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Molecular Characterization of a Catalase Gene (*VsCat*) from *Vicia sativa*

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Abstract

In plants, catalases are encoded by a multigene family and are predominantly localized in the peroxisomes and glyoxysomes for scavenging H_2O_2. A full-length cDNA encoding *Catalase* (*VsCat*) was isolated from *Vicia sativa* using RT-PCR. The cDNA consisted of 1485 bp open reading frame (ORF) encoding a 494 amino acid polypeptide with a predicted molecular mass of 57.03 kDa and an estimated pI of 6.56. The predicted protein was highly homologues to other catalases from legume plants and contained typical catalytic active site, calmodulin binding domain, and internal peroxisomal targeting signal. Phylogenetic analysis revealed that VsCat is evolutionary close to faba bean and pea Cat1. The expression patterns of *VsCat* under different abiotic stresses and exogenous phytohormones were determined by quantitative RT-PCR**.** Compared to control plants, *VsCat* was differentially up-regulated in response to abiotic stresses and phytohormones. The expression analysis suggested that *VsCat* is involved in different abiotic stress responses. In future experiment, transgenic plants overexpressing *VsCat* might be a good choice to increase tolerance of forage plants to environmental stresses.

Keywords: abiotic stress, catalase, phytohormone, *Vicia sativa*.

1. Introduction

Crop plants are greatly affected by environmental stresses. Plants subjected to biotic and abiotic stress factors exhibit different protective biochemical mechanisms, including decreased stomatal conductance and increased photoreduction of molecular oxygen, photorespiration, and excitation of electron transport chain at photosystem II (Asada, 1999; Ort & Baker, 2002). These protective mechanisms lead to increased formation of highly reactive forms of oxygen known as reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), peroxyl radical ($HOO^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet ($^1O_2$; Asada, 1999; Mittler, 2002). These reactive molecules are potentially dangerous to cellular components and metabolites. Increased ROS production can lead to lipid peroxidation in membranes, protein denaturation and DNA damage. These effects are collectively referred to as oxidative stress (Halliwell, 2006; Møller, Jensen, & Hansson, 2007).

In order to cope with oxidative stress, plants have evolved an intrinsic antioxidant system to sequester the increased ROS levels according the cellular capacity and needs (Ahmad, Sarwat, & Sharma, 2008). This antioxidant system consists of ROS-scavenging enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX), as well as non-enzymatic antioxidants like glutathione, ascorbate and carotenoids (Apel & Hirt, 2004).

Hydrogen peroxide is the most stable ROS and produced in different cellular compartments (Varanová, Inzé, & Van Breusegem, 2002). H_2O_2 carries no net charge and can permeate membranes and move from one compartment to another (Henzler & Steudle, 2000; Bienert, Schjoerring, & Jahn, 2006). H_2O_2 is produced in the chloroplast via the Mehler reaction in which SOD catalyzed the dismutation $O_2^{\cdot-}$ into H_2O_2. In plant mitochondria, the over-reduction of the electron transport chain enhanced the accumulation of H_2O_2 (Ślesak, Libik, Karpinska, Karpinski, & Miszalski, 2007). The peroxisome is a major cellular compartment where the production rate of H_2O_2 is normally high and can even be enhanced under environmental stresses (Nyathi & Baker, 2006). H_2O_2 is produced in peroxisomes during photorespiration and β-oxidation of fatty acids. Moreover, H_2O_2 is generated in peroxisomes during glycolate oxidation by peroxisomal glycolate oxidase (Wingler, Lea, Quick, & Leegood, 2000; Foyer & Noctor, 2003).

Plant catalases (E.C. 1.11.1.6) are mainly localized in peroxisomes. Catalase is the principal scavenging enzyme which catalyzes the conversion of toxic H_2O_2 to dioxygen and water (Asada, 1999). In plants, catalases are encoded by a multigene family and are differentially regulated in various tissues during development, and by a variety of environmental stimuli (Frugoli et al., 1996; Du, Wang, Chen, & Song, 2008; Mhamdi et al., 2010; Purev, Y. Kim, M. Kim, Pulla, & Yang, 2010).

