



The GH3 Protein from the Clubroot Pathogen *Plasmodiophora brassicae* Causes Hormone-Related Phenotypes in *Arabidopsis thaliana*

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Abstract

In the genome of the obligate biotrophic protist *Plasmodiophora brassicae*, which causes clubroot disease in the Brassicaceae family, a single gene was identified with homology to the group of GH3 proteins synthesizing amino acid conjugates with carboxylic acids. In comparison to plant members the *P. brassicae* GH3 protein seems to be very promiscuous with respect to the substrates converted. In vitro assays showed conversion of three different auxins (indole-3-acetic, IAA; indole-propionic, IPA; and indole-3-butyric acids, IBA), jasmonic acid (JA) and also 12-oxo-phytodienoic acid (OPDA). Auxin and Methyl-JA responsive elements were found in the promoter sequence of *PbGH3*. For further analysis *Arabidopsis thaliana* was transformed with *PbGH3*. Seedlings with confirmed constitutive PbGH3 protein production were grown to adult plants that were shown to produce fewer inflorescences per plant. Growth on 10 μM IAA or JA levels also altered the phenotype of the seedlings, but not so much between wild types and transgenic lines. The hormone profiles in seedlings and adult plants were examined for IAA, JA, OPDA, and respective conjugates. Transgenic seedlings displayed changes that could be partially attributed to the overexpression of a *GH3* gene like lower levels of free IAA and the JA precursor *cis*-OPDA under control conditions, and higher levels of some auxin conjugates accumulated after IAA treatment compared to the corresponding wild types. However, the hormone patterns in adult plants were more complex and varied based on different tissue types analyzed. After inoculation with *P. brassicae* the transgenic plants did not show any changes in infection rate and disease index, but had higher shoot and lower root weight in infected plants compared to controls. Based on the above findings we discuss a role for PbGH3 during pathogenesis.

Keywords Auxin · *Arabidopsis thaliana* · Conjugate formation · Clubroot disease · Jasmonate · *Plasmodiophora brassicae*

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Introduction

Clubroot disease is among the most devastating diseases of *Brassica* crops worldwide (Dixon 2009). The disease is caused by the biotrophic protist *Plasmodiophora brassicae*, which has a complicated life cycle (Kageyama and Asano 2009) during which it transforms the host roots into a strong metabolic sink that eventually also affects the upper plant parts (Ludwig-Müller et al. 2009a). The life cycle of *P. brassicae* is divided into two stages, one within the root hairs and the second colonizing the entire root cortex (Kageyama and Asano 2009). During the latter, a strong hypertrophy and hyperplasia of the host tissue can be observed that is coordinated by the pathogen (Malinowski et al. 2019). Plant pathogens that alter growth responses, such as *P. brassicae*, need to either reorganize the host tissue or supplement the tissue through such promoting factors by themselves (Ludwig-Müller 2014a). The changes in cell division rate and cell elongation go along with many molecular changes in the host, among them plant hormones (Schuller and Ludwig-Müller 2016). Since growth-promoting hormone homeostasis plays a role in clubroot disease symptom development, we have chosen one protein from the *P. brassicae* genome predicted to target auxin (Schwelm et al. 2015).

The control of auxin homeostasis is important for all growth and developmental processes. However, during plant–pathogen interactions auxin can either act as a signal in defense responses or contributes to the disease symptoms in the case of tumors or galls (Ludwig-Müller 2014a). For the interaction of *P. brassicae* with its host it was shown that three different kinds of growth-promoting hormones play a role: auxins, cytokinins, and brassinosteroids (Siemens et al. 2006; Jahn et al. 2013; Schuller et al. 2014). For these hormone classes it is known how the concentrations in the plants can be regulated during the development of clubroots (Siemens et al. 2006; Jahn et al. 2013; Schuller et al. 2014). Phenotypes of mutants in hormone genes show smaller galls, but also smaller shoots. We have recently shown that the simultaneous response to auxin and cytokinin is essential for proper gall development and a promoter only responsive to both hormones was activated specifically in clubs (Hurný et al. 2020).

P. brassicae is difficult to study due to its obligate biotrophic nature. The availability of the genome sequence of *P. brassicae* allowed some deduction on possible genes or proteins involved in hormone homeostasis (Schwelm et al. 2015), of which one has been characterized as a methyltransferase for salicylic acid (Ludwig-Müller et al. 2015; Bulman et al. 2019; Djavaheri et al. 2019). Another gene / protein was found to be homologous to plant GH3 proteins (Schwelm et al. 2015). The family of GH3 (Gretchen

Hagen 3) proteins of amino acid conjugate synthetases was first characterized in *Arabidopsis thaliana* to contain activities that conjugate carboxylic acids to a set of amino acids in an energy-dependent reaction. The carboxylic acids consist of jasmonic acid (JA; Staswick et al. 2002; Delfin et al. 2022), indole-3-acetic acid (IAA; Staswick et al. 2005), indole-3-butyric acid (IBA; Sherp et al. 2018), and salicylic acid (SA; Zhang et al. 2007). It was recently demonstrated that *Arabidopsis* GH3s conjugate also oxIAA with aspartate (Asp) in vitro (Brunoni et al. 2023a). A different role for a specific GH3 protein (GH3.12) was found in the biosynthesis of SA, in which it catalyzed the conjugation of isochlorogenic acid to glutamate (Rekhter et al. 2019). The proteins from higher plants seem to have a somewhat restricted substrate preference for the carboxylic acid as well as for the amino acids added. A broader substrate preference was found in the moss *Physcomitrium patens*, where two GH3 proteins act on IAA and JA using a wider set of amino acids (Ludwig-Müller et al. 2009b).

The activity of the afore mentioned GH3 protein from *P. brassicae* was examined in a preliminary experiment as a heterologously produced protein in *E. coli* and found to adenylate IAA as well as JA to a large range of proteinogenic amino acids (Schwelm et al. 2015), thus differing with respect to their substrate range from the plant proteins. Interestingly, the ability to conjugate IAA to amino acids must have evolved at least twice in phytopathogens, since *Pseudomonas syringae* can conjugate IAA to lysine using a completely different enzyme and with a high preference for the carboxylic acid and the corresponding amino acid as well (Ludwig-Müller 2020).

Another putative auxin-related protein from *P. brassicae* was found by sequence homology, but the possible function was not confirmed. This gene was hypothesized to encode a homolog to indole-3-acetaldehyde dehydrogenase that could be involved in the biosynthesis of IAA and thus constitute a pathogenicity factor (Galindo-González et al. 2021). Only a few putative pathogenicity factors have been tested for their function, such as cyclophilins (Singh et al. 2018). Other possible pathogenicity factors could include proteins involved in the synthesis of other hormones or needed for nutrient synthesis or acquisition (Ludwig-Müller et al. 2009a, b; Rolfe et al. 2016).

To act in the host cell, the respective proteins from *P. brassicae* have to be secreted across the plasmodial membrane. Generally, the prediction of *P. brassicae* effectors with bioinformatic methods has resulted in a long list of putative candidates from different authors with such secretion sequences, but not so many have been experimentally verified up to now (Pérez-López et al. 2018, 2020). In some experiments, artificial yeast systems were used to verify the secretion of *P. brassicae* proteins (Djavaheri et al. 2019; Yu et al. 2019). The methyltransferase PbBSMT targets the

SA pathway (Ludwig-Müller et al. 2015), while other *P. brassicae* effectors have shown targets such as host plant kinase (Pérez-López et al. 2020; Chen et al. 2021), E3 ubiquitin ligase (PbRING1) that could be involved in targeting proteins for ubiquitination and thus degradation (Yu et al. 2019), and an apoplastic protein inhibiting cruciferous papain-like cysteine proteases (Pérez-López et al. 2021). Others were shown to have more unspecific plant targets, for example modulation of the PAMP-triggered plant immunity pathway (Hossain et al. 2021) and induction or suppression of cell death in a non-host *Nicotiana benthamiana* (Zhan et al. 2022).

The two classes of hormones that could be substrates for PbGH3 *in vitro* (Schwelm et al. 2015) have diverse roles in development or defense responses. IAA is involved in root (Overvoorde et al. 2010; Alarcon et al. 2019) and shoot architecture (Gallavotti 2013; Wang et al. 2018) and also in the development of clubroot symptoms (Jahn et al. 2013). The role of JA in developmental processes has only recently been recognized (Gasparini et al. 2015; To et al. 2019). The JA-dependent defense against clubroot seems to be important during later time points of club development (Lemarié et al. 2015).

The role of PbGH3 *in planta* was of interest due to the adverse possible functions of the predicted *in vitro* substrates, one for growth, the other targeting a defense pathway. Therefore, we conducted experiments to get a more detailed picture on the substrates that are conjugated by PbGH3 *in vitro* and *in planta*. Based on the preferred *in planta* substrates, PbGH3 could either act as a pathogenicity factor by regulating auxin levels or an effector by changing jasmonate-dependent defense. Since *P. brassicae* cannot be routinely transformed, we have instead transformed the host *A. thaliana* with the *PbGH3* gene. A similar approach has been successfully applied for the gene encoding the methyltransferase PbBSMT (Bulman et al. 2019; Djavaheri et al. 2019), PbRING1 (Yu et al. 2019) and PBZF1 which targets a protein kinase involved in plant defense (Chen et al. 2021). In both experimental approaches changes in the plant's susceptibility to *P. brassicae* itself were found. In this work we transformed *A. thaliana* with a construct constitutively expressing *PbGH3* and analyzed the resulting transgenic population for its phenotype, infection with *P. brassicae* and determined the hormone profile.

Materials and Methods

Recombinant Expression of the PbGH3 Gene in *E. coli*

The *PbGH3* coding sequence (CDS) without the sequence of the signaling peptide was cloned in the expression vector

pGEX 4T3 (Amersham Bioscience, Freiburg, Germany) to give a glutathione S-transferase (GST) fusion protein as previously described (Ludwig-Müller et al. 2009b; Schwelm et al. 2015). Cloning was done by Life Technologies GmbH (Carlsbad, CA, USA). The expression of the GST::PbGH3 fusion protein in *E. coli* BL21 (DE3) codon plus cells (Stratagene, La Jolla, CA, USA) was induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) for 3 h at 22 °C. Subsequently the cells were collected and subjected to three freeze/thaw cycles (30 s liquid Nitrogen/10 min 37 °C) after being resuspended in lysis buffer (1xPBS Buffer with 1 mg/ml lysozyme, 10 mM MgCl₂, 10 U/ml DNase1). After centrifugation (10 min; 13000 g) the purification of GST::PbGH3 protein was done using Glutathione Sepharose 4B (Bulk GST Purification Module; Amersham Bioscience, Freiburg, Germany) according to the manufacturer's protocol. Quality and quantity of the eluted fusion protein were verified using sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Conjugate Synthetase Test

The carboxylic acids IAA, IBA and indole-3-propionic acid (IPA), JA, (all from Merck, Darmstadt, Germany) and cis-(+)-12-oxo-phytodienoic acid (OPDA; Cayman Chemical, Ann Arbor, MI, USA) (1 mM each) were used as substrates for the activity tests (Ludwig-Müller et al. 2009b). The enzyme assays were performed according to Staswick et al. (2005) for 12 h at room temperature. Each assay contained 10 μ g purified GST::PbGH3 fusion protein in 20 μ l of 50 mM Tris–HCl, pH 8.6; 3 mM MgCl₂; 3 mM ATP; 1 mM DTT; and 1 mM of each amino acid. The whole reaction were spotted on silica gel 60 F₂₅₄ plates (Merck, Darmstadt, Germany) and developed in chloroform:ethylacetate:formic acid (35:55:10; v/v/v) with the exception of the IAA-His, -Ser, -Thr, -Arg, -Lys, and -Cys conjugates which were developed in 2-propanol:ammonium hydroxide:H₂O (8:1:1, v/v/v). Indoles were stained with van Urk-Salkowski Reagent and jasmonates (JA and OPDA) with Vanillin reagent (Staswick and Tiryaki 2004). To determine the relative activity the TLC plates were scanned after staining and the individual spots assayed with ImageJ (Schindelin et al. 2015). For each TLC plate an area that did not contain active enzyme and assay components, but was sprayed with the reagent, was used as a negative control and subtracted from the spot values for that respective plate. Since for each set of experiment the same enzyme amount was used, the individual experiments are comparable between each other.

