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ORIGINAL PAPER

Identification of putative quantitative trait loci associated with a flavonoid related to resistance to cabbage seedpod weevil (*Ceutorhynchus obstrictus*) in canola derived from an intergeneric cross, *Sinapis alba* × *Brassica napus*

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Abstract
Key message **Kaempferol 3-*O*-sinapoyl-sophoroside 7-*O*-glucoside was putatively identified as the major component of a characteristic HPLC peak previously correlated with the reduction of cabbage seedpod weevil larval infestation in a novel canola genotype.**
Abstract The cabbage seedpod weevil (*Ceutorhynchus obstrictus* [Marsham]) (CSPW) is a serious pest of brassicaceous oilseed crops such as canola in both Europe and more recently in North America. At present, the only control strategy against CSPW is the application of insecticides. As an alternative more environmentally-friendly control strategy, we developed novel canola germplasm resistant to weevil attack through introgression of *Sinapis alba* DNA into *Brassica napus* by making the wide cross followed by embryo rescue and backcrossing to the *B. napus* parent. We have previously characterized resistant canola lines by metabolic profiling and were able to correlate reduction of larval infestation to the presence of a characteristic HPLC peak. In this study, we have putatively identified the major component in the peak using mass spectrometry as kaempferol 3-*O*-sinapoyl-sophoroside 7-*O*-glucoside (KSSG). We have also identified quantitative trait loci (QTL) associated with this HPLC peak in a mapping population consisting of more than 200 individual doubled haploid (DH) lines derived from a cross between CSW428 (the resistant parent) and SC030686 (the susceptible parent). This QTL accounted for approximately 9.5 % of the phenotypic variation in KSSG content. The observation that only one QTL was identified as surpassing the LOD threshold of 3.0 suggests that both parents may possess the positive alleles for other QTL that have not been detected in our study. This finding also indicates a complex regulatory mechanism for KSSG levels and provides an appropriate explanation for the large transgressive segregation observed in the DH lines of the QTL mapping population.

Abbreviations

CSPW	Cabbage seedpod weevil
KSSG	Kaempferol 3-*O*-sinapoyl-sophoroside 7-*O*-glucoside
DH	Doubled haploid
QTL	Quantitative trait loci

Introduction

The cabbage seedpod weevil (*Ceutorhynchus obstrictus* [Marsham]) is a serious pest of canola (*Brassica napus* L.) in Europe and North America. It has been reported that cabbage seedpod weevil (CSPW) infestation of canola has resulted in economic losses on the average between 20–40 %; however, up to 70 % decrease of the potential yield has been documented (Weisse et al. 2009). Canola is the most valuable field crop in Canada, adding $14 billion annually to Canada's economy (Canola Council of Canada 2012).

CSPW infestation can cause losses in seed yield in several ways. Overwintering adults emerge from the ground in spring and feed on developing flower buds reducing potential yield. In the early summer, larvae can feed on immature

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seeds within developing pods. Exit holes produced by larvae can also lead to secondary fungal infections. The second-generation adults which emerge in late summer damage the immature pods of late-seeded canola before overwintering (Dosdall et al. 2001).

All host plants of the CSPW belong to the Brassicaceae. While *B. napus* and *B. rapa* are susceptible to CSPW infestation, *Sinapis alba* (white or yellow mustard) has been shown to sustain fewer CSPW larvae and is more resistant to direct damage associated with CSPW larvae (Kalischuk and Dosdall 2004). *S. alba* can, therefore, potentially be a source of resistance genes that can be transferred to elite canola varieties to create a weevil-resistant canola.

At present, the application of insecticides is the only control strategy against CSPW. As an alternative more environmentally sustainable strategy, we have developed novel canola germplasm resistant to weevil attack through introgression of *S. alba* DNA to *B. napus*. This difficult intergeneric cross was achieved using embryo rescue and the resulting progeny was backcrossed to the *B. napus* parent for three generations and subsequent doubled haploid (DH) lines were produced (Dosdall and Kott 2006). Larval exit hole counts in duplicated field trials in Alberta and Ontario confirmed that the *B. napus* × *S. alba* hybrid genotypes inherited resistance to CSPW (Dosdall and Kott 2006; Shaw et al. 2009).

The molecular basis contributing to this resistance to CSPW remains unknown. There is much evidence suggesting that plants resist insect herbivory by employing both constitutive mechanical defenses (e.g., thorns, trichomes, and cuticles) as well as induced chemical defenses (e.g., production of glucosinolates, phenolics, protease inhibitors, and terpenoid volatiles) to modulate the feeding and oviposition behaviors of insects (Mithoefer and Boland 2012).

