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Overexpression of *Brassica napus* Myrosinase-Associated Protein 1 improved *Sclerotinia* tolerance in *Arabidopsis*

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Abstract

Canola (*Brassica napus*) productivity is severely affected by various biotic and abiotic stresses, such as insects, pathogens, drought and cold. Characterization of genes with specific effects on particular stress responses is one of the long-term goals for *Brassica* genetic improvement. Here we explored the roles of *Brassica napus* Myrosinase-Associated Protein 1 (*BnMyAPI*), which was highly represented (expressed) in a *Brassica napus* subtractive library developed after leaf damage by the crucifer flea beetle (*Phyllotreta cruciferae*). Expression of *BnMyAPI* was under different developmental control in *B. napus*, but it was co-induced in *B. napus* by flea beetle feeding, *Sclerotinia* infection, drought, and cold. A total of 20 *Brassica napus* Myrosinase-Associated Proteins (*BnMyAPs*) were represented in different *B. napus* stress and development EST libraries and indicated larger, diversified families than known earlier. Overexpression of *BnMyAPI* in *Arabidopsis* resulted in increased survival ratio (63-90%) compared to that of controls (15-20%) after *Sclerotinia* infection, which suggests that *BnMyAPI* could be used to protect *Brassica* oilseed and vegetable from *Sclerotinia* disease.

Key words: *BnMyAP*, *Brassica napus*, flea beetle, *Sclerotinia*

Abbreviations: *BnMyAPI*, *Brassica napus* Myrosinase-Associated Protein 1

Introduction

Glucosinolates are secondary metabolites characteristic of the *Brassicaceae* and feeding stimulants to some insects (Louda and Mole 1991; Rask et al. 2000). In contrast, some of their hydrolysis products are claimed to function in defense against generalized herbivores, and both substrate and product are implicated as factors in host-plant recognition by specialized insects (Giamoustaris and Mithen 1995). Glucosinolates possibly also affect interactions between plants and fungi (Mithen 1992; Giamoustaris and Mithen 1997; Manici et al. 1997).

Myrosinases (thioglucoside glucohydrolases, EC 3.2.3.1) catalyze the hydrolysis of glucosinolates, which decompose to isothiocyanates, thiocyanates, epi-thiocyanates, or nitriles depending on the epi-specifier protein involved (Tookey 1973; Kuchernig et al. 2012). Proteins binding to myrosinase-like myrosinase-binding proteins (*MBPs*) have been implicated in the stability or enzymatic properties of myrosinase (Lenman et al. 1990; Falk et al. 1995; Taipalensuu et al. 1996, 1997; Andreasson et al. 1999), and *MBPs* are necessary for myrosinase isozyme complex formation in rapeseed seeds (Eriksson et al. 2002). However, down-regulation of *MBP* does not affect cotyledon feeding damage by *Phyllotreta undulata* flea beetles (Eriksson et al. 2002) and volatiles in leaves of *B. napus* var AC Excel (eg., benzene propane nitrile and phenylacetaldehyde) are neutral or cause only weak responses by crucifer flea beetles (*P. cruciferae*) (Pivnik et al. 1992; Gruber et al. 2009). Moreover, the role of other *myrosinase-associated proteins* (*MyAPs*) with affinity for myrosinase complexes in biotic stress and the extent of their relationship with glucosinolates are completely unknown.

Canola (*Brassica napus*) is an important oil crop in Canada and its production is severely affected by various biotic and abiotic stresses, including flea beetle, *Sclerotinia*, drought and cold. Therefore, the long-term goals of *Brassica* improvement include identification of specific genes with effects on particular stress responses. Previous study has showed that a *Myrosinase-Associated Protein* (*BnMyAPI*) was highly represented (expressed) in a *Brassica napus* subtractive library developed after leaf damage by the crucifer flea beetle (*Phyllotreta cruciferae*) (Gruber et al. 2012). Here, we further explored the roles of the glucosinolate-related *BnMyAPI* and found that it was not only highly induced by flea beetle feeding, but also strongly induced under *Sclerotinia* infection, drought and cold conditions. However, the transgenic *Arabidopsis* plants with overexpression of *BnMyAPI* only improved survival after *Sclerotinia* infection.

Materials and Methods

Plant material

Expression analysis, bioassays, and EST library development for *Brassica napus* were conducted using the double haploid line DH12075 (derived from a cross between the blackleg-resistant canola cultivar Cresor and the susceptible cultivar Westar (G. Seguin Schwartz and G. Rakow, formerly of AAFC Saskatoon Research Centre). Transgenic *Arabidopsis* overexpression (OE) lines were developed in a Columbia (Col-0) ecotype.

