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Research Note

Cloning and *in silico* analysis of two genes, stearoyl-ACP desaturase (*PtSAD*) and small heat shock protein (*PtsHSP*), in response to heat stress of *Pinellia ternata*

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Abstract

High temperature has become a major deleterious factor affecting the production of *Pinellia ternata*. Here, we report the cloning of two genes that have been confirmed to be involved in the response of *P. ternata* to heat stress. The expression patterns of stearoyl-ACP desaturase gene and small heat shock protein gene of *P. ternata* during heat stress at 35℃ were investigated via real-time quantitative RT-PCR, and then the two genes were cloned employing RACE strategy by using the heat-treated materials with peaked mRNA level. The stearoyl-ACP desaturase gene, named *PtSAD*, was slightly down-regulated within the first four hours of heat stress and up-regulated whereafter, and reached a peak level 48 h later. The small heat shock protein gene, named *PtsHSP*, was up-regulated since the 4th h of heat stress, with a peaked mRNA level at the 72nd h. The full length of *PtSAD* gene (GenBank Accession JQ390410) was 1595 bp containing an open reading frame (ORF) of 1185 bp. The full length of *PtsHSP* cDNA (GenBank Accession JQ390411) was 852 bp containing an ORF of 468 bp. *In silico* analysis indicated that both PtSAD and PtsHSP are stable hydrophilic proteins. Our study would be helpful for uncovering the functions of *PtSAD* and *PtsHSP*, further facilitating us to understand the responses of *P. ternata* to heat stress.

Keywords: Gene cloning; *in silico* analysis; *Pinellia ternata*; Rapid-amplification of cDNA ends; Stearoyl-ACP desaturase; Small heat shock protein.

Abbreviations: cDNAs_complementary deoxyribonucleic acid; ESTs_expressed sequence tags; pI_isoelectric point; RACE_rapid-amplification of cDNA ends; SAD_stearoyl-ACP desaturase; sHSP_small heat shock protein; SSH_suppression subtractive hybridization.

Introduction

Pinellia ternata (Araceae) is a perennial medicinal herb that has been used widely for thousands of years in China. Its tuber has been recognized for antitussive, antiemetic, analgesic and sedative actions (Luo et al., 2000). Owing to the broad usage, *P. ternata* is now among the top ten most commonly used medicinal herbs in China (Liu et al., 2010). The yearly market demand of *P. ternata* reaches 5—6 million kilograms, while the production of wild and artificially cultivated *P. ternata* can just meet 1/3 of the market demand (Zhang and Tan, 2010). This suggests that it is urgently required to boost the output of *P. ternata.* High temperature has become a major deleterious factor limiting field production of *P. ternata*. When exposed to the temperature above 30 ºC, *P. ternata* would wither and droop rapidly, which is called sprout tumble (Xue et al., 2004). Commonly, *P. ternata* may suffer from sprout tumble for 2-3 times from May to October every year and germinate again after each sprout tumble (Zhang et al., 2004). It largely shortens the growth period and consumes the nutrients in tuber when re-germinating, severely inhibiting the growth of *P. ternata*.

The response of *P. ternata* to high temperature is a very complex process and phenotypically assumes as sprout tumble. *P. ternata* sprout tumble has been confirmed as a non-physiological process caused by high temperature rather than light intensity, and it can be avoided by cultivating at an appropriate temperature (Zhang et al., 2004). Thus, improving heat tolerance is a very important approach to enhance the yield of *P. ternata*. However, the molecular mechanism underlying the response of *P. ternata* to high temperature still remains unclear yet. Previously, the differentially expressed genes in *P. ternata* in response to heat stress were investigated via suppression subtractive hybridization (SSH), using deoxyribonucleic acid (cDNAs) of heat-stressed plants as the tester and that of unstressed plants as the driver (Lu et al., 2013). Resultantly, 303 ESTs including small heat shock protein (sHSP) and stearoyl-ACP-protein desaturase (SAD) were obtained. In the present study, we conducted real-time quantitative RT-PCR on sHSP and SAD, cloned the full length of *sHSP* gene and *SAD* gene and performed *in silico* analysis on the two genes.

