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Constitutive expression of an endogenous sugar transporter gene *SWEET11* in Indian mustard (*Brassica juncea*) and its effect thereof on mustard aphids

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ABSTRACT

One of the major oil yielding crops Indian mustard [*Brassica juncea* (L.) Czern. & Coss.] is highly susceptible to mustard aphid, a hemipteran sap sucking insect-pest. Leaf-transcriptome of mustard treated with different aphid species as host and non-host revealed variable expression of three sugar transporter genes. One of these transporters *BjSWEET11* was constitutively expressed under a CaMV35S promoter in *B. juncea* through *Agrobacterium*-mediated plant transformation. The transgenic plants after requisite molecular analysis for the presence and expression of the introduced gene were assayed for their deterring effects on the infestation by mustard aphid (*Lipaphis erysimi*). Attenuating effect of the enhanced *BjSWEET11* expression on multiplication and population growth of mustard aphids demonstrated likely involvement of this transporter in endogenous plant defense mechanism.

Key words: Indian mustard, Mustard aphid, Nonhost response, Sugar transporter, SWEET

Indian mustard [*Brassica juncea* (L.) Czern. & Coss.] is one of the most important oilseed crops in India and it occupies the largest acreage among the Brassica group of oilseed crops. The productivity of Brassica oilseeds is severely limited by a number of insect-pests and diseases. Among the major insect-pests, mustard aphid (*Lipaphis erysimi*) may cause average yield loss of 80-97.6% depending on severity of infestation (Patel *et al.* 2004). By specialized feeding mechanism and parthenogenetic mode of reproduction, aphids rapidly colonize the host and cause excessive diversion of phloem sap. Aphids also transmit many viral diseases causing indirect damage to the host plants (Hogenhout *et al.* 2008). Mustard aphid, being a specialist aphid species, feeds exclusively on the rapeseed-mustard species in India (Arora and Dhawan 2013).

Breeding efforts for developing aphid resistant mustard varieties is stalled because of non-availability of resistance source. Thus, crop protection from aphid infestation solely depends on indiscriminate application of systemic insecticides, which are hazardous and, in many instances, led to insecticide-resistance in aphid populations (Gould 1996). Recently, variable level of aphid resistance has been identified among a few wild accessions or *Brassica* coenospecies; however, the genetics of such resistance still remains obscure (Atri *et al.* 2012; Sarkar *et al.* 2016). Progress through transgenic strategy, in developing aphid resistant plant types, is limited due to paucity of effective transgenes (Rani *et al.* 2017; Das *et al.* 2018). Thus, the status quo in this area largely remained confined to attempts towards understanding plant-aphid interaction in order to devise novel strategies of aphid resistance (Bhatia *et al.* 2011). Studies on gene expression with reference to plant-aphid interaction have been carried out in Arabidopsis against peach-potato aphid, *Myzus persicae* or cabbage aphid and *Brevicoryne brassicae* by using microarray of selected defense genes or other genomic resources (Moran *et al.* 2002; Kusnierczyk *et al.* 2008; Jaouannet *et al.* 2015). However, only limited information is available on Brassica-mustard aphid interaction (Koramutla *et al.* 2014).

Aphid species vary in their host range. For example, soybean aphid, *Aphis glycines* specifically lands on soybean grown among the other nonhost plants (Du *et al.* 1994). Similarly, while mustard aphid rapidly infest Indian mustard, cowpea aphid, *A. craccivora* when released on mustard plants fails to multiply and eventually eliminated. Significant amount of studies is available on how the plants respond as a non-host against a pathogen (Gill *et al.* 2015). However, studies on gene expression of nonhost in case

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of plant-aphid interaction is very limited (Jaouannet *et al.* 2015). Recently, a few genes, viz. *bak1, vsp1, AtrbohF* etc. have been shown as the key regulator of nonhost resistance against aphid in Arabidopsis (Prince *et al.* 2014; Jaouannet *et al.* 2015). In general, non-host resistance involve genes related to cellular signalling, ROS homeostasis, secondary metabolites, components of primary metabolism including sucrose flux (Nuernberger and Lipka 2005; Uma and Podile 2014). The SWEETs (Sugars Will Eventually be Exported Transporters) are one of the recently discovered sugar transporter family in plants involved in pathogen virulence (Chen *et al.* 2010). Recently, increased attention has been focused on SWEETs as they are involved in the phloem loading of sugars and thus likely in plant-aphid interaction.However, functional analysis of SWEETs has been mostly confined within model plants only (Chen 2014).

