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The Effects of Polyphenol Oxidase and Cycloheximide on the Early Stage of Browning in *Phalaenopsis* Explants

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Abstract

Explant browning is one of the major problems in the tissue culture process, and polyphenol oxidase (PPO), is the major proteases involved in plant tissue browning. We investigated the effects of polyphenol oxidase on the early stage of browning in explants of the orchid *Phalaenopsis*. Our results show that PPO activity was significantly higher in explants cultured for 3 d than in the 0 h control. The levels of *PPO* transcripts and PPO protein were significantly higher in explants cultured for 6 h compared to the 0 h control; these high expression levels were maintained over increasing cultivation time. Cycloheximide (CHX) treatment reduced *PPO* transcript levels, PPO protein levels, and PPO enzyme activity. High levels of *PPO* mRNA and PPO protein were detected in the cytoplasm and vascular bundles of *Phalaenopsis* explants cultured for 6 h compared to explants cultured for 0 h, 24 h, and 3 d. CHX treatment did not significantly affect the distribution of *PPO* mRNA and PPO protein in explant tissues, but their levels were significantly lower than those of the untreated control.

Keywords: *Phalaenopsis*; PPO; explant browning; CHX; gene expression

1. Introduction

Enzymatic browning of plant tissues causes unappealing changes in fruits and vegetables such as cut apples and potatoes, and can cause explant death and failure of regeneration in explant culture for propagation of ornamental species such as orchids. Enzymatic browning mainly results from the oxidation of polyphenols to quinones, catalysed by browning enzymes such as polyphenol oxidase (PPO, EC 1.14.18.1) (Luo et al., 1999). PPO activity *in vivo* typically occurs in damaged plant tissues that have lost cellular compartmentalisation. The total phenolic content, as well as the expression and activity of PPO increase during tissue browning in apple and litchi (Leng et al., 2009; Di Guardo et al., 2013, Wang et al., 2014). Overexpressing *PPO* in transgenic sugarcane results in seriously browning and an increase in PPO content in the sugarcane juice (Vickers et al., 2005). Potatoes and apples with low levels of *PPO* expression exhibit low rates of browning and a delayed onset of browning (Bachenm et al., 1994; Murata et al., 2000; Coetzer et al., 2001; Arican et al., 2003; Wang et al., 2007). Using artificial microRNA technology, Chi et al. (2014) found that reducing the expression of *StuPPO1* and *StuPPO4* reduced the browning rate in transgenic potatoes. These findings help confirm the notion that the expression of *PPO* affects tissue browning.

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During tissue culture of apple (Murata et al., 2001) and Cangxi pear (Li and Yan, 2001) explants, browning rate is associated with PPO activity and total phenolic content. Using antisense RNA technology, an antisense *PPO* DNA sequence was transferred into apple by *Agrobacterium rhizogenes*-mediated transformation. The resulting transgenic callus exhibited a low browning rate, which is consistent with low PPO activity (Laukkanen et al., 1999), suggesting that PPO is involved in the browning of explants. PPO activity also increases during the browning of *Phalaenopsis* explants (Xu and Li, 2006). Analysis of PPO isozymes in *Phalaenopsis* revealed new PPO isozyme bands that occurred before explant browning; this isoform also had higher PPO activity (Xu and Li, 2006). The PPO activity in *Phalaenopsis* explants with severe browning exceeds that of explants with slight browning (Zhao et al., 2006; Huang et al., 2007). The browning of *Phalaenopsis* explants can be controlled, to some extent, by inhibiting PPO activity (Chen et al., 2009; Lai et al., 2010). These findings suggest that PPO activity is correlated with the browning of *Phalaenopsis* explants. Unfortunately, little attention has been paid to the factors initiating explant browning and to the underlying molecular mechanisms. The time period during which PPO influences the occurrence of browning is difficult to determine at the physiological level. Moreover, PPO in plants is in pro-enzymatic form (Gooding et al., 2001; Michael et al., 2009), making it important to determine whether explant browning is induced by PPO pro-enzymes and/or if PPO enzyme is biosynthesised later in the browning process.