EST subtraction library development, screening, and bioinformatics

Total RNA was isolated with guanidine hydrochloride from undamaged 8-week-old *B. napus* leaves and from flea beetle-damaged *B. napus* leaves of the same age. Leaves were damaged to 10-20% of the tissue mass after a 24 h laboratory feeding bioassay using wild *Phyllotreta cruciferae* flea beetles collected from a turnip field in Saskatoon. The two sets of RNA were treated with RQ1 RNAase-free DNAase (Promega, Madison, Wisconsin, USA) and poly (A)⁺ RNA isolated using Oligotex (Qiagen, Toronto, Ontario, Canada) following the manufacturer's instructions. The cDNA synthesis and subtraction were performed according to the protocol provided in the PCR-select cDNA Subtraction Kit from Clontech Laboratories (Mountain View, CA, USA). In brief, the tester (damaged tissue) and driver (undamaged tissue) cDNA populations were digested separately with Rsa I to obtain shorter (~100-1200 bp), blunt-ended molecules. Tester cDNA was ligated to different adaptors to create two tester populations, which were hybridized separately with excess driver cDNAs to generate the templates required for PCR amplification of differentially expressed cDNAs. PCR subtractive amplicons were cloned in a pGEM-T easy vector (Promega, Madison, WI, USA). The subtraction library was annotated by BLAST analysis to the *Arabidopsis* database (TAIR) and analyzed by bioinformatics according to Gruber et al. (2012). Other *B. napus* stress or development EST libraries identified in Gruber et al. (2012) were developed using a modified pSPORT vector and the SuperScript Plasmid System with Gateway Technology. All EST libraries were deposited at <http://brassicagenomics.ca>. *BnMyAP* sequences detected in the FB leaf subtractive library were assessed for representation and homologues in other EST libraries using reciprocal best hit (RBH) BLAST analysis (Moren-Hagelsieb and Latimer 2008). Alignments of translated sequences for *BnMyAPs* were performed using the BioEdit multiple sequence alignment program (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

Transformation of Arabidopsis with BnMyAPI binary vectors

BnMyAPI full-length coding regions (GenBank access no. KM821274) were amplified by PCR from cDNA of 8-week-old *B. napus* leaves using primers described in Supplementary Table S1. Amplicons were sequenced and those specifying *BnMyAPI* were then cloned as XbaI-SstI fragments downstream of the CaMV 35S promoter in binary transformation vector pBI121 (Clontech). The binary plasmids were introduced into *Agrobacterium tumefaciens* by electroporation. For plant transformation, wild type *Arabidopsis* seeds were sown on a wet masked soil mixture in pots. Pots were placed in the dark at 4°C for 2 days and then moved to an environmentally controlled greenhouse at 22 °C with 16 h light and 8 h dark supplemented with halogen lamps. For plant transformation, flowering *Arabidopsis* plants were transformed with the *Agrobacterium* using the floral dip method (Clough and Bent 1998).

Molecular Analysis

T₁ transgenic *Arabidopsis* plants were selected by growth on kanamycin and detection of the transgene fragment after PCR analysis with specific primers (Supplementary Table S1) using genomic DNA isolated from rosette leaves by alkaline lysis (Klimyuk et al. 1993). Seed from 10 independently transformed T₁ plants (*BnMyAPI-OE*) selected by PCR of the transgene were used to develop T₂ homozygous plants used for bioassays.

For Northern blot analysis of transgenic or non-transgenic plants, tissue samples were collected from a range of developmental stages or stress treatments (detailed below), then frozen immediately in liquid N₂. Total RNA was extracted from tissues as described by Sambrook et al. (1989) and separated on 1% agarose gels (30 µg per lane for *B. napus*; 10 µg for *Arabidopsis*). Northern membranes were prepared and hybridized as described by Sambrook et al. (1989). Probes were prepared from PCR products developed using specific primers detailed in Supplementary Table S1, then labeled with ³²P-dCTP as above. Membranes were washed twice at 65°C in 2X SSC-0.5% SDS for 15 min each and then for 30 min at 65°C in 0.2X SSC-0.5% SDS with gentle shaking, followed by exposure to X-ray film for 3 h. The Northern blots were replicated twice using independent RNA preparations.

Flea beetle feeding assays

Adult *Phyllotreta cruciferae* flea beetles (from a spring population) were collected and maintained in white-walled cabinets (housed in clear plastic cages) on cabbage and water for up to one week at 20 °C, 16 h photoperiod, 100 µmol m⁻²s⁻¹, then starved for 24 h prior to using them. To obtain FB damaged leaves (for subtraction library development and Northern blots) and cotyledons (for Northern blots), *B. napus* plants were grown in a soil-less mixture in 10 cm pots in a greenhouse supplemented with halogen lamps for 8 weeks (leaves). To test *B. napus* seedling damage by FB, one week-old seedlings were grown in small cylinders (for cotyledons). Plants and seedlings were pre-selected for uniformity and then evenly spaced in a foam-based arena placed inside a 50×50×50 cm clear plastic cage and exposed to 400 flea beetles per cage for 0-24 h in an uniformly lit, white-walled, controlled environment chamber (20 °C, 16 h

photoperiod, $100 \mu\text{mol m}^{-2} \text{s}^{-1}$; 65% relative humidity). Damage on leaves or cotyledons was scored using a rating scale from 0 (denoting no damage) to 10 (denoting the entire tissue destroyed) as described in Palaniswamy et al. (1992). Thus, a rating of 1 was assigned if approximately 10% of the area of the tissue was damaged. Leaf and cotyledon tissues were collected and frozen in liquid N_2 , then stored at -80°C . For bioassays on transgenic *Arabidopsis* expression lines and non-transgenic lines, eight (8) T_2 seedlings per transgenic line, plus WT and empty vector control lines, were grown in randomized rows for 12 days in the same white-walled growth cabinet in 96-well micro-titre plate arenas; then bio-assayed with 50 FBs per arena fitted with a modified top and scored after 24 h for damage as described in Hallett et al. (2005). Each bioassay experiment was repeated 3 times.