Plant Material, Seedling Growth, and Hormonal Treatments

Seeds of the wild type *Arabidopsis thaliana* ecotype Col-0 and Ws-2 (originally obtained from Seed Stock Centre;

Nottingham, UK), and overexpressor lines *35S::PbGH₃-His₆* 52, *35S::PbGH₃-His₆* 64, *35S::PbGH₃-His₆* 34, *35S::PbGH₃-His₆* 47 were surface sterilized with 70% ethanol (v/v) for 1 min, followed by 1% Izosan G (w/v) (Pliva, Zagreb, Croatia) for 10 min, washed five times with sterile distilled water, sown on the full-strength MS medium including vitamins, 1% (w/v) plant agar, 1% (w/v) sucrose, 2.5 mM MES monohydrate, (pH 5.7). After stratification for 3 days at 4 °C, plates were transferred to a growth chamber, vertically positioned, at 22 °C under 16 h light at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ / 8 h dark conditions. Four-day-old seedlings were transferred on MS agar plates (control) or the same medium supplemented with either 10 μM IAA or 10 μM JA (Merck Sigma Aldrich, Darmstadt, Germany). Seedlings were further cultured for 7 days at 22 °C under 16 h light at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ / 8 h dark conditions. Plates were then scanned (HP scanner; HP München, Germany) and root images analyzed with ImageJ software (NIH, Bethesda, MD, USA) for primary root (PR) length and lateral root (LR) number measurement. Seedlings were pooled and collected for biomass measurement, as well as for hormone analysis, frozen in liquid nitrogen and stored at – 80 °C until further processing.

Transformation of *Arabidopsis thaliana* with PbGH3

To generate the transgenic plants the original *PbGH3* CDS with signal peptide as described in Schwelm et al., (2015) was commercially synthesized by Life Technologies (Carlsbad, CA, USA), amplified using PCR with specific primers (Table S1) and inserted via the Gateway cloning technology into the pMDC32 (Curtis and Grossniklaus 2003). In this plant expression vector, the *PbGH3* gene is under the control of the constitutive Cauliflower Mosaic Virus 35S promoter (35S::CaMV) and a C-terminal 6 \times His tag was added to the sequence during cloning (Table S1). The correct insertion was confirmed by sequencing.

A. thaliana plants, ecotypes Col-0 and Ws-2, were transformed via floral-dip method (Clough and Bent 1998) using *Agrobacterium tumefaciens* strain GV3101 (pMP90) and binary vector pMDCPbGH3His. Transgenics were selected on plates containing MS medium supplemented with 50 mg/l hygromycin B (Merck Sigma-Aldrich, Darmstadt, Germany). Each transformant (transgenic line) was transferred into soil (Steckmedium KLASMANN; Klasmann-Deilmann, Geeste, Germany) and after self-fertilization from each plant seeds were collected. Transgenic lines with 75% germination rate on MS medium with 50 mg/l hygromycin B (one T-DNA insertion) were further self-fertilized for homozygous plants selection used for downstream experiments.

Transgene Detection by PCR

Transgenic seedlings were used for genomic DNA isolation (Kasajima et al. 2004) and transgene detected by PCR using EmeraldAmp Max PCR Master Mix (TaKaRa, Saint-Germain-en-Laye, France) and FwPbGH3 and RevHis6 primers (Table S1). For transgene expression quantification RNA was isolated from 10-days-old seedlings by using RNeasy plant mini kit (Qiagen, Hilden, Germany) and 500 ng total RNA was transcribed to cDNA with RevertAid First Strand cDNA Synthesis Kit and oligo dT₁₈ primer (Thermo Scientific, Waltham, MA, USA). Quantification of *PbGH3* gene expression was performed with 10 ng cDNA, Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and gene-specific primers (Table S1). Reaction was conducted with Applied Biosystems 7300 Real-Time PCR system. Mean $\Delta\Delta\text{Ct}$ values were calculated and normalized to *AtEXP* (AT4G26410) reference gene expression. Overexpression level was expressed in regard to native *AtGH3.3* (AT2G23170) gene expression in each line.

Western Blot

PbGH3 protein synthesis in transgenic lines was verified by Western blot and immunodetection. Proteins were extracted from fresh leaf tissue with extraction buffer (20 mM NaH₂PO₄ pH 8, 500 mM NaCl, 10 mM β -mercaptoethanol, 1% Triton X-100, 10 mM imidazole, 1 mM PMSF). His-tagged PbGH3 was purified on Ni-NTA agarose beads (Qiagen, Hilden, Germany) according to the manufacturer's instructions and finally eluted with 400 mM imidazole. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunodetected with primary AntiHis antibody (Proteintech, Manchester, UK), secondary Antimouse horseradish peroxidase (HRP) antibody (Merck Sigma-Aldrich, Darmstadt, Germany) and Luminata Forte Western HRP substrate (Merck Millipore, Darmstadt, Germany).

Plant Inoculation

The single spore isolate e3 from *P. brassicae* was used (Fähling et al. 2003) of which the genome was sequenced (Schwelm et al. 2015). *P. brassicae* resting spores were isolated from *Brassica rapa* subsp. *pekinensis* clubroots stored at – 20 °C according to Siemens et al. (2002). Briefly, resting spores were extracted by homogenizing mature clubroot galls of this Chinese cabbage, followed by filtering through gauze (25 μm pore width) and two centrifugation steps (10 min for 2500 g). The cultivation of *A. thaliana* ecotypes Col-0 and Ws-2 for infection was done in soil:sand mixture (3:1, w/w) (Einheitserde Type P; Hermina-Maier, Regensburg, Germany; commercially available children's playing sand, local commercial supplier) for 14 days under

controlled environment conditions (21 °C, 16 h light, 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Fourteen-day-old plants were inoculated by applying 2 ml of resting spore suspension (10^6 spores ml^{-1}) suspended in 50 mM KH_2PO_4 buffer, pH 5.8, into the soil around each plant (Siemens et al. 2002). Control plants were treated with the same buffer. Harvesting of roots from soil for molecular and hormone analyses was done by carefully removing soil particles in water using a painter's brush, as described in Ludwig-Müller et al. (2017).

Phytopathological Methods

Ca. 40 *A. thaliana* plants per biological replicate were analyzed. Root fresh weight of control and inoculated plants was determined and from that the root index calculated, which indicates the ratio of fresh weight inoculated vs control roots (Siemens et al. 2002). To analyze upper plant part-associated symptoms, the shoot index (Siemens et al. 2002) was likewise determined. The shoot and the root index display the fresh weight of infected plants divided by the fresh weight of control plants. Groups of two to six plants for each line were pooled for statistical calculations. To evaluate the disease severity, two parameters were calculated based on the assignment of the infected roots 28 days after inoculation (dai) into five classes (CI) according to Siemens et al. (2002): 0 = no symptoms; 1 = minor swellings at main root but root system intact; 2 = main roots visibly thickened and lateral roots affected; 3 = galls clearly visible at the primary and secondary roots and the root system strongly reduced; 4 = roots and/or hypocotyls reduced to severe galls. The infection rate was calculated based on the number of all roots from categories 1 to 4 in relation to those without symptoms (0). The disease index (DI) was calculated using the following equation: $\text{DI} = (1 \cdot \text{CI1} + 2 \cdot \text{CI2} + 3 \cdot \text{CI3} + 4 \cdot \text{CI4}) + 100 / 4 \cdot \text{number infected plants}$.

Transcription of *PbGH3* in Plant Tissues

The plant material was harvested at the respective time points, the roots cleaned from soil and frozen and homogenized in liquid nitrogen. Total RNA was isolated using RNeasy RLT (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. The RNA was further treated with DNaseI (Thermo Scientific, Waltham, MA, USA) and cDNA synthesis was carried out with the Maxima First Strand Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) following the manufacturer information. As reference gene *AtYLS8* (At5g08290) was used and to determine successful inoculation we employed *PbActin* (AY452179). Briefly, for all genes except *PbGH3* 25 PCR cycles were performed. The RT-PCR was carried out at the same temperatures, but for *PbGH3* with different cycle numbers (25, 30, and 35 cycles).

Visualization was done with ethidium bromide staining. In Table S1 all primers are given. The primers used for RT-PCR, except for *PbGH3*, and conditions have previously been published (Jäschke et al. 2010; Ludwig-Müller et al. 2017; Bulman et al. 2019).

Quantification of Auxins and Jasmonates

Extraction and purification of IAA, JA, OPDA and their respective conjugates was performed according to the method described by Široká et al. (2022). Briefly, approximately 1 mg of freeze-dried tissue was extracted in 1 mL of cold 1 mol/L formic acid in 10% aqueous methanol containing mixture of stable isotope-labeled internal standards. After the centrifugation (25 200 g, 20 min, 8 °C), 200 μl of supernatant was purified by in-tip micro Solid Phase Extraction (microSPE) using self-assembled columns. After elution, samples were evaporated under reduced pressure and resuspended in 30 μl of 10% aqueous methanol. Ten μl of each sample was injected onto a Kinetex C18 column (50 mm \times 2.1 mm, 1.7 μm ; Phenomenex) and analyzed by a single LC–MS run using a 1260 Infinity II HPLC system coupled to a 6495 Triple Quad detector (Agilent Technologies, USA) by the method described in Hladík et al. (2023) that was modified according to Široká et al. (2022) in order to enable simultaneous profiling of IAA and JA conjugates for which the MS system was operated in ESI positive and negative ionization mode simultaneously. Plant materials were collected in bulk and then divided. For the three biological replicates, plants from different tables in the greenhouse or agar plates, grown at the same time, were used.

Statistical Analysis

For all the experimental conditions using seedlings data for five biological replicates with five seedlings each were collected ($n = 25$). The data were analyzed with the XLSTAT program. ANOVA was used to analyze the relevant factors, and values were considered to be significant at $p < 0.05$. Post hoc multiple mean comparison (Tukey's HSD test) was used for multiple comparisons.

Bioinformatic Analysis

For similarity searches the gene number PBRA_002260 from the e3 reference genome (Schwelm et al. 2015) was used. The gene structure was determined by comparing the genomic with cDNA sequences. Genomes of *P. brassicae* were searched with this sequence using BLAST (Altschul et al. 1997) or specifically for other protists (<https://protists.emsembl.org>). The promoter was analyzed in a range of 323 bp in front of the transcription start using a plant promoter database (Lescot et al. 2002). The number of base

pairs was determined by the transcription end of the predicted gene before the promoter. The plant database was deliberately used for analysis because *P. brassicae* as an intracellular organism could experience regulation of promoters by chemical plant signals. The protein sequence was scanned for putative secretion signals by SignalP – 5.0 (Petersen et al. 2011; Nielsen et al. 2019), iPSORT (Nakai and Horton 1999), SOSUisignal software (Gomi et al. 2004), and for non-classical pathways SecretomeP – 2.0 (Dyrlov Bendtsen et al. 2004). TargetP (Armenteros et al. 2019) was used to determine possible subcellular localization using ‘no plant protein’ as query. The protein was found as accession PLBR_LOCUS6067 in the Uniprot (The UniProt Consortium 2023) and A0A0G4J340 in the Alphafold databases (Jumper et al. 2021; Varadi et al. 2022). Models of plant GH3 proteins for different substrate preferences were based on the searches in the protein databank (PDB, <https://www.rcsb.org>; Bernstein et al. 1977). A summary of all bioinformatic websites used can be found in Table S2.

Results

Bioinformatic Analysis of the PbGH3 Gene

Homology search in the e3 reference genome (Schwelm et al. 2015) revealed a single copy sequence that was further analyzed by bioinformatic methods and annotated as GH3 superfamily (Fig. S1A). Genomes of other protists and *P. brassicae* were further analyzed for the presence of the entry PBRA_02260 for *PbGH3*. There are only four possible

matches in other protists, but neither are known plant pathogens (Fig. S1C) and whether the respective homologs have an enzyme activity has yet to be determined. The BLAST search against “whole genome shotgun contigs” revealed that all 50 genome sequences of *P. brassicae* contain the single *GH3* homolog (Fig. S1D). Only two isolates seem to differ more, AAFC-SK-Pb6 from Canada and ZJ-1 from China.

The *PbGH3* gene has three small introns and, based on the next annotated gene as a putative protein, a very short promoter which contains all necessary regulatory elements (Fig. 1). The promoter sequence was highly conserved in all isolates as well. It was therefore screened for plant regulatory elements since *P. brassicae* completes its life cycle within the host and is therefore in close contact to plant signaling molecules. The promoter contains several plant recognition sequences for the induction, e.g., by auxin and jasmonate (Fig. 1). The gene size is 1860 bp and the CDS 1698 bp, indicating that the dispersed introns are very short (Fig. 1).