Results

Remarkable changes in mRNA levels of PtSAD and PtHSP during heat stress

During the heat stress process, *PtSAD* gene was slightly down-regulated within the first four hours of heat stress, and then up-regulated gradually, with a peaked level by 15-fold at 48th h. While *PtsHSP* gene was up-regulated since the 4th h of

Table 1. Primers for real-time quantitative RT-PCR and RACE.

Gene	Primer sequences
Tubulin	Tubulin F 5'-AACCTACACGAACCTCAACCG-3'
	Tubulin R 5'-ATGGATGAAGGCTCAAACGCA-3'
PtSAD	*PtSAD* F 5'-GAGCGTGTTGAGCATAGTCTGA-3'
	PtSAD R 5'-TTGAGAGAAAGGGCTAAGGAGA-3'
PtsHSP	*PtsHSP* F 5'-CGATCACACAGCACTCAACAA-3'
	PtsHSP R 5'-CTCTCCAGTCCCAACATCCT-3'
5'-GSP of *PtSAD*	5'-CCCGTTTCATCTCTAACGCCATCG-3'
3'-GSP of *PtSAD*	5'-CTGCCCACATATCAGACTATGCTC-3'
3'-GSP of *PtsHSP*	5'-CCGGAGGAGGTGGAGAAGGCCTTC-3'
Full length *PtsHSP*	*PtsHSP*-F 5'-ATGGACGTCAGGATGTTGGGACT-3'
	PtsHSP-R 5'-TTAGACGACCTTGACTTCGACGG-3'
Full length *PtSAD*	*PtSAD*-F 5'-ATGGCATTGAGGGTATCGCCCCTGA-3'
	PtSAD-R 5'-TCAAAGCAGAACTTCCCTGTCGT-3'

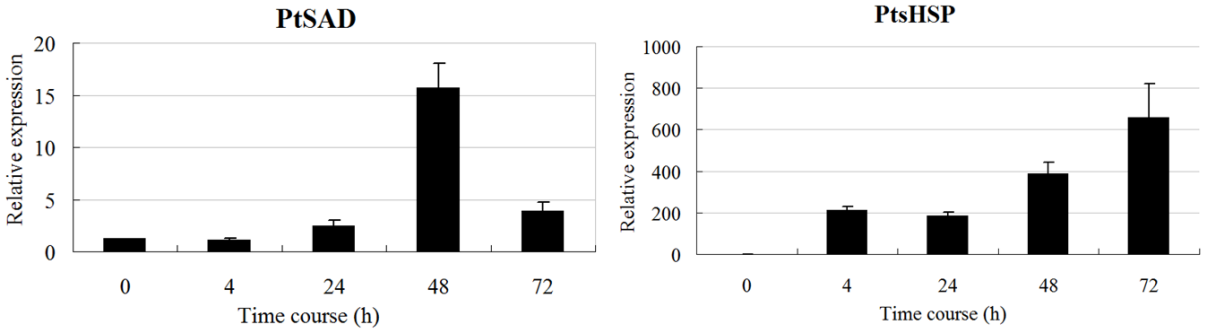

Fig 1. Remarkable changes in mRNA level of *PtSAD* and *PtsHSP* genes observed during heat stress.

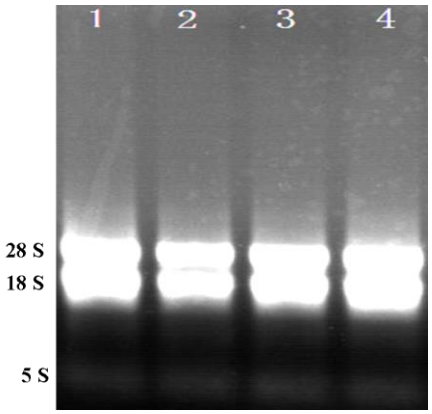

Fig 2. Electrophoretogram of total RNAs displayed clear 5S, 18 S and 28 S bands.

heat stress and reached the peaked level at the 72nd h (Fig 1.). These changes in mRNA level imply that both *PtSAD* and *PtsHSP* genes may be involved in the response of *P. ternata* to heat stress.

