

ORIGINAL ARTICLE

The barley HvSTP13GR mutant triggers resistance against biotrophic fungi

Caroline Ines Skoppek¹ | Wilko Punt¹ | Marleen Heinrichs¹ | Frank Ordon² |
Gwendolin Wehner² | Jens Boch¹ | Jana Streubel¹ 

¹Department of Plant Biotechnology,
Institute of Plant Genetics, Leibniz
Universität Hannover, Hanover, Germany

²Institute for Resistance Research and Stress
Tolerance, Julius Kühn Institute – Federal
Research Centre for Cultivated Plants,
Quedlinburg, Germany

Correspondence

Jana Streubel, Department of Plant
Biotechnology, Institute of Plant Genetics,
Leibniz Universität Hannover, Hanover,
Germany.

Email: jana.streubel@genetik.uni-hannover.
de

Present address

Wilko Punt, Institute for Plant Sciences,
University of Cologne, Cologne, Germany

Marleen Heinrichs, Department of Cellular
Biochemistry, University Medical Center
Göttingen, Göttingen, Germany

Abstract

High-yielding and stress-resistant crops are essential to ensure future food supply. Barley is an important crop to feed livestock and to produce malt, but the annual yield is threatened by pathogen infections. Pathogens can trigger an altered sugar partitioning in the host plant, which possibly leads to an advantage for the pathogen. Hampering these processes represents a promising strategy to potentially increase resistance. We analysed the response of the barley monosaccharide transporter *HvSTP13* towards biotic stress and its potential use for plant protection. The expression of *HvSTP13* increased on bacterial and fungal pathogen-associated molecular pattern (PAMP) application, suggesting a PAMP-triggered signalling that converged on the transcriptional induction of the gene. Promoter studies indicate a region that is probably targeted by transcription factors downstream of PAMP-triggered immunity pathways. We confirmed that the nonfunctional *HvSTP13GR* variant confers resistance against an economically relevant biotrophic rust fungus in barley. Our experimental setup provides basal prerequisites to further decode the role of *HvSTP13* in response to biological stress. Moreover, in line with other studies, our experiments indicate that the alteration of sugar partitioning pathways, in a host–pathogen interaction, is a promising approach to achieve broad and durable resistance in plants.

KEYWORDS

barley, fungi, *HvSTP13*, PAMP, resistance, sugar

1 | INTRODUCTION

Barley is not only one of the major crops to feed livestock and an essential ingredient of beer, it has also gained a growing importance in food production. However, infection with fungal pathogens is one of the most devastating threats to annual yields, causing severe damage to various organs (Liu et al., 2011; Park et al., 2015; Wolfe & McDermott, 2003). To ensure sustainable and high-yield crop

production, an ongoing improvement towards resistant varieties is the most important challenge for research and plant breeding.

An ecologically sustainable method of crop protection is the identification of, and breeding with, naturally occurring resistance genes that originate in related wild varieties or landraces (Dinh et al., 2020). However, many resistance genes are mainly effective against certain races of one fungal species or have already been overcome (Clifford, 1985; König et al., 2012; Niks et al., 2000). The

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identification of new resistance mechanisms is therefore a major future challenge. One promising approach is to eliminate the fundamental requirements for a successful pathogen infection. The availability of sugars to cover their energy demand is a major need for plant pathogens (Bezruczyk et al., 2018). Thus, uncovering the routes of nutrient utilization by fungal pathogens and developing methods that block feeding could represent a highly promising strategy to engineer durable and broad-spectrum resistance.

Sugars are the basal energy source for living organisms and their acquisition and partitioning are key steps to conduct physiological processes. In plants, sugars are produced in photosynthetically active source tissues and distributed to supply energy to consuming sink tissues. Specialized sugar transporters are crucial players in these delivery pathways and belong to three major groups: the SWEETs (Sugars Will Eventually be Exported Transporters), SUTs (sucrose H⁺ co-transporters) and STPs (sugar transport proteins). Members of the SWEET family typically consist of seven transmembrane (TM) domains and transport a variety of substrates. SUTs and STPs belong to the major facilitator superfamily (MFS) with typically 12 TM domains (Büttner, 2010; Julius et al., 2017). Sucrose-exporting SWEETs and SUTs control the key step of phloem loading and long-distance transport. STPs, on the other hand, transport monosaccharides, like glucose or fructose, over short distances, thereby providing ready-to-use sugars to feed energy-consuming cells and cell compartments (Büttner, 2010; Eom et al., 2015; Julius et al., 2017).

As the low sugar content in the plant apoplast might constrain their growth, pathogens utilize the sugar reservoirs of their host to convert the infection area into an artificial sink (Pommerrenig et al., 2020). SWEETs are well-known targets that are exploited by diverse fungal and bacterial pathogens, for example *Botrytis cinerea*, *Blumeria graminis*, *Ustilago maydis*, *Pseudomonas syringae*, and various *Xanthomonas* species (Breia et al., 2020; Chen et al., 2010; Doidy et al., 2012; Sosso et al., 2019; Timilsina et al., 2020). In response, plants can activate the expression or enhance the transport activity of SUTs or STPs to counter the pathogen-induced leakage of sugars (Bezruczyk et al., 2018; Pommerrenig et al., 2020).

One of the most prominent STPs involved in plant-pathogen interactions is STP13, but its role in the outcome of the respective interaction seems a double-edged sword. On the one hand, increased STP13 activity supports basal resistance, as shown for the interaction of the necrotrophic fungus *B. cinerea* with *Arabidopsis thaliana*. Hence, it was hypothesized that increased *AtSTP13* expression might remove glucose from the infection site to slow down the infection process (Lemonnier et al., 2014). Notably, *AtSTP13* is also involved in interactions with bacterial pathogens. On detection of the pathogen-associated molecular pattern (PAMP) flg22 by the plant receptor FLS2, *AtSTP13* is expressed and phosphorylated by BAK1 (Yamada et al., 2016). This modification enhances the sugar uptake of the transporter, possibly causing it to compete with the pathogen for sugar availability in the apoplast.

In contrast, an overexpression of the glucose transporter TaSTP13 supports the virulence of biotrophic fungi in wheat and *Arabidopsis* whereas silencing of *TaSTP13* in wheat increases resistance (Huai

et al., 2020). Because biotrophic fungi feed from living cells, an increased STP13 activity might enhance the glucose availability in the plant cytoplasm and thereby feed the pathogens via their haustoria. In line with this assumption is the observation that the glucose transport-deficient TaLr67res (G144 to R144 exchange, hereafter named TaSTP13GR) variant from wheat increases the resistance of wheat and *TaSTP13GR*-transgenic barley against the biotrophic fungi *Puccinia hordei* and *B. graminis* (Milne et al., 2019; Moore et al., 2015). Moreover, recently it was shown that a nonfunctional MtSTP13 G to R variant also enhances the resistance of *Medicago truncatula* to the biotrophic powdery mildew fungus *Erysiphe pisi* (Gupta et al., 2021). This suggests that an impeded glucose import from the plant apoplast to the cytoplasm might starve the fungal haustoria, thus preventing progression of a severe infection.

The barley ortholog *HvSTP13* harbouring the G144 to R144 mutation (hereafter named *HvSTP13GR*) also lacks glucose transport activity but whether this mutant can confer resistance against biotrophic pathogens in barley has not been analysed, as was observed for TaSTP13GR (Milne et al., 2019). If that were the case, it would open the way for barley plants that are resistant against a broad spectrum of biotrophic fungi.