While the glucosinolates, a major defense compound against insect attack in Brassicaceae (Textor and Gershenzon 2009) have been relatively well studied, the mechanism of action at a molecular level for many of the other classes of defense compounds such as the flavonoids remains unclear. Flavonoids and their conjugates make up one of the largest groups of plant secondary metabolites, which has been implicated to be involved in many aspects of plant growth and development, such as acting as attractants to pollinators, and in plant responses to various biotic and abiotic stress conditions such as disease, nutrient deficiency, wounding, cold, UV, and herbivory (Cartea et al. 2011). Various flavonoids, especially quercetin and kaempferol derivatives, have been shown to play a role in both the feeding and oviposition behavior of insects (Simmonds 2001, 2003). However, as flavonoids are rarely found in the aglycone form in plants but more commonly exist as complex *O*-glycosides acylated by various hydroxycinnamic acids (Cartea et al. 2011), the relationship between flavonoid structure and function has not been well studied. There is some evidence that methoxylation and composition of sugar residues can influence insect feeding activity (Simmonds 2001).

In recent years, there has been an increasing interest in the use of metabolite composition for phenotyping and for selection in crop improvement (as reviewed by Fernie and Schauer 2008; Carreno-Quintero et al. 2012). This interest in metabolomics-assisted breeding is in part due to decreasing costs for analysis, advances in data mining, improvements in instrumentation, and a non-requirement for a sequenced genome.

We have recently reported that biochemical polymorphisms correlated with CSPW resistance in the *B. napus* × *S. alba* hybrid genotypes were found by HPLC profiling (Shaw et al. 2009). In extracts from inflorescence bracts, one polymorphic HPLC peak significantly correlated with larval infestation data. *B. napus* lines with greater amounts of this peak had lower larval exit hole counts. In this present paper, we further analyzed the composition of this peak by liquid chromatography–mass spectrometry (LC–MS) and have evidence that a major component of this peak may be kaempferol 3-*O*-sinapoylsophoroside 7-*O*-glucoside (KSSG). SSR markers linked to the quantitative trait loci (QTL) associated with elevated KSSG levels were identified and should be extremely useful as a first step in understanding the molecular genetic basis of CSPW resistance in *B. napus*. Identification of the secondary metabolite correlated with decreased CSPW infestation may also prove useful as a biomarker for the breeding of insect-resistant varieties of *B. napus*.

Materials and methods

Plant material

A representation of the breeding strategy for the development of the DH mapping population is shown in Fig. 1. Intergeneric hybrids were derived from crosses between *S. alba* and *B. napus* using embryo rescue as described previously (Dosdall and Kott 2006). The F_1 hybrids were then backcrossed (BC) with the *B. napus* recurrent parent for two generations. Six BC2 seeds from one cross were the source of donor plants for bulked pollen used in the DH production of 517 BC2 DH lines. The BC2 DH line CSW428 exhibited superior resistance to CSPW in several field trials for multiple years and was selected for subsequent crossing with the DH breeding line SC030686 to produce the BC3 generation (Dosdall and Kott 2006). Again, six BC3 seeds from one cross were used as the source of donor plants for bulked pollen to ultimately

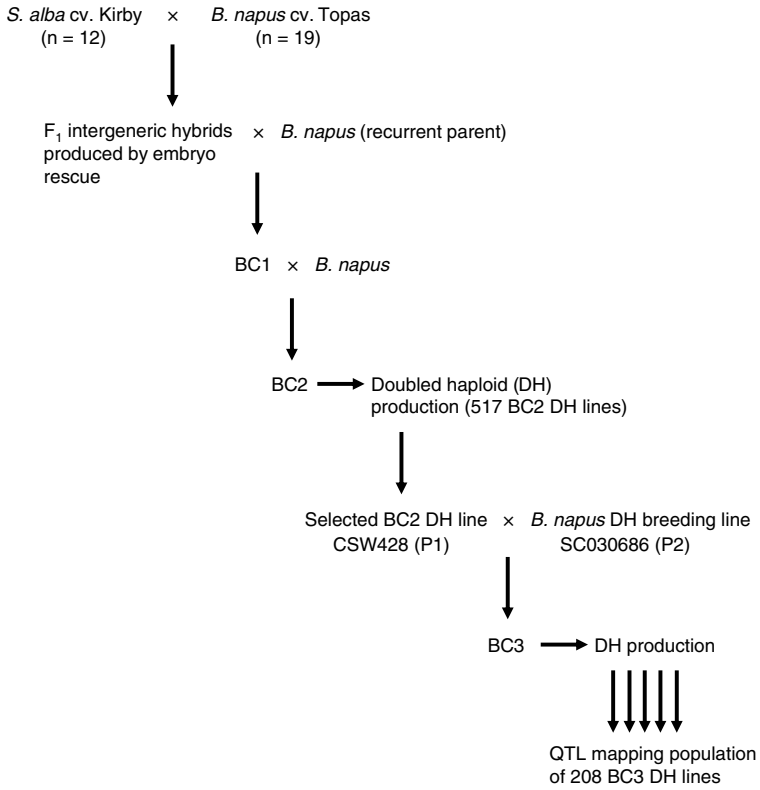

Fig. 1 Schematic representation of the breeding strategy for the development of the DH QTL mapping population. CSW428 is the CSPW-resistant parent and SC030686 is the CSPW-susceptible parent used in the cross to generate the QTL mapping population at the BC3 DH generation

generate 208 BC3 DH lines making up the QTL mapping population. This QTL mapping population along with the parental lines and checks (*B. napus* cultivars Q2, Hurricane, Avalanche, 45A65, and *S. alba*) were grown under controlled conditions in the growth room with 200 µmol $s^{-1}m^{-2}$ light source and with 22 °C day/18 °C night cycles (18-h photoperiod) in LA4 Professional Growing Mix and fertilized with 0.2 g/L All Purpose 20:20:20 fertilizer (Plant Products Co. Ltd., Brampton, Canada).