Full length cloning of PtSAD and PtHSP genes

As indicated in Fig 2., 5 S, 18 S and 28 S ribosomal RNA bands were clearly seen in the electrophoretogram. It suggests that the RNAs are relatively intact and highly pure. The yielded RNAs were reverse-transcribed into cDNA for RACE. By splicing the sequences from 5'RACE and 3'RACE of *PtSAD*, a 1595 bp long fragment containing a 1185 bp ORF was obtained (acc. no. JQ390410). The full length cDNA of *PtsSAD* was obtained by PCR amplification (Fig 3. A). For *PtsHSP*, solely the 3'RACE yielded a 852 bp long fragment containing a 468 bp ORF (acc. no. JQ390411). The full length cDNA of *PtsHSP* was obtained by PCR amplification (Fig 3. B).

General characters PtSAD and PtsHSP proteins

PtSAD gene encodes 394 amino acid residues. Functional prediction using ExPASy Proteomics Server showed that the molecular formula of PtSAD was $C_{2006}H_{3141}N_{557}O_{587}S_{17}$, with molecular weight of 45.0 kDa, theoretical pI of 6.34, and a grand average of hydropathicity (GRAVY) value of −0.449 (positive value for hydrophobicity, and the negative for hydro-

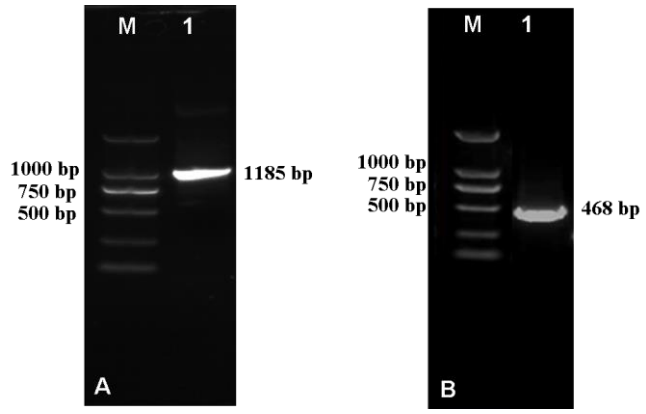

Fig 3. cDNA cloning of *PtsSAD* gene and *PtsHSP* gene. M, DL2000 DNA Marker; 1, PCR product. A, a 1185 bp cDNA fragment of PtsSAD obtained; B, a 1185 bp cDNA fragment of PtsHSP obtained.

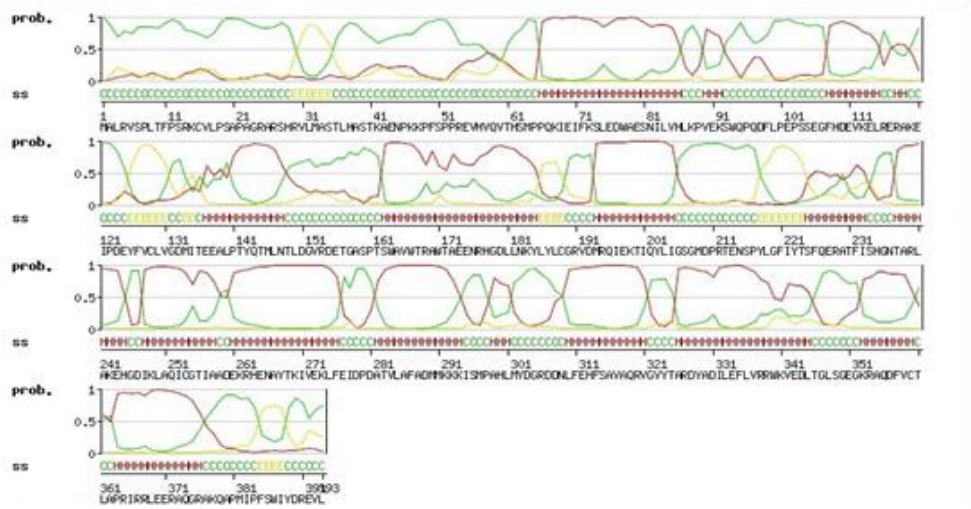

Fig 4. The predicted secondary structure of PtSAD. H, helix; E, β-sheet; C, coil. PtSAD contained 49.4% α-helix, 7.4% β-sheet and 43.2% random coil.