In our study we have identified a set of sugar transporter genes which are differentially activated in *B. juncea* in response to feeding by cowpea aphid *A. craccivora* as non-host response. Subsequently, one of these transporter genes, *SWEET11* has been constitutively expressed under a CaMV35S promoter in *B. juncea* and the transgenic plants have been assayed for their altered host response against mustard aphids.

MATERIALS AND METHODS

The plants of Indian mustard, *Brassica juncea* cv. Varuna were grown and mustard aphids were maintained on these plants as described by Koramutla *et al.* (2014). The cowpea aphids, *Aphis craccivora* was maintained on cowpea seedlings grown under similar conditions as above except the growing temperature set at 24±1°C. Four-week old *B. juncea* plants were individually infested with 100 adult aphids by the two aphid species and were allowed to settle and feed on the plants for 24h. After 24h of infestation, aphids were removed and leaf samples were collected in liquid N_2 and stored at -80°C until further use.

Total RNA was isolated from the collected plant tissues using RNAiso Plus following the manufacturer's instructions. RNA samples were treated with DNaseI. cDNA was synthesized from 2 µg of RNA using PrimeScript^{TM} 1st strand cDNA synthesis kit and diluted 20 times with nuclease free water before use in qPCR. qRT-PCR was performed onStepOne Plus Real-time PCR machine using SYBR green detection chemistry. The qRT-PCR reaction (20 µl) contained 10 µl 2X SYBR Premix ExTaq II, 0.4 µl each of the forward and reverse primer (10 µM), 0.4 µl ROX reference dye, 2 µl diluted cDNA and 6.8 µl nuclease free water. The steps in qPCR were programmed as follows: initial denaturation at 95^{0}C for 30s followed by 40 repeated cycles at 95^{0}C for 10 s, 60^{0}C for 30 s, and 72^{0}C for 30 s. The relative level of gene expression was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). *GAPDH* gene was used as normalizer. The list of primers used in qRT-PCR analysis is provided as Table 1.

In annotated transcriptome data of *B. juncea* in response to different aphid species (unpublished) sugar transporter genes were searched based on reported literature in *Arabidopsis thaliana* (Chen *et al.* 2010; Yamada *et al.* 2016). Consequently, three transcripts encoding *STP1* (*Sugar transport protein 1*), *STP4* (*Sugar transport protein 4*) and *SWEET11* (*Sugars Will Eventually be Exported Transporter 11*) were found differentially expressed in response to different aphid species. Specific primers were designed and their expressions were validated by qRT-PCR analysis.The *SWEET11* gene was PCR amplified from leaf-cDNA *B. juncea* using gene specific primers (Forward 5'-GGGGTACCATGCCTCTCTTCGACACTCAC-3' and Reverse 5'-TTGTCGACTCATGTAGGTGAT-GCGGAAG-3') and cloned into pGEM-T Easy vector (Promega, USA). The insert was validated by restriction digestion and Sanger sequencing.The insert was further taken out by *Kpn*I and*Sal*I digestion and sub-cloned into a binary vector pBINAR linearized with *Kpn*I-*Sal*I. The recombinant pBIN-SWEET11 construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 by freeze thaw method (Weigel and Glazebrook 2006) and used for plant transformation.Transformation of Indian mustard (*B. juncea* cv. Varuna) was performed using the floral spray method as described by Aminedi *et al.* (2019).