In this study we analysed the expression pattern of the barley hexose transporter *HvSTP13* during various biotic stresses. We observed that *HvSTP13* was induced in response to several fungal and bacterial pathogens as a result of the recognition of certain PAMPs. By using β -glucuronidase (GUS) reporter studies, we dissected the *HvSTP13* promoter and identified a region that is probably responsible for the induced expression. With transgenic plants we analysed the effect of the *HvSTP13GR* mutant and found that it confers resistance against an economically relevant biotrophic rust fungus. In summary, our study contributes new insights into the molecular trigger for *HvSTP13* expression and its potential to confer broad-spectrum resistance against biotrophic pathogens.

2 | RESULTS

2.1 | The expression of *HvSTP13* is induced by fungal and bacterial PAMPs

In barley, *HvSTP13* is induced on treatment with the leaf rust fungus *P. hordei* (Ph) and the powdery mildew fungus *B. graminis* pv. *hordei* (Bgh) (Milne et al., 2019). To further dissect the *HvSTP13* expression pattern under diverse biotic pressures, we infected barley seedlings with the biotrophic fungi Ph, Bgh, and *Puccinia striiformis* f. sp. *hordei* (Psh), or with the necrotrophic fungus *Pyrenophora teres* pv. *teres* (Ptt). Leaf samples were harvested at 24, 48, and 72 hr postinoculation (hpi) and total RNA was extracted for reverse transcription quantitative PCR (RT-qPCR). The infection was confirmed by analysing the expression of a plant chitinase (*HvPR3*) that is known to respond to fungal infection (Figure S1; Ali et al., 2018). In contrast to mock-inoculated plants, all four fungal pathogens elicited an increase in *HvSTP13* expression between 24 and 72 hpi (Figure 1a). To analyse whether *HvSTP13* is also induced on bacterial

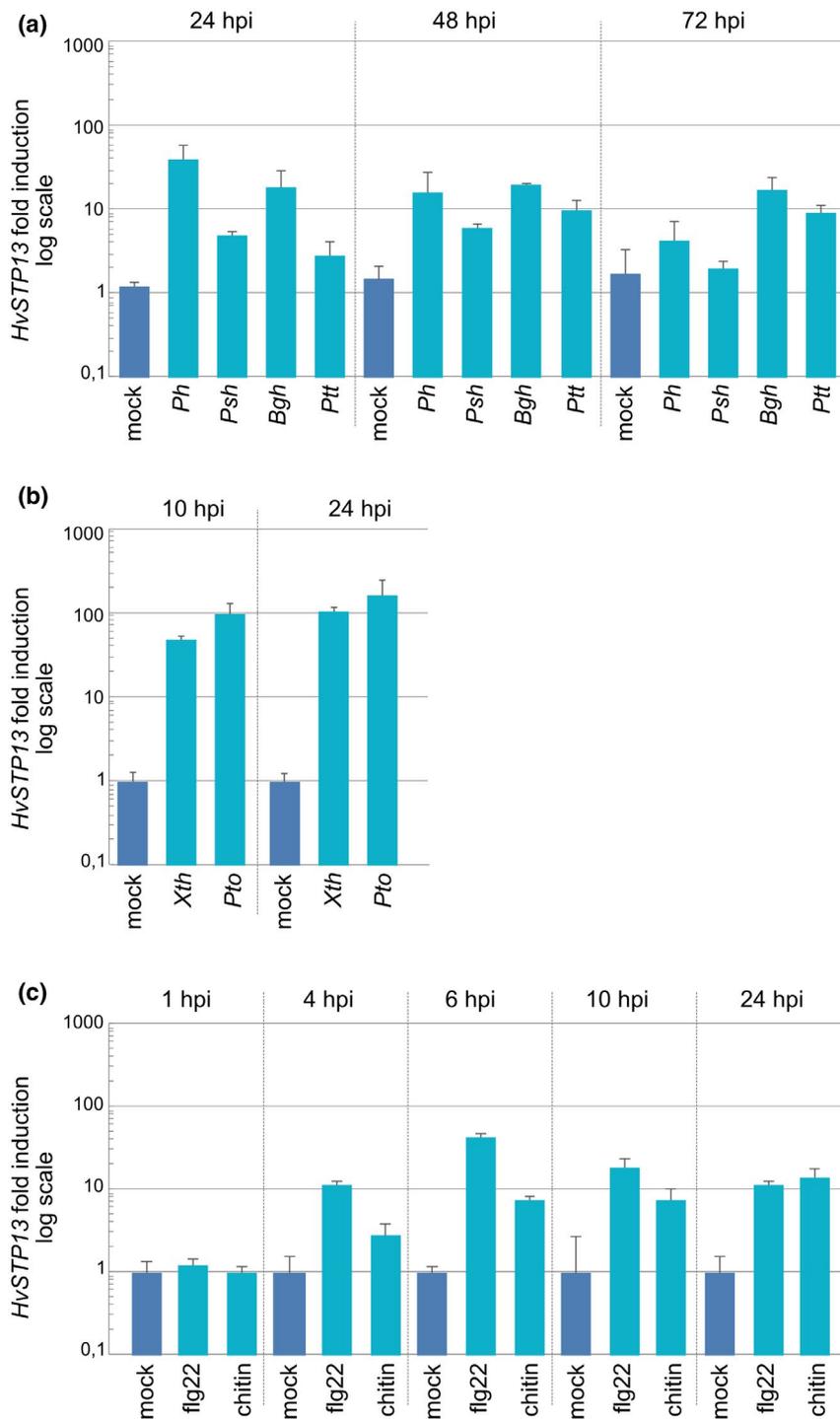


FIGURE 1 Transcript level of *HvSTP13* after treatment with pathogens or pathogen-associated molecular patterns (PAMPs). Barley plants were inoculated with (a) *Puccinia hordei* (Ph), *Puccinia striiformis* f. sp. *hordei* (Psh), *Blumeria graminis* pv. *hordei* (Bgh), and *Pyrenophora teres* pv. *teres* (Ptt), (b) *Xanthomonas translucens* pv. *hordei* UPB820 (Xth) and *Pseudomonas syringae* pv. *tomato* DC3000 (Pto), or (c) the PAMPs flg22 and chitin. (a–c) Each bar represents three biological replicates. Mock, inoculation medium; hpi, hours postinoculation. Error bars represent the standard deviation

treatment, we inoculated the host pathogen *Xanthomonas translucens* pv. *hordei* UPB820 (Xth) as well as the nonhost pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pto) into barley and found an increase of *HvSTP13* expression for both bacterial pathogens (Figure 1b). A detailed time curve, performed with Xth, indicated that *HvSTP13* induction started between 1 and 4 hpi (Figure S2a). Next, we analysed the expression of *HvSTP13* in barley after inoculation with the PAMPs chitin, flg22, β -1,3-glucan, and laminarin. An increase of *HvSTP13* transcript was detected with flg22 and chitin treatment not later than 4 hpi (Figure 1c). In contrast, laminarin and β -1,3-glucan did not activate the expression of *HvSTP13* (Figure S2b). These results confirm not only that *HvSTP13* is

induced as a consequence of fungal and bacterial PAMP-signalling, but also suggest a potential role in PAMP-triggered immunity (PTI)-related processes.