Cold and freezing bioassays

One-week-old transgenic and non-transgenic *Arabidopsis* seedlings grown in MS were placed in a 4°C chamber with a 16 h photoperiod (approx. $100 \mu\text{mol m}^{-2} \text{sec}^{-1}$) for 24 h. Non-acclimated 7-day-old T_2 transgenic *Arabidopsis* seedlings and cold-acclimated transgenic plants were grown in petri dishes with MS. Plants were placed at -2°C in the dark in controlled temperature freezing chamber for 3 h, after which freezing of the plates was nucleated with ice chips as previously described (Vogel et al. 2005). The plants were then incubated an additional 21 h at -2°C , followed by -5°C for 24 h. The temperature was then raised to 4°C for 24 h and plants allowed to recover at 24°C for a further 48 h in continuous light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$). The entire experiment was repeated 3 times in a randomized design and then scored for survival.

Mechanical wounding assay

Seeds of *B. napus* were sown in potting medium and grown in greenhouse pots at 22 °C with 16 h light and 8 h dark supplemented with halogen lamps for 4 weeks. Fully expanded leaves of the plants were punctured with a sterile forceps and post-wounded plants incubated for one hour.

Drought tolerance bioassay

Transgenic and non-transgenic *Arabidopsis* plants were grown in a soil-less potting mixture (one plant per pot; 40 pots per flat) in a greenhouse at 22 °C with 16 h light and 8 h dark supplemented with halogen lamps. After 3 weeks of growth with one watering per day (until draining), water was withheld for 9 days and then all pots were re-watered daily (until draining) and plant re-growth scored 4 days later (Haake et al. 2002). The bioassay was laid out in a randomised design, with twenty plants per line and four biological replicates.

Sclerotinia Infection Test

A modified *Sclerotinia* infection method was used based on a spray bioassay previously described by Pedras and Ahiahinu (2004). Transgenic and control *Arabidopsis* seedlings were grown in a potting mixture (5 seedlings in one pot representing one line) with pots placed in a randomized design in 50 cm x 25 cm trays (4 pots per line; total of 50 pots per tray). *Sclerotinia sclerotiorum* was obtained from Dr. Fengqun Yu at the Saskatoon Research Centre, and liquid cultures were initiated with five sclerotia per 100 ml of potato dextrose broth (PDB) media and shaken (110 rpm) at 20°C for 7 days. Trays of three-week-old *Arabidopsis* plants were

uniformly sprayed for 1 min with the fresh *Sclerotinia* inoculum (500 ml per tray) using a hand sprayer. The pots were then incubated in a growth chamber at 24 °C for 7 days and examined visually for disease symptoms and survival.

Statistical Analysis

Analysis of variance was conducted using LSD tests in SAS ver 9.0 (SAS Institute, Inc. Cary, NC, USA). Means ($\pm$ standard error) were separated using *t*-tests at $p < 0.05$.

Results

A survey was conducted of genes induced in a FB damaged leaf subtractive EST library developed from eight-week-old *B. napus* leaves damaged by crucifer flea beetle feeding. ESTs coding for a *Myrosinase-Associated Protein BnMyAPI* were much more strongly represented in this library than in a wide range of other *B. napus* tissue-specific or stress-responsive EST libraries (Table 1). In fact, *BnMyAPI* from the FB leaf subtractive library (0.62%) were also weakly represented in two *B. napus* etiolated seedling libraries (0.07% and 0.02%), an apical meristem library (0.02%), a leaf drought library (0.03%) and a flea beetle damaged cotyledon library (0.03%); moderately expressed in *Sclerotinia* infected stem library (0.18%) and mechanical-wound leaf subtractive library (0.43%). The closest homolog *BnMyAP2* (0.08% in FB leaf subtractive library) of *BnMyAPI* weakly represented in two etiolated seedling libraries (0.02% and 0.02%), a mature flower bud drought library (0.03%) and a cotyledon library (0.05%); moderately expressed in a mechanical-wound leaf subtractive library (0.32%). We predicted that the *BnMyAPI* gene as the major representative of *BnMyAPs* in the FB leaf

subtraction library and the unique representative in the *Sclerotinia* infected stem library, which may function mainly in flea beetle host plant interactions and may also function in fungus (*Sclerotinia*) tolerance.

MyAP genes comprise gene families in Brassica napus

The *B. napus* flea beetle damaged leaf subtractive library included a total of 85 MyAP ESTs. These ESTs were classified into distinct genes using the criteria that ESTs represented the same gene if sequences were $\geq 90\%$ identical (out of a total of 200 bp) (Bailey et al. 1998). The FB leaf subtractive library contained two MyAP proteins that clustered into one sub-group within 4 major groups comprising all *BnMyAP* proteins found in this study (Fig. 1A). *BnMyAPI* (with the most highly represented set of ESTs) appeared equally similar to an A genome ancestor protein from *Brassica rapa* (*BrMyAPI*; Bra037954; <http://brassicadb.org/brad/>; Supplementary Table S2), whereas *BnMyAP2* appeared most similar to one of the C genome ancestor proteins from *Brassica oleracea* (*BoMyAPI*, Bol039076, <http://brassicadb.org/brad/>; Supplementary Table S2). And these four proteins are closest to *Arabidopsis* gene AT1G54020.2, which suggests they are derived of same ancestor gene (Fig. 1B). Eighty-five (85) MyAP ESTs represented a family of 20 proteins spread across the libraries (Table 1; Fig. 1A; Supplementary Figure S1; Supplementary Table S2). MyAPs were not represented in the cold acclimated libraries, and the most diverse sets of MyAPs occurred in libraries made from roots, etiolated seedlings and drought stress (Table 1).

