Genes, Genomes and Genomics ©2009 Global Science Books

Gibberellic Acid Inhibits Browning, Enzyme Activity and Gene Expression of Phenylalanine Ammonia-Lyase in *Phalaenopsis* Leaf Explants

Wenling Zhou[†] • Rufang Tan[†] • Chuanjun Xu • Yanyan Lai • Dongyin Chen • Ling Li[*]

College of Life Sciences, South China Normal University, Guangdong Provincial Key Lab of Biotechnology for Plant Development, Guangzhou, 510631, P. R. China
[†] These authors contributed equally to this work

Corresponding author: * **liling@scnu.edu.cn**

ABSTRACT

The effects of gibberellic acid (GA_3) on browning, enzyme activity and gene expression of phenylanine ammonia lyase (PAL, EC 4.3.1.5) in *Phalaenopsis* (*Doritaenopsis* Queen Bee "Red Sky") leaf explants during tissue culture were investigated. GA_3 at 0 (control), 1, 5, 10, 25, 30, 50 and 70 mg L^{-1} were used to treat the explants. The percentage browning and tannin content of explants treated with 25 mg L^{-1} GA_3 were far lower than of control explants cultured for more than 4 days. Paclobutrazol (PBZ), a gibberellin biosynthetic inhibitor, at 5 mg L^{-1}, increased the browning percentage, which implied that it was endogenous GA_3 that repressed browning. PAL activity and *PAL* gene expression were reduced by GA_3 treatment but were increased by treatment with PBZ. These results indicate that GA_3 is responsible for the browning of leaf explants through the regulation of *PAL* gene expression and enzyme activity in *Phalaenopsis* tissue culture.

Keywords: enzymatic browning, GA_3, tannin, phenylalanine ammonia-lyase

INTRODUCTION

Explant browning is one of the most common problems in the early stages of plant tissue culture, and it can be classified as either an enzymatic or non-enzymatic process. In enzymatic browning, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), polyphenol oxidase (PPO), and peroxidase (POD) are involved (Bhat and Chandel 1991; Ye *et al.* 2004). PPO participates in redox reactions involving phenolic or flavonoid compounds resulting in enzymic browning. There is a correlation between POD activity and cellular browning in the browned pericarp of harvested litchi fruit in which higher POD activity was observed (Sarni-Manchado *et al.* 2000). The substrates of PPO and POD enzymes are mainly polyphenolic compounds. The biosynthesis of polyphenol is thus considered to be a limiting factor for enzymatic browning of explants.

L-Phenylalanine is converted to cinnamic acid by the action of PAL (Hisaminato *et al.* 2001). Hydroxycinnamic acid derivatives are formed from cinnamic acid through the phenylpropanoid pathway and are generally induced by wounding or cutting of plant tissue. *O*-Diphenols such as dicaffeoyltartaric acid and 5-caffeoylquinic acid formed in the phenylpropanoid pathway are oxidized by PPO to form a brown pigment when plant tissue is damaged by wounding (Hahlbrock and Scheel 1989). This metabolic pathway also exists in the tissue culture of *Phalaenopsis* leaf explants (Xu and Li 2006; Xu *et al.* 2007; Tan *et al.* 2009). Tannins are a type of complex phenolic compound widely distributed in plants. Research on tannins has made great progress in recent years, and more than 400 new tannins and polyphenolic compounds have been identified. The chemical structures of tannins are quite different, depending on the plant source (Singleton and Kratzer 1973). They can be divided into three categories: hydrolysable, condensed, and complex. Among them, catechin tannins derived from the condensation of catechol and their derivatives, are representatives of condensed tannins. Our previous report demonstrated that with the browning of explants, the content of total phenol compounds and the activity of PAL were increased (Xu *et al.* 2005; Xu and Li 2006) and *PAL* gene expression was up-regulated after 3 days of tissue culture, and maintained at a high level until 8 days after culture (Xu *et al.* 2007). However, whether PAL is involved in browning induction is unknown.

