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RESEARCH PAPER

Members of the germin-like protein family in *Brassica napus* are candidates for the initiation of an oxidative burst that impedes pathogenesis of *Sclerotinia sclerotiorum*

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Abstract

Germin-like proteins (GLPs) are defined by their sequence homology to germins from barley and are present ubiquitously in plants. Analyses of corresponding genes have revealed diverse functions of GLPs in plant development and biotic and abiotic stresses. This study describes the identification of a family of 14 germin-like genes from *Brassica napus* (*BnGLP*) designated *BnGLP1–BnGLP14* and investigated potential functions of BnGLPs in plant defense against the necrotrophic fungus *Sclerotinia sclerotiorum*. Sequence alignment and phylogenetic analyses classify the 14 BnGLPs into four groups, which were clearly distinguished from known germin oxalic acid oxidases. Transcriptional responses of the *BnGLP* genes to *S. sclerotiorum* infection was determined by comparing cultivars of susceptible *B. napus* 'Falcon' and partially resistant *B. napus* 'Zhongshuang 9'. Of the 14 *BnGLP* genes tested, *BnGLP3* was transcriptionally upregulated in both *B. napus* cultivars at 6 h after *S. sclerotiorum* infection, while upregulation of *BnGLP12* was restricted to resistant *B. napus* 'Zhongshuang 9'. Biochemical analysis of five representative BnGLP members identified a H_2O_2-generating superoxide dismutase activity only for higher molecular weight complexes of BnGLP3 and BnGLP12. By analogy, H_2O_2 formation at infected leaf sites increased after 6 h, with even higher H_2O_2 production in *B. napus* 'Zhongshuang 9' compared with *B. napus* 'Falcon'. Conversely, exogenous application of H_2O_2 significantly reduced the susceptibility of *B. napus* 'Falcon'. These data suggest that early induction of BnGLP3 and BnGLP12 participates in an oxidative burst that may play a pivotal role in defence of *B. napus* against *S. sclerotiorum*.

Key words: *Brassica napus*, germin-like proteins (GLPs), oxidative burst, plant disease resistance, *Sclerotinia sclerotiorum*, superoxide dismutase (SOD).

Introduction

Proteins with sequence homology to germins from wheat and barley have been identified in mosses and mono- and dicotyledonous plants and thus have been named germin-like proteins (GLPs). Initially, germins were found to accumulate in germinating wheat embryos (Lane *et al.*, 1992), and later Lane *et al.* (1993) demonstrated that this germin degraded oxalic acid to H_2O_2 and CO_2 by an oxalate oxidase (OXO) activity. So far, only germins from barley have been proven to share this OXO activity (Dumas *et al.*, 1993; Lane *et al.*, 1993; Whittaker and Whittaker, 2002), and this distinguishes these proteins from GLPs for which no OXO activity has yet been found. Instead, some GLPs have been shown to possess superoxide dismutase activity, which converts superoxide

Abbreviations: EST, expressed sequence tag; GLP, germin-like protein; OXO, oxalate oxidase; PDA, potato dextrose agar; qPCR, quantitative real-time PCR; SE, standard error; SOD, superoxide dismutase.

to H_2O_2 and O_2 (Christensen *et al.*, 2004; Gucciardo *et al.*, 2007), while others remain elusive in terms of enzymatic activity, or function as an auxin receptor (Woo *et al.*, 2002; Yin *et al.*, 2009), reflecting the high functional diversity among GLPs.

Genome and transcriptome analysis of rice (Manosalva *et al.*, 2009), barley (Zimmermann *et al.*, 2006), wheat (Schweizer *et al.*, 1999), maize (Breen and Bellgard, 2010), *Physcomitrella patens* (Nakata *et al.*, 2004), *Arabidopsis thaliana* (Carter *et al.*, 1998), and peanut and soybean (Chen *et al.*, 2011) have revealed that GLPs are encoded by gene families with multiple gene members. For example, the *A. thaliana* genome contains 32 sequences annotated as 'germin-like' genes (www.uniprot.org). Expression of germin-like genes is not restricted to germinating seeds, as initially ascribed to wheat germin, but is found in leaves (Membré *et al.*, 2000; Fan *et al.*, 2005; Banerjee and Maiti, 2010), stems (Minic *et al.*, 2009; Banerjee and Maiti, 2010), flowers (Fernández *et al.*, 2003; Yang *et al.*, 2006) and roots (Zimmermann *et al.*, 2006; Gucciardo *et al.*, 2007). The exact function of these proteins during plant development is unclear, but their apoplastic localization in combination with H_2O_2 generating superoxide dismutase (SOD) activity offers a role in cell-wall fortification through the cross-linkage of proteins and carbohydrates (Schopfer, 1996; Barceló, 1998; Banerjee and Maiti, 2010). Additionally, some GLPs bind to the plant hormone auxin and mediate auxin-induced physiological responses during plant development (Inohara *et al.*, 1989; Robert *et al.*, 2010; Effendi *et al.*, 2011). Gene expression analysis in different species has revealed that many germin-like genes are regulated following abiotic and biotic stresses. Ke *et al.* (2009) identified a GLP among ten drought-induced proteins in rice, and GLPs from wheat, barley and *Barbula unguiculata* are regulated under salt or metal stress conditions (Hurkman *et al.*, 1991; Berna and Bernier, 1999; Nakata *et al.*, 2002; Caliskan, 2009).

Pathogen infection is one of the major triggers inducing germin-like gene expression. Corresponding sequences have been identified after pathogen challenge in barley (Wei *et al.*, 1998; Hückelhoven *et al.*, 2001), wheat (Berna and Bernier, 1999), rice (Manosalva *et al.*, 2009; Banerjee and Maiti, 2010), pepper (Park *et al.*, 2004), *A. thaliana* (Collins *et al.*, 2010), sugar beet (Knecht *et al.*, 2010), and grape (Ficke *et al.*, 2004; Godfrey *et al.*, 2007). Accordingly, germin and GLP activities significantly contribute to plant defence reactions against different pathogens. Heterologous expression of barley or wheat germin was found to lead to increased resistance against *Sclerotinia* sp. fungus in rape (Dong *et al.*, 2008), peanut (Livingstone, 2005), sunflower (Hu, 2003), and tomato (Walz *et al.*, 2008), as well as in transgenic poplar leaves against *Septoria musiva* (Liang *et al.*, 2001). However, OXO activity is not necessarily essential for the defence function. Expression of mutated germin *gf-2.8* and germin-like *TaGLP2a*, which both lack OXO activity, from wheat, conferred like native *gf-2.8* increased resistance in wheat leaves against *Blumeria graminis* (Schweizer *et al.*, 1999). Knecht *et al.* (2010) enhanced the resistance in *A. thaliana* plants against the fungal pathogens *Verticillium longisporum* and *Rhizoctonia solani* through transgenic expression of germin-like *BvGLP-1* from sugar beet, and silencing of *GLP* genes in rice intensified the development of fungal rice blast and sheath blight diseases (Manosalva *et al.*, 2009). Furthermore, tobacco plants silenced for a germin-like gene (*NaGLP*) showed increased susceptibility against two insect herbivores (Lou and Baldwin, 2006), indicating a basal function of GLPs beyond microbial pathogens.