BnMyAPs are differentially regulated during development and stress

The *BnMyAPI* (8 ESTs) was most enriched in flea beetle damaged subtractive leaf library and unique expressed in *Sclerotinia* infected stem library, compared with other members of the *MyAP* gene families. Hence, we were curious to find out how this particular gene responded to other forms of stress. A number of different-sized RNA species (bands) were detected in undamaged *B. napus* leaves with the *BnMyAPI* probe (Fig. 2). All of these RNA species were strongly detected in flea beetle damaged leaves and moderately detected in *Sclerotinia*-infected and mechanically wounded leaves (Fig. 2). One of the transcripts was also induced by dehydration and by 7 h of continuous cold shock, while a different sized band was transiently induced early on during cold treatment and not detected at a later time point (Fig. 2). Neither of the cold-acclimated EST libraries showed any representation of *MyAP* genes. These results indicated that the *BnMyAP* genes were differentially regulated, but that indeed responded strongly to flea beetle feeding and *Sclerotinia* infection, which is consistent with representation in the EST libraries (Table 1).

Since cotyledons are critical tissues impacted by flea beetle damage to the canola crop (Lamb 1984), we also conducted additional laboratory bioassays and Northern blots on cotyledons fed upon by flea beetles. Here, the expression of *BnMyAPI* was steadily increased from 2 h to 4 h in cotyledons after flea beetle feeding; then expression peaked between 4 h and 16 h and declined by 24 h of feeding (Fig. 3).

To confirm whether *BnMyAPI* was also subject to tissue or development constraints, Northern blots were conducted on a range of tissues. A number of RNAs for *BnMyAPI* were very strongly

accumulated in undamaged cotyledons, seedling leaves and stem, open flowers, and a fewer number of these bands were also strongly expressed in mature 8-week leaf petioles (Fig. 4). However, *BnMyAPI* was barely detectable in seedling root and 8-week-old fully expanded leaves and not in vegetative stems (Fig. 4). These results were only partially consistent with representation in the development EST libraries. Here, *BnMyAPI* appeared in apical meristem and not in any other tissues (likely because of different tissues), while a broad assortment of *MyAP* family members appeared in the root and flower bud libraries (Table 1). These data indicate that *BnMyAPI* transcription is under mainly strict, developmental control in different tissues, but has over-lapping stress response profiles.

Expression of BnMyAPI in transgenic Arabidopsis and impact on flea beetle feeding, dehydration, and cold

Since *BnMyAPI* was most highly induced by flea beetle-feeding and moderately induced by drought and cold temperatures, we tested whether it can impact plant responses to these three types of stress when expressed in transgenic *Arabidopsis*. Therefore, 54 *BnMyAPI* overexpression lines (*BnMyAPI-OE*) were developed by transfection of *Arabidopsis* with *A. tumefaciens* binary vector (Supplementary Figure S2). These plants were confirmed by PCR using primers that amplified the transgene fragment (a small number of representative lines shown in Supplementary Figure S3). We manipulated this model plant rather than *B. napus*, since *Arabidopsis* is responsive to the crucifer flea beetle (Hallett et al. 2005), genetically close to *B. napus* (Parkin et al. 2005), and grows more quickly than the crop *Brassicas*. Representative Northern blots showed that the expression of transgene in 10 selected single transgene *BnMyAPI-OE* expression lines varied strongly and the *BnMyAPI* probe showed two transcripts

in most of the transgenic lines (Fig. 5). However, FB feeding, drought, and freezing bioassays on these lines showed no phenotype differences compared with WT control *Arabidopsis* or transgenic control *Arabidopsis* expressing an empty vector binary vector (data not shown). Since no Salk knockout lines were available to test *Arabidopsis* MyAP phenotypes at the time of investigation, there was no Salk lines were tested for At1g54020, which is the *Arabidopsis* orthologous of *BnMyAPI* (Fig. 1B).

Expression of BnMyAPI improves Arabidopsis resistance to Sclerotinia

In addition to transcript enhancement after flea beetle feeding, drought, and cold, *Sclerotinia* infection also caused very strong increases in *BnMyAPI* expression in *B. napus* seedlings. Therefore, bioassays were conducted to determine whether transgenic *Arabidopsis* expressing this *B. napus* gene could improve tolerance to *Sclerotinia*. All three *BnMyAPI-OE* transgenic lines (No. 22, 54 and 60 with moderate-to-strong transgene expression) showed greater seedling survival after *Sclerotinia* infection compared with wild type or empty vector transformed plants (Fig. 6). The strongest resistance was provided by the no. 22 line (mean of 90% survival), followed by no. 54 line (mean of 68.0% survival), and the no. 60 line showed mean of 63% survival rate in response to *Sclerotinia*. Variation for seedling survival was higher in individual transgenic lines than in control lines (Fig. 6).

Discussion

Tissue/stress specificity and diversity of the BnMyAP gene family

A survey of expressed genes in a *Brassica napus* flea beetle damaged leaf EST library revealed an abundance of transcripts for *myrosinase-associated proteins*. An expanded search for these genes within our *Brassica* EST libraries revealed a gene family consisting of at least 20 *MyAP* genes (derived from ESTs) that each are differentially expressed in different tissues, developmental stages or in response to different stresses. This gene family is comprised of four sub-groups of genes. This data indicates much larger gene family for *MyAP* than previously reported in the crucifers (Taipalensuu et al. 1996, 1997). Individual sequences may be diversified to fit local conditions in individual tissues or under specific stress conditions. However, more closely related *MyAPs* may also have some over-lapping roles or functional redundancy, since our data shows that representation of individual *BnMyAP* genes is not completely unique to each tissue or stress.

