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ORIGINAL ARTICLE

Brassica napus possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defense hormone treatment

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Abstract Most plants encode a limited set of polygalacturonase inhibitor (PGIP) genes that may be involved in aspects of plant development, but more importantly in the inactivation of polygalacturonases (PG) secreted by pathogens. Previously, we characterized two *Brassica napus* PGIP genes, *BnPgip1* and *BnPgip2*, which were differentially expressed in response to pathogen infection and wounding. Here we report that the *B. napus* genome encodes a set of at least 16 PGIP genes that are similar to *BnPgip1* or *BnPgip2*. This is the largest *Pgip* gene family reported to date. Comparison of the BnPGIPs revealed several sites within the xxLxLxx region of leucine rich repeats that form β-sheets along the interacting face of the PGIP that are hypervariable and represent good candidates for generating PGIP diversity. Characterization of the regulatory regions and RT-PCR studies with gene-specific primers revealed that individual genes were differentially responsive to pathogen infection, mechanical wounding and signaling molecules. Many of the *BnPgip* genes responded to infection by the necrotic pathogen, *Sclerotinia sclerotiorum*; however, these genes were also induced either by jasmonic acid, wounding and salicylic acid or some combination thereof. The large number of PGIPs and the differential manner in which they are regulated likely ensures that *B. napus* can respond to attack from a broad spectrum of pathogens and pests.

Keywords Polygalacturonase inhibitor protein · *Brassica napus* · *Sclerotinia sclerotiorum* · Wounding · Salicylic acid · Jasmonic acid · Promoter · Gene expression

Abbreviations

BAC	Bacterial artifical chromosome
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
GUS	β-Glucuronidase
JA	Jasmonic acid
MS	Minimal salts
NTP	Nucleoside triphosphate
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PG	Polygalacturonase
PGIP	Polygalacturonase inhibitor protein
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic acid
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride–sodium citrate

Introduction

Pectin is the main structural component providing support for the cellulose microfibrils within the middle lamella of the plant cell wall. It is primarily composed of an $\alpha(1 > 4)$-linked homopolymer of galacturonic acid with sporadic $\alpha(1 > 2)$ linkages to rhamnogalacturonan to which a variety of galactans, arabinans, and arabinogalactans are attached. Pathogens can deploy several types of enzymes to breach

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this barrier, the most common being exo- and endo-polygalacturonases and pectin lyases functioning in concert with pectin methyl and acetyl esterases (Prade et al. 1999) to degrade pectin. Of these, the polygalacturonases (PG) have received the most attention and are considered to be key pathogenicity determinants of fungal pathogens. The relative importance of PGs in the infection process is evident from the number of PG isoforms produced by such organisms. Genome sequence information (http://www.broad.mit.edu/annotation/fungi/fgi/) reveals that *Neurospora crassa* (NCU02369.1 and NCU06961.1) and *Magnaporthe grisea* (MG08938.3 and MG08752.3) each possess two genes encoding proteins containing the conserved glyco_hydro_28 domain found in PGs, whereas the *Aspergillus nidulans* genome encodes eight (AN2206.1, AN3389.1, AN4372.1, AN6656.1, AN8327.1, AN8761.1, AN8891.1, and AN9045.1). The type *Sclerotinia sclerotiorum* strain 1980 genome encodes five endo-PGs, four of which are expressed in culture or during the infection (Li et al. 2004), while *Botrytis cinerea* expresses six distinct PGs (ten Have et al. 2001). A set of 19 PG genes encoding enzymes with seemingly diverse biochemical properties were isolated from *Phytophthora cinnamomi* (Gotesson et al. 2002). Targeted mutagenesis in *Aspergillus flavus* (Shieh et al. 1997), *B. cinerea* (ten Have et al. 1998), *Fusarium oxysporum* (Garcia-Maceira et al. 2001) and *Penicillium olsonii* (Wagner et al. 2000) has also provided direct evidence to support this concept that PGs are important pathogenicity determinants.

The short (10–12 residues) oligogalacturonides released by endo-PG and pectin lyase activity are potent elicitors of the plant defense response (Darvill et al. 1994; De Lorenzo et al. 1994). Inhibitors of cell wall degrading enzymes, including polygalacturonase inhibitory proteins (PGIPs) belonging to the super-family of leucine-rich repeat proteins, form part of the plant defense system and are localized in the plant cell wall (Juge 2006). It has been proposed that inhibition of polygalacturonase activities by PGIPs may prolong the accumulation of oligogalacturonides and accentuate the defense response (De Lorenzo and Cervone 1997; Federici et al. 2006) but PGIPs can also directly impede pathogen invasion by inactivating PGs. PGs are also capable initiating programmed cell death in the host leading to tissue necrosis (Kars et al. 2005; Zuppini et al. 2005), a phenomenon that is circumvented by PGIPs (Zuppini et al. 2005).

Arabidopsis thaliana possesses two PGIP genes, both of which are induced in response to *B. cinerea* infection but by different signal transduction pathways. Over-expression of either leads to enhanced resistance (Ferrari et al. 2003) whereas anti-sense expression increased disease symptoms (Ferrari et al. 2006). Cotton, *Gossypium hirsutum*, (James and Dubery 2001) and pear, *Pyrus communis*, PGIPs (Tamura et al. 2004) were shown to inhibit fungal PG activity. The latter also increased resistance to *B. cinerea* when expressed in transgenic tomato, *Lycopersicon esculentum*, (Powell et al. 2000) and grapevine, *Vitus vinifera*, (Aguero et al. 2005). Expression of grapevine PGIP greatly increased resistance to *B. cinerea* in tobacco (Joubert et al. 2006). As a result of such studies, PGIP genes have also been characterized from several important agricultural crop species (De Lorenzo et al. 2001). Four PGIP genes have been identified in soybean, *Glycine max*; however, only a single member is capable of inhibiting fungal PG activity (D'Ovidio et al. 2006). The known complement of PGIP genes in the common bean, *Phaseolus vulgaris*, consists of a single cluster of four genes, two of which were capable of inhibiting PG activity from both fungi and phytophagous insect pests (D'Ovidio et al. 2004). The PGIP genes in rice and wheat are also represented by small gene families (Janni et al. 2006). Two *Brassica napus* PGIP genes have been extensively characterized and were found to be differentially regulated in response to various biotic and abiotic stresses (Li et al. 2003). Here we expand on this study and report that the *B. napus* genome encodes an even greater number of at least 16 PGIP genes, quite unlike that of other dicotyledonous plants studied to date. In addition, we show that groups of *Pgip* genes are differentially responsive to pathogen infection, wounding, salicylic acid and jasmonic acid treatment, which would allow the plant to selectively respond to attack from a broad spectrum of fungal pathogens.

Materials and methods

Plant and fungal materials

Brassica napus DH12075 is a doubled haploid line derived from a cross between 'Crésor' and 'Westar'. *Sclerotinia sclerotiorum* isolate '100' was used for all inoculations and was originally collected from *B. napus*. Fungal mycelia were stored at −80°C in 25% glycerol and recovered on potato-dextrose agar (PDA).