In performing molecular analysis genomic DNA was extracted from leaves as described in Edwards *et al.* (1991). The transformants were identified by PCR of genomic DNA using a forward primer BIN35S-F (5′-TGACGCACAATCCCACTATC-3′) targeted to CaMV35S promoter and a gene specific reverse primerq SWEET11-R (5′-GGACAAGCTAAAGGGCATGTA-3′). Gene expression study of *BjSWEET11* as well as glucosinolate biosynthetic genes and other defense-related genes was carried out by qRT-PCR using the sequence specific primers (Table 1).

In insect bioassay, *L. erysimi* nymphs of assorted age were used. The healthy leaves of two months old transgenic *B. juncea* plants were inoculated with five nymphs of *L. erysimi* with the help of a soft paint brush. The aphids were confined on the leaves with the help of clip cages.The total number of aphids was recorded after seven days of insect release. Data on aphid bioassay was collected from at least five replicates for each plant.The data was analyzed using one-way ANOVA, mean separations and significant difference in mean was assessed by Student's t-test (p<0.05). PCR negative plant (Var 55C) was used as control.

RESULTS AND DISCUSSION

Identification of sugar transporter genes

Flux of sugar plays an important role not only in primary metabolism but also in diverse physiological processes including response to biotic and abiotic stresses (Singh *et al.* 2011; Sami *et al.* 2016). It is directly involved in source-sink balances as well sequestration of toxic compounds during stress conditions. Therefore, sugar transporter genes namely, *SWEET11* (*Sugars Will Eventually be Exported Transporter*

Table 1 List of primers used in qRT-PCR analysis

Gene	Description	Primer sequences (5'-3')
STP1	Sugar transport protein 1	F- TGACGATGCTCTGCCATTT R- CTTTCGTCTCCGGCAAGAATA
STP4	Sugar transport protein 4	F- TAGCAAAGCCTCGCTCTTATC R-ACTCTTCTTCCAAACCTATCCAC
SWEET11	Sugars Will Eventually be Exported Transporter 11	F- TCTGTGTCGGATTCTCTGTTTG R- GGACAAGCTAAAGGGCATGTA
MAM1	Methylthioalkylmalate synthase1	F- GGTCGTGATGGCTTTGAAATG R- CTGGCTCCAACTATGGGTTTAT
GSTF11	Glutathione S-transferase F11	F- GACCAAGGAACGGACCTATTG R- CAACGTCGAACTTGGGTGTA
CYP83A1	Cytochrome P450 83A1	F- ACTGAAGACGACGTGAAGAAC R- ATGCAAGCACGAGGGATAAG
CYP83B1	Cytochrome P450 83B1	F- TCCGACCCGTTAGAGAAGAA R- AGTTGGTGAAGGACAAGAGAAG
SUR1	Supperroot 1	F- TTGTCCCTGGATGGAAGATTG R-AGTGGAAGGGTCAGGAGTTA
CYP81F1	Cytochrome P450 81F1	F- CTGGATTAGGGAGGAGGATAGT R- TCTGCACATAGCCCGTAAAG
ABCG36	ABC transporter G family member 36	F- GATTCCTGAGTGGTGGAGATG R- GGCTTGTGCTGTTATCGAATG
CBP	Calmodulin binding protein	F- GAAGGCAAACCTCCGTTACT R- CTAGCACCTAACCGGAACATC
PR1	Pathogensesis-related protein1	F-GGGTTAACGAGAAGGCTAACTATAA R- GCTTTGCCACATCCAATTCTC
WRKY70	WRKY transciption factor 70	F-AGTATCACCCAAGATCAAGCC R- CAAGTCACTCTCAGTGGAAGAA
LOX	Lipoxygenase	F- GAGGTTCGACAAGGAAGGTTTA R- TAGTGCATCCCACAGCATTAG
OPR3	12-Oxophytodienoate reductase 3	F- CAAGGCAGTGATGAGGAAGAA R- CTTGCTGAATGGCTTGCATAC
GAPDH	Glyceraldehyde-3-phospho dehydrogenase	F-TCAGTTGTTGACCTCACGGTT R- CTGTCACCAACGAAGTCAGT

11), *STP1* (*Sugar Transport Protein 1*) and *STP4* (*Sugar Transport Protein 4*) showing variable expression in leaf transcriptome data of *B. juncea* treated independently with *L. erysimi* and *A. craccivora* were identified. The qRT-PCR analysis further validated the similar pattern of expression (Fig 1).