HPLC metabolite profiling

For HPLC analysis, the upper two inflorescence bracts were harvested from plants maintained in the growth room with 2–5 open flowers and dried in an oven at 105 °C for 45 min. Inflorescence bracts were used because they may be the source of olfactory signals that attract or repel weevils (Shaw et al. 2009). In addition, bracts are easier to stage for breeding purposes. The tissue was crushed to a powder and stored at –20 °C until use. For the preparation of extract, 50 mg of bract tissue powder was suspended in 1 ml of water, incubated for 7 min at 99 °C, and particulates were sedimented using a microcentrifuge. The clarified supernatant was collected and used directly for HPLC analysis. HPLC conditions were based on the protocol developed by Lee et al. (2006) with modifications as described by Shaw et al. (2009). Mean peak height were calculated from a minimum of two HPLC runs.

LC–MS

LC–MS analyses were performed on a Dionex UHPLC UltiMate 3000 liquid chromatography interfaced to an amaZon SL ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) at the Advanced Analysis Centre, University of Guelph. A C18 column (Phenomenex Kinetex 2.6 micron particle size, 150 mm × 4.6 mm) was used for chromatographic separation. For LC–MS analysis, the flow rate was reduced from 1 ml/min to 0.4 ml/min. As a consequence, to maintain the integrity of the separation, the timings of each segment of the gradient were adjusted using an online method transfer calculator (http://www.sigmaaldrich.com/analytical-chromatography/hplc/method-transfer-calculator.html). At the lower flow rate, the mobile phase consisted of a mixture of 30 mM ammonium acetate adjusted to pH 5 with 95 % formic acid (solvent A) and 100 % methanol (solvent B) with 0 % B at 0–12.5 min, a linear gradient reaching 70 % B at 62.5 min, 100 % B at 62.75–77 min, and returning to 0 % B at 78 min. The mass spectrometer electrospray capillary voltage was maintained at 4.5 kV and the drying temperature at 220 °C with a flow rate of 10 L/min. Nebulizer pressure was 40 psi. Nitrogen was used as both nebulizing and

drying gas and helium was used as collision gas at 60 psi. The mass-to-charge ratio was scanned across the m/z range 70–1,200 in enhanced resolution negative and positive ion auto MS/MS mode. The Smart Parameter Setting was used to automatically optimize the trap drive level for precursor ions. The instrument was externally calibrated with the ESI TuneMix (Agilent).

Direct infusion MS

Multistage MS was also performed using direct infusion of extract into the ESI source at the rate of 4 μL/min. The mass spectrometer was set for negative ion mode scanning at speed 9,000 m/z/s. The capillary voltage and nitrogen gas temperature and flow rate were set as for LC–MS run. After recording of the MS spectra, manual MS^n mode was chosen to fragment MS^2, MS^3, MS^4, and if intensity was enough up to MS^6 spectra. A 2 amu isolation and 50–100 % relative collision energy fragmentation of the most first 2–3 intense ions from the MS^{n-1} spectrum was chosen. At least 10 scans for each fragmentation were collected. The 10 data points were averaged using a three consecutive rolling average method.

Molecular markers analysis

Public primer pair sequences for 661 simple sequence repeat (SSR) markers were obtained from the Multinational Brassica Genome Project (http://www.brassica.info) and included microsatellites from the Biotechnology and Biological Sciences Research Council (BBSRC) Microsatellite Programme (Lowe et al. 2004), Bell and Ecker (1994), Horticulture Research International (HRI) (Smith and King 2000), Kresovich et al. (1995), Szewc-McFadden et al. (1996), Lagercrantz et al. (1993), Suwabe et al. (2002, 2003), Uzunova and Ecke (1999), the Agriculture and Agri-Food Canada (AAFC) Consortium, the Celera AgGen *Brassica* Consortium (Piquemal et al. 2005), and FITO (Iniguez-Luy et al. 2009). Primer pairs were synthesized by either Sigma-Aldrich Canada or University of Guelph Laboratory Services (Guelph, ON).

Lyophilized leaf material was used for genomic DNA preparation. Freeze-dried leaves were ground to a powder using stainless steel beads in a FastPrep FP120 homogenizer (Bio101/Savant) at speed 4 for 40 s. Genomic DNA was extracted from 20 mg of tissue powder using a commercially available kit (GenElute Plant Genomic DNA Miniprep Kit, Sigma-Aldrich). DNA was quantitated using a NanoDrop ND-1000 spectrophotomer (NanoDrop Technologies). In general, PCR was performed with 30 ng genomic DNA, 4 % trehalose, 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq DNA polymerase (JumpStart Taq DNA polymerase, Sigma), and 5 pmol each of both forward and reverse primers in a total reaction volume of 15 μl. PCR was performed at 95 °C for 5 min, followed by 35 cycles of 95 °C for 60 s, 53 °C or 49 °C for 90 s, and 72 °C for 90 s and a final extension of 72 °C for 10 min. PCR products were separated by high-resolution agarose (A4718, Sigma) gel electrophoresis.

