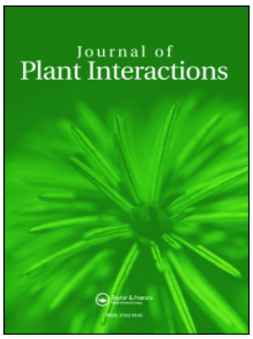

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Identifying receptor-like kinases that enable *Caulobacter* RHG1 to promote plant growth in *Arabidopsis thaliana*

Amber Lampens[a,b,c,d], Michiel Vandecasteele[a,b], Marjon Braem[a,b], Viktor Devlieghere[a,b], Judith Van Dingenen[a,b], Kris Gevaert[c,d] and Sofie Goormachtig[a,b]

[a]Department of Plant Biotechnology and Bioinformatics, Ghent University Zwijnaarde, Belgium; [b]Center for Plant Systems Biology, Zwijnaarde, Belgium; [c]Department of Biomolecular Medicine, Ghent University, Zwijnaarde, Belgium; [d]Center for Medical Biotechnology, Zwijnaarde, Belgium

ABSTRACT

Plants express an array of receptor-like kinases (RLKs) to control development and communicate with their environment. Many RLKs are uncharacterized and some of them are expected to regulate plant responses to plant growth-promoting rhizobacteria (PGPR). Despite documented effects induced by *Caulobacter* RHG1, the underlying signaling pathways and the involved RLKs remain uncharted. Through a targeted RLK mutant screening, we aimed to decipher the receptors that steer the *Caulobacter* RHG1-induced growth promotion in *Arabidopsis thaliana*. We identified four RLKs that are pivotal in the RHG1-*Arabidopsis* interaction, including the coreceptors SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1) and BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1/SERK3), which act redundantly in the RHG1-Arabidopsis interaction, possibly by interplaying with the unknown RLK AT3G28040 and the immunity-related ELONGATION FACTOR-TU RECEPTOR (EFR). These results shed new light on the molecular dynamics orchestrating plant responses to PGPR, and concurrently contribute a crucial piece to the intricate puzzle of RLK interactions.

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KEYWORDS
Caulobacter; mode-of-action; PGPR; RLK; stress response; immunity

Key policy highlights

- Four RLKs; BAK1, SERK1, EFR, and AT3G28040 (RRHG) are involved in the RHG1-*Arabidopsis* interaction.
- BAK1 and SERK1, two well-described co-receptors, act redundantly and play a pivotal role in the RHG1-driven growth promotion, possibly by interplaying with the unknown RLK AT3G28040 and the immunity-related RLK EFR.
- Most known development – and immunity-related RLKs barely influence RHG1-driven plant growth promotion in *Arabidopsis*.

Introduction

Plant growth-promoting rhizobacteria (PGPR) offer sustainable alternatives to chemical fertilizers in agriculture, contributing to enhanced plant growth and development (Lugtenberg and Kamilova 2009; Vacheron et al. 2013). Among these, *Caulobacter* RHG1 (RHG1), which has been isolated from *Zea mays* (maize) roots (Beirinckx et al. 2020), has been shown to instigate a plant growth-promoting (PGP) effect in *Arabidopsis thaliana* (Arabidopsis), colonizing both leaf and root surfaces (Luo et al. 2019). Furthermore, RHG1 stimulates Arabidopsis shoot and root growth by enhancing leaf initiation and increasing cell division in the leaf and in the root apical meristem of the primary root (Luo et al. 2019). Mode-of-action studies demonstrated that brassinosteroid (BR) biosynthesis and signaling pathways are required for the PGP effect, whereas other classical plant growth hormones such as auxin and cytokinins are not involved (Luo et al. 2019).

Such intricate plant signaling networks are often initiated by receptor-like kinases (RLKs), an extensive protein family with more than 600 members in Arabidopsis (Lehti-Shiu et al. 2009; Taj et al. 2010; Lehti-Shiu and Shiu 2012; Zipfel 2014; Zhang et al. 2018; Jose et al. 2020). Many of these RLKs exert functions in processes that are influenced by RHG1, including microbial recognition and immunity, growth and development and abiotic stress responses (Jose et al. 2020). Despite their importance, the majority of RLKs remain functionally uncharacterized (Wu et al. 2016).

RLKs can be subdivided in various types or families based on the signatures present in their extracellular domain. The best described families include the leucine-rich repeat (LRR), lectin (Lec), wall-associated kinase (WAK), self-incompatibility domain (S-domain) and lysin motif (LysM) families (Jose et al. 2020). Distinct RLK families can also be discerned based on their functional specialization. Pattern recognition receptors (PRRs) are RLKs that elicit immunity responses in the plant upon the recognition of microbe-associated or damage-associated molecular patterns (MAMPs or

CONTACT Sofie Goormachtig sofie.goormachtig@psb.vib-ugent.be Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Zwijnaarde, Belgium

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DAMPs) (Van Wees et al. 2008; Newman et al. 2013; Zipfel 2014; Saijo et al. 2018; Thoms et al. 2021). One of the best characterized immunity eliciting PRRs is the ELONGATION FACTOR-TU RECEPTOR (EFR), which recognizes elongation factor-Tu (EF-Tu) and more specifically, the peptide epitope elf18 (elf18) (Kunze et al. 2004; Zipfel et al. 2006). Other microbial ligands such as flagellin, peptidoglycan, chitin, oligogalacturonides or lipopolysaccharide, are recognized by specific RLK complexes including FLAGELLIN-SENSITIVE2 (FLS2), LYSM DOMAIN PROTEIN (LYM) 1, LYM3, and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), LYSM CONTAINING RECEPTOR-LIKE KINASE (LYK) 4, LYK5 and LYM2 or CELL WALL ASSOCIATED KINASE 1 (WAK1) or S-DOMAIN 1–29 (SD1-29), respectively (Gómez-Gómez and Boller 2000; Miya et al. 2007; Brutus et al. 2010; Willmann et al. 2011; Wan et al. 2012; Cao et al. 2014; Ranf et al. 2015; Buendia et al. 2018).

In addition, RLKs play important roles in plant development, like the well-known BR receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Nam and Li 2002; Russinova et al. 2004; Gou and Li 2020). BRs are steroid hormones with essential roles in plant growth and development by regulating for example cell elongation and cell division (Nolan et al. 2020). Furthermore, RLKs contribute to the perception of abiotic stresses, such as drought and osmotic stress (Osakabe et al. 2013; Ye et al. 2017).

