β-Glucosidase BGLU42 is a MYB72-dependent key regulator of rhizobacteria-induced systemic resistance and modulates iron deficiency responses in *Arabidopsis* roots

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Received: *2 May 2014*
Accepted: *4 July 2014*

New Phytologist (2014) **204:** 368–379
doi: 10.1111/nph.12980

Key words: beneficial microbes, glucoside hydrolase, induced systemic resistance (ISR), iron deficiency response, MYB transcription factors, *Pseudomonas fluorescens*, rhizosphere.

Summary

- Selected soil-borne rhizobacteria can trigger an induced systemic resistance (ISR) that is effective against a broad spectrum of pathogens. In *Arabidopsis thaliana*, the root-specific transcription factor MYB72 is required for the onset of ISR, but is also associated with plant survival under conditions of iron deficiency. Here, we investigated the role of MYB72 in both processes.
- To identify MYB72 target genes, we analyzed the root transcriptomes of wild-type Col-0, mutant *myb72* and complemented *35S:FLAG-MYB72/myb72* plants in response to ISR-inducing *Pseudomonas fluorescens* WCS417.
- Five WCS417-inducible genes were misregulated in *myb72* and complemented in *35S:FLAG-MYB72/myb72*. Amongst these, we uncovered β-glucosidase BGLU42 as a novel component of the ISR signaling pathway. Overexpression of *BGLU42* resulted in constitutive disease resistance, whereas the *bglu42* mutant was defective in ISR. Furthermore, we found 195 genes to be constitutively upregulated in *MYB72*-overexpressing roots in the absence of WCS417. Many of these encode enzymes involved in the production of iron-mobilizing phenolic metabolites under conditions of iron deficiency. We provide evidence that BGLU42 is required for their release into the rhizosphere.
- Together, this work highlights a thus far unidentified link between the ability of beneficial rhizobacteria to stimulate systemic immunity and mechanisms induced by iron deficiency in host plants.

Introduction

Plants nurture a large community of commensal and mutualistic microbes that provide them with essential services, such as enhanced mineral uptake, nitrogen fixation, growth promotion and protection from pathogens (Lugtenberg & Kamilova, 2009; Berendsen *et al.*, 2012). These plant microbiota are predominantly hosted by the root system, which deposits up to 40% of the plant's photosynthetically fixed carbon into the rhizosphere (Bais *et al.*, 2006). Selected root-associated mutualists promote plant health by stimulating the plant's immune system, a phenomenon called induced systemic resistance (ISR) (Pieterse *et al.*, 2014). The molecular mechanisms underpinning ISR have been studied extensively in the interaction between the model plant *Arabidopsis thaliana* (Arabidopsis) and the plant growth-promoting rhizobacterium *Pseudomonas fluorescens* WCS417 (hereafter referred to as WCS417) (Van der Ent *et al.*, 2009b; Zamioudis & Pieterse, 2012; Pieterse *et al.*, 2014). WCS417-mediated ISR is effective against a broad variety of pathogens and even insect herbivores (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Ton *et al.*, 2002; Van Oosten *et al.*, 2008; Pineda *et al.*, 2010). WCS417-ISR requires functional jasmonic acid and ethylene signaling pathways and its expression in foliar tissues is controlled by the transcriptional regulators NPR1 and MYC2 (Pieterse *et al.*, 1998; Pozo *et al.*, 2008). Large-scale gene expression analyses have revealed that the establishment of WCS417-ISR in foliar tissues is not associated with major changes in gene expression (Verhagen *et al.*, 2004; Pozo *et al.*, 2008). Instead, ISR-expressing leaves are primed for accelerated jasmonic acid/ethylene-regulated gene expression (Verhagen *et al.*, 2004; Pozo *et al.*, 2008) and enhanced deposition of callose-rich papillae at the sites of pathogen entry (Van der Ent *et al.*, 2009a), responses that become apparent only on pathogen attack. This phenomenon is known as priming and provides a cost-effective mechanism of protection against pathogens and pests (Conrath *et al.*, 2006; Van Hulten *et al.*, 2006; Conrath, 2011; Vos *et al.*, 2013).

In contrast with leaves, roots reprogram the expression of a large set of genes in response to colonization by WCS417

(Verhagen *et al.*, 2004). Amongst them, the root-specific R2R3-type MYB transcription factor MYB72 has emerged as an important component in the onset of ISR (Van der Ent *et al.*, 2008). T-DNA knockout mutants *myb72-1* and *myb72-2* are abolished in their ability to develop ISR against a broad range of pathogens. Despite the essential role of MYB72 in ISR, constitutive expression of *MYB72* does not lead to increased levels of disease resistance in the absence of rhizobacteria, suggesting that either MYB72 undergoes post-translational activation or acts in concert with other factors in response to WCS417 (Van der Ent *et al.*, 2008). Interestingly, not only beneficial rhizobacteria, but also the beneficial fungus *Trichoderma asperellum* T34, utilize MYB72-mediated signaling for ISR elicitation, pointing to a conserved mechanism that operates locally in roots during ISR triggered by different types of beneficial microbe (Segarra *et al.*, 2009).

In addition to its role in ISR, *MYB72* is induced in the roots of Arabidopsis under iron limitation and growth conditions that distort iron uptake, such as high zinc concentrations (Colangelo & Guerinot, 2004; De Mortel *et al.*, 2008; Buckhout *et al.*, 2009). In Arabidopsis, iron limitation induces a set of coordinated responses, collectively referred to as Strategy I, which foster iron mobilization and uptake by the roots (Walker & Connolly, 2008). Iron mobilization is realized by members of plasma membrane-localized H^+-ATPases, which secrete protons to acidify the rhizosphere and thereby enhance the solubility of ferric iron (Fe^{3+}) in the soil. Ferric iron is reduced to ferrous iron (Fe^{2+}) via the plasma membrane protein FERRIC REDUCTION OXIDASE 2 (FRO2) and subsequently transported from the soil environment to the root interior via the high-affinity iron transporter IRON-REGULATED TRANSPORTER 1 (IRT1) (Walker & Connolly, 2008). Recently, iron mobilization from alkaline substrates has been shown to be facilitated by phenolic compounds that are produced and excreted by the roots of Arabidopsis under conditions of iron deficiency (Rodríguez-Celma *et al.*, 2013; Fourcroy *et al.*, 2014; Schmid *et al.*, 2014). Iron uptake under conditions of iron deficiency is regulated by the basic helix-loop-helix (bHLH) transcription factor FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) (Colangelo & Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005; Bauer *et al.*, 2007), which forms heterodimers with members of the Ib subgroup of the bHLH gene family (bHLH38, 39, 100 and 101), resulting in the upregulation of *FRO2* and *IRT1* (Yuan *et al.*, 2008; Wang *et al.*, 2013). The FIT transcription factor also regulates the expression of *MYB72* under conditions of iron deficiency (Colangelo & Guerinot, 2004; Sivitz *et al.*, 2012). Recently, MYB72 and its paralog MYB10 have been shown to be required for plant survival in alkaline soils in which iron availability is greatly restricted. Amongst their target genes are *NICOTIANAMINE SYNTHASE 2* (*NAS2*) and *NICOTIANAMINE SYNTHASE 4* (*NAS4*), which are involved in the biosynthesis of the iron chelator nicotianamine (NA) (Palmer *et al.*, 2013).

