BIOLOGIA PLANTARUM 56 (2): 321-329, 2012

Hydrogen peroxide and nitric oxide promote reproductive growth in *Litchi chinensis*

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Abstract

Vegetative growth and reproductive growth strongly competes with each other during panicle development in litchi (*Litchi chinensis* Sonn.). We herein investigated the roles of hydrogen peroxide and nitric oxide in the competition between growth of rudimentary leaves and panicle development. The results show that the chilling-induced flowering increased H_2O_2 and NO contents in the mixed buds. Treatments with sodium nitroprusside (SNP), the NO donor, and methyl viologen dichloride hydrate (MV), the superoxide generator, increased NO and H_2O_2 contents in the mixed buds. MV and SNP treatments promoted abscission of rudimentary leaves and encouraged panicle development before or at the stage of panicle emergence. The nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) and the H_2O_2 trapper dimethylthiourea (DMTU) inhibited a chilling-induced flowering. SNP promoted the expression of litchi *LEAFY* homolog (*LcLFY*). These promotive effects were suppressed by the NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide (PTIO) and the H_2O_2 trapper, DMTU. The results suggest that H_2O_2 and NO promote reproductive growth by inhibiting the growth of rudimentary leaves as well as by promoting the expression of the flower related gene, *LcLFY*.

Additional key words: flowering, *LEAFY*, methyl viologen, sodium nitroprusside.

Introduction

At a developmental stage, flowering can be regarded as a transition from vegetative to the reproductive phase. In evergreen woody trees, the division between vegetative and reproductive phases might not always be evident. In the tropical evergreen tree mango (*Mangifera indica*), cool temperature is a factor known to induce flowering, but cool temperature alone does not ensure floral initiation, because there are important interactions with vegetative growth (Wilkie *et al.* 2008). Many evergreen woody trees have to experience environmental stresses such as chilling, drought or oxidative stress to induce flowering. Chilling stress induces flowering in orange (*Citrus sinensis*) and litchi (*Litchi chinensis* Sonn.) (Menzel and Simpson 1988, Ali and Lovatt 1995). Water stress induces flowering in mango (Nunez-Elisea and Davenport 1994). Potassium chlorate treatment, which results in oxidative stress, is efficient in flower induction year-around in longan (*Dimocarpus longan*) (Manochai *et al*. 2005). Hydrogen peroxide is one of the major reactive oxygen species (ROS). It generates as a result of oxidative stress, which can arise from environmental stresses such as excess excitation energy, cold and drought (Bartosz 1997, Dat *et al*. 2000). Nitric oxide, a gaseous free radical and reactive nitrogen species (Arasimowicz and Floryszak-Wieczorek 2007), accumulates when plants are subject to environmental stresses (Gould *et al*. 2003). H_2O_2 and NO are key signaling molecules involved in plant responses to both biotic and abiotic stresses (García-Mata and Lamattina 2001, Neill *et al*. 2002, Uchida *et al*. 2002).

Received 8 November 2010, *accepted* 17 May 2011.
Abbreviations: ABA - abscisic acid; 4AF-DA - 4-aminofluorescin diacetate; DAF-2DA - diaminofluorescein diacetate; DCFH-DA - dichlorofluorescin diacetate; DMTU - dimethylthiourea; *LcLFY* - *LEAFY* homolog; L-NAME - N^{ω}-nitro-L-arginine methyl ester; MV - methyl viologen dichloride hydrate; PTIO - 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; SNP - sodium nitroprusside.
A*cknowledgements*: We thank P. O'Malley and Y.Q. O'Malley for proofreading. This study was funded by the National Natural Science Foundation (30571283, 31071760), 948 Projects of Agricultural Ministry (2006G31), and the Agricultural Industry Project (nyhyzx07-031) by the Ministry of Agriculture.
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Litchi is an evergreen woody tree cultivated in subtropical and tropical regions, and produces arillate fruits with sweet, translucent, juicy flesh. However, irregular bearing attributed to unreliable flowering is an ongoing problem for litchi producers. Previous studies indicate that floral differentiation in litchi is induced by low temperatures and is enhanced by autumn and winter drought (Menzel and Simpson 1988, Chen and Huang 2005). After a period of inductive low temperature and drought, apical buds of the shoots may break and elongate when air temperature and soil moisture increase. Then axillary or apical panicle primordia emerge and become visible as "whitish millets" (Huang and Chen 2005). At this stage (the "millet stage"), floral buds are mixed buds with axillary or apical panicle primordia, leaf primordia and rudimentary leaves. The fate of the mixed buds is largely dependent on environment. If air temperature is not too warm, growth of panicle primordia may prevail and the rudimentary leaves will abscise automatically. However, if the trees are exposed to high temperatures, the rudimentary leaves may develop to fully expanded leaves and the panicle primordia may cease to develop and shrink. Hence, strong competition between vegetative growth (growth of rudimentary leaves) and reproductive growth (development of panicle) occurs during floral differentiation. Growers remove or kill the rudimentary leaves by spraying with ethephon to encourage continuative development of the panicle. The authors hypothesized that the stress-induced NO and H_2O_2 accumulation inhibit vegetative growth and promote reproductive growth. The authors herein mainly determined H_2O_2 and NO concentrations in buds of litchi during the stress-induced flowering treatment, and used methyl viologen dichloride hydrate (MV) to induce the production of H_2O_2 and sodium nitroprusside (SNP) to produce NO. The purpose for this was to investigate whether exogenous application of these two molecules would increase the number of flowers in the panicles, inhibit growth of rudimentary leaves, promote *LcLFY* expression, and to find out the role of H_2O_2 and NO in flowering of litchi.