Out of the three above mentioned sugar transporter genes, *SWEET11* showed significant upregulation in case of nonhost response of *B. juncea* to *A. craccivora*. The coding sequence (CDS) of *SWEET11* gene in *B. juncea* was retrieved and named as *BjSWEET11*. Using a pair of gene specific primer, the 858 bp CDS of *BjSWEET11* was cloned by PCR amplification from cDNA of *B. juncea* leaves. Nucleotide BLAST searches reveal edits 99% homology to *Brassica rapa SWEET11-like* (XM_009151930.1), *Brassica napus SWEET11-like* (XM_013841813.1) and *Brassica oleracea SWEET11-like* (XM_013766530.1), followed by

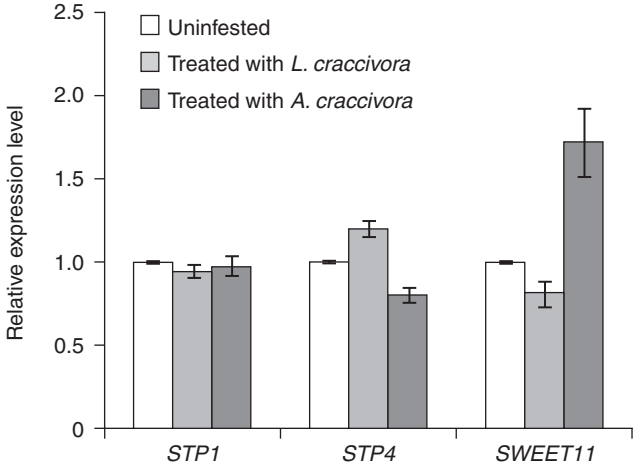

Fig 1 Expression of three sugar transporter genes of *B. juncea* in response to *L. erysimi* and *A. craccivora.*

93% to *Brassica oleracea SWEET11* (XM_013749327.1), *Brassica napus SWEET11* (XM_013871605.1) and *Brassica rapa SWEET11*(XM_009151552.1) and 90% to *Arabidopsis thaliana SWEET11*(NM_114733.3) nucleotide sequences. Similarly, the protein sequence alignment also showed that *BjSWEET11* shared 90% amino acid sequence identity with *A. thaliana* SWEET11(NP_190443.1), 99% with *B. napus* SWEET11-like (XP_013697267.1) and *B. oleracea* SWEET11-like (XP_013621984.1), and 100% with *B. rapa* SWEET11-like (XP_009150178.1) proteins.However, no BLAST hit was found from *B. juncea* indicating that the isolated gene is probably the first report in *B. juncea*. On close sequence inspection it was found that there was a single nucleotide polymorphism at position 126 ('A' in *B. juncea* and 'C' in *B. rapa*) in *BjSWEET11* and *B. rapa SWEET11*-like (XM_009151930.1) but without any change in the amino acid it coded.To assign the Clade in which the isolated BjSWEET11 possibly belongs in the global family of SWEETs, a phylogenetic tree was constructed based on the amino acid sequences of *A. thaliana* SWEET proteins retrieved from the TAIR database (https://www.arabidopsis.org/). The result showed that the isolated gene is closest with the AtSWEET11 and fell in the same Clade, i.e. Clade III (Fig 2). The *A.thaliana* has 17 SWEET proteins, and AtSWEET10 to15 belongs to the Clade III of the AtSWEET family (Chen *et al.* 2010). Clade III SWEETs are involved in export of sucrose and are responsible for the first step in phloem loading (Chen *et al.* 2010). Clade III SWEETs of rice such as OsSWEET11 and 14 are targeted by the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) during host-infection (Chen *et al.* 2010) for activating sugar transport. Mutations in the effector binding sites in SWEET promoters of Clade III SWEETs led to resistance to *Xoo* in a wide spectrum of rice lines (Chen *et al.* 2010). Therefore, SWEETs are the key elements of phloem translocation machinery that the pathogens reprogramme for gaining access to the plant's energy resources at the site of infection.