Isolation and analysis of *BnPgip* genes

A bacterial artificial chromosome (BAC) library with an approximate 7.5 fold coverage of the *B. napus* DH12075 genome was used (I. Parkin, unpublished). Membranes containing the entire BAC library were probed with cDNA fragments corresponding to either *BnPgip1* or *BnPgip2* (Li et al. 2003) labeled with [α-^{32}P] dCTP using the Prime-A-Gene Labeling System (GIBCO-BRL). Hybridizations were conducted in 0.5 M $NaHPO_4$ buffer (pH 7.2) and 7% SDS at 65°C overnight. The membranes were washed twice with 2× SSC/0.1% SDS at 65°C for 15 min, twice with

0.2× SSC/0.1% SDS at 65°C for 15 min and finally with 0.1× SSC at RT for 10 min. The DNA from individual BAC plasmids was isolated and compared by restriction enzyme analysis with either *Bam*HI or *Eco*RI and confirmed to contain *BnPgip*-related genes by Southern blot and PCR analysis. BACs containing *BnPgip1* or *BnPgip2*-related genes were pooled and sub-BAC libraries prepared in Lambda ZAP II (*Eco*RI, <10 kb) and packaged using the Gigapack III Gold Packaging Extract (Stratagene). The sub-BAC libraries were screened with the appropriate *BnPgip* cDNA probes and groups of individual clones containing similar genomic DNA regions were identified by restriction enzyme analysis prior to sequencing.

Signal peptides were identified using SignalP and *N*-linked or *O*-linked glycosylation sites using NetNGlyc 1.0 and NetOGlyc 2.0 (www.expasy.org), respectively. Intron–exon boundaries were inferred using NNSPLICE 0.9 (www.fruitfly.org/seq_tools/splice.html). Dendograms arising from phylogenetic analysis were constructed according to the neighbor-joining method and confidence values for the branches determined using bootstrap analysis where 100 trees were generated from randomly resampled data generated by CLUSTAL W.16 as provided in PHYLIP version 3.5C (distributed by Felsenstein at www.cbr.nrc.ca/cgi-bin/WebPhylip/index.html).

Plant transformation and characterization of *BnPgip* promoter activity

The regulatory regions from ten distinct types of promoters associated with the *BnPgip* genes were fused to the *β*-glucuronidase gene in the pORE-R1 plant transformation vector (Coutu et al. 2007). The size of the regulatory regions used for this analysis was dictated by their proximity to upstream genes or information available in the genomic DNA clone as follows; *BnPgip1* (1,684 bp), *BnPgip2* (1,663 bp), *BnPgip3* (1,198 bp), *BnPgip5* (1,147 bp), *BnPgip6* (783 bp), *BnPgip8* (289 bp), *BnPgip9* (1,253 bp), *BnPgip10* (1,151 bp), *BnPgip11* (250 bp) and *BnPgip15* (773 bp).

Arabidopsis thaliana cv. Columbia was transformed using the floral dip method (Clough and Bent 1998) modified to include a 30 s exposure to vacuum (25 mm Hg) immediately after dipping. Transformed lines were identified by selection on 0.5 MS salts, 1× B5 vitamins, 0.7% agar and 50 μg/mL kanamycin (pH 5.7) for 7–20 days. *β*-glucuronidase (GUS) activity was detected in flowers, siliques and roots and in stems and leaves after mechanical wounding or infection with *S. sclerotiorum*. A solution of 1 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl glucuronide in 100 mM KPO_4 (pH 7.4) buffer was infiltrated into the tissue under vacuum (25 mm Hg) for 10 min. The tissues were incubated at 37°C overnight and treated in 100% ethanol prior to observation. Ten independent transformed lines were analyzed for each of the ten constructs.

Effect of pathogen infection, wounding and treatment with signaling compounds

Eight-week-old *B. napus* DH12075 leaves were inoculated with a single 3 mm *S. sclerotiorum* mycelial plug excised from the actively growing margin of a colony growing on PDA. The plug was affixed to the leaf surface with Parafilm and remained in contact with the leaf surface for 18 h. Plants were maintained at room temperature and covered with plastic bags to provide adequate humidity for infection. To examine the effect of exposure to signaling compounds, leaves were sprayed with either distilled water, a 50 mM solution of salicylic acid (SA) (Sigma) or a 100 μM solution of jasmonic acid (Sigma) as per (Li et al. 2003). The high concentration of SA provided consistent results and may be required due to a more extensive cuticle. To simulate wounding from insect feeding, leaves were mechanically damaged by crushing a small area with sterile serrated forceps. These leaves were collected at 1 and 6 h post-treatment and immediately frozen in liquid nitrogen.

Total RNA was isolated by grinding 1 g of leaf tissue in liquid nitrogen followed by extraction with 8 ml Trizol reagent (Invitrogen) according to the manufacturer's procedure and hydrating the washed RNA precipitate in 200 μl DEPC-treated dH_20. Residual phenol and other contaminants were removed using the Illustra RNAspin Midi kit (GE Healthcare) and eluted in 500 μl RNAase-free dH_2O. mRNA was converted to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) followed by PCR using primer pairs specific to each of the *BnPgip* genes (Table 1). Whenever possible, the gene-specific primers were designed so as to span the intronic regions to differentiate amplified products derived from cDNA or genomic DNA. The basic PCR reaction mixture contained 2.5 μl 10× buffer, 2 μl dNTPs (5 mM), 2 μl forward primer (2.5 mM), 2 μl reverse primer (2.5 mM), 0.5 μl rTaq polymerase I, 2 μl of a 1/20th dilution of the cDNA and 14 μl dH_20. A 2 μl aliquot of genomic DNA (40 ng/μl) was used as a control. The PCR conditions used to detect transcripts from the *BnPgip1, 2*, *3*, *5*, *6*, *7*, *9*, *11*, *14* and *16* were as follows: initial denaturation at 94°C for 5 min followed by 27 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension of 72°C for 7 min. An annealing temperature of 57°C was used for *BnPgip10* and *13*, 43°C for *BnPgip8* and 50°C for *BnPgip12*, *15* and *17*. To confirm induction of genes by salicylic acid treatment, primers specific to *B. napus pathogenesis-related protein 1* (*BnPR1*) with an annealing temperature of 50°C and 23 cycles of PCR were used.