Because MYB72 seems to function as a node of convergence in root signaling pathways that regulate the onset of rhizobacteria-mediated ISR and adaptive responses to iron deficiency, we set out to investigate the rhizobacteria-responsive target genes of this transcriptional regulator. By employing genome-wide microarray analyses, we identified five genes that are positively regulated in the roots by MYB72 in response to WCS417. Amongst the MYB72-depedent genes, we uncovered the β-glucosidase BGLU42 as an important novel component that acts downstream of MYB72 in the ISR signaling pathway. In addition, we provide evidence for a novel function of MYB72 in the iron deficiency response Strategy I. We show that MYB72 regulates the expression of genes associated with the production of iron-mobilizing phenolic metabolites, and that BGLU42 plays an important role in the secretion of these compounds in the root vicinity. This work uncovers a mechanistic link between the ability of beneficial microbes from the root microbiome to simultaneously trigger ISR and stimulate iron nutrition in the host plant.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. accession Col-0 was used as wild-type plant genotype. The *myb72-2* mutant has been described previously (Van der Ent *et al.*, 2008). Knockout mutant *bglu42* (SALK-034026), carrying a T-DNA insertion in the 12th gene exon, was obtained from the SALK collection (Alonso *et al.*, 2003). Primers for the confirmation of the T-DNA insertion were designed with the iSect tool (http://signal.salk.edu/tdnaprimers.2.html). The following transgenic lines overexpressing the indicated genes under the control of the *35S* cauliflower mosaic virus (CaMV) promoter were used: *35S:FLAG-MYB72* (oxMYB72) in the *myb72-2* background, and *35S:YFP-MYB72*, *35S:YFP-BGLU42* (oxBGLU42 lines L1, L2 and L3), *35S:YFP-At5g55620* (oxAt5g55620 lines L1, L2 and L3), *35S:YFP-CYP71B5* (oxCYP71B5 lines L1, L2 and L3), *35S:bHLH39-GUS* (oxbHLH39) (Yuan *et al.*, 2008) and *35S:NRT1.8* (oxNRT1.8 lines L1 and L18) (Li *et al.*, 2010) in the Col-0 background. The promoter-GFP-GUS reporter line *pBGLU42:GFP-GUS* was constructed in the Col-0 background.

For the microarray experiment, seeds of Col-0, *myb72-2* and oxMYB72 were surface sterilized and sown on 1 × Murashige and Skoog (MS) agar-solidified medium supplemented with 1% sucrose (Murashige & Skoog, 1962). After 2 d of stratification at 4°C, the Petri dishes were positioned vertically and transferred to a growth chamber (22°C; 12 h : 12 h light : dark; light intensity, 100 $\mu mol\ m^{-2}\ s^{-1}$). Uniform 5-d-old seedlings were transferred to new plates containing agar-solidified Hoagland medium composed of KNO_3 (5 mM), KH_2PO_4 (2 mM), $Ca(NO_3)_2$ (5 mM), $MgSO_4$ (2 mM), KCl (50 μM), H_3BO_3 (50 μM), $MnSO_4$ (10 μM), $ZnSO_4$ (2 μM), $CuSO_4$ (1.5 μM), $(NH_4)_6Mo_7O_{24}$ (0.075 μM) and MES (2.5 mM). The concentration of Fe(III)EDTA was adjusted to 50 μM and the pH of the medium to 5.8. For experiments performed in soil, seeds were sown in quartz sand and, 2 wk later, seedlings were transferred to 60-ml pots containing sand/potting soil mixture (autoclaved twice for 20 min with a 24-h interval).

Cloning procedures and the generation of transgenic lines

The 1.7-kb genomic region upstream of the start codon of *BGLU42* was amplified from genomic Col-0 DNA, captured into the pDONR221-pGEMT-Easy vector using the BP reaction and recombined into the destination pBGWFS7.0 vector using the LR reaction according to the manufacturer's instructions (Invitrogen). The coding sequences of *MYB72*, *BGLU42*, *AT5G55620* and *CYP71B5* were amplified from wild-type Col-0 cDNA and captured into the pDONR221-pGEMT-Easy vector. The corresponding cDNAs were then recombined into either the pEarleyGate202 (35S-FLAG-Gateway-OCS-3') or pEarleyGate104 (35S-YFP-Gateway-OCS-3') plasmid (Earley *et al.*, 2006). The primers used for cloning are provided in Supporting Information Table S1. For the generation of transgenic lines, recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90. *Agrobacterium tumefaciens*-mediated plant transformation was performed using the floral dip method (Clough & Bent, 1998) in Col-0 plants, except for the *35S:FLAG-MYB72* construct which was transformed in the *myb72-2* genetic background.

Pseudomonas fluorescens WCS417 treatments

Pseudomonas fluorescens WCS417 was cultured at 28°C on King's medium B (KB) agar plates supplemented with 50 μg ml^{-1} of rifampicin. After 24 h of growth, cells were collected in 10 mM $MgSO_4$, washed twice by centrifugation for 5 min at 5000 ***g*** and finally resuspended in 10 mM $MgSO_4$. For *in vitro* assays, the bacterial titer was adjusted to the optical density at 600 nm (OD_{600}) of 0.01 (10^7 colony-forming units (CFU) ml^{-1}). Ten microliters of bacterial suspension were then applied on each root of 17-d-old seedlings, immediately below the hypocotyl. Induction of ISR with WCS417 was performed by mixing ISR-inducing rhizobacteria through the soil as described previously (Pieterse *et al.*, 1996; Van Wees *et al.*, 2013).

Pathogen cultivation and bioassays

Botrytis cinerea strain B0510 was cultivated on half-strength potato dextrose agar (PDA) plates for 10 d at 22°C. *Botrytis cinerea* spores were collected and resuspended in half-strength potato dextrose broth to a final density of 5×10^5 spores ml^{-1}. Five-week-old plants ($n = 24$) were inoculated by applying 5-μl drops of spore suspension per leaf. Symptoms were scored 4 d after inoculation. Disease ratings were expressed as the percentage of leaves showing spreading lesions as described previously (Van der Ent *et al.*, 2008; Van Wees *et al.*, 2013).

Pseudomonas syringae pv. *tomato* DC3000 was cultured on KB agar plates supplemented with 50 μg ml^{-1} of rifampicin at 28°C. After 24 h of growth, cells were collected in 10 mM $MgSO_4$, washed twice by centrifugation for 5 min at 5000 ***g*** and finally resuspended in 10 mM $MgSO_4$. Plants were inoculated at 5 wk old by spraying leaves until runoff with a solution of 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 containing 10^8 CFU ml^{-1} *P. syringae* pv. *tomato* DC3000 bacteria. Four days after inoculation, disease severity was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The disease index was calculated by determining the proportion of leaves with disease symptoms per plant ($n = 24$) as described previously (Van Wees *et al.*, 2013).

For *Hyaloperonospora arabidopsidis* bioassays, 3-wk-old Arabidopsis seedlings ($n = 24$) were misted with an *H. arabidopsidis* spore suspension containing 5×10^4 sporangiospores ml^{-1}. Disease symptoms were scored at 10 d after inoculation. Disease ratings were expressed as the severity of disease symptoms and pathogen sporulation on each leaf: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered by sporangia; IV, > 50% of the leaf area covered by sporangia, with additional chlorosis and leaf collapse, as described previously (Van der Ent *et al.*, 2008).

