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Original Article

Identification of Nitric Oxide Responsive Genes in the Rudimentary Leaves of *Litchi chinensis*

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Abstract

Litchi (*Litchi chinensis Sonn.*) is an evergreen woody fruit tree widely cultivated in subtropical and tropical regions. Warm winter and hot spring often leads to abnormal floral differentiation in litchi. Under this condition, the rudimentary leaves in the floral buds expand and the inflorescences will stop developing. Thus, how to promote abortion of rudimentary leaves in litchi inflorescence are important for floral development. Previous study indicated that nitric oxide (NO) produced by sodium nitroprusside (SNP) promoted flowering and abortion of rudimentary leaves in litchi. In the present study, a suppression subtractive hybridization (SSH) was used to identify NO responsive genes. As a result, 16 high homologous ESTs were obtained from the SSH library of the SNP treated rudimentary leaves. The ESTs were classified into three groups. They are disease/defensive, protein destination and storage, and protein synthesis. Quantitative reverse transcription PCR (qRT-PCR) analysis indicated that 6 out of the 7 randomly selected ESTs'expression showed an increasing trend from 0 h to 10 h of SNP treatment. It is suggested that the litchi homologs 18S ribosomal RNA gene, *cytochrome P450 like TBP*, and the senescence-associated protein, chaperone protein, and a hypothetical protein encoding genes may be involved in the NO-induced senescence in litchi rudimentary leaves. *LcERD15-like* may be a key gene involved in this process.

Keywords: abortion; nitric oxide; rudimentary leaves; suppression subtractive hybridization

Introduction

Litchi (*Litchi chinensis*) is an important subtropical fruit tree cultivated in Southeast Asia. Previous studies showed that low temperature is needed for floral initiation in litchi (Menzel and Simpson, 1988; Chen and Huang, 2005). However, abnormal warm winter and hot spring frequently happened in the past few decades, resulting in poor flowering in litchi. Litchi floral buds are mixed buds with apical or axillary panicle primordia, leaf primordia and rudimentary leaves (Huang and Chen, 2005). Under warm winter condition, the rudimentary leaves may expand quickly and the panicle primordia may cease to develop and shrink (Yang *et al*., 2017). Hence, warm winter turn to be a harsh challenge for litchi production. Control the growth of rudimentary leaves is important for litchi flowering.

Nitric oxide (NO) is an important signal molecule in plant cells (Beligni *et al*., 2001; Palavan-Unsal *et al*., 2009). Together with reactive oxygen species (ROS), NO accumulated under abiotic or biotic stress. NO and ROS are involved in programmed cell death (PCD) processes which are related with leaf abortion (Wang *et al*., 2013a, b).

Our previous studies showed that NO and ROS are involved in the low temperature-induced flowering and leaf abortion (Zhou *et al*., 2012). Further, we have identified many ROS responsive genes in floral bud and rudimentary leaves (Liu *et al*., 2013; Lu *et al*., 2014), as well as NO responsive genes in floral buds (Liu *et al*., 2015). In this study, we identified NO responsive genes in the rudimentary leaves by suppression subtractive hybridization (SSH), with the aim to provide basic materials for controlling the growth of rudimentary leaves and promoting flowering in litchi.

Materials and Methods

Plant material and experiment procedures

Sampling trees were grown at the experimental orchard of South China Agricultural University. Thirty-year-old litchi (*Litchi chinensis* Sonn. cv. Nuomici) trees grafted on the rootstock 'Huaizhi' were selected. About 6 cm length of branches with new flushes were cut off from the trees and immediately placed in water. The cuttings were treated with water or solutions containing 0.5 mM sodium nitroprusside (SNP) (Sigma, USA) according to the method of Zhou *et al*. (2012). All the cuttings were placed in a growth chamber

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with 160 μmol m^{-2} s^{-1} photosynthetic photon flux density at 20 °C. The third and the fourth of the rudimentary leaves (Fig. 1) were collected at 0 h, 5 h and 10 h of SNP treatment. The leaves were frozen in liquid nitrogen and stored at -80 °C for construction of SSH library and quantitative reverse transcription PCR (qRT-PCR) analysis. The proximal angle α, and the distal angle β of the third rudimentary leaves as shown in Fig. 1 were measured according to the method of Lu *et al*. (2014). The angular dimension was measured by degree.