Although the specific biochemical function of *MyAPs* is not clear, the literature reported for these genes is consistent with the stress and development profiles we saw in our EST libraries and expression studies. In previous reports, *MyAP4* (not same as *BnMyAP4*) and *MyAP5* (not same as *BnMyAP5*) transcripts were shown to be wound- and methyl jasmonate-inducible, and wound-inducible transcripts of *iMyAP9* (not same as *BnMyAP9*) and *iMyAP12* (not same as *BnMyAP12*) were developmentally regulated in various vegetative organs in *B. napus* (Supplementary Figure S4; Taipalensuu et al. 1996, 1997). Moreover, diverse transcript sizes were recovered in expression analyses using the *BnMyAP1* probe. This could be due to our probe detecting transcription of multiple genes represented in many of the EST libraries. However, multiple transcript bands in *Sclerotinia* infected seedlings may also come from the same gene,

since transcripts for only one *MyAP* gene (*BnMyAPI*) were recovered in a *Sclerotinia* stem infected library. Multiple transcripts from other *MyAP* genes are also known in the *Arabidopsis* genome (<http://www.arabidopsis.org/>). *MyAPs* have been found in high-molecular-weight complexes composed of *MBP* and myrosinase isozymes which vary in a temporal and tissue-specific manner (Härtel and Brandt. 2002). Each protein of our 20 recovered *BnMyAPs* may potentially associate with a specific myrosinase and glucosinolate to produce a unique, biologically active product. For example, *BnMyAPI* may potentially produce a leaf-specific end-product that is either attractive to flea beetles or at least not defensive against flea beetle feeding (Gruber et al. 2009), since this gene appeared with high frequency in the flea beetle damaged leaf library and *B. napus* leaves are a host tissue for flea beetles (Feeney et al. 1970). Diversity of myrosinase genes, *MBPs*, epi-specifier proteins, and glucosinolate profiles is already known for cruciferous plants (Thangstad et al. 1993; Fahey et al. 2001; Reichelt et al. 2002).

Expression of BnMyAPI improves tolerance to Sclerotinia infection

Although *BnMyAPI* were nearly unique among their gene family members in their strong response to flea beetle feeding, transgenic *Arabidopsis* plants expressing *BnMyAPI* did not improve resistance to flea beetle feeding. And also it did not improve tolerance to drought or cold, although it was strongly induced by these conditions in *B. napus*. In contrast, expression of *BnMyAPI* transgene in *Arabidopsis*, strongly improved survival after *Sclerotinia* infection. It may be because *BnMyAPI* is completely unique in response to *Sclerotinia* infection. But its unproven biochemical function limits our understanding. However, the binding of *BnMyAPI* to myrosinases in enzyme complexes, coupled with strong tolerance to *Sclerotinia* in the

BnMyAPI-OE lines suggest that *BnMyAPI* may play a role in *Sclerotinia* tolerance through regulating indole-GS levels/patterns. One study showed one of the TU mutants with altered glucosinolate patterns, TU3 line had a lower infection rate than the wild type to clubroot disease caused by the obligate biotrophic fungus *Plasmodiophora brassicae* (Ludwig-Muller et al. 1999).

Testing individual *BnMyAP* gene knockout Salk lines and other *Arabidopsis* lines transformed with remaining members of the *BnMyAP* gene family, plus bimolecular complementation studies to find other interacting proteins, are important to determine the full scope of involvement of the *BnMyAP* family in *Sclerotinia* resistance and other tolerances, such as flea beetle feeding, cold and drought.

Conclusion

ESTs for a *Myrosinase-Associated Protein* (*BnMyAPI*) were highly represented (expressed) in a *Brassica napus* subtractive library developed after leaf damage by the crucifer flea beetle (*Phyllotreta cruciferae*). Expression of this gene was under different developmental control in *B. napus*, and it was co-induced in *B. napus* by flea beetle feeding, *Sclerotinia* infection, drought, and cold. A total of 20 *BnMyAP* genes represented in different *B. napus* stress and development EST libraries indicated larger, diversified families than known earlier. The increased survival after *Sclerotinia* infection in transgenic *Arabidopsis* suggests that *BnMyAPI* plays a role in *Sclerotinia* tolerance through regulating indole-GS levels/patterns. Our transgenic lines and ESTs now provide tools with which to open a door into understanding *BnMyAPs*' functions and relationship. The maintenance of *Sclerotinia* tolerance in overexpression transgenic *Arabidopsis*,

also points to a practical use for this gene and a need for testing its ability to protect *Brassica* oilseed and vegetable from fungus disease.

Author contribution statement

L. Wu, M. Gruber and D. Hegedus designed the research; L. Wu, M. Yu and J. Holowachuk performed the experiments; L. Wu, M. Gruber, D. Hegedus, A. Sharpe and Derek Lydiate wrote the paper.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

References

- Andreasson, E., Taipalensuu, J., Rask, L., and Meijer, J. 1999. Age-dependent wound induction of a *myrosinase-associated protein* from oilseed rape (*Brassica napus*). *Plant Mol. Biol.* 41: 171–180.
- Bailey, L.C., Searls, D.B., and Overton, G.C. 1998. Analysis of EST-driven gene annotation in human genomic sequence. *Genome Res.* 8: 362–376.
- Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735–743.
- Eriksson, S., Andréasson, E., Ekbom, B., Granér, G., Pontoppidan, B., Taipalensuu, J., Zhang, J., Rask, L. and Meijer, J. 2002. Complex formation of myrosinase isoenzymes in oilseed rape seeds are dependent on the presence of myrosinase-binding proteins. *Plant Physiol.* 129: 1592–1599.
- Fahey, J.W., Zalcman, A.T. and Talalay, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry.* 56: 5–51.
- Falk, A., Taipalensuu, J., Ek, B. and Rask, L. 1995. Characterisation of rapeseed myrosinase-binding protein. *Planta.* 195: 387–395.
- Feeney, P., Pauwwe, K.L. and Demong, N.J. 1970. Flea beetles and mustard oils: host plant specificity of *Phyllotreta cruciferae* and *P. striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 63: 832–841.