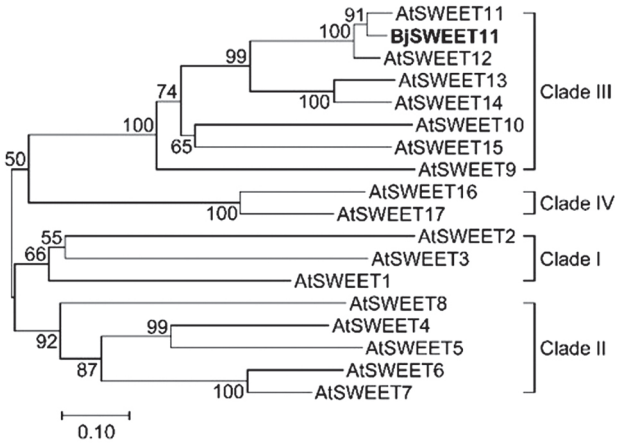

Fig 2 Dendrogram depicting the relationship of BjSWEET11 with AtSWEET family proteins.

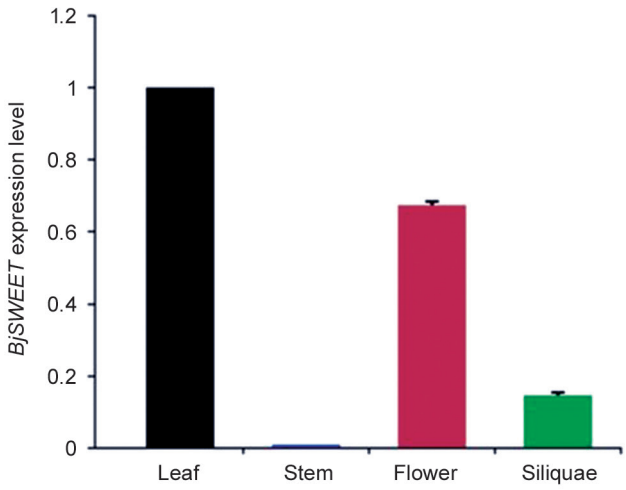

Fig 3 qRT-PCR based analysis of tissue wide gene-expression of *BjSWEET11*.

Protein sequence analysis of BjSWEET11

The protein sequence analysis at ExPaSy (http://www.expasy.org) indicated that the BjSWEET11 transporter protein consists of 285 amino acids, with a predicted isoelectric point (pI) of 9.32 and molecular weight of 31.58kDa. The predicted instability index (II) is 43.08 and thus it is considered as an unstable protein (II >40).The

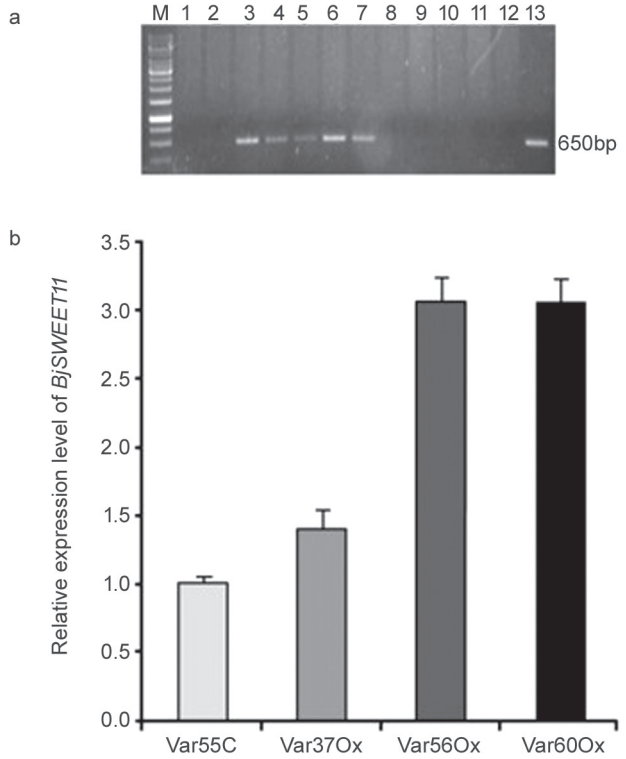

Fig 4 (a) Screening of putative transgenic mustard plants by PCR. Lane M: 1kb DNA ladder; Lane 1: wild-type plant; Lane 2-12: putative transgenic plants; Lane 13: positive control. (b) qRT-PCR based analysis of the transcripts levels of *BjSWEET11* in different transgeniclines of mustard.

calculated aliphatic index of BjSWEET11 is 108.07.The aliphatic index is the relative volume occupied by aliphatic side chains such as alanine, valine, isoleucine and leucine in a protein.The protein grand average of hydropathicity (GRAVY) is 0.501 suggesting that BjSWEET11 is a hydrophobic protein. The total number of negatively (Asp+Glu) and positively (Arg+Lys) charged residues is 16 and 28, respectively. The transmembrane prediction (http://www.cbs.dtu.dk/services/TMHMM/) shows that BjSWEET11 has 7 transmembrane helices at amino acid positions 10-32, 44-63, 73-95, 102-124, 134-153, 166-188and 192-214 which is in agreement with the number of transmembrane helices present in other homologs of SWEET proteins (Chen *et al.* 2010).

Tissue wide gene expression analysis of BjSWEET11

In order to detect tissue specific expression pattern of *BjSWEET11,* its transcript level was assessed across the various tissues of *B. juncea* through qRT-PCR. The results demonstrated that *BjSWEET11* is expressed in tissues such as leaf, stem, flower, and siliquaes at variable level (Fig 3). The highest expression was detected in leaf, followed by flower, siliquae and stem. Similar trend in relative expression was also reported in case of *SWEET* genes of other plants (Chen *et al.* 2010; Yang *et al.* 2018).

Development of transgenic B. juncea over-expressing BjSWEET11 gene

The pBIN-SWEET11 binary construct was used for *Agrobacterium*-mediated transformation of *B. juncea* cv. Varuna using floral spray transformation method (Aminedi *et al.* 2019). The putative transgenic plants were identified by PCR screening using a CaMV35S specific forward primer and a gene specific reverse primer. The amplification of 650 bp amplicon from genomic DNA of the transformed *B. juncea* confirmed their transgenic nature (Fig 4a). No amplification was obtained in case of untransformed *B. juncea* plants. Since the forward primer was specific to CaMV35S promoter it was not expected to bind the endogenous copies of the *SWEET11* gene in the control plants.Three transgenic lines were further used for gene expression study and aphid bioassay.

qRT-PCR based expression analysis of transgenic mustard plants

For qRT-PCR analysis, total RNA isolated from young leaves of transgenic lines as well as PCR negative control plants of *B. juncea* was used. The qRT-PCR results showed variable transcript levels among the transgenic lines (Fig 4b). The highest transcript level was detected in Var 60Ox followed by Var 56Ox and Var 37Ox. The

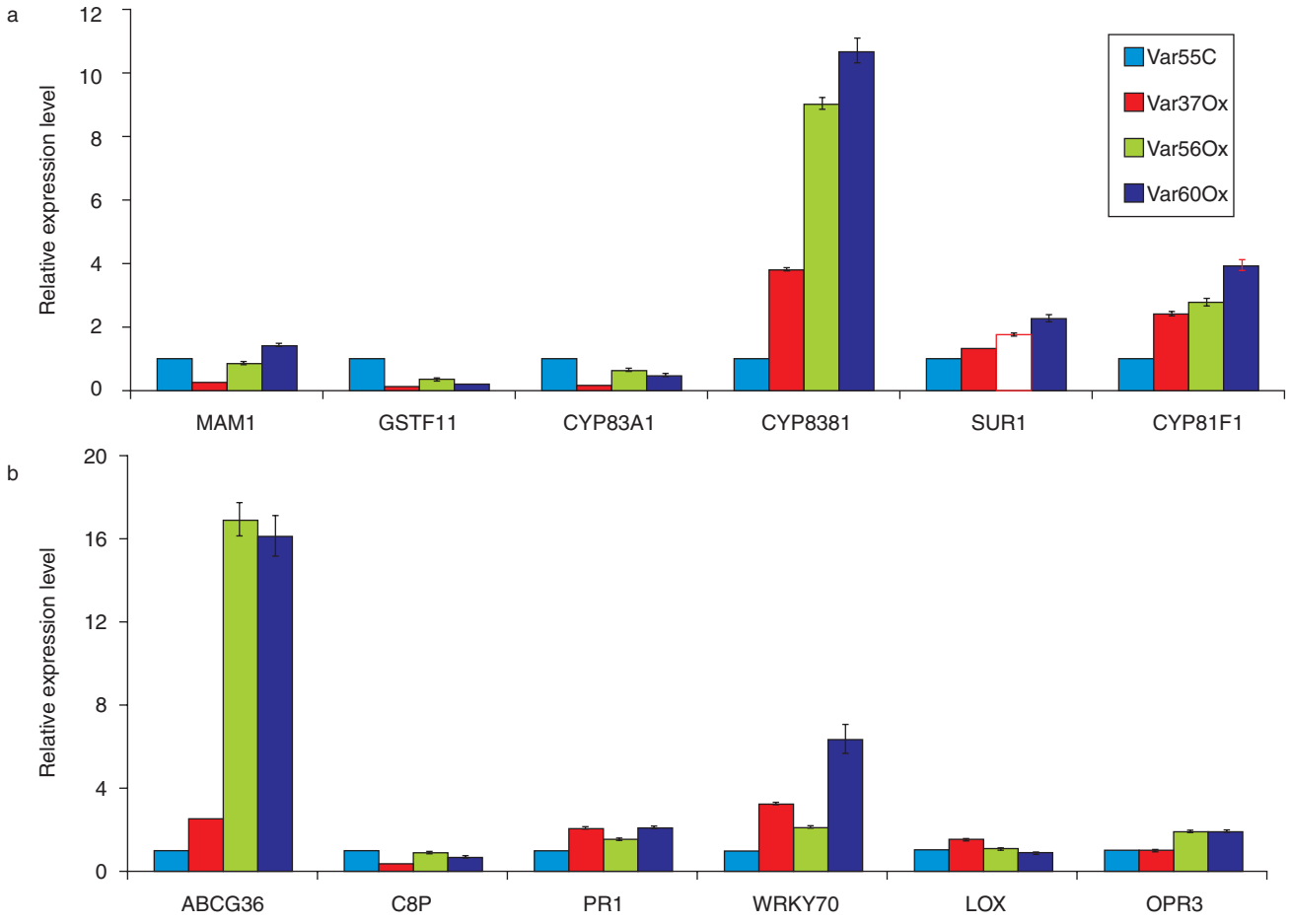

Fig 5 (a) Expression analysis of glucosinolate biosynthetic genes and (b) other defense-related genes in transgenic mustard lines.