Table 1 Primers used to determine *BnPgip* gene expression by RT-PCR

BnPgip gene	Forward primer	Reverse primer
BnPgip1	CTTGCTCTTGTTCGCTCTCCTCT	TGAGGTTAGGGAGTTTGCGGAG
BnPgip2	GTTCAATACCGAGTTCTCTG	TCTAGAGAGATCGAACTGT
BnPgip3	CGTTGAGTGCGGCAACGCAT	GGGTATCGAACCGGAGAGC
BnPgip5	GCCTCGTGGGACCCTCA	CAAAGATAGAGAACTCGGTACT
BnPgip6	CGACGACGCCACCGTTAACCG	GAGGTTAACTGTCTTAGCA
BnPgip7	GCCTTATCTACAATACCTCACG	GGCTCCAAACAACATCGACGCG
BnPgip8	GACCTCTCTGGTTCCATACCTAC	GAGGTTAACTGTCTTAGCA
BnPgip9	GCTCCTGGTACTGCCTCGAA	CTGACCGGTGATGTTAGTGATCC
BnPgip10	GCTCTTGCTCTTGTTCGCTCTCG	GGTGGGTGGGATTTCACCT
BnPgip11	TATGGGATTTACCTATCG	CTGAAGGTCACCTCCCTGA
BnPgip12	GGATCGATTTCTCAAGGAACAG	GGGAAAACTAGAGATAAGCTTGG
BnPgip13	GCTCTTGCTCTTGTTCGCTCTCC	GGTGGGTGGGATTTCACCT
BnPgip14	CTTTCAATAAACTCTCTGGC	GGCTCCAAACAACATCGACGCG
BnPgip15	ATGGATAAGATAACGACTACAT	GAGCTTGGCGATAGTAGGTT
BnPgip16	GTCAGCTCATGGATCTTGCC	ATGTCTTAGGGATCTCAACACG
BnPgip 17	CCAAAATCACTAGGCAACA	TCGGGATAGTCCCTGTGAGT

Results

Isolation of additional members of the *BnPgip* gene family

Previously, we isolated two cDNAs encoding PGIPs (*BnPgip1* and *BnPgip2*) from *B. napus* and demonstrated that they were differentially expressed in response to abiotic and biotic stresses (Li et al. 2003). The two cDNA fragments were used separately to screen a *B. napus* DH12075 BAC library with 7 and 9 BACs hybridizing to the *BnPgip1* and *BnPgip2* probes, respectively. Two BAC clones were confirmed by Southern blotting and PCR analysis to contain *Bnpgip1* and four to contain *BnPgip2*. Each of the six BACs had different restriction enzyme patterns. BACs hybridizing to each probe were pooled and used to construct two sub-BAC libraries to isolate individual *BnPgip1* or *BnPgip2*-related genes. Screening of the libraries yielded 22 clones representing five restriction digest groups for *BnPgip1* and 12 clones representing six groups for *BnPgip2*. Several clones from each group were sequenced in their entirety resulting in the identification of the original *BnPgip1* and *BnPgip2* genes as well as 14 additional *BnPgip* genes denoted *BnPgip1* through *BnPgip17* (Fig. 1). Earlier attempts to characterize additional members of the *B. napus Pgip* gene family identified *BnPgip4* (Li et al. 2003); however, this gene was isolated from a *B. napus* cv. Westar genomic library (one of the DH12075 parents) and was not found among the *BnPgip* genes isolated from the doubled haploid *B. napus* DH12075 line. *BnPgip1* and *BnPgip15* were found within a 6 kbp region separated by a *copia* retroviral element, while *BnPgip11* and *BnPgip10* were more tightly linked. *BnPgip3, BnPgip7, BnPgip13, BnPgip14* and *BnPgip17* were located on separate fragments. The latter four were closely related and possessed similar regulatory and 3′ untranslated regions (UTR) (Table 2) flanked by conserved *Eco*RI sites. Of the six additional genes identified using the *BnPgip2* probe, *BnPgip5* was the most closely related and was situated on a separate fragment (Fig. 1). *BnPgip2* was closely linked to other *BnPgip* genes in one of three configurations, with *BnPgip6* alone, with *BnPgip12, BnPgip9* and *BnPgip8* or with *BnPgip16* and *BnPgip8.*

The sub-BAC libraries were prepared using pools of BACs hybridizing to either the *BnPgip1* or *BnPgip2* DNA probe; therefore, we attempted to determine the physical location of *BnPgip* genes on individual BACs using gene-specific PCR primers (Table 2). All of the *BnPgip1*-related genes were present on BAC 75O2 while BAC 20J13 encoded all genes apart from *BnPgip13* and *BnPgip15*. Four BACs hybridized to the *BnPgip2* probe. Amplification with the *BnPgip2* gene-specific primers yielded a band of the expected size from BAC 35A2 but a higher molecular weight band from the other BACs. This was denoted *BnPgip2′* and may represent an additional very similar gene. Due to the presence of an *Eco*R1 site within the *BnPgip2* gene, an intact gene was not identified and only a single type of cDNA has been isolated (Li et al. 2003). Similarly, the expected and a higher molecular weight product were amplified with the *BnPgip5* gene-specific primers and likely represent an additional gene as well; however, three BACs had both the *BnPgip5* and *BnPgip5′* forms, whereas the presence or absence of *BnPgip2* and *BnPgip2′* was mutually exclusive.

Sequences were obtained from the ends of each BAC to identify the homologous region within the *A. thaliana* genome (Table 2). One end of BAC 20J13 contained a