- Giamoustaris, A. and Mithen, R. 1995. The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus ssp. oleifera*) on its interaction with specialist and generalist pests. *Ann. Appl. Biol.* 126: 347-363.
- Giamoustaris, A. and Mithen, R. 1997. Glucosinolates and disease resistance in oilseed rape (*Brassica napus ssp. oleifera*). *Plant Pathol.* 46: 271-275.
- Gruber, G., Wu, L., Links, M., Gjetvaj, B., Durkin, J., Lydiate, D. and Hegedus, D. 2012. Analysis of expressed sequence tags in *Brassica napus* cotyledons damaged by crucifer flea beetle feeding. *Genome.* 55: 118-133.
- Gruber, M.Y., Xu, N., Grenkow, L.F., Onyilagha, J., Soroka, J.J., Westcott, N.D. and Hegedus, D.D. 2009. Responses of the crucifer flea beetle to brassica volatiles in an olfactometer, *Environ. Entomol.* 38: 1467-1479.
- Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F. and Zhang, J.Z. 2002. Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. *Plant Physiol.* 130: 639-648.
- Hallett, R.H., Ray, H., Holowachuk, J., Soroka, J.J. and Gruber, M.Y. 2005. Bioassay for assessing resistance of *Arabidopsis thaliana* L. (Heynh.) to the adult crucifer flea beetle, *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae). *Can. J. Plant Sci.* 85: 225-235.
- Härtel, F.V. and Brandt, A. 2002. Characterization of a *Brassica napus* myrosinase expressed and secreted by *Pichia pastoris*. *Protein Expres. Purif.* 24: 221-226.
- Klimyuk, V.I., Carroll, B.J., Thomas, C.M. and Jones, J.D.G. 1993. Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *Plant J.* 3: 493-494.

- Kuchernig, J.C., Burow, M. and Wittstock, U. 2012. Evolution of specifier proteins in glucosinolate-containing plants. *Evol. Biol.* 12: 127.
- Lamb, R.J. 1984. Effects of flea beetles, *Phyllotreta* spp. (Chrysomelidae: Coleoptera), on the survival, growth, seed yield and quality of canola, rape and yellow mustard. *Can. Entomol.* 116: 269–280.
- Lenman, M., Rödin, J., Josefsson, L.G. and Rask, L. 1990. Immunological characterisation of rapeseed myrosinase. *Eur. J. Biochem.* 194: 747–753.
- Louda, S. and Mole, S. 1991. Glucosinolates: Chemistry and ecology. In Rosenthal GA & Berenbaum MR, eds. *Herbivores, their interaction with secondary plant metabolites.* Academic Press, San Diego CA, vol. 1, 2nd edn. pp. 123-164.
- Ludwig-Müller, J., Pieper, K., Ruppel, M., Cohen, J.D., Epstein, E., Kiddle, G. and Bennett, R. 1999. Indole glucosinolate and auxin biosynthesis in *Arabidopsis thaliana* (L.) Heynh. glucosinolate mutants and the development of clubroot disease. *Planta.* 208:409-419.
- Manici, L.M., Lazzeri, L. and Palmeri, S. 1997. In vitro fungitoxic activity of some glucosinolates and their enzyme-derived products towards plant pathogenic fungi. *J. Agric. Food Chem.* 45: 2768–2773.
- Mithen, R. 1992. Leaf glucosinolate profiles and their relationship to pest and disease resistance in oilseed rape. *Euphytica.* 63: 71 -83.
- Moreno-Hagelsieb, G. and Latimer, K. 2008. Choosing BLAST options for better detection of orthologs as reciprocal best hits. *Bioinformatics.* 24: 319-324.

- Palaniswamy, P., Lamb, R.J. and McVetty, P.B.E. 1992. Screening for antixenosis resistance to flea beetles, *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae) in rapeseed and related crucifers. *Can. Entomol.* 124: 895-906.
- Parkin, I., Gulden, S.M., Sharpe, A.G., Lukens, L., Trick, M., Osborn, T.C. and Lydiate, D.J. 2005. Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. *Genetics*. 171: 765-781.
- Pedras, M.S.C. and Ahiahonu, P.W.K. 2004. Phytotoxin production and phytoalexin elicitation by the phytopathogenic fungus *Sclerotinia sclerotiorum*. *J. Chem. Ecol.* 30: 2163-2179.
- Pivnik, K.A., Lamb, R.J. and Darwin, R. 1992. Response of flea beetles, *Phyllotreta* spp., to mustard oils and nitriles in field trapping experiments. *J. Chem. Ecol.* 18: 863-873.
- Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. and Meijer, J. 2000. Myrosinase: gene family evolution and herbivory defense in *Brassicaceae*. *Plant Mol. Biol.* 42: 93-113.
- Reichelt, M., Brown, P.D., Schneider, B., Oldham, N.J., Stauber, E., Tokuhisa, J., Kliebenstein, D.J., Mitchell-Olds, T. and Gershenzon, J. 2002. Benzoic acid glucosinolate esters and other glucosinolates from *Arabidopsis thaliana*. *Phytochemistry*. 59: 663-671.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular cloning: A laboratory Manual*. II edn. Cold Spring Harbour Laboratory. Press, Cold Spring Harbour, N.Y.
- SAS Institute, Inc. 2001. *The SAS system*. Version 9.0. SAS Institute, Inc., Cary, NC.