Vicia sativa, known as vetch, is one of the most important annual forage legumes and is widely cultivated for animal feed in the rain-fed Mediterranean region (Sullivan, 2003; Hueze, Tran, Baumont, 2011). This plant has received great attention among plant scientists not only because it offers a valuable source of protein and minerals for cattle, but also it presents a natural plant cover in many arid regions around the world (Álvarez-Martínez et al., 2009; Uzun, Gucer, Acikgoz, 2011). With the exception of pea, little is known about antioxidant defense genes in legumes. Therefore, this proposed research aims at identification and characterization of a CAT gene from *Vicia sativa*.

2. Materials and Methods

2.1 Plant Material

Seeds of *Vicia sativa* cv. Mahali were received from the National Center for Agricultural Research and Extension (NCARE), Ministry of Agriculture, Jordan. *V. sativa* plants were grown in peatmoss and perlite-filled plastic pots (two plants per pot) in a greenhouse under natural light and dark cycle. Pots were irrigated daily with tap water.

2.2 Gene Cloning

Total RNA of *V. sativa* leaves was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. First-strand cDNA was prepared from 2 μg of total RNA using PrimeScript™ RT Master Mix (Takara, Japan).

A pair of sense and antisense specific primers (Table 1) were designed based on contig sequence of *Vicia faba* catalase gene (v.faba_csfl_reftransV1_0058332) available from Cool Season Food Legume Genome Database (https://www.coolseasonfoodlegume.org). PCR was performed in a 25 μL reaction volume containing 12.5 μL of 2x PCR master mix solution (iNtRON, Korea), 200 ng of template cDNA, 0.1 μL of each primer (10 μmol/L), and ddH_2O up to 25 μL. The amplification reaction was performed in a T-Professional Basic Thermal Cycler (Biometra, Germany) using an initial denaturation step at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 57 °C for 40 s and 72 °C for 2 min and final extension at 72 °C for 10 min. The amplified product was run on 1% agarose gel and purified with gel extraction kit (iNtRON, Korea). The purified PCR product was then cloned into pGEM-T Easy vector (Promega, USA) and sequenced.

2.3 Bioinformatics Analysis

2.3.1 Sequence Analyses

The open reading frame (ORF) analysis was carried out using ORF finder of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.htm) and the full length nucleotide sequence of *VsCat* was translated using Translate tool (http://web.expasy.org/translate/). The theoretical molecular weight (MW) and isoelectronic point (pI) of the protein encoded by *VsCat* were estimated using ProtParam (http://web.expasy.org/protparam/). The prediction of protein domains was carried out using NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2009) and literature references.

Table 1. List of primers used for the cDNA cloning and expression analysis of *VsCAT* gene from *Vicia sativa*

Primer name	Sequence (5′–3′)	Purpose	Amplicon (bp)
VsCatF	TCCCTATCTTCTCCTCCACCAC	Gene Cloning	1697
VsCatR	GTAACAGCTCAACGAGTTGCAT	Gene Cloning	
VsCatqF	ATCCCCAGACTCACATCCAGG	qRT-PCR	173
VsCatqR	TTTCCCAGCCTTGTTGAGCAG	qRT-PCR	
VsActinqF	CAATCCAGGCCGTCTTGTCTC	qRT-PCR	157
VsActinqR	TCTGTTAAATCACGCCCAGCA	qRT-PCR	

2.3.2 Phylogeny

Sequence comparisons and multiple sequence alignment were performed using Clustal-Omega program (Sievers et al., 2011). For phylogenetic analysis, peptide sequences of catalase proteins were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). Together with VsCat, the deduced protein described in the present study, and 41 catalase proteins from other plant species, a phylogenetic tree was constructed using the neighbor-joining method with the MEGA 5.0 program (Tamura et al., 2011). In Mega 5.0, The distance matrices were generated using the pairwise deletion option with the Poisson correction amino acid matrix; all other parameters were set to default. The bootstrap percentages were calculated based on 1000 replications to estimate the confidence of nodes in the tree.

2.4 Treatments and Gene Expression Analysis

One-month old *V. sativa* plants were subjected to different abiotic stresses and phytohormones for different time periods. For salt stress treatment, plants were irrigated with approximately 200 ml of 300 mM NaCl for one time. To simulate dehydration stress, the whole plant seedlings were dried on filter paper at room temperature and under light. For hydrogen peroxide treatment, plants were sprayed with 10 mM H_2O_2 for one time. For creating cold and heat stresses, plants were exposed to 4 and 42°C, respectively. For phytohormone treatments, plants were sprayed with solution of 0.15 mM abscisic acid (ABA), 1 mM salicylic acid (SA), 0.2 mM methyl jasmonate (MeJA), or 0.75 g/L ethephon (ET).