There was no predicted sequence for a secretion peptide when using three different prediction tools, two for prediction of classical secretion pathways SignalP and iPSORT, but also one tool for the prediction of non-classical protein secretion, SecretomeP (Table S2). However, with SOSU-Isignal (Gomi et al. 2004) a 27 amino acid stretch was identified as a possible secretion signal which is underlined in the box in Fig. S1B. The method predicts a signal peptide with different parameters recognizing three-domain structures by three modules of the software system (Gomi et al. 2004). Alternatively, the protein could be localized in the

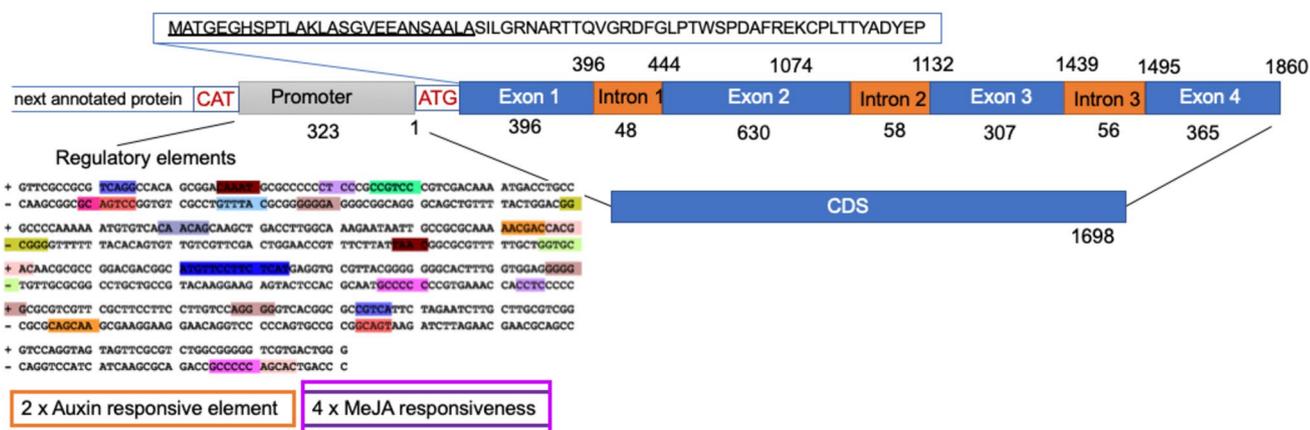


Fig. 1 Some features of the *PbGH3* gene showing that it has three introns and four exons. The presence of plant regulatory elements has been investigated using a plant database (PlantCARE; Lescot et al. 2002) and the two auxin responsive elements and four MeJA responsive elements are highlighted orange and purple, respectively. This was done since *P. brassicae* resides within the host and therefore, the promoter region of the *GH3* gene could be activated by plant signals.

The complete list of putative regulatory sequences of the *PbGH3* promoter is displayed in Fig. S2. The ATG is symbolizing the start of the coding sequence (CDS). A part of the *PbGH3* protein sequence from the N-terminus is shown in the blue rectangle with the putative secretion signal as predicted by SOSUisignal (Gomi et al. 2004) underlined. The complete protein sequence can be found in Fig. S1

plasmodia of *P. brassicae*. TargetP (Armenteros et al. 2019) was used to determine possible subcellular localization using ‘no plant protein’ as query, this revealed no specific subcellular prediction.

Characterization of the Enzyme Activity in more Detail

Similarities between *P. brassicae* and plant GH3 nucleotide sequences were not detected via BLAST search on the nucleotide level. A search with the BLAST query ‘translated nucleotide vs. protein’ resulted in *Vitis vinifera* (GH3.1) and *A. thaliana* (GH3.5, GH3.11 = JAR1) proteins as the only hits with E-values of 2e-29, 6e-25, and 2e-24, respectively. The protein sequence resulted in reasonable similarity when compared to available protein models in PDB for GH3 proteins from *A. thaliana* (Fig. 2A) and *V. vinifera*. PDB curates experimentally determined 3D models based on their crystal structure (Bernstein et al. 1977). These structures are used by other bioinformatic web-based tools such as AlphaFold (Jumper et al. 2021; Varadi et al. 2022) to obtain predicted models for protein queries. The putative PbGH3 protein model from AlphaFold was oriented to fit best with the protein models for host *A. thaliana* GH3 proteins with different carboxylic acids as substrates. The best fits seem to be with AtGH3.5 that conjugates IAA and SA (Zhang et al. 2007) and with AtGH3.12 that is involved in SA biosynthesis from the precursor CA (Rekhter et al. 2019), but also some similarity with GH3.15 which conjugates IBA (Sherp et al. 2018) and AtGH3.11 conjugating JA to Ile (Staswick et al. 2002) (Fig. 2A). Nevertheless, modeling does not substitute for the determination of enzymatic activities.

Preliminary results have shown that PbGH3, when produced as a heterologous protein in *E. coli*, was active with IAA and JA as carboxylic acid substrates (Schwelm et al. 2015). The characterization of the *in vitro* enzymatic activity with more substrates was carried out with an attempt to semi-quantify the results of a set of different experiments (Fig. 2B). The protein was active with a wide variety of substrates, both with the amino acid and the carboxylic acid that was attached. This is very unusual, since plant GH3 enzymes have a broad, but still some specificity concerning substrates. Based on the models predicted, JA would have been the least probable substrate, but the activity was also quite high. OPDA as a substrate for PbGH3 has only once very recently been reported for a plant GH3 activity (Brunoni et al. 2023b, even though OPDA amino acid conjugates have been described (Arnold et al. 2016; Fu et al. 2022). The compound was also conjugated to at least nine different amino acids (Fig. 2C), although it had lower values for conversion compared to all other substrates (Fig. 2B). In contrast to some plant GH3 proteins PbGH3 does not conjugate SA to any of the proteinogenic amino acids

tested (Schwelm et al. 2015). A summary of all amino acids accepted by PbGH3 is summarized in Fig. 2D.

PbGH3 Expression During Clubroot Development

After *A. thaliana* Col-0 root infection with *P. brassicae* the transcript abundance of *PbGH3* during infection in roots at different time points was analyzed to confirm its expression (Fig. 3). *AtYLS8* was used as the host plant reference gene and for *P. brassicae* *PbActin* was employed. The host reference gene expression was uniform in all the samples, and *PbActin* expression was detected, as expected, only in the inoculated plants. The transcript of *PbGH3* was prominently found at later stages of infection, and its earliest expression was detected 14 dai with stronger expression present between 18 and 23 dai, when gall formation starts to be visible on infected plants.

The Overexpression of PbGH3 Results in a Hormone-Related Plant Phenotype

We further transformed *A. thaliana* with the *PbGH3* gene and the molecular and physiological phenotype of transgenic lines was investigated. The use of a constitutive promoter was also reported in other work where *A. thaliana* was transformed with *P. brassicae* genes to verify function (Djavaheri et al. 2019; Yu et al. 2019; Chen et al. 2021). To rule out that the transformation event is integration specific, two independent transgenic lines from two different *A. thaliana* accessions, Col-0 and Ws-2 with a confirmed integration of the transgene and synthesis of the PbGH3 protein *in planta* were analyzed. Three different result categories for the transgenic lines were detected: (1) changes present in all lines compared to wild type, (2) changes that are ecotype or line specific, and (3) changes found in control vs. hormone treatments in all lines. The lines PbGH3-34 and PbGH3-47 were regenerated from Ws-2 ecotype and had *PbGH3* transgene expression 10 and three times higher than the native *AtGH3.3* gene, respectively. The lines PbGH3-52 and PbGH3-64 regenerated from the Col-0 ecotype had very different *PbGH3* expression levels compared to the native *AtGH3.3* gene, namely the first had similar values and the second 58 times higher expression, respectively (Fig. 4). The amount of recombinant protein synthesized in the independent lines was detected by Western blot using the anti-His-tag antibody (Fig. 4). The highest amount of PbGH3 protein was accumulated in PbGH3-34 line of Ws-2 ecotype. Since not in all transgenic plant lines a signal was detected, the PbGH3-His6x proteins were purified over Ni-NTA columns which resulted in recombinant protein enrichment and proved the presence of PbGH3 in all transgenic lines examined (Fig. 4A). Therefore, some differences between the four transgenic lines could be explained by the differences

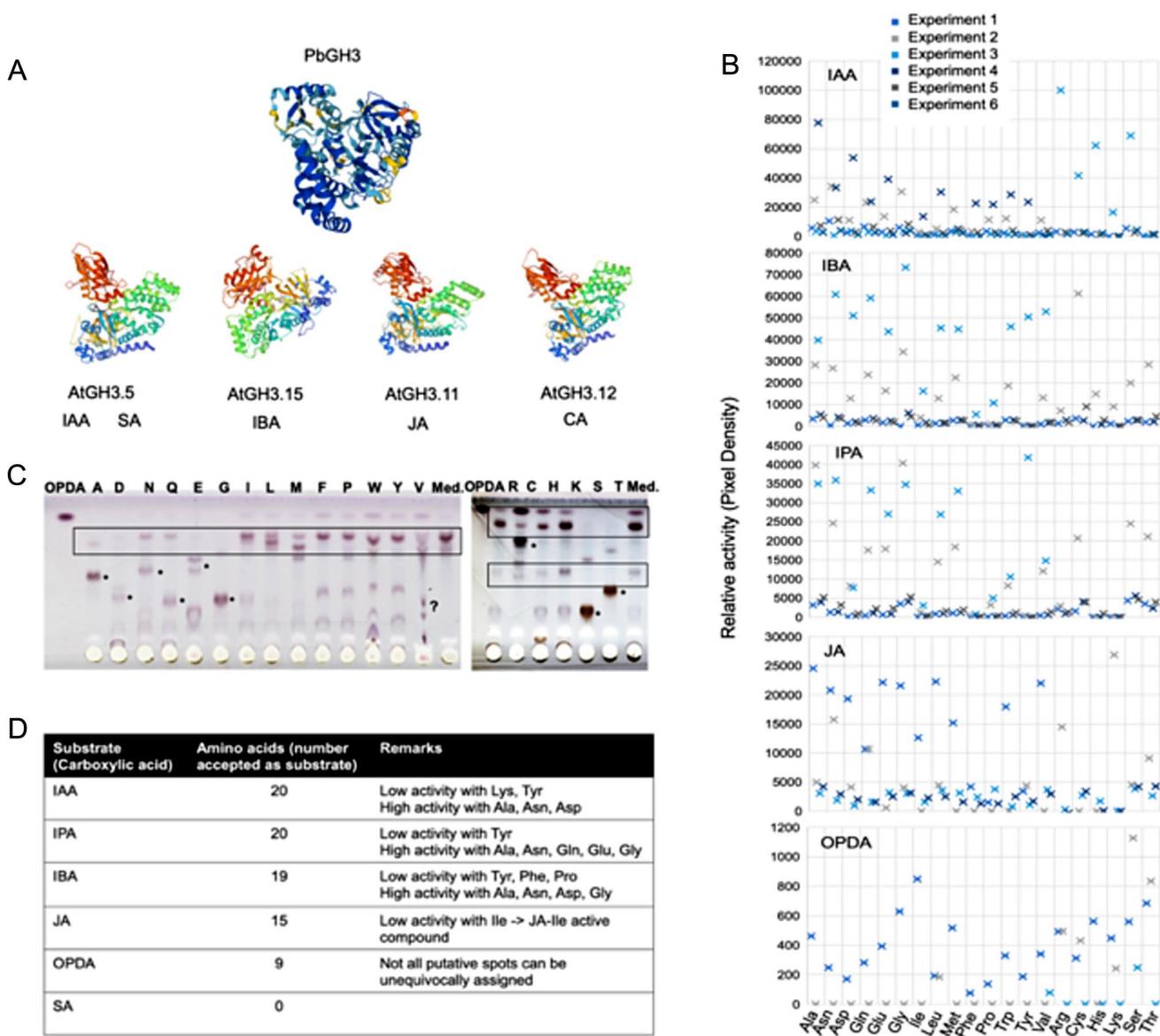


Fig. 2 **A** Structure of the putative PbGH3 protein as determined by AlphaFold (<https://alphafold.ebi.ac.uk/entry/A0A0G4J517>; accessed 26.1.2023) databases (A0A0G4J340). The PbGH3 protein molecule was oriented in a way that it has a similar orientation as the plant PDB models. In the lower row four plant GH3 structures are shown with their respective carboxylic acid substrate given below the name of the protein. While IAA, IBA, JA, and SA are plant hormones, CA is the precursor in one biosynthetic pathway to SA. CA chorismic acid, IAA indole-3-acetic acid, IBA indole-3-butyric acid, JA jasmonic acid. **B** The activity of PbGH3 with IAA, IBA, and IPA yielded a