The complexity of RLKs is further extended by the fact that they form complexes with other receptors and coreceptors, triggering trans-autophosphorylation and activating downstream signaling pathways (Chakraborty et al. 2019). The coreceptors of the largest LRR-RLK family belong to the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) subfamily, represented by five members in Arabidopsis (SERK1-5) (Chakraborty et al. 2019). SERK proteins are highly homologous and often execute similar functions in a plethora of processes, such as development and immunity (Li 2010; Ma et al. 2016; Gou and Li 2020). SERK3, also known as BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1), is the most extensively described LRR-RLK coreceptor and associates with FLS2 and EFR, which are involved in immunity as well as with BRI1 to activate BR signaling (Russinova et al. 2004; Sun et al. 2013; Ma et al. 2016; Zhou et al. 2019). The pleiotropic, redundant roles of coreceptors complicate research on their involvement in specific processes. Interestingly, mutant alleles specifically impaired in one function of the coreceptor are available, such as *bak1-5* that is impaired in the immunity signaling activities of BAK1 but not in BR signaling or cell death control (Schwessinger et al. 2011).

Despite the fact that the downstream phenotypic effects and the molecular changes that take place upon RHG1 treatment have been described, the upstream receptors that steer these responses remain elusive. To identify the RLKs involved in RHG1-induced PGP, we assembled a *rlk* mutant library based on RLK characteristics that were relevant for the RHG1-Arabidopsis interaction, such as expression in the root and involvement in processes modulated by RHG1 (i.e. growth and development, immunity and abiotic stress responses). To broaden our range and increase the possibility in identifying a novel RLK with a role in *Caulobacter*-induced growth promotion, we included unknown RLKs in our library. By focusing on changes in fresh weight increase, which is in our opinion the most relevant plant parameter with regard to possible biostimulant applications, we phenotypically screened a *rlk* mutant library. This way, we identified potential receptors involved in RHG1-induced PGP and found the coreceptor(s) necessary to initiate the RLK-dependent signaling networks steered by RHG1.

Materials and methods

Bacterial strains and growth conditions

Caulobacter RHG1 is an in-house GFP-labeled bacterial strain containing a kanamycin resistance gene (Luo et al. 2019; Beirinckx et al. 2020). The *Bacillus* sp. control strain is part of the same in-house bacterial collection (Beirinckx et al. 2020). To create a bacterial inoculum, strains were grown overnight in a liquid culture of R2A medium (0.5 g/l proteose peptone, 0.5 g/l casamino acids, 0.5 g/l yeast extract, 0.5 g/l dextrose, 0.5 g/l soluble starch, 0.3 g/l dipotassium phosphate, 0.05 g/l magnesium sulfate heptahydrate, 0.3 g/l sodium pyruvate; pH 7) at 28°C, diluted the next morning and again grown for 3–4 h to reach the exponential growth phase. The culture was centrifuged at 2500 rcf for 10 min and the bacterial pellet was resuspended and diluted to an optical density (OD_{600}) of 0.01 with phosphate buffered saline (PBS).

Arabidopsis genotypes and growth conditions

Seeds of *Arabidopsis thaliana* mutant lines (*serk1-1*, *serk2-1*, *serk4*, *serk5*, *shrk2*, *shrk1shrk2*, *lym1-1*, *lym1-2*, *lym3-1*, *lym3-2*, *cerk1-2* and *wak1*) were obtained from the NASC germplasm stock center, from dr. ir. Heidstra (Wageningen University and Research, Wageningen, The Netherlands; collection of 121 lines), from prof. dr. Russinova (VIB-UGent Center for Plant Systems Biology, Ghent, Belgium) (*bak1-5*, *bri1*, *bri1-301*, *fls2*, *efr-1*, *fls2efr-1* and *cpd*), from prof. dr. Zipfel (University of Zurich, Zurich, Switzerland) (*serk2-2;bak1-4*, *serk1-3;bak1-4*, *bak1-5;bkk1-1* and *fls2efr-1cerk1-2*), prof. dr. Lipka (Georg-August-Universität Göttingen, Göttingen, Germany) (*cerk1-4*) or from prof. dr. Ranf (Technical University of Munich, Munich, Germany) (*sd1-29*) (see Dataset S1 and Supplementary Table S1 for details) and upscaled together with their respective wild type (WT) seeds. Seeds were surface-sterilized with chlorine gas followed by stratification at 4°C in the dark for 2 days. Seeds were sown on agar-solidified plant growth medium (2.3 g/l MS, 0.5 g/l MES, 8 g/l plant tissue culture agar; pH 5.7) and incubated vertically in a tissue culture room at 21°C under long-day conditions (16 h light/8 h dark).

Establishment of the rlk mutant library

The *rlk* mutant library was assembled based on a literature search focusing on root-expressed RLKs, as these form the most likely interaction point with RHG1 in the rhizosphere. Our *rlk* mutant library comprised 145 lines corresponding to 81 *RLK* genes belonging to five different RLK families (LRR, LysM, S-domain, Lectin and WAK) (Supplementary Table S1, Dataset S1). For 38 out of 81 candidate *RLK* genes, multiple (two to six) independent single mutant lines were included, whereas for nine out of 81 candidate *RLK* genes, (one to three) double mutant

lines were included. To guarantee the correct identity of the mutants in the library, 10 mutants were randomly genotyped and mutants that were selected for further research were also genotyped.

Next to classifying the RLKs according to their extracellular ligand-binding domain, we also categorized our library according to function (Supplementary Table S1). From the 145 lines, 60 lines (corresponding to 30 genes) were shown to be impaired in developmental processes (Scholl et al. 2000; Alonso et al. 2003; Xu et al. 2008; Jaillais et al. 2011; Ten Hove et al. 2011; Lv et al. 2018; Luo et al. 2019). Thirty-three lines (corresponding to 18 genes) were found to be impaired in PAMP/DAMP recognition and immunity processes (Scholl et al. 2000; Alonso et al. 2003; Rosso et al. 2003; Zipfel et al. 2006; Gimenez-Ibanez et al. 2009; Robinson et al. 2009; Schwessinger et al. 2011; Ten Hove et al. 2011; Kleinboelting et al. 2012; Ranf et al. 2015). Eleven mutant lines (corresponding to five genes) were reported to be impaired in (abiotic) stress responses (Scholl et al. 2000; Alonso et al. 2003; Ten Hove et al. 2011). Next, our library contained 12 lines (corresponding to six genes) impaired in multifunctional coreceptors (Rosso et al. 2003; Roux et al. 2011; Schwessinger et al. 2011; Ten Hove et al. 2011; Kleinboelting et al. 2012; Petutschnig et al. 2014). Finally, 51 lines (corresponding to 31 genes) impaired in RLKs with a yet unknown function were included and thus provide potential candidates in our quest for (novel) signaling networks involved in beneficial plant-microbe interactions.