This study reports on the identification and characterization of germin-like genes in the rapeseed (*Brassica napus*) genome and evaluated their potential in defence against the fungal pathogen *S. sclerotiorum*, the causal agent of *Sclerotinia* stem rot disease. *S. sclerotiorum* is a necrotrophic pathogen that thrives on more than 400 plant species (Bolton *et al.*, 2006) and poses a considerable threat to rape farming. Efforts to increase rape resistance against *S. sclerotiorum* to the generation of varieties with increased tolerance (Wang *et al.*, 2004; Liu *et al.*, 2005; Li *et al.*, 2009) and intensive research is ongoing. In the available sequence databases, we identified a germin-like gene family in *B. napus* composed of 14 members designated *BnGLP1–BnGLP14*. To identify *BnGLP* members with a putative function in defence against *S. sclerotiorum*, we measured the corresponding transcripts by quantitative real-time PCR (qPCR) in rape after infection and compared the susceptible *B. napus* 'Falcon' variety with the more tolerant *B. napus* 'Zhongshuang 9' cultivar. Biochemical characterization of five selected BnGLP proteins revealed a H_2O_2-generating SOD activity for two members, while none showed OXO activity. by analogy, *S. sclerotiorum* evoked an oxidative burst in the plant at 6 h after infection, and H_2O_2 itself was shown to augment plant tolerance against *S. sclerotiorum*. Taken together, these results describe for the first time the family of germin-like genes in *B. napus* and demonstrate that early upregulation of SOD-active BnGLPs correlates with H_2O_2 formation in plants and may play a pivotal role in resistance of *B. napus* to *S. sclerotiorum* infection.

Materials and methods

Plant material and growth conditions

The commercial rape variety *B. napus* 'Falcon' was provided by the Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Hohenlieth, Germany) and *B. napus* 'Zhongshuang 9' was obtained from Professor Wang Hanzhong (Oil Crops Research Institute, Wuhan, China). Plants of *A. thaliana* and *B. napus* were cultivated in growth cabinets with 10 h light d^{-1} at 300 µmoles m^{-2} s^{-1} and the temperature set to 22 and 20 °C during day and night periods, respectively.

Infection with S. sclerotiorum

The *S. sclerotiorum* isolate used throughout this work was obtained from Professor W. Qian (Mei *et al.*, 2011). *S. sclerotiorum* mycelium was grown on potato dextrose agar (PDA) plates (26.5 g l^{-1} potato dextrose, 15 g l^{-1} agar, pH 5.6) for 2 d at 22 °C. Infection of fully expanded leaves from 5–6-week-old plants was performed as described by Zhao and Meng (2003). In brief, scissored leaves were arranged in moistened trays and inoculated with *S. sclerotiorum*-grown PDA plugs cut with a 0.6 cm cork borer from the margin of the expanding mycelium. Trays were sealed with plastic foil and incubated at 22 °C. Developing lesions were measured with a caliper at the indicated time points. To measure lesion formation following water or H_2O_2 infiltration, leaves were not cut from the plant but were wrapped with plastic foil after inoculation to prevent drying of the agar plugs. Infiltration of ~20 µl solution was applied from the bottom side of the leaf at the site of plug infection. In the case of plant sampling for gene transcript analysis, leaves were also not cut from the plant.

Infection of *A. thaliana* was performed with *S. sclerotiorum* grown in 70 ml liquid Czapek Dox medium (33.4 g l^{-1} Czapek Dox, 2 g l^{-1} yeast extract, 2 g l^{-1} malt extract, pH 5.5) for 2 d at 22 °C. The mycelium was homogenized for 3 s using an Ultra-Turrax, centrifuged at 6000 *g* for 10 min, and the sedimented mycelium resuspended in 10 mM $MgCl_2$ to a concentration of 0.7 g in 50 ml. *A. thaliana* plants were grown in 9 × 9 cm pots filled with peat soil and four plants per pot. Fully expanded leaves of 5–6-week-old soil-grown plants were infected with 30 µl drops of the mycelium suspension and covered with a plastic lid to maintain a high humidity. At 3 d after infection, lesion development was divided in three classes: 1, necrotic spot, poor fungal expansion; 2, round wet lesion; and 3, macerated leaf. Based on each class, a disease index was calculated with the formula: (0.5 × class 1/ total number of drops+class 2/total number of drops +2× class 3/total number of drops) × 100. Two independent experiments were performed to validate the results.

For *S. sclerotiorum* growth inhibition assays, 20 µl of H_2O_2 solution was applied to Whatman paper discs (0.8 cm diameter) in the centre of a PDA plate before inoculation and incubated for 2 d.

RNA extraction and qPCR

Plant samples were excised with a 0.8 cm cork borer at the sites of *S. sclerotiorum* agar plug infection and immediately frozen in liquid nitrogen. Agar plugs without mycelium were used as controls. Total RNA was extracted with TRIzol Reagent (Invitrogen; www.invitrogen.com/) from ground tissue of three leaf discs per sample and three samples were processed for each treatment. Reverse transcription of mRNA into cDNA was performed from 2 µg of total RNA using SuperScriptIII (Invitrogen) following the manufacturer's instructions. qPCR was conducted with a 7300 Real Time PCR System (Applied Biosystems; www.appliedbiosystems.com) using MAXIMA®SYBR Green Master Mix (Fermentas; www.fermentas.de) for gene amplification. The primer combinations to amplify *BnGLP1–BnGLP 14* and *β-tubulin* as the reference gene are given in Supplementary Table S1 at *JXB* online. All PCR products were sequenced to verify specificity for the respective gene, and invariant expression of the *β-tubulin* reference gene in response to *S. sclerotiorum* infection was assured beforehand. Data were analysed using 7300 System SDS software by the comparative $\Delta\Delta C_t$ method (Supplementary Fig. S1 at *JXB* online).

Gene cloning and transient expression in Nicotiana benthamiana

Full-length sequences of *BnGLP3*, *BnGLP7*, *BnGLP8*, *BnGLP10* and *BnGLP12* were amplified from genomic DNA of *B. napus* 'Zhongshuang 9' using *Pfu* polymerase (Fermentas). As control genes, *GFP* was amplified from pGWB5 (Nakagawa *et al.*, 2007) and wheat germin *gf-2.8* template DNA was kindly provided by Dr Bornemann (Gucciardo *et al.*, 2007). The respective primers contained *attB* sites at their 5' ends to allow cloning of the PCR products via the Gateway®BP recombination reaction (Invitrogen) into the pDONR201 Entry vector. The primer sequences are listed in Supplementary Table S2 at *JXB* online. For each *BnGLP* gene, at least six independent clones were sequenced to confirm consistent sequence identity. In the cases of *BnGLP3* and *BnGLP12*, two copies of these family members were amplified with nucleotide polymorphisms. Subsequently, genes were transferred into the Gateway compatible binary pGWB414 vector (Nakagawa *et al.*, 2007) in frame with a C-terminal triple haemagglutinin (HA)-tag coding sequence. For transient plant transformation, the binary vector constructs were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90RK and infiltrated with a 1 ml syringe without a needle into fully expanded leaves of *N. benthamiana*, as described by Witte *et al.* (2004). Tissue for protein extraction was harvested at 6–8 d post-infiltration and frozen at –80°C until further processing.

Generation of transgenic A. thaliana

Transformation of *A. thaliana* 'Columbia-0' was conducted according to Clough and Bent (1998). Transgenic plants (T_1) were selected on half-strength Murashige and Skoog medium (Duchefa; www.duchefa.com) supplemented with 50 g l^{-1} kanamycin and later transferred to soil for seed setting. Infection experiments were conducted with T_2 plants selected for kanamycin resistance as above.