2.2 | The promoter of *HvSTP13* harbours PAMP-responsive elements

To identify elements that might be required for the pathogen-mediated transcriptional activation, we used PLACE and PlantCARE to predict *cis*-regulatory elements (CREs) in an approximately 2 kb

region of the *HvSTP13* promoter (Higo et al., 1998; Lescot et al., 2002). In accordance with the response of the *STP13* expression to multifaceted biotic and abiotic factors, the in silico analysis predicted a variety of different CREs within the *HvSTP13* promoter region (Tables S1 and S2). Abscisic acid (ABA) has been shown to activate the expression of *STP13* in *Arabidopsis*, barley, wheat, grapevine, and *M. truncatula* (Gupta et al., 2021; Hayes et al., 2010; Huai et al., 2020; Milne et al., 2019; Yamada et al., 2011). Consistent with this, the prediction revealed the presence of several ABA-responsive elements (ABREs) in the *HvSTP13* promoter. Additionally, the in silico prediction identified several promising binding sites for WRKY transcription factors (W-box) and one sugar-responsive element (SURE), two CRE types that are involved in immunity and sugar signalling pathways (Tables S1 and S2, and Figure S3a; Chen et al., 2019; Sun et al., 2003).

To analyse which promoter regions are required for the PAMP-triggered induction of *HvSTP13*, we amplified four truncated *HvSTP13* promoter variants that lack certain CREs. We labelled them according to the number of nucleotides upstream of the transcription start site (TSS). This results in promoter fragments p-887, p-445, p-222, and p-93. All used promoter fragments additionally included the 301 bp of the 5' untranslated region (UTR) (Figures 2a and S3a). The promoter variants were fused to a promoterless β -glucuronidase (*GUS*) reporter gene and transformed into barley embryos

to generate transgenic *GUS* reporter lines. For each construct, three independent T_1 lines, with six plants each, were analysed. The presence of the transgene was verified by PCR. Negative siblings (indicated with -), as well as wild-type Golden Promise plants, were used as negative controls. Leaf discs were infiltrated with flg22, chitin, or $MgCl_2$. The resulting *GUS* activity was analysed after 24 h. The two fragments p-887 and p-445 displayed an increase in *GUS* activity after treatment with flg22 and chitin, indicating an induction after PAMP signalling. For the two shorter fragments, p-222 and p-93, no difference in *GUS* activity was observed between transgenic and nontransgenic siblings after flg22 or chitin treatment (Figure 2b). This indicates that the region between -445 and -222 bp upstream of the TSS possibly contains important *cis*-regulatory elements, which could be bound by regulatory transcription factors and thus allow for PAMP-mediated induction.

2.3 | The glucose transport-deficient *HvSTP13GR* mutant is localized in the plasma membrane in *N. benthamiana*

The *HvSTP13* wild type and the *HvSTP13GR* mutant were cloned into yeast expression vectors and transformed into the yeast strain EBY.

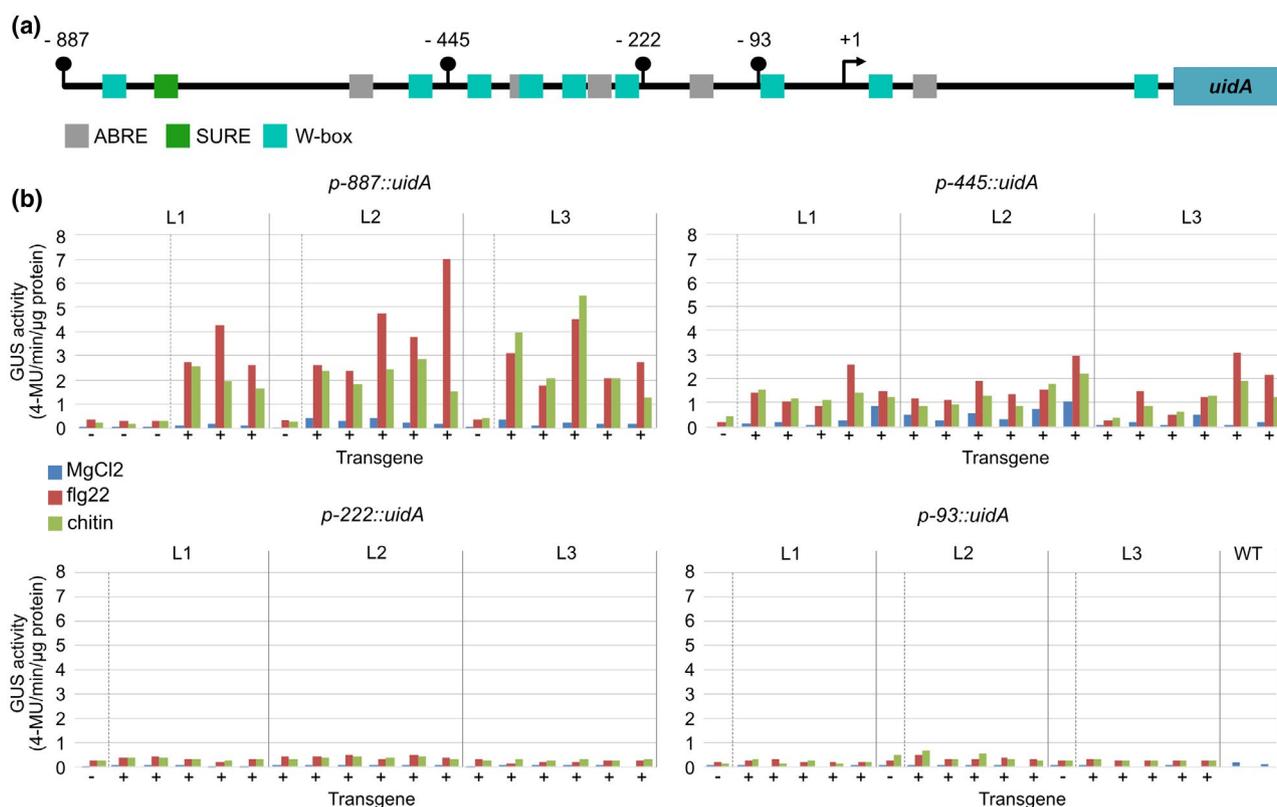


FIGURE 2 Analysis of the pathogen-associated molecular pattern (PAMP)-triggered activation of truncated *HvSTP13* promoter fragments. (a) Schematic overview of the truncated *pHvSTP13* fragments. The black arrow marks the transcription start site (+1) as annotated in Phytozome. The length of each truncated promoter fragment is marked by a black dot. Predicted ABA-responsive elements (ABREs) (grey), sugar-responsive elements (SUREs) (green) or W-boxes (turquoise) are indicated. The truncated promoter fragments were fused to the *uidA* (β -glucuronidase, *GUS*) reporter gene. (b) For each reporter construct six individual plants of three T_1 lines (L1, L2, L3) were used. For each plant the presence of the transgene was analysed by PCR (- no transgene, + transgenic). Leaf discs of each plant were vacuum infiltrated with PAMPs or $MgCl_2$. *GUS* activity was measured 24 h postinoculation. Each bar represents the mean of the technical replicates

VW4000 to confirm the reported glucose transport-deficiency for the *HvSTP13GR* mutant (Milne et al., 2019; Wieczorke et al., 1999). The strain EBYVW.4000 lacks 18 hexose and 3 additional transporter genes and does not grow on glucose, fructose, galactose, or mannose as carbon source. The growth of EBY.VW4000 on maltose is not affected. Thus, EBY.VW4000 is frequently used to analyse the hexose transport function of heterologous proteins. As positive control we included the yeast hexose transporter *Hxt1*, which is known to restore growth of EBY.VW4000 on glucose-containing medium. *eGFP* (enhanced green fluorescent protein) served as negative control (Wieczorke et al., 1999). The resulting yeast strains were spotted on selective medium containing either glucose or maltose as carbon source. On maltose-containing medium, all strains showed comparable growth, thus providing a growth control (Figure S4). However, on glucose-containing medium, only the heterologous expression of the wild-type *HvSTP13* transporter and the positive control *Hxt1* conferred growth. In contrast, neither *HvSTP13GR* nor the negative control *eGFP* conferred growth on glucose-containing medium. This demonstrates that only *HvSTP13* complemented the glucose uptake deficiency of the yeast strain EBY.VW4000, whereas the *HvSTP13GR* mutant is incapable of transporting glucose.