Microarray and data analysis

RNA samples for microarray analysis were collected 2 d after the induction treatment with WCS417. RNA purity and integrity were confirmed using an RNA 6000 Nano Assay (Agilent Technologies, Waldbronn, Germany) and gel electrophoresis. cRNA labeling, hybridization, washing and scanning of Affymetrix Arabidopsis ATH1 GeneChips (Affymetrix) were performed according to Affymetrix OneCycle Lab protocols (Affymetrix, Santa Clara, CA, USA). Data were analyzed statistically using the R language environment for statistical computing version 2.11.1 and Bioconductor release 2.6 (Gentleman *et al.*, 2004). Data were normalized using the Robust Multichip Average (RMA) expression measure in the Affy package (Gautier *et al.*, 2004). Differentially expressed genes were identified using the LIMMA package (Smyth, 2004). The *P* values obtained were corrected for multiple testing errors using the BH procedure (Benjamini & Hochberg, 1995), yielding *q* values. Lists of *q* values were transferred to Microsoft Excel™ and sorted. For gene annotations into biological categories, the AmiGO Term Enrichment software was used (Carbon *et al.*, 2009).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted using the RNeasy kit (Qiagen), according to the manufacturer's instructions, and treated with Ambion TURBO™ DNase. Subsequently, cDNA was synthesized using SuperScript-III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Cycle thresholds were determined in duplicate per transcript in three biological replicates per sample using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Netherlands) and SYBR Green I as reporter dye. The data were normalized using *Actin7*. The primers used for qRT-PCR are provided in Table S1.

Confocal microscopy

Confocal laser-scanning microscopy on the *35S:YFP-MYB72* and *pBGLU42:GFP-GUS* reporter lines was performed with a Leica SP2 inverted microscope (Leica Microsystems, Wetzlar, Germany) as described previously (Zamioudis *et al.*, 2013). As counterstain, roots were stained in 10 μg ml^{-1} propidium iodide (PI) solution for 2 min. Chromophores were excited using the 488-nm argon laser and fluorescence was detected at 500–550 nm (for green fluorescent protein (GFP)), 550–615 nm (for yellow fluorescent protein (YFP)) and 570–620 nm (for PI).

Detection of fluorescent phenolic compounds in the root exudates

The production and/or secretion of fluorescent phenolic compounds was monitored under UV light (365 nm) in 6-d-old seedlings germinating on agar-solidified medium with the composition described above, with either 10 μM Fe(III)EDTA and pH 5.8 (available iron) or 10 μM $FeCl_3$ and pH 7.0 (non-available iron) (Rodríguez-Celma *et al.*, 2013). The secretion of fluorescent phenolic compounds was quantified using a 96-well microplate assay in which seeds were allowed to germinate within individual wells (one seed per well) containing either available or non-available iron (200 μl agar-solidified medium per well). Fluorescence emitted by root exudates (excitation at 360 nm; emission at 528 nm) was measured with a Synergy™ Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) after carefully removing the seedlings from the growth medium.

Results

WCS417-inducible genes that are regulated by MYB72 encode components of the iron deficiency response

Confocal imaging of independent transgenic lines overexpressing the YFP-MYB72 chimeric protein (*35S:YFP-MYB72*) revealed that, consistent with its function as a transcription factor, MYB72 is localized in the nucleus (Fig. 1a). The same pattern was also observed on root bacterization by WCS417 (data not shown). In order to gain insights into the role of MYB72 in plant responses to beneficial WCS417 bacteria, we first set out to identify WCS417-responsive genes that are regulated by MYB72 in Arabidopsis roots. To this end, we generated global gene expression profiles of mock- and WCS417-treated roots of wild-type Col-0 and mutant *myb72-2* seedlings. We also included in our analysis a transgenic *35S:FLAG-MYB72* line, which overexpresses *MYB72* in the *myb72-2* mutant background (hereafter referred to as oxMYB72). Two days after WCS417 application, root material was harvested from three

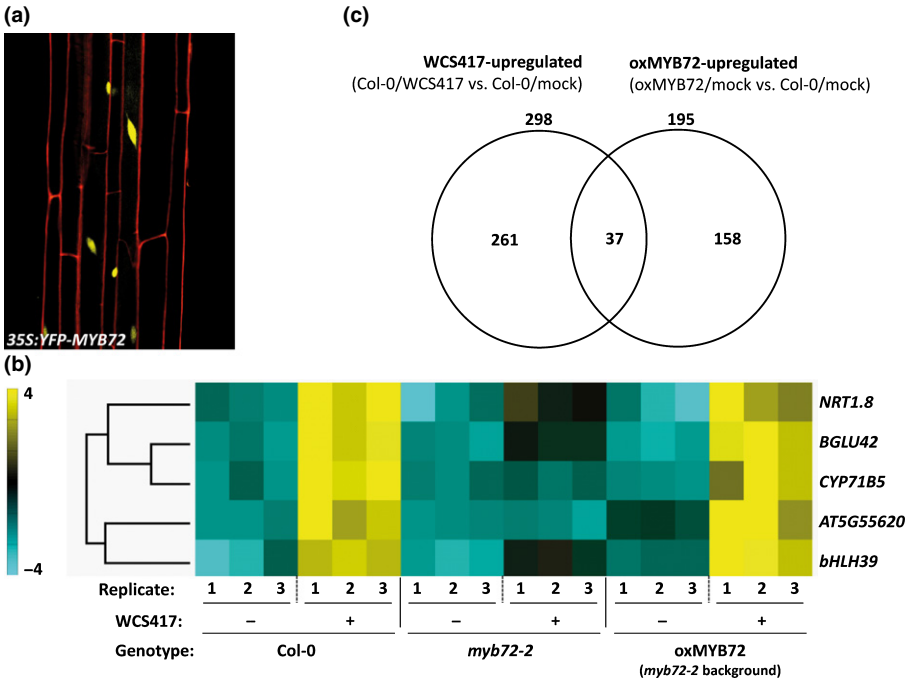

Fig. 1 *Pseudomonas fluorescens* WCS417-induced genes that are regulated in a MYB72-dependent manner in Arabidopsis roots. (a) Nuclear localization of MYB72 in roots of 35S:YFP-MYB72 transgenic plants. A representative confocal image is shown. (b) Heat map of the expression of genes that are induced by *P. fluorescens* WCS417 in a MYB72-dependent manner in Arabidopsis roots, as revealed by microarray analysis. Seventeen-day-old Col-0, *myb72-2* and oxMYB72 seedlings growing in Hoagland medium were inoculated with WCS417 by applying a 10-μl bacterial suspension (10^7 CFU ml^{-1}) to the primary root of each seedling. Forty-eight hours later, three biological replicates of mock- and WCS417-treated roots were harvested for microarray analysis. Genes that are induced more than two-fold in wild-type Col-0 roots and show more than two-fold deregulation in the *myb72-2* mutant are depicted ($P < 0.05$). (c) Overlap between WCS417-upregulated genes in Col-0 and constitutively upregulated genes in oxMYB72 ($P < 0.05$ and more than two-fold induction; see gene lists in Supporting Information Tables S2–S4).