Growth promotion bioassay and statistical analysis

One four-day-old WT and four different four-day-old mutant seedlings (five plants of different genotypes per plate, 15 plates per condition) were transferred to new plates and inoculated by pipetting 8 µl of the bacterial inoculum (OD_{600} = 0.01) or PBS in control conditions (Mock) on the root tip and further grown vertically in the tissue culture room. At 14 days post inoculation (dpi), the ratio of mutant total fresh weight versus WT total fresh weight was calculated per plate, to account for a plate effect. Next, these values were compared between mock and RHG1 conditions by a one-way ANOVA or Kruskal–Wallis test (if the assumptions for a one-way ANOVA were not met). We screened for mutants showing a significant increase, decrease or complete loss of the RHG1-induced PGP phenotype when compared with RHG1-treated WT plants. Initially, all 146 mutant lines (Dataset S1) were subjected once to the RHG1-induced PGP bioassay. Mutant lines showing a significant change in the RHG1-induced PGP effect were subsequently subjected to multiple independent follow-up experiments.

RNA extraction and qRT-PCR analysis

Hundred WT Col-0 seeds were sown on plates (two plates per treatment-time point condition), stratified for two days in the dark and incubated vertically in a tissue culture room for four days. Each row consisting of 20 four-day-old seedlings was inoculated by pipetting 160 µl of *Caulobacter* RHG1, the *Bacillus* sp. strain or PBS along the root tips and further grown vertically in the tissue culture room. At 12 h post inoculation (hpi) and at 1, 2, 3, 5, 7 and 9 dpi roots were cut and snap-frozen in liquid nitrogen. The cells were disrupted by 3-mm metal beads in 2-ml tubes (Eppendorf) with a mixer mill 400 (Retsch) for 3 min at 20 Hz. RNA was extracted with the Relia Prep RNA tissue MiniPrep System (Promega) and RNA concentrations were measured with a ND-1000 Spectrophotometer (Thermo Fisher Scientific Nanodrop) and 1 µg was reverse transcribed with the qScript cDNA SuperMix (Quantabio). The primer sequences (Supplementary Table 2) were obtained from literature or designed with the Primer-BLAST tool from the National Center for Biotechnology Information (NCBI). The primers were diluted with water to a concentration of 2.5 µM. All qRT-PCR experiments were performed in three technical replicates on 384-multiwell plates with SYBR Green detection. Reaction mixtures were composed by the Janus Robot (PerkinElmer) with a final volume of 5 µl and a 10% cDNA fraction with the SYBR Green Master Mix (PerkinElmer). The Roche Lightcycler 480 system (Roche Diagnostics) was used to execute all qRT-PCR reactions with the following settings: 1x preincubation (95°C for 5 min), 45× amplification (95°C for 10 s, 60°C for 10 s and 72°C for 10 s), 1× melting curve (95°C for 5 s, 65°C to 97°C for 1 min) and 1× cooling down (40°C for 10 s). Threshold cycle and efficiency values were determined by the Lightcycler 480 software and analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The obtained expression data were normalized to the expression levels of *TUBULIN2* (*TUB2*) and *PROTEIN PHOSPHATASE 2* (*PP2A*). The experiment was repeated three times.

Ethics

This study did not require ethical approval or permission. No animal or human participants were used.

Results

Exploring the involvement of development – and immunity-related RLKs in RHG1-driven plant growth promotion

Because PGPR, including RHG1, are able to modulate development responses in the plant, we included 60 mutant lines impaired in genes with a known role in development in our *rlk* library. Prior the library screen, we confirmed that application of RGH1 on WT Arabidopsis Col-0 plants resulted in an increase in root, shoot and total fresh weight (Supplementary Figure S1). Following an initial library screen, seven mutant lines impaired in five genes (RH33, RH62, RH65, RH67, RH68, RH86 and RH89, impaired in *ZAR1, BIR3, BRI2, RGI5* and *CEPR2*) involved in development exhibited a significant change in the RHG1-induced PGP compared to the WT. However, because of the known variability inherent to PGPR research (Luo et al. 2019), the seven selected mutants were subjected to multiple independent repeats of the bioassay to evaluate the consistency of the observed significant change in PGP. None of these lines showed a consistent significant change in RHG1-induced PGP in independent follow-up experiments (Dataset S1; *P*-value >0.05) and therefore we did not execute additional follow-up experiments. Interestingly, we were not able to confirm the results of Luo et al. 2019, since the null *bri1* and the weaker *bri1-301* mutants, previously suggested to be involved in RHG1-induced PGP (Luo et al. 2019), did

not show a significant average change in RHG1-induced PGP in total fresh weight compared with WT plants (−22% and −23%, respectively) over all experiments, i.e. the initial library screen and four independent follow-up experiments (Figure 1A–B, Supplementary Figure S2A–B; *P*-value > 0.05). Furthermore, Luo et al. 2019 also suggested the involvement of BR biosynthesis in the RHG1-induced PGP phenotype. As we were not able to confirm the BR signaling data, we were wondering if this would also be the case for the BR biosynthesis data. Therefore, although it is not an *rlk* mutant, we decided to subject the BR biosynthesis mutant *cpd* to our bioassay (Szekeres et al. 1996). The *cpd* mutant exhibited a significant average decrease of 38% in the RHG1-induced PGP effect in total fresh weight compared with WT plants over all experiments, i.e. the initial library screen and four independent follow-up experiments (Figure 1(C), Supplementary Figure S2C, *P*-value < 0.001). Taken together, our results show that none of the development-related RLKs tested are involved in RHG1-induced PGP and further suggest that BR biosynthesis but not BR signaling might be important for RHG1 to induce a PGP phenotype.