The barley *HvSTP13* transporter and its *HvSTP13GR* mutant are postulated to localize to the plasma membrane, but this has not been shown experimentally so far. To verify that the lost glucose transport function is not based on a disturbed protein localization, we cloned *HvSTP13* and *HvSTP13GR*, both including the *pHvSTP13* 5' UTR, under control of a short 35S promoter and monitored the subcellular localization after transient expression in *Nicotiana benthamiana* leaves (Figure 3a). As positive control *OsSWEET4*, a plasma membrane-localized sugar transporter from rice, was used (Sosso et al., 2015), while free *eGFP* served as negative control. Free *eGFP* localized unspecifically into the nucleus and the cytoplasm, whereas *OsSWEET4* exhibited a distinct localization at the edges of each cell that is typical for plasma membrane proteins (Figure 3b). The wild-type *HvSTP13* displayed a clear localization to the plasma membrane comparable to *OsSWEET4*. The expression of the *HvSTP13GR* mutant appeared more patchy compared to the *HvSTP13* wild type, but also localized predominantly to the plasma membrane (Figure 3b). This suggests that the lost glucose transport activity of the *HvSTP13GR* mutant is not due to mislocalization or protein degradation. Consequently, we used the *HvSTP13GR* variant for resistance analysis.

2.4 | The barley *HvSTP13GR* mutant confers resistance to a biotrophic fungus

In previous studies the transport-deficient *TaSTP13GR* or *MtSTP13GR* mutants were introduced as a transgene and exerted a dominant negative effect on the wild-type *STP13* copies in the respective genomes, thus conferring resistance in wheat, barley, and *M. truncatula* (Gupta et al., 2021; Milne et al., 2019; Moore et al., 2015). Presumably, the barley *HvSTP13GR* mutant would

also confer resistance to biotrophic fungi when introduced as a transgene. However, this has not been analysed yet.

To analyse the impact of the *HvSTP13GR* mutant on various pathogen infections, we generated transgenic barley plants that carry the genomic sequence of either the *HvSTP13* wild type or the *HvSTP13GR* mutant under control of the native *HvSTP13* promoter. To analyse their resistance phenotype, we infected T₁ plants of three independent transgenic *HvSTP13* or *HvSTP13GR* lines as well as the wild-type Golden Promise with the biotrophic rust fungus *Ph* and monitored the infection progress. Two and 4 days after infection, we harvested leaf sections and stained the fungal structures. At 2 days postinfection (dpi) all plants showed an even dispersion of spores on the leaf surface and only minimal differences in their overall developmental stage (Figure S5). Strikingly, at 4 dpi, significant differences in the *Ph* infection development became apparent between *HvSTP13* and *HvSTP13GR* transgenic plants (Figures 4 and 5). Plants from the *HvSTP13* transgenic lines displayed a comparable size of infection sites as the wild type, with a branched hyphal net, indicating a successful infection (Figure 4). For *HvSTP13GR* transgenic lines, on the other hand, we observed a retarded infection progress, with infection sites being significantly smaller compared to the wild type or the *HvSTP13* transgenic lines (Figure 4). Moreover, the infection sites never exceeded the formation of only a few haustorial mother cells (Figure 5). Ten days after infection, the leaves of the remaining plants were evaluated regarding their rust pustule formation (Figure 6). For the *HvSTP13*-transgenic and wild-type Golden Promise plants a heavy formation of pustules was observed on the surface of the infected leaves. In contrast, for the *HvSTP13GR* transgenic plants, only chlorotic spots but no pustules were observed (Figure 6). We confirmed an adequate induction of *HvSTP13* at 48 hpi via RT-qPCR, indicating that the resistance phenotype, observed for the *HvSTP13GR* transgenic plants, is not based on an impaired expression of either the genomic or the transgenic copy of *HvSTP13* (Figure S6a,b). Instead, it is conceivable that the presence of the transport-deficient *HvSTP13GR* mutant dominantly affects the function of the wild-type *HvSTP13*, comparable to the reported resistance mechanism for *TaSTP13GR* and *MtSTP13GR*.

3 | DISCUSSION

In wheat the presence of the glucose transport-deficient *TaSTP13GR* mutant confers resistance against the two rust fungi *Puccinia triticina* and *P. striiformis* f. sp. *tritici* as well as against the powdery mildew fungus *B. graminis* f. sp. *tritici* (Moore et al., 2015). Transferring the *TaSTP13GR* transgene into barley mediated resistance against the respective barley rust fungus *Ph* and the powdery mildew fungus *Bgh* (Milne et al., 2019). The barley ortholog *HvSTP13* has already been cloned and an equivalent *HvSTP13GR* mutant has been described as glucose transport-deficient in heterologous yeast studies (Milne et al., 2019). However, it has not been analysed whether the *HvSTP13GR* mutant confers resistance to biotrophic fungi comparable to *TaSTP13GR*. Here we

