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IiSDD1, a gene responsive to autopolyploidy and environmental factors in *Isatis indigotica*

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Abstract In plants, stomata play a pivotal role in the regulation of gas exchange and are distributed throughout the aerial epidermis. *SDD1*, a gene isolated from *Arabidopsis thaliana* has been demonstrated to specialize in stomatal density and distribution. In our present study, a comprehensive survey of global gene expression performed by using an *A. thaliana* whole genome Affymetrix gene chip revealed *SDD1* tends to be significantly lower in tetraploid *Isatis indigotica* than in diploid ones. To intensively investigate different SDD1 expression in response to polyploidy, a full-length cDNA clone (*IiSDD1*) encoding SDD1 was isolated from the traditional Chinese medicinal herb *I. indigotica* cDNA library. *IiSDD1* shared a high level of identity with that from *A. thaliana*, containing some basic features of subtilases: D, H and S regions, as well as a substrate-binding site. Real-time quantitative PCR analysis indicated that *IiSDD1* was constitutively expressed in all tested tissues, including roots, stems and leaves, both in tetraploid and diploid *I. indigotica*, and with the highest expression in leaves. In addition, *IiSDD1* was also found to be down-regulated by signalling molecules for plant defence responses, such as abscisic acid (100 μM) and gibberellin (100 mg/L), as well as by environmental stresses including salt, darkness, coldness and drought. Our study, for the first time, indicates SDD1 participates not only in the defense/stress responsive pathways, but also probably involves in plants polyploidy evolution.

Keywords Environmental stresses · *IiSDD1* · *Isatis indigotica* · Polyploidy evolution · Stomata density and distribution

Introduction

Isatis indigotica Fort., belonging to the family Cruciferae, is a biennial herbaceous plant species distributed widely in China [1]. The root of *I. indigotica* (Radix Isatidis), named "Ban–Lan–Gen" as a traditional Chinese medicine, is used for the treatment of influenza, epidemic hepatitis and epidemic encephalitis B for 100 of years in China [2]. As one of the most popular Chinese medicinal herb, the tetraploid *I. indigotica* ($2n = 28$) with better yield and enhanced resistance had been obtained from their natural diploid progenitor ($2n = 14$) through artificial chromosome doubled after 5 years of field selective breeding [3]. A second report by Qiao and Li [4] presenting the results of comparative cultivation of a high-yielding tetraploid line and its diploid parents by evaluating seed and fruit quality as well as compounds accumulation, showed that the results favored the tetraploids. Since extremely limited molecular research of *I. indigotica* has been reported, the underlying molecular basis of the significantly qualitative difference

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between tetraploid and diploid *I. indigotica* remains unclear.

Arabidopsis thaliana, of which genome sequencing project has been finished by 2000 [5], is a well studied organism model. A comprehensive survey of global gene expression can therefore be implemented by *A. thaliana* whole genome Affymetrix gene chip (ATH1), containing 22,810 probe sets, which can respond to about 80% of the gene sequences on a single array. This method has been successfully used in findings in *Brassica*, *Arabidopsis* and wheat [6–8]. In our earlier research, it was used to survey the expression changes between tetraploid and diploid *I. indigotica* on a large scale, and changes in gene expression between tetraploid and diploid *I. indigotica* has also been observed by applying microarray analysis [9]. By analyzing the expression profile, it was found that the expression of SDD1 of *I. indigotica* (*Ii*SDD1) tends to be significantly lower in tetraploid *I. indigotica* than in diploid ones.