Next, 30 mutants in our library were known to play a role in plant immunity. Only one mutant, *efr-1*, affected in recognition of the MAMP EF-Tu, showed a significant average increase of 31% in the RHG1-induced PGP phenotype in total fresh weight compared with WT plants over all experiments, i.e. the initial library screen and three independent follow-up experiments (Figure 2(A), Supplementary Figure S3A, *P*-value < 0.001). The *efr-1* allele was also present in the double mutant *fls2efr-1*, which exhibited inconsistent changes (−50%, +32%, – 38% and +9%) during the initial screen and three independent follow-up experiments and therefore did not show a significant average change (−3%) in RHG1-induced PGP in total fresh weight compared with WT plants over all experiments (Figure 2(C), Supplementary Figure 3C, *P*-value > 0.05). In accordance, the *fls2* mutant showed inconsistent changes (+30%, – 26%, +2% and +2%) during the initial screen and three independent follow-up experiments and thus did not exhibit a significant average change (+9%) in RHG1-induced PGP in total fresh weight in comparison with WT plants over all experiments (Figure 2(B), Supplementary Figure 3B, *P*-value > 0.05). Furthermore, the *efr-1* allele was also present in the triple mutant *fls2efr-1cerk1-2*, which did not show a significant change in the RHG1-induced PGP phenotype in total fresh weight compared with WT plants in the initial library screen and was therefore not subjected to follow-up experiments (Dataset S1, *P*-value > 0.05). Additionally, one (of the four) *pepr1* allele (designated RH91), affected in peptide ligand recognition, exhibited a significant (−41%) and a non-significant change (+8%) in the RHG1-induced PGP phenotype in total fresh weight in comparison with WT plants in the initial library screen and an independent follow-up experiment, respectively, and therefore we did not perform additional follow-up experiments (Dataset S1; *P*-value < 0.05). The remaining 25 mutant *rlk* lines that are part of the immunity category, among which the MAMP recognition mutants *lym1-1*, *lym1-2*, *lym3-1*, *lym3-2*, *sd1-29* and *wak1*, did not show a significant change in the RHG1-induced PGP phenotype in total fresh weight compared with WT plants in the initial library screen and were therefore not subjected to follow-up experiments (Dataset S1, *P*-value > 0.05).

Together, these results demonstrate that from the development and immunity-related *rlk* mutants, only *efr-1* plants showed a consistent change in the RHG1-induced PGP phenotype compared with WT, suggesting that knocking-out known immunity RLKs only has a mild influence on RHG1-induced PGP.

LRR coreceptors deliver further clues to unravel unique RHG1-induced signaling pathways

As coreceptors have been shown to exert pleiotropic roles in diverse signaling networks, we included 12 lines impaired in one or several of the six LRR or LysM domain coreceptors genes (*SERKs* and *CERKs*, respectively) in our library (Albrecht et al. 2008; Li 2010; Ma et al. 2016; Buendia et al. 2018; Gou and Li 2020). For four of the six coreceptor genes, *SERK1*, *SERK2*, *SERK4* and *SERK5*, we had one single

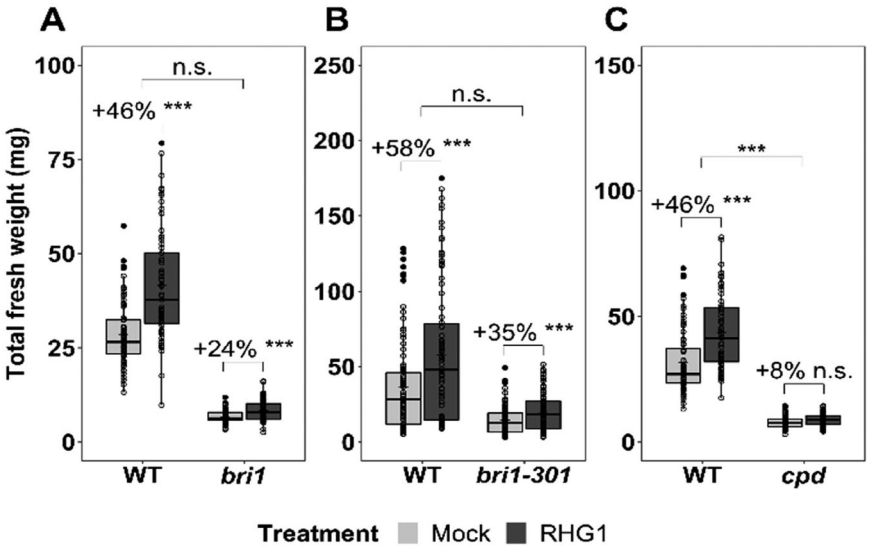

Figure 1. RHG1-induced plant growth promotion in selected BR signaling and biosynthesis mutants. Four-day-old wild type (WT) and mutant (*brassinosteroid insensitive 1* (*bri1*, **A**), *bri1-301* (**B**) and *constitutive photomorphogenic dwarf* (*cpd*, **C**)) seedlings were treated with a mock (light gray) or RHG1 (dark gray) solution (OD 0.01) and total fresh weight values were determined at 14 days post inoculation (dpi). Values represent boxplots of five biological repeats with their mean (+) and median (horizontal line). Asterisks indicate significant differences between mock and inoculated plants or between RHG1-induced effects in WT and mutant plants (one-way ANOVA or Kruskal–Wallis test; ***$P < 0.001$; n.s., not significant).