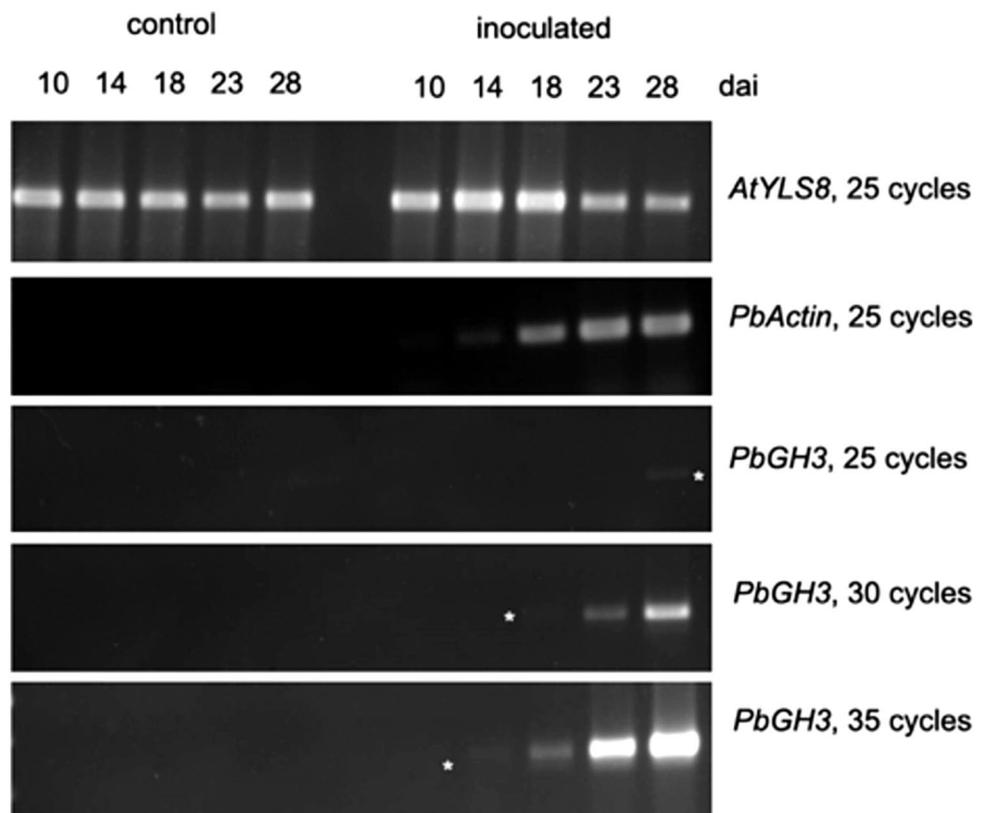
high synthesis of the amino acid conjugate with Ala. Enzyme activity is presented as relative pixel intensity of the three auxins and JA with a range of amino acids for five or six (IAA) independent experiments. SA was not converted (Schwelm et al. 2015). **C** Example of a thin layer chromatography plate with OPDA as substrate; The amino acids are given in the one letter code; Med=medium sample without enzyme; asterisks denote likely reaction products based on bands not present in medium controls. The bands that are also present in medium controls are framed. **D** Summary of all carboxylic and amino acids converted by PbGH3

in protein production (Fig. 4A). Overall, the phenotype of adult transgenic plants grown in soil revealed an altered apical dominance with a reduced number of inflorescences per plant (Fig. 4B).

Since PbGH3 can conjugate auxins as well as JA we incubated the seedlings on IAA and JA-containing media and monitored root growth and biomass accumulation (Fig. 5).

If the PbGH3 protein is also active in planta, the transgenic plants should be more resistant to IAA or JA. In contrast to wild type seedlings, addition of JA significantly reduced average biomass accumulation of all transgenic seedlings while addition of IAA significantly reduced biomass in lines PbGH3-64 (Col-0) and PbGH3-47 (Ws-2) (Fig. 5A, B). Both hormones equally reduced primary root growth of

Fig. 3 RT-PCR to confirm expression of *PbGH3* during clubroot development. As reference genes for the plant *A. thaliana* *YLS8* was used and for *P. brassicae* *actin*. The numbers indicate for the infected roots days after inoculation (dai) and for the controls the corresponding time point (+ 14 days). The cycle numbers refer to the PCR cycles carried out. The asterisks mark weakly expressed bands of *PbGH3*



all lines and wild types (Fig. 5C, D). The development of lateral roots was significantly disturbed in line PbGH3-52 (Col-0). In control conditions, PbGH3-52 had significantly reduced number of lateral roots and in contrast to other lines addition of JA significantly induced lateral root production (Fig. 5E, F).

Auxin and Jasmonate Contents of Transgenic Seedlings and Adult Plants

The levels of IAA, IAAsp, IAGlu, oxIAA, oxIAAsp, oxIAGlu, *cis*-OPDA, JA, JAIIe, and JAGlu were determined for the seedlings from the experiment for which the growth parameters were recorded (Fig. 5) keeping in mind that also OPDA is a potential substrate for PbGH3 (Fig. 2). Hormone levels are given for seedlings grown under control conditions as well as on IAA and JA (Fig. 6). For IAA the following additional amino acid conjugates were analyzed: Ala, Gly, Val, Phe, Trp and for JA Ala, Trp, and Phe, but these were below the detection limit. In a preliminary experiment we analyzed seedling roots and shoots of the Col-0 dependent transgenic set under control conditions separately. However, it was not possible to find the majority of metabolites in the separated root samples (data not shown). Therefore, auxin and jasmonate contents were analyzed in complete seedlings.

The analysis of IAA, its metabolites and degradation products is presented in Figs. 6A–F. All transgenic lines showed significantly reduced free IAA compared to the corresponding wild types in control conditions. After addition of 10 μ M IAA a reduction was found only for the two transgenic lines in the Ws-2 background compared to the corresponding wild type. oxIAA did not dramatically change under control conditions except in one Col-0 based overexpressor line (PbGH3-64) and was not altered after IAA treatment in a consistent manner. Also, the addition of 10 μ M JA did not alter the patterns of these two auxins. IAAsp which was found in the majority of samples only after IAA treatment increased in the two transgenic lines in the Ws-2 background, but not in Col-0 derived lines where a decrease was found. oxIAAsp showed the same pattern after IAA treatment. IAGlu levels decreased in Col-0 lines under control conditions and increased significantly in all transgenic lines after IAA treatment compared to the corresponding wild type. oxIAGlu rather decreased in Col-0 based lines compared to wild types under control and IAA treatment conditions, while line PbGH3-34 (Ws-2 background) accumulated significantly higher level of IAGlu and oxIAGlu in control conditions and after IAA treatment.

JA decreased significantly under control conditions only in line PbGH3-47 compared to the corresponding wild type (Fig. 6G). Treatment with IAA significantly reduced the JA

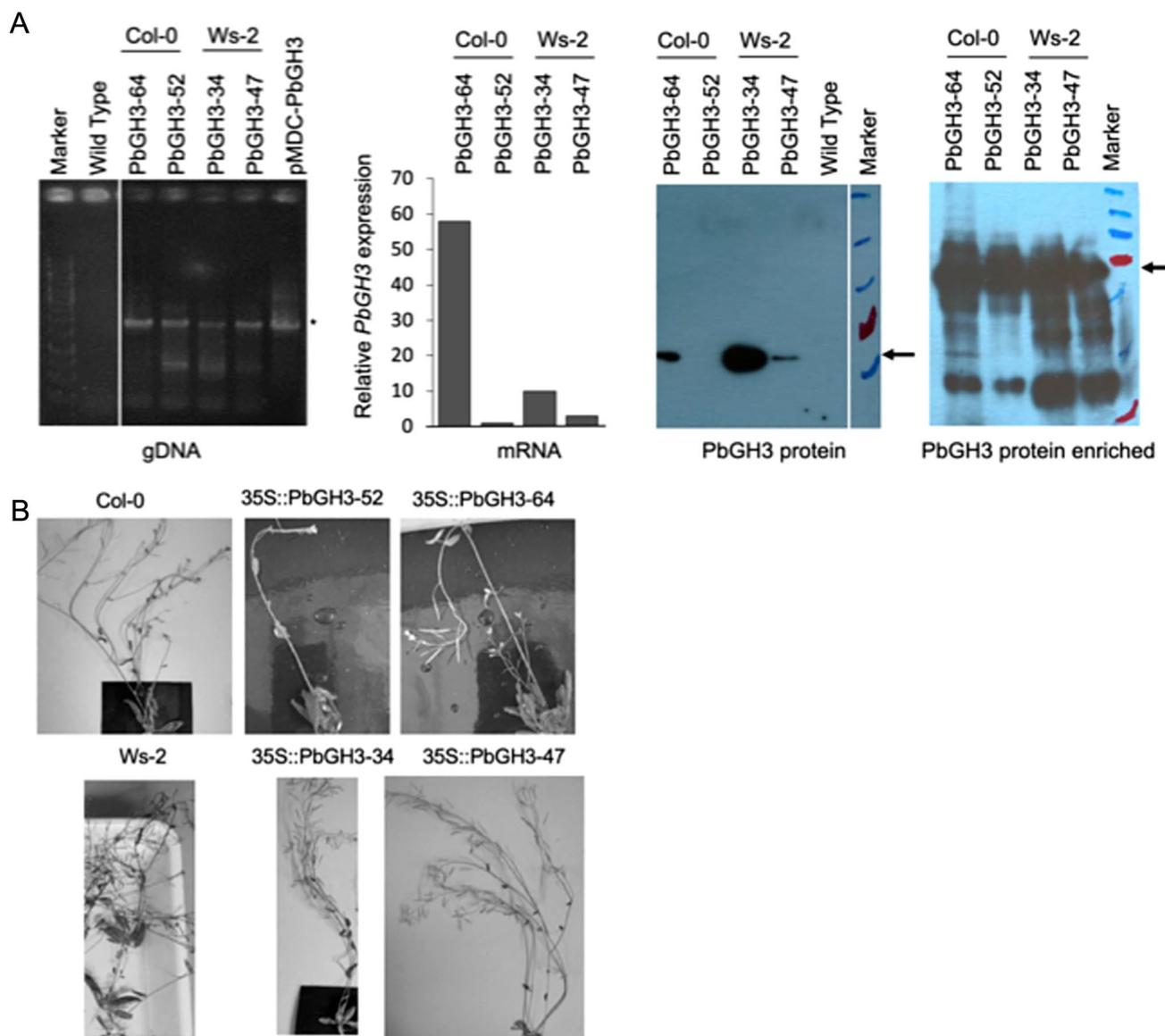


Fig. 4 *A. thaliana* transgenic lines transformation, *PbGH3* transgene expression, protein amount and inflorescence phenotype. **A** After floral dip transformation seedlings were selected on hygromycin B, and *PbGH3* transgene amplified from gDNA by PCR (*denotes 1713 bp amplicon of transgene). In transformants *PbGH3* gene is under the control of the constitutive 35S::CaMV promoter. The transgene expression was quantified by qPCR and shown as fold change in comparison to native *AtGH3.3* gene expression in each line. Identification of *PbGH3* recombinant protein production was performed by Western blot using crude protein extract. Since not in all lines *PbGH3* was

identified, proteins were enriched by Ni-NTA chromatography (right hand panel). The detection was done by using antibodies against the His-tag of the protein. The arrowheads denote recombinant *PbGH3* of 62 kDa. **B** Phenotype of two transgenic lines overexpression *PbGH3* in comparison to wild type (=transformed with an empty construct) for two different *A. thaliana* accessions as backgrounds. On soil the adults showed a reduction of the number of inflorescences per plant. The phenotype was stronger in the lines that displayed higher protein production (**A**)

level in wild type Col-0 and induced JA in *PbGH3*-34 (Ws-2) line. After treatment with JA the levels of endogenous JA increased dramatically, but not with a pattern that would be expected if the overexpressors convert JA to amino acid conjugates. JA_{le} was only detectable after JA treatment and the levels did not change in a transgene-dependent pattern (Fig. 6K). As a second JA conjugate we found JAGlu

in very high amounts after JA treatment, but also for this conjugate no pattern was found that would be consistent for all transgenic lines. Only line *PbGH3*-34 showed an increased level of JAGlu compared to wild type. The JA precursor *cis*-OPDA was also analyzed since it seemed to be a substrate for *PbGH3*. Under control conditions all transgenic lines showed a strong reduction of OPDA. This was

