (OD = 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).

Fig 6. Relative expression of immune-related genes in the roots of KD-hvnpr1 vs. WT barley in the presence or absence of RrF4. 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 RrF4 (OD = 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).
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$_2$O 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 ($\alpha$ = 0.05).

Together, these results both confirm our prior demonstration that HvNPR1 is required in the inoculated leaf for basal resistance to _BghA6_ (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 _Psj_ 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 signaling pathways.

**Rf4-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 _BghA6_ 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 _PR_ gene expression upon _BghA6_ 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 _BghA6_ 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).
**KD-hvnpr1 plants have a higher biomass but are compromised for RrF4-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 RrF4 (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 RrF4 over a growth period of 3 weeks. RrF4-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 RrF4-inoculated WT plants (Fig. 9; Fig. S5). In comparison to WT plants, however, the FW of RrF4-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

**Fig 9.** Root and shoot biomass of 3-week-old WT and KD-hvnpr1 plants after colonization with RrF4 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 ($α = 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]
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 RrF4 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 RrF4 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 RrF4-inoculated WT plants. Based on these findings, we hypothesize that RrF4-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 RrF4-induced root-initiated systemic resistance to BghA6. Together, these findings suggest that HvNPR1 plays important roles in both modulating the tissue-specific capacity for successful RrF4 colonization, as well as transducing the signal for RrF4-induced immune responses in barley. Finally, HvNPR1 function negatively interferes with the growth of barley roots and shoots, however, reinforces RrF4-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 RrF4 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 (Ø12 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 1/2 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} benimidazole (Merck-Schuchardt, Munich, Germany) and inoculated with BghA6 (5 conidia/mm² 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 cm² segment. Comparisons between groups were performed via ANOVA + Tukey with a 95% family-wise confidence level.
gentamicin) at 28°C and 150 r.p.m. GUS-expressing RfF4 was cultured in the presence of 100 μg ml⁻¹ spectinomycin. Bacterial cells were collected by centrifugation (3200g, 10 min), washed and resuspended in a 10 mM MgSO₄ 7H₂O solution. Roots of 3-day-old barley seedlings were dip-inoculated for 2–3 h in RfF4 suspensions (OD₆₀₀ = 1.4–2). Control seedlings were dipped into 10 mM MgSO₄ 7H₂O. 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^−ΔΔCT 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). The phylogenetic tree was used for the protein structure and phylogenetic analyses. 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). The phylogenetic tree was used for the protein structure and phylogenetic analyses.

**Microscopy**

Visualization of root colonization by RfF4. The colonization of plant roots was visualized using GUS-expressing RfF4 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.** *Rf4* colonization pattern and strength in WT and *Kd-hvpr1* roots. Primary root segments colonized by *GUS*-expressing *Rf4* at 2 dpi, 4 dpi and 10 dpi. The number of bacteria was reduced in roots of *Kd-hvpr1* as compared to WT plants (methods see Fig. 3).

**Fig. S4.** Scatterplot with trends on the relative systemic expression of defence-related genes upon *Bgh* inoculation in non-colonized WT vs. *Kd-hvpr1* 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 replications (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-hvpr1* 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 bar-
ley 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% rel-
ative 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 differ-
ence of the group means against WT mock (**$p < 0.01$). Let-
ters represent statistical difference among all group
means ($\alpha = 0.05$).

**Table S1.** List of primers used in the study.