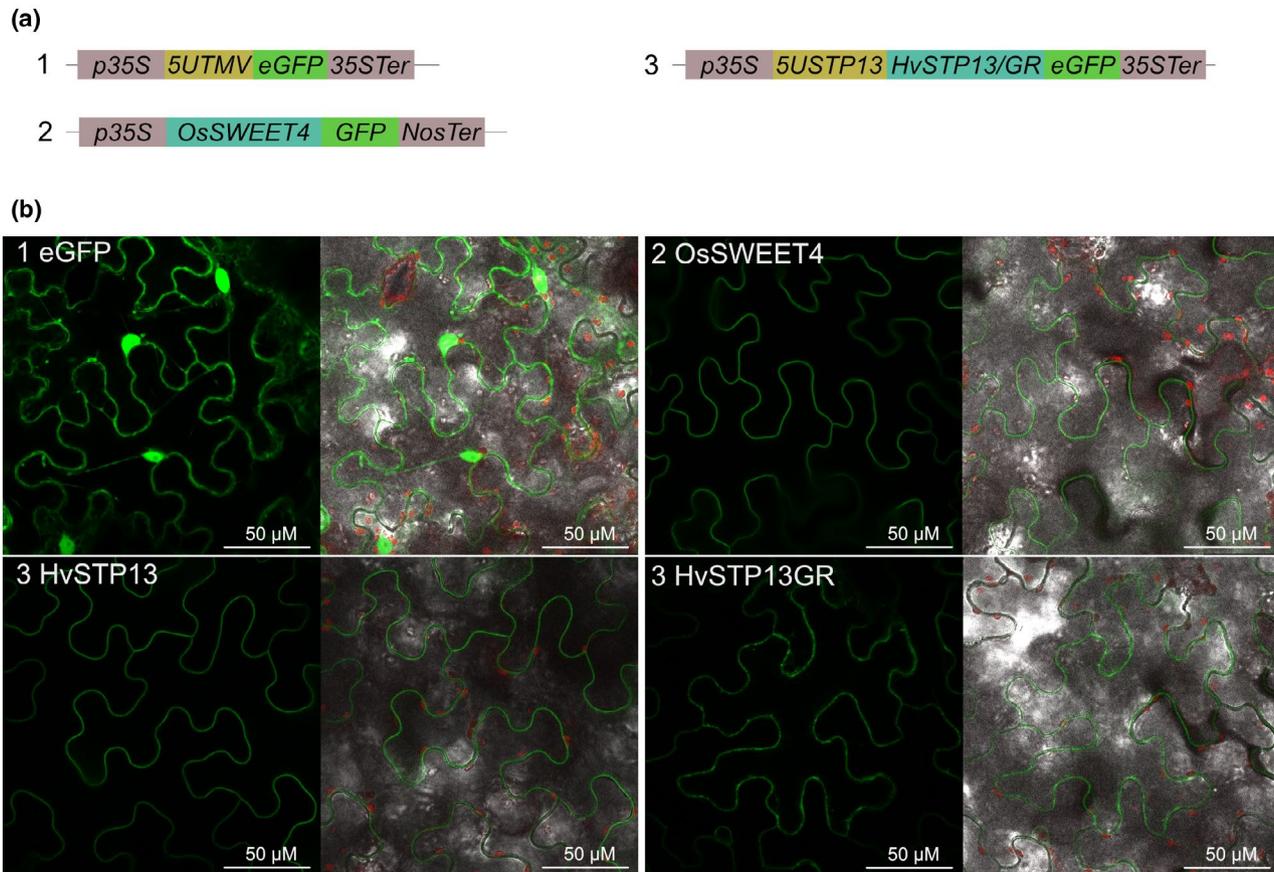


FIGURE 3 Localization of HvSTP13 and HvSTP13GR in planta. (a) Overview of the used constructs. (b) Constructs were transiently expressed in *Nicotiana benthamiana* and visualized 48 h postinoculation using a Leica SP8 confocal microscope (40× magnification and 1.5× zoom). Left panels show eGFP fluorescence and the respective right panels merged pictures (brightfield, chlorophyll autofluorescence, and eGFP)

demonstrated that the glucose transport-deficient HvSTP13GR variant confers resistance against the economically relevant rust fungus Ph, thus behaving in a similar way to the previously described TaSTP13GR variant (Milne et al., 2019). Because this resistance is not based on a gene-for-gene relationship, but possibly on the reduced availability of nutrients for the pathogen, or on increased defence signalling, the manipulation of the STP13 glucose transporter might present a promising way for the generation of a durable and broad-spectrum resistance, not only for barley but also for other important crops.

Our detailed microscopic analysis of the infection progress for Ph demonstrated that the presence of the HvSTP13GR transgene does not disturb the initial phase of fungal development until the first haustorial mother cells (HMCs) are formed. However, while the development of Ph progresses in HvSTP13 transgenic and wild-type plants, the fungus revealed an arrested development in the presence of the nonfunctional HvSTP13GR transporter. Based on our and other findings, different scenarios are conceivable to explain the impact of the glucose transport-deficient STP13GR variant on resistance. On the one hand, the glucose that is imported by STP13 could be an essential carbon source for the fungus, especially in the beginning of the infection. After a spore attaches to the plant

surface it germinates and forms infectious structures. In this first developmental phase biotrophic fungi feed from their own nutrient store, but internal stores are limited and the host plant is needed as nutrient source to energize further developmental processes (Divon & Fluhr, 2007). For this, biotrophic fungi form specialized organs called haustoria that invaginate the plant cell membrane and form a tight contact to the plant cytoplasm. There is emerging evidence that the composition of these organs is unique and provides all components needed for an efficient nutrient or signal transfer between the fungus and the plant (Bozkurt & Kamoun, 2020; Kwaaitaal et al., 2017; Polonio et al., 2020). Thus, the establishment of this tight interaction platform to gain nutrients from the host might be a key step that decides the failure or success of the disease. Support for this scenario comes from the observation that diverse fungal pathogens express one or several haustorial glucose transporters that are important for fungal virulence. Furthermore, it was hypothesized that glucose is not only used as an energy source but also functions as a signal molecule for fungal growth (Chang et al., 2020; van der Linde & Göhre, 2021; Sosso et al., 2019; Voegelé et al., 2001). Additionally, fungi secrete invertases, whose activity might further increase the supply of monosaccharides at the interaction surface (Chang et al., 2017; Voegelé et al., 2006). If this glucose supply is

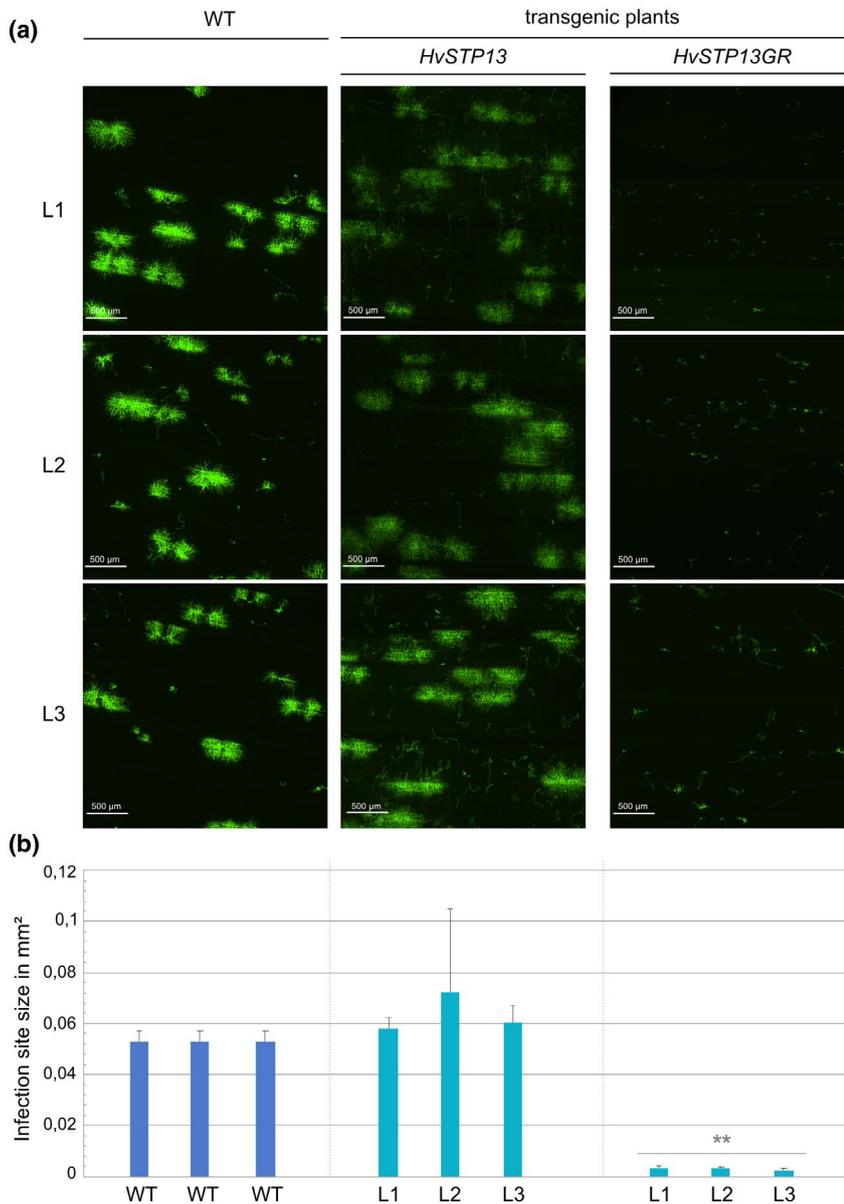


FIGURE 4 Histological analysis of *Puccinia hordei* growth in wild-type and transgenic plants at 4 days postinfection. (a) Three wild-type Golden Promise (WT) and three independent *HvSTP13* or *HvSTP13GR* transgenic lines (L1–L3) were infected with *P. hordei*. The stained fungal structures were visualized with a Nikon Ti Eclipse at 4× magnification using the GFP fluorescence filter. Images are representatives for three independent infection experiments. (b) Calculation of infection site size. The values represent the mean of two independent samples and 10 infection sites per sample. The error bars represent the positive standard deviation. Asterisks indicate a significant difference of *HvSTP13GR* transgenic plants from *HvSTP13* transgenic plants and the WT (Student's *t* test; ***p* < 0.01)