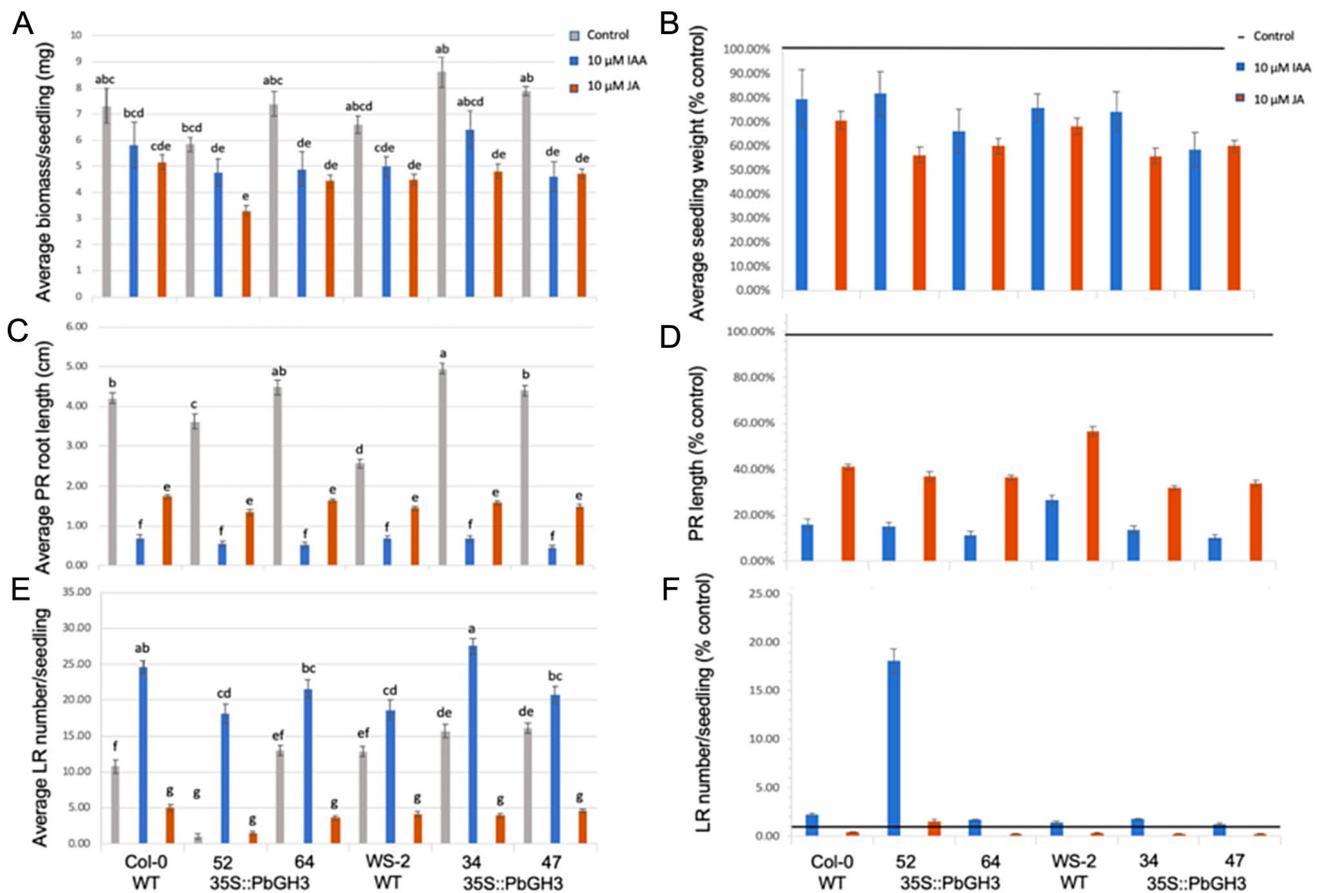


Fig. 5 Physiological traits of the wild type *Arabidopsis thaliana* ecotype Col-0 and WS-2, and overexpressor lines 35S::PbGH3-52, 35S::PbGH3-64, 35S::PbGH3-34, 35S::PbGH3-47. Seedlings were grown on MS medium (control) and on supplemented media with 10 μM IAA or 10 μM JA. **A** Average biomass per seedling; **B** Average biomass calculated as percent of control; **C** Average primary root (PR) length per seedling; **D** Average primary root length calculated as

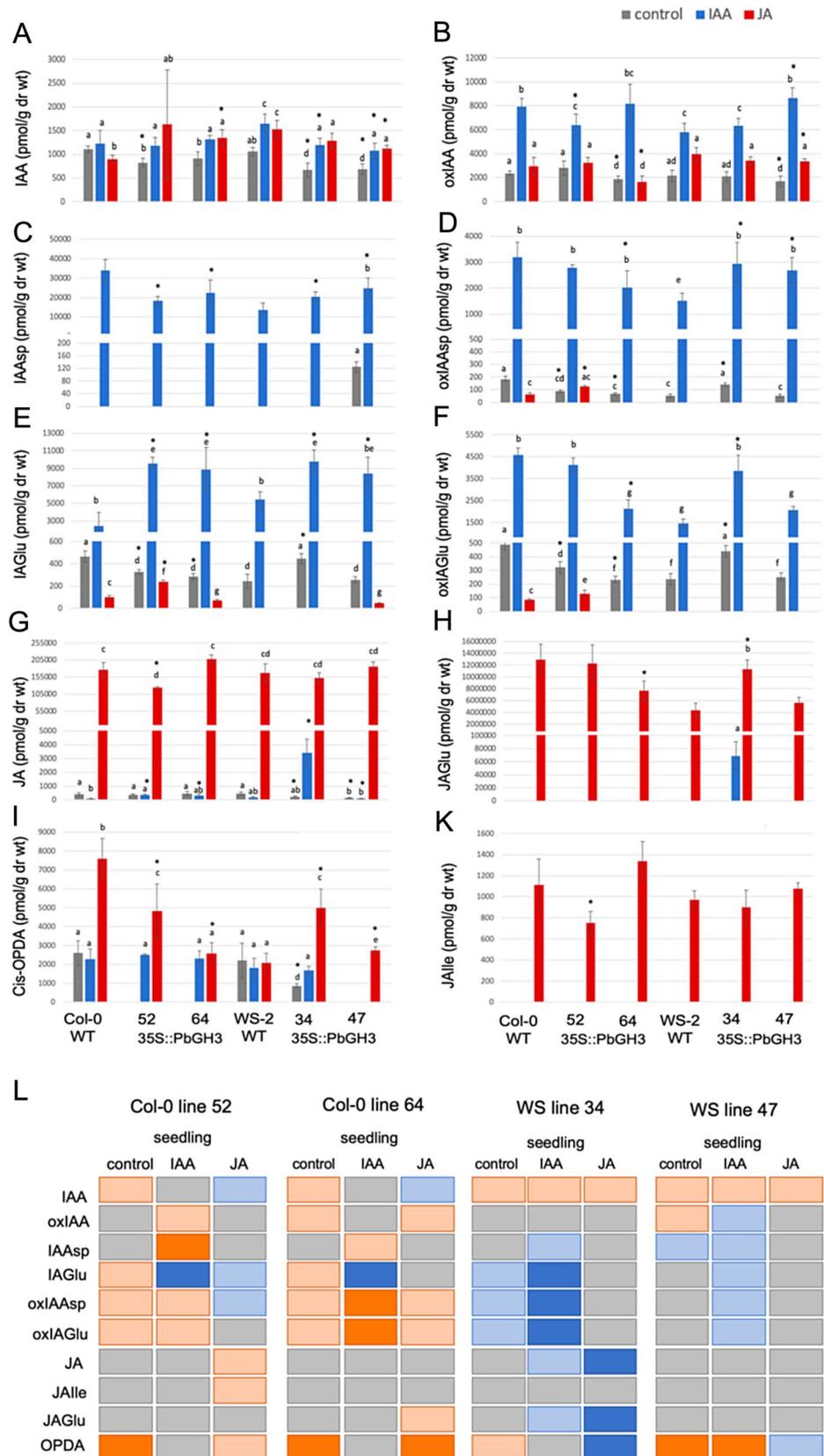
percent of control; **E** Average lateral root (RT) number per seedling; **F** Average lateral root number calculated as percent of control. Black line represents controls. Data are presented as mean ± SE (n=25). Data were analyzed by ANOVA with post hoc multiple mean comparison (Tukey's HSD test) and are labeled with different letters if differing significantly among lines and treatments at $p < 0.05$

found for the Col-0-derived lines also after JA treatment. Based on these metabolite patterns PbGH3 is probably able to conjugate IAA and OPDA also *in planta*, even though trends for the two backgrounds differed as Col-0 based lines accumulated significantly lower, whereas WS-2 lines higher OPDA levels after JA treatment. The conjugation of JA to Ile and Glu occurred only after JA treatment in all lines and also in PbGH3-47 after IAA treatment. JAGlu increased significantly only in line PbGH3-47. For better visualization of the data a heat map for control and hormone treated transgenic lines compared to the wild types was generated (Fig. 6L). These data allow the conclusion that all Pb-GH3 overexpressor lines have lower level of free IAA and the JA precursor *cis*-OPDA under control conditions compared to the corresponding wild types. Furthermore, after IAA treatment the Col-0 based overexpressor lines (52 and 64) accumulated higher levels of IAGlu compared to the wild type, while WS-2 based overexpressor lines (34 in a higher

manner than 47), accumulated besides IAGlu and IAAsp also higher levels of both OxIAA conjugates compared to the wild types. JA treatment resulted in non-consistent and eco-type specific results; while Col-0 based lines decreased their *cis*-OPDA level, WS-2 based lines increased the level of *cis*-OPDA and particularly line PbGH3-34 increased also JA and JAGlu levels.

The hormone changes were analyzed separately in adult plant roots, rosettes, and inflorescences (Fig. 7). These changes differed also in the two accessions. In Col-0 wild type vs. the two transgenic lines in all three different issues free IAA increased unexpectedly in the transgenics, while in the WS-2 background in roots and rosettes the free IAA declined, only in the inflorescences the IAA increased. oxIAA as a degradation product increased in almost all transgenic lines compared to wild type in both accessions. Only two IAA conjugates could be determined, IAAsp and IAGlu, in inflorescence of both ecotypes and rosette of Col-0. Both

Fig. 6 The levels of IAA (A), oxIAA (B), IAAsp (C), oxIAAsp (D), IAGlu (E), oxIAGlu (F), JA (G), JAGlu (H), cis-OPDA (I), JAIIe (K) in whole seedlings grown on agar plates without and with IAA or JA addition determined by LC–MS. Other conjugates were below the detection limit. The levels of detection for all compounds analyzed here are given in Šíroká et al. (2022). When there are ‘zero’ values that means only that these conjugates are below the level of detection. Asterisks indicate statistically significant difference in over-expressor line versus the wild type in a paired Student’s *t*-test ($p < 0.05$) and different letters indicate statistical differences between control and treated plants ($p < 0.05$). **L** Relative hormone levels in transgenic lines compared to the respective wild types after treatments with IAA or JA. The heat map shows two blue shades (light blue twofold; dark blue > twofold) as upregulated compared to wild type and two orange shades (light orange – twofold; dark orange <—twofold) as down-regulation; gray no regulation or in one of the samples no value was determined



conjugates slightly increased in Ws-2 transgenic lines compared to wild types, except IAAsp which was reduced in inflorescence of Col-0 based lines. JA decreased in Col-0 lines, whereas in the Ws-2 background free JA increased in both transgenic lines. Here there was no consistent pattern. Its conjugate with Ile (JAIIe) could only be determined in inflorescence and rosette of Ws-2 wild type and in inflorescences of PbGH3-52 (Col-0) and PbGH3-34 (Ws-2) lines (Fig. 7). None of the conjugates were determined in root tissue. OPDA was not found in these adult plants.

Phytopathological Analysis of Transgenic Plants After Inoculation with *P. brassicae*

The transformants were analyzed for their phenotype after infection by *P. brassicae*. We determined values that represent disease severity, namely the ratios of root and shoot weight for infected vs. control plants (Fig. 8A), the infection rate and the disease index (DI) (Fig. 8B). The DI consists of a system of four classes, of which examples for individual roots in the respective class is shown in Fig. 8B on the right side. High values for infection rate and DI represent strongly infected plants. Generally, no difference between the wild type and the two transgenic lines in terms of infection rate and disease severity as indicated by the DI was found (Fig. 8B). Also, the overall disease classes did not reveal any significant differences that would be the same for both ecotypes (Fig. 8). The experiment was repeated with independently cultivated plants that showed the same results (Fig. 8B). The root and shoot indices, however, differed for both transgenic lines compared to wild types, and in most cases the differences were significant (Fig. 8A). Here, all transgenic lines were reduced compared to wild type accessions. A high shoot index indicates that plants are performing better. However, this shoot phenotype was only obvious in the photographs of all Ws-2 based lines (Fig. S3). A lower root index would indicate that root clubs are reduced. This could be expected if a protein (PbGH3) that constitutively conjugates IAA to inactive amino acids is present *in planta* at all times. Indeed, we found lower levels of free IAA in infected transgenic plants, whereas IAA was elevated in *P. brassicae* infected roots (Fig. S4).

Auxin and Jasmonate Contents in Wild Type and Transgenic *P. brassicae* Infected Plants

The same free and conjugated hormones as for the seedlings were analyzed in *P. brassicae* infected plants compared to the mock controls, separately in inflorescence (when possible), rosettes and root (Fig. S4, Fig. 9). The analysis of IAA and JA metabolites in both wild type ecotypes showed strong inductions of IAA and JA in

rosettes and roots of infected plants compared to controls (Fig. S4) while no comparisons can be done for inflorescences of Col-0, because these were not present in inoculated plants. In inflorescences of infected Ws-2 plants there was a slight increase in IAA but a decrease in JA compared to controls. These patterns were not found for the degradation metabolite oxIAA, namely Col-0 had an increase in roots, but not rosettes, and Ws-2 vice versa, and the metabolite increased in infected Ws-2 inflorescences. Only in infected root and rosette tissues of Col-0 plants there was a considerable increase in the IAA conjugate with Glu, and since IAAsp was not found in inoculated tissues, a decrease in rosettes is reported. In Ws-2 neither conjugate was present in roots or rosettes, but in inflorescences where only IAAsp increased in infected ones. JAIIe was not present in any Col-0 sample in detectable amounts, but in infected Ws-2 root tissues an increase of this defense signal was seen whereas rosettes and inflorescences did not show such an induction (Fig. S4). The JA response occurs later in the development of the disease, while a response to SA is occurring earlier. This late activity could be in line with the expression levels of *PbGH3* that were found at later time points in Col-0 plants (Fig. 3). Ecotype specific defense hormone changes in clubroot diseased plants have been observed for Bur-0 vs. Col-0 roots (Lemarié et al. 2015).

