Changes of Antioxidant Enzyme and Phenylalanine Ammonia-Lyase Activities during *Chimonanthus praecox* Seed Maturation

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Changes in peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and phenylalanine ammonia-lyase (PAL) activities were studied during *Chimonanthus praecox* seed maturation. According to our findings the protein content increased steadily from 8 to 12 weeks after flowering, and thereafter decreased significantly. Similarly, SOD and POD activities increased gradually up to 12 weeks after flowering and then declined. PAL activity declined gradually during seed maturation. CAT activity, however, showed no changes during seed maturation. By means of polyacrylamide gel electrophoresis (PAGE), SOD and POD isoenzymes were observed during seed maturation. The staining intensities of SOD and POD isoenzymes correlated well with SOD and POD activities as obtained by an assay in solution. These findings suggest that POD, SOD and PAL may be involved in the growth and development during *Chimonanthus praecox* seed maturation.

Key words: Antioxidant Enzymes, *Chimonanthus praecox*, Phenylalanine Ammonia-Lyase

Introduction

Seed development is a crucial part of the lifecycle of higher plants, providing the link between two distinct sporophytic generations and, thus, maintenance of the species (Wobus and Weber, 1999). Developing seeds are a well-defined system, allowing physiological and biochemical approaches to be combined with an analysis of the underlying developmental processes (Weber *et al*., 2005).

Reactive oxygen species (ROSs) are known to occur during seed development, where they have a dual function being either cytotoxic or playing a role in development, dormancy breakage, and defense against biotic and abiotic stresses (Gapper and Dolan, 2006). They can react with lipids, proteins, nucleic acids, pigments and other important cellular components, causing their degradation (Mittler *et al*., 2004). Plant cells prevent such macromolecular oxidative damage by maintaining the ROSs levels under tight control through an elaborate and highly redundant plant ROSs network. Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) constitute the major enzymatic systems through which cells catabolize free radicals and H_2O_2, thus limiting the potential for oxidative damage (Apel and Hirt, 2004). SOD catalyzes the conversion of superoxide to O_2 and H_2O_2. POD and CAT then catalyze the breakdown of H_2O_2 to H_2O and O_2 and hence preventing the production of more free radicals from H_2O_2 (Mittler *et al*., 2004). Phenylalanine ammonia-lyase (PAL) is the initial rate-controlling enzyme in the phenolic synthesis, and many phenolic compounds in plants possess antioxidant activity (MacDonald and D'Cunha, 2007).

Chimonanthus praecox is a multi-stemmed shrub with a fountain-like shape growing up to 2.5–3 m in height and width. It is in flower from December to the following early March, and its seeds ripen from May to June. Its seeds are packaged in pods containing 6–12 seeds. The plant is used as a traditional fragrant flower plant, and in Chinese traditional medicine for the treatment of rheumatic arthritis, heatstroke, nausea and measles (Zhang and Liu, 1998). The flowers of *C. praecox* are rich in volatile flavour and are a potential source of essential oil (Deng *et al*., 2004). *C. praecox* flower tea has recently been produced in several areas of China. To the best of our knowledge, there are no present published works that focus on the changes in the antioxidant enzyme defense system during seed maturation in *Chimonanthus praecox*. The main objective of this research was to elucidate the changes in SOD, POD, CAT and PAL activities during different phases of seed maturation.

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Materials and Methods

Plant materials and chemicals

Seed development in *Chimonanthus praecox* can be roughly divided into two stages. 1) Incubation stage: fertilization forms young seed; seed steadily accumulates nutrients, and the coat does not form. 2) Maturation stage: seed continues to develop to coat forms; seed changes colour from yellow-white to light browns; seed coat changes from white to yellow, to brown, then harden; seed pod changes from green to yellow, then brown, soften and eventually split (Zhang and Liu, 1998).

Developing seeds were harvested in 2007 from one approx. 10-year-old tree, growing on the grounds of Sichuan University, Chengdu, China. Seeds were collected at 8, 10, 12 and 15 weeks after flowering from the same tree in the experiment. At each stage seeds were inspected, the seed coat was separated from the embryo, and immediately frozen with liquid nitrogen. The embryos in a plastic box (labeled with No. 20070502) were deposited at −20 °C until processing. Methionine, l-phenylalanine, and nitroblue tetrazolium were purchased from Sigma (St. Louis, MO, USA). Other reagents used were of reagent grade or higher.

Protein extraction and estimation

Developing seeds were homogenized using a chilled pestle and mortar under liquid nitrogen. Then they were extracted in 50 mM sodium phosphate buffer (pH 7.0, 1/10, w/v) containing 1 mM EDTA and 0.15 M NaCl. The homogenized suspension was obtained by centrifugation at $15294 \times g$ for 10 min at 4 °C. The supernatant was used for determining the protein content and enzyme activity assays. Protein content was measured according to Lowry *et al.* (1951). Each sample was extracted once, and each extract was assayed three times. All assays and measurements were carried out three times.

Polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed with 10% acrylamide gels under non-denaturing conditions. The isoenzyme pattern of POD was obtained according to Ros Barcelo (1987). The gels were immersed in a solution containing 0.06% (v/v) H_2O_2, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature until they turned brown. The isoenzyme pattern of SOD was estimated by the method of Wang and Yang (2005). The gels were incubated in the dark for 30 min at room temperature in an assay mixture containing 50 mM sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.05 mM riboflavin, 0.3% *N,N,N,N*-tetramethyl ethylenediamine (TEMED) and 0.1 mM nitroblue tetrazolium. The gels were then rinsed with water and exposed to light for 30 min at room temperature, until the colourless bands in the purple-stained gel became visible.

Assay of antioxidant enzymes

The SOD activity was assayed in a 3 ml mixture containing 50 μl enzyme extract, 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium, 0.15 M NaCl and 2 μM riboflavin (Chen and Pan, 1996). One SOD unit (U) was defined as the amount of enzyme needed to inhibit the reduction of nitroblue tetrazolium by light by 50%.

The POD activity was measured by absorbance increase at 470 nm (Sakharov and Bautista, 1999). One POD unit (U) was taken as the amount of enzyme required to increase the absorbance by 1.0 per min.

The CAT activity was determined by the decrease in the absorbance at 240 nm due to the decomposition of H_2O_2 (Montavon *et al.*, 2007). One unit (U) of CAT was defined as the amount of enzyme needed to catalyze the breakdown of 1 μmol of H_2O_2 per min at 25 °C.

Results were expressed as SOD, POD and CAT units per gramme fresh weight of seed, respectively.

Enzyme extraction and PAL activity assay

Samples were homogenized with chilled Tris [tris(hydroxymethyl) aminomethane]-HCl (50 mM, pH 8.8, 2 ml/g fresh weight), supplemented with 10% polyvinylpolypyrrolidone and 0.1 mM EDTA. The crude enzyme was obtained by centrifugation. PAL activity was measured by the increase in the absorbance at 290 nm (D'Cunha *et al.*, 1996). The assays were performed in 3 ml reaction solution containing 50 mM Tris-HCl buffer (pH 8.8), 20 mM L-phe and 50 μl enzyme solution. The mixtures were incubated for 30 min at 30 °C, and the reaction was stopped by addition of 0.5 ml trichloroacetic acid (10%). One PAL unit was defined as the amount of enzyme needed to cause an increase

Table I. Changes in total protein content, SOD, POD and PAL activities during seed maturation.

Developmental stage [weeks after flowering]	Protein concent [mg/g fresh weight]	SOD activity [U/g fresh weight]	POD activity [U/g fresh weight]	PAL activity [U/g fresh weight]
8	21.4 ± 1.13	87.6 ± 4.08	234 ± 11.22	336.1 ± 15.31
10	23.7 ± 1.21	112.5 ± 5.33	345.76 ± 16.89	242.6 ± 11.73
12	63.3 ± 2.82	180.7 ± 8.74	475.3 ± 20.65	229.2 ± 11.06
15	52.1 ± 2.36	175.3 ± 8.48	348.2 ± 17.21	211 ± 9.95

Data are displayed as mean ± standard deviation for three replications.

in the absorbance of 0.01 per min. Results were expressed as PAL units per gramme fresh weight.

Statistical analysis

All data are reported as means ± SD and were analyzed by Student's *t* test. *P* values less than 0.05 were considered to be statistically significant.

Results and Discussion

Changes in total protein content, SOD, POD and PAL activities during seed maturation are shown in Table I. The protein content increased progressively during seed maturation up to 12 weeks after flowering, reaching 63.3 mg/g fresh weight; then it declined significantly at 15 weeks after flowering. In the later stages of seed maturation, the protein content remained 52.1 mg/g fresh weight. Seed storage proteins are usually accumulated in the vacuole or as membrane-bound protein bodies within the cell during the maturation stage (Hoekstra *et al*., 2001). Seed storage proteins such as albumins and globulins are expressed more during the accumulation phase through the middle of the maturation phase (Wobus and Weber, 1999). Our finding suggested that the synthesis of these storage proteins continues into the last stage of development but increasingly slows down until the end of seed development. The mechanism of protein synthesis during the maturation stage is complex and requires further study.