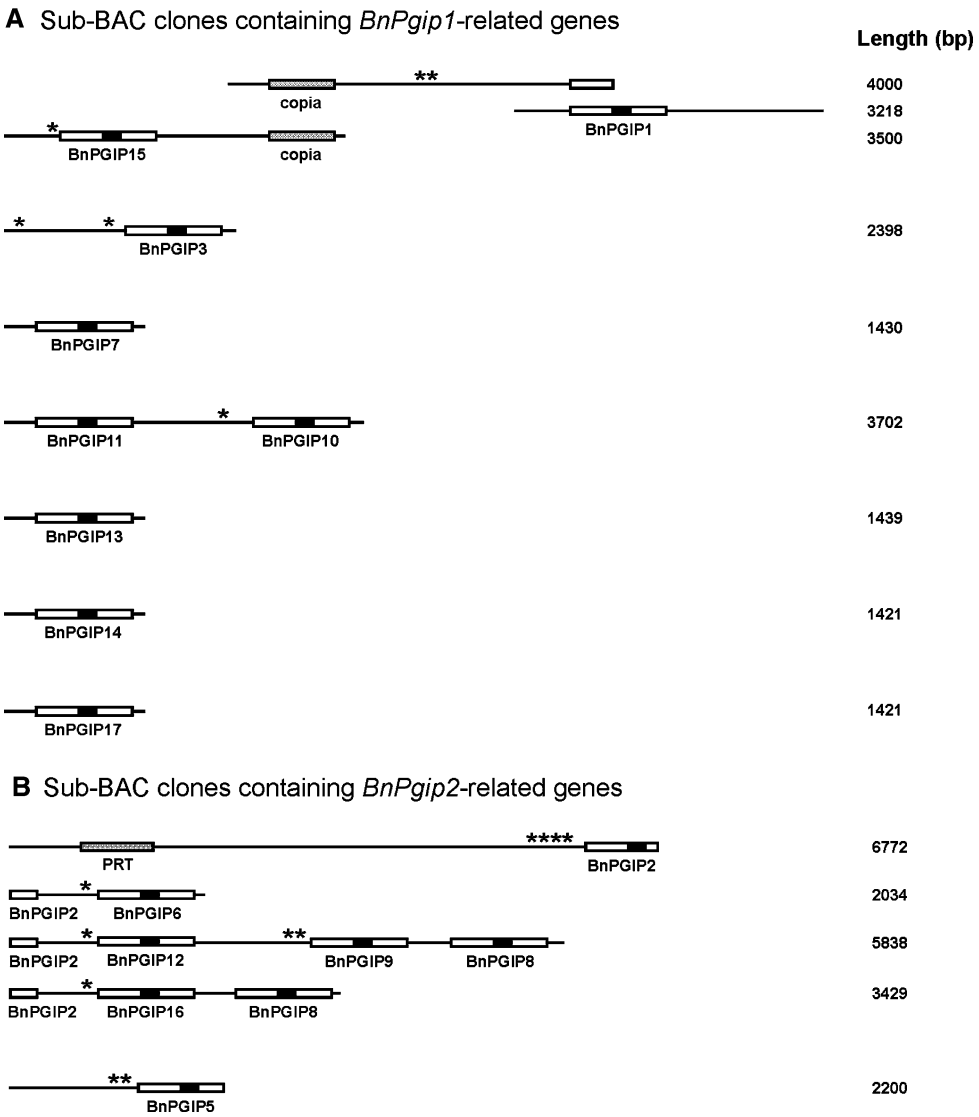


Fig. 1 Organization of *B. napus* DH12075 genes found on clones from sub-BAC libraries hybridizing to either *BnPgip1* (**a**) or *BnPgip2* (**b**) DNA probes. The location of WRKY transcription factor binding sites (*), a copia retroviral element and a phosphoribosyl transferase (*PRT*) are also shown. *Black bars* show location of introns

portion of *BnPgip15* and similarly BAC13J12 a portion of *BnPgip5*. However, the ends of BACs 75O2, 35A22, 73P4 and 28K18 contained genes homologous to those flanking *AtPgip1* (At5g06860) and *AtPgip2* (At5g06870), suggesting these BACs encompassed the collinear region in *B. napus*. We were able to assign several of the *BnPgip* genes to either the *Brassica* A or C genome based on identity (accounting for likely allelic variation) to sequences made available by the *B. rapa* genome sequencing project (http://www.brassica.info) or *B. rapa* (A genome) or *B. oleracaea* (C genome) cDNAs (A. Sharpe, unpublished data). A 20 kbp region from BAC KBrB046M04 (AC189367) was found to contain a region collinear with the *A. thaliana AtPgip* genomic segment that was flanked by genes corresponding to At5g06850 and At5g06920. The *B. rapa* segment encodes in the following order *BnPgip4*, *BnPgip11*, a fragmented gene related to *BnPgip7/13/10* and *BnPgip3*. The *BnPgip* genes were interspersed with a fragment related to At5g24840 and several retroelements. This strongly suggests that both the *BnPgip1* and *BnPgip2*-related genes arose by successive duplication and subsequent rearrangement of an ancestral gene related to *AtPgip1* and *AtPgip2*, probably through a combination of whole genome and segmental duplication.

Phylogenetic analysis of BnPGIPs

Phylogenetic analysis revealed that the BnPGIPs consisted of two clusters, one cluster comprising the proteins related to BnPGIP1 and a second cluster related to BnPGIP2 (Fig. 2). The 16 BnPGIPs share 50.4% identity at the amino acid level, with the BnPGIP1 and BnPGIP2 groups being more similar, 77.5 and 63.4%, respectively. The genes encoding proteins in the BnPGIP1 cluster shared many related non-coding elements, for example, all possessed similar regulatory regions with those from *BnPgip7, BnPgip11, BnPgip13, BnPgip14* and *BnPgip17* being identical within the 250 bp of sequence available (Table 3). Intron

Table 2 Characterization of *B. napus* 12075 BACs containing *BnPgip* genes

BAC Identifier	Corresponding *A. thaliana* Syntenic region[a, b]	*BnPgip* genes[c]								
		1	3	7	10	11	13	14	15	17
A. *BnPgip1*-related										
7502	At5g06850–At5g07350	+	+	+	+	+	+	+	+	+
20J13[d]	At5g06870–n/a	+	+	+	+	+	−	+	−	+
Located on A or C genome		A	A	?	A	A	?	?	C	?
BAC Identifier	Corresponding *A. thaliana* Syntenic region	*BnPgip* genes present								
		2	2′	5	5′	6	8	9	12	16
B. *BnPgip2*-related										
35A22	At5g06800–At5g06940	+	−	+	+	+	+	+	+	+
73P4	At5g06839–At5g07360	−	+	+	−	+	+	+	+	+
28K18	At5g06820–At5g07630	−	+	+	+	+	−	+	+	−
13J12[e]	At5g06865–(At1g20640)	−	+	+	+	−	−	−	−	−
Located on A or C genome		C	?	C	?	A	?	C	A	A

[a] Based on comparison of BAC end sequences to the *A. thaliana* genome

[b] The duplicated *A. thaliana Pgip* genes reside at At5g06860 and At5g06870

[c] Presence (+) or absence (−) on the BAC was determined using gene-specific PCR primers

[d] One end of BAC 20J13 corresponded to a portion of *BnPgip15*

[e] One end of BAC 13J12 corresponded to a portion of *BnPgip5*

length was reasonably well conserved of which three types were present. This group of proteins generally exhibited neutral to slightly alkaline pIs. Both of the *A. thaliana* PGIPs fell within the BnPGIP2-related cluster, which was more diverse than the BnPGIP1-related cluster, and contained proteins having alkaline pIs. BnPGIP8 and BnPGIP15 were intermediate between the two larger groups and shared features in common with both the BnPGIP1 and BnPGIP2 groups. The intron and 3′UTR of *BnPgip8* were identical to that of *BnPgip6*. A region of the *BnPgip15* intron was similar to that from *BnPgip12* and *BnPgip16* (not shown); however, BnPGIP15 possessed a carboxy-terminal extension, the function of which is not known, that was very similar to that of BnPGIP1 and BnPGIP11 (Fig. 3). Recall that *BnPgip15* and *BnPgip1* are in close physical proximity to one another in a genomic region separate from that of *BnPgip12* and *BnPgip16*. BnPGIP15 was the only PGIP to possess an acidic pI. The BnPGIPs had 10 leucine-rich repeats, the collection of which was flanked by amino- and carboxy terminal domains each containing four conserved cysteine residues. All were predicted to possess a signal peptide, and therefore likely to be secreted, with the mature proteins being N-glycosylated at anywhere from three to six sites (not shown).

Expression and regulation of *BnPgip* genes

Ten distinct types of regulatory regions (promoters) were identified among the 16 *BnPgip* genes (Table 3). These regions were fused to the β-glucuronidase (GUS) reporter gene and introduced into *A. thaliana*. The promoters associated with *BnPgip1*, *BnPgip3*, *BnPgip5*, *BnPgip6*, *BnPgip8* and *BnPgip11* directed GUS expression in all floral parts whereas *BnPgip15* was restricted to the filament (Fig. 4). GUS expression was not detected in flowers with the constructs containing the promoters from *BnPgip2*, *BnPgip9* or *BnPgip10*. The promoters from *BnPgip3*, *BnPgip5* and *BnPgip8* directed expression throughout the silique. Expression from the *BnPgip6* region was detected in the silique and pedicel but was restricted to the vasculature. The promoters from *BnPgip1*, *BnPgip11* and *BnPgip15* also directed expression in these tissues, but this was adjacent to the excision site which is indicative of induction by wounding. The promoter from *BnPgip6* was the only one to direct constitutive expression in stems. The expression was elevated at the excision site, which was also the case for the *BnPgip1*, *BnPgip3*, *BnPgip11* and *BnPgip15* promoters. All promoters, except those from *BnPgip2* and *BnPgip9*, were active in roots.