*SDD*1 is a gene involved specifically in the regulation of stomatal differentiation and pattern formation. It was firstly identified from *A. thaliana*, encoding a subtilisin-like Ser protease [10]. In the *Arabidopsis* mutant *stomatal density and distribution1-1* (*sdd1-1*), which lacks functional SDD1 protein, the establishment of the stomatal pattern is disrupted, resulting in stomata clustering and twofold to fourfold increase in stomatal density, as well as high performance in photosynthesis [11]. In contrast, *SDD1* overexpression in the wild type leads to a phenotype opposite to that caused by the *sdd1-1* mutation, with a twofold to threefold decrease in stomatal density and the formation of arrested stomata [12]. Since physiological role of SDD1 protein is particularly related to adjusting gas exchange and suiting surrounding environmental condition, the different expression level of *Ii*SDD1 in diploid and tetraploid *I. indigotica* might represent differences in the quality.

In this paper, we first report on cloning and characterization of a novel *IiSDD1* from *I. indigotica*, and examine expression levels of *IiSDD1* in different tissues and under various stresses. The different expression profile may reveal the reason for why the tetraploids are superior to the diploids.

Materials and methods

Plant materials

Two-month-old leaves of autotetraploid and diploid *I. indigotica* Fort., collected from the School of Pharmacy, Second Military Medical University, Shanghai, China, and identified by Professor Hanming Zhang of School of Pharmacy (Second Military Medical University) were used for various treatments, DNA and RNA isolation.

Various treatments

The leaves from 2-month-old tetraploid *I. indigotica* seedlings grown in small plastic flowerpots were sprayed with solution of 100 μM abscisic acid (ABA), 100 mg/L gibberellin (GA) and 100 mM NaCl, respectively followed by RNA isolation at the time of 24 h post-treatments. Another set of control plants were similarly treated with distilled water. For dark and cold treatment, the seedlings were kept in dark and at 4°C for 24 h, respectively. In addition, the 2-month-old seedlings were drought-stressed by terminating irrigation for 1 week.

RNA and DNA isolation

Roots, leaves and stems of 2 month-old tetraploid and diploid *I. indigotica* seedlings, as well as tetraploid *I. indigotica* leaves with various treatments were used for RNA isolation. Total RNAs were extracted using TRIzol Reagent (GIBCO BRL) according to the manufacturer's instruction [13]. The genomic DNA of *I. indigotica* was isolated using a Cetyl trimethyl ammonium bromide (CTAB)-based method [14]. The quality and concentration of RNA and DNA samples were examined by EB-stained agarose gel electrophoresis and spectrophotometer analysis.

ATH1 microarray assay

Microarray hybridization and related data analysis strictly followed the methods as described by Lu et al. [9]. Particular attention had been paid to genes with significantly increased expression in the autotetraploid sample such as phenylalanine ammonia-lyase gene (designated as *IiPAL*) [15] and calcium-dependent protein kinase gene (designated as *IiCPK2*) [16]. On the other hand, several groups of data among near 4.3% chosen and meaningful analysis results from all probe sets also indicated that there was opposite variance in gene expression between tetraploid and diploid samples. One group of the most noticeable data is focus on the probe set of 264319_at, locus identifier At1g04110 (Table 1), i.e., the *SDD1* gene identified by map-based cloning which encodes a subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *A. thaliana* [10].

Molecular cloning of *IiSDD1* full-length cDNA

Molecular cloning of *IiSDD1* from *I. indigotica* was carried out by Rapid amplification of cDNA ends (RACE) method using a SMART TM RACE cDNA Amplification Kit (Clontech, USA).

For 3′ RACE of *IiSDD1*, about 100 ng of total RNA was reverse transcribed with 3′-CDS primer by BD PowerScript

Table 1 Functional classification of some of significantly up-regulated and down-regulated genes in autotetraploid