Plants were maintained at these different treatments for a range of time periods (0, 2, 4, 6, 10 and 24 h). At each time point, leaves were harvested and immediately frozen in liquid nitrogen. Control group plants were grown in parallel and were harvested at the same time points as those of the other treatment groups. Total RNA was isolated from 250 mg leaf tissue, and cDNA was synthesized as described previously and used as the template for qRT-PCR.

Quantitative real-time PCR (qRT-PCR) was carried out with a CFX96 Real-Time PCR Detection System (Bio-Rad) using KAPA SYBR® FAST qPCR Kit (KAPA BIO, USA). according to the manufacturer's instructions. Primers used in qRT-PCR experiments are listed in Table 1. Thermal cycling consisted of enzyme activation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 3 s and annealing and extension at 60°C for 30 s. Data collection was carried out during the 60°C step.The $2^{-\Delta\Delta Ct}$ method was adopted to analyze the relative changes in gene expression from qRT-PCR experiments (Schmittgen & Livak, 2008). The data are presented as the fold change in transcript level normalized to the *V. sativa Actin* gene (GenBank accession No. GU946218), and set relative to that in control plants. The relative quantification data were expressed as mean and standard error of the mean (±SE) of three biological replicates. The differences in gene expression between control and treated plants were statistically analyzed using a two-tailed *t*-test ($P \leq 0.05$).

3. Results and Discussion

3.1 Cloning and Bioinformatics Analysis

The goal of the present study was to clone and characterize a *Catalase* gene from the forage legume *V. sativa*. Using sequence information of *V. faba* catalase gene obtained from the contig v.faba_csfl_reftransV1_0058332 available from Cool Season Food Legume Genome Database (https://www.coolseasonfoodlegume.org), primers were designed to amplify a *Catalase* gene from *V. sativa* by RT-PCR. The full-length cDNA of *VsCat* (GenBank accession no. KX090583) was 1697 bp in length and contained a continuous ORF of 1485 bp with a 48-bp 5′ UTR (untranslated region) and a 164-bp 3′ downstream UTR (Figure 1). The *VsCat* ORF is predicted to encode a polypeptide of 494 amino acid residues, with a predicted MW of 57.03 kDa and a hypothetical pI of 6.56.

Domain and motif search via NCBI Conserved Domain Database revealed the presence of catalytic active site, calmodulin binding domain, and internal peroxisomal targeting signal (PTS1) in VsCat putative protein. The 15 amino acids at the position of 54th Phe (F) to 68th Gly (G) are putatively involved in the catalytic activity VsCat. Calmodulin binding domain is presented by amino acid residues from 415th Ala (A) to 451st Val (V). Different potential proteins were reported to interact with catalase proteins, including calmodulin, nucleoside disphosphate kinase, and *s*alt *o*verly *s*ensitive 2, a SNF-related kinase (Yang & Poovaiah, 2002; Fukamatsu, Yabe, & Hasunuma, 2003; Verslues et al., 2007)

The C-terminal tri-peptide 482nd Gln (Q), 483rd Lys (K) and 484th Leu (L) represents the putative internal peroxisomal targeting signal PTS1 in VsCat putative protein. The signal sequence QKL was reported to direct the interaction between Cat protein and PTS1 receptor PEX5 in the cytosol (Kamigaki et al., 2003). The present data suggest that *VsCat* in *V. sativa* encodes a putative peroxisomal catalase, that is likely regulated and activated by calmodulin and calcium.