With the exception of that from *BnPgip15*, none of the promoters were found to be active constitutively in young leaves, but several were responsive to mechanical wounding and/or *S. sclerotiorum* infection (Fig. 5). The promoters from *BnPgip1*, *BnPgip3*, *BnPgip5*, *BnPgip6*, *BnPgip8* and *BnPgip11* were highly induced within 2–4 h after mechanical wounding, whereas those from *BnPgip2* and *BnPgip9* were slightly induced. The *BnPgip15* promoter was induced by wounding but in the leaf its expression was limited

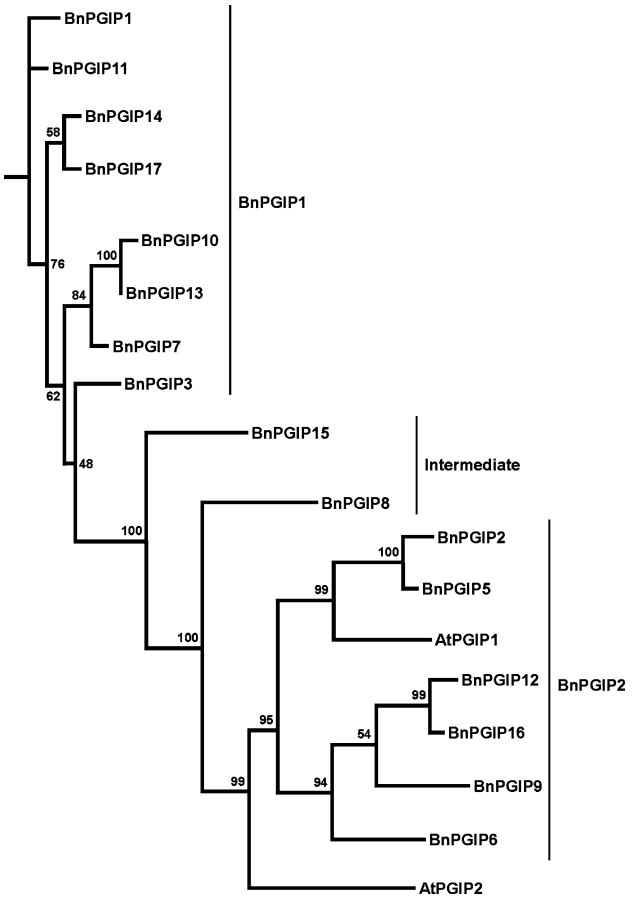


Fig. 2 Phylogenetic relationship between PGIPs from *B. napus* DH12075. The proteins used for alignment were BnPGIP1–BnPGIP3 (GenBank Accession # EU142023-EU142025), BnPGIP5–BnPGIP17 (EU142026-EU142038), AtPGIP1 (*Arabidopsis thaliana*, AF229249) and AtPGIP2 (AF229250). Proteins have been grouped according to their similarity to BnPGIP1, BnPGIP2 or as intermediate

to the vasculature. For all constructs, the background or constitutive level of GUS expression was found to be elevated in older leaves and stems (not shown); therefore, the effect of *S. sclerotiorum* infection was assessed using young tissues. GUS expression was detected in the healthy tissues immediately adjacent to the necrotic zone in leaves and stems of plants transformed with the *BnPgip3*, *BnPgip5*, *BnPgip6*, *BnPgip8*, *BnPgip11* and *BnPgip15* promoter constructs. Older leaves and stems of plants containing the *BnPgip2* and *BnPgip10* promoter constructs exhibited GUS expression (data not shown), including induction by infection, but younger tissues were unresponsive.

The analysis of promoter activity using the GUS fusions was limited as the size of the regulatory regions was determined by their proximity to upstream genes or the end of the sub-BAC library clone. To assess more accurately the regulation of individual *BnPgip* genes in *B. napus* in their natural genomic context, we conducted RT-PCR experiments with gene specific primers for 15 *BnPgip* genes (Fig. 6). The experiments were conducted with young *B. napus* leaves since in *A. thaliana* they were found to respond to *S. sclerotiorum* infection and wounding similarly to stems (Fig. 5) and were also amenable to treatment with various elicitors. The leaf tissues were sampled 1 and 6 h post-treatment. This is much earlier than the several hours to several days reported in many other studies examining defense gene expression. *BnPgip* genes induced soon after inoculation may be important for blocking pathogen establishment. In leaves, *Bnpgip* gene expression was induced by *S. sclerotiorum* infection, mechanical wounding, jasmonic acid and salicylic acid, or some combination thereof. *BnPgip1* possessed a type A1 promoter and was the only gene among the *BnPgip1* group to be highly induced by *S. sclerotiorum* infection. It was also induced by mechanical wounding and jasmonic acid treatment but not by salicylic acid. *BnPgip* genes possessing type A2 and A3 promoters were induced by mechanical wounding, jasmonic acid and salicylic acid treatment, though, *BnPgip7* was induced to a lesser extent by SA. In general, the effect of mechanical wounding and jasmonic acid treatment remained after 6 h while the response to salicylic acid was more transient. *BnPgip8* and *BnPgip10* exhibited higher basal levels of expression and limited induction in response to the various treatments. All of the *BnPgip* genes belonging to the *BnPgip2* group, as well as *BnPgip8,* were induced by *S. sclerotiorum* infection. *BnPgip2* and *BnPgip5*, which have similar type B promoters, were unaffected by mechanical wounding or jasmonic acid treatment but were induced by salicylic acid within 6 h. *BnPgip* genes possessing type C promoters were generally induced by mechanical wounding, jasmonic acid and salicylic acid treatment. Subtle differences existed as *BnPgip16* responded more to *S. sclerotiorum* infection than these other treatments and *BnPgip6* responded transiently to SA but not JA. In contrast to the *BnPgip1* group, the *BnPgip2* related genes with type C promoters remained induced by salicylic acid 6 h after treatment, whereas the response to mechanical wounding and jasmonic acid was more transient.

Discussion

Previously, we isolated and characterized two *B. napus* PGIP genes with *BnPgip1* being responsive to mechanical wounding and flea beetle feeding and *BnPgip2* being more responsive to *S. sclerotiorum* infection (Li et al. 2003). To investigate further the factors underlying these differential responses, we isolated genomic clones of these genes including their regulatory regions. At the time we presumed that only a limited number of genes were present in *B. napus*, as this was the precedent for other plant species, and

Table 3 Physical characterization of elements associated with *B. napus* PGIP genes