To prevent browning, various approaches, such as the application of adsorbent (activated charcoal) or antioxidants, have been applied to improve the efficiency of tissue culture. However, less effort has been focused on the mechanism of explant browning. Gibberellins (GAs) are diterpenoid plant hormones that promote a number of plant growth responses, including seed germination, stem elongation, leaf expansion and flowering. The effect of gibberellic acid (GA_3), which is one of active gibberellins, on explant browning during tissue culture of plants has not yet been reported. We found that GA_3 represses the browning of leaf explants in *Phalaenopsis* during tissue culture, and the effect of GA_3 and the interaction between GA_3 and PAL activity and *PAL* gene expression in browning has been investigated in this study, revealing the role of GA_3 in this process and the possible mechanisms involved.

MATERIALS AND METHODS

Plant materials and cultural conditions

Leaves of *Phalaenopsis* (*Doritaenopsis* Queen Bee "Red Sky") plants, growing in the Botanical Garden of South China Normal University, were excised as 1 cm × 1 cm segments and sequentially rinsed with water, 70% ethanol for 30 s, 0.1% $HgCl_2$ for 7 min, and three times in sterile distilled water. Sterile leaves were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 0.8% (w/v) agar at pH 6.0. All cultures were maintained under cool white fluorescent light (approximately 35 μmol m^{-2} s^{-1} photon flux density and with a 16 h photoperiod) at 25°C.

Received: 20 November, 2009. Accepted: 28 November, 2009.

Original Research Paper

Chemicals

Vanillin, hydrochloric acid and agar were purchased from Whiga (Guangzhou, China). GA_3, paclobutrazol (PBZ) and polyvinyl-polypyrroridone (PVP40) were obtained from Sigma (St. Louis, USA). All other materials were from the Sangon Bioengineering CO., Ltd. (Shanghai, China) and of analytical reagent grade.

GA_3 and PBZ treatments

Explants were treated with different concentrations of GA_3 (1 to 75 mg L^{-1}) for a varying period (5 to 40 min) and then transferred to MS medium solidified with agar. Explants were treated with GA_3 (25 mg L^{-1}), PBZ, or water (as control) under the optimized conditions above, before each segment was briefly wiped with a paper towel. The browning of the cut edges of *Phalaenopsis* leaf explants was visually evaluated during culture. The number of explants with brown pigment in the surrounding medium was counted.

Tannin content measurement

Tannin contents were determined according to the procedure of Wang and Lu (2004). Segments of leaf explants were homogenized with twice the weight of 10% methanol using a mortar and pestle and extracted for 16 h. The homogenate was centrifuged at 3,000 × *g* for 20 min and the resulting supernatant was used for the determination of tannin content. One ml of supernatant was combined with 5 ml of reaction agent (1% vanillin: 8% hydrochloric acid = 1: 1) and incubated at 30 ± 1°C for 30 min. Absorbance was detected at 500 nm using a UV-visible spectrophotometer (Shimadzu UV2450). The total content of tannin in the sample was calculated based on a standard curve.

PAL activity assay

The procedure of Engelsma (1974) was adapted for the extraction of PAL from *Phalaenopsis* leaf explants. Explant segments were homogenized with five volumes (w/v) of 50 mM boric acid buffer (pH 8.8) containing 5 mM 2-mercaptoethanol and 0.1 g PVP at 4°C. The homogenate was centrifuged at 12,000 × *g* for 20 min. The obtained supernatant was used as a crude enzyme for the determination of PAL activity. The enzyme reaction consisted of a 2 ml 100 mM boric acid buffer (pH 8.8), 1 ml 20 mM phenylalanine and 1 ml crude enzyme solution. PAL activity was measured spectrophotometrically at 290 nm to detect the decrease of phenylalanine as a substrate. An increase in the absorbance of 0.01 per min at 35°C was defined as one unit of enzyme activity. Protein content was determined according to Bradford's method (1976), using bovine serum albumin (Ameresco, USA) as a standard.

