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Research paper

Genome-wide identification and expression analysis of sucrose synthase genes in allotetraploid *Brassica juncea*

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ABSTRACT

Sucrose plays pivotal role in energy metabolism and regulating gene expression of several physiological processes in higher plants. Here, fourteen *sucrose synthase* (*SUS*) genes have been identified in the allotetraploid genome of Indian mustard, *Brassica juncea*. The identified *SUS* genes in *B. juncea* (*BjSUS*) were derived from the two-progenitor species, *B. rapa* and *B. nigra*. Intron-exon analysis indicated loss or gain of 1–3 introns in diversification of *SUS* gene family. Phylogenetic analysis revealed discrete evolutionary paths for the *BjSUS* genes, originating from three ancestor groups, SUS I, SUS II and SUS III. Gene expression study revealed significant variability in expression of the *BjSUS* paralogs across the different tissues. *BjSUS* genes showed transcriptional activation in response to defense hormones and a late response to wounding. Tissue and temporal specificity of expression revealed importance of specific SUS paralogs at different developmental stages and under different stress conditions. The study highlighted differential involvement of SUS paralogs in sucrose metabolism across the tissues and stress-responses, in a major oilseed crop *B. juncea*.

1. Introduction

Sucrose is the major carbon source, mainly synthesized in leaves and green parts of the plant and transported to different sink tissues via phloem. Flux of sucrose acts as signal to various regulatory mechanisms related to growth and development. It also influences expression of genes encoding transporters, storage proteins and stress responses (Bolouri Moghaddam and Van den Ende, 2012; Van den Ende and El-Esawe, 2014). In plants, sucrose synthases (SUS; EC 2.4.1.13) and invertases (INV; EC 3.2.1.26) are the two key enzymes involved in sucrose metabolism. SUS catalyzes reversible synthesis of sucrose from UDP-glucose and D-fructose though, their primary function *in vivo* is cleavage of sucrose (Schmalstig and Hitz, 1987). SUS enzymes are mainly found in plants and encoded by small multigene family. The number of genes in the *SUS* family differs among the plant species. For example, fifteen isoforms of *SUS* gene family have been identified in genus *Populus* (An et al., 2014); fourteen in *Nicotiana tabacum* (Wang et al., 2015); seven in cotton (Chen et al., 2012); six in each of *Arabidopsis* (Bieniawska et al., 2007), rice (Hirose et al., 2008), citrus (Islam et al., 2014), and *Hevea brasiliensis* (Xiao et al., 2014); five in *Saccharum* (Zhang et al., 2013) and four in *Hordeum vulgare* (Barrero-Sicilia et al., 2011). Irrespective of the species, the *SUS* genes contain conserved structural motifs. For example, the characteristic sucrose synthase and glucosyl transferase domains are common in all the family members. In addition, they may have a putative serine phosphorylation site, as present in the *SUS* gene family members in *Populus* and cotton (Zhang et al., 2011; Chen et al., 2012). Based on the protein sequence and phylogenetic relationship, plant SUS proteins have been categorized into three major groups viz.SUS I, II, and III (Zhang et al., 2011, 2013).

The expression pattern of the *SUS* paralogs varies within individual plants depending on the tissue and physiological conditions. For example, in tobacco *SUS2* and *SUS3* are expressed across diverse tissues whereas, *SUS5* and *SUS7* are predominantly expressed in flower buds and sepals (Wang et al., 2015). Furthermore, *SUS1* and *SUS2* both are induced in response to drought stress, whereas only *SUS2* is induced by low-temperature stress and only *SUS7* is significantly activated in response to virus infection (Wang et al., 2015). In *Hevea brasiliensis* at least one SUS isoform was abundant in each tissue type; *HbSus3* was

Abbreviations: SUS, sucrose synthase; RT-qPCR, reverse transcription- quantitative real time PCR; UDP-glucose, Uridine diphosphate glucose; ADP-glucose, Adenosine-5′-diphosphoglucose; HMM, Hidden Markov model; BRAD, Brassica database; CDD, Conserved Domain Database; dpa, day post anthesis; MeJ, methyl jasmonate; SA, salicylic acid; MeSA, methyl salicylate; ET, ethephon

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predominately expressed in latex tissue, isoform 4 in bark and isoform 5 in root tissue. *HbSus5* showed induced expression to low temperature and drought stress, suggesting its positive involvement in stress resistance mechanisms (Xiao et al., 2014). The tissue and stage specific expression of *SUS* genes have been demonstrated in many other plant species including Arabidopsis, rice, citrus, *Populus* and cotton (Bieniawska et al., 2007; Hirose et al., 2008; Zhang et al., 2011; Chen et al., 2012; Islam et al., 2014). The variable expression pattern of the *SUS* genes indicates specific functions of each member in particular physiological conditions and tissue types.

Ectopic expression of *SUS* genes led to agronomic advantages in a number of plants. Over-expression of the *PvSUS1* gene in transgenic switch grass increased the plant height, biomass and tiller number (Poovaiah et al., 2015). In cotton, transgenic-expression of potato *sucrose synthase* gene enhanced leaf growth, seed development and fibre elongation (Xu et al., 2012a). Maize transgenic plants expressing potato *StSUS4* showed enhanced levels of starch and ADP-glucose in mature seeds when compared to their non-transgenic counterparts (Li et al., 2013). Similarly, in tobacco, over expression of the *Populus PsnSuSy2* gene increased both thickening of the secondary cell wall in stem tissues and plant height (Wei et al., 2015). On the other hand, loss of function of *SUS* genes in several plant species resulted into altered phenotype. For example, suppression of *sucrose synthase* reduced fibre and seed development in cotton (Ruan et al., 2008), wood density in *Populus* (Gerber et al., 2014) and callose deposition in the sieve elements of *Arabidopsis* (Barratt et al., 2009). In contrast, some studies did not find any significant impact of suppressing SUS isoforms on plant growth and development (Bieniawska et al., 2007; Barratt et al., 2009).

Sucrose synthases are also activated in response to biotic and abiotic stresses. In grapevine and maize infection by bacterial phytoplasma activated SUS enzymes. After colonization, protoplasma in phloem sieve cells blocked sugar transporters and activated expression of the host *SUS* genes facilitating conversion of sucrose into monosaccharides (Hren et al., 2009; Brzin et al., 2011). Similarly, in *Arabidopsis*, significant up-regulation of the *AtSUS 1*, *4* and *6* genes were seen in syncitia induced by the parasitic nematode *H. schachtii* as compared to expression in the tissues of non-infected plants. Mutant analysis revealed that single *SUS* mutant did not show any effect on *H. schachtii* development or reproduction. However, in the double mutant lines *Atsus1/Atsus4* and *Atsus5/Atsus6* female infection rate was doubled and eggs per cyst rose upto 200% as compared to the wild type plants (Cabello et al., 2013). More studies across the plant species are warranted for resolving the contradictions in involvement of *SUS* paralogs in defense responses against a range of biotic stresses.

Rapeseed-mustard group of crops occupies the third position in the global oilseed production and contributes 27.8% of the Indian oilseed economy (Shekhawat et al., 2012). In India, the chief oil yielding crop in this group is Indian mustard, *Brassica juncea.* However, productivity of this crop has plateaued and is further threatened by damage due to insects and pathogens. Sucrose flux and distribution play a pivotal role in yield pathways as well in defense responses in many plants (Dubey et al., 2013; Wang et al., 2017). Therefore, identifying the key genes of sucrose metabolism and understanding their regulation are of significances in many economically important plants (Huang et al., 2017; Zhao et al., 2017). This has been facilitated by recent advances in genomics tools, draft genome sequences, and availability of robust bioinformatic tools. However, little attention has been paid to major oilseeds. *B. juncea* is an amphidiploid species evolved by acquisition of two paternal genomes of *B. rapa* (AA) and *B. nigra* (BB). Its genome sequence has been published recently by Yang et al. (2016). Here, we focussed on the make-up of the *sucrose synthase* (*SUS*) gene family in *B. juncea*.

In this study we have identified and characterized fourteen *BjSUS* genes on a genome-wide scale in *B. juncea* investigating their structure, evolutionary origin and expression profile in various tissues and stress related cues. The study has laid a foundation for understanding the potential function of individual *BjSUS* genes with reference to various physiological processes and stresses.

2. Materials and methods

2.1. Identification of sucrose synthase gene family members in Brassica juncea

In order to identify the paralogs of *sucrose synthase* (*SUS*) genes in *B. juncea*, the Hidden Markov model (HMM) profiles of sucrose synthase and glycosyl transferase domains (PF00862; PF00534) from Pfam database (https://pfam.xfam.org/) was used as a query against predicted protein sequences of *B. juncea* from Brassica database (http://brassicadb.org/brad/datasets/pub/Genomes/Brassica_juncea/V1.1/). Further, TBLASTN searches were performed against *B. juncea* genome at BRAD database (http://brassicadb.org/brad/blastPage.php) using six SUS protein sequences of each Arabidopsis and rice (Bieniawska et al., 2007; Hirose et al., 2008), seven sequences of each *B. rapa* and *nigra* (http://brassicadb.org/brad/). The putative hits of *SUS* genes in *B. juncea* were further refined by eliminating false positives and partial and redundant sequences using NCBI CDD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Nomenclature for the *SUS* genes identified in *B. juncea was* carried out in the similar way as it was followed in case of *Arabidopsis SUS* genes. Genes were named *BjSUS1* to *BjSUS7* with decimal numbers representing the number of paralogs.

2.2. Chromosomal distribution

To map the *BjSUS* genes on the chromosomes of *B. juncea*, BLASTn was performed against the *B. juncea* draft genome using BjSUS genomic sequences as the query with default parameters. The position of each gene on the chromosome was recorded manually and a chromosomal map was constructed using MapChart 2.3 (Voorrips, 2002).

2.3. Analysis of gene structure, phylogeny and cis-regulatory elements

For predicting exon/intron regions of the *SUS* genes, the coding sequences (cDNA) were aligned to their corresponding genomic DNA sequences using the Gene Structure Display Server 2.0 (Hu et al., 2015). Protein sequences of the *B. juncea* SUS members were predicted from their genomic sequences using softberry FGENESH programe (http://www.softberry.com) and SUS protein sequences from other plant species were retrieved from the NCBI database using their unique accession numbers. Multiple sequence alignment of SUS protein sequences was performed using DNAMAN (version 10) and Clustal X (version 2.0.11) with default parameters. The phylogenetic trees were constructed using the Maximum likelihood method in MEGA 7.0 with one thousand bootstrap replications. As putative promoter sequences of *BjSUS*, 1 kb upstream of start codon ATG were extracted from the *B. juncea* genomic database and *cis*-regulatory elements were identified by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database.

2.4. Plant material and growth conditions

Plants of *B. juncea* cv. Varuna were grown in 12-in. pots under net house conditions during the mustard growing season (November–February) of Delhi, India. Four different tissues from vegetative stages were collected: five-day old plants, young leaves from 21-day old plants, stems and matured leaves from 40-day old plants. Three tissues were collected from reproductive stages: buds, young pods at 7-day post anthesis (dpa) and mature pods at 30 dpa. The samples were frozen immediately in liquid nitrogen and stored at −80 °C until further use for gene expression studies.

2.5. Hormonal treatment and mechanical wounding of plants

Plants grown in net house for 3 weeks were transferred to a growth chamber maintained at 22 ± 2 °C, with 65–70% relative humidity and 16 h light (140 μmol $m^{-2} s^{-1}$)/8 h dark cycles and stabilized for at least one day-night cycle before the treatments. For hormone treatments, plants were sprayed with 100 μM methyl jasmonate (MeJ) or 1 mM methyl salicylate (SA) or 1.5 mM ethephon (ET) solutions prepared in double distilled water containing 0.1% triton X-100. Leaf samples were collected at 0, 1, 3, 8, and 24 h after spraying, quickly frozen in liquid nitrogen and stored at −80 °C until used. For wounding, a haemostat was used for repeated wounding across the mid-vein of the fifth and sixth leaves from the top of the plant. Unwounded upper systemic leaves from the wounded plants were collected at 0, 1, 8, and 24 h after wounding. The leaf samples were frozen in liquid nitrogen and kept at −80 °C until used.

2.6. RNA isolation and cDNA synthesis

RNA isolation was done using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Residual DNA, if any, were removed by TURBO DNA-free kit (Ambion, USA). Concentration and quality of RNA was determined by NanoDrop ND-1000 spectrophotometer (Nanodrop technologies, USA). RNA samples with OD 260/280 between 1.9 and 2.2 and OD 260/230 > 2.0, were used. Two μg of total RNA was used for synthesizing first stand cDNA using a cDNA synthesis kit (Takara Bio Inc., Japan). Each cDNA sample was diluted 20 times for qPCR.

2.7. RT-qPCR

Quantitative RT-PCR reactions were performed in a StepOne plus real time machine (Applied Biosystems, USA) using SYBR green detection chemistry. A reaction cocktail of 20 μl was constituted of 2 μl diluted cDNA, 10 μl 2× SYBR Premix Ex Taq II (Takara Bio Inc., Japan), 0.4 μl of ROX reference dye and 0.4 μl each of the forward and reverse primers (Table S1). PCR was carried out at an initial denaturation for 1 min at 95 °C, followed by 40 repeated cycles each consisting of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. Amplicon specificity was checked by dissociation curve analysis with constant increase of temperature between 60 °C and 95 °C. Mean Ct values were derived from at least nine replicates consisting of minimum three biological replicates and three technical replicates each time. Glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was used as reference gene for normalization.

3. Results

3.1. Data mining, identification and characterization of SUS gene family in Brassica juncea

The *SUS* genes of *B. juncea* (*BjSUS*) were identified through BLAST search querying for HMM profiles of the SUS domain in BRAD database against the *B. juncea* genome. After eliminating short and low identity sequences, 14 putative *SUS* genes were obtained. These gene sequences revealed that out of 14 genes, eight originated from AA genome of *B. rapa* and remaining six from BB genome of *B. nigra* (Table 1). We named the *SUS* genes of *B. juncea* as *BjSUS1* to *BjSUS7* with decimal numbers representing the copies of the paralogs.