Enzyme analysis and immunodetection of proteins

Unless otherwise noted, protein extraction from ~10 mg of ground tissue was performed in 110 µl extraction buffer (20 mM Tris/HCl, pH 7.5, 0.5% SDS, 5 mM DTT), except for BnGLP3.1 and BnGLP3.2, which were extracted with 20 mM Tris/HCl (pH 7.5), 2% SDS, 50 mM DTT. Subsequently, protein samples were adjusted to 2% SDS, 50 mM DTT, 10% glycerol and 0.01% bromophenol blue and loaded on an SDS-polyacrylamide gel (10% acrylamide) without boiling (semi-native) for separation. An in-gel SOD activity assay was performed following the protocol of Beauchamp and Fridovich (1971). OXO activity was tested after transferring the proteins to nitrocellulose membrane (Roth; www.carlroth.com) as described by Lane *et al.* (1993). For immunodetection, proteins were blotted on a PVDF membrane (Roche; www.roche.de) and visualized with anti-HA antibody (Roche) in combination with a Lumi-Light^{PLUS} Western Blotting Kit (Roche) following the manufacturer's instructions.

Measurement of H_2O_2 in rape leaves

Measurement of H_2O_2 from leaf tissue was performed using the FOX reagent as described by Cheeseman (2006) with slight modifications. *B. napus* leaf discs were collected with a 0.6 cm cork borer at sites of *S. sclerotiorum* infection or from control treatment plants and immediately ground in liquid nitrogen. To extract the H_2O_2, 400 µl of 25 mM HCl was added and the sample thawed on ice with shaking. After centrifugation for 5 min at 17 000 *g* at 4 °C, 100 µl of the supernatant was added to 900 µl of FOX reagent [250 µM ammonium iron (II) sulfate, 100 µM sorbitol, 100 µM xylenol orange, 25 mM H_2SO_4, 1% ethanol] and incubated for 15 min in the dark. Complex formation of Fe^{III+} with xylenol orange in the presence of H_2O_2 was measured with a photometer at 560 nm.

Results

The germin-like gene family in B. napus

To identify germin-like genes in *B. napus* (*BnGLP*), we searched genomic and expressed sequence tag (EST) databases from the NCBI, TGI (http://compbio.dfci.harvard.edu/tgi/plant.html) and the Shanghai Rapeseed database (http://rapeseed.plantsignal.cn) with an original germin sequence from barley using tblastn. ESTs provide an alternative source for gene identification in plants whose genome sequences are not fully available (Rudd, 2003), as is the case for *B. napus*. Gene candidates from genomic database were verified by EST fragments to exclude non-transcribed pseudo-genes. Putative full-length sequences matching an E-value of a maximum of 10^{-3} were selected and sequences were only considered that contained the two conserved histidines essential for binding the manganese co-factor (Fig. 1A; Woo *et al.*, 2000), giving 307 candidate sequences in total. The average sequence length of the BnGLPs was around 220 aa, matching the length of the original germins including the signal peptide, and amino acid identity ranged from 30 to 48% between the BnGLPs and HvOXO2 germin from barley. Polyploidity of *B. napus* and independent sequence donations from various *B. napus* accessions to the databases can result in redundancy of gene family members. We thus performed a protein sequence alignment and subsequent phylogenetic analysis (http://bioweb2.

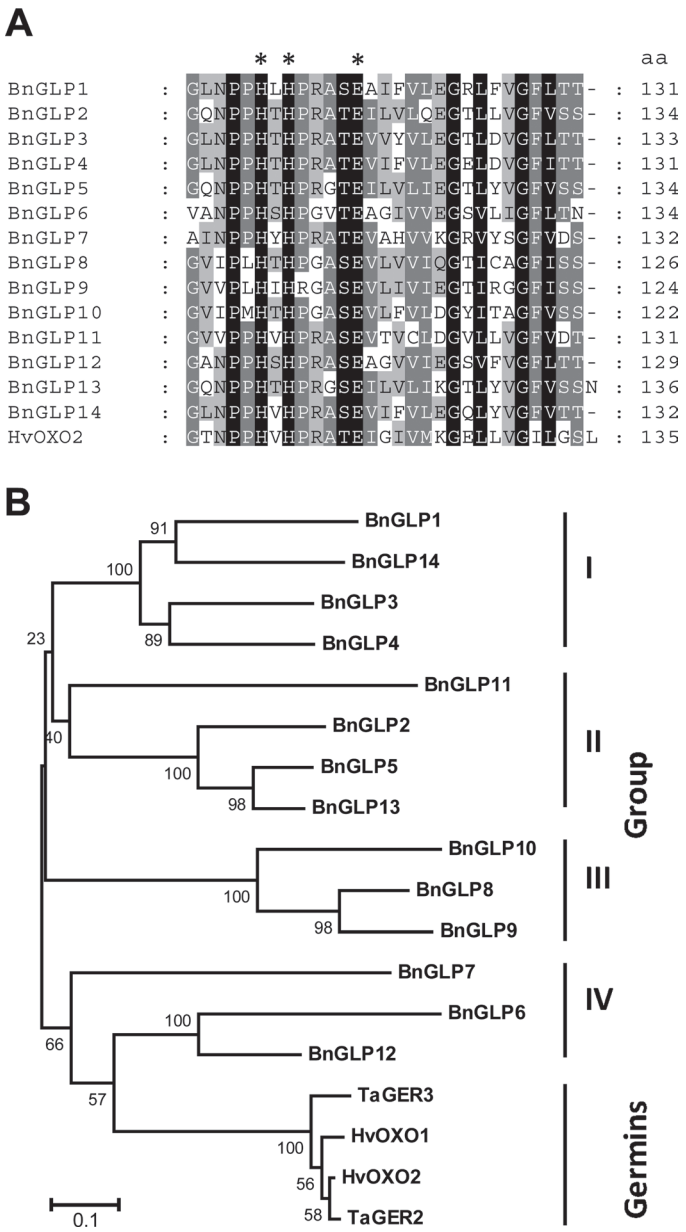

Fig. 1. Primary structure analysis of GLPs from *B. napus* (BnGLP). (A) Partial amino acid alignment of the 14 BnGLP members with HvOXO2 germin from barley. Asterisks denote conserved amino acids involved in manganese ion binding of germins (Woo *et al.*, 2000). (B) Phylogenetic tree of GLPs from *B. napus* and germins from *Hordeum vulgare* (HvOXO1 and HvOXO2) and *Triticum aestivum* (TaGER2 and TaGER3). Analysis was conducted using MEGA5 software (Tamura *et al.*, 2011) with the neighbour-joining method. The sum of branch length is 4.9. Numbers next to the branches indicate percentage of replicate trees in which the sequences clustered together in a bootstrap test (100 replicates). Bar, 0.1 amino acid substitutions per site.

pasteur.fr; Felsenstein, 1989; Thompson *et al.*, 1994) to distinguish classes of highly similar sequences. For each class, a representative BnGLP sequence was chosen that showed no more than 65% amino acid identity to any protein of the other aligned clusters. This produced a GLP family in *B. napus* comprising 14 members, which could be clustered into four groups (Fig. 1A, 1B). Comparison of the BnGLP family with the proven germin OXOs TaGER2, TaGER3, HvOXO1 and HvOXO2 did not reveal high homology and thus these were separated from the germins in the phylogenetic analysis (Fig. 1B).