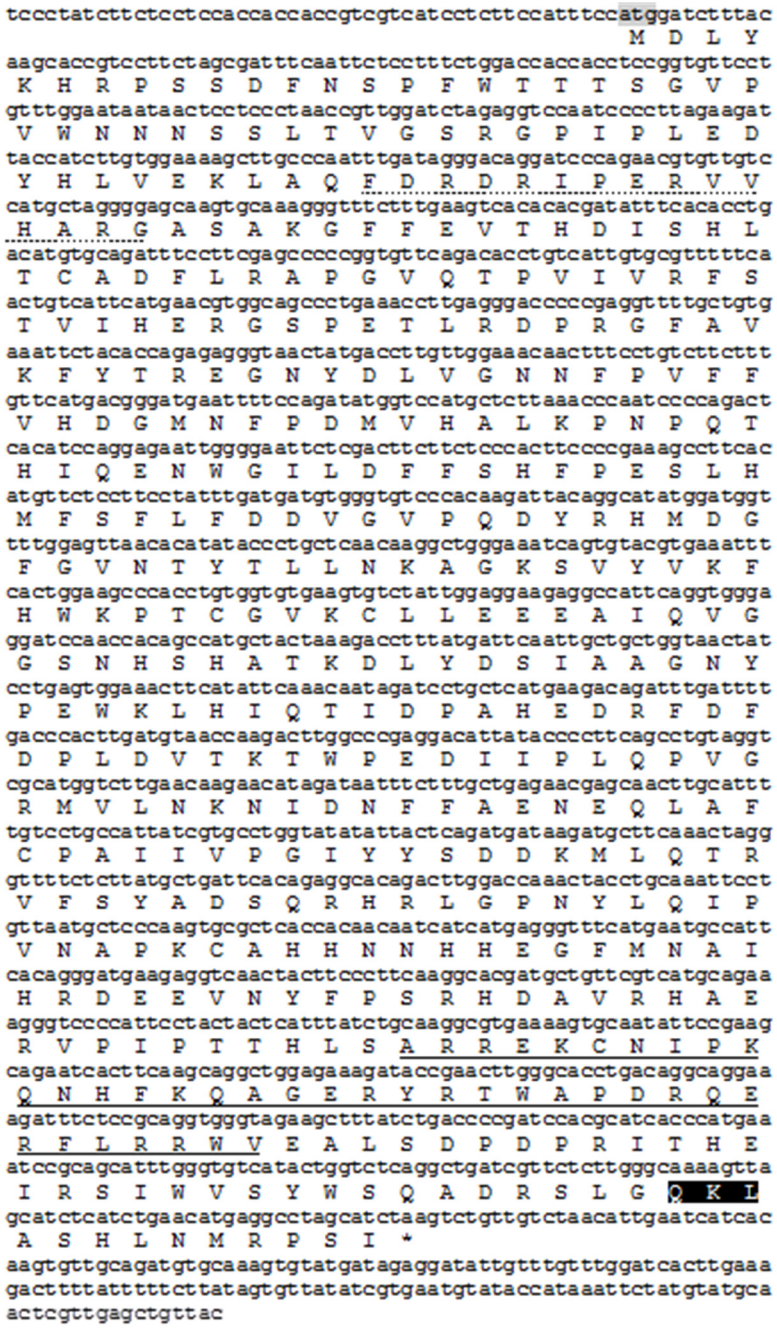

Figure 1. Full-length cDNA sequence of *VsCat1* gene. The amino acid sequence is displayed in a one-letter code under the coding sequence, with the dots underlined amino acids representing the residues involved in catalytic activity. The putative calmodulin binding domain is underlined, and the black box represents the putative conserved internal peroxisomal targeting signal PTS1. The translation start codon is marked with a gray box. The asterisk denotes the stop codon

Multiple sequence alignment shows that VsCat protein shares high degree of identity with Cat from other plant species (Figure 2). The highest identity was found to be 98 % with faba bean Cat1, 96 % with pea Cat1, and 91 % with barrel clover Cat1. The alignment also indicated the conserved calmodulin binding domain and PTS1.