Materials and methods

Plant materials and experimental procedures: Two kinds of important litchi (*Litchi chinensis* Sonn.) commercial cultivars, Feizixiao and Nuomici were used in the experiment. They require low temperature to induce flowering. Feizixiao seedlings grow fast and are easily cultivated in pots.

Three-year-old Feizixiao trees grafted onto cv. Huaizhi were grown in 30-dm^3 pots with loam, mushroom cinder and coconut chaff (v: v: v, 3:1:1). Potted trees (1 m height and with about 10 terminal shoots) were selected for the experiment. After the terminal shoots had matured (mid-November), eight trees as controls were transferred to a growth chamber at 12-h photoperiod with natural irradiance, day/night temperature of 28/23 °C and relative humidity of 75 - 85 %. The other eight trees were transferred to a growth chamber with the same irradiance and humidity, but temperature of 15/8 °C. When panicle primordia emerged, buds were sampled for H_2O_2 or NO imaging. Then the chilling chamber was re-warmed to 28/23 °C. Leaves located at the centre of the terminal shoots were sampled for the determination of H_2O_2.

In another experiment, the three-year-old potted trees cv. Feizixiao were transferred to the chamber and grown at 18/13 °C. Six replicate trees were sprayed with 2 mM SNP (*Shanghai Chemical reagent Co,* Shanghai, China) or 40 μM MV (*Sigma*, St Louis, MO, USA) after 1, 21 and 41 d. Another 6 trees were sprayed with distilled water as a control at the same time. The chamber was re-warmed to 28/23 °C after 63 d of treatment, when panicle primordia emerged. Then numbers of leaves per panicle, numbers of the axillary panicles per panicle, ratios of axillary panicles to total nodes per panicle were counted after the chamber was re-warmed for 10 d. All trees were continually grown in the chamber and panicles were packed in nylon net before flowering so that abscised flowers could be collected. Numbers of flowers per panicle were calculated after anthesis.

To investigate whether the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, *Sigma*) and the H_2O_2 trapper dimethyl-thiourea (DMTU, *Sigma*) inhibit a chilling-induced flowering, 4-year-old potted trees of cv. Feizixiao were transferred to a chamber at 15/8 °C. Six replicate trees were uniformly sprayed with 1 mM L-NAME or 3 mM DMTU per week. Another 6 trees were sprayed with distilled water as a control at the same time. The chamber was re-warmed to 28/23 °C after 63 d of treatment. All the trees were continually grown in the chamber as described above and numbers of flowers per panicle were calculated after anthesis.

To find out appropriate concentrations in SNP and MV for the control of rudimentary leaf growth, six-year-old litchi trees of cv. Nuomici grafted onto cv. Huaizhi were grown in the experimental orchard of South China Agricultural University. Four replicate trees of similar size and with leafy panicles were selected on 19 January 2006. Five different branches in one replicate tree were uniformly sprayed with water, 1 mM SNP, 3 mM SNP, 40 μM MV and 120 μM MV. Two weeks after the treatment, 10 panicles in one branch were randomly chosen for counting the numbers of leaves per panicle and numbers of the axillary panicles per panicle. In

addition, ratios of axillary panicles to total nodes per panicle were calculated.

In another experiment, three litchi trees grown in the orchard as described above were selected. Branches of the trees were uniformly sprayed with water, 2 mM SNP in the absence or presence of 800 μM NO specific scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, *Sigma*), 2 mM SNP analogue, potassium ferricyanide, which does not produce NO, 40 μM MV in the absence or presence of 1 mM H_2O_2 trapper DMTU. Numbers of leaves in the panicles were counted before and 8 d after spraying.

To determine whether SNP and MV treatment can induce NO and H_2O_2 in the mixed buds, five different branches in one replicate tree were uniformly sprayed with water, 2 mM SNP in the absence or presence of 800 μM PTIO, or 40 μM MV in the absence or presence of 1 mM DMTU. After 10 h, the mixed buds were sampled for H_2O_2 or NO imaging.