Pair wise identities among the nucleotide sequences of the 14 *BjSUS* genes and the amino acid sequences of their encoded polypeptides were calculated using the multiple sequence alignment algorithm of DNAMAN software (http://www.lynnon.com/). As shown in Table S2, the identity matrix revealed high levels of similarity between the BjSUS sequences. They could be grouped into seven pairs, having highest similarity (> 86%) within the pairs. The results suggested that, in amphidiploid genome of *B. juncea*, each *SUS* gene has two paralogs originating from each of the two progenitor genomes. Alignment of the fourteen BjSUS coding sequences confirmed this hypothesis. The highest per cent similarity was found between *BjSUS1* and *BjSUS4*, which ranged from 92.37 to 97.15% at the amino acid level and 89.38–92.73% at the nucleotide level. The alignment of amino acid and nucleotide sequences of the *SUS* genes between *B. juncea* and its two diploid progenitors, *B. rapa* and *B. nigra*, showed high levels of sequence identity, which was more in case of the same paralogs (Table S3). It was evident that for most of the *SUS* genes, except *BjSUS 3* and *4* at least one of the paralogs in *B. juncea* was derived from its diploid progenitors. Locations of the 14 putative *BjSUS* genes were distributed across 10 of the 18 chromosomes in the *B. juncea* draft genome (Fig. 1). Three *BjSUS* paralogs were found to be on chromosome B03; two each on chromosomes A09 and A10 and one each on chromosomes A03, A05, A06, A07, B02, B06 and B08.

3.2. Exon/intron organization and structural analysis of the BjSUS family

Analysis of the exon/intron structural organization provides deep insight into the evolutionary mechanisms hidden in the genesis of the gene family (Boudet et al., 2001; Lecharny et al., 2003; Wang et al., 2015). The alignment between the genomic and cDNA sequences of the *BjSUS* genes revealed that the *BjSUS* genes typically consist of 10–15 exons interspersed by 9–14 introns (Fig. 2). For example, 11 introns were identified in *BjSUS1.1*, *3.2*, *4.1*, *4.2*, *6.1*, *6.2*, *7.1* and *7.2*, 12 in *BjSUS5.1* and *5.2*, 13 in *BjSUS2.1*, 14 in *BjSUS2.2*, and 9 introns in *BjSUS1.2*. The length of the introns ranged from 50 to 481 bp, with most between 50 and 100 bp, except in one case in which an intron of *BjSUS5.2* was larger than 5 kb. Interestingly, most of the *SUS* genes in the allopolyploid genome of *B. juncea* contained closely similar numbers of introns as those seen in their ancestors.

As the *SUS* genes had high sequence similarity, high conservation of the exon/intron structures was expected. In the 14 *BjSUS* sequences, 15 intron positions conserved across *B. juncea* and its progenitor species *B. rapa* and *B. nigra* were identified (Fig. S1). Several *SUS* genes lack one or more introns suggesting the possibility of intron loss. Such intron losses were mainly associated with the 5th, 8th and 12th intron positions, leading to the formation of larger exons (Fig. 2). All the BjSUS putative protein sequences, except BjSUS2.1 and BjSUS5, contained a conserved serine residue at the N-terminal region (Fig. S2), possibly the site of phosphorylation by a calcium dependent Ser/Thr protein kinase (Huber et al., 1996; Hardin and Huber, 2004). Conserved domain analysis revealed the presence of two conserved sucrose synthase and glucosyl-transferase domains in all the BjSUS enzymes. These have been considered as signature motifs of SUS proteins (Fig. S2).

3.3. Phylogenetic analysis of B. juncea SUS genes

For deducing evolutionary relationship among the *SUS* gene families of *B. juncea* and between *B. juncea* and other plant species, alignments were made across 116 SUS amino acid sequences from14 BjSUS isoforms, 83 sequences from dicots, 28 sequences from monocot species and 5 SUS sequences of bacterial origin (Table S4). Based on ClustalX an unrooted tree was constructed using the Maximum likelihood method of MEGA7.0 with 1000 bootstrap replicates for ensuring robustness of the phylogram's topology (Fig. 3). The phylogenetic analysis showed a relatively deep evolutionary origin and recent duplications. All the amino acid sequences formed two monophyletic groups consisting of bacterial and plant sequences separately, showing their origin from respective ancestral type. Bacterial *SUS* genes were consequently used as outgroup. The plant sequences were clustered into three distinct groups based on strong statistical bootstrap values (100%). These groups were named as SUS I, II and III, respectively (Fig. 3). Deep analysis of the phylogenetic tree revealed that the protein sequences in the SUS I group were further classified into two subgroups,

Table 1
Characteristic features of sucrose synthase genes in *Brassica juncea*, *B. rapa* and *B. nigra*.

	Gene name	Gene ID	gDNA size (bp)	cDNA size (bp)	CDS size (bp)	Amino acid size	Theoretical MW (kDa)	Theoretical pI	Functional domains (start-end)	
									Sucrose synthase	Glycosyl transferase
B. juncea	*BjSUS1.1*	BjuB015313	4264	3138	2442	813	93.20	5.95	8–560	564–749
	BjSUS1.2	BjuA009339	4283	3098	2421	806	92.47	5.67	8–553	557–742
	BjSUS2.1	BjuB037515	4042	3140	2424	807	92.02	5.86	5–552	555–740
	BjSUS2.2	BjuA023848	4341	2721	2571	856	97.71	6.15	54–601	604–790
	BjSUS3.1	BjuO006586	4015	2724	2442	813	92.76	5.99	8–559	562–748
	BjSUS3.2	BjuA036504	4138	2722	2454	817	93.12	5.93	8–563	566–752
	BjSUS4.1	BjuA047153	4024	3061	2418	805	92.25	5.80	8–553	556–742
	BjSUS4.2	BjuO008945	3608	2487	2325	774	88.54	5.83	8–431	525–711
	BjSUS5.1	BjuA018844	4665	2716	2514	837	95.12	6.24	5–549	552–734
	BjSUS5.2	BjuB022852	9774	2974	2268	755	85.42	5.82	1–309	470–652
	BjSUS6.1	BjuB030220	3881	2987	2790	929	105.47	6.47	12–551	555–729
	BjSUS6.2	BjuB047347	3978	3100	2823	940	106.32	6.94	12–560	563–739
	BjSUS7.1	BjuB030962	4719	3785	2796	931	105.13	6.92	12–551	554–730
	BjSUS7.2	BjuA043452	3843	2854	2706	901	102.30	6.52	232–518	521–696
B. rapa	*BrSUS1*	Bra006578	3968	2558	2421	806	92.44	5.76	8–553	557–742
	BrSUS2	Bra037432	4342	2974	2571	856	97.76	6.20	54–601	604–790
	BrSUS3	Bra036282	4280	2793	2436	811	92.33	5.83	8–557	560–746
	BrSUS4	Bra002332	4064	3038	2361	786	90.16	6.00	3–534	537–723
	BrSUS5	Bra039502	4711	2716	2514	837	95.16	6.24	5–549	552–734
	BrSUS6	Bra003845	3930	3100	2823	940	106.31	6.94	12–560	563–739
	BrSUS7	Bra015995	3847	2981	2832	943	107.05	6.42	12–560	563–738
B. nigra	*BniSUS1*	BniB038530	4287	3115	2421	806	92.47	6.06	8–553	556–742
	BniSUS2	BniB001016	3839	2673	2424	807	92.08	6.09	5–552	555–741
	BniSUS3	BniB022086	4555	3300	2679	892	101.73	6.13	89–638	641–827
	BniSUS4	BniB026394	3603	2521	2418	805	92.31	5.85	8–553	556–742
	BniSUS5	BniB007158	4639	2717	2514	837	95.20	6.45	5–549	552–734
	BniSUS6	BniB043086-	3888	2988	2790	929	105.38	6.03	12–551	555–735
	BniSUS7	BniB013853	4016	3084	2781	926	104.81	7.75	12–551	554–729