RNA analysis and *PAL* gene expression

Total RNAs were extracted from the leaf explants at 0, 4, 8 and 12 days after culture with Trizol Reagent (Invitrogen, USA). *PAL* transcripts in RNA samples were estimated by semi-quantified RT-PCR with the Primescript one-step RT-PCR Kit (code: DRR055A, TaKaRa, Japan) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 20 µl with 1 µl of RNA (50 ng). The number of PCR cycles for optimized RT-PCR was determined to be 25 for actin and 28 for the *PAL* gene. The PCR conditions for the amplification of the *PAL* gene were 50°C for 30 min, 94°C for 2 min, followed by 28 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final elongation step of 72°C for 10 min. RT-PCR primers (Xu *et al.* 2007) for *PAL* were PAL-F (5′-GAGAGCGTGGACAAAGGAACTGAT-3′) and PAL-R (5′-TAAAGGATCCATCTCATGGAG-3′). Actin-F (5′-CTGAGCGTG AAATTGTAAGGG-3′) and Actin-R (5′-TGCTAAAATAGAACC TCCAATCC-3′) specific to a 431-bp actin cDNA were used for normalization of RT-PCR. PCR products were analyzed by electrophoresis on a 1.0% (w/v) agarose gel.

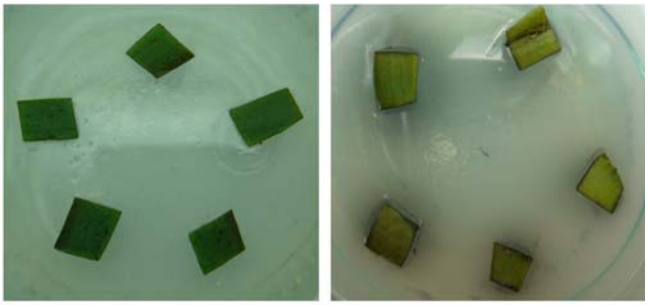

Fig. 1 Explant browning vs control with no browning.

Statistical analysis

All data on enzymatic activity were the mean values of three independent experiments. Statistical analysis was carried out using the Student's *t*-test by DPS statistical package (DPS 9.50 version). Each treatment had five replicates, and experiments were repeated three times.

RESULTS AND DISCUSSION

GA_3 inhibits browning of leaf explants *in vitro*

Phalaenopsis leaf explants gradually turned brown on cut sections after several days of tissue culture (**Fig. 1**). Compared with non-treated controls, the percentage of browning of *Phalaenolsis* leaf explants treated with GA_3 from 1 to 75 mg L^{-1} decreased after culture for 8 days (**Fig. 2A**). The browning percentage of leaf explants treated with 25 mg L^{-1} GA_3 was 22.2%, 36.1% lower than the control on day 8 (**Fig. 2A**). At a higher concentration (above 30 mg L^{-1}), GA_3 strongly inhibited callus or bud induction (**Table 1**).

The degree of browning decreased as treatment time increased from 5 to 20 min, falling to 33.1% at 20 min (**Fig. 2B**), although it increased following 30 to 40 min treatment with GA_3. Therefore, the GA_3 treatment period at 25 mg L^{-1} was restricted to 20 min in further experiments.

The leaf explants were soaked with 25 mg L^{-1} GA_3 solution for 20 min and then the percentage browning of explants cultured for 8 days was determined. Under this condition, the browning percentage of leaf explants treated with GA_3 was 41.1%, 19.1% lower than the control (**Fig. 2C**). This result indicated that soaking explants in 25 mg L^{-1} GA_3 solution for 20 min can significantly reduce the percentage of browning.

To determine endogenous GA_3's role in the browning of *Phalaenopsis* leaf explants during culture, leaf explants treated with 5 mg L^{-1} PBZ, which inhibits gibberellin biosynthesis (Hedden and Graebe 1985), caused a 6.3% increase in the browning percentage compared to the control at 7th day (**Fig. 2D**).