biological replicates and whole-genome transcript profiles were generated using Affymetrix ATH1 GeneChips. In wild-type Col-0 plants, 298 genes were significantly upregulated and showed a more than two-fold change in expression on colonization of the roots by WCS417, whereas 218 genes were significantly downregulated ($P < 0.05$ with an additional cut-off value of more than two-fold change; Table S2). To identify WCS417-inducible genes that are regulated by MYB72, we analyzed the expression of this WCS417-regulated gene set in *myb72-2* and oxMYB72 plants. Of the WCS417-upregulated genes, a small set of five genes was significantly impaired in their ability to be induced by WCS417 in the roots of *myb72-2* plants ($P < 0.05$ and more than two-fold change) (Fig. 1b). qRT-PCR analysis further validated these genes as MYB72 targets (Fig. S1). Overexpression of MYB72 in the *myb72-2* mutant background (oxMYB72) did not constitutively activate these genes, but restored their WCS417 responsiveness, indicating that the WCS417-inducible expression of these genes is MYB72 dependent (Figs 1b, S1). Amongst these five MYB72-dependent, WCS417-inducible genes, three genes have been reported previously to be controlled by FIT, the central transcriptional regulator of the iron deficiency response Strategy I in Arabidopsis roots (Colangelo & Guerinot, 2004). These three FIT- and MYB72-regulated genes encode the nitrate transporter NRT1.8 (At4g21680), the β-glucosidase BGLU42 (At5g36890) and the cytochrome P450 monooxygenase CYP71B5 (At3g 53280). The MYB72-dependent genes *bHLH39* (At3g56980), which encodes the FIT-interacting transcription factor bHLH39 (Yuan *et al.*, 2008), and At5g55620, encoding an unknown protein, are not FIT-regulated, but are also upregulated at the early stages of iron deficiency (Buckhout *et al.*, 2009). By applying less stringent criteria ($P < 0.05$ without the additional cut-off value of more than two-fold change), we identified four additional WCS417-inducible MYB72 target genes, including the iron- and FIT-regulated genes *4CL2* (At3g21240), encoding the 4-COUMARATE-COA LIGASE (4CL) isoform 4CL2, *PDR9* (At3g53480), encoding the ABC transporter PLEIOTROPIC DRUG RESISTANCE 9, and *At3g61930*, encoding a protein of unknown function (Fig. S2). The ninth MYB72 target gene encodes a member of the PHT1 family of phosphate transporters (At5g43360; PHT1;3). Together, these results indicate that the majority of the MYB72-regulated genes that are activated in the Arabidopsis root in response to colonization by ISR-inducing WCS417 bacteria encode proteins that function in the iron deficiency response.

By comparing the transcriptomes of mock-treated Col-0 and oxMYB72 plants, we further identified 195 genes that were constitutively upregulated and 66 genes that were constitutively downregulated in the roots of oxMYB72 seedlings ($P < 0.05$ with additional cut-off value of more than two-fold change; Table S3). Of the constitutively upregulated genes in oxMYB72, 37 genes were also significantly upregulated by WCS417 in Col-0 (Fig. 1c; Table S4). This, in turn, suggests that MYB72 regulates broad transcriptional programs in the roots of Arabidopsis, possibly redundantly with its closest paralog MYB10.

Overexpression of the MYB72 target gene *BGLU42* results in constitutive disease resistance

Previously, we have demonstrated that overexpression of *MYB72* does not lead to the constitutive expression of ISR in Arabidopsis (Van der Ent *et al.*, 2008), suggesting that MYB72 may act in concert with one or more other WCS417-activated factors in the onset of ISR. To identify components that function downstream of MYB72 during the establishment of ISR, we focused on the selection of five WCS417-inducible genes that were more than two-fold misregulated in the *myb72-2* mutant (Fig. 1b). None of these genes was constitutively activated in the oxMYB72 line, suggesting that MYB72 by itself is not sufficient for their expression. However, the WCS417 inducibility of all five genes was restored in the *MYB72*-complemented oxMYB72 line. We therefore hypothesized that the upregulation of one of these MYB72 targets may be the limiting step in the initiation of WCS417-ISR. If this was the case, the overexpression of such a gene should confer enhanced resistance against pathogens that are sensitive to WCS417-ISR, such as the necrotrophic pathogen *B. cinerea* (Van der Ent *et al.*, 2008) and the biotrophic pathogens *H. arabidopsidis* (Luna *et al.*, 2014) and *P. syringae* pv. *tomato* DC3000 (Pieterse *et al.*, 1996). To this end, we generated lines overexpressing *At5g55620* (oxAt5g55620), *BGLU42* (oxBGLU42) and *CYP71B5* (oxCYP71B5) and obtained transgenic lines overexpressing *NRT1.8* (oxNRT1.8) (Li *et al.*, 2010) and *bHLH39* (oxbHLH39) (Yuan *et al.*, 2008). We first tested these transgenic lines for disease resistance against *B. cinerea*. As shown in Fig. 2(a), the oxAt5g55620, oxCYP71B5, oxNRT1.8 and oxbHLH39 lines showed similar levels of susceptibility as wild-type Col-0 to *B. cinerea*. However, three independent oxBGLU42 lines displayed significantly enhanced resistance against *B. cinerea*. Overexpression of *BGLU42* also conferred resistance against the pathogens *H. arabidopsidis* and *P. syringae* pv. *tomato* DC3000 (Fig. 2b,c). Together, these results indicate that the overexpression of the MYB72 target gene *BGLU42* confers enhanced resistance against a broad spectrum of foliar pathogens.

Knockout mutant *bglu42* is defective in WCS417-mediated ISR

To examine whether *BGLU42* is required for WCS417-mediated ISR, we obtained a knockout line from the SALK collection (Alonso *et al.*, 2003) carrying a T-DNA insert in the 12th exon in sense orientation (designated *bglu42*; Fig. 3a), and tested the ability of this mutant to express WCS417-ISR against *P. syringae* pv. *tomato* DC3000. To this end, Col-0, *myb72-2* and *bglu42* plants, growing in the presence or absence of WCS417 bacteria, were inoculated with *P. syringae* pv. *tomato* DC3000. Colonization of the roots of Col-0 by WCS417 resulted in a moderate but significant reduction in the development of disease symptoms caused by the pathogen (Fig. 3b). By contrast, the *myb72-2* mutant did not develop ISR, confirming previous findings (Van der Ent *et al.*, 2008). Similar to *myb72-2*, *bglu42* plants growing in the presence of WCS417 did not mount enhanced resistance against *P. syringae* pv. *tomato* DC3000 (Fig. 3b). These data