Transcript profiling of BnGLP *genes in response to* S. sclerotiorum *infection*

B. napus is a highly susceptible host for *S. sclerotiorum* and only a few varieties exist with partial resistance, such as *B. napus* 'Zhongshuang 9' derived from native Chinese cultivars (Wang *et al.*, 2004). In order to find two *B. napus* cultivars with differential susceptibility to *Sclerotinia* disease, we compared *B. napus* 'Zhongshuang 9' with the commercial cultivar *B. napus* 'Falcon' that was previously shown to have a high susceptibility against *V. longisporum* (Eynck *et al.*, 2009). Detached leaves of both varieties were infected with *S. sclerotiorum* grown on PDA and incubated at 20–22 °C. Around the infection sites, circular lesions developed that were twice the size on *B. napus* 'Falcon' leaves at 36 and 48 h after infection compared with those on *B. napus* 'Zhongshuang 9' leaves (Fig. 2A), revealing significant differences in susceptibility to *S. sclerotiorum* infection. To verify the expression of *BnGLP* genes in leaf tissue, we first extracted RNA from non-treated leaves and measured the transcript abundance by qPCR using primers specifically amplifying fragments of each of the 14 *BnGLP* genes (Fig. 2B). Gene transcripts were detected for all *BnGLP* genes, although the relative amounts varied by three orders of magnitudes, with *BnGLP6* and *BnGLP10* being the least and most abundant transcripts, respectively. Significant differences in *BnGLP* gene expression between *B. napus* 'Falcon' and 'Zhongshuang 9' were not detected. Furthermore, we used these cultivars to test whether members of the *BnGLP* family were transcriptionally regulated in response to infection with *S. sclerotiorum* and whether differences in regulation existed between the susceptible *B. napus* 'Falcon' and the partially resistant *B. napus* 'Zhongshuang 9' lines. We chose a 6 h time point for transcript analysis as an early stage of infection, assuming that molecular effects decisive for the success of plant defence appear in the beginning and to limit secondary effects derived from massive cell degradation at later time points. In the susceptible *B. napus* 'Falcon' background, *BnGLP3* was upregulated at 6 h post-infection, while in *B. napus* 'Zhongshuang 9', in addition to *BnGLP3*, *BnGLP12* also became transcriptionally induced. None of the other *BnGLP* genes showed a significant response in gene expression, although *BnGLP8* and *BnGLP10* showed a tendency in multiple experiments to become suppressed in *B. napus* 'Zhongshuang 9' following *S. sclerotiorum* infection. Together, these data demonstrated that *B. napus* 'Zhongshuang 9' responded to the *S. sclerotiorum* infection differently in the regulation of *BnGLP* genes compared with *B. napus* 'Falcon'.

Cloning and biochemical characterization of BnGLP3, BnGLP7, BnGLP8, BnGLP10 and BnGLP12

As GLPs from *B. napus* have not been characterized to date, we cloned five representative members of the *BnGLP* gene family

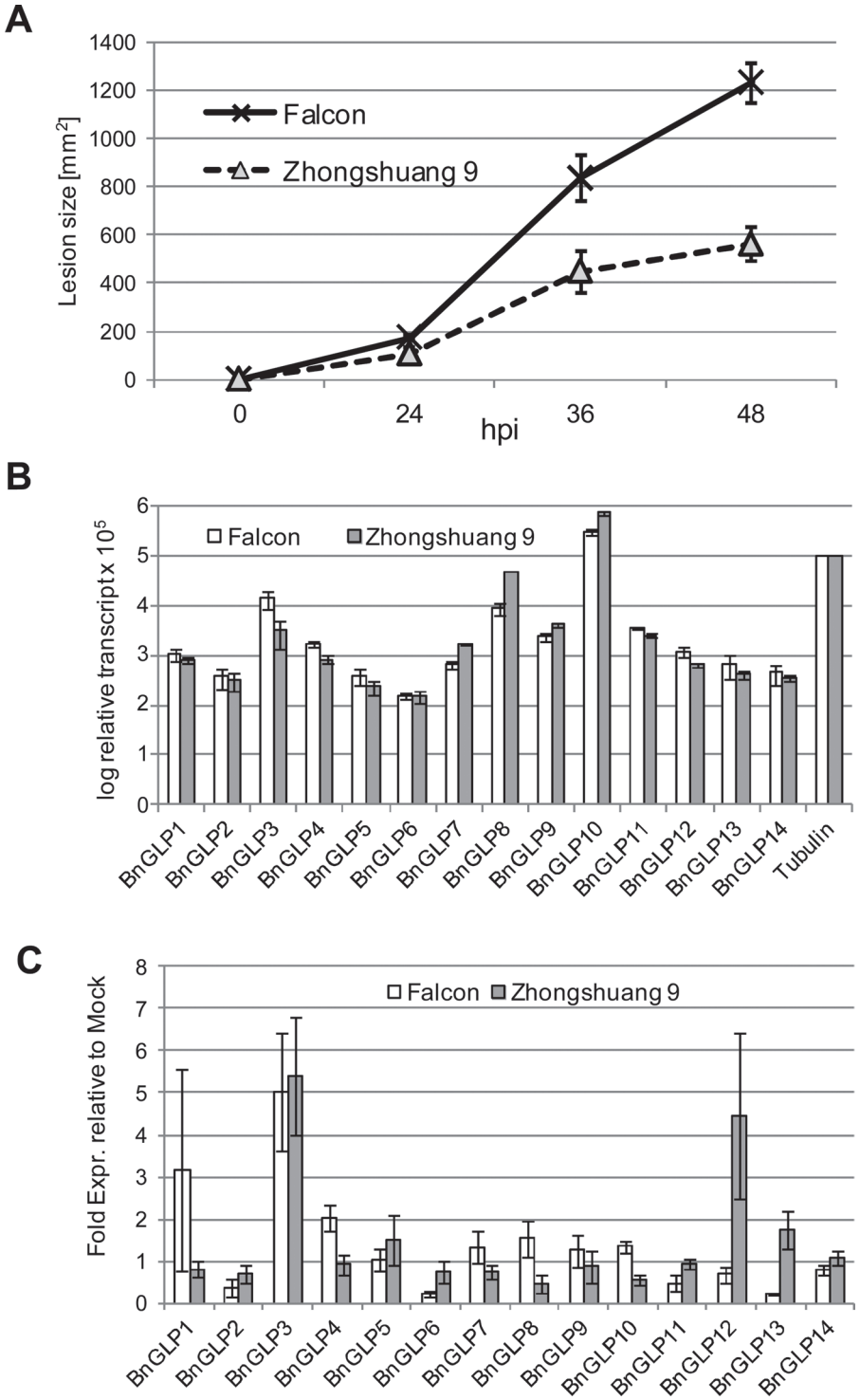

Fig. 2. *B. napus* infection with *S. sclerotiorum* and transcript analysis of *BnGLP* genes. (A) Infection of *B. napus* 'Falcon'and 'Zhongshuang 9' with *S. sclerotiorum* in a detached-leaf assay. Lesion sizes were measured at the indicated hours post-infection (hpi) and results are shown as means ± standard error (SE) ($n=8$). (B) Transcript abundance of the 14 *BnGLP* genes relative to that of β-*tubulin* in leaves of *B. napus* 'Falcon' and 'Zhongshuang 9'. Transcripts were measured by qPCR and values calculated following the equation $2C^{tTubulin}/2C^{tBnGLP}\times10^5$. Results are shown as means ± SE ($n=3$) and independent experiments showed the same trend. (C) qPCR analysis of the 14 *BnGLP* genes relative to mock treatment at 6 h after infection with *S. sclerotiorum*.