For bulked segregant analysis, equal amounts of genomic DNA from the 15 lines in the QTL mapping population with the highest peak were pooled. Similarly, DNA from the 15 lines with the lowest peak was pooled. Each pool, or bulk, was screened with SSR markers polymorphic for the parental lines.

SAS 9.3 and MapQTL6 programs were used for statistical and QTL analysis, respectively.

Results

Development of the QTL mapping population

Canola line CSW428, a DH line derived by crossing *S. alba* × *B. napus* and backcrossing the resulting progeny to *B. napus* for a number of generations (Fig. 1), was chosen as the CSPW-resistant parent to generate the QTL mapping population. Field assessments to validate the resistance of CSW428 to CSPW were performed between 2001 and 2005 and at different locations (data not shown and Dosdall and Kott 2006). When compared with *B. napus* cv. Q2, a variety known to be susceptible to CSPW infestation, CSW428 consistently exhibited significant less damage. Previous crosses made between CSW428 and the CSPW-susceptible DH breeding line SC030686 produced many DH progeny which demonstrated a greater resistance to weevil damage than Q2 in field trials (data not shown).

Metabolite profiling by HPLC

HPLC metabolite profiling was performed on parental lines and the DH lines from the mapping population. Peak heights for the peak previously identified to be significantly correlated with resistance to larval infestation were determined for all 208 lines in the QTL mapping population as previously described (Shaw et al. 2009). The population mean was 8.62 units with a minimum of 4.49 and a maximum of 13.17. The frequency distribution of mean peak heights from duplicate HPLC profiles of the 208 DH lines of the QTL population with parental checks is shown in Fig. 2. Eighty-seven lines (41.8 %) had peaks higher than that found for CSW428 while 42 lines (20.2 %) had a lower peaks than that found for SC030686.

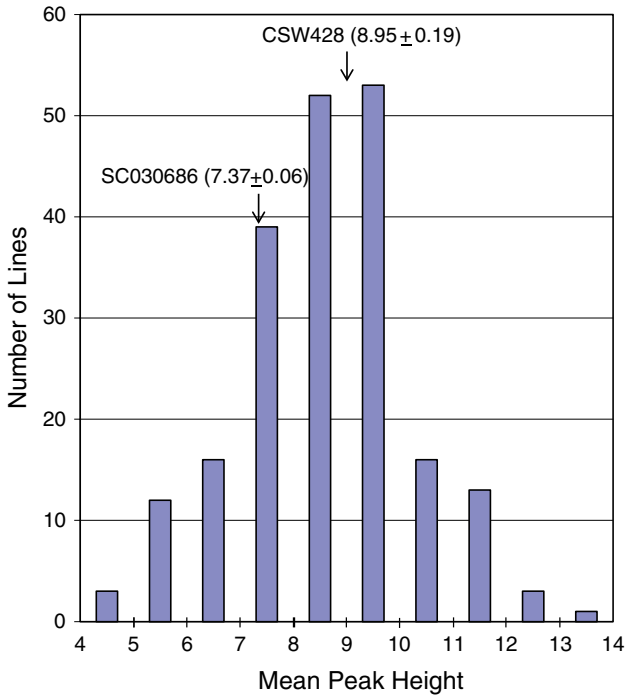

Fig. 2 Frequency distribution of mean peak height for the HPLC peak found to be positively correlated with CSPW resistance for the 208 DH lines making up the QTL mapping population derived from a cross of CSW428 and SC030686. Mean peak heights ± SD for the parental *check lines* are indicated

Analysis of ions present in the HPLC peak correlated with CSPW resistance

For LC–MS, the peak previously found to be correlated with insect resistance (Shaw et al. 2009) had a retention time of 47.7 min and a representative HPLC chromatogram is shown in Fig. 3 for line 239 of the QTL mapping population which exhibits a high peak height value. MS spectra in the negative ion mode revealed that the ions with m/z ratios of 977.5 and 488.3 were highly abundant in this peak (Fig. 4a). LC–MS analysis was carried out for extracts from ten different canola lines and similar results were obtained. Analysis of the isotopic clusters for these two compounds suggests that the compound at m/z 488.3 is the doubly charged form of the compound at m/z 977.5. The isotope cluster of the compound at m/z 488.3 show peaks that differ by about 0.5 mass units whereas the m/z difference of the isotopic peaks of the compound at m/z 977.5 is approximately 1 mass unit (Fig. 4b, c). The single-ion chromatogram shows that the bulk of M/Z 977.5 ions had a very similar retention time as that of the HPLC peak (Fig. 3b). To confirm that the reduced flow rate used for MS analysis did not significantly alter the separation, fractions were collected from an HPLC separation using the higher flow rate and subjected to MS by infusion. Again, ions with M/Z ratios of 977.3 and 488.2