hampered by the presence of the transport-deficient *STP13GR* variant, the fungus starves and lacks energy to develop further. Such a disruption of fundamental requirements would also explain the broad-spectrum resistance conferred by the *STP13GR* mutant against different biotrophic fungi and in different host plants (Gupta et al., 2021; Milne et al., 2019; Moore et al., 2015). On the other hand, various sugars have been shown to regulate cellular processes at multiple levels, including immunity reactions (Bezruczyk et al., 2018). Hence, a blocked glucose import might change the relative glucose concentrations in the apoplast and the cytosol, and activate sugar signalling pathways. This shift might be sensed by the intracellular hexokinase 1 or the extracellular G-protein-coupled receptor RGS1 (regulator of G-protein signalling protein 1). Both proteins are reported to be involved in the activation of defence pathways, including signal transduction, gene expression, stomatal closure, cell wall restructuring, or reactive oxygen species (ROS) production (Grigston et al., 2008; Jing et al., 2020; Morkunas & Ratajczak, 2014;

Xiao et al., 2000; Zhong et al., 2019). Here, the inactive *STP13GR* mutant might result in the activation of those sugar signalling pathways and promote the plant stress response. Furthermore, an altered or gain of function of the *TaSTP13GR* mutant was discussed by Milne et al. (2019) as the basis for the resistance mechanism. Indeed, the G144 to R144 mutation adds an additional positively charged amino acid in close proximity to the proton donor-acceptor pair D41 and R140 (Paulsen et al., 2019). This might result in rearrangements of the binding pocket and allow for the binding of an altered substrate that activates an immune response. However, as Huai et al. (2020) demonstrated, silencing of the *TaSTP13* transporter increased the resistance against stripe rust; therefore, it is still under debate whether or not the loss of the glucose transport activity, a gain of function, or a combination of both is the basis for the resistance phenotype of the *STP13GR* mutants. In future, alternative mutants and analysis are needed to elucidate the underlying resistance mechanism.

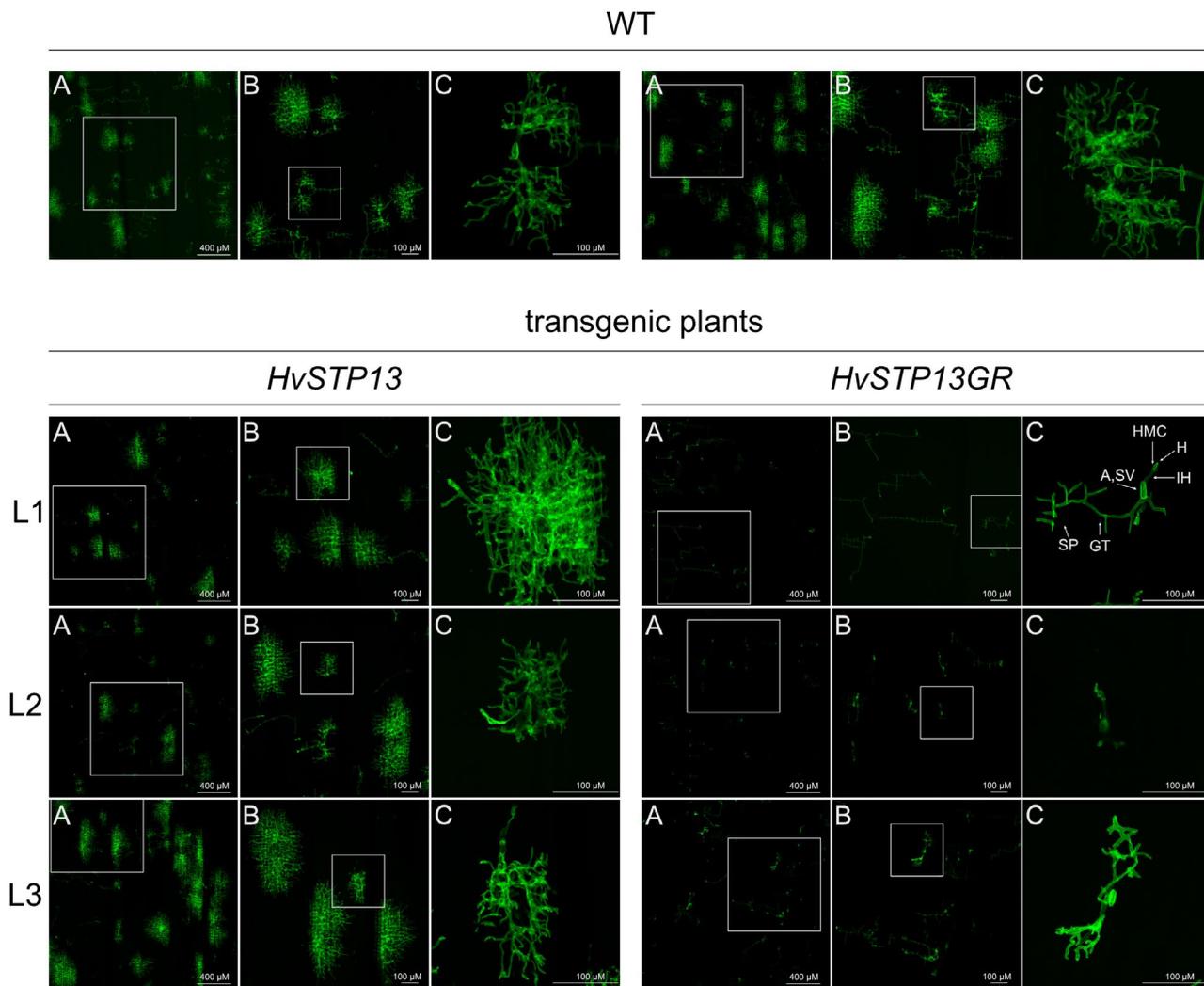


FIGURE 5 Histological analysis of *Puccinia hordei* development in wild-type and transgenic plants at 4 days postinoculation. Wild-type Golden Promise (WT) and three independent *HvSTP13* or *HvSTP13GR* transgenic lines (L1–L3) were infected with *P. hordei*. The stained fungal structures were visualized with a Leica SP8 confocal microscope at 5 \times (a), 10 \times (b; Z-stack), and 40 \times (c; Z-stack) magnification and GFP fluorescence settings. The respective enlarged area is marked with a grey square. The images are representatives of two biological replicates with at least three independent infection areas each. SP, spore; GT, germ tube; A, appressorium; SV, substomatal vesicle; IH, infection hypha; HMC, haustorial mother cell; H, haustorium