- Taipalensuu, J., Andréasson, E., Eriksson, S. and Rask, L. 1997. Regulation of the wound-induced *myrosinase-associated protein* transcript in *Brassica napus* plants. Eur. J. Biochem. 247: 963-71.
- Taipalensuu, J., Falk, A. and Rask, L. 1996. A wound- and methyl jasmonate-inducible transcript coding for a *myrosinase-associated protein* with similarities to an early nodulin. Plant Physiol. 110: 483-491.
- Thangstad, O.P., Winge, P., Husbye, H. and Bones, A. 1993. The myrosinase (thioglucoside glucohydrolase) gene family in *Brassicaceae*. Plant Mol. Biol. 23: 511–524.
- Tookey, H.L. 1973. Crambe thioglucoside glucohydrolase (EC 3:4:3:1): separation of a protein required for epithiobutane formation. Can. J. Biochem. 51: 1654–1660.
- Vogel, J.T., Zarka, D.G., Buskirk, H.A.V., Fowler, S.G. and Thomashow, M.F. 2005. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. Plant J. 41: 195–211.

Figure Legends

Fig. 1 Phylogenetic amino acid analysis of *BnMyAP* gene families and orthologues from *Arabidopsis*, *B. rapa* and *B. oleracea*. A) *MyAP* (myrosinase-associated protein) family members used in these phylogenetic trees are shown in the alignment in Supplementary Figure S1 (in the on-line version). B) Alignment of *BnMyAP1*, *BnMyAP2* and their ancestor orthologues *BrMyAP1*, *BoMyAP1* and *Arabidopsis* orthologues *ATIG54020.2*.

Fig. 2 Representative Northern blot analysis of *BnMyAP1* expression in *Brassica napus* leaves after different stress applications. Bottom Panel: Ethidium bromide stained rRNA shown as a gel loading control. 1) No stress. 2) Crucifer flea beetle feeding. 3) Mechanical wounding. 4) *Sclerotinia* infection. 5) Dehydration. 6) Cold shock 1 h. 7) Cold shock 2 h. 8) Cold shock 7 h. Probe was hybridized with membrane overnight and membrane was exposed to film for 3h.

Fig. 3 Representative Northern blot analysis of *BnMyAP1* in *Brassica napus* cotyledons fed upon by crucifer flea beetles. Bottom panels show ethidium bromide stained rRNA shown as a gel loading control. Probe was hybridized with membrane overnight and membrane was exposed to film for 1h.

Fig. 4 Representative Northern blot analysis of *BnMyAP1* expression in specific *B. napus* tissues and developmental stages. Bottom Panel: Ethidium bromide stained rRNA shown as a gel loading control. 1) 7-day cotyledon, 2) 14-day seedling leaf, 3) 14-day seedling stem, 4) 14-

day seedling root, 5) 8-week fully-expanded adult leaf, 6) 8-week adult stem, 7) fully-opened flower, 8) seed pod 20 days-after-pollination, 9) 8-week adult petiole. The probe was hybridized with membrane overnight and membrane was exposed to film for 3h.

Fig. 5 Representative Northern blot analysis of transgene expression level in seedling leaves of T₂ transgenic *Arabidopsis BnMyAPI*-OE lines. Leaves were taken from 14-day-old plants. Ethidium bromide stained rRNA shown as a gel loading control, and the probe was hybridized with membrane for overnight and membrane was exposed to film for 3h.

Fig. 6 Seedling survival after *Sclerotinia* infection in *Arabidopsis* lines expressing *BnMyAPI*. *Sclerotinia* inocula were sprayed onto 21- day old plants and leaves assessed after 7 days. WT: wild type, WT+PBI121: transgenic *Arabidopsis* plants with empty PBI121 vector. *BnMyAPI*-OE-22, *BnMyAPI*-OE-54 and *BnMyAPI*-OE-60 are three representative *Arabidopsis* transgenic lines expressing the *BnMyAPI* gene. Error bars indicate SE (n=4). ANOVA and a post-hoc *t*-test were conducted, such that the same letter indicates no significant difference of the means at $p < 0.05$.

Supplementary Figure S1 Sequence alignment of putative *myrosinase-associated proteins* from *B. napus*, *B. rapa*, *B. oleracea* and *Arabidopsis*. The sequence alignment was generated using the ClustalW Multiple alignment method in BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

Supplementary Figure S2 Construct of *BnMyAPI*-pBI121. The CDS (ATG to TAG) of *BnMyAPI* gene was constructed on pBI121 binary vector through XbaI and SstI restriction endonuclease sites.

Supplementary Figure S3 PCR confirmation of the transgenes in transgenic *Arabidopsis* plants.

Agarose gel showing PCR amplification of transgenes in three representative transgenic plants. Lane M) 1 kb plus DNA marker; Lane 1) *BnMyAPI-OE-22*; Lane 2) *BnMyAPI-OE-54*; Lane 3) *BnMyAPI-OE-60*; Lane 4) WT.

