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ORIGINAL PAPER

Changes in H_2O_2 content and antioxidant enzyme gene expression during the somatic embryogenesis of *Larix leptolepis*

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Abstract Hydrogen peroxide (H_2O_2) content and transcript levels of genes encoding superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) antioxidant enzymes were investigated during different stages of somatic embryogenesis in *Larix leptolepis*. H_2O_2 content was lowest on day 0 when embryogenic callus was incubated on Murashige and Skoog (MS) medium supplemented with polyethylene glycol (PEG). This content began to increase when callus was transferred to the same medium but containing abscisic acid (ABA), and reached a peak at day 3 following incubation. The level of H_2O_2 dropped from day 7 to day 10, peaked at day 21, then dropped again at day 24 and day 35, but increased when somatic embryos reached maturity at day 45. Transcript levels of *SOD*, *CAT*, and *APX* were lowest when somatic embryos were cultured on callus induction medium without ABA. When calli were transferred onto somatic embryo maturation medium, expression patterns of *SOD*, *CAT*, and *APX* varied, and transcript levels at all stages were higher than those at d 0. *SOD* expression was highest at day 3. Whereas, *CAT* expression levels were low during early stages of somatic embryogenesis, but increased at day 21, declined at days 24 and 35, and then began to increase again at day 45. APX gene expression patterns were highest at days 3, 21, and 45. These results suggested that ABA was essential for promoting somatic embryogenesis of *L. leptolepis*. Moreover, ABA induced production of H_2O_2 and other active oxygen species (AOS), and mediated *CAT*, *SOD*, and *APX* gene expression in somatic embryogenesis of *L. leptolepis*.

Keywords Somatic embryogenesis · Hydrogen peroxide · Catalase · Superoxide dismutase · