Pak. J. Bot., 55(4): 1383-1388, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-4(11)

CLONING AND EXPRESSION ANALYSIS OF *DELAY OF GERMINATION 1* GENE IN BUFFALOBUR (*SOLANUM ROSTRATUM* DUNAL)

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Abstract

DOG1 (*DELAY OF GERMINATION 1*) is a specific gene related to seed dormancy and the expression level determines the depth of seed dormancy. However, the site and dynamics of *DOG1* expression in *Solanum rostratum* Dunal (buffalobur), a highly dangerous exotic weed, is still largely unknown. Buffalobur has strong competitiveness and adaptability, it can only be propagated by seed and strong seed dormancy is the main constraint for its control and eradication. In this paper, *DOG1* gene was cloned and the expression characteristics were analyzed at different developmental stages of buffalobur. The results showed that *SrDOG1* transcription is seed specific, and the embryo is the main expression site. Low temperature can significantly promote *SrDOG1* transcription level in seeds, while seed imbibition can decrease the expression. In the process of plant growth, *SrDOG1* was expressed in all the reproductive organs as well as in roots, stems and leaves, although the expression is relatively low. Knowledge obtained here could help to elucidate the expression characteristics of *SrDOG1* and provide a basis for revealing the role of *DOG1* in regulating seed dormancy in buffalobur.

Key words: Buffalobur, Seed dormancy, *DOG1*, Gene expression.

Introduction

Seed dormancy and its dynamics usually depends on the interaction between genetic and environmental factors, but mainly by its internal genetic factors. The regulatory elements including a series of genes which related to ABA (abscisic acid) and GA (gibberellic acid) signalling pathways (Footitt *et al*., 2011; Shu *et al*., 2016; Klupczynska & Pawłowski, 2021). Studies have shown that *DELAY OF GERMINATION 1* (*DOG1*) played a crucial role in regulating seed dormancy, it has been classified as a major quantitative trait locus (Bentsink *et al*., 2006; Murphey *et al*., 2015).

DOG1 is required for seed dormancy and is a key component for plants to adapt to the environment, blocking the *DOG1* gene resulted in a complete absence of dormancy (Kronholm *et al*., 2012; Graeber *et al*., 2014). Environmental signals, mainly temperature, during dormancy cycling of seeds affect the *DOG1* expression and the depth of seed dormancy is highly associated with the protein level (Chiang *et al*., 2011; Kendall *et al*., 2011; Nakabayashi *et al*., 2012). It indicated that *DOG1* acts in concert with ABA to enhance seed dormancy, and may integrate into the ABA-mediated sugar-signalling pathway (Teng *et al*., 2008; Carrillo-Barral *et al*., 2015). Furthermore, *DOG1* imposes seed dormancy by enhancing the seed coat through regulating the expression of GA metabolic genes (Graeber *et al*., 2014). In addition, *DOG1* could also induce the secondary dormancy and have a certain relationship with the ABA signalling pathway (Murphey *et al*., 2015; Nee *et al*., 2015; Dekkers *et al*., 2016).

Buffalobur (*Solanum rostratum* Dunal) is an annual weed of the family Solanaceae. It mainly infests cotton, corn, soybean and other crops (Bassett & Munro, 1986; Abu-Nassar *et al*., 2022). Currently, buffalobur has spread to nearly 20 countries in Asia, Europe, Africa and Oceania (Wei *et al*., 2010; Abu-Nassar *et al*., 2022). Its strong adaptability and reproductive ability seriously affected the diversity of the ecosystem in invaded area. In addition, buffalobur contains neurotoxins solanine, it is also the host of potato leaf roll virus and Colorado potato beetle (*Leptinotarsa decemlineata*), and the latter one is the most destructive quarantine pest for many crops (Bah *et al*., 2004; Wei *et al*., 2010; Huang *et al*., 2017; Zhao *et al*., 2019).

Buffalobur is a prolific seed producer and a single plant can yield up to tens of thousands of seeds. By taking measures to reduce the soil seed bank, we can effectively control the spread of weed infestation (Buhler *et al*., 1997; Yang *et al*., 2019; Wisnoski & Shoemaker, 2022). Buffalobur has a strong seed dormancy and control the germination of seeds to block their propagation will be an important strategy for sustainable weed management.