Supplementary Figure S4 Phylogenetic amino acid analysis of *BnMyAPs* found in this study and other *MyAPs* from previous studies. The phylogenetic tree was generated using the Neighbor phylogenetic tree in BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

Supplementary Table S1: Primers for *BnMyAPI* coding sequences and Northern blotting

Supplementary Table S2 Orthologs of MyAPs among *B. napus*, *B. rapa*, *B. oleracea* and *A. thaliana*

B

Representative Northern blot analysis of *BnMyAP1* expression in *Brassica napus* leaves after different stress applications. Bottom Panel: Ethidium bromide stained rRNA shown as a gel loading control. 1) No stress. 2) Crucifer flea beetle feeding. 3) Mechanical wounding. 4) Sclerotinia infection. 5) Dehydration. 6) Cold shock 1 h. 7) Cold shock 2 h. 8) Cold shock 7 h. Probe was hybridized with membrane overnight and membrane was exposed to film for 3h.

Fig. 2

137x114mm (96 x 96 DPI)

Representative Northern blot analysis of *BnMyAP1* in *Brassica napus* cotyledons fed upon by crucifer flea beetles. Bottom panels show ethidium bromide stained rRNA shown as a gel loading control. Probe was hybridized with membrane overnight and membrane was exposed to film for 1h.

Fig. 3
76x47mm (96 x 96 DPI)

Representative Northern blot analysis of *BnMyAP1* expression in specific *B. napus* tissues and developmental stages. Bottom Panel: Ethidium bromide stained rRNA shown as a gel loading control. 1) 7-day cotyledon, 2) 14-day seedling leaf, 3) 14-day seedling stem, 4) 14-day seedling root, 5) 8-week fully-expanded adult leaf, 6) 8-week adult stem, 7) fully-opened flower, 8) seed pod 20 days-after-pollination, 9) 8-week adult petiole. The probe was hybridized with membrane overnight and membrane was exposed to film for 3h.

Fig. 4

139x90mm (96 x 96 DPI)

Representative Northern blot analysis of transgene expression level in seedling leaves of T₂ transgenic *Arabidopsis BnMyAP1-OE* lines. Leaves were taken from 14-day-old plants. Ethidium bromide stained rRNA shown as a gel loading control, and the probe was hybridized with membrane for overnight and membrane was exposed to film for 3h.

Fig. 5
176x79mm (96 x 96 DPI)

Seedling survival after *Sclerotinia* infection in *Arabidopsis* lines expressing *BnMyAP1*. *Sclerotinia* inocula was sprayed onto 21- day old plants and leaves assessed after 7 days. WT: wild type, WT+PBI121: transgenic *Arabidopsis* plants with empty PBI121 vector. *BnMyAP1-OE-22*, *BnMyAP1-OE-54* and *BnMyAP1-OE-60* are three representative *Arabidopsis* transgenic lines expressing the *BnMyAP1* gene. Error bars indicate SE (n=4). ANOVA and a post-hoc t-test were conducted, such that the same letter indicates no significant difference of the means at $p < 0.05$.

Fig. 6

162x104mm (150 x 150 DPI)

Table 1. *BnMyAPs* representation in Saskatoon *Brassica napus* EST Libraries

Tissue or Development Stage												
Tissue Source	Apical Meristem	Root	Stem	Flower Bud	Mature Flower Bud	Mature Flower	Very Early Anther	Early Anther	Embryo	Cotyledon	Young Leaf	Senescent Leaf
Individual <i>BnMyAP</i> ESTs	1(1)*	5(2), 6(2), 8(1), 9(1), 11(1), 13(1), 16(5), 17(2), 19(9), 20(1)	ND	3(1), 4(2)	2(1), 4(3)	ND	ND	ND	7(2)	2(2)	ND	ND
Total ESTs	4844	11250	2798	6014	3051	6711	3680	3263	5498	3838	4763	1055
<i>BnMyAP</i> Representation	0.02%	0.22%	0.00%	0.05%	0.13%	0.00%	0.00%	0.00%	0.04%	0.05%	0.00%	0.00%
Stress												
Tissue Source	Etiolated seedling (vector 1)	Etiolated seedling (vector 2)	Cold-acclim. leaf (dark)	Cold-acclim. Leaf (light)	Drought (Root)	Drought (Leaf)	Mechanical-wound leaf Subtraction	Flea beetle-damaged leaf Subtraction	Flea beetle-damaged cotyledon	Sclerotinia-infected Stem		
Individual <i>BnMyAP</i> ESTs	1(4), 2(1), 3(1), 8(1), 12(1)	1(1), 2(1), 4(1), 6(1), 10(1), 14(1), 15(2)	ND	ND	4(1), 5(2), 6(2), 11(1), 18 (1), 19(1), 20(1)	1(2), 4(1), 7(1)	1(4), 2(3)	1(8), 2(1)	1(1)	1(2)		
Total ESTs	5551	5116	6012	7914	6577	5941	933	1292	3762	1106		
MyAP Representation	0.14%	0.16%	0.00%	0.00%	0.14%	0.07%	0.75%	0.70%	0.03%	0.18%		

*Unbracketed numbers 1 through 20 indicates 20 unique genes (out of 85 ESTs in total for all the libraries). Bracketed number () shows the frequency of each specific *BnMyAP* within each EST library (<http://brassica.ca> and <http://brassicagenomics.ca>). The coloured highlights indicate the *BnMyAPs* from the flea beetle damaged leaf subtraction library. ND, not detected.