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 BnGPL1–BnGPL14 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 were 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 BnGPL12 was restricted to resistant *B. napus* ‘Zhongshuang 9’. Biochemical analysis of five representative BnGLP members identified a H₂O₂-generating superoxide dismutase activity only for higher molecular weight complexes of BnGPL3 and BnGPL12. By analogy, H₂O₂ formation at infected leaf sites increased after 6 h, with even higher H₂O₂ production in *B. napus* ‘Zhongshuang 9’ compared with *B. napus* ‘Falcon’. Conversely, exogenous application of H₂O₂ significantly reduced the susceptibility of *B. napus* ‘Falcon’. These data suggest that early induction of BnGLP3 and BnGPL12 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 H₂O₂ and CO₂ by an oxalate oxidase (O XO) activity. So far, only germins from barley have been proven to share this O XO activity (Dumas *et al.*, 1993; Lane *et al.*, 1993; Whittaker and Whittaker, 2002), and this distinguishes these proteins from GLPs for which no O XO activity has yet been found. Instead, some GLPs have been shown to possess superoxide dismutase activity, which converts superoxide...
to H$_2$O$_2$ 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 (Membre 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$_2$O$_2$ generating superoxide dismutase (SOD) activity offers a role in cell-wall fortification through the cross-linkage of proteins and carbohydrates (Schofer, 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$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$ 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 µmole 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$_2$O$_2$ 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 MgCl₂ 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 H₂O₂ 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 reverse transcriptase (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*–*BnGLP14* and β-actin, and invariant expression of the β-tubulin products were sequenced to verify specificity for the respective gene, and peptidase, and amino acid identity ranged from 30 to 48% between the sequences are listed in Supplementary Table S2 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 Phu polymerase (Fermentas). As control genes, *GFP* was amplified from pGW5 (Nakagawa et al., 2007) and wheat germin *gp2*–8 template DNA was kindly provided by Dr Bornemann (Gucciardo et al., 2007). The respective primers contained atatt 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 pGW4B144 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::MP90RK 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 (*T₃*) 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 *T₃* 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/HC1, pH 7.5, 0.5% SDS, 5 mM DTT), except for *BnGLP3.1* and *BnGLP3.2*, which were extracted with 20 mM Tris/HC1 (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-LightPLUS Western Blotting Kit (Roche) following the manufacturer’s instructions.

**Measurement of H₂O₂ in rape leaves**

Measurement of H₂O₂ 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 H₂O₂, 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 xylene orange, 25 mM H₂SO₄, 1% ethanol] and incubated for 15 min in the dark. Complex formation of Fe³⁺ with xylene orange in the presence of H₂O₂ 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 HvO XO2 germin from barley. Polypeplicity 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.
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 different 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.
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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

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**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 $2^{\Delta C_{t\text{Tubulin}}} / 2^{\Delta C_{t\text{BnGLP}}} \times 10^5$. 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*. 
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 kDa 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 denaturing 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 *BnGLP12.2: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 CO₂ and H₂O₂ (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 H₂O₂, 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 *BnGLP12.2:HA* and their respective homologues showed a clear activity at 170 kDa and 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.](image-url)

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
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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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ production in the leaves of *B. napus* ‘Falcon’ and ‘Zhongshuang 9’ at 6 h after infection with *S. sclerotiorum* infection. Figure 5 shows the change in H$_2$O$_2$ formation relative to the control treatment. Both *B. napus* varieties generated more H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ and H$_2$O$_2$ infiltration. Thus, an external supply of H$_2$O$_2$ 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$_2$O$_2$ on *S. sclerotiorum* growth, as H$_2$O$_2$ 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).
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 graminis* 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,
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 H2O2 and CO2. 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 H2O2 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 H2O2 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 fulfill 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 H2O2 restricts S. sclerotiorum pathogenesis**

In line with the induction of SOD-active BnGLPs, we also measured an increase in H2O2 in rape leaves at 6 h after S. sclerotiorum infection (Fig. 5). Both varieties responded with an increase in H2O2, but B. napus ‘Zhongshuang 9’ significantly exceeded the H2O2 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 H2O2 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 H2O2 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 H2O2 generation, the timing and the amount of H2O2 produced. The BnGLP proteins are predicted to possess 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ on the resistance of B. napus ‘Falcon’ to S. sclerotiorum by the infiltration of 0.5 mM H₂O₂ prior to infection (Fig. 6). B. napus ‘Zhongshuang 9’ did not respond with increased S. sclerotiorum resistance, indicating that the naturally stronger induction of H₂O₂ production in B. napus ‘Zhongshuang 9’ in response to S. sclerotiorum infection (Fig. 4) is sufficient for H₂O₂-triggered defences at the applied concentration and may explain the increased resistance to S. sclerotiorum compared with B. napus ‘Falcon’. In A. italiana, L’Haridon et al. (2011) induced H₂O₂ formation by wounding leaves or exogenously applied H₂O₂ 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 H₂O₂, 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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