To study the effects of SNP and MV on the expression of *LcLFY*, terminal shoots about 6 cm long with floral buds at the "millet stage" were cut off from the six-year-old litchi trees (cv. Nuomici) and immediately placed in different solutions containing 0, 0.5, 1.0, 1.5, 2.0 mM SNP or 0, 10, 20, 30, 40 mM MV. In another treatment, the detached shoots were immediately placed in different solutions: water, 2.0 mM SNP in the absence or presence of 800 μM PTIO, 40 μM MV in the absence or presence of 1.0 mM DMTU, 2 mM SNP in the presence of 1 mM DMTU, 40 μM MV in the presence of 800 μM PTIO. All the treated shoot cuttings were placed in a growth chamber at photosynthetic photon flux density of 160 μmol m^{-2} s^{-1} and temperature of 20 °C. After 10 h, panicle primordia were sampled and frozen in liquid nitrogen and stored at -80 °C.

Hydrogen peroxide and nitric oxide: H_2O_2 content was determined according to Zhou *et al*. (2006). H_2O_2 or NO imaging was done by the method of Lü *et al*. (2005) with slight modifications. Buds or inflorescence, including a small piece of stem tissue and petiole bases, were excised, dissected lengthwise and incubated in buffer (pH 7.2, 50 mM Tris, 50 mM KCl) containing 50 μM dichlorofluorescin diacetate (DCFH-DA, *Sigma*), a H_2O_2 fluorescent probe (Zhao *et al*. 2007), or NO indicator 10 μM diaminofluorescein diacetate (DAF-2DA, *Sigma*) (Desikan *et al*. 2002, Malerba *et al*. 2005), or 10 μM 4-aminofluorescin diacetate (4AF-DA) (*Alexis*, San Diego, CA, USA), negative control compound for the fluorescent probe DAF-2DA, and 1 % (v/v) *Triton X-100* for 30 min. The tissues were washed with the buffer twice. Before incubated in the DCFH-DA buffer, the dissected buds were incubated in buffer (pH 7.2, 50 mM Tris, 50 mM KCl) containing 800 μM PTIO for 30 min, and washed with the buffer twice. All images were visualized by confocal laser scanning microscopy (excitation 488 nm, emission 515 - 560 nm; *Leica TCS SP2*, Mannheim, Germany), using *Leica* confocal software. The amounts of H_2O_2 or NO were presented as a percentage of fluorescence intensities of control.

***LcLFY* expression:** Total RNA was extracted from frozen buds using a sodiumdodecyl sulphate (SDS) protocol described by Zhou *et al*. (1999). A full length cDNA of *LcLFY* was isolated using methods of reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end PCR (RACE). Transcript levels of *LcLFY* were analyzed by Northern hybridization and semi-quantitative PCR. Northern blot analysis was done according to the method of Wang *et al.* (2006). Primers for the probe preparation were 5'-CTCTTGTGGTCATTGCAGCAGGCCA-3' (F) and 5'-GACAGACAGTAATTAGCTGCTC-3 (R). For the semi-quantitative PCR analysis, the extracted RNA was reversely transcribed into cDNA. The PCR was carried out using the same pair of primers for *LcLFY* as described above. The *litchi actin* (DQ309464) was used as a loading control. The PCR products were electrophoresed on a 1 % (m/v) agarose gel, stained with ethidium bromide, and visualized by a UV radiation.

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 at the 0.05 probability level. Differences between treatments and controls were evaluated by Student's *t*-test.

Results and discussion

All the chilled trees were induced to flower (50 to 100 % flowering terminal shoots), whereas all control trees failed to flower. H_2O_2 content in chilled and control trees fluctuated before the panicle primordia emerged and differences were not statistically significant. When the panicle primordia emerged, H_2O_2 content in chilled trees significantly increased and was considerably higher than in control trees (Table 1). The H_2O_2 content decreased to the level of the control trees after the chilled trees were re-warmed (Table 1).

As there were not enough buds for the determination of H_2O_2 and NO contents by regular methods (Zhou *et al*. 2006), H_2O_2 and NO images in buds were observed under confocal laser scanning microscope when the inflorescence primordia emerged. In this study, we used H_2O_2 sensitive fluorescent probe DCFH-DA for the determina-

Table 1. Change in H_2O_2 content [nmol g^{-1}(f.m.)] in litchi leaves during chilling-induced flowering. Panicle primordia emerged after 63 d of chilling treatment. Means ± SE, n = 4. Statistically significant differences ($P < 0.05$, Student's t-test) between the treatment and the control at a given time are indicated by *asterisk*.