Figure 2. Alignment of *Vicia sativa* Cat1 (KX090583) with related catalase proteins from *Vicia faba* (VfCat1, AFD29285), *Pisum sativum (PsCat1,* CAA42736*), Medicago truncatula* (MtCat1, XP_013462277) and *Arabidopsis thaliana* (AtCat1, AAB07026). The putative calmodulin binding domain (CBD) is marked with a gray box. The putative conserved internal peroxisomal targeting signal (PTS1) is marked by a black box. The conserved regions are indicated by '*'

To better understand the evolutionary relationships among the *CAT* genes of *Vicia sativa* and other plant species, 42 CAT protein sequences from 22 species were used to make an alignment using ClustalX. An unrooted tree was constructed based on the alignment using the Neighbor-Joining method implemented in MEGA-5. As shown in Fig. 3, VsCat was clustered together with *V. faba* Cat1 and *P. sativum* Cat 1, both belonging to the leguminous plants. Catalase proteins from dicot- and monocotyledonous plants are distributed among the phylogenetic tree (Figure 3). Frugoli et al. (1998) showed that evolutionary divergence (gene duplications) of multiple plant catalases occurred before the divergence of monocot- and dicotyledonous plants.

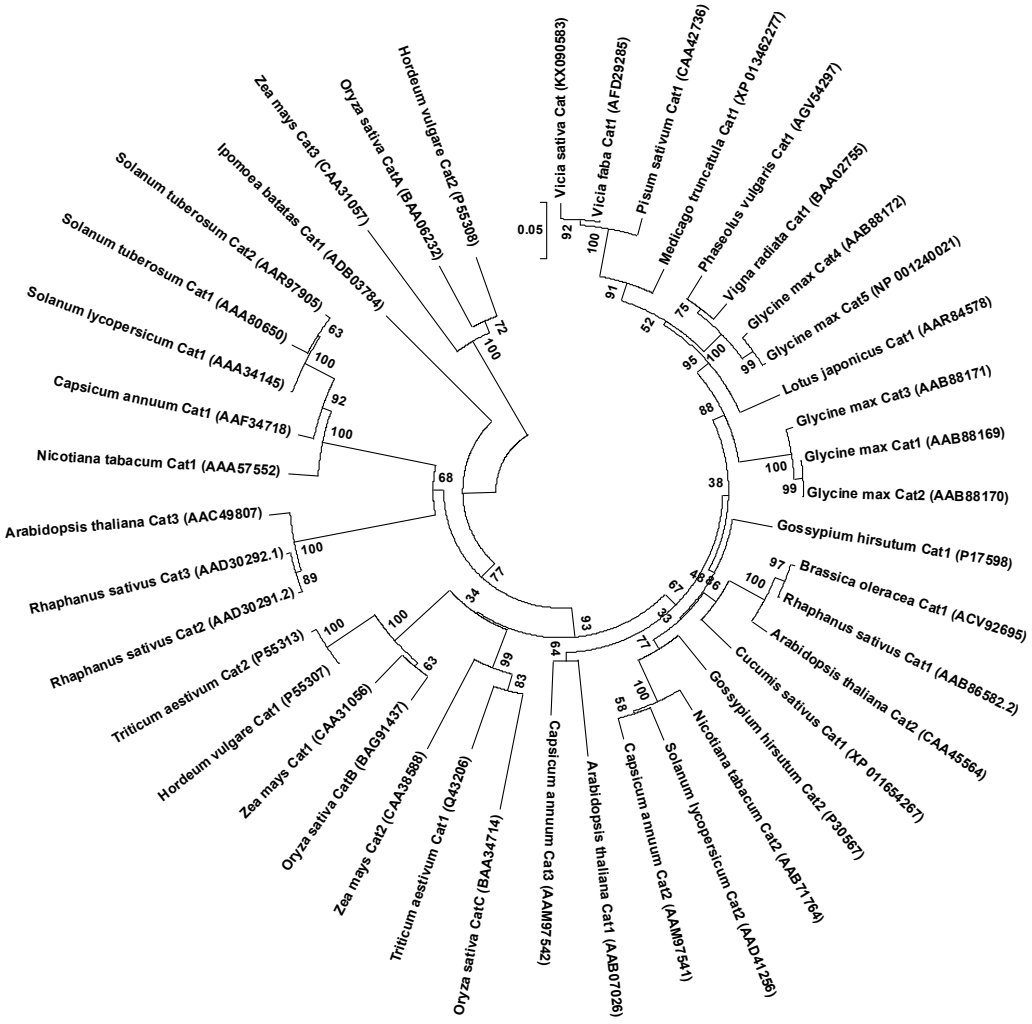

Figure 3. Phylogenetic analysis of VsCat1. The full-length amino acid sequences of VsCat1 and selected Cat proteins from other plant species were aligned by ClustalX and the phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates by MEGA 5.0. GenBank accession numbers are indicated in parentheses

3.2 Expression Profiles of VsCat

Plant catalases play a protective role against reactive oxygen species (ROS) produced in excess under environmental stress. Therefore their expression level often reflects the occurrence of stress conditions (Du et al., 2008).