Table 1 The effect of gibberellic acid (GA_3) on callus formation and bud induction.

GA_3 concentration (mg L^{-1})	The percentage of callus formation/ Total explants	Budding percentage (%)
30	32.5%/ 65	6.4%
50	8.2%/ 62	0
75	7.5%/ 68	0

GA_3 decreases tannin content of *Phalaenolsis* leaf explants

Explant browning mainly takes place in plant tissues containing relatively high levels of tannins, which was confirmed in litchi (Sarni-Manchado *et al.* 2000), chestnut (Tao *et al.* 2002), pear and Fuji apples (Mie *et al.* 2004). Our previous report demonstrated that condensed tannins in browning *Phalaenopsis* leaf explants were mainly distri-

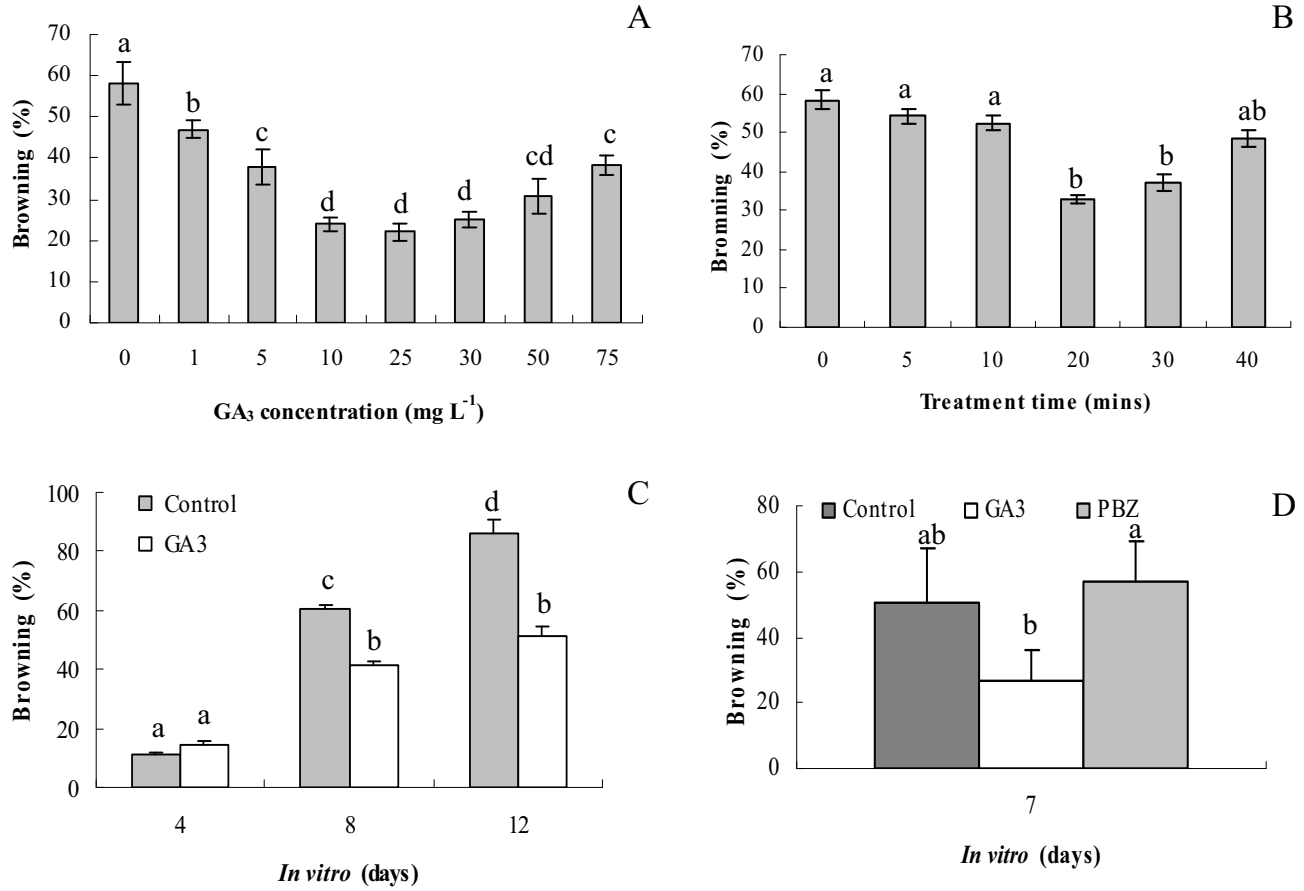

Fig. 2 Effects of gibberellic acid (GA_3) on browning percentages of *Phalaenopsis* leaf explants. (**A**) Explants treated with different concentrations of GA_3. (**B**) Explants treated with 25 mg L^{-1} GA_3 for a varying period (5 to 40 min). (**C**) Explants treated with 25 mg L^{-1} GA_3 for 20 min and then cultured for 12 days. (**D**) Explants treated with 25 mg L^{-1} GA_3, 5 mg L^{-1} paclobutrazole (PBZ), or water (control) for 20 min and then cultured for 7 days. Number of explants for each treatment = 50-70. Different letters above bars indicate significant differences at $p<0.05$ according to Tukey's test.

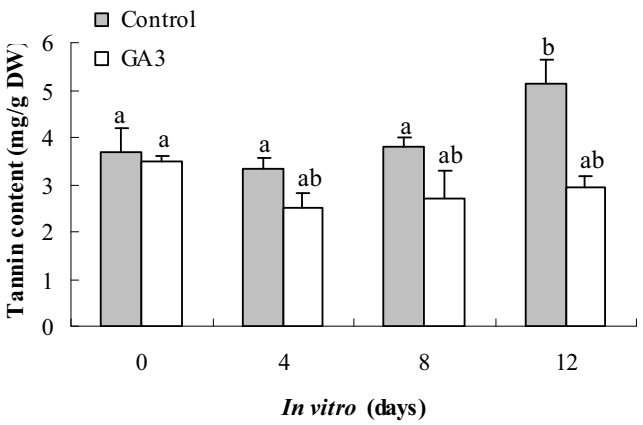

Fig. 3 Effect of GA_3 on tannin contents of *Phalaenopsis* leaf explants during tissue culture. Different letters above bars indicate significant differences at $p<0.05$ according to Tukey's test.

buted in the intracellular fractions and vascular bundles (Xu *et al.* 2005). Here, we found that the tannin content in leaf explants increased rapidly after 4 days culture and GA_3 at 25 mg L^{-1} caused a decrease of tannin in leaf explants compared to the control (**Fig. 3**). The reason may be that the tannins stopped being transferred to intercellular space or secreted into the medium.

Effect of GA_3 on PAL activity and *PAL* expression in *Phalaenopsis* leaf explants

PAL is a key enzyme in the synthetic pathway for plant secondary metabolites, and it is located in the cytoplasm, chloroplasts and mitochondria. Cantos *et al.* (2002) pointed out that PAL plays a role in potato browning after mecha-