Despite *DOG1* played an important role in seed dormancy, its expression dynamics and participation in a conserved dormancy mechanism is still unclear. The objective of this study is to clone the *DOG1* gene of buffalobur and analyze the expression pattern of *DOG1* gene with regard to seed dormancy. It will be helpful to disclose the molecular mechanism of *DOG1* in regulating seed dormancy and provide a basis for implementation of suicidal germination strategy in weed management.

Materials and Methods

Plant materials: The buffalobur seed samples of different years (2007, 2008, 2009, 2010, 2011 and 2013) were collected from the suburb in Beijing (40°24′ N, 116°50′ E). The seeds of 2013 were used in most experiments and

were soaked in water for 24 h, and then incubated in Petri dishes at 30°C in dark. Germinated seeds with different radicle length were collected in this period. The remaining seeds were planted in nutrition pot filled with peat and loam (v:v=1:1). Each pot had three to five plants. The pots were placed in the greenhouse with a day/night temperature of 28/22°C and a light/dark cycle of 12/12 h. The root, stem, leave, flower and fruit samples were collected at different growth stages. In addition, the seeds were dissected to collect the endosperm cap, endosperm and embryo, respectively.

***DOG1* gene cloning:** Using the *DOG1* sequences of *S. lycopersicum* (*SlDOG1* XM_010319033.2) and *S. tuberosum* (*StDOG1* XM_015313760) as reference sequences to design the primers (Fd: 5'-TCAACACGAAGAACTAACCGAACT-3'; Rd: 5'-CTCCTTATGCTGCTGATCCCTCT-3') for *DOG1* gene of buffalobur (*SrDOG1*). Buffalobur leaves were collected from 3-5 plants for DNA extraction which was performed with Tiangen DNA Secure Plant Kit. PCR system (20 µL total volume) included 1 µL DNA, 1 µL of both forward and reverse primers, 10 µL Taq 2× master mix and 7 µL sterilized deionized water. PCR procedures were as follows: 10 min predenaturation at 95°C; 30 s keeping at 95°C, 30 s annealing at 55°C, 50 s elongation at 72°C, totally 30 cycles; and final 10 min extension at 72°C. The fragments were analyzed on 1% agarose 1× TAE gels, stained with EB (ethidium bromide), and then gel purified by TIANgel Midi Purification Kit (Tiangen Biotech Beijing CO., LTD).

Ligation of purified product and *pEASY*-T1 vector were conducted with *pEASY*®-T1 cloning kit, and transformed into resistant competent cell of *Trans*1®-T1 Phage (TransGen Biotech Beijing Co., LTD). Then, the recombinant plasmid was isolated and verified by PCR, and sequenced by TSINGKE Biological Technology Beijing Co., LTD. The positive results were analyzed by Vector NTI software. The complete sequence of *SrDOG1* gene was obtained by using Genome Walking Kit (TaKaRa biotechnology Dalian CO., LTD.).

Homology analysis: Neighbor-joining method was used to establish the phylogenetic tree in MEGA 6 software. Protein sequence of the DOG1 was downloaded from the NCBI, a total of 25 species, including *Arabidopsis thaliana* (*AtDOG1* AED95304.2), *Brassica napus* (*BnDOG1* AJE25535.1, *BnDOG1a* AJE25536.1, *BnDOG1b* AJE25537.1), *Brassica rapa* subsp. *pekinensis* (*BrDOG1* ACV41802.1), *Capsella rubella* (*CrDOG1* XP_006281774.1), *Dorcoceras hygrometricum* (*DhDOG1* KZV51842.1), *Hordeum vulgare* (*HvDOG1L1* BAJ05340.1), *Lactuca sativa* (*LsDOG1* ALS87232.1, *LsDOG1-1* ALS87233.1, *LsDOG1-2* ALS87234.1), *Lepidium papillosum* (*LpDOG1a* AGG09198.1, *LpDOG1c* AGG09199.1), *L. sativum* (*LesaDOG1* ACV41800.1), *Nicotiana sylvestris* (*NsDOG1* XP_009762839.1), *Oryza sativa* (*OsDOG1* EAY72627.1), *Populus trichocarpa* (*PtDOG1* XP_002310543.2), *Sesamum indicum* (*SiDOG1* XP_011085982.1), *Sisymbrium officinale* (*SoDOG1* AIN76390.1), *Solanum lycopersicum* (*SlDOG1* XP_010317335.1), *S. pennellii* (*SpDOG1* XP_015065335.1), *S. tuberosum* (*StDOG1* XP_015169246.1), *Theobroma cacao* (*TcDOG1* XP_007025355.1), *Triticum aestivum* (*TaDOG1* BAO79416.1) and *Zea mays* (*ZmDOG1* ACG36725.1).