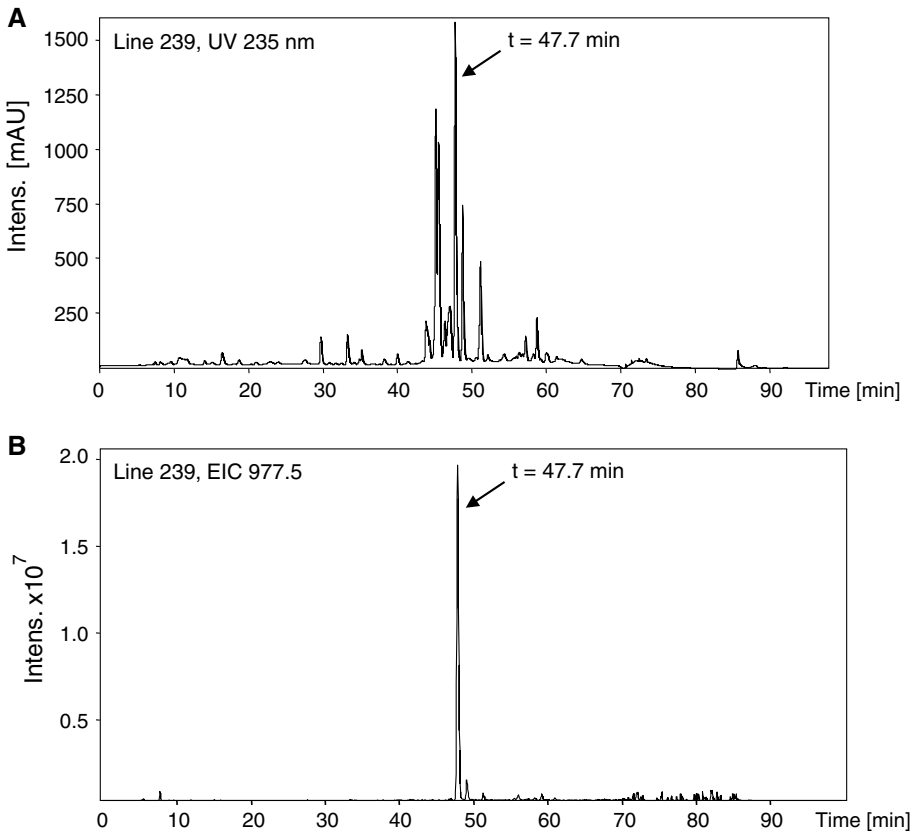

Fig. 3 HPLC chromatograms from LC–MS analysis. A representative HPLC UV chromatograms monitored at 235 nm is shown for an extract from canola line 239 of the QTL mapping population (**a**). The peak found to be positively correlated with CSPW resistance (*arrow*) has a retention time of 47.7 min. The extracted ion chromatogram for m/z 977.5 for line 239 is shown in (**b**) and indicates that the majority of m/z 977.5 ions appear with the same retention time as the HPLC peak

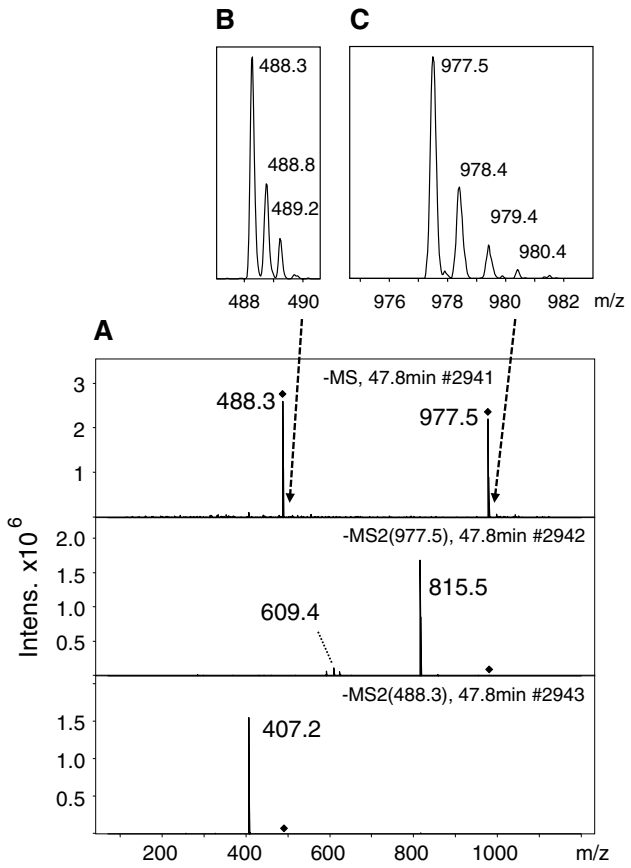

Fig. 4 MS analysis of the HPLC peak found to be positively correlated with CSPW resistance for canola line 239 from the QTL mapping population (**a**). The MS spectrum indicates that two major ions are present. Magnified views of isotope cluster for the two ions (**b** and **c**) show that m/z 488.3 is the doubly charged form of m/z 977.5

were found in the fraction containing the peak of interest (data not shown).