The transgenic lines exhibited a more complex pattern after inoculation (Fig. 9). Here, it needs to be taken into account that in the infected tissues the activities of the native and transgenic enzymes are present. In the Col-0 background all transgenic plants did not develop inflorescences in the infected set of plants. In brief, many ecotype- and tissue-specific changes were observed in hormones upon infection in the transgenic lines. Overall, different intensities were observed in different lines of the same ecotype as well (Fig. 9). For example, the free IAA content was decreased in roots of both Col-0 based lines compared to wild type, while it increased in both Ws-2 based lines. IAA and oxIAA followed the same pattern of changes with sometimes non-significant decrease or increase in all lines compared to the wild types. The levels of IAA conjugates were unchanged or followed the pattern of IAA in roots of both ecotype lines, namely IAAsp and IAGlu were both induced in the Ws-2 background, but not in the Col-0-based lines compared to wild types. In the transgenic lines JA was decreased in roots and rosettes of all lines together with an unchanged level of JAIIe in most lines or an increased level of JAIIe in line PbGH3-47. Inflorescences, formed only by Ws-2 based plants upon infection showed a small decrease in IAA and an increase in JA. For better visualization of the data a heat map for control and hormone treated transgenic lines compared to the wild types was generated (Fig. 9C).

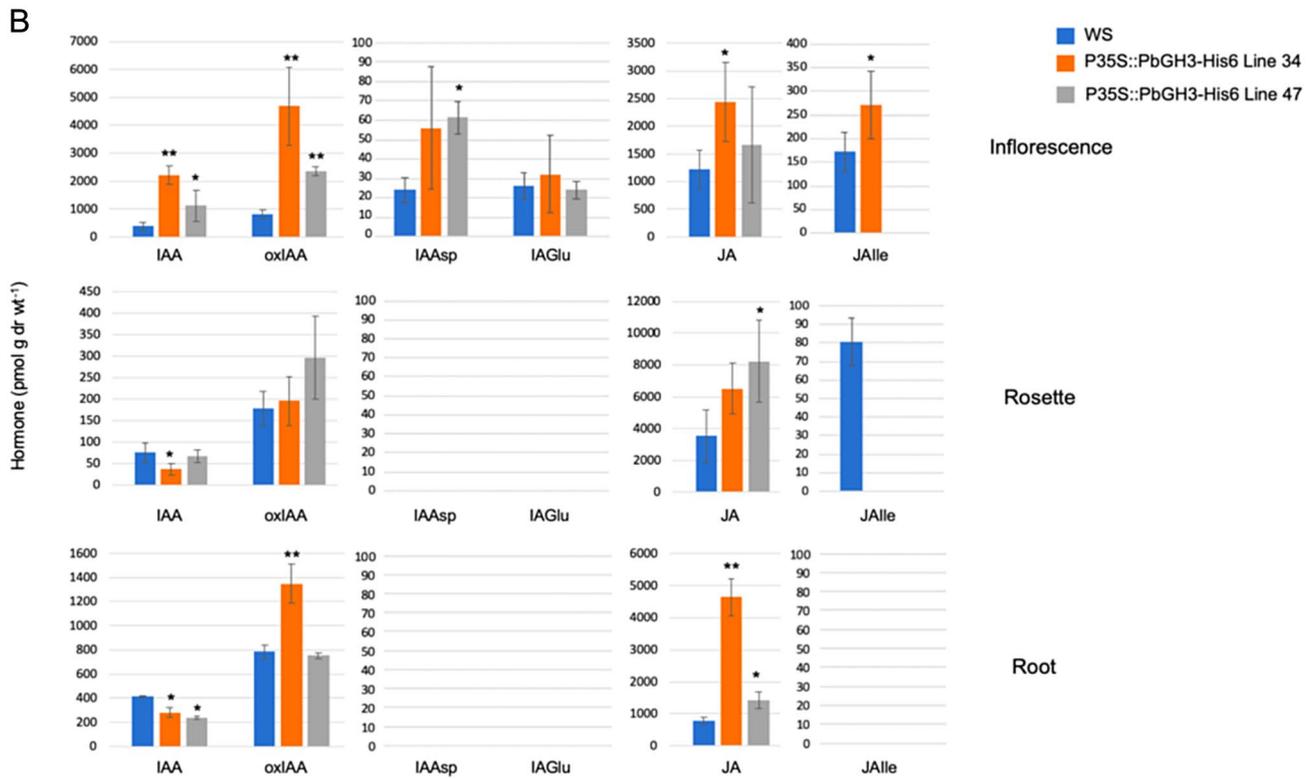
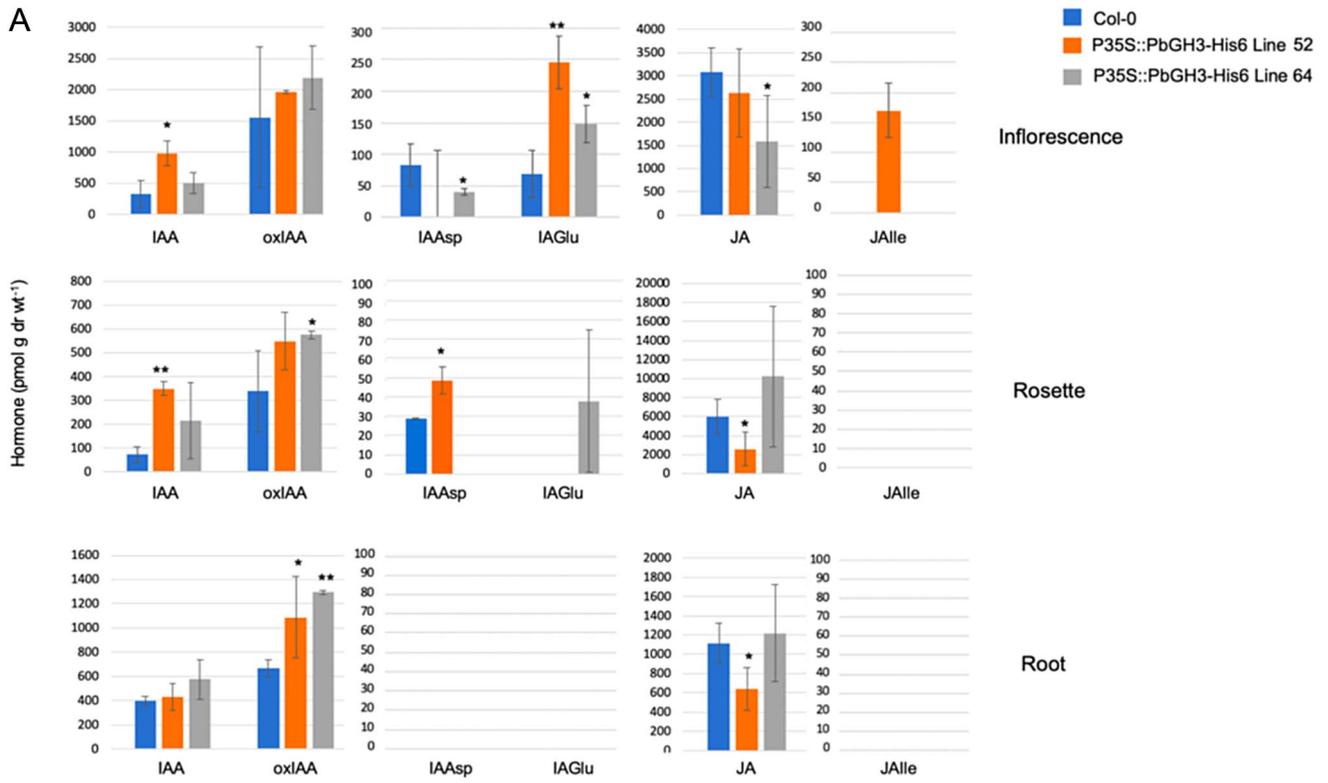


Fig. 7 The levels of IAA, JA, and selected amino acid conjugates in inflorescences, leaves, and roots of plants grown on soil determined by LC–MS. These were the same experimental batch as the inoculated plants (Fig. 9). Other conjugates were below the detection limit. The levels of detection for all compounds analyzed here are given in Široká et al. (2022). **A** Col-0 background and two transgenic lines; **B** Ws-2 background and two transgenic lines in roots, rosette leaves, and inflorescences. When there are ‘zero’ values that means only that these conjugates are below the level of detection. Asterisks indicate statistically significant difference in overexpressor line versus the wild type in a paired Student’s *t*-test (* $p < 0.05$, ** $p < 0.01$)

Discussion

The Clubroot Pathogen has a Functional Plant Hormone-Metabolizing Protein

For micro-organisms that interact with host plant tissues, IAA can be either beneficial through growth-promoting effects, or detrimental by being involved in plant defense (Duca et al. 2014). The control of plant hormone levels by a biotrophic organism can constitute a possible mechanism to interfere with the plant’s response, especially, if the respective hormone is needed for full symptom development or involved in defense responses. Since auxins play a role in the formation of clubroot symptoms in various host species (Ando et al. 2006; Ishikawa et al. 2007; Jahn et al. 2013; Wei et al. 2021), the single *P. brassicae* GH3 gene product could therefore have a role in controlling IAA homeostasis. The origin of this gene is not clear, it could be evolved in the protist, or having been transferred by horizontal gene transfer. The latter might be possible, since it was shown in one publication that *P. brassicae* can integrate host DNA (Bryngelsson et al. 1988). Furthermore, *P. brassicae* can also integrate foreign DNA as shown by the analysis of GFP that had been integrated via zoospores, but a foreign protein had not been produced (Feng et al. 2013).

The sequence for the *PbGH3* CDS as well as the promoter was highly conserved over all *P. brassicae* isolate sequences worldwide (Fig. S1). Since the promoter sequences were also conserved, a screen for putative regulatory elements using a plant-specific database was carried out (Fig. 1), presuming that the majority of the life cycle of *P. brassicae* takes place in the host tissues and the *PbGH3* promoter would be accessible to host signals. Indeed, two IAA- and four JA-responsive elements were found in the *PbGH3* promoter. Other elements were identified as being regulated by stressors or light (Fig. S2). In plants, not all but many GH3 genes encoding proteins that convert IAA to amino acid conjugates are auxin inducible (Liu et al. 1994; Ulmasov et al. 1995). These could be activated if too much of the plant hormone is intracellularly present. Such regulatory elements could also be expected in other plant pathogenic organisms, especially biotrophic ones that live in close proximity to

their host. Tomato spotted wilt tospovirus (TSWV) has such an auxin-responsive element (AuxRE) in the promoter region that was shown to be active in *A. thaliana* since a mutation in the AuxRE abolished the sensitivity to auxin (Zhai et al. 2019). The alignment of more TSWV sequences from GenBank revealed the presence of AuxRE sequences in 14 out of 56 genomes, which may be a significant number because the authors could not find similar elements in the promoters of other known tospoviral genomes (Zhai et al. 2019).

The family of plant GH3 proteins catalyzes the formation of conjugates with hormones such as IAA, IBA, SA, or JA with a selected set of proteinogenic amino acids, thereby inactivating the hormone (Piotrowska and Bajguz 2011). An exception is the formation of the conjugate of CA with Glu, that has a role in the biosynthesis pathway of SA (Rekhter et al. 2019). Two classes of substrates were identified that showed high activity with a set of proteinogenic amino acids. Its broad substrate range toward the corresponding amino acids is different from that of land plant members. While plant GH3 enzymes show a broad, but still more specific substrate preference for the carboxylic acids (Piotrowska and Bajguz 2011), the *PbGH3* has a high promiscuity for accepting all auxin and JA derived carboxylic acids together with almost all amino acids as substrates (Fig. 2), except SA which is not converted (Schwelm et al. 2015).

Functional Analysis of *PbGH3* in the Host Plant Shows Alterations in Phenotypes

Overexpression of *PbGH3* in *A. thaliana* leads to phenotypes that can be partially attributed to the in vitro enzyme activities (Fig. 2). Since several hormones are substrates, cross-talk between the respective hormones has to be considered. The IAA and JA effects on lateral root formation are often antagonistic (Ishimaru et al. 2018; Xu et al. 2020), but there are also examples of synergism like the JA induced auxin biosynthesis via anthranilate synthase1 (Sun et al. 2009). JA also partially controls the angle of lateral roots together with auxin (Sharma et al. 2022). High IAA levels could be detrimental for plant growth and development since both, shoot and root development, depend on the respective IAA concentration (Gallavotti 2013; Overvoorde et al. 2010) and can be regulated by, for example, GH3 proteins in plants (Park et al. 2007). Jasmonates also control root architecture in addition to auxin (Han et al. 2023). Furthermore, the application of JAs inhibits various aspects of seedling growth, including primary root growth, leaf expansion, and hypocotyl elongation (Huang et al. 2017).