In the present study, qRT-PCR was used to perform a time course analysis of the transcript levels of *VsCat* following exposure to abiotic stresses. *VsCat* was up-regulated in response to all treatments, although varying expression pattern in response time and type of stress were noticed (Figure 4). Under salt stress, about 5.2-fold increase in transcript level over control was noticed at 4 h in *VsCat* and then the level increased afterward to reach the peak of expression at 10 h post treatment. The expression level of *VsCat* in plants suffering from dehydration stress increased from 5.6-fold at 2 h to 11.2-fold at 4 h and then the level declined over time. Salinity and drought are known to up-regulate *Cat* expression in higher plants (Kwon & An, 2001; Lee & An, 2005; Xing, Jia, Zhang, 2007; Liu Hu, Yao, Xu, & Xing, 2016).

Under H_2O_2-induced oxidative stress, the expression of *VsCat* gradually increased and reached a peak of 14.5-fold at 6 h post treatment. Jithesh et al. (2006) have reported enhanced transcript levels of grey mangrove *CAT1* by exogenous H_2O_2 application. Similar observations were reported in maize (Polidoros & Scandalios, 1999) and ginseng (Purev et al., 2010).

The expression of *VsCat* rapidly increased under heat stress and scored an early peak of 9.1-fold at 2 h post treatment. However, the expression of this gene remained unchanged at 2 h when seedling were exposed to cold stress. Under this condition, the expression of *VsCat* reached the peak of 9.2-fold at 6 h post treatment. Enhanced transcript levels of *Cat* genes in response to temperature stress were previously reported in different species (Prasad, Anderson, Martin, & Steward, 1994; Willekens et al. 1994; Figueroa-Yáñez et al., 2012). Heat stress was shown to enhance broccoli *Cat* transcripts and enzymatic activity (Lin, Huang, & Lin, 2010). In *Arabidopsis*, only *Cat2* was shown to be up-regulated in cold-stressed plants (Soitamo, Piippo, Allahverdiyeva, Battchikova, & Aro, 2008). Moreover, transgenic rice plants overexpressing wheat *Cat* gene showed improved tolerance against low temperature stress which might be attributed to the effective detoxification of H_2O_2 by the enhanced catalase activities (Matsumura, Tabayashi, Kamagata, Souma, & Saruyama, 2002).