with regard to their transcriptional regulation after *S. sclerotiorum* infection (Fig. 2B). These comprised the inducible *BnGLP3* and *BnGLP12* and the non-induced *BnGLP8*, *BnGLP10* and *BnGLP7* genes. The full-length open reading frames were cloned behind a 35S promoter and fused to the coding sequence of a triple HA tag (3×HA) to allow later immunodetection of

the expressed proteins. Six to ten clones of each gene were sequenced to verify sequence consistency and to identify gene copies in the *Brassica* genome. While *BnGLP7*, *BnGLP8* and *BnGLP10* were cloned as single genes, amplification of *BnGLP3* and *BnGLP12* each yielded two homologues coding for proteins with 95% identities and were named *BnGLP3.1* and *BnGLP3.2*, and *BnGLP12.1* and *BnGLP12.2*, respectively. The homologous genes were included in the further biochemical analysis to test for alterations resulting from the amino acid differences. Additionally, we generated 3×HA-tagged fusion proteins of wheat germin gf-2.8 (Lane *et al.*, 1993) and GFP to serve as positive and negative controls, respectively. The recombinant proteins were transiently expressed in *N. benthamiana*, and total protein extracts were separated by semi-native SDS-PAGE omitting a reducing agent in the loading buffer and without boiling before loading the sample (Fig. 3A). The calculated molecular weight of monomeric germin and all BnGLPs fused to the 3×HA tag was ~29 kDs and that of GFP:HA was ~32.8 kDa, matching the band sizes seen at the bottom of the HA-specific immunoblot. The larger proteins between 35 to 40 kDa and around 170 kDa were not observed under full denaturating conditions (data not shown) and thus probably reflect different oligomerizations of the proteins. The active gf-2.8 enzyme is a hexameric complex and has been shown to migrate at ~125 kDa by semi-native SDS-PAGE (Lane *et al.*, 1993; Walz *et al.*, 2008). In Fig. 3A, the majority of gf-2.8:HA protein migrated at between 25 and 40 kDa and a minor amount also formed a complex at ~170 kDa. Taking the mass of the 3×HA tag into account, the latter probably represents the hexameric gf-2.8:HA complex. By analogy, of the seven investigated BnGLPs, both homologues of BnGLP3:HA and BnGLP12:HA, and BnGLP8:HA migrated as monomers and also formed higher-molecular-weight complexes. In contrast, BnGLP7:HA and BnGLP10:HA were expressed as monomeric proteins only and did not form higher-molecular-weight complexes.

Originally, germins from wheat and barley were found to oxidize oxalic acid to CO_2 and H_2O_2 (Dumas *et al.*, 1993; Lane *et al.*, 1993), defining them as true germins. This OXO activity has so far not been shown for any of the GLPs present in mono- and dicotyledonous plant species and mosses. Instead, some GLPs are SODs, reducing superoxide to H_2O_2, while the enzyme activity of other GLPs remains elusive (Yamahara *et al.*, 1999; Christensen *et al.*, 2004; Nakata *et al.*, 2004; Zimmermann *et al.*, 2006; Gucciardo *et al.*, 2007; Banerjee and Maiti, 2010). We thus tested for OXO and SOD activities of the transient expressed proteins (Fig. 3B, 3C). In the OXO activity assay, the gf-2.8:HA protein gave a clear signal at the size of the higher-molecular-weight complex of ~170 kDa, confirming the OXO activity for recombinant gf-2.8:HA protein (Fig. 3B). Under the same conditions, none of the BnGLPs or GFP:HA displayed an OXO activity at any protein complex size. However, when we tested for SOD activity, the protein complexes of BnGLP3:HA and BnGLP12:HA and their respective homologues showed a clear activity at 170 kDa or higher (Fig. 3C), but none of the other tested BnGLPs or gf-2.8:HA and GFP:HA appeared to possess SOD activity under the experimental conditions used. Notably, despite higher-molecular-weight complex formation, BnGLP8:HA did not show any OXO or SOD activity.

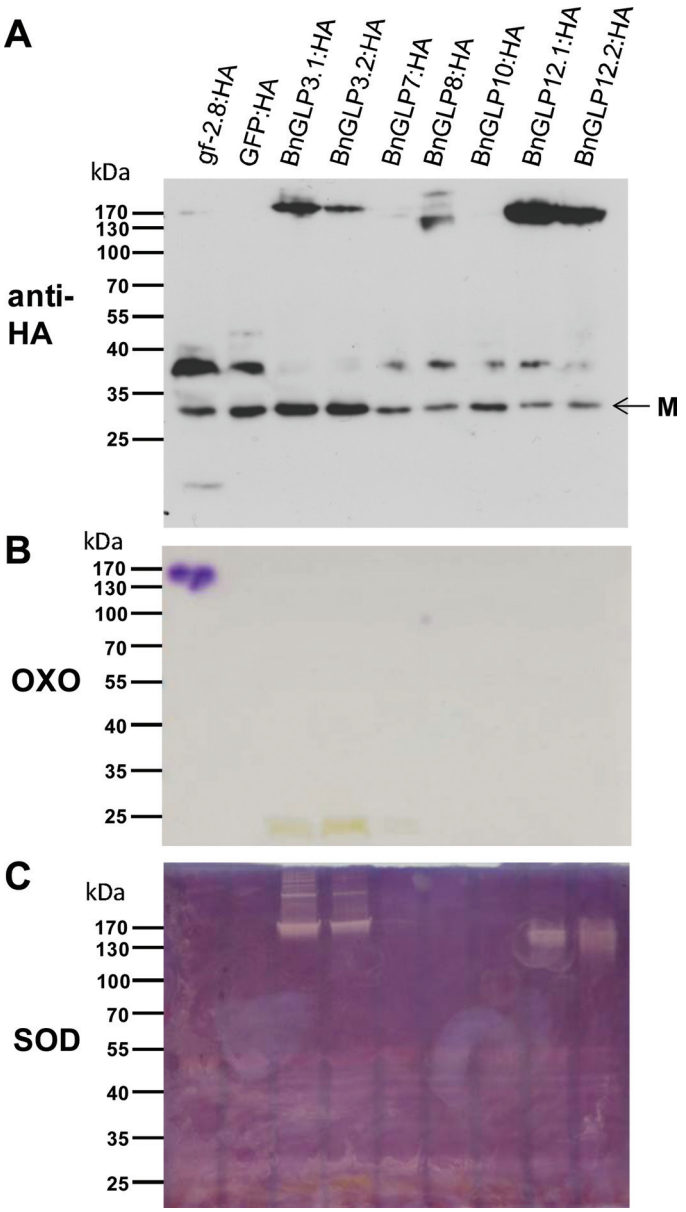

Fig. 3. Biochemical characterization of the fusion proteins BnGLP3.1:HA, BnGLP3.2:HA, BnGLP7:HA, BnGLP8:HA, BnGLP10:HA, BnGLP12.1:HA, BnGLP12.2:HA, gf-2.8:HA and GFP:HA transiently expressed in *N. benthamiana*. (A) Immunodetection of recombinant proteins using HA-specific antibody in total protein extracts. Samples were loaded without DTT in the loading buffer and without prior boiling (semi-native). M indicates the monomer. (B) Protein extracts separated as in (A) were blotted on nitrocellulose membrane and assayed for OXO activity. (C) Protein extracts separated as in (A) were assayed for SOD activity.

We also expressed BnGLP10 as native protein to test for potential interference of the 3×HA tag with enzyme function but again did not observe SOD activity (data not shown).