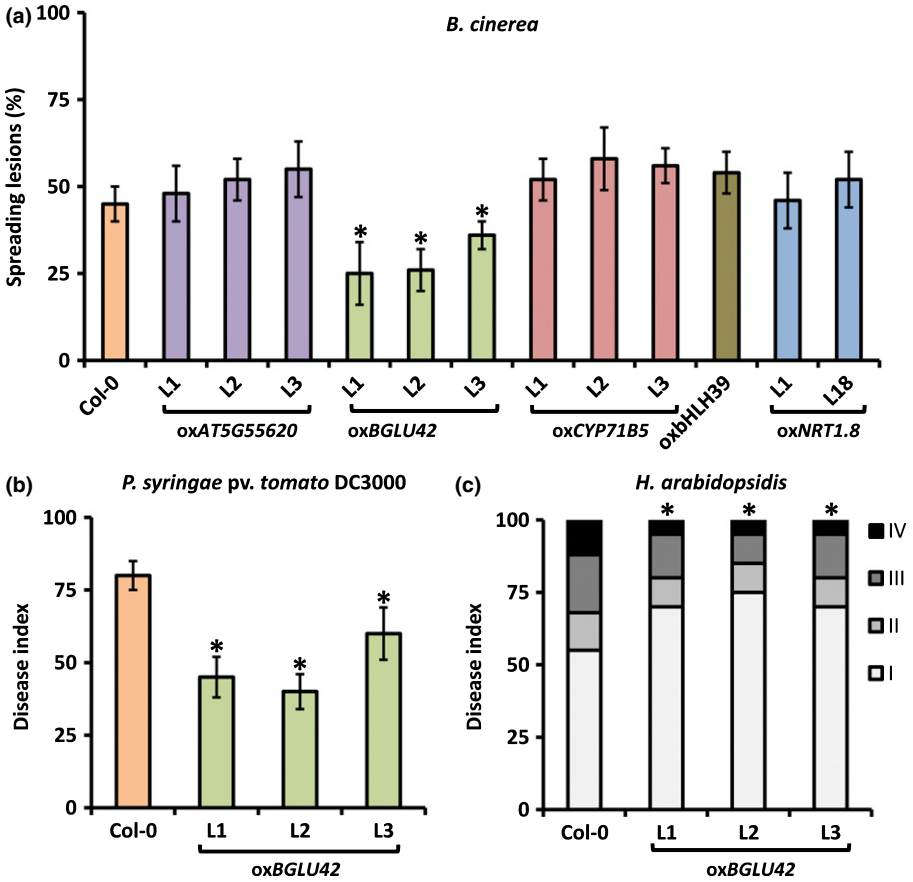

Fig. 2 Level of disease resistance in transgenic Arabidopsis lines overexpressing the MYB72 target genes *At5g55620*, *BGLU42*, *CYP71B5*, *bHLH39* and *NRT1.8*. Levels of disease severity in Col-0 and independent transgenic lines (L1, L2, L3 or L18) overexpressing *At5g55560*, *BGLU42*, *CYP71B5*, b*HLH39* or *NRT1*.8 on inoculation with (a) *Botrytis cinerea*, (b) *Pseudomonas syringae* pv. *tomato* DC3000 or (c) *Hyaloperonospora arabidopsidis*. For *B. cinerea* infections, disease symptoms were determined 5 d after inoculation. Disease ratings are expressed as the percentage of leaves showing spreading lesions. For *P. syringae* pv. *tomato* DC3000 infections, disease symptoms were determined 4 d after inoculation. Disease ratings are expressed as the percentage of leaves showing necrotic or water-soaked lesions surrounded by chlorosis. For *H. arabidopsidis* infections, disease severity was determined 10 d after inoculation. Disease ratings are expressed as the percentage of leaves in disease severity classes: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered with sporangia; IV, > 50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. Asterisks indicate statistically significant differences compared with Col-0 (a and b, Student's *t*-test; $P < 0.05$; c, χ^2-test; $\alpha = 0.05$). Error bars, ± SD.

indicate that BGLU42 is an essential component for the onset of WCS417-mediated ISR.

To investigate the tissue-specific expression pattern of *BGLU42* on root colonization by WCS417, we generated transgenic *pBGLU42:GFP-GUS* lines expressing a GFP-GUS fusion protein under the control of the 1.7-kb promoter region of the *BGLU42* gene, and examined GFP expression patterns in roots under basal and WCS417-induced conditions. Confocal imaging of independent lines showed that, in the absence of WCS417 bacteria, the *BGLU42* promoter is active at low levels predominantly in the epidermal cells (Fig. 3c). However, on colonization of the roots by WCS417, strong *pBGLU42:GFP-GUS* expression was detected in root trichoblasts and, to a lesser extent, in cortical cells (Fig. 3d). This, in turn, suggests that MYB72-depedent transcriptional changes occurring in the root epidermis in response to colonization by WCS417 bacteria are critical for the establishment of ISR.

MYB72 upregulates biosynthesis genes of secondary metabolites involved in iron uptake

Under iron deficiency conditions, MYB72 and its paralog MYB10 have recently been shown to regulate the expression of the NA synthase genes *NAS2* and *NAS4*, involved in the biosynthesis of the iron chelator NA (Palmer *et al.*, 2013). Considering the dual role of MYB72 in the onset of ISR and the iron deficiency response, we decided to take a closer look at the genes whose expression was constitutively activated in the roots of oxMYB72 plants in the absence of WCS417 bacteria (Fig. 1c). Classification of the 195 MYB72-upregulated genes (Table S3) into biological categories using AmiGO Term Enrichment Software revealed a clear over-representation of secondary metabolic processes related to phenylpropanoid metabolism (Table S5). In particular, amongst the 195 upregulated genes in the oxMYB72 line, many genes encode enzymes of the shikimate and general phenylpropanoid pathway, and enzymes involved in coumarin biosynthesis (Fig. 4a,b). In the phenylpropanoid pathway, the conversion of caffeoyl CoA to feruloyl CoA, the biosynthetic precursor of coumarins, is catalyzed by caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT), which utilizes S-adenosyl-methionine (SAM) as methyl donor. In addition to its important function in transmethylation reactions, SAM has a critical role in the metabolism of iron-deficient roots, because it is the precursor of the iron chelator NA (Lan *et al.*, 2011). SAM is synthesized from L-methionine (L-Met) by S-methionine (S-Met) adenosyltransferases (SAM synthetases), also known as methionine adenosyltransferases (MATs), whereas the conversion of SAM to NA is mediated by NA synthases (Lan *et al.*, 2011). In addition to *NAS2*, which is upregulated in the roots of oxMYB72 plants, two SAM synthetases were also constitutively upregulated (Fig. 4c). This, in turn, indicates that MYB72 transcriptionally regulates the biosynthetic steps involved in the production of NA.

Fluorescent phenolic compounds that are produced via the phenylpropanoid route and excreted in the root vicinity via the

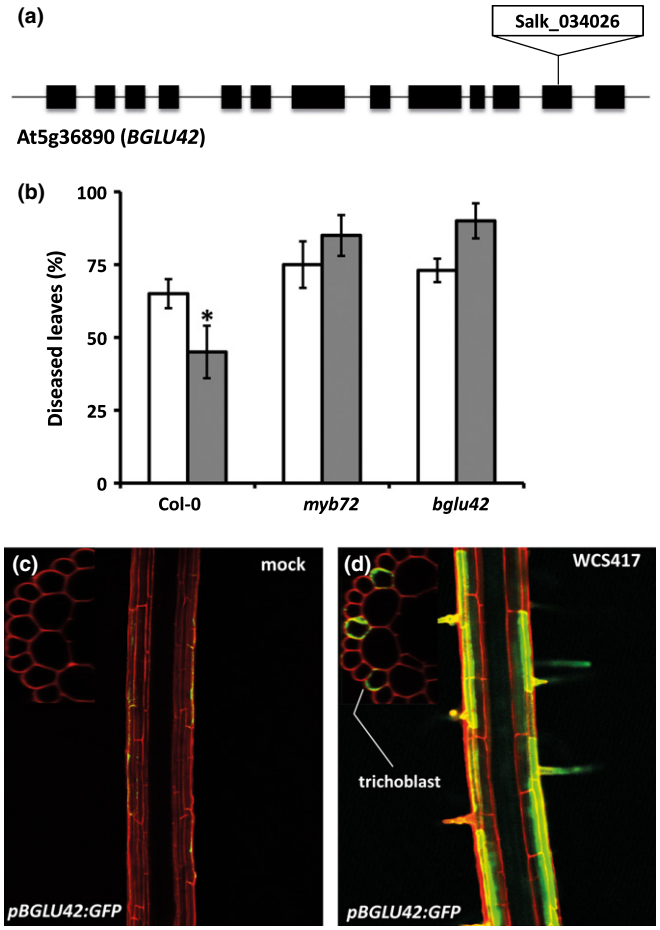

Fig. 3 WCS417-induced systemic resistance (ISR) against *Pseudomonas syringae* pv. *tomato* DC3000 is blocked in the *blgu42* mutant. (a) Genomic structure of the *BGLU42* gene and position of the T-DNA insertion in the *bglu42* mutant. Exons are indicated as black boxes. (b) Level of disease severity in control (open bars) and WCS417-treated (closed bars) Col-0, *myb72-2* and *bglu42* plants on inoculation with *P. syringae* pv. *tomato* DC3000. ISR was induced by growing plants for 3 wk in soil containing *Pseudomonas fluorescens* WCS417 bacteria. Five-week-old plants were challenge inoculated with a *P. syringae* pv. *tomato* DC3000 bacterial suspension. The percentage of diseased leaves was assessed 4 d after inoculation. Asterisks indicate statistically significant differences compared with non-induced plants (Student's *t*-test; $P < 0.05$). (c, d) Tissue-specific expression pattern of *BGLU42*. Representative confocal images of longitudinal and transverse optical sections of roots of *pBGLU42:GFP-GUS* seedlings that were either mock treated (c) or treated with WCS417 bacteria (d). Error bars, ± SD.