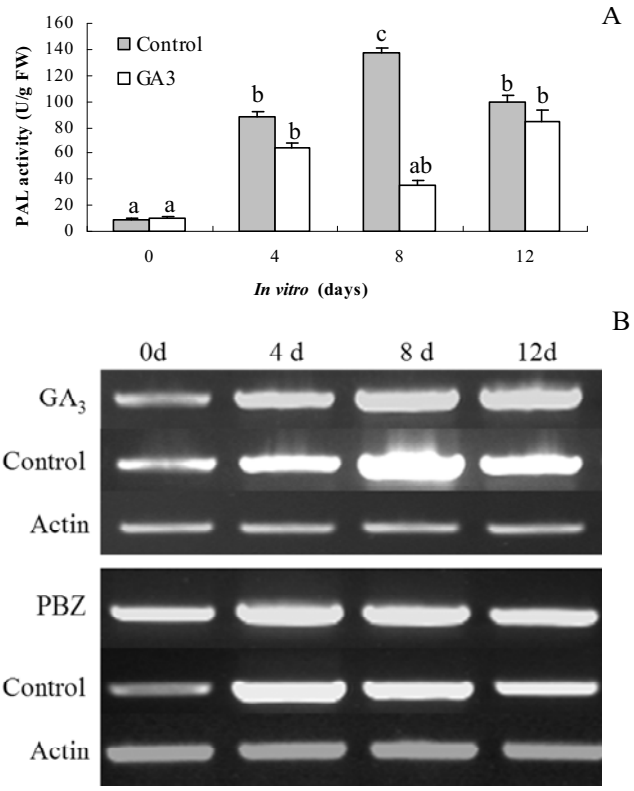

Fig. 4 Effects of GA_3 treatment on PAL activity (A) and *PAL* gene expression (B) of *Phalaenopsis* leaf explants during tissue culture. *PAL* gene expression was analyzed by semi-quantitative RT-PCR. Number of explants for each treatment = 50-70. Different letters above bars indicate significant differences at $p<0.05$ according to Tukey's test.

Table 2 PAL activities of *Phalaenolsis* leaf explants treated with water (as control), gibberellic acid (GA_3) or paclobutrazole (PBZ).

Treatment (mg L^{-1})	PAL activity (U/g FW)	
	4^{th} day	8^{th} day
Control	82.3 ± 12.7 b	125.2 ± 24.2 c
GA_3 (25)	54.8 ± 16.2 a	76.5 ± 19.6 b
PBZ (5)	98.0 ± 21.2 b	138.3 ± 32.9 c

The number of explants for each treatment = 50-70. The different letters ind significant differences at $p < 0.05$.

nical injury. PAL activity of explants treated with GA_3 was far lower than that of the control during tissue culture (**Fig. 4A**). Especially on day 8, PAL activity of explants decreased 48.7% when treated with GA_3 but increased 13.1% when treated with PBZ compared with the controls (**Table 2**), which demonstrated that the decrease in PAL activity was due to the inhibition of endogenous GA_3 by PBZ.

To confirm the effect of GA_3 on *PAL* gene expression, semi-quantitative RT-PCR was performed to evaluate the levels of *PAL* mRNAs in various samples. GA_3-treated explants did not show any marked deviation compared to the control at day 0. However, the mRNA level of *PAL* gene of explants treated with GA_3 was lower than the controls at day 8, which revealed that GA_3 can down-regulate *PAL* gene expression (**Fig. 4B**). The level of *PAL* gene mRNAs of explants treated with PBZ were up-regulated compared to the control (**Fig. 4B**), which is consistent with the PAL activity shown in **Table 2**.

Recent years, our group has made some progress on the browning of *Phalaenopsis* leaf explants and the main findings were summarized in **Table 3**, which would play an important role in the tissue culture of *Phalaenopsis*.

CONCLUSIONS

From the results obtained in our work, it can be concluded that 25 mg L^{-1} GA_3 can effectively repress the browning of *Phalaenopsis* leaf explants, which was confirmed using the biosynthetic inhibitor of endogenous GA_3. The mechanism may be that GA_3 regulated *PAL* gene expression and reduced PAL activity, in which PBZ can repress endogenous GA_3 and enhance PAL activity. We thus suggest that GA_3 could become an important browning inhibitor with special reference to tissue culture in horticultural plants. However, how GA_3 regulated *PAL* gene expression and whether it had a relationship with PPO and POD activities in the browning of *Phalaenopsis* leaf explants needs further investigation.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Science and Technology Program of Guangdong Province of China (No. 2005B 20901019).

