DOI: 10.1007/s10535-014-0466-x BIOLOGIA PLANTARUM 59 (1): 115-122, 2015

Identification of nitric oxide responsive genes in the floral buds of *Litchi chinensis*

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Abstract

Litchi (*Litchi chinensis* Sonn.) is an important tropical and subtropical evergreen woody fruit tree, and it has been shown that nitric oxide (NO) could promote litchi flowering. NO responsive genes of litchi (cv. Nuomici) primordia were identified through a suppression subtractive hybridization (SSH) library screen. We obtained 1 563 expressed sequences tags (ESTs) that were enriched in the NO treated inflorescence primordia. We then used a reverse Northern analysis to identify 728 true NO responsive ESTs, the sequences of which have been further analyzed. They represent 70 litchi unique genes that could be classified into 9 categories: 14 % of them were involved in transport facilitation, 7 % in transcription regulation, 9 % in stress response, 7 % in sugar metabolism, 9 % in secondary metabolism, 10 % in intracellular signalling, and 44 % in other metabolism, whereas 11 % were genes with unknown functions, and 7 % were genes with no hit found. Next, we performed a real-time quantitative polymerase chain reaction (RT-qPCR) to determine the expression of selected candidate genes during a time-course of NO treatment and of normal floral tissue development.

Additional key words: expressed sequences tags, litchi flowering, suppression subtractive hybridization.

Introduction

Litchi (*Litchi chinensis*) is one of the most important subtropical fruits in southern China. Low winter temperature is indispensable for litchi floral induction and it directly correlates with fruit sets in the following year (Menzel and Simpson 1988, Chen and Huang 2005). Under normal winter conditions with a prolonged exposure to low temperature, the litchi apical bud breaks when air temperature and soil moisture increase. Next, the axillary or apical panicle primordia emerge and become visible as "whitish millets" (Huang and Chen 2005). At the "millet stage", the buds are mixed buds with axillary or apical panicle primordia, leaf primordia, and rudimentary leaves. As the air temperature increases gradually from winter to spring, the panicle primordia develop into panicles, and the rudimentary leaves abscise. In the context of global warming and climate change, a high winter temperature is a major challenge for litchi production and sudden increase in air temperature causes the rudimentary leaves to develop into fully expanded leaves and the panicle primordia may be ceased to develop and shrink.

It has been shown that abiotic stresses could promote flowering in *Citrus sinensis*, *Mangifera indica*, *Dimocarpus longana*, and *L. chinensis* (Menzel and Simpson 1988, Nunez-Elisea and Davenport 1994, Ali and Lovatt 1995, Manochai *et al.* 2005). Such a

Submitted 4 March 2014, *last revision* 2 August 2014, *accepted* 5 August 2014.
Abbreviations: ANR - anthocyanidin reductase; CHS - chalcone synthase; DIG - digoxin; EST - expressed sequences tags; GA14 - gibberellin-regulated protein 14-like; IAA - indole-acetic acid; IPTG - isopropyl-β-D-thiogalactopyranoside; LB - Luria Bertani; *LFY* - *LEAFY*; ROS - reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; SNP - sodium nitroprusside; X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
Acknowledgements: We thank Dr. B.C. Sarker for proofreading. This study was funded by the National Natural Science Foundation (31071760) and the Agricultural Industry Project (CARS-33-08) by the Ministry of Agriculture.
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mechanism to hasten reproduction when a plant is subjected to an environmental stress has an evolutionary advantage to ensure the survival of the species under adverse conditions (Kocsy *et al.* 2013). Nitric oxide (NO) has been proved to be involved in plant growth and development (Wu *et al*. 2014). It is also well documented that stress induces reactive oxygen species (ROS) and NO accumulation in plants (Dat *et al.* 2000, Gould *et al.* 2003). H_2O_2 and NO are key signalling molecules involved in plant responses to both biotic and abiotic stresses (García-Mata and Lamattina 2001, Neill *et al.* 2002, Uchida *et al.* 2002, Hermes *et al*. 2013).