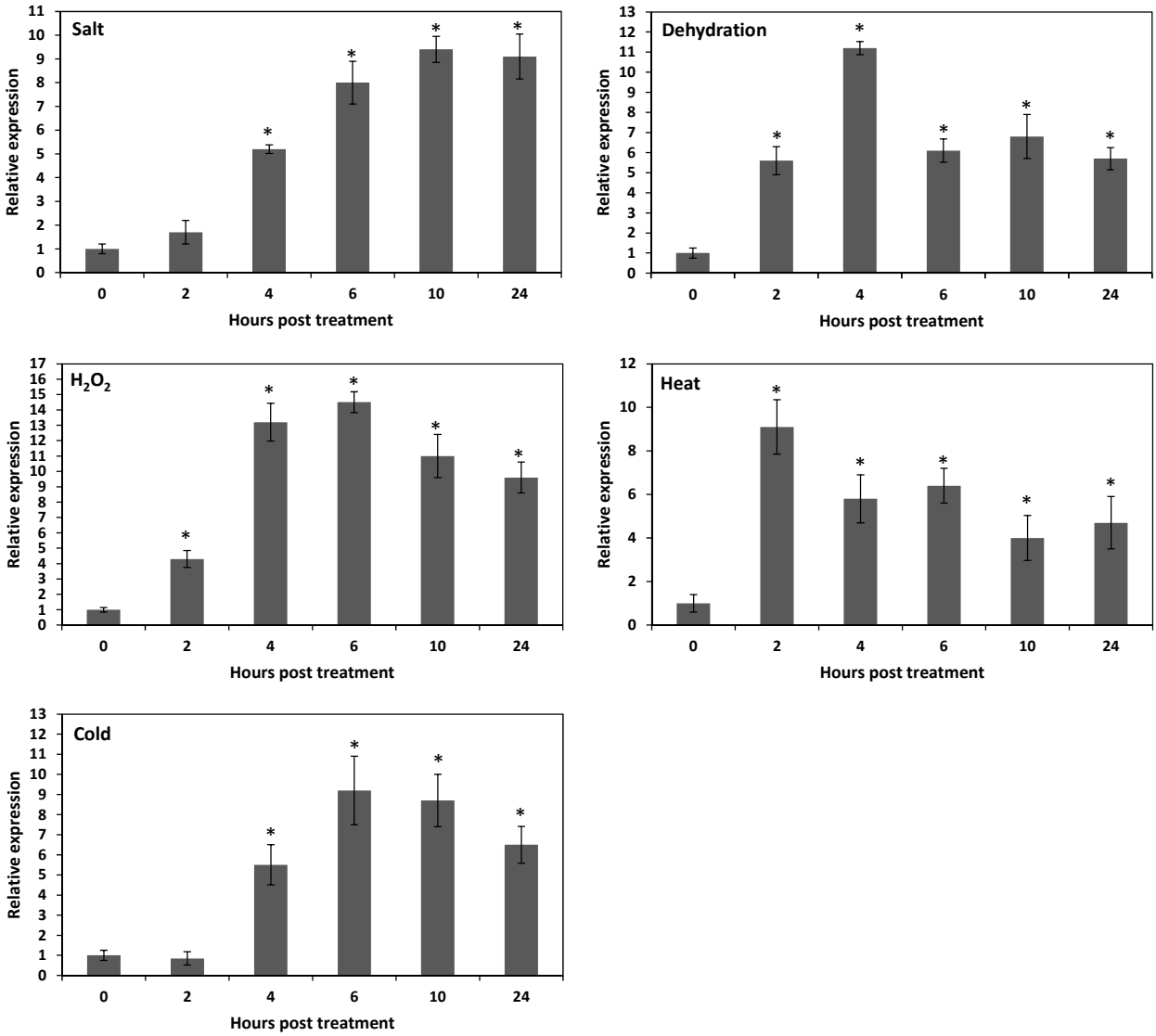

Figure 4. Expression of *VsCAT* under different abiotic stress conditions in *Vicia sativa*. One-month old *V. sativa* plants were treated with salt, dehydration, H_2O_2, heat, and cold stresses. The amount of each mRNA in *V. sativa* leaves was measured by qRT-PCR. The expression level of *VsCAT* was normalized to the *Actin* reference gene and expressed as a ratio relative to the control seedlings (0 h), which was set at 1. Each value represents the averages of three biological replicates, and the error bars indicate the standard error. The asterisks indicate significant changes in the expression compared with the control conditions (*t*-test, * $P \leq 0.05$)

To investigate the effect of various phytohormones on expression of *VsCat*, plants were treated with ABA, SA, MeJA, and ET in a time course study. Compared with the control plants, *VsCat* expression increased in response to all these phytohormones used (Figure 5).

When plants were sprayed with ABA, the expression level of *VsCat* gradually up-regulated and showed 8.2 fold increase at 4 h and reached the peak value at 6 h. Exogenous ABA was reported to enhance the expression of *Cat* genes in several plant species. In maize, promoter regions of *Cat1* gene were shown to possess an antioxidant responsive element (ARE) and an ABA responsive element (Rushmore, Morton, & Pickett, 1991; Guan & Scandalios, 1993; 1998). Exogenously applied ABA was shown to increase cellular H_2O_2 levels in maize, leading to enhanced *ZmCat1* transcript level through the ARE motif in the *ZmCat1* promoter (Guan & Scandalios, 2000).

In response to SA, the expression of *VsCat* exhibited steady increase and reached the highest expression level at 6 h. This elevated expression level almost remained during the latter periods. The expression of *VsCat* was early activated in response to MeJA. The expression level reached 5.1- and 5.6-fold at 2 and 4 h post treatment with MeJA, respectively. Then the expression of *VsCat* fluctuated between 2.8- and 4.8-fold during the rest of the treatment time. Similar results were reported in other plan species, where SA and JA induced the expression of *Cat* genes in ginseng (Purev et al., 2010) and maize (Guan & Scandalios, 1995). ET resulted in gradual increase in the expression level of *VsCat* which peaked at 6 and 10 h post treatment. Similarly, *Cat1* transcripts in sweet potato were up-regulated by ethephon treatment (Chen et al., 2012).