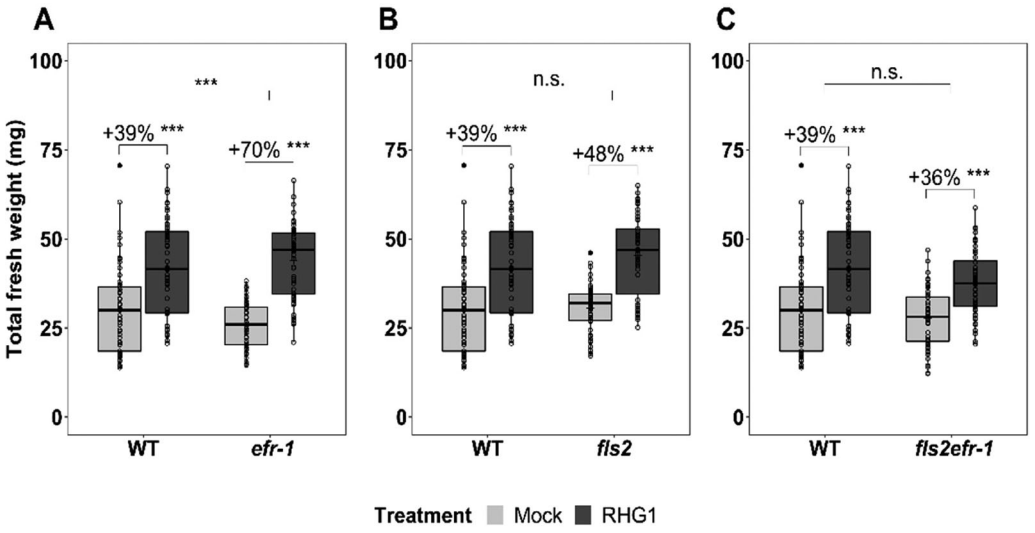

Figure 2. RHG1-induced plant growth promotion in selected immunity *rlk* mutants. Four-day-old wild type (WT) and mutant (*elongation factor-Tu receptor 1* (*efr-1*, **A**), *flagellin-sensitive 2* (*fls2*, **B**) and *fls2efr-1* (**C**)) seedlings were treated with a mock (light gray) or RHG1 (dark gray) solution (OD 0.01) and total fresh weight values were determined at 14 days post inoculation (dpi). Values represent boxplots of four biological repeats with their mean (+) and median (horizontal line). Asterisks indicate significant differences between mock and inoculated plants or between RHG1-induced effects in WT and mutant plants (one-way ANOVA or Kruskal–Wallis test; ***$P < 0.001$; n.s., not significant).

mutant line available, i.e. *serk1-1*, *serk2-1*, *serk4* (*bkk1-1*) and *serk5*, respectively. For *CERK1*, we possessed two independent single mutant lines, *cerk1-2* and *cerk1-4*, and for *BAK1* we collected the immunity-comprised *bak1-5* allele and two other single mutant alleles, *bak1-3* and *bak1-4*. The mutant line *bak1-3* contains a hypomorphic intronic T-DNA insertion and is therefore not a null allele, whereas *bak1-4* represents a true null allele (Wierzba and Tax 2016). As SERK coreceptors are known to operate in a redundant way, we included three double coreceptor mutant lines, i.e. *serk1-3;bak1-4*, *serk2-2;bak1-4* and *bak1-5;bkk1-1,* in our library.

Over all experiments, i.e. the initial library screen and eight independent follow-up experiments, *bak1-4* plants showed a significant average decrease of 29% in RHG1-induced PGP in total fresh weight compared with WT plants (Figure 4(A), Supplementary Figure S6A-B; *P*-value < 0.001). Mutant lines *serk1-3;bak1-4* showed a significant average decrease of 32% in RHG1-induced PGP in total fresh weight in comparison to WT plants, over all experiments, i.e. the initial library screen and two independent follow-up experiments (Figure 4(B); Supplementary Figure S6C, *P*-value < 0.001). Additionally, *bak1-5* exhibited a significant increase (+45%, *P*-value < 0.01) in the RHG1-induced PGP phenotype in total fresh weight in comparison with WT plants in the initial library screen, but none of these showed a significant change in RHG1-induced PGP in three independent follow-up experiments (Dataset S1; Figure 4(C); Supplementary Figure S6D). The remaining single coreceptor mutants (*serk1-1*, *serk2-1*, *serk4* (*bkk1-1*), *serk5*, *cerk1-2, cerk1-4* and *bak1-3*) and two double coreceptor mutants (*serk2-2;bak1-4* and *bak1-5; bkk1-1*) did not exhibit a consistent significant change in RHG1-induced PGP in total fresh weight compared with WT conditions in the initial library screen and were therefore not subjected to follow-up experiments (Dataset S1; *P*-value > 0.05).

In summary, the single coreceptor mutant *bak1-4* and the double coreceptor mutant *serk1-3;bak1-4* exhibited a significant decrease in the RHG1-induced PGP phenotype in total fresh weight in comparison with WT plants. Interestingly, the decreased PGP phenotype in *bak1-4* plants exhibited a certain degree of variability throughout the nine independent experiments, while a similar decreased PGP phenotype could be observed in all three independent experiments in *serk1-3;bak1-4* plants (Supplementary Figure S6A-C). The consistent change in PGP phenotype in the double mutant compared with the single mutant could be due to functional redundancy which is characteristic for SERK family members (Albrecht et al. 2008; Li 2010; Sun et al. 2013; Ma et al. 2016; Gou and Li 2020). Our data thus suggest a role for the LRR coreceptors BAK1 and SERK1 in signaling processes elicited upon RHG1 inoculation that influence the PGP phenotype.

An unknown RLK, seemingly involved in abiotic stress, plays a role in RHG1-induced PGP

Because RHG1 has been isolated from the roots of maize plants grown at chilling temperatures and as several PGPR strains, including RHG1, are known to help plants cope with abiotic stresses, we included 11 *rlk* mutant lines impaired in genes known to be involved in stress responses in our library (Cohen et al. 2008; Yang et al. 2009; Ilangumaran and Smith 2017; Beirinckx et al. 2020; Goswami and Deka 2020; Arora et al. 2020). One mutant line (*cepr2* mutant RH89) showed a significant change (−39%) in the RHG1-induced PGP phenotype in total fresh weight compared with WT plants in the initial library screen. However, in an independent follow-up experiment, a non-significant change (−18%) was observed (Dataset S1). Therefore, this line was not selected for further experiments.

As the majority of the RLKs exert yet unknown functions and might be interesting targets to identify (novel) signaling networks at play during beneficial plant-microbe interactions, we included 51 mutant lines impaired in root expressed RLKs with a yet unknown function in our mutant *rlk* library (Dataset S1) (Wu et al. 2016). Following an initial screen of the library, 10 mutant lines impaired in unknown *RLK* genes (RH6, RH7, RH31, RH43, RH54, RH55, RH83,

RH101, RH102, RH131, impaired in *AT5G58300*, *AT5G58300*, *AT2G15300*, *AT5G58150*, *AT3G28040*, *AT1G28440*, *AT2G33170*, *AT2G33170*, and *AT4G03230*, respectively) exhibited a significant change in the RHG1-induced PGP phenotype in total (or root in the case of RH83) fresh weight in comparison with WT plants. Only two mutant lines, *at3g28040a* and *at3g28040b* showed a significant average decrease of −29% and −36%, respectively, in follow-up experiments (Figure 3, Supplementary Figures S4-5; *P*-value < 0.01). AT3G28040 is an LRR-RLK and part of the Ser/Thr protein kinase family (Figure 3(C)). This protein was shown to interact with AT4G34220, also known as RECEPTOR DEAD KINASE 1 (RDK1), an RLK involved in ABA-mediated seedling development and drought tolerance (Szklarczyk et al. 2019; Kumar et al. 2017). The remaining 43 *rlk* mutants impaired in functionally unknown RLKs that did not show a significant change in RHG1-induced PGP in total fresh weight in comparison with WT plants in the initial library screen were not subjected to follow-up experiments (Dataset S1).