Total seedling fresh weight was reduced in all lines on both hormones compared to control conditions (Fig. 5A, B). It was not altered on IAA when all transgenic lines were compared to the wild type, but reduced in one Col-0 based

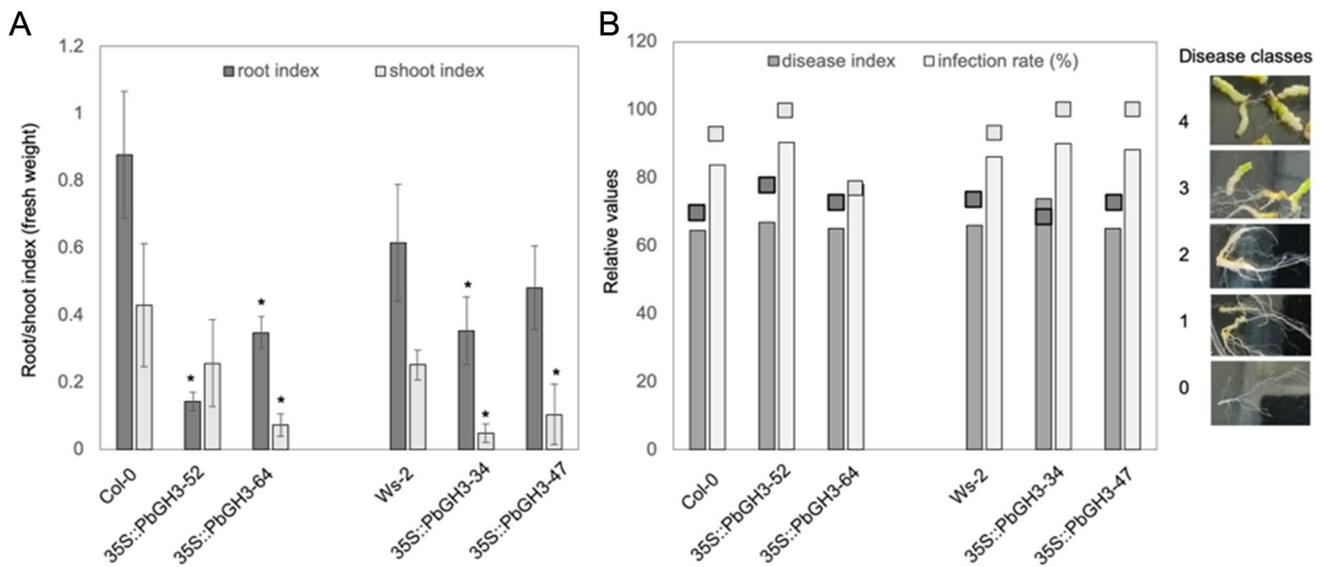


Fig. 8 Phytopathological analysis of *PbGH3* overexpressor lines compared to their respective background lines. **A** Root and shoot index, both reflecting the weight of infected vs. control plants. Asterisks indicate statistically significant changes ($p < 0.05$) between wild type and transgenic lines of 2–6 pools of independently harvested plants; **B** disease index that reflects the severity of the disease and infection rate (%) were determined. The histograms and squares rep-

resent data from separate experiments carried out in different annual seasons. Examples for the individual disease classes that were used to determine the disease indices and infection rates are shown next to panel B. The upper plant parts and roots of typical trays are shown in Fig. S3 as well as the fresh weight that was used to calculate the root and shoot indices

line on JA (PbGH3-52; Fig. 5A, B). Primary root growth of all lines was inhibited on IAA- and JA-containing media in comparison to the control conditions. On control medium the two Ws-2 based lines showed an increased primary root length, whereas no consistent effect concerning primary root length was found for the Col-0 based lines (Fig. 5C, D). The number of lateral roots on IAA increased for all seedlings compared to the control conditions and specifically the huge increase observed for line PbGH3-52 is probably due to the extremely low number of lateral roots of this line under control conditions (Fig. 5E, F). Therefore, the same line showed an increased number of lateral roots on JA-containing medium compared to control treatments. Under control conditions both Ws-2 based transgenics had enhanced lateral root formation when compared to wild type. For the ecotype Col-0 the wild type and line PbGH3-64 were similar, but line PbGH3-52 had significantly reduced lateral root initiation. There were some significant changes between wild types and transgenic lines on IAA-containing medium, but again without a consistent pattern. On JA there were no differences for all plant sets compared to their respective wild types (Fig. 5E, F). Overall, our research employing seedlings revealed that only a small set of the parameters investigated showed a trend that could be linked to the expression of *PbGH3*, while many observations seem related to the effect of the hormone treatments or were even ecotype or line-specific.

Some apparent phenotypes from the adult transgenic plants can be attributed to alterations in IAA like fewer branches in some experiments on soil and changes in the number of lateral roots (Figs. 4, 5). These are reminiscent of *A. thaliana* plants overexpressing *GH3* genes encoding proteins involved in IAA conjugation which show reduced shoot and root growth and the number of branches (Park et al. 2007), whereas mutations in *GH3* genes do not lead to obvious phenotypes unless they are subjected to changes in growth conditions (Casanova-Saez et al. 2022).

The infection of transgenic plants did not much alter the phenotype, indicating that the *PbGH3* protein is rather a ‘mild’ factor in pathogenesis. Overexpression resulted in somewhat smaller root galls, as indicated by the root index (Fig. 8). *A. thaliana gh3* double mutants resulted in a slightly more susceptible phenotype after inoculation with *P. brassicae* (Jahn et al. 2013).

Hormone Profiles of Transgenic Plants are Complex

In the transgenic lines there were expected, but also unexpected changes in the hormone profiles (Figs. 6, 7). Transgenic seedlings revealed a reduction in free IAA for all lines tested, and this was observed partially for the IAA amino acid conjugates specifically after growing the seedlings on high IAA concentrations (Fig. 6). oxIAA is a degradation product that may also involve conjugation via IAAsp and

IAGlu, while other IAA conjugates could be hydrolyzed back to free IAA (Mellor et al. 2016). Since oxIAA was always increased in the transgenic lines, together with the IAA conjugates IAAsp and IAGlu, this could indicate a degradation pathway that is induced in the PbGH3 lines, at least in the seedling stage. Only recent analyses have broadened the knowledge on the formation of oxidized forms of IAA and also shown conjugate formation of oxIAA metabolites with Asp and Glu (Hayashi et al. 2021; Müller et al. 2021; Brunoni et al. 2023a; Hladík et al. 2023). In adult plants free IAA increased in some transgenic lines and tissues compared to controls, which we would not expect. However, other pathways such as IAA hydrolysis could also contribute to the overall outcome of free IAA levels (Ludwig-Müller 2011; Savić et al. 2009), even though additional IAA conjugates important for the hydrolysis back to free IAA, such as IAAla, were not found in our analyses.

As for JA, the results seem more consistent for a role in the conjugation to conjugates other than Ile. In the seedlings we found also high amounts of JAGlu, but no major differences between the transgenic lines and wild types. On medium with JA the transgenic lines based on the Col-0 background had reduced, while Ws-2 based transgenic lines had increased JA levels compared to corresponding wild types indicating a different JA metabolism in the two ecotypes. Finally, OPDA was reduced in all transgenic seedlings when grown on control medium. This could be an indication for the formation of OPDA conjugates. Like IAA conjugates, conjugates with JA can be hydrolyzed to yield the free hormone (Widemann et al. 2013). In adult plants, JA was mainly higher in the Ws-2 based transgenic lines and JA Ile was only present in inflorescence tissues (Fig. 7), but conjugates other than JA Ile in the samples were not found, probably due to the low detection limits for these. As reported by Široká et al. (2022) the LODs for JA were 0.75 fmol, for OPDA 2.5 fmol, for JA Ile also 2.5 fmol, and details on all other LODs for the compounds determined in our work are also reported in that study. The infected transgenic lines exhibited a more complex metabolite pattern (Fig. 9), possibly due to the presence of the plant GH3 proteins that might be independently regulated. Indeed, an upregulation of GH3 genes in *A. thaliana* during club formation was found in different transcriptome datasets (Jahn et al. 2013; Ludwig-Müller 2014b). Only the reduction of JA in the roots of transgenic plants compared to controls seems to be consistent in all lines. When the hormone levels in controls vs. *P. brassicae* infected tissues were compared in wild type plants free IAA increased in the root and rosettes of both accessions (Fig. S4). The JA content rather decreased indicating a possible function for defense suppression (Lemarié et al. 2015). oxIAA did not change or decreased in infected vs. control tissues. The patterns for IAA conjugates were not consistent between the wild types and tissues. JA

increased in infected roots and a bit in the rosettes, while it decreased in the inflorescences, but JA Ile did not increase in infected vs. controls (Fig. S4). The *PbGH3* gene is expressed rather late during infection (Fig. 3). Again, this could point to a function to suppress JA-related defense, since the JA-dependent defense against clubroot is induced at later time points, whereas SA-related defense is induced early on (Lemarié et al. 2015).

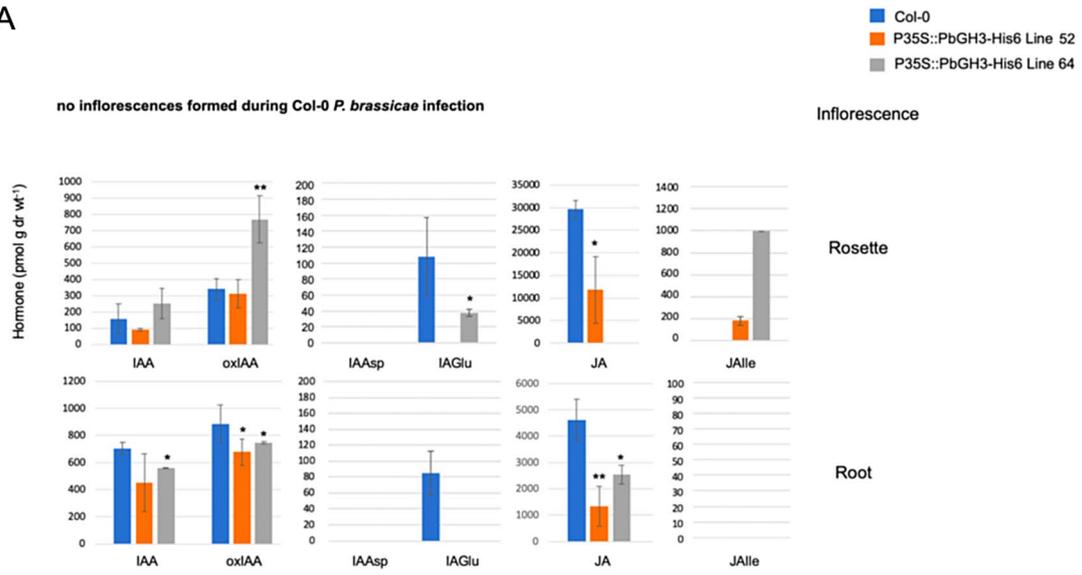
PbGH3 Function may be Connected to Localization in Host or Protist

The localization of the PbGH3 protein either in the protist or in the host could tell something about a putative role (Fig. 10). First, PbGH3 could localize within *P. brassicae* to regulate too high hormone concentrations if IAA (or other hormones) could enter the plasmodia (Fig. 10). Alternatively, a specific auxin conjugate can act as a signal in pathogenesis. This has been described for two completely unrelated plant pathogenic organisms, *Pseudomonas syringae* and *Botrytis cinerea* where IAAsp is a virulence signal (González-Lamothe et al. 2012). The conjugate specifically induced expression of such genes. Consequently, treatment with IAAsp increased susceptibility, while in *gh3* mutants of *A. thaliana* the effect was diminished (González-Lamothe et al. 2012). However, this might not be the most likely role for PbGH3 since the protein can synthesize a high number of amino acid conjugates.

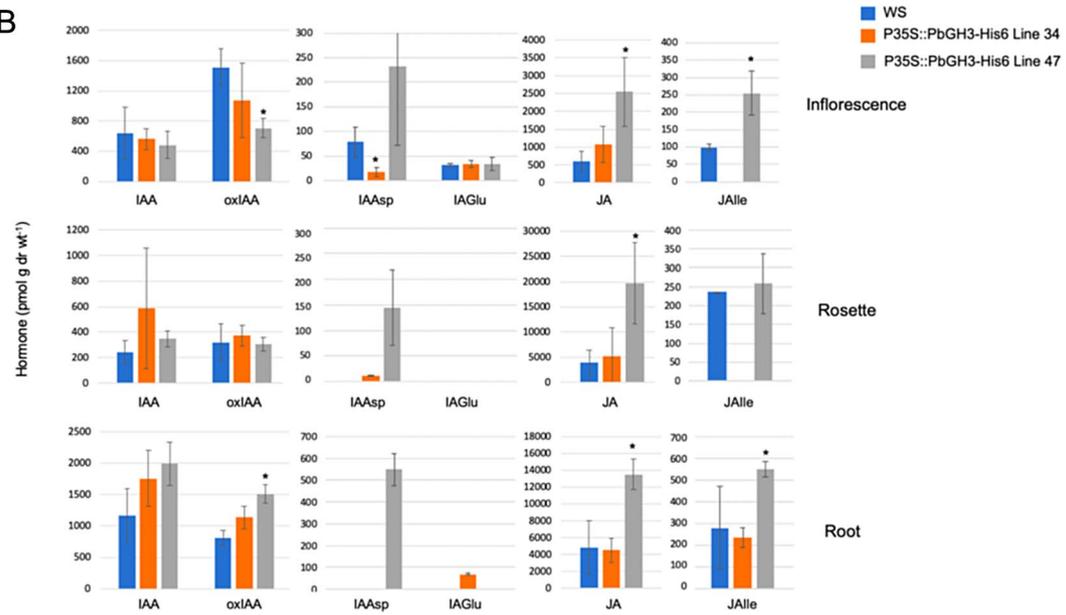
Second, PbGH3 is located in the host cell, where its role may lie in controlling of the auxin and/or jasmonate concentration. Concerning auxin, PbGH3 may be involved in the control of auxin vs. cytokinin levels during the phases of tissue proliferation. This would fit to the late expression of *PbGH3* in developing clubs (Fig. 3). Recently, it was shown that the simultaneous response to auxin and cytokinin is essential for proper gall development and a promoter only responsive to both hormones was activated specifically in clubs (Hurný et al. 2020). In addition to IAA, IBA has also been found in various plant species, but IPA was reported only in a few. An auxin conjugate hydrolase gene from *Brassica rapa* is expressed during pathogenesis (Schuller and Ludwig-Müller 2006) and the corresponding enzyme has a much higher activity to cleave IBA and even more IPA conjugates with alanine (Savić et al. 2009). However, the corresponding substrates have to be available.