Analysis of *SrDOG1* gene expression: Using the β-actin gene of buffalobur (*SrACT*) as a reference gene. Based on *SrDOG1* and *SrACT* coding sequences, the primers designed for *SrDOG1* are 5'-GGCAAGTAATTCAAA TAACAGTAAGCG-3' (forward) and 5'-CTACTGATTA TGCTCCTGATCCCT-3' (reverse); for *SrACT* are 5'-TGACAATGGAACTGGAATGGT-3' (forward) and 5'-ACAATGAAAGCACA GCCTGGA-3' (reverse). The RNA was extracted from seeds and leaves of buffalobur according to the manufacturer's instructions, and cDNA was produced using *TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech Beijing Co., LTD). PCR system and procedure are the same as the above, except that the template is cDNA. We examined the PCR products using gel electrophoresis and ligated it into *pEASY*®-Blunt Zero Cloning Vector (TransGen Biotech Beijing Co., LTD). Then, the recombinant plasmids transformed into the resistant competent cell. The positive clones were screened and cultured in LB (10 mL) with ampicillin for 5 h. The recombinant plasmids were obtained by plasmid DNA extraction kit of TSINGKE Biological Technology Beijing Co., LTD. The purity and concentration of plasmids were determined by UV spectrophotometer, and the copy number can be obtained. A series of gradient dilution standard plasmids (threefold serial dilution) were used to construct a standard curve, the amplification efficiency (90%-110%) and correlation coefficient (≥0.99) were used to determine the quality of the standard plasmid. The quantitative real-time PCR (qPCR) reaction mixture (25 µL) was conducted in the ABI 7500 PCR with the conditions set as follows: One cycle of 95°C for 10 min; 95°C for 20 s and 60°C for 1 min, totally 40 cycles; finally increasing 0.5°C every 5 s to achieve the melt curve of product.

Primer pair FMY (AGAAGAGAGGAGGATTACG ACCA) and RMY (ATGCGTTT GCTAAGTTCATCCAA) was designed as qPCR primer for *SrDOG1*, and the primer pair for *SrACT* was FNY (GGAATGGTTAAGGCA GGATTTG) and RNY (TTCATCACCCACATAGG CATC). The RNA was extracted from seeds (collected from different years), embryos, endosperm, endosperm caps, stems, leaves, roots, flowers and fruits. The extraction of RNA and synthesis of cDNA have been described above. The copy number of *SrDOG1* and *SrACT* was measured by qPCR. There are two standard curves (template is standard plasmid, threefold serial dilution) for each of the 96-well plates, one for *SrDOG1*, and the other for *SrACT*. According to the CT value of the sample and the standard curve, the copy number of the test samples can be obtained directly. The copy number of *SrDOG1* divided by that of *SrACT* is the *SrDOG1* relative expression level of the sample. The results were compared and the analysis of variance were performed using Duncan's test at the significance level of 0.05 in SPSS (SPSS, Chicago, USA).

Results and Discussion

***SrDOG1* sequence and homology:** In this study, the fragment of *DOG1* gene of buffalobur was cloned and then by genome walking method, the complete sequence of *SrDOG1* was obtained. The full-length of *SrDOG1* gene is 753 bp with no introns, and totally encoding 250 amino acids (GenBank accession number: ON988322). It was found that *SrDOG1* showed highest identities to *SlDOG1* (81%) of tomato and *StDOG1* (83%) of potato.