In order to evaluate whether BnGLP3 and BnGLP12 represent SODs with redundant functions in the plant, we investigated their solubility under different extraction conditions (Fig. 4). Both proteins exhibited maximum solubility when extracted with

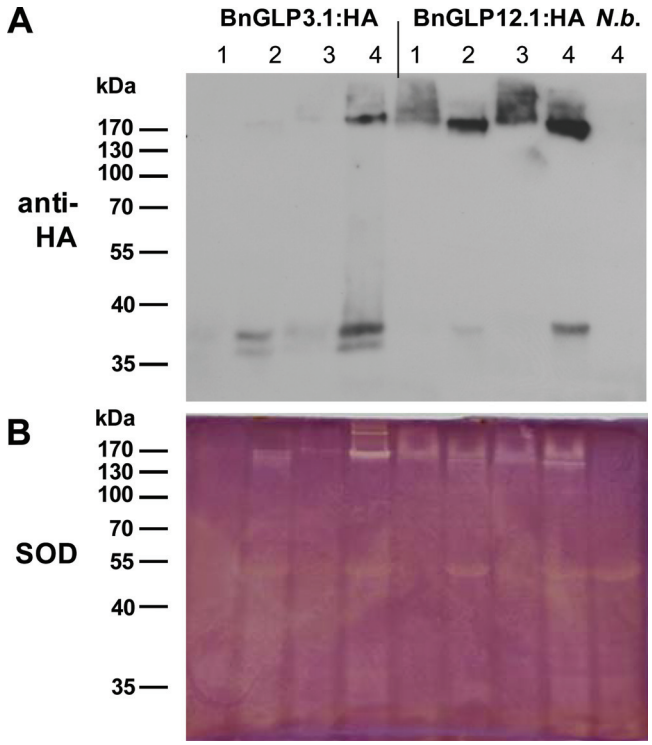

Fig. 4. Total proteins from leaves of *N. benthamiana* expressing BnGLP3.1:HA, BnGLP12.1:HA or no (*N.b.*) recombinant protein. Extraction was performed as follows: lane 1, 50 mM Tris/HCl (pH 7.5); lane 2, 50 mM Tris/HCl (pH 7.5), 50 mM DTT; lane 3, 50 mM Tris/HCl (pH 7.5), 2% SDS; lane 4, 50 mM Tris/HCl (pH 7.5), 50 mM DTT, 2% SDS. Prior to SDS-PAGE loading, all samples were adjusted to the same concentration of DTT and SDS but not boiled. (A) Immunodetection of transblotted proteins with HA-specific antibody. (B) In-gel SOD activity assay.

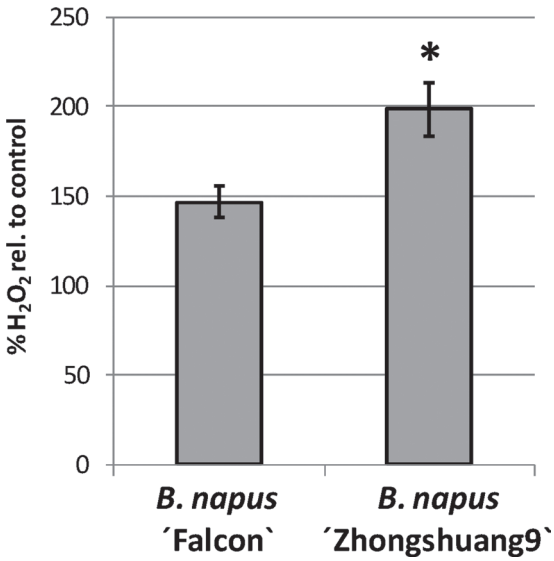

Fig. 5. H_2O_2 formation at 6 h after *S. sclerotiorum* infection on leaves of *B. napus* 'Falcon' and 'Zhongshuang 9' relative to control treatment (100%). Results are shown as means ±SE ($n = 3$) and the asterisk indicates a statistically significant difference (*P <0.05, Student's t-test). One of three independent experiments with similar results is shown.

50 mM Tris/HCl (pH 7.5), 2% SDS and 50 mM DTT. In contrast, 50 mM Tris/HCl (pH 7.5) without detergent and reducing agent extracted BnGLP12.1:HA but not BnGLP3.1:HA, as shown by anti-HA immunodetection and SOD activity assay (Fig. 4, lane 1). The addition of either 50 mM DTT or 2% SDS (Fig. 4, lanes 2 and 3) slightly increased the solubility of both proteins, although the majority of BnGLP3.1:HA was extracted with the combination of both. Together, these results are in line with earlier findings that disproved an OXO activity for GLPs, and instead we have demonstrated SOD activity for two members of five tested GLPs from *B. napus*. Interestingly, it was BnGLP3 and BnGLP12, which were transcriptionally induced in response to *S. sclerotiorum* infection, that displayed SOD activity.

Impact of H_2O_2 on defence of B. napus *against* S. sclerotiorum

The correlation between *BnGLP3* and *BnGLP12* gene transcript activation in response to *S. sclerotiorum* infection and the SOD activity of the corresponding proteins prompted us to further investigate the role of H_2O_2 as one reaction product of SOD activity during the defence response of *B. napus* to *S. sclerotiorum* infection. The *BnGLP* transcript analyses shown in Fig. 2C revealed gene induction of SOD *BnGLP3* and *BnGLP12* at 6 h post-infection. We thus measured H_2O_2 production in the leaves of *B. napus* 'Falcon' and *B. napus* 'Zhongshuang 9' at 6 h after infection with *S. sclerotiorum* infection. Figure 5 shows the change in H_2O_2 formation relative to the control treatment. Both *B. napus* varieties generated more H_2O_2 at 6 h post-infection, but the increase in the partially resistant *B. napus* 'Zhongshuang 9' variety was significantly higher compared with *B. napus* 'Falcon'. Thus, H_2O_2 production is part of the early plant defence against *S. sclerotiorum* infection and its magnitude may contribute to the resistance phenotype of *B. napus* 'Zhongshuang 9'. To strengthen this idea further, we infiltrated leaves of *B. napus* 'Falcon' and 'Zhongshuang 9' from the bottom side with 0.5 mM H_2O_2 or water as a control and infected the infiltration sites from the top with *S. sclerotiorum* grown on PDA plugs. At 29 h post-infection, we measured the lesion sizes of the spreading *S. sclerotiorum* fungus to evaluate whether artificial supply with H_2O_2 could influence the progress of *S. sclerotiorum* infection (Fig. 6). Lesion sizes were significantly smaller on *B. napus* 'Zhongshuang 9' compared with *B. napus* 'Falcon' when only water was infiltrated, in line with the detached-leaf assay shown in Fig. 2A. In contrast, infiltration of H_2O_2 resulted in significantly smaller lesion formation on *B. napus* 'Falcon', and was approximately equal to that observed on the more tolerant 'Zhongshuang 9' genotype. Lesion formation was not significantly different on *B. napus* 'Zhongshuang 9' between H_2O and H_2O_2 infiltration. Thus, an external supply of H_2O_2 can increase the resistance of *B. napus* 'Falcon' to *S. sclerotiorum* infection and this observation was not caused by a direct toxic effect of H_2O_2 on *S. sclerotiorum* growth, as H_2O_2 did not induce a further lesion size reduction on *B. napus* 'Zhongshuang 9' and did not affect *S. sclerotiorum* growth *in vitro* (data not shown).

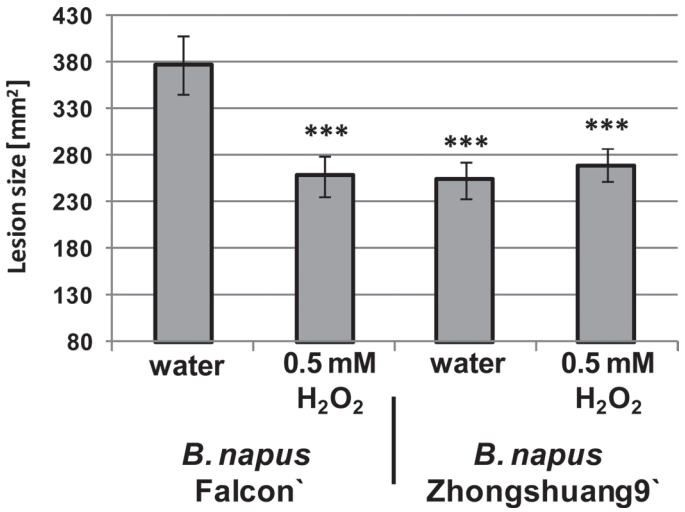

Fig. 6. Infection of *B. napus* 'Falcon' and 'Zhongshuang 9' with *S. sclerotiorum* mycelium plugs at sites infiltrated with water or 0.5 mM H_2O_2. Lesion sizes were measured at 31 h post-infection. Results are shown as means ±SE ($n = 8$) and asterisks indicate statistically significant differences compared with Falcon water treatment (***$P < 0.01$, Students t-test). One of three independent experiments with similar results is shown.