The MS^2 scan of the compound at m/z 977.5 produced fragments with m/z ratios of 815.5 and 609.4 (Fig. 4a). MS^2 fragmentation of the doubly charged compound at m/z 488.3 resulted in the appearance of m/z 407.3 which again appeared to be the doubly charged form of the compound at m/z 815.5 (Fig. 4a). The MS^3 product ions of m/z 815.5 included major fragments at m/z 623.3, 609.3, 591.3, 429.2, 299.2, 285.2, and 284.1 (Fig. 5b).

Based on the interpretation of the mass spectra and searches of metabolic databases, the compound present in the peak is tentatively identified as kaempferol 3-*O*-sinapoyl-sophoroside 7-*O*-glucoside (KSSG). Database searches of KSSG also resulted in two alternative structures with the sinapoyl moiety attached to either the proximal (see the Human Metabolome Database http://www.hmdb.ca/metabolites/HMDB29267) or the distal (Fig. 5a, see the Arita Lab database http://metabolomics.jp/wiki/FL5FAAGL0097) sugar of the sophorose disaccharide.

QTL associated with the HPLC peak correlated with CSPW resistance

The parental lines were first screened with all 661 SSR markers for polymorphism. From this first initial screen, 152 markers were selected for use in the bulked segregant analysis. In the end, five SSR markers (Na10-D09, sN11516, Ol10-B02, A48350, and Ra2-G08) were found to be polymorphic between the bulks for the variation in the height of the peak previously found to be correlated with CSPW resistance. Only one QTL (LOD = 4.6) was detected. This QTL was linked to SSR marker Ra2-G08 found on linkage group N7 (Piquemal et al. 2005) and accounted for 9.5 % of the total phenotypic variation for the trait. The other four other markers produced LOD values below two and were not further considered in this study.

Discussion

As *S. alba* was previously shown to be resistant to CSPW (Kalischuk and Dosdall 2004), it was hoped that this important trait could be transferred to *B. napus* by performing a wide cross. In early-generation intergeneric DH lines derived by crossing *S. alba* × *B. napus*, genomic slot-blot hybridization showed that between 0.28 and 0.42 % *S. alba* DNA was indeed present in these hybrids (Dosdall et al. 2000). These hybrid DH lines also consistently exhibited significant resistance to CSPW damage over a number of field seasons (Dosdall and Kott 2006). The BC2 DH line CSW428 developed from this wide cross was selected as the CSPW-resistant parent to generate the QTL mapping population because it exhibited superior resistance to CSPW (Dosdall and Kott 2006). Previous crosses made with CSW428 had resulted in DH progeny which also demonstrated a greater resistance to weevil damage (data not shown). All these results support the hypothesis that *B.napus* can inherit resistance to CSPW from *S. alba*. In hopes of elucidating the nature of this resistance, metabolite profiling and QTL analysis were performed in this present study.

Metabolic profiling

The HPLC peak previously identified to be a possible biochemical marker for resistance to CSPW (Shaw et al. 2009) appears to contain a compound with a mass of 978 which negatively ionizes to give both a singly (m/z 977.5) and a doubly (m/z 488.3) charged species (Fig. 4a). Subsequent fragmentation patterns of this compound suggest that it is the flavonoid glycoside kaempferol 3-*O*-sinapoyl-sophoroside 7-*O*-glucoside (KSSG) based on MS data