Homology basically reflects the genetic relationship among species. The homology of 26 DOG1 proteins (including SrDOG1) were analyzed and phylogenetic tree was established (Fig. 1). Results showed that the phylogenetic tree had common motif compositions for closely related species, indicating high sequence similarity of DOG1 proteins in the same family. The distribution of motif also revealed that the evolution of *DOG1* genes may be conserved. The neighbor-joining tree was divided into two main clusters. One cluster comprised of DOG1 sequences of dicotyledonous species and was subdivided into six families, Brassicaceae (D1), Asteraceae (D2), Solanaceae (D3), Sterculiaceae, Gesneriaceae and Pedaliaceae. Buffalobur, *S. lycopersicum*, *S. pennellii*, and *S. tuberosum* belong to Solanaceae family, their sequence similarity is higher, and the result agrees with the phylogenetic relationship. The second major cluster mainly formed by monocotyledonous species including the DOG1 protein from *Triticum aestivum, Zea mays, Oryza sativa,* and *Hordeum vulgare*.

Expression of *SrDOG1* in seeds with different storage time: A positive correlation between seed dormancy and the levels of *DOG1* transcription in seeds was observed in previous studies (Nakabayashi *et al*., 2012; Footitt *et al*., 2015; Murphey *et al*., 2015). Our results indicated that the expression of *SrDOG1* was increased gradually with prolonged storage time, and also in accordance with the germination rate of buffalobur. Moreover, low temperature during seed maturation could promote the expression of DOG1 protein (Chiang *et al*., 2011; Footitt *et al*., 2015). In this study, the expression of *SrDOG1* in cold storage seeds was 2 times higher than that of normal temperature, indicating that low temperature could promote the *SrDOG1* expression in mature seeds. This result also suggested that *SrDOG1* transcript level in seeds might be affected by environmental temperature (Fig. 2).