Treatment	10 d	20 d	30 d	40 d	50 d	63 d	71 d
Control	34.5 ± 8.1	59.6 ± 24.1	117.8 ± 46.9	110.9 ± 14.9	134.5 ± 23.0	130.3 ± 33.4	130.0 ± 16.3
Chilling	39.5 ± 7.4	127.2 ± 46.3	79.5 ± 26.9	117.4 ± 46.3	97.5 ± 54.2	225.4 ± 20.7*	162.9 ± 50.7

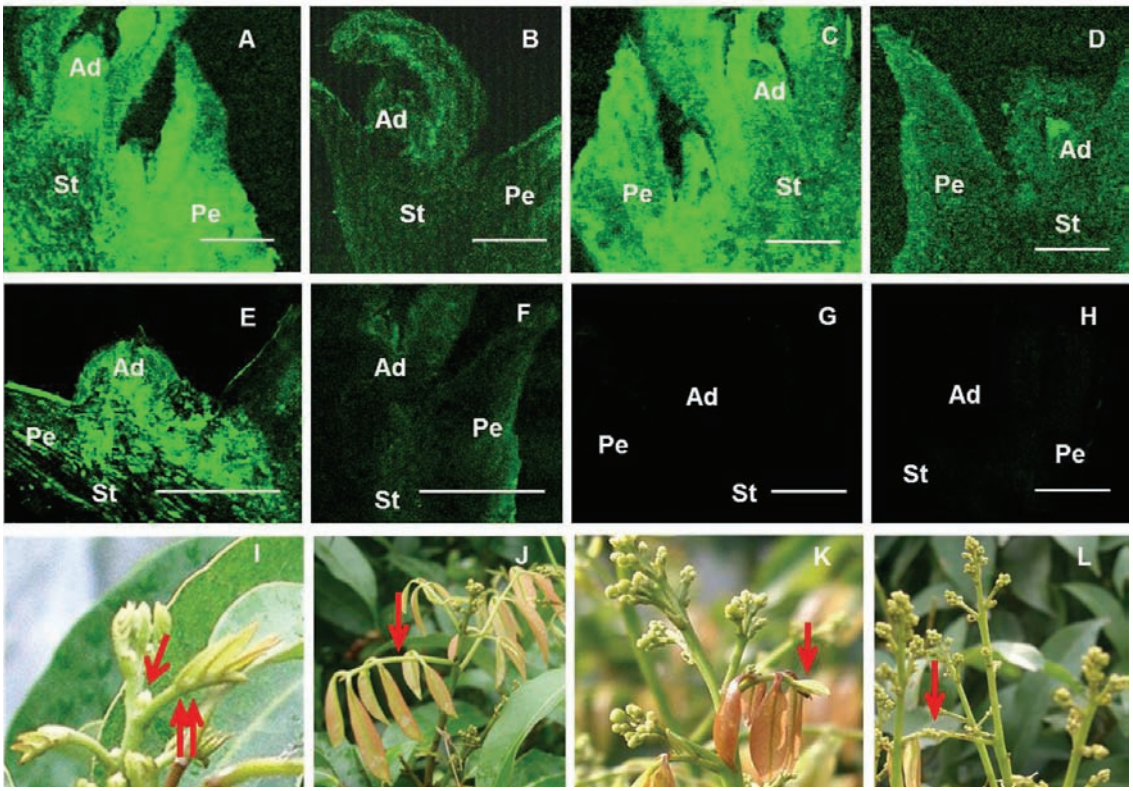

Fig. 1. H_2O_2 and NO productions in response to chilling treatment in litchi buds (*A* - *H*) and panicles (*I* - *L*) with or without MV or SNP treatments. *A* - DCFH-DA fluorescence of the chilled buds without pretreatment of PTIO. *B* - DCFH-DA fluorescence of the control buds without pretreatment of PTIO. *C* - DAF-2DA fluorescence of the chilled buds. *D* - DAF-2DA fluorescence of the control buds. *E* - DCFH-DA fluorescence of the chilled buds with pretreatment of PTIO before staining. *F* - DCFH-DA fluorescence of the control buds with pretreatment of PTIO before staining. *G* - 4AF-DA fluorescence of the chilled buds. H - 4AF-DA fluorescence of the control buds. *I* - A panicle with axillary panicle primordia (*arrow*) and expanding rudimentary leaves (*double arrows*) before sprayed with SNP or MV. *J* - Panicles without SNP or MV treatments (control) in which some rudimentary leaves were expanding (*arrow*). *K* - Panicles after two weeks of SNP treatment in which some rudimentary leaves had abscised with incomplete leaves left (*arrow*). *L* - Panicles after two weeks of MV treatment in which some rudimentary leaves had abscised, and some of the leaflets in the compound leaf had abscised (*arrow*). Images of A to H were visualized under confocal laser scanning microscope (excitation 488 nm, emission 515 - 560 nm). *Bar* = 300 μm. Ad - apical dome, Pe - petiole, St - stem.

tion of H_2O_2. However, the DCFH-DA is not specific for H_2O_2 and also reacts with NO (Hempel *et al*. 1999, Bright *et al*. 2006). Hence, the DCFH-DA fluorescence by NO should be deducted before determining H_2O_2 accumulation. In the chilled or control trees, the fluorescent intensity of the buds pretreated with NO specific scavenger, PTIO, was always weaker than that of non-pretreated buds, suggesting that the interference from NO was effectively removed (Fig. 1*A*,*B*,*E*,*F*). Based on DCFH-DA fluorescence, H_2O_2 contents in the chilled petiole, apical dome and stem were higher by 169, 96 and 127 % than those in the control (Table 2). To determine NO accumulation, we used the NO specific fluorescent probe DAF-2DA. The results showed that chilling increased DAF-2DA fluorescence (Fig. 1*C*,*D*). By contrast, no fluorescence was observed in the chilled buds and control buds when they were stained with the negative probe 4AF-DA (Stöhr and Stremlau 2006, Zhang *et al.* 2007) (Fig. 1*G*,*H*), demonstrating that DAF-2DA is suitable for monitoring NO production in litchi