Construction of suppression subtractive hybridization (SSH) cDNA library

Total RNA was extracted from frozen leaves according the method of Zhou *et al*. (1999). RNA extracted from rudimentary leaves treated with water for 10 h was used as driver and those treated with SNP for 10 h was used as tester. PolyATract mRNA isolation system III (Promega, USA) was used to isolate mRNA from total RNA, and subtracted library was prepared using PCR-SelectTM cDNA subtraction kit (Clontech, USA) according to the manufacturer's instructions. After tester cDNA was digested with Rsa I and ligated to adaptors, two rounds of hybridization and PCR were run to enrich the differentially expressed fragments. The second-round PCR products of subtracted cDNA was cloned into pMD19-T Vector (TaKaRa, Japan) and then transformed into *Escherichia coli* DH5α. Positive transformants were selected on LB medium supplemented with 50 mg L^{-1} ampicillin, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside).

Identification of the insert fragments by colony PCR

White clones were picked, and then inoculated into LB medium. PCR amplification was done using the bacterial suspension as template, the nested primer 1 and primer 2R as primers were provided by the PCR-SelectTM cDNA subtraction kit (Clontech, USA). PCR amplification by a thermal cycler (Bio-Rad, USA) were run as follows: 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s.

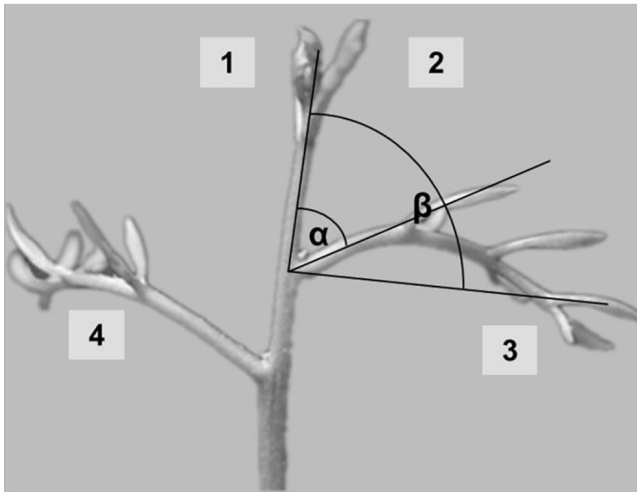

Fig. 1. Image of a new flush showing the first to forth rudimentary leaves. α, proximal angle of the rudimentary leaves; β, distal angle of the rudimentary leaves; numbers from 1 to 4 indicate the first, the second, the third, and the fourth rudimentary leaves respectively

Reverse Northern analysis for positive clone selection

Reverse Northern blot was performed to screen positive clones. According to the manufacturer's instruction for DIG High Prime DNA labelling and detection starter kit II (Roche, Switzerland), two cDNA probes (driver and tester) were prepared using random primers and were labelled with digoxin (DIG). 4 μL of colony PCR products were denatured at 100 °C for 5 min and quickly placed on ice for 5 min, then mixed with 0.6 M NaOH respectively. Denatured DNA was blotted onto two nylon membranes (Roche, Switzerland). Saturated with 1.0 M Tris-HCl (pH 7.5), the two membranes were placed on the chromatography paper, dried at room temperature and fixed at 80 °C for 2 h. The labeled 'tester' cDNA probe was hybridized to one membrane, and the labeled 'driver' probe to the other. After overnight hybridization and stringency washes, a chromogenic reaction was carried out. The hybridized membranes were exposed to X-ray film (Kodak XBT-1; 12.7 cm*17.8 cm) for more than 30 min at 15-25 °C after incubate for 10 min at 37 °C.

DNA sequencing and analysis

Differentially expressed clones confirmed by the reversed Northern blot were selected and sequenced by Majorbio Co., Ltd. (Shanghai, China).The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih. gov/BLAST/) at NCBI. Functional classification of the clones was according to the method of Bevan *et al.* (1998).

Analysis of EST expression by qRT-PCR

Total RNA of the 0 h, 5 h and 10 h SNP-treated rudimentary leaves for qRT-PCR were extracted using kits from Huayueyang Biotechnology Co., Ltd. (Beijing, China), according to the manufacturer's protocol. First-strand cDNA was generated from 1 μg total RNA using the superscript first-strand synthesis system (Invitrogen, USA). Primers for qRT-PCR were designed using Primer Premier 5.0 software (Premier, Canada) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All the primers are shown in Table 1. Litchi homologue *actin* (accession number HQ588865.1) was the reference gene. qPCR was performed on an iQ5 optical system (Bio-Rad, USA) using a SYBR Green based qPCR assay. Each reaction mixture was 20 μL containing 6 μL of diluted first-strand cDNAs (40-80 ng) and 250 nM primer, SYBR Green PCR Master Mix (TaKaRa, Japan) 10 μL. The qPCRs were run as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s in 96-well optical reaction plates (Bio-Rad, USA). Each qRT-PCR analysis was performed in triplicate. Expression levels of the tested reference genes were determined by CT values and calculated by $2^{-\triangle\triangle CT}$ (Livak and Schmittgen, 2001).

Statistical analysis

All data were subjected to analysis of variances using a SPSS program (SPSS Inc. Chicago, IL, USA). The differences among treatment means were evaluated by Duncan's multiple range test ($P \leq 0.01$).

Table 1. Primer sequences of the reference gene and ESTs for qRT-PCR

Clone	Description in NCBI database	Forward primer (5'→3')	Reverse primer (5'→3')
	β actin	AGTTTGGTTGATGTGGGAGAC	TGGCTGAACCCGAGATGAT
1008	protein EARLY RESPONSIVE TO DEHYDRATION 15-like	TGGCTCCAGGAATGCTAC	ACATCGGCTTCTCAAACG
397	putative senescence-associated protein	TGATAGGAAGAGCCGACA	AATACGAACCGTGAAAGC
372	cytochrome P450 like_TBP	AAAGCATTGCGATGGTCC	CGTTCCCTTGGCTGTGGT
457	vegetative storage protein	TGACCCTGTCGTAAATCC	GCTTCCGAGCCGTTGTAT
11	18S ribosomal RNA gene, partial sequence	CCGTTGCTCTGATGATTC	CTGCCTTCCTTGGATGTG
261	Chaperone protein dnaJ 10	TACTCCAAGGTGCCAAAT	CAACTGAAACTGTCTACGATGA
987	hypothetical protein GUITHDRAFT_79647	AACATCTAAGGGCATCACA	GACCTCAGCCTGCTAACT

Results

SNP induced abortion of rudimentary leaves

Our previous work revealed an early sign of abortion of rudimentary leaves which was the downward growth of the leaves (Zhou *et al.*, 2008). We also established method for measuring the degree of the downward growth by proximal angle and the distal angle (Lu *et al.*, 2014). In this study, to confirm the effect of the SNP-generated NO on abortion of rudimentary leaves, shoot cuttings were treated with water or SNP in a growth chamber. Besides, our preliminary study confirmed that detached shoots could survive in water without wilting for at least 2 d. The proximal angle (α) and the distal angle (β) of the third leaves were measured (Fig. 1). The results showed that proximal angle α significantly increased at 5 h of SNP-treatment, and remained at similar level at 10 h of treatment. The distal angle β continually increased during the whole stage of the treatment (Table 2). The SNP-treated rudimentary leaves showed epinasty as characterized by downward curvature of leaves (Fig. 2A, B, C), presenting an early sign of abortion (Zhou *et al.*, 2008).

Differential screening of SSH library and functional classification of differentially expressed clones

Our SNP-treated SSH library was constructed for identifying NO responsive genes. To determine insert fragment size of the 1350 randomly picked clones, colony-PCRs were performed with the nested primers 1 and 2R. We obtained 746 positive clones then, and the insertion fragments were 150-800 bp (Fig. 3). Reverse northern blot was used to screen the differentially expressed EST clones. Finally, 180 clones that showed a differentially expressed pattern were obtained (Fig. 4). Their DNAs were subjected to sequencing.The results showed that the 180 clones were only ligated to 16 high homologous ESTs attributed to the high presenting times of the clones. As shown in Table 3, 7 of them were presented more than 5 times. Clone 987 encoding hypothetical protein GUITHDRAFT_79647 and clone 1008 encoding early responsive to dehydration 15-like (ERD15-like) protein were presented more than 40 times. According to the method of Bevan *et al.* (1998), 5 ESTs were classified to disease/defensive group, 3 ESTs were found to be related to protein destination and storage, and 3 ESTs were identified as protein synthesis (Table 4).