one for the dicot and the other for the monocot sequences. The *BjSUS* genes were distributed in the dicot clades of three major SUS groups. BjSUS1 and 4 grouped in the dicot subgroup SUS I, BjSUS2 and 3 in SUS II and BjSUS5, 6 and 7 in SUS III. Exon/intron organization patterns of the BjSUS paralogs were consistent with the phylogenetic tree (Table S2, Fig. S1). The BjSUS isoforms were more closely clustered to their putative SUS orthologs from progenitor *B. rapa* and *B. nigra*, and were also closely related to sequences from *Arabidopsis*, a member of the same family. This reflects conservation of the SUS isoforms in the evolution through their plant lineages. Even though *BjSUS* paralogs shared high sequence similarity, limited diversification occurred within the members of *BjSUS* family possibly indicating their discrete biological roles and diverse evolutionary histories.

3.4. Analysis of cis-regulatory elements in BjSUS promoters

The upstream promoter regions are crucial in spatio-temporal specificity of gene expression (Liu et al., 2013). For identifying the *cis*-regulatory elements in the promoter regions of the BjSUS genes, 1 kb upstream sequences of the start codon were analyzed using the plantCARE database. A total of 78 *cis*-regulatory elements were detected in the putative promoter regions of the *BjSUS* genes. Excluding the minimal promoter elements TATA and CAAT boxes, the remaining elements were categorized into five groups based on their functional association, viz. response to light, hormones, stress, tissue specific expression and others. Among these, the light responsive elements formed the biggest group (34%) followed by stress responsive (20%), hormone responsive (12%) and tissue responsive elements (Fig. 4A). All fourteen BjSUS promoters contained at least one or more *cis*-regulatory element (s) for each of the light, stress and hormone responsive categories (Fig. 4B). However, presence of tissue specific elements was not universal. For example, promoters of *BjSUS1.2*, *4.1* and *7.1* contained no tissue specific elements. Each BjSUS promoter contained 2–11 different *cis*-regulatory elements, responsive to stresses including heat, pathogen infection and wounding, sucrose, and plant defense (Table S5). In addition, *BjSUS* gene promoters also contained several hormone-responsive elements; such hormones include methyl jasmonate (MeJ), salicylic acid (SA) and gibberellin (GA). Among these, MeJ and SA elements were prevalent in these promoters.

3.5. Gene expression analyis of BjSUS paralogs

For analysing tissue specific expression of the *BjSUS* genes specific primers for each gene instead of each paralog were developed (Table S1). RT-qPCR analysis showed differential expression patterns of the *BjSUS* genes across a wide range of tissues (Fig. 5). *BjSUS1*and *BjSUS2* showed much higher levels of expression in stems, buds and young pods compared to their low expression in either young or mature leaves. Interestingly, only 2–3 *BjSUS* genes were strongly expressed in any given tissue type. For example, young leaves showed higher expression of *BjSUS5*, *6*, *7* and young pods higher expression of *BjSUS1*, *2*, *3* compared to other *BjSUS* genes. However, within an individual tissue the expression profile of the *SUS* genes changed as the plant matured. For example, the expression of *BjSUS5*, *6*, *7* rapidly declined as leaves matured. Nevertheless, the wide range of differential expression of the *BjSUS* genes across tissues at different temporal points led us to hypothesize their tissue specific role under various physiological conditions.

3.6. Expression of BjSUS genes in response to stress hormones and wounding

Activation of gene expression by defense related hormones such as MeJ, ethylene (ET), SA etc. has been used for associating gene functions to defense responses. Plant *SUS* genes have been demonstrated to be induced by plant hormones abscisic acid and GA (Harada et al., 2005; Tang et al., 2009; Bai et al., 2014). We studied the activation patterns of the *BjSUS* genes in *B. juncea* in response to applications of MeJ, SA and

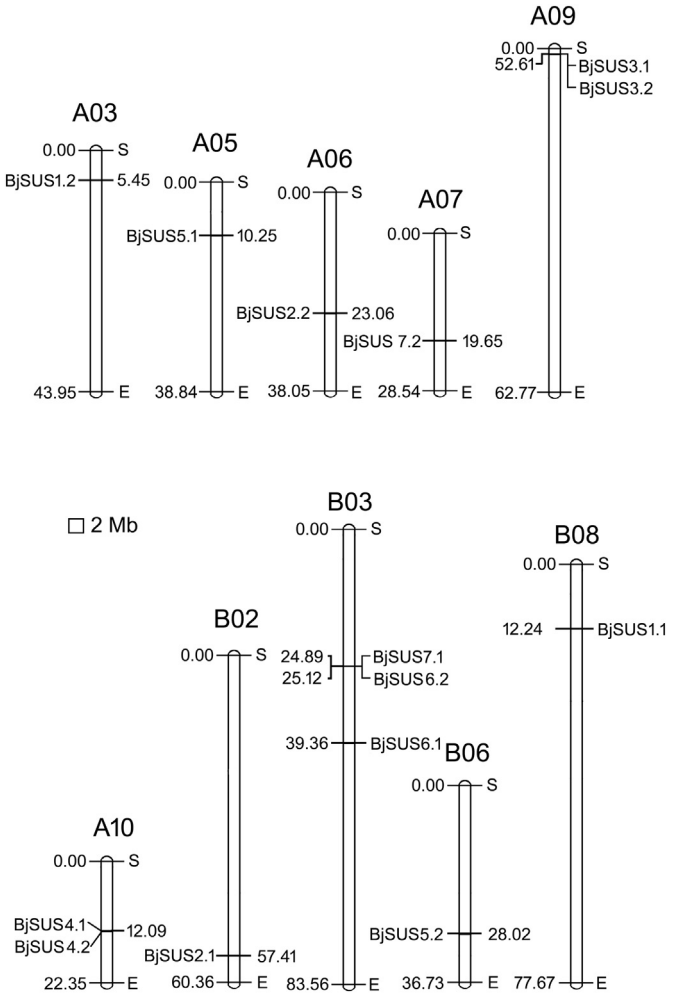

Fig. 1. Chromosomal location of SUS gene family members in *Brassica juncea*. Scale represents 2 Mb chromosomal distances. Chromosome sizes are indicated at the bottom and chromosome numbers at the top ends of each scaffold.

ET and to mechanical wounding in a time-course manner. In the case of MeJ treated samples, a gradual increase in gene expression was observed in case of all the paralogs of the *BjSUS* genes except *BjSUS2* which showed no significant induction (Fig. 6A). However, the inductive response was strongest in *BjSUS4*, with a 17.6-fold increase at 24 h followed by *BjSUS5*, *6*, and *7*. With SA, the activation was more prominent in *BjSUS1*, *2*, *3*, *4* compared to others (Fig. 6B). In all these activation patterns, gene expression gradually increased and reached the maximum at 3 h following the treatment. The highest activation was shown by *BjSUS1* with a maximum of 22.13-fold. The activation profile of the *BjSUS* genes due to application of ET was similar to that in response to MeJ, though the extent of activation was less (Fig. 6C). Mechanical wounding activated all the *BjSUS* genes except *BjSUS2* and *3* at 24 h of treatment (Fig. 7).

4. Discussion

A draft genome sequence of the allopolyploid oil seed crop, *Brassica juncea* has been recently published (Yang et al., 2016). Based on the genome sequence, 14 *SUS* genes were identified in this amphidiploid species. A basic set of six *SUS* genes have been identified in *Arabidopsis* genome (Baud et al., 2004). *B. rapa*, one of the progenitors of *B. juncea* has undergone whole genome triplication since it diverged from *Arabidopsis* lineage (Lysak et al., 2005; Fang et al., 2012). Therefore, up to eighteen *SUS* genes could be expected in *B. rapa*. However, only seven *SUS* genes have been identified in *B. rapa* genome (Table 1) suggesting that > 60% of the duplicated *SUS* genes were lost due to genomic fractionation following the whole genome triplication. Similar results were observed in *B. nigra*. In *B. juncea* all the 14 *SUS* genes were contributed by AA and BB genomes of its diploid progenitors. These findings are consistent with the mesopolyploid nature of *B. rapa* and *B. nigra* and their evolutionary relationship with amphidiploid *B. juncea*.

The *SUS* gene family in *B juncea* has been found to the second largest, next to *Populus* which has fifteen *SUS* genes (An et al., 2014). The 14 SUS proteins of *B. juncea* shared two common domains, sucrose synthase and glycosyltransferase, as previously characterized in other SUS proteins (Huber et al., 1996; Hardin and Huber, 2004; Li et al., 2015; Wang et al., 2015). Phylogenetic analysis of *BjSUS* genes demonstrated that at least one *BjSUS* gene belonged to each of the three groups SUS I, SUSII and SUS III which supports the hypothesis that all three groups are represented in higher plant species (Hirose et al., 2008). *BjSUS1* (1.1 and 1.2) and *BjSUS4* (4.1 and 4.2) were clustered together with *SUS* homologs of *Arabidopsis* and soybean in the dicot specific sub group of SUSI. This result supports the idea that the multiple gene duplication events might have occurred after the divergence of monocot and dicot lineages, and gene duplication events may have occurred after the separation of *Brassica*/Arabidopsis/soybean from a common ancestor. The presence of *BjSUS2* and *3* in the SUS II group and *BjSUS5*, *6* and *7* in the SUS III group representing both monocot and dicot species, indicate that these *BjSUS* genes are evolutionarily older than the *BjSUS1* and *4* genes of the SUS I dicot sub group.

Structural divergence of gene family members arises due to exon/intron loss or gain, insertion/deletion and exonization/pseudo-exonization mechanisms. Analyses of exon/intron structures are therefore important in revealing the evolutionary foot prints of gene families (Lecharny et al., 2003; Xu et al., 2012a). The present study showed that the number and positions of introns in the *SUS* genes of *Brassica* species remained highly conserved. Members of the three major SUS groups were known to contain 14 introns which are highly conserved across a range of plant species (Chen et al., 2012; Li et al., 2015; Wang et al., 2015). Those 14 introns were found to be present in most of the *SUS* genes of *Brassica* sp. It strengthens the hypothesis that divergence of these three SUS group lineages may have occurred before evolution of the angiosperms and prior to the segregation of eudicot and monocot species (Tang et al., 2008).

Loss of introns 5 and 12 was observed in *SUS1* and *SUS4* genes of *Brassica sp*., without exception. This characteristic signature in *SUS* genes was observed only in other dicot species belonging to the SUS I dicot subgroup (*Arabidopsis*, cotton, tobacco etc.) (Baud et al., 2004; Chen et al., 2012; Wang et al., 2015). Such intron loss was not observed in monocot species (Chen et al., 2012). The largest number of introns lost is in *BjSUS1.1* belonging to the SUSI dicot group. It indicates more selection pressure on *BjSUS1.1* compared to other paralogues of *BjSUS1* and *4*. *BjSUS2* genes, similar to *SUS2* group genes *OsSus2*, *3* and *ZmSh1* from monocot species contain 14 introns (Chen et al., 2012). In the SUS III group of *B. juncea* genes, such as, *SUS 5*, *6*, and *7*, we observed previously identified signature feature of either extended or additional exons in the 3′ region. This lends support to the idea of non-homologus recombination between the progenitors of the SUS III group.

Gene expression analysis of *sucrose synthase* genes in many plants such as *Arabidopsis*, rice, maize, cotton, tobacco, populus etc., revealed discrete expression patterns of the *SUS* genes within a species with respect to a range of factors (Baud et al., 2004; Hirose et al., 2008; Chen et al., 2012; Wang et al., 2015). This can be due to specificity of tissues, developmental stages, stress types, plant signal hormones and combination of these factors. However, expression analysis of *SUS* genes in *B. juncea* had never been undertaken. SUS isozymes are dominant in sink organs as they participate in the regulation of sink strength in plants (Baroja-Fernández et al., 2009; Li et al., 2013). Coherent with expression behaviour of other *SUS* genes, *BjSUS* exhibited differential expression in different tissues, and in response to plant hormones and

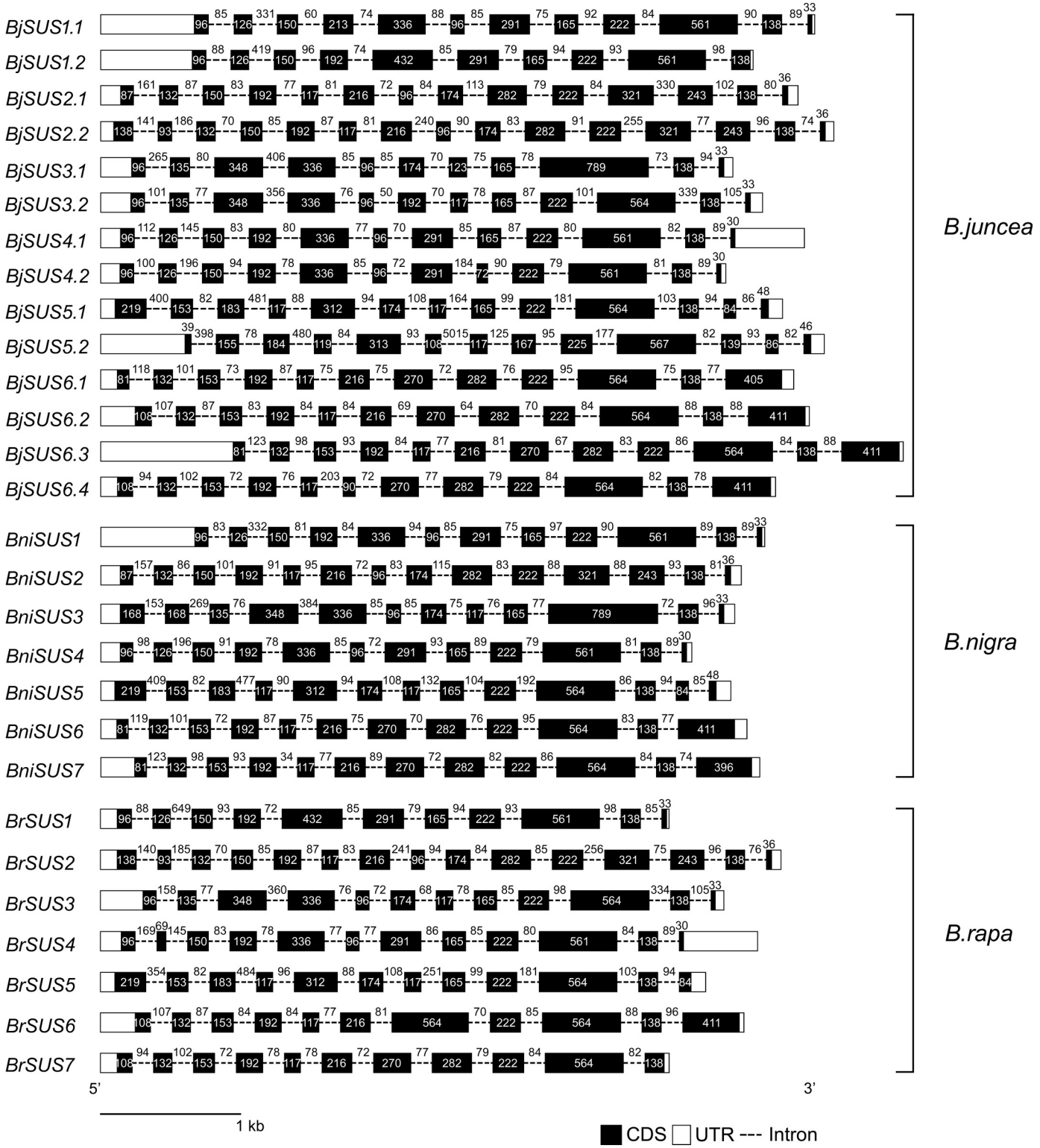

Fig. 2. Exon/intron structural organization in the *SUS* genes of *B. juncea*, *B. rapa* and *B. nigra*. Exons and introns have been identified by aligning genomic and predicted coding sequences. The 5′ and 3′ untranslated regions are represented by hatched boxes, exons with coding regions are shown in black boxes and the connecting lines represent introns. Numbers above the boxes and lines represent the size (bp) of the corresponding exons and introns, respectively.

artificial wounding. Gene expression studies revealed variable expression levels of the *BjSUS* genes in different tissues; though none of the genes showed expression across all the tissues. The overall transcript level of *SUS* genes was much higher in sink tissues such as stems, buds and young pods compared to their expression in source tissues as reported in in several earlier studies (Islam et al., 2014; Zhu et al., 2017; Huang et al., 2017). Higher expression of *BjSUS2* in buds and young pods indicates its likely involvement in energy supply during the pod formation process. Significantly high expression of *BjSUS 5*, *6* and *7* only in young leaves, and not in any other tissues, indicates their exclusive role in leaf development. Similar spatio-temporal specificity in expression of *SUS* genes has been observed in other plants also (Islam

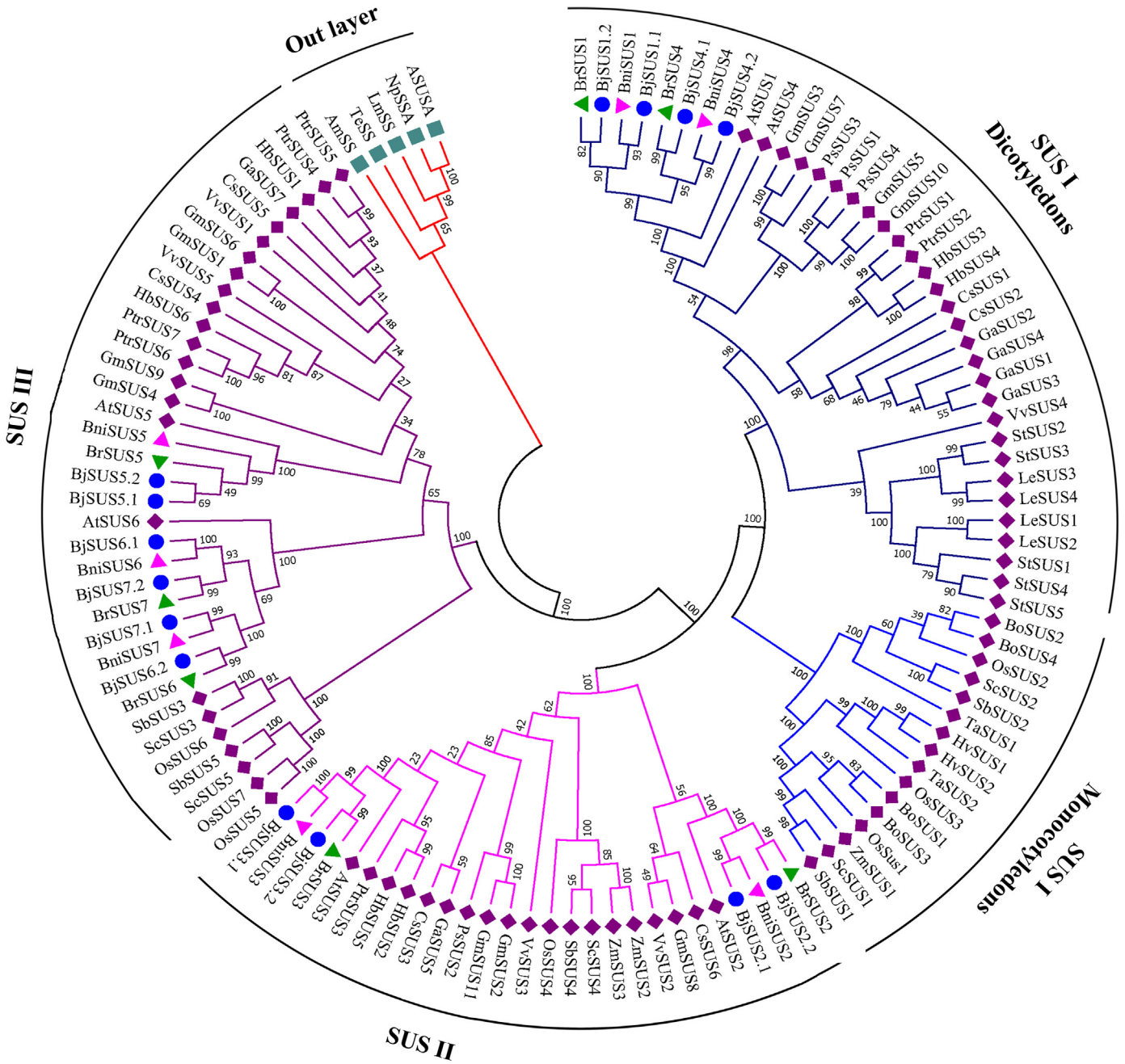

Fig. 3. Phylogenetic analysis of *B. juncea* SUS proteins with other SUS homologs in plants. Unrooted phylogenetic tree of plant SUS proteins was constructed using the Maximum-likelihood method with 1000 bootstrap value. The protein sequences and the source plants are listed in Table S4.