ABC transporter PDR9 have been shown to play critical roles in iron acquisition by facilitating iron mobilization from alkaline substrates (Rodríguez-Celma *et al.*, 2013; Fourcroy *et al.*, 2014; Schmid *et al.*, 2014). Interestingly, we found *PDR9* to be amongst the WCS417-induced genes that are regulated in a MYB72-dependent manner (Fig. S2). To examine whether the *myb72-2* mutant shows defects in the biosynthesis and/or secretion of fluorescent phenolic compounds, we grew Col-0, *myb72-2* and oxMYB72 seedlings on medium with sufficiently available iron (10 μM Fe(III)EDTA, pH 5.8) or on medium with very low iron availability (10 μM $FeCl_3$, pH 7.0) (Rodríguez-Celma *et al.*, 2013). We also included in this experiment the ISR-defective mutant *bglu42* in order to examine whether BGLU42 is involved in the production or secretion of root-derived phenolics. By employing qRT-PCR analysis on the iron deficiency markers *FRO2* and *IRT1*, we validated the induction of the iron deficiency response in this setup (Fig. S3). As shown in Fig. 5(a), under conditions of sufficient iron, fluorescent phenolic compounds typically accumulated at low levels predominantly in the root interior of Col-0, *myb72-2* and *bglu42* roots, and strongly within the roots of oxMYB72 seedlings. Under conditions of low iron availability, wild-type Col-0 plants accumulated higher levels of fluorescent compounds in the root interior, and secreted more of these compounds into the medium, relative to Col-0 seedlings growing on medium with available iron (Fig. 5a). By contrast, the roots of the *myb72-2* seedlings accumulated and secreted less fluorescent compounds than wild-type seedlings under the same conditions. Both the production and secretion of these compounds were restored in the oxMYB72 line under conditions of iron deficiency (Fig. 5a). Interestingly, the *bglu42* mutant accumulated more phenolics in the root interior than Col-0, and showed reduced excretion of phenolics (Fig. 5a). To further validate the role of MYB72 and BGLU42 in the production and secretion of root-produced phenolics under conditions of iron deficiency, we employed a 96-well plate assay in which we quantified the fluorescence emitted by root exudates under conditions of available and non-available iron. As shown in Fig. 5(b), the *myb72-2* and *bglu42* mutants secreted, under conditions of iron deficiency, significantly less fluorescent phenolics relative to Col-0, whereas the oxMYB72 line fully complemented the *myb72-2* genotype in terms of secretion. These results suggest that MYB72 is involved in the biosynthesis of phenolic compounds that are produced in roots under low iron conditions, and that MYB72-mediated induction of *BGLU42* plays a role in their secretion.

Discussion

Iron deficiency: the root of rhizobacteria-mediated ISR?

Immune elicitors from beneficial soil-borne microbes have long been considered as molecular determinants for the elicitation of ISR (Meziane *et al.*, 2005; Bakker *et al.*, 2007). However, ISR is a complex phenomenon in which multiple bacterial determinants have been shown to differentially activate various signaling pathways involved in disease resistance (De Vleesschauwer & Höfte, 2009). The identification of the root-specific MYB72 transcription factor as an essential early signaling component of *Pseudomonas*- and *Trichoderma*-mediated ISR (Van der Ent *et al.*, 2008; Segarra *et al.*, 2009; Pieterse *et al.*, 2014), combined with its role in iron deficiency responses in Arabidopsis roots (Colangelo & Guerinot, 2004; De Mortel *et al.*, 2008; Buckhout *et al.*, 2009; Palmer *et al.*, 2013), pointed to the possibility that selected ISR-eliciting microbes may recruit iron deficiency signaling to trigger ISR. In this study, we provide clear evidence for this notion by our observation that the majority of the WCS417-inducible genes that are regulated by MYB72 have putative or demonstrated functions in iron homeostasis. Of the five