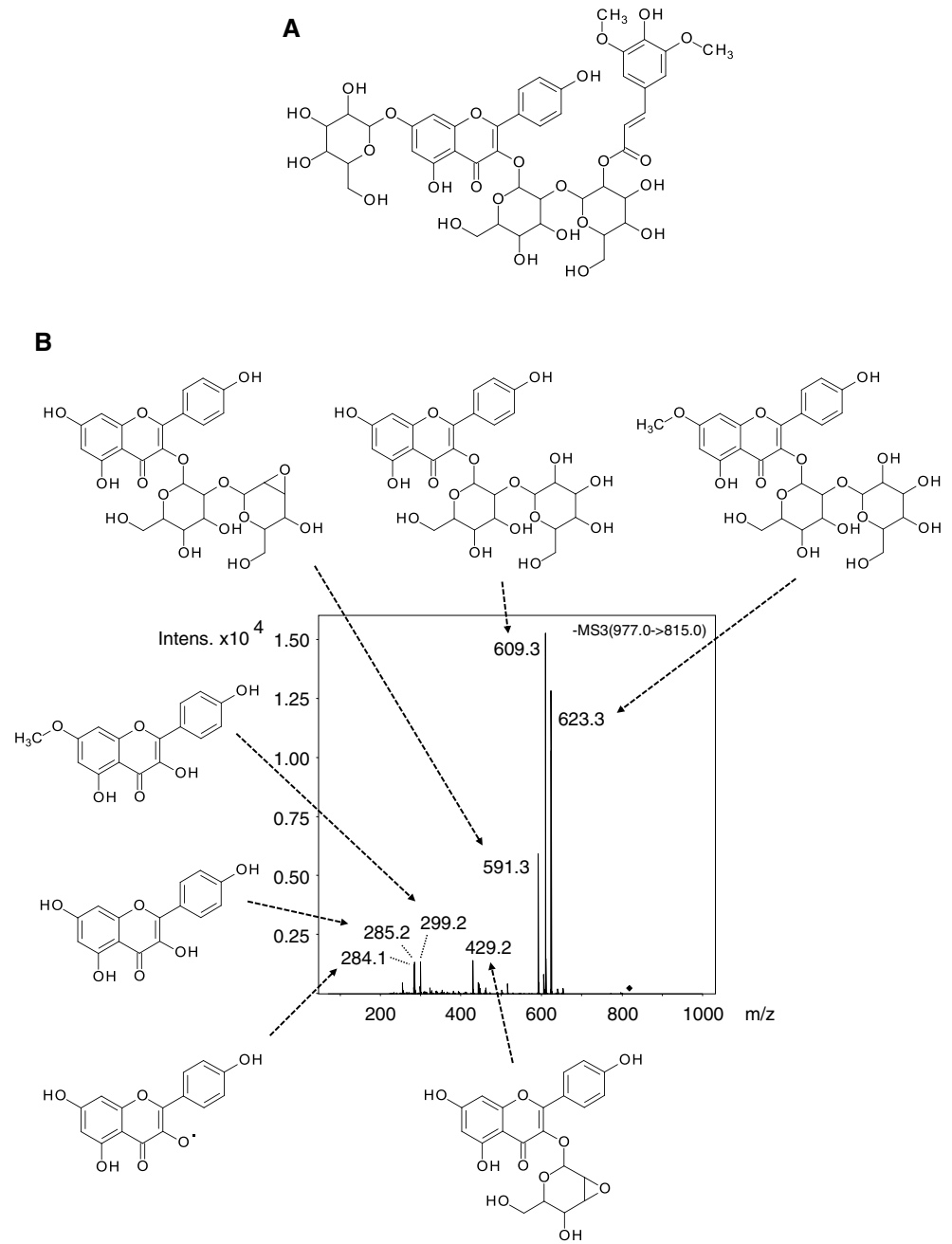

Fig. 5 A proposed structure for kaempferol 3-*O*-sinapoyl-sophoroside 7-*O*-glucoside (KSSG) from the Arita laboratory Metabolomics database (http://metabolomics.jp/wiki/Main_Page) is shown in (**a**). The MS^3 spectrum for KSSG and proposed structures for the major peaks is shown in (**b**)

as previously described in the literature (Velasco et al. 2010). MS^2 fragmentation of m/z 977.5 resulted in a m/z 815.5 fragment which is likely kaempferol 3-*O*-sinapoyl-sophoroside resulting from the loss of the 7-*O* hexose from KSSG (Fig. 4a). The loss of glycosidic residues at position seven of the flavonoid molecule appears to always be more favored over glycosidic residues at position three (Tomas-Barberan and Ferreres 2012). The further loss of the sinapoyl moiety would be expected to produce a kaempferol 3-*O*-sophoroside with an m/z of 609.4 (Fig 4a). The MS^2 spectra of the doubly charged m/z 488.3 give an ion of m/z 407.2 which is the doubly charged form of m/z 815.5 (Fig. 4a).

The MS^3 product ions of m/z 815.5 included fragments of m/z 609.3 and m/z 285.2 in agreement with data previously reported by Velasco et al. (2010) and likely represent

the characteristic kaempferol diglucoside and aglycone kaempferol ions, respectively (Fig. 5). We also observed a number of additional major fragments in the MS^3 spectra (Fig. 5) which have been tentatively assigned structures based on fragmentation patterns reported for other flavonoid glycosides. The m/z 623.3 ion likely is 7-methoxy-kaempferol diglucoside resulting from the $^{0,1}X^-$ fragmentation of the 7-*O* glycosidic residue of the parent compound (March et al. 2006) and further loss of all sugar moieties would result in the aglycone 7-methoxy-kaempferol ion with an m/z of 299.2. Loss of water from kaempferol diglucoside and kaempferol monoglucoside would result in the formation of m/z 591.3 and m/z 429.2 ions, respectively. It has also been reported that in MS/MS with high collision energies, the formation of aglycone flavonol radical ions is commonly observed (Hvattum and Ekeberg 2003; Cuyckens and Claeys 2005). The m/z 284.1 ion is likely the kaempferol radical anion. As all the major ions seen in the MS^3 spectra can be formed as a result of KSSG fragmentation, this strongly suggests that the structure of the compound giving rise to the HPLC peak is indeed KSSG. As database searches of KSSG suggested two alternative structures for this compound, further work will be necessary to resolve the exact structure of KSSG.