REFERENCES

Bhat SR, Chandel KPS (1991) A novel technique to overcome browning in tissue culture. *Plant Cell Reports* **10**, 358-361

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248-254

Cantos E, Tudela JA, Gil MI, Espin JC (2002) Phenolic compounds and related enzymes are not rat e-limiting in browning development of fresh-cut potatoes. *Journal of Agriculture and Food Chemistry* **50**, 3015-3023

Engelsma G (1974) On the mechanism of the changes in phenylalanine ammonialyase activity induced by ultraviolet and blue light in hypocotyls. *Plant Physiology* **54**, 702-705

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 347-369

Hedden P, Graebe JE (1985) Inhibition of gibberellin biosynthesis by paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm and *Malus pumila* embryos. *Journal of Plant Growth Regulation* **4**, 111

Hisaminato H, Murata M, Homma S (2001) Relationship between the enzymatic browning and phenylalanine ammonia-lyase activity of cut lettuce, and the prevention of browning by inhibitors of polyphenol biosynthesis. *Bioscience, Biotechnology Biochemistry* **65**, 1016-1021

Mie LC, Sun JS, Xin B, Lü XQ (2004) Studies on phenolic composition and polyphenol activity in apple fruits. *Acta Horticulturae Sinica* **31**, 502-504

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Sarni-Manchado P, Le Roux E, Le Guernevé C, Lozano Y, Cheynier V (2000) Phenolic composition of litchi fruit pericarp. *Journal of Agriculture and Food Chemistry* **48**, 5995-6002

Singleton VL, Kratzer FH (1973) Plant phenolics. In: Committee on food production (Eds) *Toxicants Occurring Naturally in Foods*, Springer, Washington DC, p 309

Tao YL, Lin Y, Xiang XJ, Zhou SP, Zhang XH, Zheng XY (2002) The browning substrate and its contents in different tissues of chestnut seeds. *Hebei Journal of Forestry and Orchard Research* **17**, 121-124

Tan RF, Zhou WL, Xu CJ, Li L (2009) Effect of catechin on browning of leaf explants in tissue culture of *Phalaenopsis* sp. *Northern Horticulture* **3**, 65-68

Wang K, Lu J (2004) Study oil determination of tannin in crude drugs. *Chinese Pharmaceutical Affairs* **18**, 361-365

Xu CJ, Li H, Li L (2007) Phenylalanine ammonia lyase gene expression correlated with *Phalaenopsis sp.* leaf explants browning. *Journal of Tropical and Subtropical Botany* **15**, 50-54

Xu CJ, Li L (2006) Changes of total phenol content and the activities of PPO, POD and PAL during the browning in *Phalaenopsis* explants *in vitro*. *Acta Horticulturae Sinica* **33**, 671-674

Xu CJ, Li L, Li H, Zhang MG (2005) Preliminary studies on the elements of browning and the changes in cellular texture of leaf explants browning in *Phalaenopsis*. *Acta Horticulturae Sinica* **32**, 1111-1113

Ye M, Wang BC, Duan CR (2004) Advances of explant browning in plant tissue culture. *Letters in Biotechnology* **15**, 426-428

Table 3 Different findings of our studies.

Treatment	Browning degree	Main findings	Reference
No	++	With the browning of explants, the content of total phenol compounds and the activity of PAL were increased.	Xu *et al.* 2005
No	++	PAL gene expression was associated with the browning of *Phalaenopsis* sp. leaf explant.	Xu *et al.* 2007
Catechin	+++	Leaf explants treated with catechin increased tannin contents resulted in promotation of browning development.	Tan *et al.* 2009
GA_3	+	GA_3 is responsible for the browning of leaf explants through the regulation of *PAL* gene expression and enzyme activity in *Phalaenopsis* tissue culture.	This study

"+" indicate the browning degree is light; "++" indicate the browning degree is moderate; "+++" indicate the browning degree is serious. GA_3, gibberellic acid