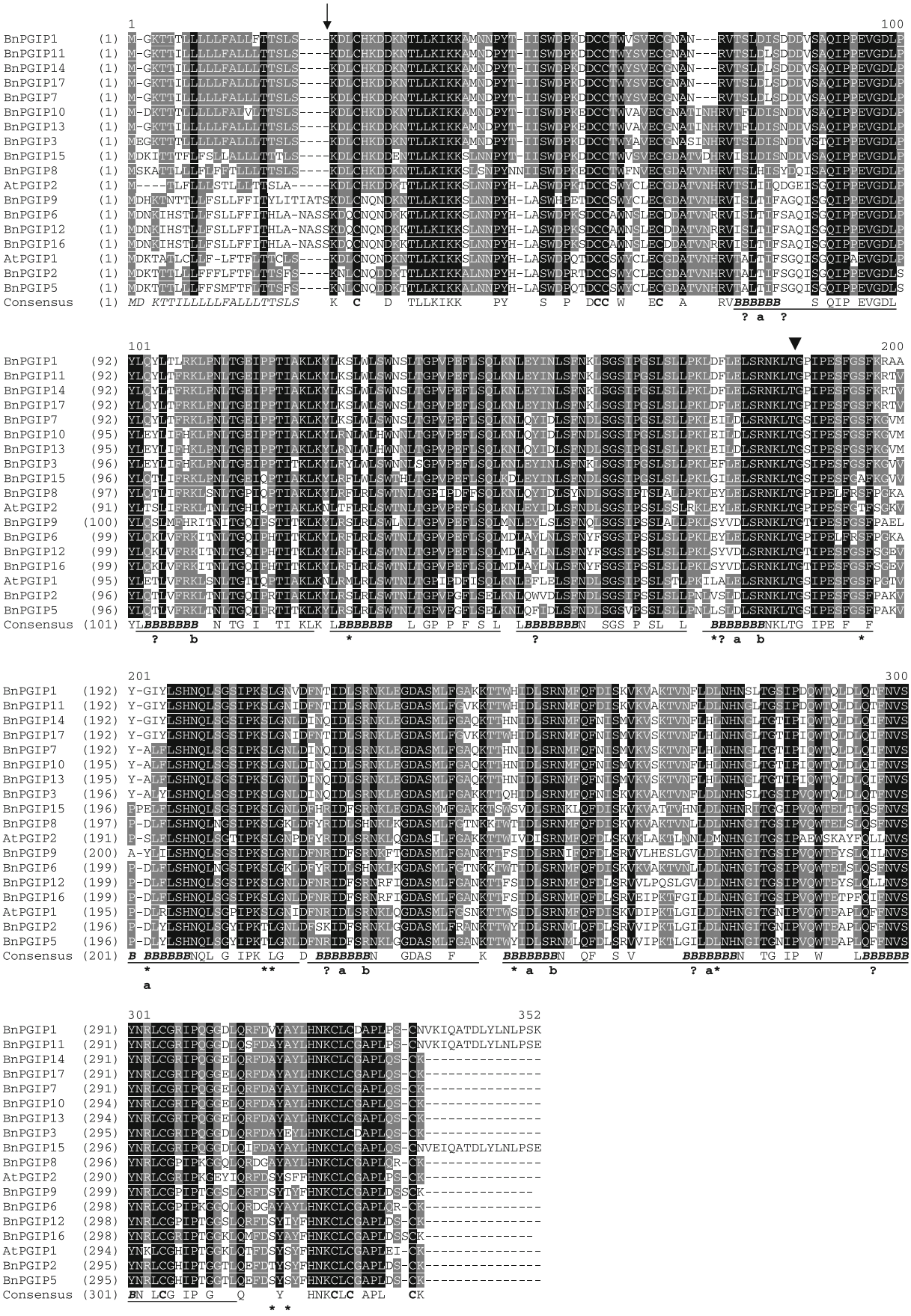
PGIP gene	Promoter type	Intron length (bp)	Intron type	3′UTR length (bp)[a]	3′UTR type	Mature peptide (aa)	MW (Da)	pI
BnPGIP1	A1	86	A	68	A	321	36,021	8.07
BnPGIP3	A2	87	B		B1	309	34,909	7.61
BnPGIP7	A3	95	C		B2	306	34,307	7.60
BnPGIP11	A3	86	A		C	321	36,036	7.20
BnPGIP13	A3	95	C		B2	309	34,778	7.34
BnPGIP14	A3	86	A		B2	306	34,435	8.25
BnPGIP17	A3	86	A		B2	306	34,542	8.40
BnPGIP10	A4	95	C		B2	309	34,778	7.34
BnPGIP2	B1	72	D1	176	D	309	34,512	8.50
BnPGIP5	B2	119	D2	–	–	309	34,445	8.21
BnPGIP6	C1	69	E		E	309	34,881	9.40
BnPGIP12	C1	101	F1		F1	309	34,650	8.57
BnPGIP16	C1	101	F2		F2	310	34,879	8.70
BnPGIP9	C2	150	G		F3	310	34,563	7.85
BnPGIP8	D	69	E		E	310	35,044	9.08
BnPGIP15	E	351	F3		G	325	36,316	6.14

[a] Untranslated region as determined from cDNA

our Southern blotting data revealed only a small number of bands. The *A. thaliana* genome contains only two *Pgip* genes linked in tandem on chromosome 5 and the high degree of synteny between the *A. thaliana* and *B.napus* genomes (Parkin et al. 2005) suggested that a similar arrangement might occur with *B. napus*. Our initial experiments, however, underestimated the number of genes encoded by the *B. napus* genome since, as we have now shown, they are present in clusters and possess conserved restriction enzyme sites that yield similar sized products upon digestion. The *Pgip* gene family in *B. napus* consists of at least 16 genes as the doubled haploid line we used excludes allelic variants. The large number is not due to a genomic rearrangement that might have occurred during doubled haploidization since all DNA fragments hybridizing to the *BnPgip1* and *BnPgip2* probes were present in either or both of the parents, cv. Westar and Cresor (Li et al. 2003). Indeed, the total number may be even greater since only six of the 16 BACs that hybridized to the *BnPgip* probes were examined and an additional gene, *BnPgip4*, was found to be linked in tandem to *BnPgip3* in *B. napus* cv. Westar (Li et al. 2003). The assertion that PGIPs are encoded by small gene families in plants is based on a limited number of species and experiments (De Lorenzo et al. 2001; Li et al. 2003; D'Ovidio et al. 2004, 2006; Janni et al. 2006). It will be interesting to determine whether this holds true once the genomes of these and other more complex plants are fully sequenced.

Some PGIPs are involved in regulating aspects of plant development such as floral organization (Jang et al. 2003), but they have been more widely studied for their role in plant defense against pathogens (De Lorenzo et al. 2001; Gomathi and Gnanamanickam 2004). The expansion of the *BnPgip* gene family was likely driven by strong selective pressures to evolve new PGIP variants to combat the diverse array of PGs produced by pathogens, since a similar degree of diversity among related plant species would be expected if PGIPs were primarily involved in conserved aspects of plant development. The BnPGIPs are typical of extracellular leucine rich repeat (LRR) proteins, each having 10 repeats exhibiting some variation of the LxxLxLxx-NxLt/sGxIPxxLxxLxxL 24 amino acid consensus. The LRRs form a curved right-handed superhelix or solenoid flanked by cysteine-rich amino- and carboxy-terminal domains that are stabilized by internal disulfide bonds. The xxLxLxx regions in these motifs form 10 parallel β-sheets along the concave surface of the structure and are considered the main sites for PG interaction (Di Matteo et al. 2003, 2006). Computational and mutational analyses have