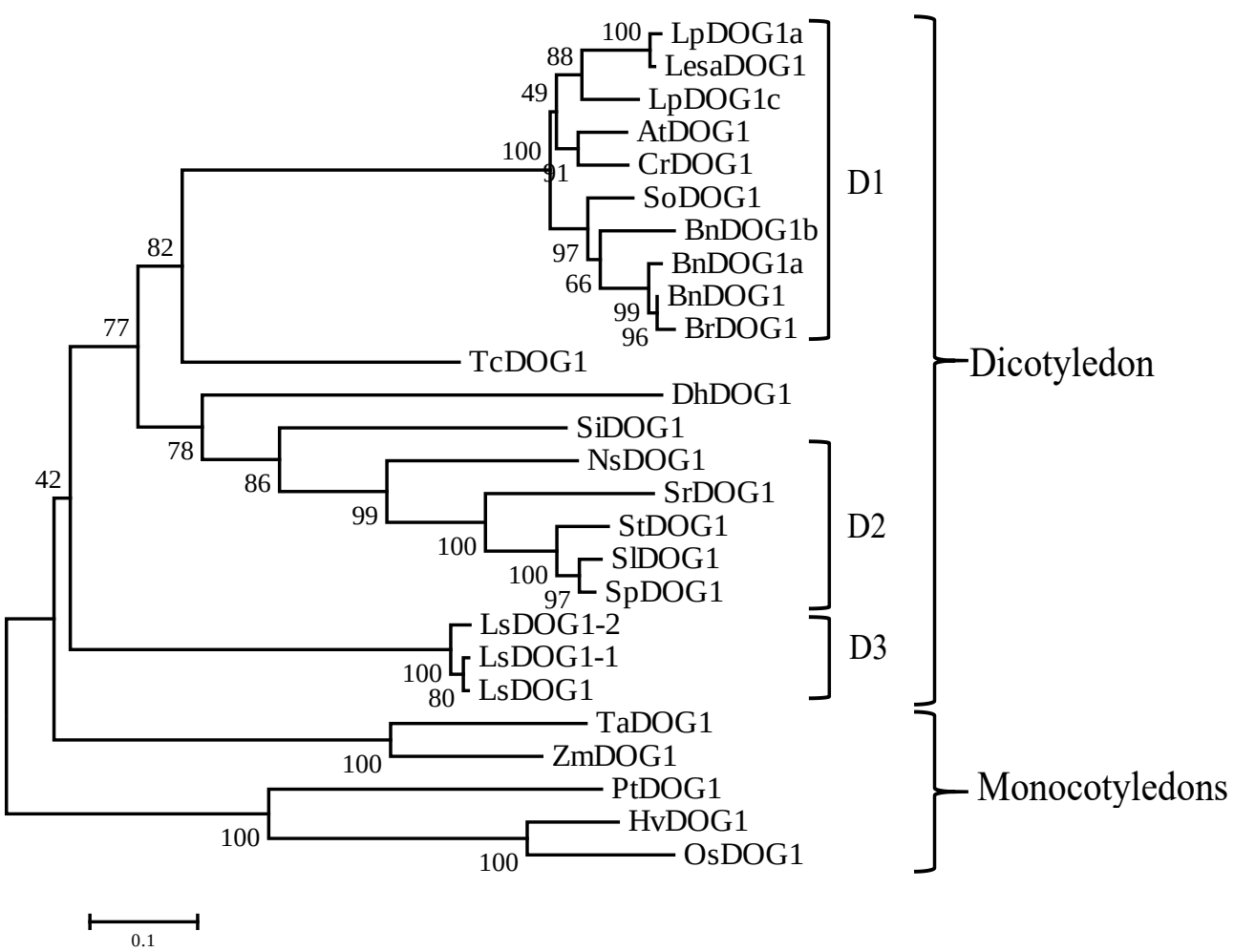

Fig. 1. Phylogenetic tree of DOG1 proteins. Amino acid sequences were used to construct the neighbor-joining tree in MEGA 6 supporting with 1,000 bootstrapped values. The accession number of the sequences are listed above. The number at the branch point is bootstrap value. The scale bar indicates the substitution of 0.1 amino acid per site.

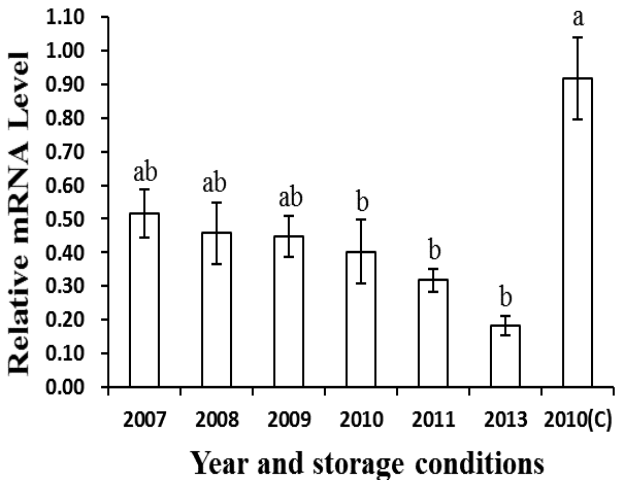

Fig. 2. Relative expression of *SrDOG1* gene in seeds collected in different years. 2007, 2008, 2009, 2010, 2011 and 2013 represent the year seeds collected and stored at room temperature; 2010(C) represents the seeds stored at 4°C collected in 2010. The error bars represent the standard error.

Expression of *SrDOG1* during seed germination: The *SrDOG1* expressions were examined before and after buffalobur seed germination. The level of *SrDOG1* expression reduced by 90% in seeds after being soaked in water for 24 h, suggesting that seed imbibition can significantly change the expression of *SrDOG1* in seeds (Fig. 3A). These results were in accordance with previous reports that *DOG1* transcripts are largely presented in *Arabidopsis* seeds and disappeared rapidly upon imbibition (Nakabayashi *et al*., 2012). After seed soaking, there was a slight increase of *SrDOG1* expression when the seed imbibed for 48 h. It was speculated that there might be some clips or other unknown function of *DOG1* transcripts at this stage (Nakabayashi *et al*., 2015). When seeds germinated, the expression of *SrDOG1* was significantly reduced, and was only 5% of that in dry seeds. Along with the radicle elongation to 2 cm, the *SrDOG1* transcript level remained essentially unchanged (Fig. 3B). Graeber *et al*., (2014) also showed that there was a strict association of *LesaDOG1* transcript abundance with seed germination, suggested that *DOG1* may have additional functions apart from dormancy, such as regulate the weakening of endosperm cap.