Table 2. Hydrogen peroxide (DCFH-DA fluorescence) and NO production (DAF-2DA fluorescence) in response to chilling treatment in litchi buds. Potted treees of cv. Feizixiao were subjected to a chilling after the terminal shoots had matured. Buds were sampled after 63 d when panicle primordia emerged. Means ± SE, *n* = 3, * - significant differences between the treatment and the control.

Treatment	DCFH-DA fluorescence [% control]			DAF-2DA fluorescence [% control]		
	petiole	apical dome	stem	petiole	apical dome	stem
Control	100.0 ± 13.9	100.0 ± 13.9	100.0 ± 6.6	100.0 ± 20.2	100.0 ± 30.6	100.0 ± 7.0
Chilling	268.9 ± 33.3*	196.5 ± 13.7*	227.3 ± 22.0*	384.5 ± 21.4*	331.0 ± 69.9*	304.0 ± 30.1*

buds. Based on DAF-2DA fluorescence, NO contents in the chilled petiole, apical dome and stem were higher by 284, 231 and 204 % than in those of the control (Table 2).

Both MV (40 or 120 μM) and SNP (1 or 3 mM) reduced the numbers of leaves per panicle. More reduction was generated by 120 μM MV and 3 mM SNP treatments, in which the numbers of leaves per panicle declined to half of those of the control panicles (Table 3). SNP and MV treatments enhanced the number of axillary panicles per panicle and the ratios of axillary panicles to total nodes per panicle, though the increases were not statistically significant. In fact, the panicles in the MV or SNP treated trees had longer axillary panicles than those of control ones, and developed into high quality panicles with less rudimentary leaves and more flowers. Furthermore, most of the remained rudimentary leaves in the treated panicles were incomplete (Fig. 1*K,L*), in which some of the leaflets had abscised. As 1 - 3 mM SNP were effective to promote flowering, we used 2 mM for further investigation. MV in 40 - 120 μM was able to promote flowering, but the numbers of axillary panicles per panicle in 120 μM MV treated trees were less than those in 40 μM MV treated ones, so 40 μM MV was used in further experiments.

Previous studies have shown that SNP is a useful NO donor and can deliver NO for many hours, but cyanide is also generated (Bethke *et al.* 2006). To find out whether the effect of SNP on the abscission of rudimentary leaves

Table 3. Effects of SNP and MV on number of leaves and axillary panicles on a panicle of litchi cv. Nuomici 2 weeks after treatment. Four replicate trees grown in the field with similar size and with leafy panicle were selected. On 19 January 2006, five different branches in one replicate tree were uniformly sprayed with water, 1 mM SNP, 3 mM SNP, 40 μM MV, 120 μM MV. Values are means ± SE from 4 replicate trees. Each value in one replicate is calculated from 10 panicles. Significant differences ($P < 0.05$, Student's *t*-test) between the treatment and the control are indicated by *asterisks.*

Treatments	Number of leaves [panicle^{-1}]	Number of axillary panicles [panicle^{-1}]	Ratio of axillary panicles to total nodes
Control	3.9 ± 1.2	7.0 ± 3.5	0.58 ± 0.30
1 mM SNP	2.5 ± 1.5	12.3 ± 4.9	0.73 ± 0.25
3 mM SNP	1.8 ± 1.0*	13.7 ± 2.8	0.87 ± 0.09
40 μM MV	3.2 ± 0.5	11.0 ± 1.7	0.77 ± 0.15
120 μM MV	1.9 ± 1.2 *	8.5 ± 2.6	0.74 ± 0.17

Table 4. Effects of SNP and MV and their scavengers on the number of rudimentary leaves. Values are means ± SE, *n* = 3. Different letters indicate significant difference at $P < 0.05$ level between the given treatment time according to Duncan's multiple range test.

ime of treatment	Control	SNP	SNP + PTIO	Potassium ferricyanide	MV	MV + DMTU
Before treatment	4.8 ± 0.2 a	4.4 ± 0.2 a	4.9 ± 0.3 a	4.3 ± 0.3 a	4.8 ± 0.4 a	4.6 ± 0.3 a
8 d after treatment	4.1 ± 0.2 a	2.5 ± 0.1 b	4.3 ± 0.2 a	3.8 ± 0.1 a	2.7 ± 0.5 b	3.8 ± 0.4 a

Table 5. Amounts of H_2O_2 and NO in litchi buds presented as percentages of DCFH-DA or DAF-2DA fluorescence intensities of controls. Values are means ± SE, *n* = 3. Different letters indicate significant difference at $P < 0.05$ level among treatments according to Duncan's multiple range test.