Up-regulated genes					Down-regulated genes				
Functional characterization	Probe set	AGI	Ratio of autotetraploid vs. diploid	Change *P*-value*	Functional characterization	Probe set	AGI	Ratio of diploid vs. autotetraploid	Change *P*-value*
Myb family transcription factor	259751_at	At1g71030	1.6	0.999508	Stomatal density and distribution	264319_at	At1g04110	5.3	0.999417
LRR transmembrane protein kinase family	259958_at	At1g53730	22.6	0.999654	Subtilisin-like protease	248961_at	At5g45650	2.5	0.999226
Calcium-dependent protein kinase, CDPK	251636_at	At3g57530	16	0.998799	Low temperature and salt responsive protein	253627_at	At4g20850	9.2	0.997635
Calcium-dependent protein kinase isoform	246955_at	At5g04870	3.2	0.996645	Receptor-like kinase, similar to TMK	257202_at	At3g46840	2	0.999382
Caffeic acid *O*-methyltransferase	248200_at	At5g54160	3.7	0.997968	Putative fibrillin	255364_s_at	At4g04020	11.3	0.999977
Phenylalanine ammonia-lyase 1	263845_at	At2g37040	2.5	0.996959	Putative protein	251221_at	At3g62550	3.7	0.997968

* Change *P*-value > 0.9955

Reverse Transcriptase (Clontech, USA). Universal Primer A Mix (UPM), Nested Universal Primer A (NUP) (Clontech, USA), gene-specific primers SDD1-3′-1 [5′-TCGGC GTGCTTGATACTGGAGTTTGG-3′, as 3′ RACE first amplification primer] and SDD1-3′-2 [5′-ATCTGTGCA GCTGGTAACAACGGTCC-3′, as 3′ RACE nest amplification primer] were used. The PCR was conducted in accordance with the protocol provided by the manufacture (Clontech, USA). The nested amplified PCR product was purified and cloned into PMD18-T vector (TaKaRa, Japan) and then sequenced.

For 5′ RACE of *IiSDD1*, about 100 ng of total RNA was reverse transcribed with 5′-CDS primer and SMART TM II A Oligonucleotide (Clontech, USA). Universal Primer A Mix (UPM) and gene-specific primers SDD1-5′-anti1 (5′-ACTCCACGGTCACATATCACCATCTTGC-3′, as 5′ RACE amplification primer) were used. The PCR was conducted in accordance with the protocol provided by the manufacture (Clontech, USA). The amplified PCR product was purified and cloned into PMD18-T vector (TaKaRa, Japan) and then sequenced.

By aligning and assembling the products of 3′ and 5′ RACE, the full-length *IiSDD1* from *I. indigotica* was deduced and subsequently amplified by proof-reading PCR amplification with primers *Ii*SDD1-F (5′-AAAGCGAA ACTTTTCTTCACTCCTTC-3′) and *Ii*SDD1-R (5′-TCAA TGATTCTTTGAGGTTACCGAGATTGG-3′). The PCR procedure was conducted under the following conditions: 5 min at 94°C, 5 cycles (35 s at 94°C, 35 s at 65°C, 3 min at 72°C), 35 cycles (35 s at 94°C, 35 s at 60°C, 3 min at 72°C) and 10 min at 72°C. The amplified PCR product was purified and cloned into PMD18-T vector (TaKaRa, Japan) followed sequencing.

Isolation of genomic sequence of *IiSDD1*

In order to detect whether there exist introns within the *IiSDD1*, PCR amplification was carried out using the same reaction system as that for the cloning of the full-length cDNA except that the template was substituted by 1.5 μg of total genomic DNA. The PCR procedure was conducted under the following conditions: 5 min at 94°C, 5 cycles (35 s at 94°C, 35 s at 70°C, 3 min at 72°C), 5 cycles (35 s at 94°C, 35 s at 65°C, 3 min at 72°C), 35 cycles (35 s at 94°C, 35 s at 58°C, 3 min at 72°C) and 10 min at 72°C. The PCR product was cloned into the pMD18-T-vector (TaKaRa) and then sequenced.

Genome Walker DNA libraries were constructed using the Universal Genome Walker Kit (Clontech, USA). The genomic DNA was completely digested with different blunt-end restriction enzymes (*Eco*RV, *Pvu*II, *Stu*I and *Dra*I) (Takara, Japan) and DNA fragments were ligated

separately to the Genome Walker adaptor using the DNA Blunting Kit (Takara, Japan).