In summary, our data suggest a potential role for an unknown RLK, *AT3G28040*, seemingly involved in abiotic stress response, in eliciting signaling pathways that are necessary for a RHG1-induced PGP phenotype.

Transcript levels of selected RLKs do not show major changes in response to RHG1 inoculation

It has been shown that the expression of the RLKs *FLS2* and *WAK1* are induced upon treatment with flagellin or oligogalacturonides, respectively (Shiu and Bleecker 2001; Zipfel et al., 2006; Denoux et al., 2008). To explore the involvement of the four *RLK* genes impaired in the mutant lines that showed a significant change in RHG1-induced PGP in total fresh weight compared with WT plants in multiple repeats of the bioassay (*EFR1*, *BAK1*, *SERK1* and *AT3G28040*, respectively impaired in the mutants *efr-1*, *bak1-4*, *serk1-3; bak1-4* and *at3g28040a* and *at3g28040b*), we performed expression analyses of the selected genes at different time points after treatment with RHG1 using qRT-PCR analysis. In the qRT-PCR analyses, we included treatment with a neutral *Bacillus* sp. strain (Bacillus) without a phenotypic effect on Arabidopsis growth as an extra control to filter out general plant responses to bacteria. Roots of four-day-old WT seedlings were harvested at 12 h post inoculation (hpi) and at 1, 2, 3, 5, 7 and 9 days after treatment with mock, Bacillus or RHG1, RNA was extracted and used for qRT-PCR analyses. During the entire time course (at 12 h and 1, 2, 3, 5, 7 and 9 days after treatment), no consistent changes in the gene expression levels of *EFR1*, *BAK1*, *SERK1* and

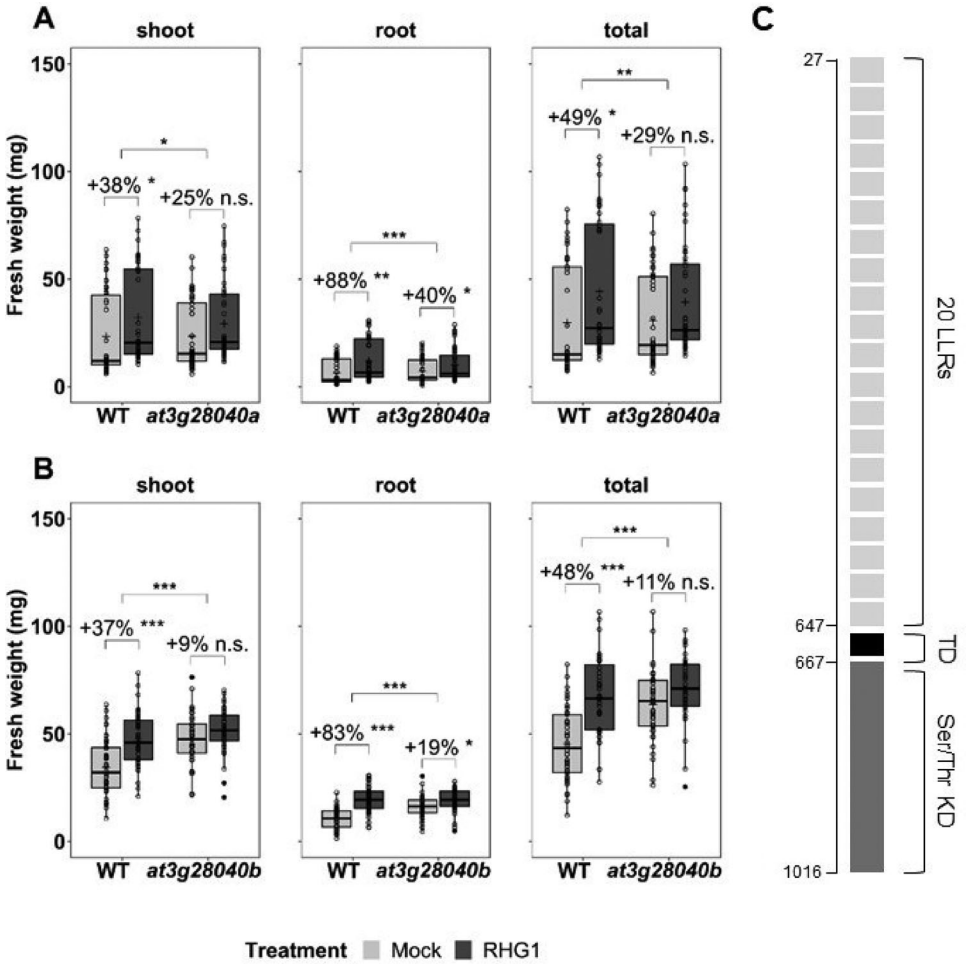

Figure 3. RHG1-induced plant growth promotion in selected two unknown *rlk* mutants. Four-day-old wild type (WT) and mutant (*at3g28040a* (**A**), *at3g28040b* (**B**)) seedlings were treated with a mock (light gray) or RHG1 (dark gray) solution (OD 0.01) and total fresh weight values were determined at 14 days post inoculation (dpi). Values represent boxplots of three biological repeats with their mean (+) and median (horizontal line). Asterisks indicate significant differences between mock and inoculated plants or between RHG1-induced effects in WT and mutant plants plants (one-way ANOVA or Kruskal–Wallis test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001; n.s., not significant). (**C**) Schematic representation of AT3G28040 protein structure with amino acid number marked for the different domains. LRRs: leucine-rich repeats, TD: transmembrane domain, KD: kinase domain.