Seed or fruit maturation is accompanied by alterations in the physical properties of the membrane lipid matrix and changes in the activity of membrane-bound enzymes (Wang and Jiao, 2001). It has been reported that the tolerance of plants to damage-causing conditions may be associated with their greater ability to remove ROS through ROS-detoxifying enzymes, such as SOD, POD and CAT (Apel and Hirt, 2004). In the present study, the SOD activity increased significantly up to 12 weeks after flowering, and then declined slightly at 15 weeks after flowering, representing 175.3 U/g fresh weight in the mature seeds (Table I). Our results clearly showed that increased SOD activity appears to effectively mop up oxygen radicals during the early stage of maturation. Similarly, an increased SOD activity has been observed during seed maturation (Bailly *et al*., 2004). Analysis with native PAGE suggested there are five SOD isoforms visualized during seed maturation, but different isoform patterns were observed. The differences in the isoenzyme presence were accompanied by quantitative changes in the isoenzyme activity as assayed in solutions during seed maturation (Table I and Fig. 1). The SOD activity increased with seed maturation, indicating that the SOD activity may play an important role in combating oxidative stress.

The POD activity increased continuously up to 12 weeks after flowering, while the activity decreased significantly at 15 weeks after flowering (Table I). Similar changes in the peroxidase activity were observed in other plant species during seed or fruit development (Fernandez-Trujillo *et al*., 2003; Montavon and Bortlik, 2004). The pres-

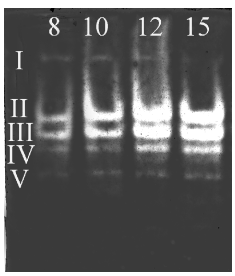

Fig. 1. Isoenzyme pattern of superoxide dismutase (SOD) isoenzymes during seed maturation. Enzyme samples were extracted from developing seeds harvested 8, 10, 12 and 15 weeks after flowering. About 20 μl extract from each sample were loaded.

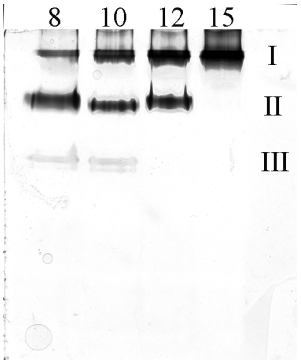

Fig. 2. Isoenzyme pattern of peroxidase (POD) isoenzymes during seed maturation. Enzyme samples were extracted from developing seeds harvested 8, 10, 12 and 15 weeks after flowering. About 10 μl extract from each sample were loaded.

ence of POD isoenzymes has been demonstrated in numerous plant species, and several polymorphic forms of POD as well as a high diversity of *in vitro* substrates have been detected (Passardi *et al*., 2005). As shown in Fig. 2, three bands of POD isoenzymes were found during seed maturation, but only one isoenzyme band of POD was detected at 15 weeks after flowering. The staining intensities of the isoenzyme band thus correlated well with the increase in the POD activity as assayed in solution (Table I and Fig. 2). Taken together these results indicated that changes in the POD activity strongly depend on different stages of seed maturation. Moreover, the changes in peroxidase activity also corresponded with changes in the protein content and SOD activity. Increases of the POD activity and its isoenzymes have been shown to occur in response to stress, for example that caused by wounding, ozone, and mechanical injury, or in relation to seed development (Fernandez-Trujillo *et al*., 2003; Montavon and Bortlik, 2004). Changes in the specific SOD and POD activities were not simply a consequence of changes in the seed protein content during the seed maturation, and further research is required.

CAT, one of the most important enzymes involved in removing reactive oxygen species in plant cells, is responsible for scavenging H_2O_2 formed by POD (Mittler *et al*., 2004). The CAT activity has been shown to increase during seed development and is dependent on seed moisture content in sunflower (Bailly *et al*., 2004). However, no changes in the CAT activity were found during seed maturation, and the activity remained low, representing only 16 U/g fresh weight.

The PAL activity decreased steadily with seed maturation for up to 15 weeks after flowering, ranging from 336.1 to 211 U/g fresh weight (Table I). These results show a clear inverse relationship between protein content and PAL activity, suggesting that the synthesis of these enzymes might be inhibited during seed maturation. In previous investigations, the PAL activity has been shown to be stimulated by chilling, wounding, UV-B light, ozone, pathogen invasion and plant hormones. Thus, PAL can be considered as part of the plant defense system (MacDonald and D'Cunha, 2007). These findings suggest that PAL may play an essential role in modulating the resistance of plant tissues to such stresses during the early stages of seed maturation.

These findings suggest also that SOD, POD and PAL activity in *Chimonanthus praecox* seeds are dependent upon the stage of seed maturation. The seeds' ability to cope with oxidative stress depends on its ability to remove ROSs. Increases in SOD and POD activities during the early stages of seed maturation suggest an increased oxidative stress. It appears that SOD and POD play a major role in regulating the level of ROSs, whereas PAL plays a role in the defensive response, especially in the early stage of seed maturation.

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