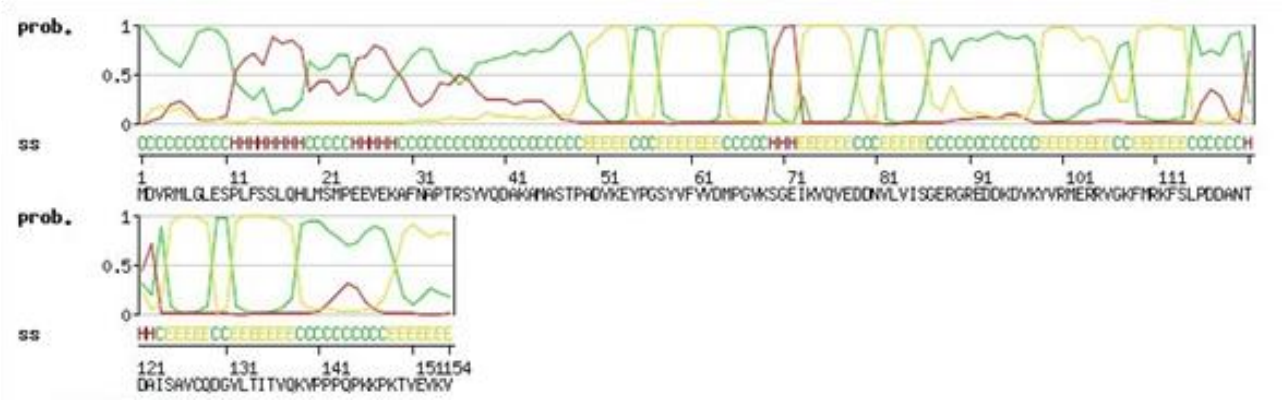

Fig 5. The predicted secondary structure of PtsHSP. H, helix; E, β-sheet; C, coil. PtHSP contained 12.3% α-helix, 36.4% β-sheet and 51.3% random coil.

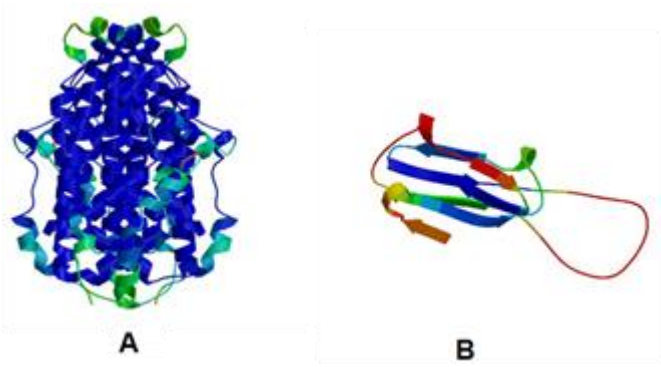

Fig 6. *In silico* modelling of three-dimensional structure of PtSAD and PtsHSP. A, PtSAD; B, PtsHSP. Blue helices represent α-helix, yellow arrows represent β-sheet, and green filaments represent random coils.

philicity). Therefore, PtSAD was supposed as a hydrophilic protein. Meanwhile, PtSAD was presumed to be a stable protein due to the instability coefficient of 43.61.

PtsHSP gene encodes 155 amino acid residues. Functional prediction showed that the molecular formula of PtsHSP was $C_{758}H_{1228}N_{204}O_{234}S_9$, with molecular weight of 17.2 kDa, theoretical pI of 5.52, and a grand average of hydropathicity (GRAVY) value of − 0.368. Therefore, PtsHSP was supposed as a hydrophilic protein. Meanwhile, PtsHSP was presumed to be a stable protein due to the instability coefficient of 42.77. Secondary structure prediction showed that α-helix and random coil were the main elements of PtSAD (Fig 4.), with the percentage of 49.4% and 43.2%, respectively. And β-sheet and random coil were the main elements of PtHSP (Fig 5.), with the percentage of 36.4% and 51.3%, respectively. Three dimensional structure modeling showed that PtSAD and PtsHSP contain highly conserved domains (Fig 6., A and B). These results indicate that both PtSAD and PtsHSP are stable hydrophilic proteins which can be used for genetic improvement of *P. ternata*.