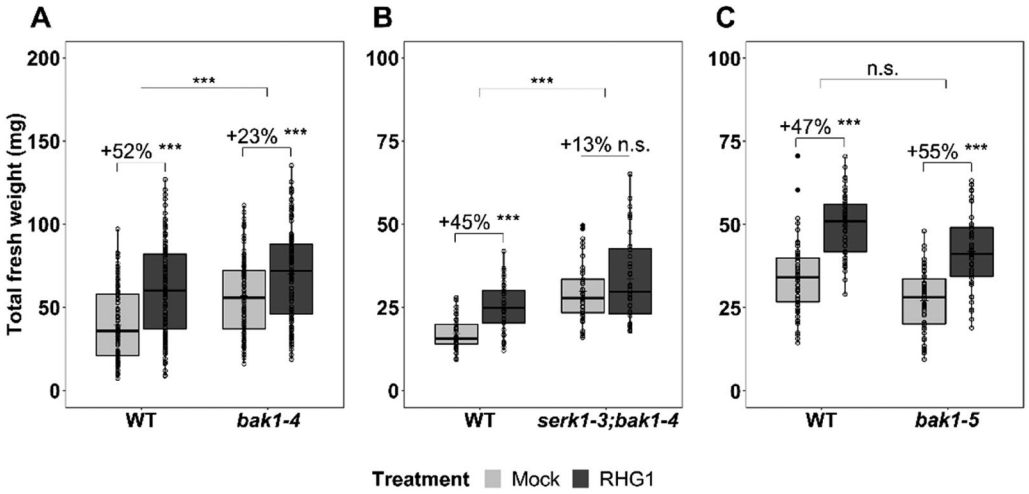

Figure 4. RHG1-induced plant growth promotion in selected *rlk* coreceptor mutants. Four-day-old wild type (WT) and mutant (*brassinosteroid insensitive 1-associated kinase 1–4* (*bak1-4*, **A**), *somatic embryogenesis receptor-like kinase 1-3; bak1-4* (*serk1-3;bak1-4*, **B**) and *brassinosteroid insensitive 1-associated kinase 1–5* (*bak1-5*, **C**)) seedlings were treated with a mock (light gray) or RHG1 (dark gray) solution (OD 0.01) and total fresh weight values were determined at 14 days post inoculation (dpi). Values represent boxplots of nine (A) three (B) or four (C) biological repeats with their mean (+) and median (horizontal line). Asterisks indicate significant differences between mock and inoculated plants or between RHG1-induced effects in WT and mutant plants (one-way ANOVA or Kruskal–Wallis test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; n.s., not significant).

AT3G28040 could be determined between mock, RHG1 – and Bacillus-treated samples (Supplementary Figure 7A–D).

Together, these results indicate that although EFR1, BAK1, SERK1, and AT3G28040 are suggested to play a role in RHG1-induced PGP, their gene expression levels do not show consistent RHG1-induced changes during a time frame from 12 hpi until 9 dpi.

Discussion

In this study, we investigated the perception and signaling networks influenced during the beneficial RHG1-Arabidopsis interaction by phenotypically screening a *rlk* mutant library consisting of 145 mutant lines corresponding to 81 root-expressed *RLK* genes, mainly belonging to the LRR-RLK subfamily. Since we know from previous research that *Caulobacter* triggers plant responses in different physiological areas (Luo et al. 2019; Beirinckx et al. 2020; Berrios and Ely 2021), we included RLKs involved in development, microbial recognition and immunity responses, abiotic stress responses, multifunctional coreceptors and functionally unknown RLKs. In an initial library screen, 27 mutant lines (7 involved in development, 5 in immunity responses, 4 in coreceptors, 1 in abiotic stress responses and 10 in unknown RLKs, see also Dataset S1) were picked up that showed a significant change in the RHG1-induced PGP phenotype compared with WT plants. After multiple independent follow-up experiments with these 27 mutant lines, only five mutants showed a consistently changed PGP phenotype. These mutant lines are the immunity-linked PRR mutant *efr-1*, the multifunctional coreceptor mutants *bak1-4* and *serk1-3;bak1-4* and the unknown RLK mutants *at3g28040a* and *at3g28040b*.

We showed that *efr-1* exhibited a significant increase in RHG1-induced PGP, hinting at a possible negative role for plant immunity in the RHG1-induced PGP effect. However, another immunity-related RLK mutant, *fls2*, did not show a consistent change in RHG1-induced PGP. Next to *efr-1* and *fls2*, we tested additional *rlk* mutants impaired in microbial perception and immunity, including the higher order mutants *fls2efr-1* and *fls2efr-1cerk1-2* and the immunity-compromised coreceptor allele *bak1-5*. None of these mutants showed an altered PGP response, again illustrating the complex, pleiotropic nature of RLK interactions. This observation might as well be caused by the fact that RHG1 mainly colonizes the root surface epiphytically (Luo et al. 2019) and therefore elicits only a confined immune response and/or RHG1 might be able to dampen immune responses, a second strategy often applied by PGPR (and pathogens) to overcome immunity and allow interaction with the host plant (Abramovitch and Martin 2004; Teixeira et al. 2019; Yu et al. 2019a; Yu et al. 2019b; Colaianni et al. 2021; Ma et al. 2021). We therefore conclude that bacterial recognition and immunity barely influence RHG1-induced PGP effects in Arabidopsis. It is important to note that multiple perception/immunity receptors might be involved in this interaction which could all be linked to the initiation of growth promotion in yet-to-be revealed downstream pathways.

We identified two coreceptor mutants, *bak1-4* and *serk1-3; bak1-4*, showing a significantly reduced RHG1-induced PGP. Remarkably, the single mutant *bak1-4* exhibited variation in this phenotype throughout independent experiments, whereas the double mutant *serk1-3;bak1-4* showed a consistent loss of the PGP effect. These data show that BAK1 and SERK1 act redundantly and fulfill a crucial role in the RHG1-Arabidopsis interaction. Notably, the *serk1-1* single mutant in our library did not show a significantly altered PGP phenotype upon RHG1 treatment. A similar observation was made by Van Esse et al. (2016), who described that loss of *SERK1* only affects root growth in the absence of *SERK3* (*BAK1*). Furthermore, it has also been shown that the compromised immunity phenotype in a *serk3* single mutant is enhanced in a *serk3; serk4* double mutant, while the *serk4* single mutant is not affected (Chinchilla et al. 2007; Heese et al. 2007; Roux et al. 2011). Together, these observations highlight the complexity and redundancy of RLK signaling which could mask putative RLK involvement in the observed phenotype. To tackle this problem, CRISPR libraries could be used that target multiple RLK in a single multiplex gene editing event (Jacobs et al. 2017).