Fig. 3 Alignment of *B. napus* and *A. thaliana* PGIPs arranged according to their phylogenetic relationships. Regions identical in all (*white lettering on black background*) or most (*white lettering on gray background*) are indicated with a consensus provided below the alignment. The putative signal peptide cleavage site (*arrow*), 10 distinct leucine-rich repeats (*underlined*), conserved cysteine residues flanking the LRR region (**C**) and location of the intron (*arrowhead*) are indicated. The β-sheet regions comprising the xxLxLxx motif within the LRRs (***B***) are shown with acidic (*a*), basic (*b*) and variable residues (**?**) below. Residues with an increased likelihood of being specificity determinants as reported by Stotz et al. (2000) are indicated (*)


BnPGIP1
BnPGIP11
BnPGIP14
BnPGIP17
BnPGIP7
BnPGIP10
BnPGIP13
BnPGIP3
BnPGIP15
BnPGIP8
AtPGIP2
BnPGIP9
BnPGIP6
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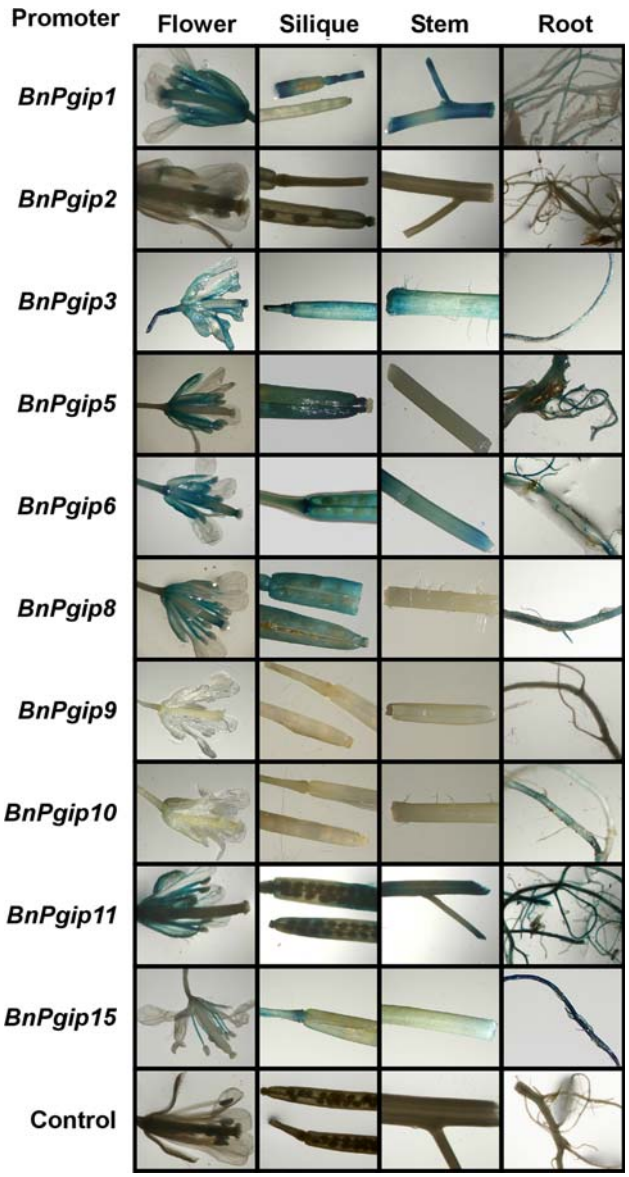

Fig. 4 β-glucuronidase activity in excised tissues of transgenic *A. thaliana* lines transformed with various *BnPgip* promoter-GUS fusions

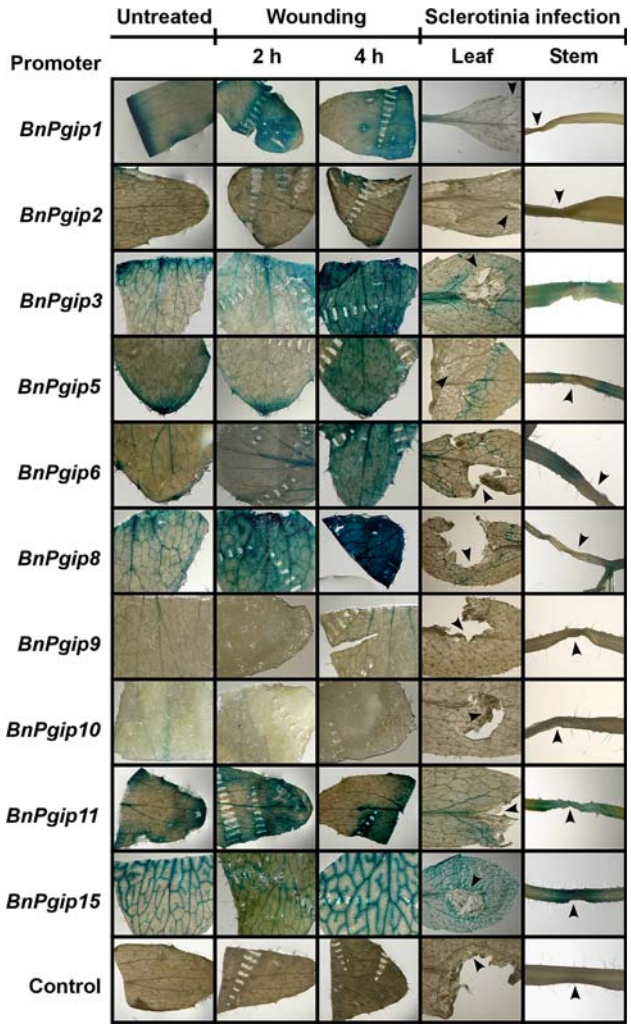

Fig. 5 β-Glucuronidase activity in transgenic *A. thaliana* lines transformed with various *BnPgip* promoter-GUS fusions. Leaves were mechanically wounded by crushing with serrated forceps and examined after 2 or 4 h as indicated. The staining observed in some untreated leaves is associated with the tissues adjacent to the excision site required to remove leaf samples. Stems and leaves were inoculated with PDA plugs (site indicated by *arrowhead*) containing *S. sclerotiorum* and sampled after 18–24 h

identified residues within and near this region that impart specificity upon PG–PGIP interactions. Stotz et al. (2000) used codon evolution models compiled from 22 dicot PGIPs to identify nine residues subject to substitutions, four of which resided within xxLxLxx regions. *Phaseolus vulgaris* PvPGIP1 and PvPGIP2 differ in their ability to interact with *Fusarium moniliforme* PG (FmPG) and five of the eight amino acid differences between them lie within this region of the LRRs. Mutation of any of the five PvPGIP2 residues reduced interaction with FmPG; however, a single point mutation changing K-253 of PvPGIP1 to the glutamine found in PvPGIP2 conferred ability to inhibit FmPG (Leckie et al. 1999). Similarly, conversion of the PvPGIP2 Val-152 to the corresponding glycine found in PvPGIP1 resulted in a dramatic loss of inhibitory activity against *B. cinerea* PG1 (Sicilia et al. 2005). Comparison of the BnPGIP LRRs revealed that the first and/or second positions of the xxLxLxx region were hypervariable (Fig. 3); these corresponded with sites identified through computational analysis that were positively selected for mutational events (Stotz et al. 2000; Bishop 2005) and would be ideal candidates for generating BnPGIP diversity.