Organ	DCFH-DA fluorescence [% control]			DAF-2DA fluorescence [% control]		
	control	MV	MV + DMTU	control	SNP	SNP + PTIO
Petiole	100.0 ± 32.3 a	381.9 ± 59.1 b	124.3 ± 20.4 a	100.0 ± 17.8 a	232.1 ± 19.4 b	109.2 ± 17.0 a
Apical dome	100.0 ± 14.0 a	283.8 ± 5.4 b	144.4 ± 18.7 a	100.0 ± 12.0 a	207.6 ± 4.3 b	84.6 ± 15.7 a
Stem	100.0 ± 5.9 a	308.0 ± 1.3 b	122.9 ± 1.0 a	100.0 ± 10.5 a	205.7 ± 8.3 b	114.0 ± 22.0 a

is attributed to NO, the cyanide analogue potassium ferricyanide was used. To further test the effects of NO and H_2O_2 on abscission of rudimentary leaves, MV or in combination with the H_2O_2 trapper DMTU, SNP or in combination with the NO specific scavenger PTIO were used. Panicles had similar numbers of rudimentary leaves before treatment. The numbers of leaves in the potassium ferricyanide-treated panicles were similar to those of control, indicating that potassium ferricyanide had no effect on the leaves. SNP promoted abscission of rudimentary leaves, but this effect was suppressed by the NO scavenger PTIO. The results of treatments with potassium ferricyanide and SNP in the absence or presence of PTIO suggest that the effect of SNP is attributed to NO not the cyanide, and NO certainly promotes abscission of rudimentary leaves. MV promoted abscission of rudimentary leaves, but this effect was suppressed by the H_2O_2 trapper DMTU. This result further proves that H_2O_2 promotes abscission of rudimentary leaves.

MV increased H_2O_2 fluorescence and SNP increased NO fluorescence in apical dome, petiole of the rudimentary leaves and stem, but these increases were arrested by DMTU and PTIO (Table 5), indicating that MV increased H_2O_2 content and SNP increased NO content in the buds.

Table 6. Effects of MV and SNP on panicles of cv. Feizixiao. After the terminal shoots had matured, trees were transferred to growth chamber at day/night temperature 18/13 °C (12-h photoperiod), and were sprayed uniformly with water, 2 mM SNP and 40 μM MV after 1, 21 and 41 d. The chamber was re-warmed to 28/23 °C after panicle primordia emerged. Values are means ± SE from 6 replicate trees. Each value in one replicate is calculated from 4 to 7 panicles. Significant differences ($P < 0.05$) according to Student's *t*-test between the treatment and the control are indicated by *asterisks*.

Treatments	Number of leaves [panicle^{-1}]	Number of axillary panicles [panicle^{-1}]	Ratio of axillary panicles to total nodes	Number of flowers [panicle^{-1}]
Control	5.0 ± 2.4	9.4 ± 3.9	0.61 ± 0.58	448.0 ± 98.4
2 mM SNP	1.1 ± 0.2*	16.9 ± 3.2	0.92 ± 0.01	1043.9 ± 182.0*
40 μM MV	0.9 ± 0.1*	15.1 ± 0.3	0.92 ± 0.01	1404.3 ± 34.2*

Table 7. Effects of DMTU and L-NAME on flowering under chilling-induced flowering treatment. After the terminal shoots had matured, trees of cv. Feizixiao were transferred to a chamber at 15/8 °C (12-h photoperiod). Six replicate trees were uniformly sprayed with water as control, 3 mM DMTU or 1 mM L-NAME per week. The chamber was re-warmed to 28/23 °C after 63 d of treatment. All the trees were continually grown in the chamber. A percentage of flowering terminal shoots was counted and numbers of flowers per panicle were calculated after anthesis. Significant differences ($P < 0.05$, Student's *t*-test) between the treatment and the control are indicated by *asterisks*.

Treatments	Flowering terminal shoots [%]	Number of flowers [panicle^{-1}]
Control	96.0 ± 3.6	1301.0 ± 127.6
DMTU	38.3 ± 12.1*	515.4 ± 150.6*
L-NAME	35.7 ± 14.0*	221.4 ± 81.7*

Potted litchi trees were transferred to a growth chamber with day/night temperature of 18/13 °C and sprayed with SNP or MV before the "millet stage". We used 18/13 °C for the induction treatment because a previous study showed that the temperatures were not as effective for floral induction as 15/8 °C, and trees under 18/13 °C might be easily induced to produce leafy panicles (Chen 2002). MV and SNP promoted flowering when the trees were treated before the "millet stage" (Table 6). SNP and MV decreased the numbers of leaves in one panicle. Numbers of axillary panicles per panicle and ratios of axillary panicles to total nodes per panicle were increased by these treatments. Numbers of axillary panicles per panicle in the SNP and MV treated trees were higher by 80 and 61 % than those of the control, respectively. SNP and MV also increased the numbers of flowers per panicle, the numbers in the SNP and MV treated panicles were higher by 133 and 213 % than those in the control. The results suggested that SNP or MV promoted flowering when the litchi trees were treated before "millet stage".