Discussion

Plants can activate or suppress the expression of some genes to adapt to various stresses. The stearoyl-ACP desaturase (SAD) is the first rate-limiting enzyme that catalyzes the desaturation of fatty acids. SAD decides the saturation degree of fatty acids to a large extent, *i.e.*, ratio of saturated fatty acids to desaturated fatty acids (Zhang *et al*., 2011). The ratio is closely related with the fluidity, permselectivity of membrane, as well as various physiological functions. To date, *SAD* gene has been cloned from various plants including soybean (Li et al., 2007), *Cinnamomum longepaniculatum* (Luo *et al.*, 2009), peanut (Shilman *et al*., 2011), *Ginkgo biloba* (Wang *et al*., 2013) and *Cocos nucifera* (Gao *et al*., 2014). The *SAD* gene has been confirmed effective in regulating the saturation degree of fatty acids (Ruddle *et al.*, 2014), enhancing tolerance to low temperature (Byfield and Upchurch, 2007) and improving the resistance of crops to diseases and insects (Song *et al.*, 2013). However, less is known on the role of *SAD* gene in temperature stress, particularly in *P. ternata*. During the process of heat stress at 35 ℃, *PtSAD* gene was 15-fold-upregulated at the 48th h, which indicates that it may take an important role in adaptation process of *P. ternata* to heat stress. The expression pattern of *PtSAD* gene is in accordance with the sprout tumble process as reported previously (Lu et al., 2013). This implies that suppression of *PtSAD* gene may to some extent protect seedlings from sprout tumble during heat stress, thus reducing the yield loss of *P. ternata*. Small proteins are known to perform varieties of relevant functions and participate in regulation of various biological processes (Jia et al., 2015). *HSP* genes would be activated immediately to alleviate the injury caused by high temperature (Jiang et al., 2009). Of various HSPs, the sHSP, ranging in size from 12 to 42 kDa, has been confirmed most important for plants in response to heat stress (Waters, 2013; Koo et al., 2015). sHSP contains an α-crystallin consisting of 80—100 amino acid residues at the C terminus which can specifically recognize and bind with the denatured proteins and prevent the irreversible denaturation (Mogk et al., 2003; Basha et al., 2004). Moreover, overexpression of *sHSP* gene can enhance the heat tolerance of various plants, including grapefruit (Rozenzviega et al., 2004), *Nelumbo nucifera* (Zhou et al, 2012) and *Primula malacoides* Franch. (Zhang et al., 2013). Moreover, documents also indicated that sHSP can bind with membrane lipid and modulate the fluidity of membrane (Koo et al., 2015). The *PtsHSP* gene cloned is the first report of *HSP* gene of *P. ternata*, and it may take a key role during sprout tumble process.

Materials and Methods

Materials and reagents

The *Pinellia ternata* seedlings, provided by Key Laboratory of Plant Biology Resources in Anhui Province, were grown at 25 ±1 ºC with light intensity of 3000 lx under a photoperiod scheme of 12 h/d illumination. When grown to 15 cm in length, the seedlings were divided into two portions, with one transferred to an artificially controlled incubator (35±1 ºC) for heat stress and the other one maintained at 25 ±1 ºC as control. The sampling was performed at the 4th, 24th, 36th and 48th h of heat treatment, and that in the control group was collected at corresponding time course separately. The collected samples were immediately frozen in liquid nitrogen and preserved at −70 ºC for RNA isolation. The main reagents used included RACE System (Invitrogen, USA), *Taq* plus DNA polymerase (Tiangen, China), cloning vector pGEM-T easy (Promega, USA). The primers were synthesized by Sangon Biotech (Shanghai, China).

RNA isolation and cDNA synthesis

The total RNAs were extracted following a previously published protocol (Wu et al., 2008). The yielded RNA samples were separated on a 1.0% agarose gel for qualitative analysis. The first complimentary strand DNA was synthesized by using PrimeScript reverse transcriptase following to the detailed reaction conditions listed in

PrimeScript reverse transcription kit (TaKaRa, Japan).