Expression of *SrDOG1* at different growth stages: The levels of *SrDOG1* transcript in roots, stems and leaves at different growth stages of buffalobur were analyzed. The expression in root was the highest at the cotyledon stage, and then decreased significantly and no changes were found after the 3-leaf stage. The expression level in stem was lower than that in root, and showed no significant changes through the whole plant growing period except for the 1-leaf stage. In leaves, the *SrDOG1* expression was highest at the cotyledon stage; it showed a similar trend but relatively lower expression with that of in root (Fig. 4). In general, *SrDOG1* transcripts were detected in all test organs (root, stem and leaf), even though the expression level was very low. The expression of *SrDOG1* in buds, flowers and fruits of buffalobur were detected, it indicated that the expression in fruit collected from fructescence stage (FFr) was notably higher than the other parts (Fig. 5). These results are consistent with previous studies demonstrating the presence of *SoDOG1* in most organs (i.e. flowers and seeds) as well as in roots, stems and leaves (Chiang *et al*., 2012; Ashikawa *et al*., 2013; Carrillo-Barral *et al*., 2015). Based on the expression of *SrDOG1* in organs other than the seeds, it indicates that *SrDOG1* may participate in the induction and maintenance of seed dormancy, and also may be related to other process during vegetative and reproductive development of buffalobur. For instance, the *DOG1* has been proved participated in the flowering regulation through the microRNA pathways (Huo *et al*., 2016).

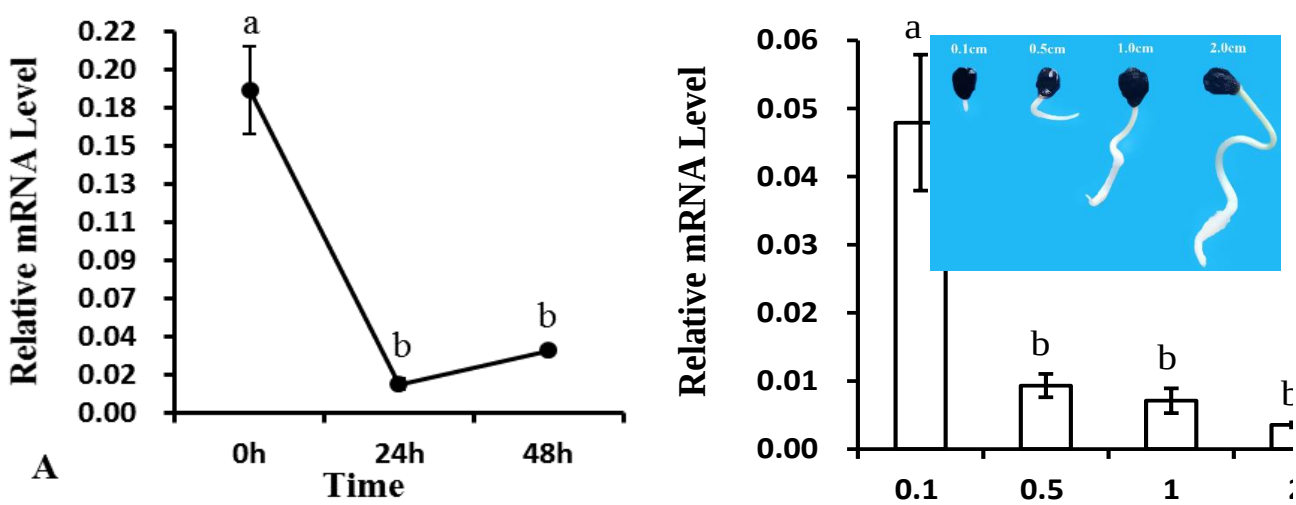

Fig. 3. Relative expression of *SrDOG1* gene during seed germination. A: *SrDOG1* expression during seed imbibition (before germination). 0 h represents seed stored at room temperature; 24 h represents the seed soaked in water for 24 h; 48 h represents the seed imbibed for 48 h in Petri dish. B: *SrDOG1* expression during radicle elongation. 0.1, 0.5, 1, and 2 represent the length of radicle in cm. The error bars represent the standard error.

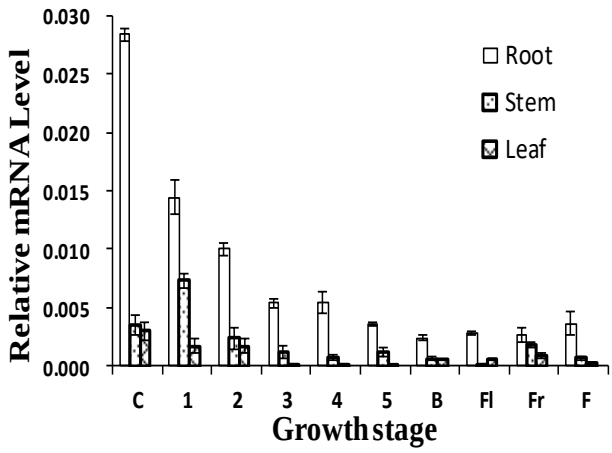

Fig. 4. Relative expression of *SrDOG1* at different growth stage. C represents the cotyledon stage; 1-5 represent 1-5 leaf stage; B represents the bud stage; Fl, Fr and F represent florescence, fruiting and fruit maturity period, respectively. The error bars represent standard error.