The amplification of upstream sequence of the known sequence consists of two PCR amplifications. The primary PCR uses the outer adaptor primer AP1 provided in the kit and an outer, gene specific primer 5′ GSP1 (5′-GAAGA AAGACGATGCTGAGAAAATAAG-3′). The amplification was performed in a GeneAmp PCR System 2400 for 7 cycles with 25 s at 94°C, 3 min at 72°C and then for 32 cycles with 25 s at 94°C, 3 min at 67°C. After the final cycle, the amplification was extended for 7 min at 67°C. The primary PCR mixture was then diluted and used as a template for nested PCR with the nested adaptor primer AP2 provided in the kit and a nested gene-specific primer 5′ GSP2 (5′-TGCTGAGAAAATAAGTTTTGGGTTCC A-3′). The conditions of the PCR reaction were the same as mentioned above. The PCR product was cloned into the pMD18-T-vector (TaKaRa) and then sequenced.

Sequence analyses of *IiSDD1*

ORF translation and Genbank BLASTs were carried out on NCBI (http://www.ncbi.nlm.nih.gov/). Alignment of sequences of the characteristic domains of various subtilisin-like serine proteases was performed strictly as described by Berger and Altmann [10]. Promoter was analyzed using Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Expression profile of *IiSDD1* in different tissues and under various stresses

Total RNA from different tissues (roots, stems and leaves) of two forms of *I. indigotica*, as well as tetraploid *I. indigotica* with various stresses was reversely transcribed by using AMV reserve transcriptase (Takara, Japan) to generate cDNA. Gene specific primers (5′-TTCACCCTAAT AGCGAAACCG-3′ and 5′-AAATCCTTCAAATGCAGA GCC-3′) were designed according to the corresponding sequences of *I. indigotica*. Partial of polyubiquitin gene was amplified with primers (5′-ACCCTCACGGGGAAGACC ATC-3′ and 5′-ACCACGGAGACGGAGGACAAG-3′) as a control. The Real-time quantitative PCR was performed according to manufacturer's instruction (Takara, Japan) under the following condition: 30 s pre-denaturation at 95°C, 1 cycle; 10 s denaturation at 95°C, 20 s annealing at 59°C, 20 s collection fluorescence at 72°C, 40 cycles. The products of real-time quantitative PCR were run on 1.5% agarose gel electrophoresis and showed an equal-sized band as predicted. Quantification of the gene expression was done with comparative CT method. Each data represents the average of three experiments.

Results and discussion

Microarray analysis of ploidy-responsive *IiSDD1*

Analysis of genome duplication event in *Arabidopsis* revealed that some classes of genes, such as those involved in transcription and signal transduction, had been preferentially retained, whereas other classes, including those involved in DNA repair and those for organellar proteins, had been preferentially lost [17]. Consequently, it may have been expected that genes in tetraploid *I. indigotica* would have higher expression levels than its diploid parents because of dosage effects. However, microarray analysis indicated *IiSDD1* expression in tetraploid *I. indigotica* was down-regulated by 5.3-fold compared to its diploid progenitor (Table 1).

Previous study had demonstrated that the lack or silence of functional SDD1 protein would result in elevation of stomata density in the leaves of the plants, thus, prompt higher stress tolerance and higher growth performance. In the *Arabidopsis* mutant *Sdd1-1*, which lacks functional SDD1 protein, increased stomatal densities were observed, enabling 30% higher CO_2 assimilation rates compared to the wild type when exposed to high light intensities. And after 2 days under high light conditions leaves of *sdd1-1* accumulated 30% higher levels of starch and hexoses than wild-type plants [11]. In potato plant (*Solannum tuberosum*), the expression of the RNAi construct of the *SDD1* gene increases the number of stomata in the leaves of the plants. Under high light or high temperature conditions, these plants showed an increased tuber production compared to the untransformed parent line (http://gmoinfo.jrc.ec.europa.eu/gmp_report.aspx?CurNot=B/DE/04/159). Therefore, it is reasonable to believe lower expression level of *Ii*SDD1 in tetraploid *I. indigotica* is one of reasons for why the tetraploids are superior to the diploids. However, the underlying molecular processes that how genome duplication induced SDD1 silence is not clear, which is now under intensive investigation in our laboratory.