In wheat, the expression of the closely related ortholog *TaSTP13* is induced by pathogen infection as well as abiotic treatments, including ABA (Huai et al., 2020; Moore et al., 2015). The comparison of the *HvSTP13* promoter with an equivalent portion of the *TaSTP13* promoter resulted in an identity of 65%–68% (Figure S3b) and it would be interesting in future to analyse if certain CREs are shared by *TaSTP13* and *HvSTP13*. We demonstrated that the expression of *HvSTP13* is increased on recognition of bacterial and fungal PAMPs, and we identified a promoter region that is probably required for the induced expression. In this promoter region two interesting CREs were predicted, namely two ABREs and a cluster of four W-boxes. ABA, which activates the binding of transcription factors to ABREs, is mainly related to the abiotic stress response, but emerging evidence suggests a cross-talk of ABA with pathogen infection. The role of ABA to support or repress immunity reactions largely depends on the pathogen

and the phase of infection (Cao et al., 2011). Notably, in grapevine, the promoter of the *STP13* ortholog *VvHT5* also employs a unique cluster of ABREs that connects the expression of *VvHT5* to ABA signalling pathways, thus linking it to both wounding and pathogen infection (Hayes et al., 2010). However, whether this observation is connected to PAMP recognition or other effects of pathogen infection is unknown. In comparison, the presence of a W-box cluster in the *HvSTP13* promoter indicates an intriguing link to a PAMP-induced response, as WRKY transcription factors are key players in downstream processes of the PTI (Chen et al., 2019). The interaction of *AtSTP13* with *FLS2* and its phosphorylation via *BAK1* further supports such a connection between *STP13* activity and PTI signalling, thus rendering WRKYs as promising agents for *HvSTP13* induction (Yamada et al., 2016). Increasing research characterized WRKY transcription factors in important crops as wheat and barley, and different roles for

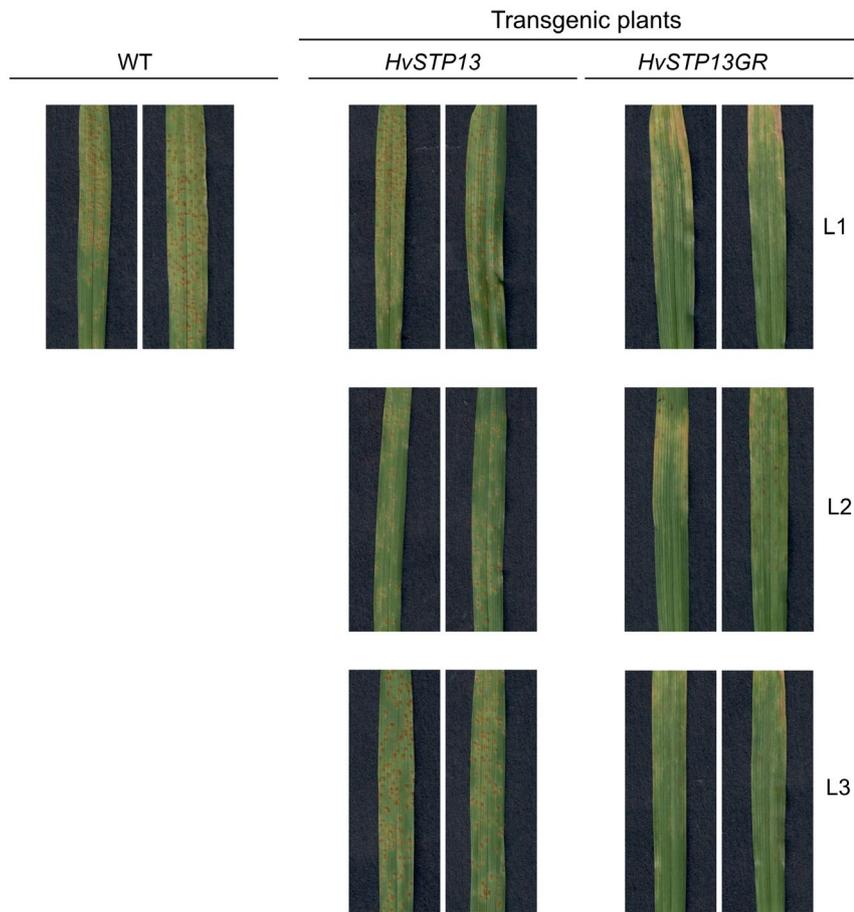


FIGURE 6 Disease phenotypes of *Puccinia hordei*-infected barley plants at 10 days postinoculation. Wild-type Golden Promise (WT) and three independent *HvSTP13* or *HvSTP13GR* transgenic lines (L1–L3) were infected with *P. hordei*. The pictures are representative of three independent infection experiments

WRKY transcription factors in modulating resistance or susceptibility were presented. For example, *HvWRKY10*, *HvWRKY19*, and *HvWRKY28* have been shown to act as positive regulators in the resistance response of barley to Bgh (Meng & Wise, 2012). For wheat it has been shown that the transient expression of the barley WRKYs *HvWRKY6*, *HvWRKY40*, and *HvWRKY70* increases resistance against the biotrophic leaf rust fungus *P. tritricina* and a follow-up study confirmed the resistance of wheat expressing *HvWRKY6* and *HvWRKY70* against *P. striiformis* f. sp. *tritici* and *B. graminis* f. sp. *tritici* (Gao et al., 2018; Li et al., 2020). Furthermore, *HvWRKY23* is activated on PAMP recognition by *HvCERK1* and probably supports resistance against the hemibiotrophic head blight pathogen *Fusarium graminearum* (Karre et al., 2017, 2019). These examples support the hypothesis that the PAMP-dependent activation of the *HvSTP13* promoter is a result of PTI signalling via WRKY transcription factors to combat the artificial sink that is generated by pathogens (Pommerrenig et al., 2020). In conclusion, *HvSTP13* might be an important regulatory checkpoint in the multifaceted response of the plant on both biotic and abiotic stresses.

Our experimental approaches confirm the potential of the *HvSTP13GR* mutant for resistance breeding and identified promoter regions that are relevant for regulating transcription factors. Consequently, our work contributed to further decode the complex regulation network in which *STP13* is probably a major player.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial infection of barley

Pto strain DC3000 and Xth strain UPB820^{Rif} were grown for 2 days at 28°C using peptone sucrose agar (PSA) plates. The strains were resuspended in 10 mM MgCl₂, adjusted to OD₆₀₀ of 0.2 and inoculated in 7-day-old barley plants. The plants were cultivated in a phytochamber with 16/8 h light and 18/12°C day/night conditions.

4.2 | Fungal infection for *HvSTP13* expression analysis

Fungal infection experiments for *HvSTP13* expression analysis were performed at the Institute for Resistance Research and Stress Tolerance (Federal Research Centre for Cultivated Plants, Julius Kühn Institute, Quedlinburg, Germany).

4.2.1 | Rust infection

Fresh uredospores for Ph isolate I-80 were propagated by using the highly susceptible barley cultivar Großklappige as previously described in Fazlikhani et al. (2019). Seven-day-old Golden Promise plants were

infected with Ph according to Wehner et al. (2019) and cultivated with 16/8 hr light and 20/17°C. Psh isolate 24 was propagated by using Hakai. The infection of Golden Promise plants was performed as described for Ph and plants were cultivated with 16/8 h light and 16/13°C day/night conditions in a greenhouse.

4.2.2 | Powdery mildew infection

Infection was performed as described in Šurlan-Momirović et al. (2016) with the isolate Freising, which was propagated on leaf segments of the susceptible cultivar Igri. Whole plants were infected in the settling tower. Pots with infected plants were placed in a tray with moist sand and covered with transparent plastic boxes. After 3 days, the air supply was increased by placing small clay pots under the rim of the plastic boxes. Incubation took place at 18/15°C, 16/8 h day/night cycle.