The second substrate group that could be targets of PbGH3 in the host cell contains jasmonates, specifically JA which is a signal for the activation of the defense pathway when conjugated to isoleucine (Xie et al. 1998; Guo and Stotz 2007), and OPDA, a jasmonate precursor, but also a signal on its own (Stintzi et al. 2001; Liu and Park 2021). OPDA is a novel substrate for a GH3 protein and only very recently it was shown that GH3 proteins can act on this

A



B



C

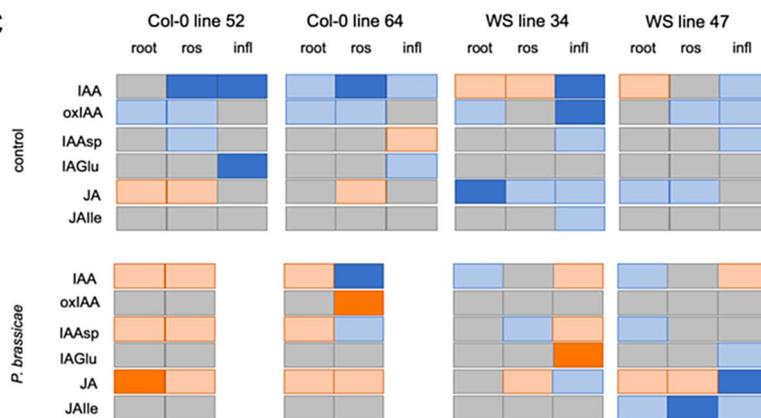


Fig. 9 The levels of IAA, JA, and selected amino acid conjugates in inflorescences, leaves, and roots of plants grown on soil which were inoculated with *P. brassicae* 10 days after sowing determined by LC–MS. Other conjugates were below the detection limit. The levels of detection for all compounds analyzed here are given in Široká et al. (2022). **A** Col-0 background and two transgenic lines; **B** Ws-2 background and two transgenic lines in roots, rosette leaves, and inflorescences. When there are ‘zero’ values that means only that these conjugates are below the level of detection. Asterisks indicate statistically significant difference in overexpressor lines versus the wild type in a paired Student's t-test ($*p < 0.05$, $**p < 0.01$). **C** The levels of IAA and JA and their metabolites determined in inflorescences, leaves, and roots of adult plants grown in soil. The plants were mock-inoculated (controls; data Fig. 7) or inoculated with *P. brassicae*. A comparison of hormone measurements comparing infected tissues of wild type plants only can be found in Fig. S4. Relative hormone levels in transgenic lines compared to the respective wild types are given. The heat map shows two blue shades (light blue twofold; dark blue > twofold) as upregulated compared to wild type and two orange shades (light orange—twofold; dark orange <—twofold) as down-regulation; grey no regulation or in one of the samples no value was determined

compound (Brunoni et al. 2023b). OPDA conjugates, specifically with Ile, were shown to act in defense either dependent or independent of COII (Wasternack and Hause 2016; Liu and Park 2021). PbGH3 does not conjugate Ile to OPDA (Fig. 2B, C), thus avoiding to activate that possible defense pathway. In *A. thaliana*, two GH3 proteins have been shown to conjugate JA to amino acids, both specific to conjugate Ile (Staswick and Tiryaki 2004; Delfin et al. 2022). The activity of PbGH3 with Ile is low compared to other amino acid substrates (Fig. 2), so maybe by competing with other amino acids available, the JA is less conjugated to the active JA-Ile signal that interacts with the receptor COII. Indeed, while JA is dramatically increased in infected roots, JA-Ile is not found in ecotype Col-0 and in very low amounts in Ws-2 plants (Fig. S4). It was shown that in addition to SA a JA-dependent pathway contributed to partial resistance against clubroot in *A. thaliana* (Lemarié et al. 2015). In contrast to the SA-dependent defense, the JA levels increase at a late time point or at least genes for the JA pathway are induced later (Siemens et al. 2006; Agarwal et al. 2011; Lemarié et al. 2015; Prerostova et al. 2018). Also, this would fit to the late expression of the *PbGH3* gene.

The PbGH3 protein has to be secreted if its role as an effector lies in the control of plant hormones within the host. Motifs including RxLR and PEXEL-like which are hallmarks for other biotrophic plant pathogens, such as oomycetes (Liu et al. 2014) or functional equivalent to translocation domains of the malaria parasite *Plasmodium* spp. (Bhattacharjee et al. 2006), were found in predicted effector sequences from *P. brassicae* (Perez-Lopez et al., 2020). Candidate effectors containing the RXLR motif functioned in the cytoplasm and endomembrane of plant cells (Hossain et al. 2021). A different type of effector was predicted for the methyltransferase PbBSMT able to derivatize

salicylic acid and thereby interfere with the plant's immune system (Ludwig-Müller et al. 2015; Bulman et al. 2019). The secretion of PbBSMT has been demonstrated in vitro (Djavaheri et al. 2019) and its overexpression compromised plant defense responses (Bulman et al. 2019; Djavaheri et al. 2019). PbGH3 does not possess a ‘classic’ secretion signal. However, with SOSUIsignal (Gomi et al. 2004) a 27 amino acid stretch was identified as a possible secretion signal (Fig. 2) that would need a different secretion pathway as compared to the classical pathways. Proteins that lack signal peptides can access the cell exterior via at least four different unconventional protein secretion pathways documented in animal, plant, bacterial, and yeast cells (Nickel 2010; Ding et al. 2012) such as extracellular vesicles (Zhou et al. 2022). For plant pathogenic fungi unconventional secretion systems have been described, e.g., the rice blast fungus *Magnaporthe oryzae* has two systems to target effectors during plant infection, of which one can be inhibited by brefeldin A, the other involves exocyst components and t-SNAREs (Giraldo et al. 2013). Another unconventional secretion pathway has been described for effectors from *Phytophthora sojae* and *Verticillium dahliae* that target salicylic acid-mediated defense induction in the host plants (Liu et al. 2014) that were predicted by SecretomeP 2.0 (Dyrløv Bendtsen et al. 2004) and experimentally confirmed (Liu et al. 2014).

Conclusion

The identification of a protein from *P. brassicae* that is able to adenylate a large number of acidic plant hormones to their respective amino acid conjugates triggered our interest in exploiting this observation further. Even though the PbGH3 protein did not show high similarities to plant proteins on the amino acid levels, the protein model was quite similar to plant GH3 proteins with different substrate preferences. The activity of PbGH3 in vitro assayed in *E. coli* showed that the protein, in contrast to plant GH3 proteins, has a very broad substrate range that it can convert, both at the carboxylic acid side, and also for the amino acids accepted.

To be active as a pathogenicity or virulence factor, it has to be localized to the host cell (Fig. 10). The transcription of *PbGH3* occurred at later time points during disease development where the galls were already visible, and it might be regulated by plant hormones since several auxin- and jasmonate-responsive elements were identified by bioinformatic predictions. Future experiments need to be directed to show the localization of PbGH3 in either pathogen stages and/or hosts to further elucidate its function. For this, a specific antibody would be needed, but this might be complicated due to the high number of highly conserved GH3 proteins present in the plant. The pathogen's GH3 protein may not be structurally different enough as indicated by the

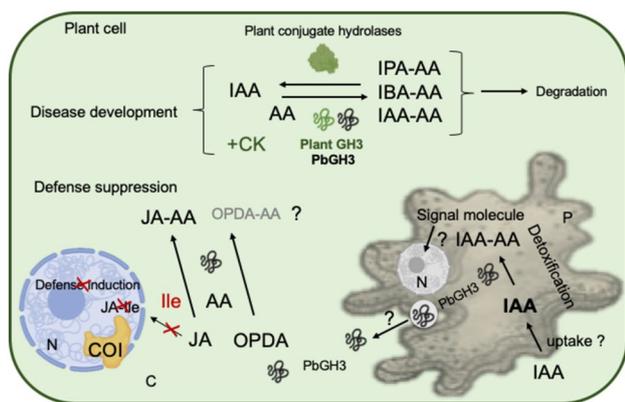


Fig. 10 Model on the possible roles of PbGH3 in the protist or in plant cells. PbGH3 catalyzes the conversion of IAA (and other auxins), JA, and OPDA to different amino acids. The first possibility is that PbGH3 is localized to *P. brassicae* structures to regulate too high hormone concentrations if IAA (or other hormones) could enter the organism and would be toxic. Also, a specific auxin conjugate could act as a signal as shown for other pathogens (see discussion for more information). Second, PbGH3 is located in the host cell, where its role may lie in controlling the auxin and/or jasmonate concentrations. For IAA, PbGH3 could be involved in controlling the endogenous ratios of auxin to cytokinin since these are necessary for proliferation. For JA the activity of PbGH3 with Ile is low compared to other amino acid substrates, so maybe by competing with other amino acids available, JA is less conjugated to the active JAIle signal that interacts with the receptor COI1 to start a defense response. Such a function would then be important for defense suppression. OPDA is also conjugated *in vitro*, so we cannot rule out that this compound is also a substrate in planta for PbGH3 and thus another defense suppression mechanism. Since *P. brassicae* resides inside the host cell, all proteins need to be secreted, as symbolized by the shape around the PbGH3 protein symbol. AA amino acid, C cytoplasm, CK cytokinin, COI coronatine insensitive, IAA indole-3-acetic acid, IBA indole-3-butyric acid, Ile isoleucine, IPA indole-3-propionic acid, JA jasmonic acid, N nucleus, OPDA 12-oxo-phytyldienoic acid, P plasmidium of *P. brassicae*, ?=feature/pathway hypothetical

overlays of the protein models. Finally, its localization could be inferred from translational fusions of the promoter with genes encoding fluorescent proteins.

For further investigations, we have expressed the *PbGH3* gene under the control of a constitutive promoter. These were analyzed for the growth of seedlings on plates under control conditions and after the addition of IAA and JA as well as adult plants grown in soil. The majority of observations seem related to the effect of the hormone treatments of the seedlings or were even ecotype or line-specific. However, adult transgenic plants show a reduction in shoot number. The latter were also inoculated with *P. brassicae*, and showed alterations in root and shoot weight that were affected in the transgenic lines compared to wild types, but the disease severity was not altered.

In addition to phenotypes, hormone profiles for a selection of IAA, IAA degradation products, IAA conjugates,

JA and two of its conjugates as well as OPDA were determined. While auxins are thought to be involved in growth responses, JA is usually classified as a defense hormone. Since PbGH3 conjugates auxins, JA and OPDA *in vitro*, it is of interest to look further into the conjugate levels *in planta*. Some changes were more consistent over all lines such as an increased IAAGlu level in seedlings after treatment with IAA, whereas IAAsp was upregulated only in WS-2 background lines. On the contrary, JA conjugates did not show a consistent pattern in seedling controls compared to transgenic lines, but both were upregulated in all lines after JA treatment. There was not such a clear trend for the adult plants, especially since we have separated the plants into three organs and also analyzed control and *P. brassicae* infected tissues. Altogether, both groups of hormones, growth and defense, seem to be affected by the overexpression of *PbGH3*.

Some changes in the transgenic plants may result from overlapping activities of endogenous PbGH3 and/or plant GH3 proteins, but this cannot be distinguished from these results. Due to this strong similarity on the protein structure level, the recently identified GH3 activity inhibitor kakeimide (Hayashi et al. 2021; Fukui et al. 2022) would not work as a selective agent in the respective assays. The same can be assumed for jarin-1, that targets the *A. thaliana* AtGH3.11 (JAR1) synthetase (Meesters et al. 2014). Another possibility would be to express *PbGH3* in multiple *gh3* mutants for the different classes (Casanova-Saez et al. 2022). The development of more specific inhibitors, that may be able to distinguish between plant and pathogen GH3 proteins, could be a promising approach.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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