Molecular cloning and characterization of *IiSDD1*

The cDNA clone of *IiSDD1* (GenBank Accession No.: DQ407741) was 2,615 bp long and the sequences from two forms of *I. indigotica* (tetraploid and diploid) were found to be exactly same. *IiSDD1* has a 2,334 bp ORF encoding a predicted protein of 778 amino acids, with a predicted molecular mass of 83.9 kDa (data not shown). Similar to *SDD1* from *A. thaliana* [10], there was no intron detected in the genomic sequence of *IiSDD1*. Comparison of *Ii*SDD1 protein sequence with that of *A. thaliana* showed that the amino-acid homology was 94.2%, the high degree of similarity indicated that they shared similar functions. The typical domains of subtilisin-like serine proteases

D region

			*	
Ii SDD1	139	GQGTIIGVL	DTGVWPESPS	FGDTGM
SDD1	137	GQGTIIGVL	DTGVWPESPS	FDDTGM
Ag12	136	GEDVIIGVI	DSGVWPESDS	FKDDGM
LeP69	137	GKGVIIGVI	DTGILPDHPS	FSDVGM
Cucumisin	131	ESNIVVGVL	DTGIWPESPS	FDDEGF
Furin/PACE	144	GHGIVVSIL	DDGIEKNHPD	LAGNYD
PC1/PC3	158	GKGVVITVL	DDGLEWNHTD	IYANYD
KEX2	166	GAGVVAAIV	DDGLDYENED	LKDNFC
BPN'	130	GSNVKVAVI	DSGIDSSHPD	LKVAGG

H region

		*		
Ii SDD1	216	ISARDSTGHG	THTASTAGGS	SVSMA
SDD1	214	ISARDSTGHG	THTASTVGGS	SVSMA
Ag12	208	NSARDTLGHG	THTASTAAGN	YVNGA
LeP69	195	GSPIDDDGHG	THTASTAAGA	FVNGA
Cucumisin	196	NGPRDTNGHG	THTASTAAGG	LVSQA
Furin/PACE	186	YTQMNDNPHG	TRCAGEVAAV	ANNGV
PC1/PC3	200	YDPTNENKHG	TRCAGEIAMQ	ANNHK
KEX2	205	KPRLSDDYHG	TRCAGEIAAK	KGNNF
BPN'	163	NPFQDNNSHG	THVAGTVAAL	NNSIG

Substrate binding site

			*	
*Ii*SDD1	315	EQGISVVCAA	GNNGP	
SDD1	313	ERGISVICAA	GNNGP	
Ag12	307	EKGVVVSTSA	GNAGP	
LeP69	295	ERGILVSCSA	GNNGP	
Cucumisin	296	ERGILTSNSA	GNGGP	
Furin/PACE	284	GLGSIFVWAS	GNGGR	
PC1/PC3	298	GKGSIFVWAS	GNGQR	
KEX2	303	SKGAIYVFAS	GNGGT	
BPN'	251	ASGVVVVAAA	GNGGT	

S region

		*		
*Ii*SDD1	549	VMSGTSMS	CPHVSGITAL	IRS
SDD1	547	VMSGTSMS	CPHVSGITAL	IRS
Ag12	532	MVSGTSMA	CPHASGVAAL	LKA
LeP69	527	IISGTSMS	CPHLSGVRAL	LKS
Cucumisin	520	IISGTSMS	CPHITGIATY	VKT
Furin/PACE	363	SHTGTSAS	APLAAGIIAL	TLE
PC1/PC3	377	THTGTSAS	APLAAGIFAL	ALE
KEX2	380	SHGGTSAA	APLAAGVYTL	LLE
BPN'	323	AYNGTSHA	SPHVAGAAAL	ILS