4.2.3 | Net blotch infection

The mycelium was propagated on potato dextrose agar (PDA) plates. One gram of the conidia-bearing mycelium was scraped off the agar plates and 100 ml of sterile water, containing 0.01% Tween 20, was added. The suspension was homogenized for 3 min with a stick blender, filtered through gauze, and sprayed evenly onto 3-week-old plants. The plants were covered with a plastic hood moistened with water for 48 h. Three days after infection, the plants were exposed to light (16/8 h day/night cycle, 60% humidity) and the temperature was raised from 18°C to 20°C according to Novakazi et al. (2019).

4.3 | Ph infection for resistance analysis in transgenic barley plants

Ph infections for resistance analysis were performed at the Department of Plant Biotechnology in Hanover. Fresh uredospores of Ph were propagated in climatic chambers (Polyklima) as described before and mixed with activated (10 min at 42°C, 1 h at room temperature) frozen spores in a 1:5 ratio. The spores were mixed with clay in a 1:3 ratio. Ten-day-old plants were sprayed with 0.02% Tween 20 using the GANZTON SP180K airbrush system. The spores were applied using a pipe cleaner. Plastic cups were moistened with 0.02% Tween 20 and used to cover the plants for 48 h. The plants were kept in climatic chambers in darkness for 24 h followed by a 16/8 h day/night cycle at 20°C for Ph. The infection experiment was repeated three times with 30 plants per transgenic line and 30 wild-type plants each (120 plants per infection).

4.4 | PAMP treatment of barley seedlings

Seven-day-old barley seedlings were inoculated with the PAMPs laminarin (200 µg/µl; Sigma-Aldrich), β-1,3-glucan (200 µg/µl;

Sigma-Aldrich), chitin (100 µg/ml; Sigma-Aldrich), and flg22 (2 µM; Genscript). All PAMPs were dissolved in 10 mM MgCl₂. The negative control was 10 mM MgCl₂. Plants were cultivated in a phytochamber (16/8 h light, 18/12°C day/night). Inoculations were done in biological triplicate.

4.5 | RNA extraction and gene expression analysis

Total RNA was isolated using a RNeasy Plant Mini Kit (Qiagen). Leaf material was disrupted using a TissueLyser II (Qiagen). On-column DNase digestion was performed using a RNase-Free DNase Set (Qiagen). RNA concentration and purity were determined by using a Spark 10M (Tecan). cDNA was synthesized from 2 µg of RNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). All qPCR analyses were performed in technical replicates, including no-RT controls, using the CFX Connect Real-Time PCR Detection System (Bio-Rad). Reactions contained Luna Universal qPCR Master Mix (NEB), 0.25 µM of each primer, and 100 ng of template cDNA in 20 µl final volume. Primers used for RT-qPCR are listed in Table S3. The ubiquitin-protein ligase (*HORVU4HrG004090*) served as a reference gene (Hua et al., 2015). *HvPR3* (*HORVU1Hr1G052430*; Scheler, Schnepf, et al., 2016) served as infection control. Primer efficiencies were determined using a serial cDNA dilution. The fold induction was calculated to the mock control for each time point. Each experiment includes at least two biological replicates and was repeated at least twice.

4.6 | Construction of STP13 modules

The generated level 0 MoClo modules are shown in Figure S7a. The *HvSTP13* (*HORVU1Hr1G052430*) modules were amplified from Golden Promise cDNA or gDNA (Table S3). The yeast *pPMA1* promoter, *ADH2* terminator, and *Hxt1* coding sequences were amplified from EBY.VW4000 or already existing constructs (authors' unpublished results). Level 1 transcription units were cloned into pICH47742 (plants; Weber et al., 2011; Werner et al., 2012) or in pAGT572 or pAGT573 (yeast; Scheler, Brandt, et al., 2016). For GUS reporter constructs the MoClo module pICH75111 was used (Engler et al., 2014). All multigene constructs for the generation of transgenic plants contained a hygromycin resistance gene and were generated using the destination vector pAGM8031. For transient expression in *N. benthamiana*, *HvSTP13* and *HvSTP13GR* were cloned under control of the short CaMV 35S promoter and included the *HvSTP13* 5' UTR.

4.7 | Generation of transgenic plants and transgene analysis

Immature embryos of the barley cultivar Golden Promise were transformed with *Agrobacterium tumefaciens* AGL1 according to

the protocol of Harwood et al. (2009) with the following modifications. The selection period was reduced to 30 days, and 800 mg/L L-cysteine (Hensel et al., 2007) and 0.1 mM acetosyringone were added to the cocultivation medium. After a 10-day transition period, calli were placed on transition medium containing a 50% reduced hygromycin concentration (25 mg/L) for 4 days. The hygromycin concentration of the regeneration medium was also reduced to 50%. The transgene status of all regenerated T_0 plants and following generations (T_1 , T_2) was analysed by extracting the gDNA with either innuPREP Plant DNA Kit (Analytik Jena; T_0) or REExtract-N-AMP plant Kit (Sigma Aldrich; T_1 , T_2). The transgene was detected by amplifying the Ocs terminator of the hygromycin resistance gene cassette (Table S3).

4.8 | Analysis of promoter variants via GUS assay

For each GUS reporter construct, three independent T_1 transgenic lines with six plants each were used. Two leaf discs (5 mm) were harvested from 10-day-old plants and vacuum infiltrated with 10 mM $MgCl_2$, 1 μ M flg22, or 100 μ g/ml chitin. The leaf discs were incubated in the phytochamber as described before. After 24 h the leaf discs were frozen in liquid nitrogen and ground using the TissueLyser II. The GUS activity was measured in the Spark 10M reader as described previously (Kay et al., 2007). The measurements were performed in technical replicates. The experiment was performed twice.

4.9 | In planta localization studies

Four- to 6-week-old *N. benthamiana* plants were inoculated with *A. tumefaciens* GV3101 strains using a needleless syringe and an OD_{600} of 0.4. The localization of the resulting proteins was analysed at 2 dpi using a Leica True Confocal Scanner SP8 microscope equipped with an HC PL APO CS2 40x/1.10 water immersion objective (Leica Microsystems). Pictures for GFP fluorescence were taken with a HyD detector (488 nm, 502–516 nm) and pictures for chlorophyll autofluorescence with a PMT2 detector (658–708 nm). Pictures show 1.5x zoom of 40x magnification.

4.10 | Yeast sugar uptake analysis

All constructs were cloned under the control of the *pPMA1* promoter and *ADH2* terminator, and transformed into EBY.VW4000 (Wieczorke et al., 1999). Positively transformed colonies were identified on selective medium with 2% maltose. Yeast cells were re-suspended in sterile water and adjusted to an OD_{600} of 0.4. Three microlitres of a serial 10-times dilution were pipetted on selective media with 2% maltose or 2% glucose. Plates were incubated at 28°C for 2 days and growth was documented with the BioRad Chemidoc.

4.11 | Histological analysis of fungal growth

Staining of harvested leaves was performed with Alexa Fluor 488 conjugate of wheat germ agglutinin according to Redkar et al. (2018) and the stained infection sites were examined using a Ti fluorescence microscope (Nikon). The size of the infection sites was calculated using the object count function of the NIS Elements software. The calculation is based on a one-point threshold definition. With the GFP filter and 10x magnification, the hue of a characteristic fluorescence signal in the respective infection site was selected to define the threshold for object detection. Based on this calibration, the software calculates the fluorescing area in μm^2 . Detailed pictures were taken with a Leica True Confocal Scanner SP8 microscope using the HyD detector settings for GFP. Z-stacks represent up to 30 panels depending on the growth depth of the fungal structures.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Jana Streubel  <https://orcid.org/0000-0001-8155-511X>

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