Table 2. Effects of SNP on angles of the rudimentary leaves

Time of treatment	Proximal angle α (°)	Distal angle β (°)
0 h	53.80±1.28 b	81.56±2.06 c
5 h	58.20±1.26 a	112.17±2.72 b
10 h	59.83±1.78 a	122.33±2.65 a

Notes: Values are means ± SE from 30 rudimentary leaves. The differences among all the treatment means were evaluated by Duncan' s multiple range tests at a 0.01 probability level using a SPSS program. Different lower-case letters indicate significant differences.

Fig. 2. Images of the new flushes. (A) Image of a new flush in 0 h of SNP treatment, (B) Image of a new flush in 5 h of SNP treatment, (C) Image of a new flush in 10 h of SNP treatment

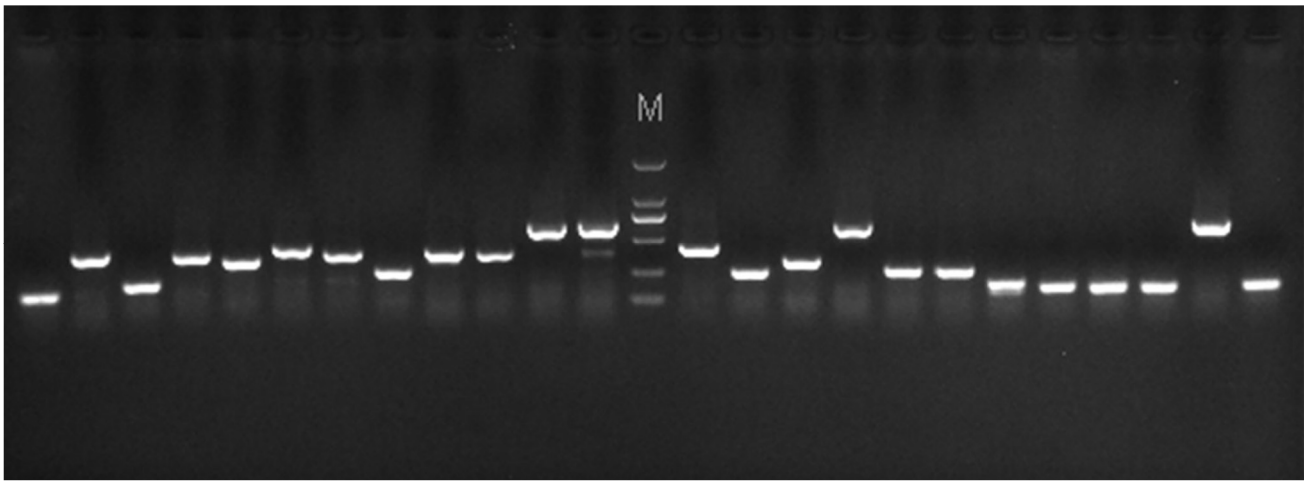

Fig. 3. PCR identification of the insert size in randomly selected clones from the SSH cDNA library. M, DNA marker 2000