Fig. 1 Alignment of the sequences of the characteristic domains of various subtilisin-like serine proteases and *Ii*SDD1. The D, H, and S regions, which together form the catalytic triad, and the substrate-binding site of different subtilisin-like serine proteases—SDD1 from *A. thaliana* [10], Ag12 from *A. glutinosa* [34], LeP69 from tomato [35], cucumisin from melon [36], FURIN/PACE [37] and PC1/PC3 [38] from human, KEX2 from *Sacharomyces cerevisiae* [39], and subtilisin BPN' from *B. amyloliquefaciens* [40] are shown. Three characteristic catalytic domains (*D*, *H* and *S* regions) and a substrate-binding site (*N*) are marked with an *asterisk*

were also found in *Ii*SDD1, containing three characteristic catalytic domains (D, H and S regions) and a substrate-binding site (Fig. 1). Proteases of this type have been demonstrated to activate precursors of hormones, growth factors, or receptors involved in the control of various developmental processes [18–20]. Accordingly, SDD1 has been proposed to process factor(s) involved in the control of stomatal development [10].

About 1.5 kb 5′ flanking fragment has been obtained from *I. indigotica* genome using genome walking technology. PlantCARE database search program was used to predicate the putative promoter motifs in this region. TATA box and CAAT box are common elements existing in the 5′ flanking region of eukaryotic genes. They are critical for eukaryotic transcription initiation [21, 22]. According to the result, several sequences analogous to TATA box were found in *IiSDD1* and the most probable TATA box was located at −30. In addition, there were 15 CAAT boxes identified (data not shown).

Beside the core *cis*-acting elements described above, several *cis*-acting elements with relation to stress resistance were also found in *IiSDD1* promoter: (1) one site of ABRE, *cis*-acting element involved in abscisic acid responsiveness, TACGTG; (2) one site of HSE, *cis*-acting element involved in heat stress responsiveness, AAAAAATTTC; (3) one sites of LTR, *cis*-acting element involved in low-temperature responsiveness, CCGAAA; (4) one site of MBS, MYB binding site, CGGTCA; (5) three site of TC-rich repeats, *cis*-acting element involved in defense and stress responsiveness, ATTTTCTTCA; (6) three sites of TCA-element, *cis*-acting element involved in salicylic acid responsiveness, GAGAAGAATA; (7) one site of circadian, *cis*-acting regulatory element involved in circadian control, CAAAGATATC. In addition, a crowd of light responsiveness-related elements were also found in this region, including 1 ACE, 2 AE-box, 1 Box I, 1 CATT-motif, 2 G-Box, 4 G-box, 1 GA-motif, 2 GAG-motif, 1 GT1-motif, 1 I-box, 1 Sp1, 3 TCT-motif (data not shown). Since SDD1 is known as a functional protease involved in the regulation of stomatal density and distribution [10], the existence of these *cis*-acting elements in this region suggested that signaling components (e.g. abscisic acid and salicylic acid), as well as environmental factors (e.g. temperature and light intensity) may affect stomatal density or distribution by regulating the expression of SDD1. Coincidently, this has been supported by several previous reports, which indicated that stomatal density is modulated in response to environmental factors such as humidity [23], temperature [24], or light intensity [11, 25–27].