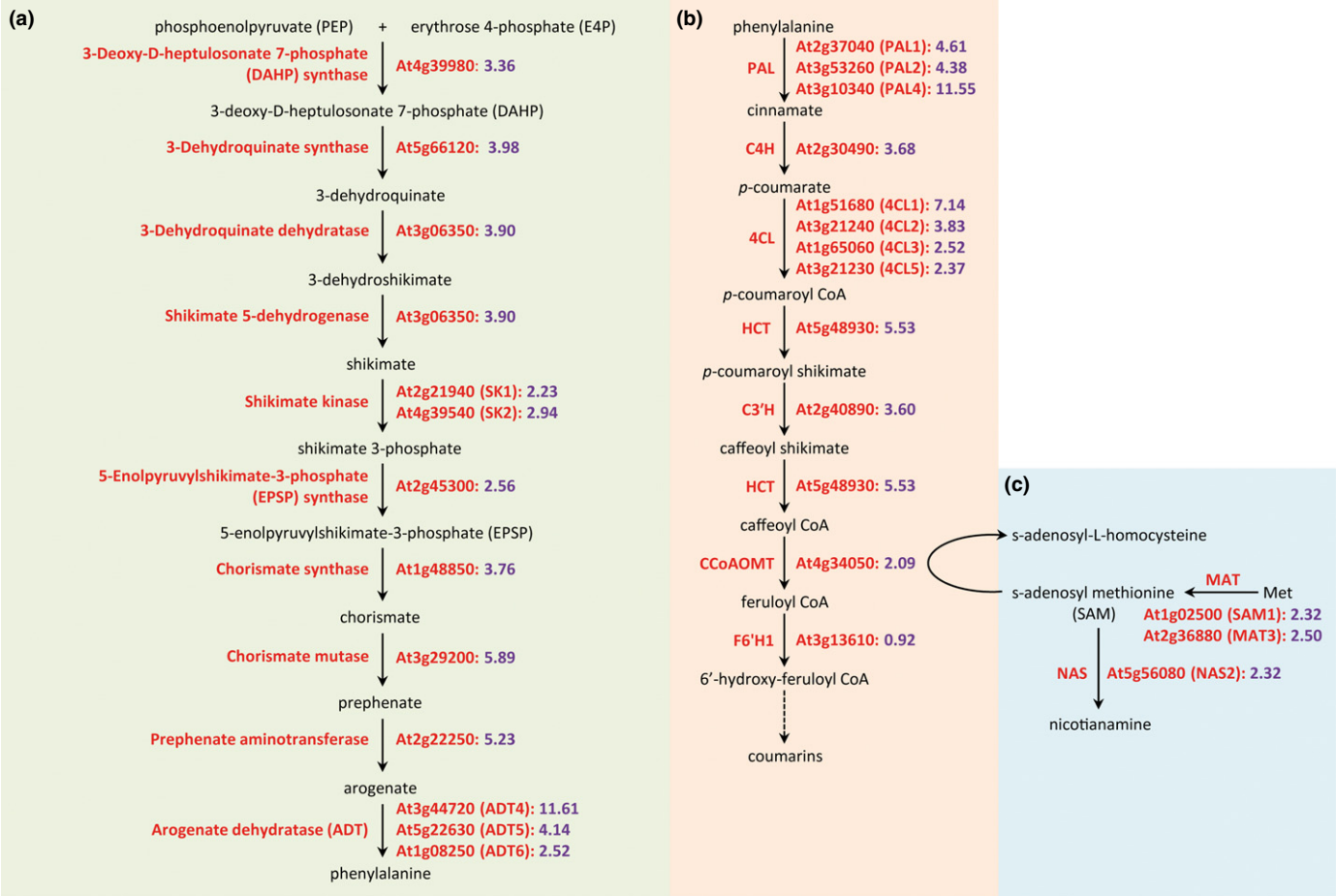

Fig. 4 MYB72 overexpression upregulates biosynthetic genes of the shikimate, phenylpropanoid and nicotianamine biosynthesis pathways. Coordinated induction of Arabidopsis genes encoding key enzymes of the shikimate (a), phenylpropanoid (b) and nicotianamine (c) biosynthetic pathways in the roots of 17-d-old oxMYB72 seedlings. Depicted are the major intermediates and enzymes, and the average fold induction (numbers in purple) of the corresponding genes compared with wild-type Col-0 as revealed by microarray analysis (Table S3). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, hydroxycinnamoyl-coenzyme A shikimate:quinate hydroxycinnamoyl-transferase; C3′H, *p*-coumaroyl shikimate 3′ hydroxylase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; F6′H1, feruloyl-CoA 6′-hydroxylase1; MAT, methionine adenosyltransferase (SAM synthetases); NAS, nicotianamine synthase.

WCS417-inducible MYB72 targets, *BGLU42*, *CYP71B5* and *NRT1.8* are controlled by the central regulator of the iron deficiency response FIT (Colangelo & Guerinot, 2004), suggesting that these genes are not primary targets of FIT, but are indirectly regulated via MYB72. The other two targets, *At5g55620* and *bHLH39*, the latter of which codes for the FIT-interacting transcription factor bHLH39 (Yuan *et al.*, 2008), are also activated under conditions of iron limitation (Buckhout *et al.*, 2009). How are root-colonizing rhizobacteria capable of activating iron deficiency-regulated mechanisms in plant roots? Soil-borne Pseudomonads are known to produce and secrete iron-chelating siderophores that may deplete iron from the rhizosphere (Bakker *et al.*, 2007; Lugtenberg & Kamilova, 2009), thereby triggering iron deficiency responses in the roots. Alternatively, the activation of iron deficiency responses may stem from a sophisticated manipulation of the host's iron sensing systems. Future studies focusing on the mechanisms by which ISR-inducing microbes activate iron deficiency-inducible mechanisms in host plants are needed to address these issues.

BGLU42 is a novel regulator of rhizobacteria-mediated ISR

Of the identified WCS417-inducible MYB72 target genes, we pinpointed the β-glucosidase gene *BGLU42* as a novel regulator of ISR in Arabidopsis roots (Fig. 3b). Although mutation of the *BGLU42* gene blocked WCS417-ISR, overexpression of *BGLU42* resulted in a broad-spectrum disease resistance (Fig. 2), suggesting that MYB72-mediated induction of *BGLU42* on colonization of the roots by WCS417 is sufficient for the onset of ISR. *BGLU42* encodes a β-glucosidase of the glycoside hydrolase (GH) family 1 (Xu *et al.*, 2004). GHs hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Glucosidases play important roles in diverse aspects of plant physiology and have critical roles in plant defense responses by liberating either chemical effectors or signaling molecules from conjugated glucosides (Morant *et al.*, 2008; Bednarek *et al.*, 2009; Clay *et al.*, 2009; Ahmad *et al.*, 2011). In this study, we provide evidence that functional BGLU42 is important for the secretion of fluorescent

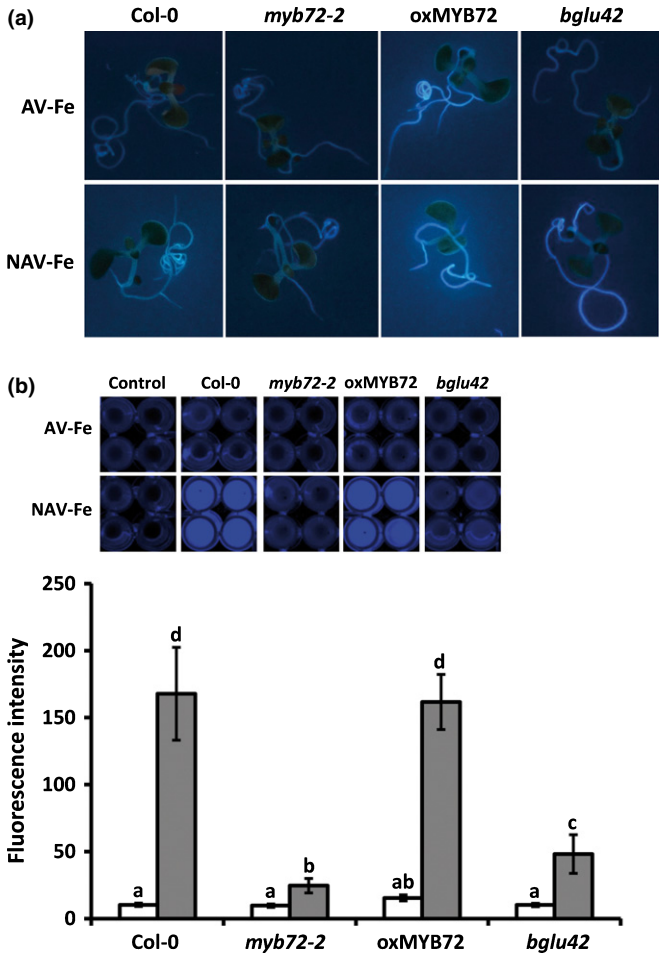

Fig. 5 MYB72 is required for the production and secretion of fluorescent phenolic compounds under iron-limited conditions. (a) Visualization of fluorescent phenolic compounds that are produced and secreted by the roots of 6-d-old Arabidopsis seedlings of the indicated genotypes grown under conditions of sufficiently available iron (AV-Fe: 10 μM Fe(III)EDTA, pH 5.8) and low iron availability (NAV-Fe: 10 μM $FeCl_3$, pH 7.0). Photographs were taken after excitation with 365-nm UV light. (b) Relative quantification of fluorescent phenolic compounds in the medium of 6-d-old seedlings grown in a 96-well plate under the indicated conditions. Fluorescence was quantified in a 96-well microplate reader (excitation at 360 nm; emission at 528 nm) after removing seedlings from the growth medium. AV-Fe, open bars; NAV-Fe, closed bars. Data represent the mean fluorescence intensity in the root exudates of 20 seedlings ± SD. Different letters indicate statistically significant differences (Tukey's honestly significant difference (HSD) test; $P < 0.05$).

phenolic compounds, which are produced and secreted by the roots on iron limitation to mobilize iron from insoluble sources. In line with this function of BGLU42, we found *pBGLU42:GFP-GUS* to be predominantly expressed in the root hairs and trichoblasts of WCS417-colonized roots. Secondary metabolites with potential toxicity for the plant cell are commonly produced and/or stored in the form of inactive glucosides and secreted after being processed by GHs (Morant *et al.*, 2008). Thus, it is reasonable to speculate that BGLU42 functions in the processing of glucosylated phenolic compounds as an essential step for their secretion in the root vicinity.