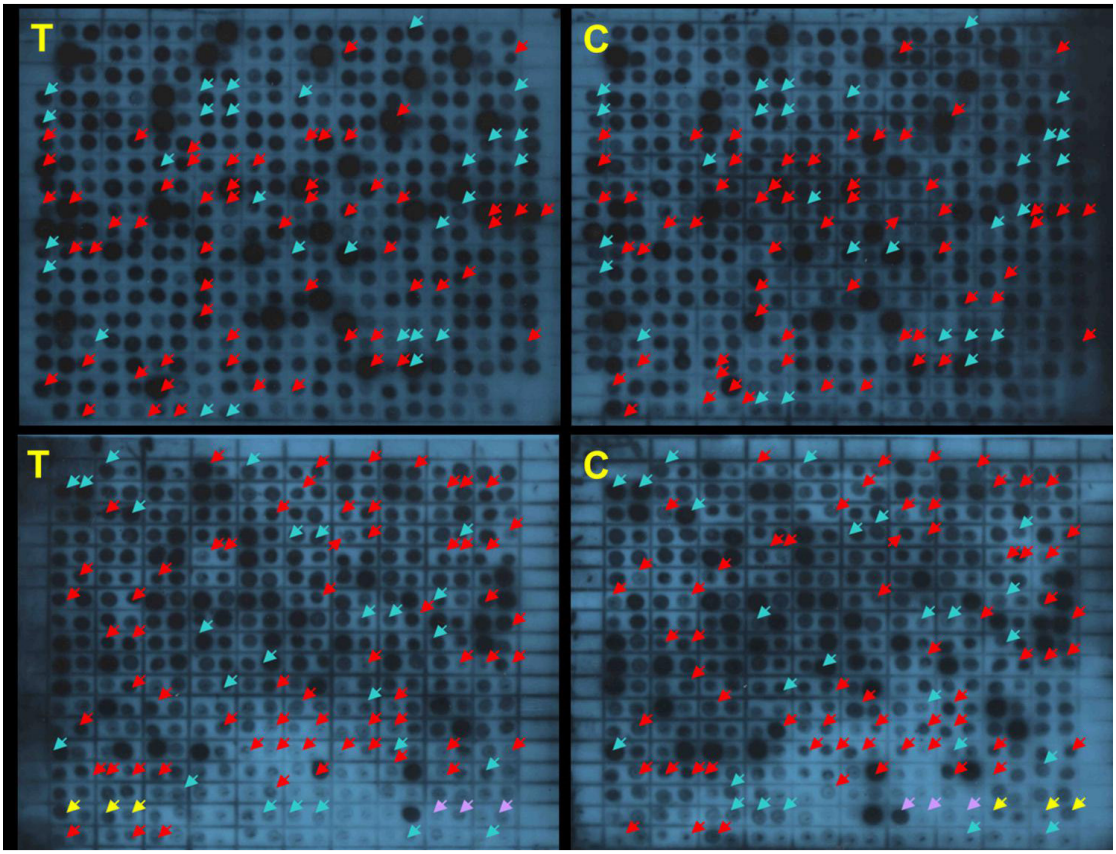

Fig. 4. Differential screening the SSH library by a reverse Northern analysis. Arrays of 746 clones were hybridized with DIG labeled tester (T) and driver (C). The spots marked with color arrows were recognized as differentially expressed clones

Table 3. Differentially expressed cDNA clones represented multiple times in SSH library

Code	Similarity	Number of isolates	Functional classification
987	hypothetical protein GUITHDRAFT_79647	48	Unclear classification or unclassified
1008	protein EARLY RESPONSIVE TO DEHYDRATION 15-like	43	Disease/Defence
247	PREDICTED: transcription factor HBP-1b(c38)	23	Transcription
372	cytochrome P450 like_TBP	20	Secondary metabolism
92	26S ribosomal RNA gene	19	Protein synthesis
457	vegetative storage protein	6	Protein destination and storage
11	18S ribosomal RNA gene, partial sequence	5	Protein synthesis

Table 4. 16 differentially expressed cDNA clones of SNP-treated SSH library in litchi

Code	Accession	Description in NCBI database	E-value	Functional classification
1008	XP_015877468.1	protein EARLY RESPONSIVE TO DEHYDRATION 15-like (*Ziziphus jujuba*)	5.00E-08	Disease/Defence
C16	XP_013442968.1	senescence-associated protein (*Medicago truncatula*)	2.00E-52	Disease/Defence
397	ABO20851.1	putative senescence-associated protein (*Lilium longiflorum*)	1.00E-56	Disease/Defence
468	BAB33421.1	putative senescence-associated protein (*Pisum sativum*)	2.00E-15	Disease/Defence
B15	AHF81484.1	small GTP Rab5 (*Mangifera indica*)	4.00E-38	Disease/Defence
261	XP_002512946.1	chaperone protein dnaJ 10 (*Ricinus communis*)	3.00E-04	Protein destination and storage
1220	XP_002884233.1	expressed protein (*Arabidopsis lyrata subsp. lyrata*)	3.00E-12	Protein destination and storage
457	ABG47463.1	vegetative storage protein (*Litchi chinensis*)	9.00E-25	Protein destination and storage
11	JF759906.1	18S ribosomal RNA gene, partial sequence (*Litchi chinensis*)	0.00E+00	Protein synthesis
92	AF479111.1	26S ribosomal RNA gene (*Loeseneriella barbata*)	2.00E-112	Protein synthesis
65	HM135404.1	26S ribosomal RNA gene, partial sequence (*Citrus unshiu*)	9.00E-29	Protein synthesis
372	BAA10929.1	cytochrome P450 like_TBP (*Nicotiana tabacum*)	2.00E-45	Secondary metabolism
247	XP_002532112.1	PREDICTED: transcription factor HBP-1b(c38) (*Ricinus communis*)	2.00E-07	Transcription
41	XP_010044420.1	PREDICTED: mechanosensitive ion channel protein 6-like (*Eucalyptus grandis*)	3.00E-69	Transporters
849	ACR56615.1	plasma intrinsic protein 2;5 (*Eucalyptus grandis*)	1.00E-21	Cell structure
987	XP_005822623.1	hypothetical protein GUITHDRAFT_79647 (*Guillardia theta CCMP2712*)	9.00E-27	Unclear classification or unclassified