Tissue-specific expression profile of *IiSDD1* in tetraploid and diploid *I. indigotica*

Total RNAs isolated from roots, stems, and leaves of two kinds of *I. indigotica* (tetraploid and diploid) seedlings were used to investigate tissue-specific expression profiles

of *IiSDD1* by real-time quantitative PCR. Results indicated that *IiSDD1* expressed constitutively in all examined tissues, and the strongest expression pattern was seen in leaves, followed by stems, with the lowest in roots, both in tetraploid and diploid *I. indigotica*, which was consistent with that found in *A. thaliana* [10]. For diploid *I. indigotica*, the expression level of *IiSDD1* in leaf were 1.3 and 1.5-fold higher than that in stem and root, respectively, whereas for tetraploids, the fold was 1.1 and 1.2. Furthermore, it was also found that *IiSDD1* expression in tetraploid *I. indigotica* was generally lower than its diploid progenitor (Fig. 2a), paralleled with the findings observed in previous microarray analysis.

Effects of applied stresses on *IiSDD1* transcription in tetraploid *I. indigotica*

It is well-known that stomatal differentiation and pattern formation in plants is set according to the environmental conditions prevailing during leaf development, they adapt to local and global changes on all timescales from minutes to millennia [28]. SDD1 is proposed to act as a processing protease involved in the mediation of a signal that controls the stomatal density and distribution [10], therefore, it is speculated that SDD1 may also be modulated by environmental factors (stresses) such as ABA, GA, salt, darkness, coldness or drought. In the present study, real-time quantitative PCR was performed to investigate the expression profiling of *IiSDD1* in tetraploid *I. indigotica* under various stresses.

The plant hormone ABA plays important roles in seed maturation and dormancy and in adaptation to a variety of environmental stresses. One of important role of ABA is to accelerate to close stomata [29, 30]. In addition, there has been report indicating ABA-treated *Tradescantia virginiana* plants had significantly smaller stomata and higher stomatal density in their lower epidermis, compared with non-treated plants [31]. In this study, exogenous ABA (100 μM) was found to dramatically down-regulate *IiSDD1* transcript (5-fold lower than untreated control) of