Percentage of flowering terminal shoots and number of flowers per panicle in the L-NAME and DMTU treated trees were fewer than those of the control (Table 7), suggesting that reducing H_2O_2 and NO levels could suppress flowering to some extent, though the treatments could not completely suppress flowering.

LFY is known to affect floral meristem in *Arabidopsis* (Blazquez *et al*. 1997). A full length cDNA of *LcLFY*, whose deduced amino acid sequences show 96 % identities to *Dimocarpus longon* LEAFY-like protein, was isolated using methods of RT-PCR and rapid amplification of cDNA end PCR. Transcript levels of *LcLFY* were analyzed by Northern hybridization and semi-quantitative PCR. It was previously found that *LcLFY* was strongly expressed at the "millet stage" and SNP and MV promoted *LcLFY* expression 10 and 20 h after the treatments (Li 2008). Hence the buds were

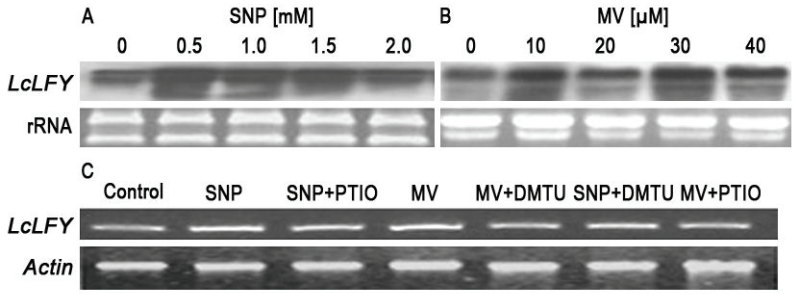

Fig. 2. Effects of MV and SNP on transcript level of *LcLFY*. *A* - Buds were treated with 0 - 2.0 mM SNP. *B* - Buds were treated with 0 - 40 μM MV. *C* - Buds were treated with water, 2.0 mM SNP in the absence or presence of 800 μM PTIO, 40 μM MV in the absence or presence of 1 mM DMTU, 2 mM SNP in the presence of 1 mM DMTU, 40 μM MV in the presence of 800 μM PTIO.

treated with SNP and MV at this stage and buds were collected 10 h after treatments for *LcLFY* expression analysis. SNP (0.5 - 2.0 mM) promoted *LcLFY* mRNA accumulation (Fig. 2*A*). MV also promoted expression of the *LcLFY*. Compared with the control buds, the buds treated with 10 - 40 μM MV evidently accumulated higher levels of *LcLFY* mRNA (Fig. 2*B*). To confirm whether H_2O_2 or NO is the direct player that promotes the expression of *LcLFY*, the effect of SNP was compared with that of SNP plus NO scavenger PTIO, and the effect of MV was compared with that of MV plus the H_2O_2 trapper DMTU. In agreement with the results described above, 2.0 mM SNP and 40 μM MV promoted mRNA accumulation of *LcLFY*, but these inductive effects were suppressed by the NO scavenger, PTIO and the H_2O_2 trapper, DMTU (Fig. 2*C*). To investigate whether H_2O_2 promotion of mRNA accumulation of *LcLFY* is dependent on NO, or NO promotion of the mRNA accumulation is dependent on H_2O_2, the effect of SNP treatment was compared with that of SNP plus the H_2O_2 trapper DMTU, and the effect of MV was compared with that of MV plus the NO scavenger PTIO. The results showed that the inductive effects of SNP were reduced by DMTU, and the inductive effects of MV were suppressed by PTIO (Fig. 2*C*), suggesting that NO promotion of the mRNA accumulation might be dependent on H_2O_2 production, and H_2O_2 promotion of expression of the *LcLFY* might be dependent on NO production.

Many kinds of species need chilling treatment to attain the ability to flower (*e.g.* Filek *et al.* 2010). Apart from chilling stress, many evergreen woody trees have to experience environmental stresses such as drought and oxidative stress to induce flowering (Menzel and Simpson 1988, Ali and Lovatt 1995, Nunez-Elisea and Davenport 1994, Manochai *et al*. 2005). Floral differentiation in litchi is induced by low temperatures and enhanced by autumn and winter drought (Menzel and Simpson 1988, Chen and Huang 2005). In the present study, potted litchi trees were induced to flower by chilling stress in growth chambers. It was found that a transient increase in H_2O_2 content in leaves occurred at the "millet stage", a stage of primordium emergency (Table 1). The result is consistent with our previous determination in leaves under the chilling- and drought-induced flowering treatment in the field (Zhou *et al*. 2010a). H_2O_2 content in buds at the "millet stage" was also assayed, in which vegetative growth (the growth of rudimentary leaves) and reproductive growth (panicle development) strongly competes with each other. The results indicate that buds accumulated highest H_2O_2 content at this stage (Fig. 1, Table 2). In the previous study, abscissing rudimentary leaves had higher contents of H_2O_2 than the non-abscissing leaves which subtended a vegetative bud (Zhou *et al*. 2008). Physiological comparison of abscissing rudimentary leaf and developing panicle indicated that a high content of H_2O_2 might be associated with senescence and abscission of rudimentary leaves (Zhou *et al*. 2010b). In *Arabidopsis*, H_2O_2 content is elevated in leaves right at the floral transition (Zimmermann *et al.* 2006). It is likely that this developmentally controlled H_2O_2 burst generates a signal in leaves associated either with the induction of flowering or with leaf senescence (Bañuelos *et al*. 2008). The authors suspected that H_2O_2 may also serve as a signal for senescence and at last abscission of rudimentary leaves in panicle, as a result, the development of the competing panicles is promoted. This may be one of the roles that H_2O_2 plays in flowering.

Both H_2O_2 and NO are synthesized as signaling molecules in response to stresses (Neill *et al.* 2002, Uchida *et al.* 2002, Desikan *et al*. 2004). Determination of NO in the mixed buds under chilling-induced flowering treatment indicated that NO content increased by the treatment, similar to that of H_2O_2. Hence, we hypothesized that high contents of H_2O_2 and NO in the buds under the stress conditions result in senescence and abscission of rudimentary leaves. In fact, at the "millet stage", exogenous application of SNP promoted abscission of rudimentary leaves as MV did. MV can generate superoxide by accepting an electron from PS 1 (Dodge 1971). Superoxide is then transformed to H_2O_2 by superoxide dismutase (Kraus and Fletcher 1994). SNP is a NO donor used in plant experimental systems (Wang *et al*. 2010). 0.5 mM SNP can produce 2 μM NO (Delledonne *et al* 1998). Similar result has been found when growers remove the rudimentary leaves in panicles

by hand. Hence, the authors suggest that H_2O_2 and NO promote flowering at least partially by reducing the leaves in the panicles when trees are treated at the "millet stage". When trees were treated before this stage, more flowers and fewer expanding leaves in the panicle were still found in treated trees than in control ones. Moreover, reducing H_2O_2 content by spraying with DMTU and NO content by spraying with L-NAME suppressed flowering to some extents under chilling conditions (Table 7), further suggesting that H_2O_2 and NO may serve as a signal for senescence leading to abscission of rudimentary leaves, and promote flowering.

Floral development is controlled by environmental conditions and developmental regulation. The complexity of this regulation is created by an intricate network of signaling pathways. FT protein as a mobile signal has been proven to be a florigen migrated from leaves to the apical meristem to promote floral initiation in several plant species (Yang *et al.* 2007), activating the downstream flowering-related genes (Li *et al.* 2010), such as *LEAFY* (*LFY*), *APETALA1* (*AP1*) , *APETALA3* (*AP3*), *AG* (Mouradov *et al*. 2002, Wagner *et al.* 1999, Zhou *et al.* 2010c). *LFY* is one of the key genes of flowering (Blazquez *et al*. 1997). Studies on *Arabidopsis* and other plants indicated that *LFY* or its homologue is a transcriptional gene which determines the floral meristem identity and is strongly expressed in the flower buds (Ahearn *et al*. 2001, Ma *et al*. 2008). In herbaceous plants such as rice, reduction in rice *LFY* homolog (*RFL*) expression causes a dramatic delay in transition to flowering, with the extreme phenotype showing no flowering. Conversely, *RFL* over expression triggers precocious flowering (Rao *et al*. 2008). In woody trees such as citrus (Pena *et al*. 2001) and aspen (Weigel and Coupland 1995), constitutive expressing of *Arabidopsis LFY* promoted flowering. Our results show that both NO and H_2O_2 promoted mRNA accumulation of *LcLFY* and this promotion is dependent on the interaction of these compounds (Fig. 2). However, flowering in litchi is a complicated process. The relationship between the two signals should be investigated by more genes expression and physiological processes. We are trying to clone more genes for the future studies.

Under field conditions, temperature and moisture, which are important factors for flowering, are changing. Litchi cultivated areas usually have warm winters due to global warming, which encourages growth in rudimentary leaves during floral differentiation. The results presented may provide a hint on how to control rudimentary leaves and to promote flowering.

In brief, our study suggests that chilling-induced flowering treatment increases H_2O_2 and NO contents at the "millet stage". H_2O_2 and NO promote reproductive growth by inhibiting the growth of rudimentary leaves as well as by promoting the expression of *LcLFY*. The results for the first time provide evidences showing the role of stress signals in flowering in the evergreen woody tree, litchi, whose floral regulation is quite different from that of herbaceous plants.

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