Transgenic expression of BnGLP12:HA in A. thaliana *increases tolerance to* S. sclerotiorum

The experiments performed in this study so far strongly suggested that members of the germin-like family in *B. napus* with SOD activity contribute to reduce the spread of *S. sclerotiorum*. To confirm a direct link between the upregulation of GLPs and resistance to *S. sclerotiorum*, we transformed *A. thaliana* with *BnGLP7:HA* and *BnGLP12:HA* representing GLPs without and with SOD activity, respectively. *A. thaliana* is a host for *S. sclerotiorum* and is closely related to *B. napus*. Following infection with *S. sclerotiorum*, transgenic *Arabidopsis* expressing BnGLP7:HA did not reveal any difference in susceptibility compared with wild-type Col-0, while two of three plants expressing BnGLP12:HA were significantly ($P < 0.05$) more resistant (Fig. 7).

Discussion

GLP family in B. napus

Germin-like proteins are present in many if not all plants and are encoded by gene families in the respective genomes. Here, we have presented for the first time the GLP family in rape (*B. napus*), which is represented by 14 BnGLPs (Fig. 1A, 1B). As complete sequence information of the *B. napus* genome is currently not available, our classification of GLPs was based on genomic sequences and ESTs that translate into proteins with a maximum of 65% amino acid identity. In this way, we excluded cultivar-specific variants of one gene, and probably also gene copies present in the *B. napus* genome due to genome duplication during evolution (Parkin *et al.*, 2003) and the amphidiploid nature of the *B. napus* genome (Nagaharu, 1935). The BnGLPs possessed all three conserved germin sequence boxes defined by Bernier and Berna (2001) including the PxHxHxxxxE motive (Fig. 1A) essential for OXO activity, but exhibited only 30–48% amino acid sequence identity to the original HvOXO2 germin from wheat and thus are termed 'germin-like'. Accordingly, all germins with proven OXO activity separated in a phylogenetic tree from the BnGLPs (Fig. 1B), implying that they also exhibit functional divergence. In the same analysis, the 14 BnGLPs clustered into four groups, but it was not clear whether proteins of one group shared common features.

The SODs BnGLP3 and BnGLP12 are induced in response to S. sclerotiorum *infection*

Gene expression and protein function analyses reported in the literature indicate a bias of germins and GLPs to participate in plant defence responses against pathogens, including the necrotrophic fungus *S. sclerotiorum* (reviewed by Lane, 2002; Dunwell *et al.*, 2008). *B. napus* is also a host for S. *sclerotiorum*, and most commercially available rape varieties are highly susceptible to *S. sclerotiorum*, such as *B. napus* 'Falcon'. In contrast, *B. napus* 'Zhongshuang 9' shows increased resistance against the biotrophic fungus *V. longisporum* (Eynck *et al.*, 2009) and higher tolerance to *S. sclerotiorum* infection compared with *B. napus* 'Falcon', as shown in Fig. 2A. To investigate a possible involvement of the BnGLP family in response to *S. sclerotiorum* infection, we examined the regulation of the corresponding genes. Quantitative measurements of the 14 *BnGLP* genes in non-treated leaves revealed no differences *a priori* in transcript abundance between both *B. napus* varieties (Fig. 2B), indicating conserved gene regulation under normal growth conditions. We extended the analysis to 6 h after *S. sclerotiorum* infection, assuming that early regulated genes would be directly connected to *S. sclerotiorum* invasion and probably essential for the success of plant defence. In both *B. napus* varieties, *BnGLP3* was upregulated at 6 h post-infection, while *BnGLP12* transcripts increased in *B. napus* 'Zhongshuang 9' only (Fig. 2C). Similarly, Zhao *et al.* (2007) investigated gene expression changes in rape with a microarray from *A. thaliana* and found a germin-like gene to be upregulated 4–4.8-fold in a susceptible and a semi-tolerant variety at the earliest time point of 24 h after *S. sclerotiorum* infection. The corresponding orthologue in *B. napus* (*BnGLP7*) was not upregulated under our experimental conditions; this may be due to the different time point chosen or non-specific hybridization of the microarray to other *BnGLP* members. In another experiment employing a *B. napus*-specific oligonucleotide chip, *BnGLP3* was upregulated 11-fold at 78 h after *S. sclerotiorum* infection in stem tissue of the susceptible *B. napus* 'Westar' but not in the partially resistant 'Zhongyou 821' cultivar (Zhao *et al.*, 2009). It should be noted that, in *B. napus*, BnGLP3 is regulated following *S. sclerotiorum* infection in both susceptible and tolerant *B. napus* varieties, supporting a potential role in plant basal resistance, as suggested by Zimmermann *et al.* (2006) for barley GLPs in the compatible interaction with the powdery mildew fungus *Blumeria gramins* f. sp. *hordei*. The fact that *BnGLP12* was only upregulated in the resistant 'Zhongshuang 9' following *S. sclerotiorum* infection strongly suggests that it has a role in plant resistance to *S. sclerotiorum*. Furthermore,

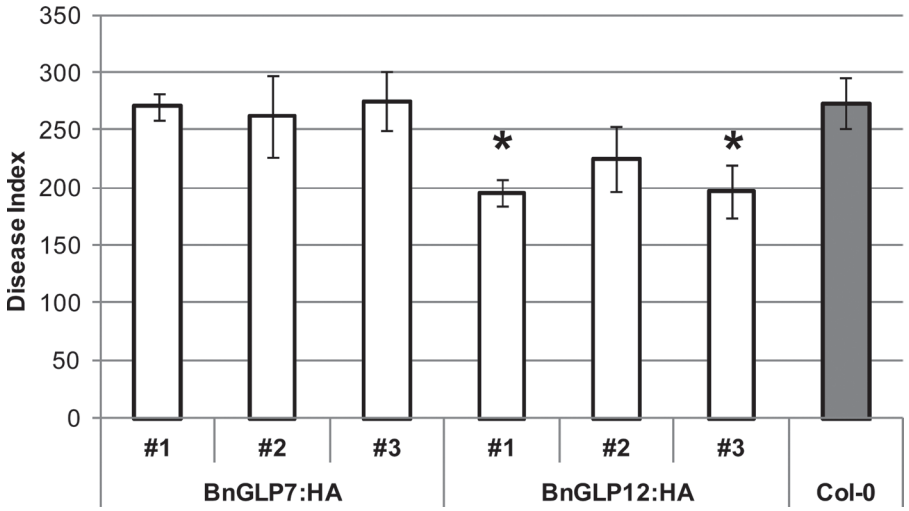

Fig. 7. Infection of *A. thaliana* with *S. sclerotiorum.* Transgenic plants expressing BnGLP7:HA or BnGLP12:HA under control of a 35S promoter compared with Col-0 *Arabidopsis* wild-type. Leaves were infected with drops of mycelia solution and evaluated after 3 d, as described in Materials and methods. Numbers denote independent transgenic lines. Results are shown as means ±SE of three pots with four plants each and asterisks indicate a significant difference at $P < 0.05$ compared with Col-0 (Student's *t*-test). Two independent experiments were carried out and showed the same trend.

we demonstrated that heterologous expression of BnGLP12:HA, but not of the SOD-inactive germin-like protein BnGLP7:HA, in *A. thaliana* increased tolerance to *S. sclerotiorum* (Fig. 7). This corroborates our interpretation of increased resistance against *S. sclerotiorum* through the upregulation of BnGLP12.