et al., 2014; Zhu et al., 2017). In tobacco *Sus2* and *3* isoforms were highly induced in the leaf tissues after detopping, indicating their role in leaf developmental processes (Wang et al., 2015). In *B. juncea* the tissue-specific expression profile of the *BjSUS* genes signified a predominant role of *BjSUS1*, *2*, *5*, *6* and *7* isoforms in growth and development.

Several reports have shown the involvement of *sucrose synthase* genes in plant responses to abiotic and biotic stresses. For example, a grape sucrose synthase gene *VvSS5* was continuously induced by high temperature, cold, salt, dark and drought conditions (Zhu et al., 2017). Similarly, expression of sucrose synthase genes were significantly induced by low temperature and drought stress (Wang et al., 2015; Barrero-Sicilia et al., 2011; Xiao et al., 2014). *CsSUS3* in cucumber was highly induced by hypoxic conditions and suppression of the *CsSUS3* gene through antisense technology led to reduced resistance to hypoxia when compared to the wild type plants (Wang et al., 2014). Differential activation of the *BjSUS* genes by the defense hormones substantiates their involvement in plant defense (Zhu et al., 2017; Wang et al., 2015). However, the mechanism of their involvement in defense response is still obscure. Nevertheless, this study has laid the foundation for further research on understanding possible physiological roles for of each *BjSUS* gene and in turn the role of sucrose metabolism in growth, development and stress-response in *B. juncea*.

5. Conclusions

In this study, we identified fourteen *sucrose synthase* genes in the genome of allotetraploid *Brassica juncea* and revealed their classification, evolutionary genesis, phylogenetic relationship, structural organization and disposition to specific expression pattern by promoter

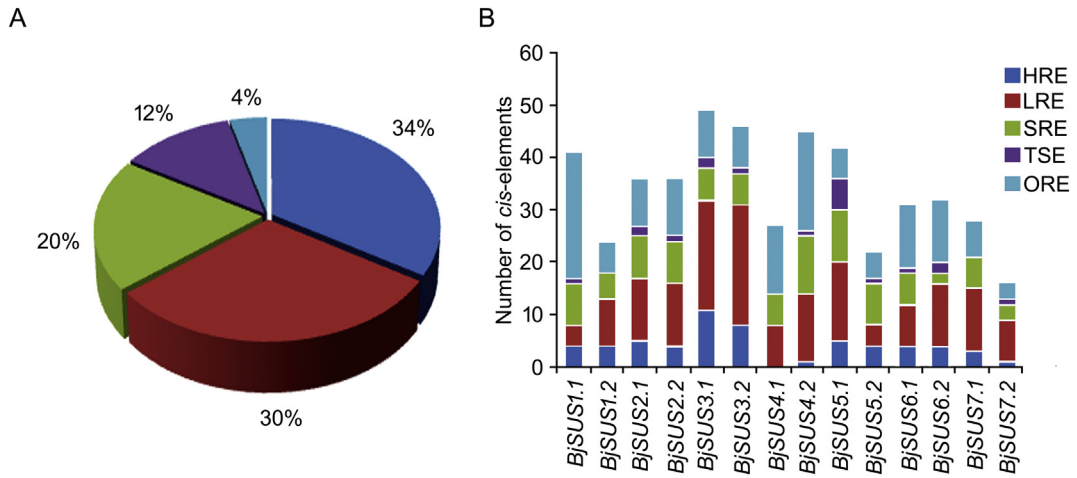

Fig. 4. *cis*-Element analysis of *BjSUS* promoters. *cis*-regulatory elements in the upstream regions of *BjSUS* genes were identified using the plantCARE database and classified into five groups. A) functional groups of *cis*-elements; HRE: hormone responsive elements, LRE: light responsive elements, SRE: stress responsive elements, TSE: tissue specific elements, ORE: other responsive elements, B) No. of *cis*-elements belonging to each functional group in individual BjSUS promoter sequences.

elements. Structural analysis demonstrated conservation of three ancestral lineages of the *SUS* gene family and diversification of the paralogs due to duplication events. Variable pattern of tissue specific expression and response to stress hormones indicated distinct involvement of the *BjSUS* paralogs in sucrose metabolism under various physiological processes and stress-responses. Sucrose metabolism being central to energy metabolism as well as diverse signalling process in plants, insight into gene-function relationship for the *SUS* paralogs remain intriguing. This study is a way forward to that and warrant further study on *SUS* genes in crop species.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

RB and MKK conceived the idea, designed the experiments and wrote the manuscript; MKK, CR, DB and AMG conducted the