In conclusion, the present study reported the cloning and characterization of a catalase gene (*VsCat*) from *V. sativa* for the first time. The *VsCat* expressions were up-regulated in response to abiotic stresses and phytohormones. The expression analysis suggested that *VsCat* is involved in different abiotic stress responses. In future experiment, transgenic plants overexpressing *VsCat* might be a good choice to increase tolerance of forage plants to environmental stresses.

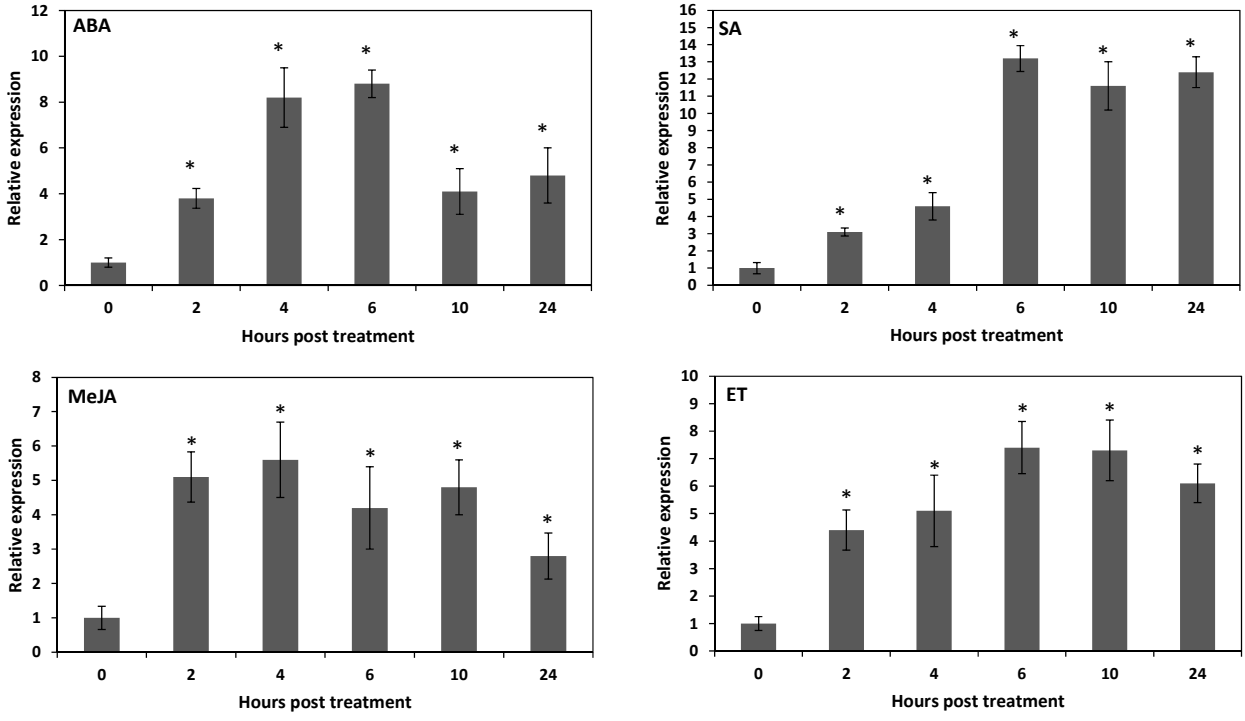

Figure 5. Expression of *VsCAT* under different phytohormone treatments in *Vicia sativa*. One-month old *V. sativa* plants were treated with ABA, SA, MeJA, and ET. The amount of each mRNA in *V. sativa* leaves was measured by qRT-PCR. The expression level of *VsCAT* was normalized to the *Actin* reference gene and expressed as a ratio relative to the control seedlings (0 h), which was set at 1. Each value represents the averages of three biological replicates, and the error bars indicate the standard error. The asterisks indicate significant changes in the expression compared with the control conditions (*t*-test, * $P \leq 0.05$)

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