We have shown that NO and ROS could promote litchi reproductive growth by inhibiting the growth of rudimentary leaves in the panicles as well as by inducing the expression of *LcLFY* gene (Zhou *et al.* 2012). However, the signalling network and molecular mechanism of NO and ROS in the control of flowering related genes and rudimentary leaves in litchi remain largely unknown. Identification of the full spectrum of downstream target genes controlled by NO and ROS would be the first step to elucidate the mechanism of stress-induced flowering in litchi. Liu *et al.* (2013) used the suppression subtractive hybridization (SSH) cDNA library screening to identify ROS responsive genes. This approach was also used to identify NO responsive genes in the litchi primordia.

Materials and methods

Plants and treatments: The experiment was carried out at the experimental orchard of the South China Agricultural University, Guangzhou, during the period of 2011 and 2012. Eleven-year-old trees of litchi (*Litchi chinensis* Sonn.) cv. Nuomici grafted onto a rootstock Huaizhi were used for the study. The terminal shoots of about 6 cm with floral buds were cut off from the trees at the "millet stage". According to the method of Zhou *et al.* (2012), they were immediately placed in solutions containing either 0 or 0.5 mM sodium nitroprusside (SNP) (*Sigma*, St Louis, MO, USA). The cuttings were placed in a growth chamber at a photosynthetic photon flux density of 160 μmol m^{-2} s^{-1} and a temperature of 20 °C. Ten hours later, the panicle primordia were sampled, frozen in liquid nitrogen, and stored at -80 °C for SSH library construction.

A real-time quantitative polymerase chain reaction (RT-qPCR) was used to confirm whether the isolated clones from the SSH library screen were differentially expressed between NO treated primordia and the control ones. Five to 10 shoot cuttings were pooled, and 3 biological replicates were used. Panicle primordia were collected after 0, 5, 10, and 15 h of the treatment, frozen in liquid nitrogen, and stored at -80 °C for RT-qPCR.

Buds were collected on 25 December 2011, 13 January, 1, 5, 12, and 20 February 2012 to determine the expression of the candidate unigenes during floral induction and differentiation stages. Samples were frozen in liquid nitrogen and stored at -80 °C for RT-qPCR analysis.

Construction of SSH cDNA library: We followed the protocol described by Zhou *et al.* (1999) to extract total RNA from frozen buds. Extracted RNA from panicle primordia treated with water for 10 h was used as driver and those treated with SNP for 10 h was used as tester. mRNA was isolated from total RNA using *PolyATract* mRNA isolation system III (*Promega*, Madison, WI, USA). A *PCR-SelectTM* cDNA subtraction kit (*Clontech*, Mountain View, CA, USA) was used to prepare the subtracted library. Two rounds of hybridization and PCR amplification were performed to enrich the differentially expressed fragments after tester cDNA was digested with *Rsa* I and ligated to adaptors. Cloning the second-round PCR products of subtracted cDNA into a *pMD19-T* vector was done and then transformed into an *Escherichia coli* strain DH5α. We then selected the white positive transformants on a Luria Bertani (LB) medium supplemented with 50 mg dm^{-3} ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and isopropyl-β-D-thiogalactopyranoside (IPTG) for sequence analysis.

Identification of the insert fragments by PCR: White colonies were picked and inoculated into 1 cm^3 of a liquid LB medium containing ampicillin and incubated at 37 °C for 12 h. A PCR amplification was performed with a bacterial suspension as template, nested primer 1 and primer 2R as primers provided by a *PCR-SelectTM* cDNA subtraction kit (*Clontech*). The length of subtractive cDNA fragment was then recorded. The PCR amplification was performed using a thermal cycler (*Bio-Rad*, Hercules, CA, USA): predenaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 10 s, 66 °C for 30 s, and 72 °C for 90 s.

Reverse Northern analysis for positive clone selection: To screen positive clones, a reverse Northern blot was performed according to the method of Liu *et al.* (2013). Two cDNA probes, one driver and the other tester, were prepared using random primers and were labelled with digoxin (DIG). The colony PCR products (0.004 cm^3) were denatured at 100 °C for 5 min and quickly placed on ice for 5 min, then mixed with 0.004 cm^3 of 0.6 M NaOH. Denatured DNA was blotted onto two nylon membranes (*Roche*, Basel, Switzerland). The PCR product of *actin* cDNA was spotted as control. 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. A chromogenic reaction was carried out according to the manufacturer's instruction of *DIG High Prime* DNA labelling and detection starter kit II (*Roche*).