Accumulating evidence suggests that the initiation of ISR results from a sophisticated dialogue between host plants and beneficial microbes during which both partners reciprocally communicate with chemical signals (Zamioudis & Pieterse, 2012; Pieterse *et al.*, 2014). For instance, gene expression studies in the ISR-eliciting fungus *Trichoderma virens* revealed that the hydrolysis of maize-derived sucrose is essential for the upregulation of Sm1, the *Trichoderma*-secreted elicitor that systemically activates defense mechanisms in maize leaves (Vargas *et al.*, 2009). Phenolics have a well-established role in plant defense as they can serve as antimicrobials against certain pathogens (Boudet, 2007). In addition to this role, various plant-derived phenolics have been shown to serve as specific substrates or to act as signaling molecules for the onset of important microbial functions in the soil, such as flavonoids in the *Rhizobium*–legume symbiosis (Shaw *et al.*, 2006; Badri *et al.*, 2013). Hence, it is tempting to speculate that, during the initiation of ISR, MYB72- and BGLU42-dependent exudation of phenolic compounds in the rhizosphere may, in turn, elicit responses in ISR-triggering rhizobacteria that are necessary for the production of ISR-eliciting molecules (systemic elicitors). In support of this hypothesis, root exudates of iron-deprived maize plants have been shown to elicit transcriptional responses in the soil bacterium *Bacillus amyloliquefaciens* FZB42 (Carvalhais *et al.*, 2013). Alternatively, the putative BGLU42-liberated phenolics may be relocated and subsequently transported to the root interior to function as short- or long-distance signaling molecules. This latter scenario is supported by the fact that the overexpression of *BGLU42* is sufficient to confer enhanced disease resistance against different pathogens. Revealing the chemical identity of the BGLU42 substrate(s) in Arabidopsis and its impact on the transcriptome and/or metabolome of ISR-inducing microbes is anticipated to provide important insights into the ISR phenomenon.

Novel functions of MYB72 in the iron deficiency response

Palmer *et al.* (2013) have recently reported that MYB72 and MYB10 have critical functions in iron homeostasis by regulating the transcription of the NA synthase genes *NAS2* and *NAS4*. Interestingly, the same study also identified *BGLU42* as a MYB72/MYB10-regulated gene (Palmer *et al.*, 2013). Our study suggests that MYB72, possibly redundantly with MYB10, has additional functions in iron homeostasis by communicating the iron deficiency response to nitrogen and phosphate homeostasis, or by orchestrating the production and exudation of iron-mobilizing metabolites. In particular, we found MYB72 to partially regulate the expression of genes encoding the nitrate transporter NRT1.8 and the phosphate transporter PHT1;3. NRT1.8 was identified as a proton-dependent, low-affinity transporter specialized to export nitrate from xylem vessels to xylem parenchyma cells (Li *et al.*, 2010). PHT1;3 belongs to the PHT1 family of phosphate transporters. It is expressed in the epidermis of lateral roots and, more specifically, in the root hair-producing trichoblasts, suggesting that this transporter is involved in the uptake of phosphate from the root vicinity. However, in the primary roots, as well as in the lateral–primary root junctions, *PHT1;3*

expression is restricted to the stele (pericycle), suggesting that PHT1;3 may play a scavenging role by re-absorbing the phosphate that leaks from the xylem vessels into the apoplastic spaces (Mudge *et al.*, 2002). Cross-talk amongst signaling pathways that mediate responses to various nutrient deficiencies frequently occurs in plants in order to retain mineral homeostasis and ensure survival. For instance, in Arabidopsis, phosphate has an antagonistic cross-talk with nitrate (Kant *et al.*, 2011). In addition, phosphate and zinc transport and homeostasis are highly co-regulated processes (Khan *et al.*, 2014). Although more research is clearly required, our findings indicate that MYB72, and probably MYB10, may have a critical function in communicating the iron deficiency response to nitrogen and phosphate homeostasis by selectively regulating the expression of genes involved in nitrogen and phosphate transport. In addition to *NRT1.8* and *PHT1;3*, we found MYB72 to regulate the expression of *At5g55620* and *CYP71B5* genes that are also strongly upregulated under conditions of iron deficiency (Buckhout *et al.*, 2009). Despite their elusive role in iron deficiency responses, both are predicted to participate in metabolic processes that occur in the plastids (Yang *et al.*, 2010). Importantly, our study demonstrates that MYB72 upregulates the expression of many genes involved in the shikimate pathway and the general phenylpropanoid pathway, metabolic routes that lead to the production and secretion of phenolic compounds that aid in iron mobilization in the root vicinity (Rodríguez-Celma *et al.*, 2013; Fourcroy *et al.*, 2014; Schmid *et al.*, 2014). This clearly suggests a function of MYB72/MYB10 in processes directly linked to iron uptake.

Phenylpropanoids encompass an important class of secondary metabolites with essential roles in a plethora of biological processes, from structural support to biotic and abiotic stress tolerance (Dixon & Paiva, 1995). In the iron deficiency response, phenolic compounds have been shown to function as iron-mobilizing compounds (Rodríguez-Celma *et al.*, 2013; Fourcroy *et al.*, 2014; Schmid *et al.*, 2014). In Arabidopsis and other plant species, several members of the R2R3-MYB family have been found to regulate the expression of genes encoding biosynthetic enzymes of the shikimate and the general phenylpropanoid pathway (Vogt, 2010). Moreover, downstream of these pathways, different MYB transcription factors have been shown to differentially (co-)regulate the expression of genes involved in different metabolic sub-pathways, suggesting a role for MYB proteins in regulating the specificity towards metabolic endproducts. For instance, the closely related MYB46 and MYB83 transcription factors (McCarthy *et al.*, 2009; Zhong & Ye, 2012), as well as the phylogenetically more distinct MYB58 and MYB63 transcription factors (Zhou *et al.*, 2009), have been shown to regulate secondary cell wall formation downstream of the NAC domain transcription factor SND1 (Zhong *et al.*, 2006, 2008; Mitsuda *et al.*, 2007) by activating the expression of lignin biosynthetic genes. In addition, the synthesis of anthocyanins in Arabidopsis is controlled by the closely related MYB transcription factors PAP1/MYB75, PAP2/MYB90, MYB113 and MYB114 that stimulate the transcription of both general phenylpropanoid- and anthocyanin-specific genes (Schwinn *et al.*, 2006).

Overall, our results uncovered BGLU42 as a novel regulator of rhizobacteria-mediated ISR that acts downstream of MYB72 in the roots of Arabidopsis. We also demonstrate that MYB72 and BGLU42 have a dual role in the onset of ISR and the activation of iron uptake mechanisms, suggesting a thus far unidentified mechanistic link between the ability of beneficial root-colonizing bacteria to stimulate systemic immunity and mechanisms induced by iron deficiency in the host roots.

Acknowledgements

We acknowledge Dr Hong-Qing Ling (State Key Laboratory of Plant Cell and Chromosome Engineering, China) for providing the *bHLH39*-overexpressing line and Dr Ji-Ming Gong (National Key Laboratory of Plant Molecular Genetics, China) for providing the *NRT1.8*-overexpressing lines. We thank Hans van Pelt for excellent technical assistance. This work was supported by ERC Advanced Grant 269072 of the European Research Council.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of MYB72-dependent genes in mock- and WCS417-treated roots.

Fig. S2 Heat map of the expression of MYB72-dependent genes that are induced by WCS417 in Arabidopsis roots, as revealed by microarray analysis.

Fig. S3 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of the iron deficiency markers *FRO2* and *IRT1* in the roots of Arabidopsis under low and high iron availability.

Table S1 List of primers used in this study

Table S2 Up- and downregulated genes in Arabidopsis Col-0 roots in response to colonization by *Pseudomonas fluorescens* WCS417 bacteria

Table S3 Up- and downregulated genes in mock-treated roots of oxMYB72 plants in comparison with mock-treated roots of Col-0 plants

Table S4 Genes that are upregulated in roots of both mock-treated ox.MYB72 and WCS417-treated Col-0 plants

Table S5 Gene ontology (GO) analysis of constitutively upregulated genes in the roots of oxMYB72 seedlings

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