We also tested another *bak1* allele (*bak1-3*) in our bioassay, which showed a normal PGP effect. However, *bak1-3* has been shown to be a weak *bak1* allele, whereas *bak1-4* is a true null allele, which might explain these allele-specific results (Li et al. 2002; He et al. 2007; Albrecht et al. 2008; Roux et al. 2011; Schwessinger et al. 2011; Gou et al. 2012; Wierzba and Tax 2016).

As Luo et al. (2019) already suggested a role for BR signaling in RHG1-induced PGP, we further investigated the importance of BR signaling by testing different *bri1* alleles and other BR receptor mutants, *brl2* and *brl3*, in our bioassay. While BRI1 is proposed to be the major BR receptor functioning throughout the whole plant in normal conditions to drive growth and development, BRLs have been suggested to regulate cell-specific BR responses activated during environmental stress conditions (Planas-Riverola et al. 2019). However, none of the tested mutants, *bri1*, *bri1-301*, four individual *brl2* lines and *brl3*, exhibited a consistent significant change in RHG1-induced PGP, which is in contrast with the previously obtained results from Luo et al. (2019). It should be noted that, in contrast to Luo et al. (2019), we inoculated 4-day-old seedlings instead of seeds and that, due to the epiphytic, 'loose' interaction of RHG1 with the root, variation in the PGP effect is to be expected. Indeed, although significant, the changes in PGP that were observed by Luo et al. (2019) in the *bri1* mutant were also variable (fold changes of 0.97, 1.06 and 1.15 in three biological repeats, respectively). Collectively, these BR receptor data suggest that the role of BAK1 in the RHG1-Arabidopsis interaction is likely also not predominantly associated with BR signaling. In view of this, we investigated a BR biosynthesis mutant, *constitutive photomorphogenic dwarf* (*cpd*), because its involvement in RHG1-induced PGP was also suggested before (Luo et al. 2019). In contrast to our data on BR signaling, we could confirm that BR biosynthesis indeed plays a role in RHG1-induced PGP, since the *cpd* mutant significantly lost the PGP effect in our bioassay. Taken together, our data suggest that BR biosynthesis, but not BR signaling, might be involved in RHG1-induced PGP. More BR biosynthesis mutants should be tested to substantiate this hypothesis.

In our quest to find other possible RLKs acting upstream of BAK1 and SERK1, we also considered the unknown RLK gene, *AT3G28040*, because corresponding mutant lines showed a significant decrease in RHG1-induced PGP in our bioassay, which was similar to the phenotype observed in the coreceptor mutants. As its function is currently not known, this receptor provides an interesting target to unravel (novel) signaling networks at play during the RHG1-Arabidopsis interaction. *AT3G28040* belongs to LRR subfamily VII and its expression in the root is mainly restricted to meristematic cortical cells (Shiu and Bleecker 2001; Winter et al. 2007; Ten Hove et al. 2011). Upon cold, osmotic, salt and heat stress, and upon ABA treatment, the expression of *AT3G28040* is downregulated and *at3g28040* mutants are suggested to be mannitol (drought stress) resistant (Winter et al. 2007; Ten Hove et al. 2011). Experimental data support protein–protein associations of AT3G28040 with RDK1, an RLK involved in ABA-mediated seedling development and drought tolerance (Szklarczyk et al. 2019; Kumar et al. 2017). These data suggest that AT3G28040 might play a role in abiotic, ABA-dependent stress responses. To further examine the exact role of this unknown RLK in RHG1-induced PGP, marker lines should be constructed to investigate its subcellular localization and expression in control and RHG1-treated samples. Furthermore, its potential interaction with BAK1 and SERK1 could be investigated by binary protein interaction analyses.

Finally, we showed that RHG1 treatment does not influence the transcript levels of the identified RLKs, suggesting that the modulation of signaling networks upon RHG1 treatment is situated at the post-translational level. A phosphoproteomics analysis should provide more insight.

In conclusion, by phenotypically analyzing a *rlk* mutant library we revealed that the coreceptors BAK1 and SERK1 play a redundant, but pivotal role in RHG1-induced PGP. Notably, our investigation reveals that the well-established roles of BAK1 in microbial recognition, immunity, and BR signaling, do not emerge as crucial for the observed PGP effect. Therefore, we hypothesize that BAK1, in cooperation with SERK1, play a yet unknown but essential role in the RHG1-Arabidopsis interaction, possibly by interacting with the uncharacterized RLK AT3G28040. Although inherent variability in observed PGP effects is a consistent challenge in studying plant-microbe interactions, which, in this study, is fueled by the enigmatic, complex nature of RLK-mediated signaling pathways, it is paramount to further explore the perception mechanisms governing plant-microbe associations. Our findings provide only a small piece of the larger puzzle and therefore, we hypothesize that the identified RLKs might be involved in novel pathways at play in the beneficial interaction between RHG1 or other PGPR, and the host plant Arabidopsis.

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Author contributions

A.L. designed, performed and analyzed the experiments. A.L. together with M.V. wrote and revised the manuscript and made the figures. Guided by A.L., M.B. and V.D. performed part of the mutant assays. J.V.D. provided suggestions during writing. A.L., K.G. and S.G. coordinated the research.

Notes on contributors

Amber Lampens obtained her Master degree Biochemistry and Biotechnology in 2017 and moved on to pursue a PhD degree in the Goormachtig lab, which she obtained in 2022.

Michiel Vandecasteele his PhD degree in Bioscience Engineering in 2019. Now he is a postdoc in the Goormachtig lab.

Marjon Braem obtained her master's degree in biology in 2021. Now, she performs her PhD study in the Goormachtig lab.

Viktor Devlieghere obtained his Biochemistry and Biotechnology Master's degree in 2022. Now he pursues a PhD degree at ILVO.

Judith Van Dingenen obtained her PhD in Plant Biotechnology in 2016 after which she performed a postdoc study in the Max Planck Institute of Molecular Plant Physiology at Potsdam, Germany. Now she is a postdoc in the Goormachtig lab.

Kris Gevaert became UGent professor in 2004 and leads the proteomics lab at the VIB Center for Medical Biotechnology. In 2018, he became head of the UGent department of Biomolecular Medicine.

Sofie Goormachtig became professor at UGent in 2005 and full professor in 2017. She leads the Rhizosphere lab at VIB Center for Plant Systems Biology and contributed to the start-up of VIB spin-off companies Protealis and Aphea. Bio. Since 2023, she became adjunct director of VIB Center for Plant Systems Biology.

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