It is not too surprising that a flavonoid compound may be involved CSPW resistance in canola. There is previous evidence to suggest that kaempferol-derived flavonols can affect the feeding and oviposition behavior of insects as reviewed by Simmonds (2001, 2003). More recently, it was found that overexpression of MYB12 in Arabidopsis led to the significant accumulation of quercitins and kaempferol flavonols (Mehrtens et al. 2005) and transgenic tobacco plants overexpressing AtMYB12 exhibited higher levels of insect resistance (Misra et al. 2010). Flavonoids are synthesized from phenylalanine via the phenylpropanoid pathway. In Arabidopsis, the transcription factor MYB12 was found to regulate two genes in this pathway, CHS (chalcone synthase) and FLS (flavonol synthase). These results further support a role for flavonoid biosynthesis in plant resistance to herbivory. While individual flavonoids have demonstrated anti-herbivory activity, there is also evidence that flavonoids and other defensive metabolites work in synergy to exert its effects on increasing insect feeding deterrence in plants (Schwachtje and Baldwin 2008). For example, while individual flavonoid fractions minimally deterred flea beetle feeding, combined flavonoid fractions were found to be more effective in feeding deterrence (Onyilagha et al. 2012). Further work is warranted to show if KSSG alone or in combination with other defensive metabolites is able to deter CSPW feeding in canola.

Both glucosinolate and flavonoid biosynthetic pathways partly share early regulatory events involving jasmonic acid signaling and it is possible that the levels of some glucosinolates will also be increased in CSPW-resistant canola. No obvious large variation of HPLC peaks corresponding to the various glucosinolates have been observed and correlated with CSPW resistance. One reason is that these peaks are less prominent in a HPLC profile and are more difficult to use as biochemical markers. We are in the process of using MS to further search for less abundant metabolites, which may be associated with CSPW resistance. This future work will allow us not only to identify new metabolites that were not apparent in the HPLC profiles, but also to enable the study of the potential co-regulation of glucosinolates and flavonoids.

Major QTLs and transgressive segregation

Analysis of the segregation of alleles for the SSR markers identified only one significant QTL with a LOD score > 3.0 which accounted for only 9.5 % of the variation in KSSG peak levels in HPLC profiles. It is interesting to note that it was the SCO30686 parent that contributed to positive alleles for high KSSG levels. However, with much of the trait still unexplained, it cannot be ruled out that the introgressed DNA from *S. alba* did not possess genes or other elements which result in increased resistance to CSPW. The fact that it is possible that both parents may possess positive alleles for the elevated levels of KSSG provides an appropriate explanation for the large transgressive segregation in both directions observed in the DH lines of the QTL mapping population. Over 40 % of the progeny had greater KSSG peak values than that seen in the CSW428 parental line. The combination of these positive alleles from both parents could result in higher levels of KSSG than is conferred by either parent alone.

The hypothesis that regulation of flavonoid accumulation is a complex trait affected by many small effect alleles is also supported by the fact that only one QTL explaining 9.5 % of the trait was found. While over 60 genes have now been characterized to be involved in flavonoid metabolism, a recent study on Arabidopsis seed flavonoid QTLs found that most loci identified did not co-localize with any known genes in the flavonoid metabolic pathway, further suggesting that the regulation of flavonoid accumulation may involve higher order transcriptional control or other yet uncharacterized limiting step such as transporter activity (Routaboul et al. 2012).

The SSR marker associated with the QTL for KSSG content is located in linkage group N7 of *B. napus*. Since *B. napus* is an allopolyploid species formed by the hybridization of ancestors of *B. rapa* with *B. oleracea*, a high degree of colinearity and sequence conservation has been retained between *B. napus* and *B. rapa* (Jiang et al. 2011). Thus, the *B. rapa* A genome which has been sequenced (Wang et al. 2011) could be used to predict the structure

of the *B. napus* A subgenome. However, we have not yet located any genes that may account for the variation of KSSG levels close to the location of SSR marker Ra2-G08 in the *B. rapa* chromosome A7 which is homologous with the *B. napus* N7. While there is a general conservation of the two genomes at the chromosomal level, genomic rearrangements between the two species do exist. In order to identify potential candidate genes associated with the QTL for KSSG content, it will be necessary to look directly at the *B. napus* genomic sequence when it becomes publically available.

A practical application of genetic markers associated with QTL is trait selection and monitoring in crop breeding. While marker-assisted selection (MAS) usually works well with simple monogenic traits or in cases where dominant genes predominate, use of MAS becomes more difficult in crop species with polyploid genomes such as *B. napus* or with complex polygenic traits involving multiple small-effect genes. Both flavonoid accumulation and insect resistance are likely complex traits as evidenced by the difficulty in finding large effect QTLs in this present work. A larger mapping population size may be required for the detection of weak QTL that may become significant. While the QTL for KSSG content by itself may have limited usefulness for breeding purposes, the use of this genetic marker in combination with metabolite biomarkers may prove to be a better strategy in the selection of complex traits in breeding material. The identification of herbivory-associated metabolites will be extremely useful as indicators (or markers) of insect resistance, which will lead to the reductions in cost and time for the breeding of new insect-resistant canola varieties.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All experiments comply with the current laws of Canada.

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