DNA sequencing and analysis: Differentially expressed clones confirmed by the reversed Northern blot were subjected to conventional Sanger sequencing. The *BLAST* program (http://www.ncbi.nlm.nih.gov/BLAST/) at the National Center for Biotechnology Information (NCBI) was used to analyze their sequences.

Analysis of gene expression by RT-qPCR: We isolated total RNA from floral buds or panicle primordia, and it was used to generate the first-strand cDNA according to the method of Liu *et al.* (2013). The expression analysis was performed at the transcript level which was determined by RT-qPCR using single-stranded cDNA as template. All the primers are shown in Table 1. The reference gene was litchi homologue *actin* (accession number HQ588865.1). qPCR was performed on an *iQ5* optical system (*Bio-Rad*) using a *SYBR Green* based qPCR assay. qPCRs were run as follows: 50 °C for 2 min, 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*). Each RT-qPCR analysis was performed in triplicate. Amplification efficiencies were calculated as $10^{(-1/slope)}$ - 1. Slope is the value obtained from the standard curve. The transcript quantifications of the candidate genes were performed in relation to *actin* and they were calculated by the $2^{-\triangle\triangle C_T}$ method (Livak and Schmittgen 2001).

Statistical analysis: Data were subjected to the analysis of variances using a *SPSS* program (*SPSS*, Chicago, IL, USA). The differences among treatment means were evaluated by the Duncan's multiple range test at a 0.05 probability level. Differences between treatments and controls were evaluated by the Student's *t*-test.

Table 1. Primer sequences of the reference gene and ESTs for RT-qPCR.

Clone	Homology gene	Forward primer (5'→3')	Reverse primer (5'→3')
	actin	AGTTTGGTTGATGTGGGAGAC	TGGCTGAACCCGAGATGAT
21	*ANR*	ACAAAAATATGGGCTCTGCAAAC	GCATGCAAATGCTGTCAGGTT
74	*CHS3*	TCAGTCGCAGCAAACTAAGCA	TTGGGCCTGGCCTTACTGT
76	*GA14*	TGCACAGATTTGGCCTTGAGT	CAGAAGGCTCATGCAGGACAT
503	*AUX/IAA*	CCTTGGCCGATGTTTATTGAC	TGGCATCGGAGCTCTTCATT
1000	*E3*	CTACGTGGTGGCATGCAGAT	AGCTCTCCACCTCCAGCGTTA

Results

We have isolated 1 563 clones from the SSH library screen. To determine insert size, these clones were PCR amplified by using nested primers 1 and 2R. The insertion fragments were found from 250 to 750 bp. Out of total clones, 938 (60 %) carried a single exogenous fragment (Fig. 1). Next, they were subjected to the reverse Northern blot to identify true differentially expressed EST clones. A number of 728 EST clones (spots marked with circles) showed a differentially expressed pattern (Fig. 2), and their DNA was isolated for Sanger sequencing.

The sequence analysis revealed that the 728 clones could be aligned to 70 unique genes and 25 clones were presented more than 3 times (Table 2 and Table 1 Suppl). These unique genes could be classified into 9 categories: 14 % of them were involved in transport facilitation, 7 %

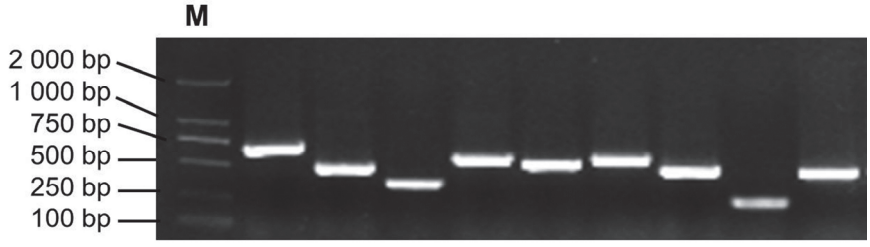

Fig. 1. PCR identification of the insert size in randomly selected clones from the SSH cDNA library. M - 100 bp DNA ladder marker.

Table 2. Differentially expressed cDNA clones presented multiple times in the SSH library.

Clone number	Number of isolates	Similarity
12	115	lipid transfer protein (LTP)
2	41	vegetative storage protein (VSP1)
10	30	chlorophyll *a-b* binding protein CP29.1 (LHCB4.1)
96	28	conserved hypothetical protein
4	26	mannose/glucose-specific lectin
86	25	aquaporin PIP1 (PIP1)
80	21	fructose-bisphosphate aldolase, class I (AT2G36460)
47	14	polyubiquitin 10 (UBQ10)
105	12	peroxidase 4
503	10	Aux/IAA protein
126	9	flavonol sulfotransferase-like protein (SOT12)
435	8	gibberellin-regulated family protein (AT2G18420)
76	8	gibberellin-regulated protein 14-like (LOC100265557),
482	9	laccase
696	6	ripening-induced protein 1-like (MRIP1)
470	6	ATP binding protein
368	6	chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase
154	6	monodehydroascorbate reductase (NADH) - like protein
579	5	oligopeptide transporter 7 (OPT7)
232	5	carbonic anhydrase
179	5	polyubiquitin (WubiG)
350	4	putative proline-rich cell wall protein
74	4	chalcone synthase CHS3
566	3	S-adenosylmethionine decarboxylase-like protein
30	3	conserved hypothetical protein

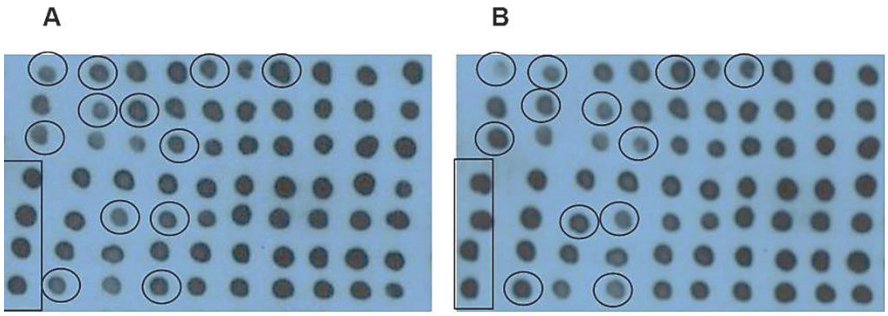

Fig. 2. Differential screening the SSH library by a reverse Northern analysis. An array of 66 clones was hybridized with a DIG-labeled tester (*A*) and driver (*B*). The spots marked with the *circles* were recognized as differentially expressed clones. The spots marked with the *rectangles* are those of the *actin* gene.

Table 3. Functional classification of NO responsive clones in litchi.

Functional classification	[%]
Transport facilitation	14
Transcription regulation	7
Stress response	9
Sugar metabolism	7
Secondary metabolism	9
Intracellular signalling	10
Others	26
Unknown protein	11
No match	7

in transcription regulation, 9 % in stress response, 7 % in sugar metabolism, 9 % in secondary metabolism, 10 % in intracellular signalling, and 26 % in other metabolism, whereas 11 % were genes with unknown functions and 7 % were genes with no hit found (Table 3).

Based on the SSH results, we analyzed five differentially expressed ESTs using RT-qPCR. The amplification efficiencies were 90 - 110 %. Transcriptions of four out of the five unique genes were significantly different between the treatment and the control (Table 4). These differentially expressed unigenes included the litchi homologues encoding anthocyanidin reductase (*LcANR*), chalcone synthase (*LcCHS3*), gibberellin-regulated protein 14-like (*LcGA14*), and

auxin-induced protein AUX/IAA (*LcAUX/IAA*). Next, they were subjected to a time-course analysis from 0 to 15 h of the NO treatment. The relative expression of *LcANR* in the treated samples was higher than that of the control in 5 h of the treatment, and lower than the control in 10 h of the treatment. The expressions of *LcCHS*, *LcGA14*, and *LcAUX/IAA* increased to the maximum at 10 h of the treatment and then decreased to a relatively low level at 15 h of the treatment (Fig. 3).

To investigate the expression of candidate NO responsive unique genes during normal floral tissue differentiation, we determined their relative expression in buds of the terminal shoots at the induction stage (25 December 2011), floral initiation stage (13 January 2012), rhachis developmental stages when apical and lateral axillary panicle began to develop (1, 5, and 12 February 2012), and floral organ developmental stage (20 February 2012) as described by Liu *et al.* (2013). The results show that the relative expression of *LcANR* at the floral induction stage was low and increased by 121.0 and 162.6 % at the floral initiation stage and the early rhachis developmental stage, respectively. *LcCHS3* was expressed at a relatively high level at the induction stage, floral initiation stage, and floral organ developmental stage, whereas *LcGA14* was expressed at the highest level at the floral initiation stage. *LcAUX/IAA* was found to be expressed at the highest level at the middle stage of rhachis development (Fig. 4).

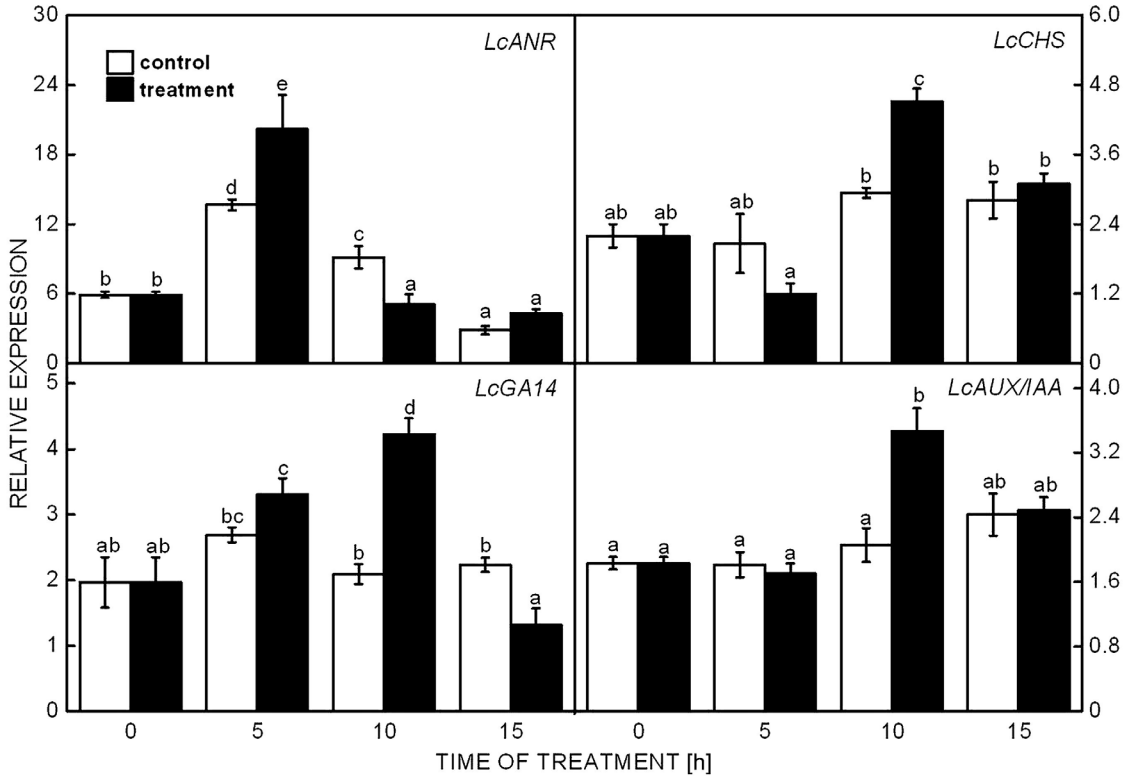

Fig. 3. Relative quantities of *LcANR*, *LcCHS3*, *LcGA14*, and *LcAUX/IAA* in floral buds from 0 to 15 h of NO treatment. Terminal shoots with floral buds at the "millet stage" were cut off from litchi trees and immediately placed in a 0.5 μM SNP solution or distilled water as control. The relative transcription was calculated by RT-qPCR using the $2^{-\Delta\Delta CT}$ method with *actin* as reference gene. Data are means of three replicates and the *bars* represent SE. Different letters above the columns indicate significant differences at $P < 0.05$ according to the Duncan's multiple range test.

Table 4. The relative expression of five unique genes after 10 h of SNP treatment. The relative transcription was calculated by RT-qPCR using the $2^{-\Delta\Delta CT}$ method with *actin* as reference gene. Values are means ± SE from three replicates. Significant differences ($P \leq 0.05$) according to the Student's t-test between the treatment and the control are indicated by the *asterisks*.

Homology gene	*ANR*	*CHS3*	*GA14*	*AUX/IAA*	*E3*
Control	9.14	2.94	2.09	2.06	1.06
Treatment	5.10*	4.51*	4.23*	3.47*	1.47

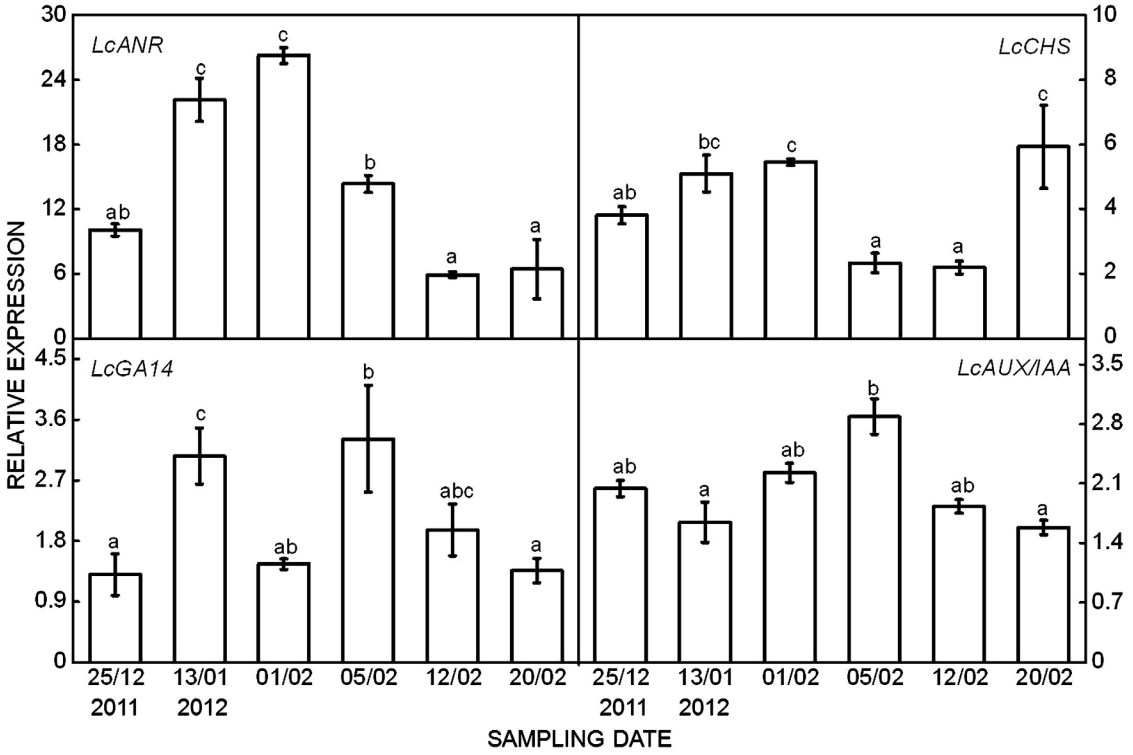

Fig. 4. The relative expressions of *LcANR*, *LcCHS3*, *LcGA14*, and *LcAUX/IAA* in floral buds. Three litchi trees were induced to flower under natural conditions. The floral buds were sampled from 25 December 2011 to 20 February 2012 before and during panicle development. The relative transcription was calculated by RT-qPCR using the $2^{-\Delta\Delta C_T}$ method with *actin* as reference gene. Data are means of three replicates and the *bars* represent SE. Different letters above the columns indicate significant differences at $P < 0.05$ according to the Duncan's multiple range test.

Discussion

It has been shown that abiotic stresses, such as chilling, drought, and oxidative stresses, could induce flowering in evergreen woody fruit trees (Nunez-Elisea and Davenport 1994, Manochai *et al.* 2005). Our previous study indicated that the stress signal of NO generated by SNP can promote flowering in litchi (Zhou *et al.* 2012). In the present study, we constructed an SSH library to identify NO responsive genes during floral differentiation. A number of 70 EST clones were identified to be responsive to NO at the "millet stage" known as critical period of floral differentiation. In the SSH library, 7 % of the clones were identified to be related to sugar metabolism (Table 3). Sugars have conventionally been viewed as resources for respiration and metabolic intermediates, as well structural or storage components (Sheen *et al.* 1999). The carbon to nitrogen ratio in plants has been considered as factor that affects flowering. The ability to sense carbon and nitrogen metabolites enables plants to regulate metabolism and development in response to their internal ratio of carbon to nitrogen (Coruzzi and Zhou 2001, Gibson 2005). Floral formation in fruit trees is promoted by ringing, root pruning, which could restrict vegetative growth resulting in sugar accumulation (Ito *et al.* 2002, Davenport and Stern 2005). We isolated five clones encoding proteins that are related to sugar metabolism. For example, clone No. 80 encodes fructose-bisphosphate aldolase class I which is a key metabolic enzyme catalyzing the cleavage of β-fructose-1,6-phosphate to D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, and reversible aldol condensation which is involved in both the glycolytic/gluconeogenic pathway and the pentose phosphate cycle in plants (Lebherz *et al.* 1984). Abiotic stimuli, such as NaCl, exogenous application of abscisic acid (ABA), and polyethylene glycol (PEG), could trigger a significant induction of fructose-bisphosphate aldolase (Fan *et al.* 2009).

NO could act as signal molecule in response to biotic and abiotic stresses (García-Mata and Lamattina 2001, Neill *et al.* 2002). In the present study, we found that NO could induce stress responsive genes in litchi flower buds. For example, clone No. 4 encoding protein is mannose/glucose-specific lectin, No. 350 encodes a homologue of proline-rich cell wall protein in *Vitis vinifera*. Furthermore, we observed additional NO

responsive genes related to intracellular signalling. For example, clone No. 503 encoding protein is homologous to the AUX/IAA protein which plays important roles in the auxin signal transduction pathway (Song and Xu 2013). Clone No. 163 encoding protein is homologue of ABA and calcium induced protein phosphatase 2C in *Fagus sylvatica*. Moreover, clone No. 12 encodes a protein homologous to the lipid transfer protein in *Dimocarpus longan* belonged to the transport facilitation group, and No. 199 encodes a protein homologous to the gibberellin-regulated family protein in *Arabidopsis thaliana*.

Chalcone synthase (CHS) is involved in flavonoid biosynthesis (Kumar *et al.* 2013). Anthocyanidin reductase (ANR) can catalyze the conversion of anthocyanidin to epicatechin (Li *et al.* 2008). It has been suggested that flavonoids can control reproductive growth by modulating the content of gibberellic acid (GA) and indole-acetic acid (IAA) or through ROS regulation (Buer *et al.* 2010). *GA14* encodes a gibberellin-regulated protein which is involved in the gibberellin signalling pathway, and *AUX/IAA* encodes an auxin-induced protein AUX/IAA which is involved in the auxin signalling pathway. Our results show that *LcCHS3*, *LcANR, LcGA14*, and *LcAUX/IAA* were expressed during floral differentiation suggesting that these NO responsive genes have the possibility to be involved in flowering. Future research may provide more insights into the molecular function of these genes and elucidate their potential roles in NO response and flowering.

In summary, we have constructed the SSH library for the NO-treated panicle primordia, and for the first time, we identified 70 NO responsive EST unique genes in the litchi floral buds. Among the unique genes, 82 % are presumably involved in transport facilitation, transcription regulation, stress response, sugar metabolism, secondary metabolism, intracellular signalling, and other metabolic pathways.

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