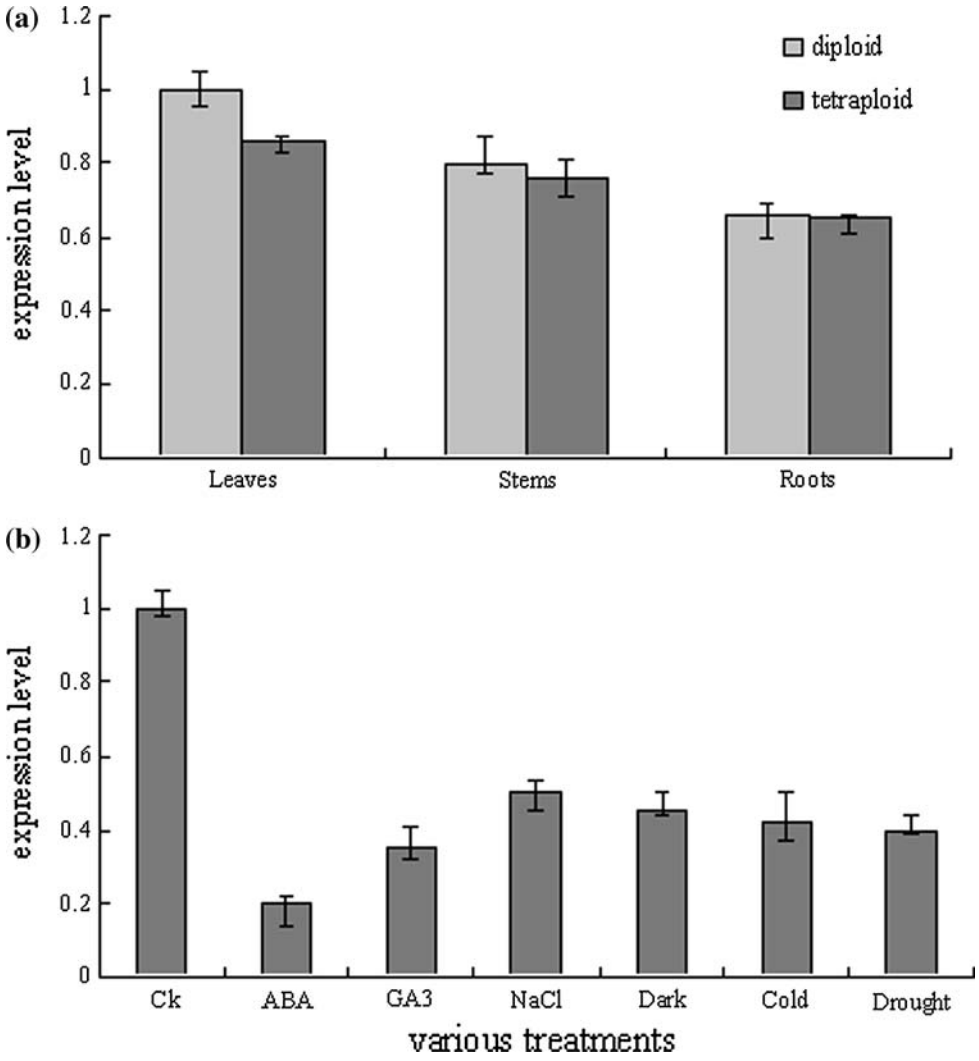

Fig. 2 Expression profiling analysis of *Ii*SDD1 (**a**) in different tissues of tetraploid and diploid *I. indigotica*, and (**b**) under different treatments (ABA, GA, NaCl, salt, darkness, coldness and drought) in tetraploid *I. indigotica*, with untreated plant as control. Data represents the mean values ± SE of three replicates

tetraploid *I. indigotica* after 24-h treatment (Fig. 2b), suggesting stomatal density or distribution of *I. indigotica* may also be modulated by ABA.

GA has been suggested the main signal inducing stomata formation in *Arabidopsis* hypocotyls [32], i.e., treatment of GA could directly result in elevation of stomatal density of plant hypocotyls. However, effect of GA on stomatal density or distribution of plant leaves has not been elucidated ever. In this study, leaves of tetraploid *I. indigotica* were treated with GA (100 mg/L) and harvested for RNA isolation at the time of 24 h after treatment. Real-time quantitative PCR analysis revealed that *IiSDD1* transcripts were down-regulate about three-fold compared to untreated control, suggesting the plant hormone GA may also involve in the modulation of stomatal density or distribution of plant leaves.

In addition, *IiSDD1* transcripts were found responsive to environmental stresses such as NaCl (100 mM), darkness, coldness and drought at various degrees. Compared to untreated control, *IiSDD1* transcript levels were down-regulated by 2, 2.2, 2.4, 2.5-fold, respectively (Fig. 2b). And the negative response of *Ii*SDD1 under dark and cold stresses was coinciding with the observations that light-responsive and heat stress responsiveness *cis*-elements existed in its promoter region.

To sum up, according to previous reports [10, 11, 33], it may have been expected that, during leaf development of tetraploid *I. indigotica*, the down-regulation or silence of SDD1 caused by these environment stresses (ABA, GA, salt, darkness, coldness or drought) would result in elevation of the number of plant stomata, thus prompt higher growth performance (e.g. improved water use efficiency, higher photosynthetic performance), as well as higher stress tolerance. However, this remains to be investigated in further study. Similarly, *Ii*SDD1 silence phenomenon caused by genome duplication thus suggested tetraploid *I. indigotica* exhibited a much stronger ability of adaptation than diploid one by altering SDD1 mRNA level. Evolution was a process of adapting to environment. Our work implies a key advantage of "fast-evolution" by artificial breeding. Why is the polyploid form more adaptable than the diploid one? A possible hypothesis is: the changes of critical genes could regulate the physiological process faster and more significantly. *SDD1* is one of such genes. Our work, for this first time, proposed that SDD1 participated not only in the defense/stress responsive pathways, but also probably implicated in plants polyploidy evolution.

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