The orchid *Doritaenopsis* Queen Bee 'Red Sky' is a *Phalaenopsis* hybrid that readily undergoes browning in tissue culture. We previously cloned the full-length *Phalaenopsis PPO* gene (GenBank accession number: EF363553.1) and obtained PPO antibody (Xu et al., 2009), providing key reagents for investigating the effects of PPO on explant browning at the molecular level. In the current study, we treated *Phalaenopsis* explants with the protein synthesis inhibitor cycloheximide (CHX) and analysed *PPO* transcript levels and PPO protein levels during the early stage of browning, in the first three days of culture. We also examined the effects of the timing and position of *PPO* expression on the occurrence of explant browning. These experiments helped us determine whether newly synthesized PPO or existing PPO causes explant browning. The results of this study increased our understanding of the mechanisms and occurrence of explant browning and may be helpful for designing methods to effectively control browning in orchid explants.

2. Materials and methods

2.1. Preparation of plant materials

Phalaenopsis (*Doritaenopsis* Queen Bee 'Red Sky') seedlings were grown on Murashige and Skoog (MS) medium containing active carbon (1 g · L^{-1}). Leaves of *Phalaenopsis* seedlings were cut into 0.5 cm × 0.5 cm segments and immediately transferred to fresh MS medium containing 6-benzylaminopurine (3 mg · L^{-1}) or supplied with CHX (0.1 mg · mL^{-1}) (Zhou et al., 2000). All cultures were maintained under cool white fluorescent lights of approximately 35 μmol · m^{-2} · s^{-1} photon flux density under a 16 h/8 h cycle at (24 ± 2) ℃. Leaf explants were collected at 0 h, 6 h, 12 h, 1 d, and 3 d, frozen immediately in liquid nitrogen, and stored at −80 ℃.

2.2. PPO activity assay

Frozen leaf explants of *Phalaenopsis* (200 mg) were mixed with 2 mL pre-cooled 0.2 mol · L^{-1} sodium phosphate buffer (pH 8.0) containing 0.1% TritonX-100. The homogenate was centrifuged at 12 000 × *g* for 10 min at 4 ℃. The supernatant, referred to as crude enzyme extract, was collected and stored at −20 ℃. For the PPO activity assay, 0.2 mL crude enzyme extract was mixed with 3 mL 2 mol · L^{-1} phosphate buffer (pH 8.0) and 0.2 mL 2% catechol. The reaction was carried out at 32 ℃. The changes in absorbance at 310 nm were recorded with a spectrophotometer (UV6000PC, Shanghai Metash Instruments Co.) every 20 s for 2 min. PPO activity is expressed as units · mg^{-1} protein. One unit of PPO activity is defined as a change in absorbance value (OD) of 0.001 min^{-1} · mg^{-1} of enzyme. The protein content was determined by the Bradford method using bovine serum albumin (BSA) as the standard.

2.3. Total phenolics assay

Total phenolics were determined according to the procedure of Luo et al. (1999). Segments of leaf explants were homogenized in a ten-fold quantity (w/w) of methanol (pH 3.0) using a mortar and pestle and extracted for 24 h at 4 ℃. The homogenate was centrifuged at 12 000 × *g* for 10 min and the resulting supernatant was used for the determination of phenolic compound contents. Absorbance was detected at 280 nm using a UV-visible spectrophotometer (Shimadzu UV2450). The total phenolic content in the sample was calculated based on a standard curve for catechol.

2.4. RNA blot analysis of PPO expression

To detect the expression of *PPO*, total RNA was isolated in Trizol Reagent (Invitrogen, CA, USA) following the manufacturer's protocol. Each 10 μg sample of total RNA was separated on a 1% agarose denaturing formaldehyde gel, transferred to a Hybond-N + nylon membrane, and fixed by UV crosslinking. Hybridisation and detection were conducted following the manufacturer's instructions (DIG Northern Starter Kit, Roche, Mannheim, Germany).