Expression analysis of 7 ESTs in rudimentary leaves

Based on the SSH results, we randomly selected 7 differentially expressed ESTs to determine their expression by qRT-PCR. They encode ERD15-like protein, senescence-associated protein, cytochrome P450 like TBP, vegetative storage protein, 18S ribosomal RNA, chaperone protein and a hypothetical protein, respectively. Six out of the 7 ESTs showed increasing trends during 0 to 10 h of NO treatment (Fig. 5). For example, the expression level of which encoding ERD 15-like protein increased almost 20-fold from 0 h to 10 h of treatment.

Discussion

NO is an important plant signal molecule. The NO responsive genes were reported to be associated with stress-response, oxidation-reduction, signal transduction, senescence, reactive oxygen metabolism, and biological processes (Polverari *et al*., 2003; Lindermayr *et al*., 2005; Besson-Bard *et al*., 2009). Our previous study indicated that NO generated by SNP can promote flowering and rudimentary leaf abortion in litchi (Zhou *et al*., 2012). In addition, we have identified NO responsive genes in the floral buds by an SSH technique (Liu *et al*., 2015). In this study, we constructed an SSH cDNA library of the NO-treated rudimentary leaves to identify NO responsive genes. We obtained 16 high homologous ESTs from the SSH library. They are mainly related with the function of disease/defensive, protein destination and storage, and protein synthesis, suggested that these processes might be involved in the NO-induced abortion in litchi rudimentary leaves. Interestingly, we found that some identified ESTs had high presented times, such as the clone 987 encoding a hypothetical protein, and clone 1008 encoding ERD15 protein. The high presented times suggested that they might be the key genes involved in the NO-induced abortion of the rudimentary leaves.

ERDs are rapidly activated proteins under dehydration stress and could be classified into 16 groups (Kiyosue *et al*., 1994; Alves *et al*., 2011a), such as the chloroplast ATP-dependent protease (ERD1), heat shock protein (ERD2,8), S-adenosyl-methionine-dependent methyltransferases (ERD3), membrane protein (ERD4), proline dehydrogenase (ERD5), hydrophilic protein (ERD15). Each kind of ERD gene has specific functions. In our SSH library, we isolated one EST encoding ERD15-like and the expression level of which increased almost 20-fold from 0 h to 10 h of SNP treatment, indicating that ERD15-like might be an important NO responsive gene involved in rudimentary leaf abortion.

ERD15 is a transcription factor that integrates several stress signaling pathways, such as abscisic acid (ABA) response, salicylic acid (SA)-dependent defense, mechanical and biological damage (Alves *et al*., 2011a). In Arabidopsis, the expression of *ERD15* is up-regulated under stress conditions. *ERD15* transgenic plants displayed hypersensitivity to ABA and enhanced tolerance to both drought and freezing (Kariola *et al*., 2006). In soybean, ERD15 functions as an upstream component of stress, and could connect endoplasmic reticulum stress with an osmotic stress-induced cell death signal (Alves *et al*., 2011b). In tomato, ERD15 could increase plant tolerance of dehydration, salinity, ABA, ethylene, and salicylic acid (Ziaf *et al*., 2016). Furthermore, transformation of wild tomato *ERD15* (*SpERD15*) into tobacco resulted in enhanced stress tolerance (Ziaf *et al*., 2011). Future work should be focused on the functional study on the litchi homologue *ERD15-like* (*LcERD15-like*).