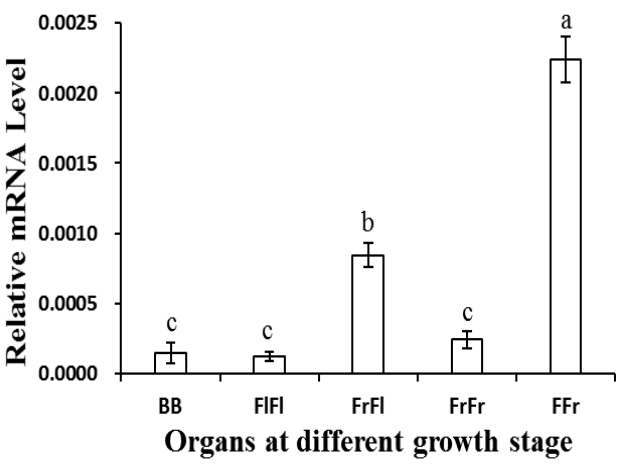

Fig. 5. Relative expression of *SrDOG1* in florescence, fruiting and fructescence period. BB represents the bud at bud stage; FlFl, FrFl, FrFr and FFr represent flowers in florescence period, flowers in fruiting period, fruits in fruiting period, and fruits in fructescence period, respectively. The error bars represent standard error.

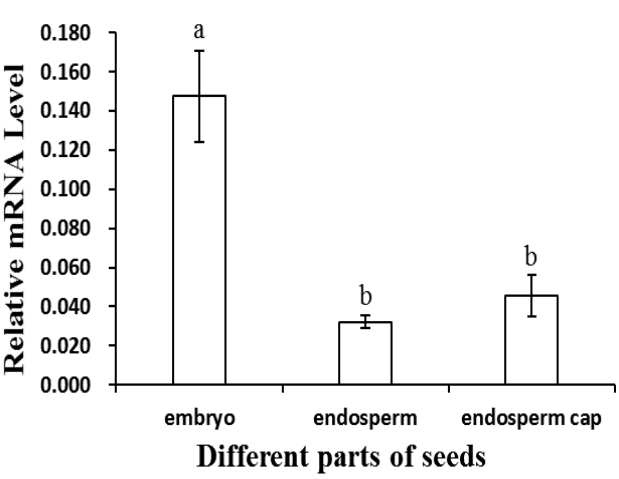

Fig. 6. Expression of *SrDOG1* in different parts of seeds. The error bars represent standard error.

Expression of *SrDOG1* in different parts of seeds: To determine the main expression sites of *DOG1*, the expression levels of *SrDOG1* in the embryo, endosperm and endosperm cap of buffalobur seeds were measured. The results showed that *SrDOG1* transcript level in embryo was 5 times higher than that in the endosperm and 3 times higher than that in the endosperm cap, but no remarkably difference was observed in the expression between the endosperm and endosperm cap (Fig. 6). This finding confirmed that the embryo may be the main expression site of *SrDOG1* in the seed, and the result was similar to that in *Arabidopsis* (Nakabayashi *et al*., 2012).

In summary, *SrDOG1* gene was mainly expressed in dormant seeds, and the embryo may be the main expression site. Cold storage can greatly promote *SrDOG1* expression, and the expression level in seeds can be altered significantly by seed soaking. After germination and subsequent growth stages, the *SrDOG1* transcript level was very low in different parts of plant. In addition, due to the extensive and appreciable expression characteristics, we speculate that the *SrDOG1* functions not only in seed dormancy regulation but also in other unknown biochemical process. In future research, much attention should be paid on the clarification of the interaction between *DOG1* and ABA or GA pathway, and the specific role of *DOG1* in plant life cycle.

Acknowledgements

This research was funded by Beijing Natural Science Foundation (6212027) and Natural Science Foundation of China (31572022).

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(Received for publication 28 January 2022)