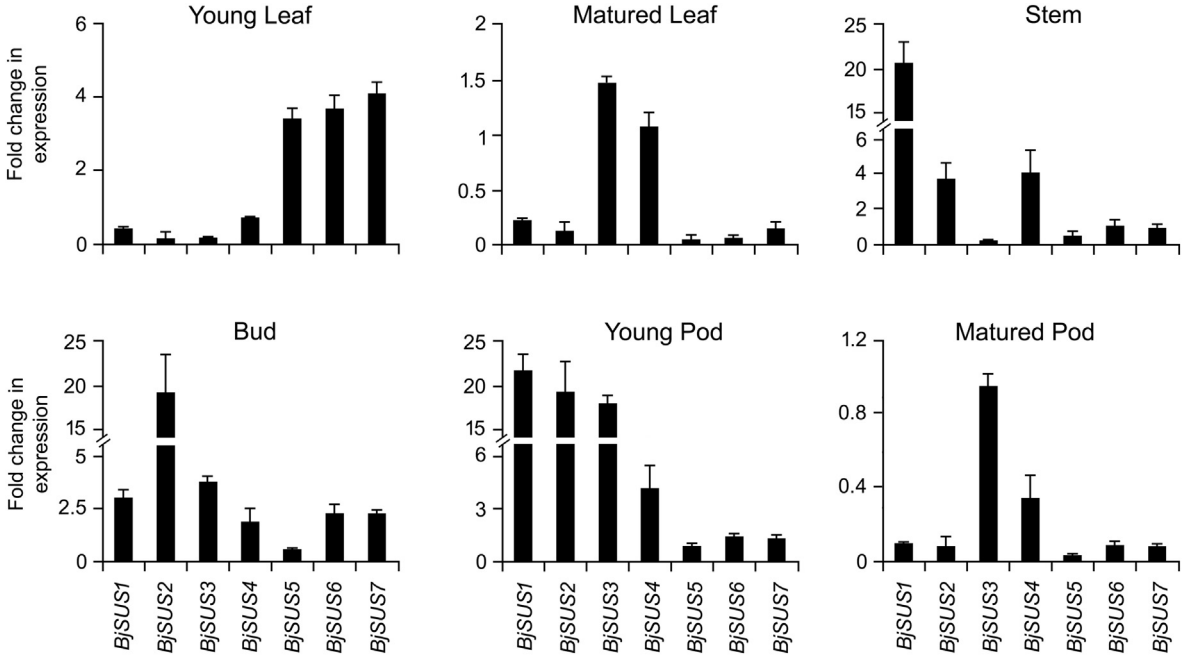

Fig. 5. RT-qPCR based expression analysis of *BjSUS* genes in different tissues. Total RNA was isolated from five-day old plants, young leaves, matured leaves, stems, buds, young pods, and matured pods. The samples were assayed for transcript levels of SUS genes by RT-qPCR. The values were shown as fold change in transcripts with respect to the transcript level in three-day old plants. Values are mean ± SE of three independent biological replicates each with three technical replicates.

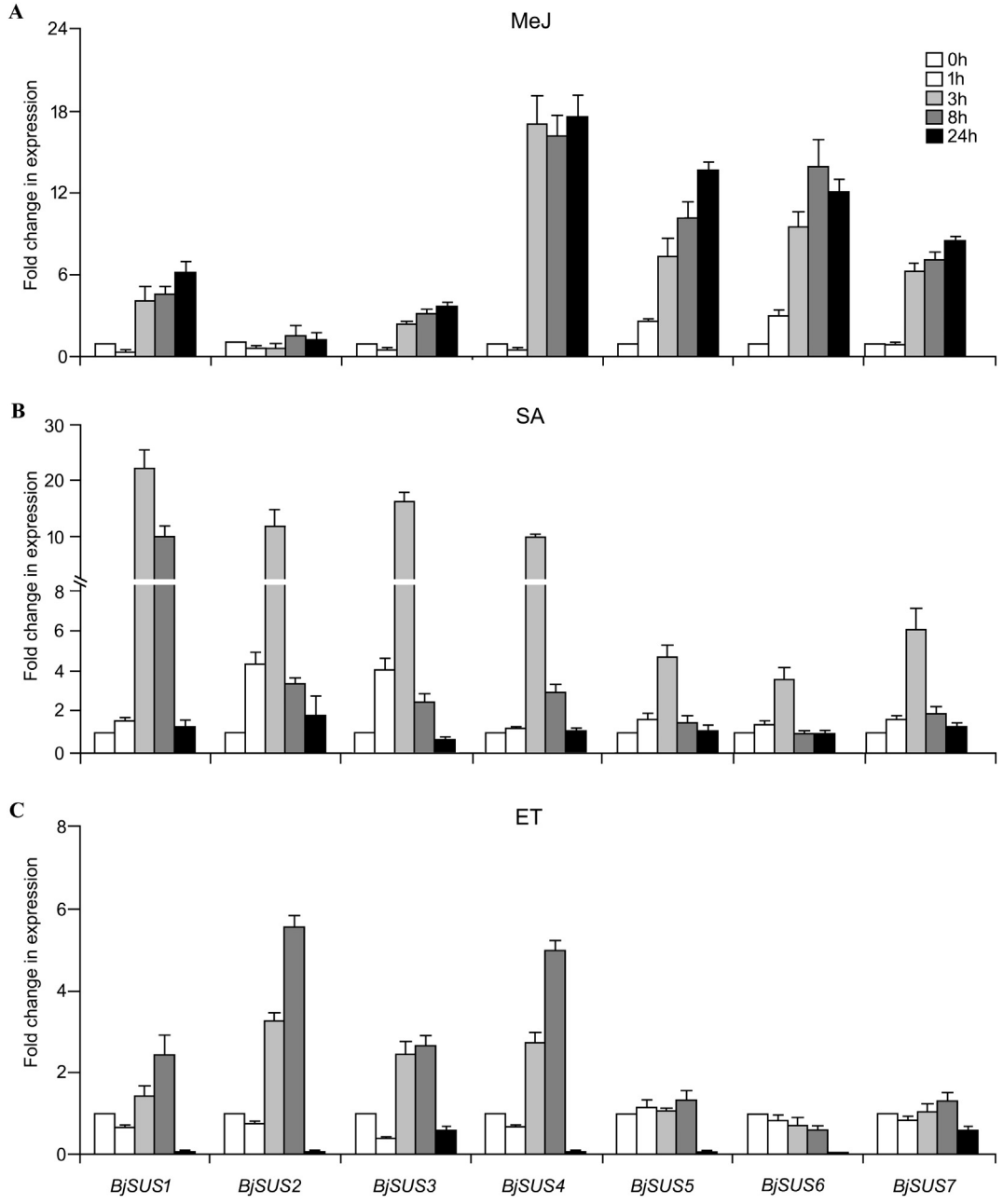

Fig. 6. Expression analysis of *BjSUS* genes in response to stress related hormones. Four-week old *B. juncea* plants were sprayed with A) methyl jasmonate (MeJ), B) salicylic acid (SA) and C) ethylene (ET) in independent experiments and leaf samples were collected at 0, 1, 3, 8, and 24 h. Total RNA was isolated and used for assaying *BjSUS* gene expression by quantitative RT-PCR. Values are mean ± SE of three independent biological replicates each with three technical replicates.

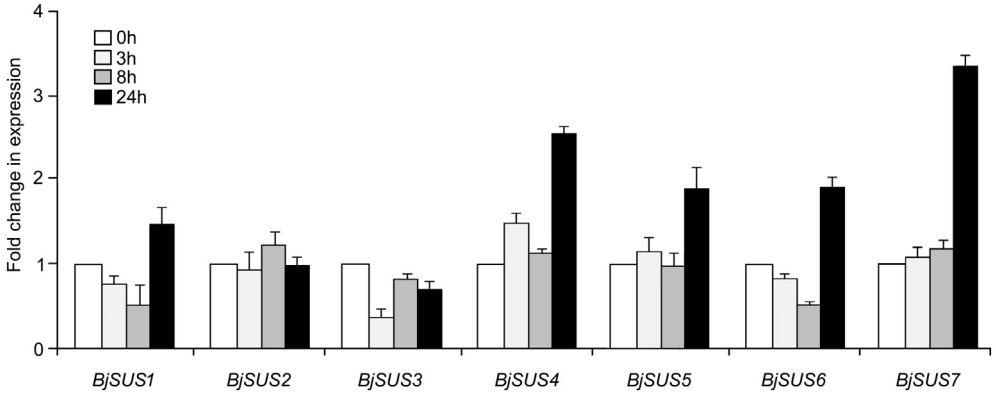

Fig. 7. Expression analysis of *BjSUS* genes in response to wounding. Four week old *B. juncea* plants were mechanically wounded across the mid-rib of the leaves using a haemostat. Total RNA was extracted at 0, 3, 8, and 24 h and used for assaying *BjSUS* gene expression. Values are mean ± SE of three independent biological replicates each with three technical replicates.

experiments and analyzed the data.

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