Dependent upon the PG–PGIP pair, inhibition can occur in either a competitive or non-competitive manner and models have been developed to depict both (Federici et al. 2006). Competitive inhibition between PvPGIP2 and FmPG was shown to occur through interaction of acidic residues within the xxLxLxx region with conserved, but

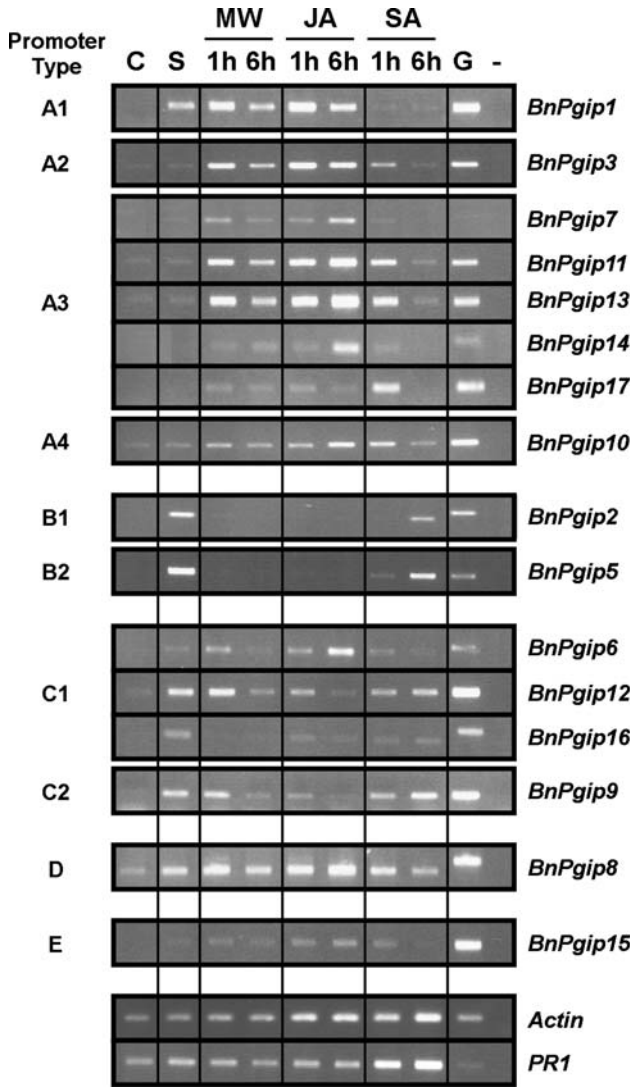

Fig. 6 RT-PCR analysis showing the effect of *S. sclerotiorum* 18 h after infection (*S*) and mechanical wounding (*MW*), jasmonic acid (*JA*) and salicylic acid (*SA*) 1 and 6 h after treatment on *Pgip* gene expression in *B. napus* DH12075 leaves as indicated. A genomic DNA control (*G*) was also included. In cases where gene-specific primer pairs spanned an intron, the presence of a single higher molecular weight band shows the absence of contaminating genomic DNA (*BnPgip2*, *BnPgip6*, *BnPgip8*, *BnPgip14*, *BnPgip16*)

non-catalytic, Lys-269 and Arg-267 in the PG active site (Federici et al. 2001; Di Matteo et al. 2003). The cluster of acidic residues was deemed to be a unique aspect of the PvPGIP structure; however, most of BnPGIPs possess aspartic or glutamic acid residues in the xLxLx positions in a majority of their exposed β-sheet regions (Fig. 3) suggesting that this may be a common mechanism for PG–PGIP interaction. Non-competitive inhibition is presumed to result from binding at other PG locations (Sella et al. 2004; Sicilia et al. 2005), but such interactions are likely to be diverse in nature and have not yet been fully elucidated. In fact, some PGIPs are capable of activating PG activity in certain ionic environments (Kemp et al. 2004). It has been proposed that inhibition of PG activity may also occur through binding of the substrate. Spadoni et al. (2006) reported that basic residues lying within the terminal variable site of a cluster of xxLxLxx regions were responsible for the ability of the PvPGIP1 and PvPGIP2 to bind pectin. It is unclear whether binding of PGIP to pectin protects the latter from degradation since the presence of PG leads to dissociation of PGIP from pectin, possibly through competitive interaction with a common site on the PGIP. All of the 16 BnPGIPs possessed a basic amino acid in the terminal xxLxLxx position in four of the ten LRRs (Fig. 3) implying that PGIPs are generally localized in the cell wall through interaction with pectin.

The role of a given PGIP is determined not only by its specificity but also by the tissue in which it is expressed and the manner by which it is regulated. Previously, we reported that *BnPgip1* and *BnPgip2* were differentially regulated in response to biotic and abiotic stresses (Li et al. 2003); however, these results were obtained via northern blot analysis using probes that we now know were unable to distinguish between the various members of these groups. Transgenic *A. thaliana* lines transformed with constructs where the GUS reporter was linked to each of the ten distinct *BnPgip* promoters revealed that many were constitutively active in various floral organs and roots, but were induced by mechanical wounding and *S. sclerotiorum* infection in leaves and stems. RT-PCR analysis provided a more accurate assessment of individual *BnPgip* gene expression in their normal chromosomal contexts. In these studies, all members of the *BnPgip2* group were markedly induced by *S. sclerotiorum* infection, whereas this was true only for *BnPgip1*, despite the fact that all members of the *BnPgip1* group share related promoter elements. The complexity of the response to *S. sclerotiorum* infection is further exemplified by the observation that *BnPgip1* responded strongly to mechanical wounding and jasmonic acid treatment but not to salicylic acid, whereas the converse was found for *BnPgip2* and *BnPgip5*. An equally confounding phenomenon was observed with the tandemly duplicated PGIP genes in *A. thaliana* (Ferrari et al. 2003). Both *AtPgip1* and *AtPgip2* were induced by *B. cinerea* infection, a necrotrophic pathogen similar to *S. sclerotiorum*; however, *AtPgip2* expression was dependent upon the jasmonate pathway, whereas *AtPgip2* was induced only by oligogalacturonides, these being generated by wounding. The regulatory regions associated with the *BnPgip2*-related genes have a greater preponderance of WRKY elements (TTGACC/T) than *BnPgip1*-related genes (Fig. 1), which may partly explain the differential responsiveness to salicylic acid (Eulgem and Somssich 2007). The interplay between various signaling pathways in regulating *BnPgip*

gene expression is evident in that most were responsive to some combination of *S. sclerotiorum* infection, mechanical wounding, jasmonic or salicylic acid treatment. This mixed mode of regulation was also observed among the gene families in *Glycine max* (D'Ovidio et al. 2006) and *P. vulgaris* (D'Ovidio et al. 2004).

In conclusion, the 16 genes identified in the *B. napus* DH12075 genome represent the largest family of *Pgip* genes described in any plant species to date. While the series of gene duplications, mutations and genomic rearrangements that led to this expansion may never be fully elucidated, it is worth noting again that all of the *BnPgip* genes described herein were expressed. This suggests that some selective pressure has maintained their activity. We have now embarked on studies to examine the spectrum of PGs that are affected by the BnPGIPs which will provide useful insights into the structural requirements underlying inhibitory activity and specificity.

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