Germin proteins from wheat and barley form homohexameric complexes and degrade oxalic acid to H_2O_2 and CO_2. Of the five BnGLPs tested here, both homologues of BnGLP3:HA and BnGLP12:HA as well as BnGLP8:HA migrated as higher-molecular-weight complexes of~170 kDa in semi-native SDS-PAGE (Fig. 3A). This is equivalent to six times the monomer mass of ~28 kDa, revealing the same complex stoichiometry and SDS stability as observed for germin proteins (Lane *et al.*, 1992; Woo *et al.*, 1998) and GLPs from *P. patens* (Nakata *et al.*, 2004), barley (Christensen *et al.*, 2004; Zimmermann *et al.*, 2006), *A. thaliana* (Membré *et al.*, 2000), and pea (Gucciardo *et al.*, 2007). The absence of higher-molecular-weight complexes observed here for BnGLP8:HA and BnGLP10:HA was also shown for AtGER1 (Membré *et al.*, 2000), revealing distinct biochemical properties with respect to either complex formation or complex stability.

The GLPs examined to date do not possess an OXO activity, but some members have been shown to produce H_2O_2 through a SOD activity. The BnGLP proteins investigated here did not show any OXO activity, but higher-molecular-weight complexes of BnGLP3:HA and BnGLP12:HA, including their homologues, were able to dismutate superoxide to H_2O_2 through SOD activity (Fig. 3B, 3C). Despite the complex formation of BnGLP8:HA, no SOD activity could be detected as for monomeric BnGLP7:HA and BnGLP10:HA. Thus, BnGLP7, BnGLP8 and BnGLP10 probably fulfil different functions in the plant compared with BnGLP3 and BnGLP12. We also expressed BnGLP10 as native protein to exclude a negative effect of the HA tag, but were again not able to detect SOD activity (data not shown). Remarkably, of the five BnGLPs tested, those that showed SOD activity were also transcriptionally induced in response to *S. sclerotiorum* infection. Despite equivalent complex formation and SOD activity of BnGLP3:HA and BnGLP12:HA, BnGLP12:HA was soluble in Tris buffer (pH 7.5) during protein extraction from plant tissue, while BnGLP3 required the addition of a reducing agent and a strong detergent to become fully soluble (Fig. 4). This indicated that BnGLP3 is associated with cellular structures via reducing/oxidizing and hydrophobic interactions and is not redundant with BnGLP12 in the plant. Thus, infection with *S. sclerotiorum* results in quantitative and qualitative differences in SOD activation between susceptible *B. napus* 'Falcon' and the tolerant *B. napus* 'Zhongshuang 9'.

Early formation of H_2O_2 restricts S. sclerotiorum *pathogenesis*

In line with the induction of SOD-active BnGLPs, we also measured an increase in H_2O_2 in rape leaves at 6 h after *S. sclerotiorum* infection (Fig. 5). Both varieties responded with an increase in H_2O_2, but *B. napus* 'Zhongshuang 9' significantly exceeded the H_2O_2 amounts of the susceptible 'Falcon' cultivar, correlating with the additional induction of *BnGLP12* in 'Zhongshuang 9'. This oxidative burst at the early state of *S. sclerotiorum*-infected rape leaves confirmed results Xu *et al.* (2009) who also found higher H_2O_2 levels in transgenic rape expressing a glucose oxidase and this plant also had restrained *S. sclerotiorum* lesion formation compared with a susceptible rape variety. Production of plant-derived H_2O_2 has been reported for compatible and incompatible plant pathogen interactions acting as a direct antimicrobial compound, to trigger signal transduction pathways that occasionally lead to a hypersensitive response or to foster cell-wall fortification (reviewed in Shetty *et al.*, 2008). This functional diversity probably relates to the site of H_2O_2 generation, the timing and the amount of H_2O_2 produced. The BnGLP proteins are predicted to posses a secretion signal (Petersen *et al.*, 2011), and this is in agreement with experimental data that localized GLPs to the cell wall (Irshad *et al.*, 2008;

Banerjee *et al.*, 2010; Komatsu *et al.*, 2010). Thus, BnGLP3 and BnGLP12 are likely to participate in the *S. sclerotiorum*-induced apoplastic formation of H_2O_2 and may act in concert with NADPH oxidases and peroxidases, which are known to execute the apoplastic oxidative burst in response to pathogen stress in different species (Torres, 2010). The target of BnGLP-derived H_2O_2 is unclear, but the work of Banerjee *et al.* (2010) suggests a role in cell-wall reinforcement. The authors expressed rice germin-like protein1 in transgenic tobacco and correlated its SOD activity with hyper-accumulation of H_2O_2 and enhanced cross-linkage of cell-wall components after infection with *Fusarium solani*, which led to higher tolerance against this fungal pathogen. Similarly, we observed a positive effect of H_2O_2 on the resistance of *B. napus* 'Falcon' to *S. sclerotiorum* by the infiltration of 0.5 mM H_2O_2 prior to infection (Fig. 6). *B. napus* 'Zhongshuang 9' did not respond with increased *S. sclerotiorum* resistance, indicating that the naturally stronger induction of H_2O_2 production in *B. napus* 'Zhongshuang 9' in response to *S. sclerotiorum* infection (Fig. 4) is sufficient for H_2O_2-triggered defences at the applied concentration and may explain the increased resistance to *S. sclerotiorum* compared with *B. napus* 'Falcon'. In *A. thaliana*, L'Haridon *et al.* (2011) induced H_2O_2 formation by wounding leaves or exogenously applied H_2O_2 and by this increased the resistance against the necrotrophic pathogen *Botrytis cinerea*. Moreover, the authors showed that the wound-induced reactive oxygen species formation and resistance against *B. cinerea* were independent of the NADPH oxidases AtRBOHD and AtRBOHF, substantiating our interpretation of BnGLP proteins as part of an oxidative burst and subsequent increase in rape resistance against necrotrophic *S. sclerotiorum*.

Taken together, we have established here the family of GLPs in *B. napus* represented by 14 BnGLP members. Gene expression profiling of this family and biochemical characterization of selected members suggested that the SODs BnGLP3 and BnGLP12 are involved in early rape defence against *S. sclerotiorum* by the initiation of an oxidative burst. We also showed that H_2O_2, either produced *in vivo* or applied exogenously, correlated with increased resistance against *S. sclerotiorum*, providing a link to the functions of BnGLP3 and BnGLP12 in rape defence. Beyond the *B. napus*/*S. sclerotiorum* system investigated here, GLPs in different species relate to increased resistance including insects (Lou and Baldwin, 2006; Collins *et al.*, 2010), nematodes (Knecht *et al.*, 2010), microbes (Wei *et al.*, 1998; Schweizer *et al.*, 1999; Hückelhoven *et al.*, 2001; Ficke *et al.*, 2004; Zimmermann *et al.*, 2006; Godfrey *et al.*, 2007; Manosalva *et al.*, 2009; Shetty *et al.*, 2009; Banerjee and Maiti, 2010) and tobacco mosaic virus (Park *et al.*, 2004), indicating basal functions of GLPs in plant resistance. Whether GLPs are also involved in R-protein-mediated or non-host resistance and what the immediate consequences of GLP derived reactive oxygen species formation are remain to be investigated.

Supplementary data

Supplementary data can be found at *JXB* online.

Fig. S1. Differential transcription of the *β-tubulin* reference gene between mock- and *S. sclerotiorum*-treated leaf samples of *B. napus* 'Falcon' and 'Zhongshuang 9'.

Table S1. Primer combinations for qPCR analysis of the *BnGLP* gene family and β-*tubulin* as a reference gene.

Table S2. Primer combinations for full-length gene cloning into the pDONR201 Gateway vector.

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