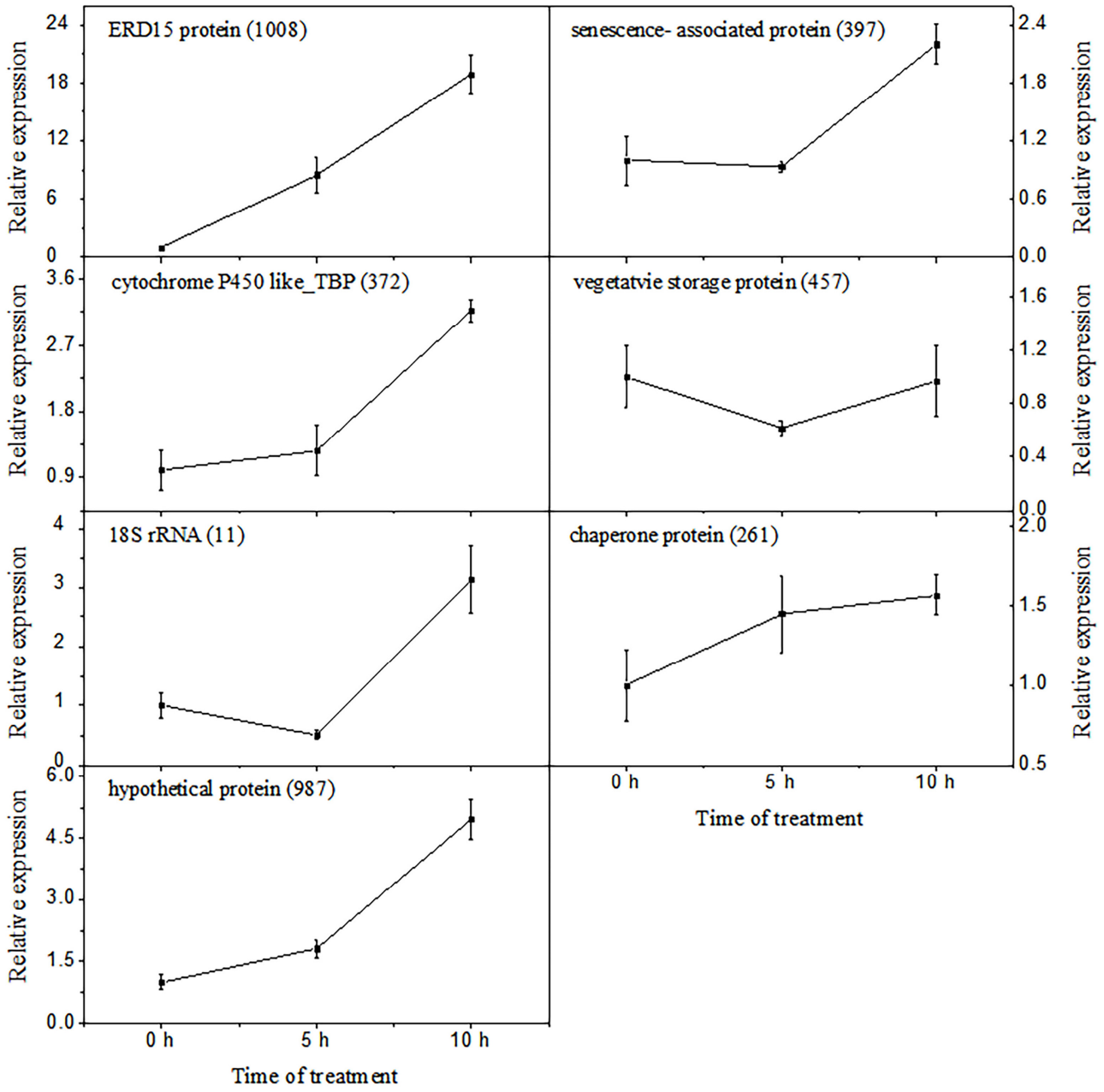

Fig. 5. Relative expression of 7 ESTs in the rudimentary leaves of 'Nuomici' litchi from 0 to 10 h of SNP treatment. The encoding proteins are indicated at the upper-left corner of the figures. The numbers marked in the brackets are the codes of the clones. Relative expression levels were calculated by qRT-PCR using the $2^{-\Delta\Delta CT}$ method with *Actin* as a reference gene. Data are means of 3 replicates and bars represent SD

Conclusions

We have constructed an SSH library for the NO-treated rudimentary leaves of litchi, and identified 16 ESTs associated with the NO-induced rudimentary leaf abortion. Most of them are functionally related with disease/defensive, protein destination and storage, and protein synthesis. The expression level of *LcERD15-like* increased from 0 h to 10 h of SNP treatment. *LcERD15-like* may be an important NO responsive genes in litchi rudimentary leaves.

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