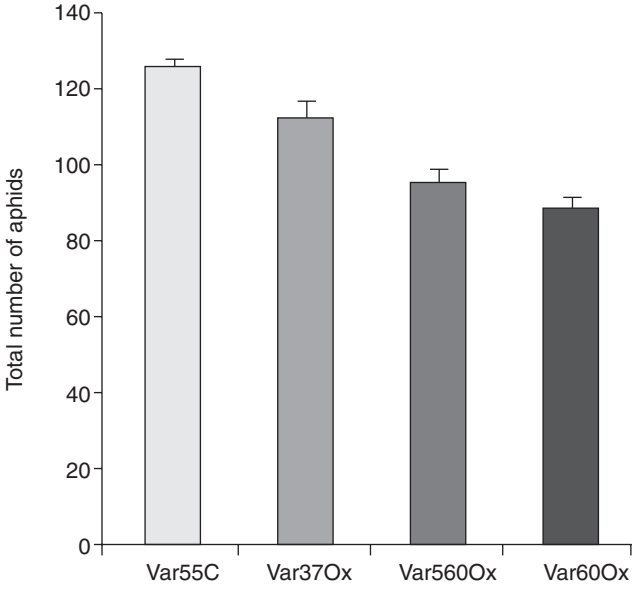

Fig 6 *In planta* aphid bioassay on different transgenic mustard lines (Var 37Ox, Var 56Ox and Var 60Ox) and untransformed control (Var 55C) plant.

transgenic lines were also analyzed for the transcript level of several glucosinolate biosynthetic genes such as *MAM1* (*Methyl thioalkylmalate synthase 1)*, *GSTF11* (*Glutathione S-transferase F11*), *CYP83A1*(*Cytochrome P450 83A1*), *CYP83B1*(*Cytochrome P450 83B1*), *SUR1*(*Supperroot 1*) and *CYP81F1*(*Cytochrome P450 81F1*). Brassicacae family members are rich reservoir of glucosinolates. Glucosinolate-myrosinase system represents an important component of plant defense mechanism in these plants (Hopkins *et al.* 2009). Thus, to hypothesize any indirect effect of higher *BjSWEET11* expressions on the activation of defense pathways in *B. juncea*, co-activation of glucosinolate biosynthetic genes were studied. The qRT-PCR based analysis showed increased transcript levels of *CYP83B1*, *SUR1* and *CYP81F1* in the transgenic lines as compared to untransformed control plants (Fig 5a). The expression levels of these glucosinolate biosynthesis genes were highest in Var 60Ox line and lowest in Var 37Ox line. The transgenic lines were further analysed for transcriptional activation of other defense-related genes, viz. *ABCG36* (*ABC transporter G family member 36*), *CBP* (*Calmodulinbinding protein*), *PR1*(*Pathogensesis-related protein 1*), *WRKY70*(*WRKY transciption factor 70*), *LOX* (*Lipoxygenase*) and *OPR3*(*12-Oxophytodienoate reductase 3*) (Koramutla *et al.* 2014; Campe *et al.* 2016). The expression of *ABCG36* and *WRKY70* was found to be significantly higher across the transgenic lines when compared to their expression in untransformed control plants (Fig 5b). Thus, the result of gene expression study empirically demonstrated transcriptional activation of several endogenous defense-related genes due to higher expression level of *BjSWEET11* in the transgenic plants.

Aphid bioassay of the transgenic lines of B. juncea

Three transgenic lines showing detectable increase in *BjSWEET11* expression compared to the untransformed controls were subjected to aphid bioassay *in planta*. On each plant five late instar nymphs of mustard aphid were released on the leaves of two months old *B. juncea* plants; and were confined to that leaf with the help of clip cages. Increase in the number of aphids was monitored and at 7th day after inoculation the total number of aphids was counted. The total numbers of aphids on each of the transgenic plants were compared with the total number of aphids scored in case of control plants. Thus, compared to the control plants the population of aphids on the transgenics were reduced by 10 to 28% (Fig 6). The maximum attenuation of aphid population was obtained on the transgenic line Var 60Ox. The result of the insect bioassay demonstrated that, enhanced level of *BjSWEET11* expression, though did not confer any insect mortality but attenuated the population growth of aphids. However, lack of much significant effect in deterring aphid population reiterated complex nature and involvement of multiple genes in endogenous defense response of plants.

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