Real-time quantitative RT-PCR

Real-time fluorescence reactions consisted of 2 X SYBR, 10 μL of Premix Ex TaqTM, 0.2 μmol/L forward and reverse primers each, 1 μL of cDNA template, ddH_2O up to 25 μL. The reaction components were reacted in an ABI 7300 quantitative real-time RT-PCR system (Applied Biosystems, USA) with a program of 50 ºC 2 min and 95 ºC 30 s, followed by 40 cycles of 95 ºC 30 s and 62 ºC 40 s. The tubulin gene was used as an endogenous control for comparison. For each pair of primers, and four biological parallel replications were contained. Gene expression levels were calculated using the 2-ΔΔCt method. The specific primers for rapid amplification of cDNA ends (RACE) were designed based on the EST sequences as reported previously (Lu et al., 2013) (Table 1.).

RACE and cloning of PtSAD gene and PtsHSP gene

The rapid amplification of cDNA ends (RACE), including 5'-RACE and 3'-RACE, of both genes were performed using a RACE System (Carlsbad, CA, USA). An antisense gene-specific primer (5'-GSP) and a sense gene-specific primer (3'-GSP) were used for 5'- RACE and 3'-RACE, respectively. Fragments generated by 5'-RACE and 3'-RACE were cloned into a pGEMTEasy Vector (Promega, USA) and sequenced by Sangon Biotech (Shanghai, China). The sequencing results of 5'-RACE and 3'-RACE were spliced into a complete fragment based on the overlapping region. The open reading frame (ORF) was predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi).

In silico analysis of PtSAD and PtsHSP

The physicochemical properties of two proteins were predicted using Protparam (http://web.expasy.org/protparam/) in ExPASy Proteomics Server. The secondary structure analysis and three dimensional modeling were performed by employing SWISS-MODEL (http://swissmodel.expasy.org/). Moreover, the functional prediction was predicted using SI-BLAST Profile search and InterproScan.

Conclusion

We cloned *PtSAD* and *PtsHSP* genes that are in response to heat stress of *P. ternata*. The full length of *PtSAD* gene is 1595 bp containing a 1185 bp ORF, and *PtsHSP* gene is 852 bp containing a 468 bp ORF. The expression patterns of *PtSAD* and *PtsHSP* genes are in accordance with the sprout tumble process of *P. ternata*. These two proteins contain typical and relatively conserved domains. *In silico* analysis indicated that both PtSAD and PtsHSP are stable hydrophilic proteins. These two genes could be used for genetic improvement of *P. ternata*.

Acknowledgments

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References

Basha E, Lee G J, Breci LA (2004) The identity of proteins associated with a small heat shock protein during heat stress *in vivo* indicates that these chaperones protect a wide range of cellular functions. J Biol Chem. 279(9): 7566-7575.

Byfield GE, Upchurch RG (2007) Effect of temperature on delta- 9 stearoyl- ACP and microsomal omega- 6 desaturase gene expression and fatty acid content in developing soybean seeds. Crop Sci. 47(4): 1698-1704.

Gao L, Sun R, Liang Y, Zhang M, Zheng Y, Li D (2014) Cloning and functional expression of a cDNA encoding stearoyl-ACP Δ9-desaturase from the endosperm of coconut (*Cocos nucifera* L.). Gene. 549: 70-76.

Jia XM, Yu J, Wu JY, Xiao JF (2015) A systematic bioinformatics analysis of small proteins in plants. Plant Omics. 8(2): 112-121.

Jiang CH, Xu JY, Zhang H, Zhang X, Shi JL, Li M, Ming F (2009) A cytosolic class I small heat shock protein, RcHSP17.8, of *Rosa chinensis* confers resistance to a variety of stresses to *Escherichia coli*, yeast and *Arabidopsis thaliana*. Plant Cell Environ. 32(8): 1046-1059.

Koo HJ, Park SM, Kim KP, Suh MC, Lee MO, Lee SK, Xinli X, Hong CB (2015) Small heat shock proteins can release light dependence of tobacco seed during germination. Plant Physiol. 167(3): 1030-1038.

Li L, Wang X, Gai J, Yu D (2007) Molecular cloning and characterization of a novel microsomal oleate desaturase gene from soybean. J Plant Physiol. 164: 1516-1526.

Liu YH, Liang ZS, Liu JL (2010) Use of protocorm-like bodies for studying alkaloid metabolism in *Pinellia ternata*. Plant Cell Tiss Organ Cult. 100: 83-89.

Lu HD, Xue T, Zhang AM, Sheng W, Zhu YF, Chang L, Song YX, Xue JP (2013) Construction of an SSH library of *Pinellia ternata* under heat stress, and expression analysis of four transcripts. Plant Mol Biol Rep. 31(1): 185-194.

Luo L, Wang JN, Kong LD, Jiang QG, Tan RX (2000) Antidepressant effects of Banxia Houpu decoction, a traditional Chinese medicinal empirical formula. J Ethnopharmacol. 73(1-2): 277-81.

Luo T, Deng WY, Zeng J, Zhang F (2009) Cloning and characterization of a stearoyl- acyl carrier protein desaturase gene from *Cinnamomum longepaniculatum*. Plant Mol Biol Rep. 27: 13-19.

Mogk A, Schlieker C, Friedrich K L, Schonfeld HJ, Vierling E, Bukau B (2003) Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. J Biol Chem. 278(33): 31033-31042.

Rozenzviega D, Elmaci C, Samach A (2004) Isolation of four heat shock protein cDNAs from grapefruit peel tissue and characterization of their expression in response to heat and chilling temperature stresses. Physiol Plantarum. 121: 421-428.

Ruddle P II, Whetten R, Cardinal A, Upchurch RG, Miranda L (2014) Effect of Δ9 stearoyl ACP-desaturase-C mutants in a high oleic background on soybean seed oil composition. Theor Appl Genet. 127: 349-358.

Shilman F, Brand Y, Brand A, Hedvat I, Hovav R (2011) Identification and molecular characterization of homeologous Δ9-stearoyl acyl carrier protein desaturase 3 genes from the allotetraploid peanut (*Arachis hypogaea*). Plant Mol Biol Rep. 29: 232-241.

Song N, Hu ZR, Li YH, Li C, Peng FX, Yao YY, Peng HR, Ni ZF , Xie CJ, Sun QX (2013) Overexpression of a wheat stearoyl-ACP desaturase (SACPD) gene TaSSI2 in *Arabidopsisssi2* mutant compromise its resistance to powdery mildew. Gene. 524: 220-227.

Wang HL, Cao FL, Zhang WX,Wang GB, Yu WW (2013) Cloning and expression of stearoyl-ACP desaturase and two oleate desaturases genes from *Ginkgo biloba* L. Plant Mol Biol Rep. 31: 633-648.

Waters ER (2013) The evolution, function, structure, and expression of the plant sHSPs. J Exp Bot. 64: 391-403.

Wu L, Xue JP, Xu YM, Tian ZD (2008) Comparison of four methods and their efficiency for total RNA extraction from leaves of *Pinellia ternata*. Chin J Chin Mater Med. 39(6): 901-905.

Xue JP, Ding Y, Zhang AM, Hu CQ (2004) The change of activity of protective enzyme around sprout tumble of *Pinellia ternata* under high temperature stress. Chin J Chin Mater Med. 29(7): 641-643.

Zhang J, Tan XH (2010) Research progress on *Pinellia ternata* resources. Chin J Infor on TCM. 7(5): 104-106.

Zhang L, Gao YK, Pan HT, Hu WJ, Zhang QX (2013) Cloning and characterisation of a *Primula* heat shock protein gene, *PfHSP17.1*, which confers heat, salt and drought tolerance in transgenic *Arabidopsis thaliana*. Acta Physiol Plant. 35(11): 3191-3200.

Zhang M, Zhong GY, Ma KS, Ding JC (2004) Experimental investigation on sprout tumble in *Pinellia ternata*. Chin J Chin Mater Med. 29(3): 273-274.

Zhang YM, Wang CC, Hu HH, Yang L (2011) Cloning and expression of three fatty acid desaturase genes from cold-sensitive lima bean (*Phaseolus lunatus* L.). Biotechnol Lett. 33(2): 395-401.

Zhou Y, Chen H, Chu P, Li Y, Tan B, Ding Y, Tsang EWT, Jiang L, Wu K, Huang S (2012) NnHSP17.5, a cytosolic class II small heat shock protein gene from *Nelumbo nucifera*, contributes to seed germination vigor and seedling thermotolerance in transgenic *Arabidopsis*. Plant Cell Rep. 31: 379-389.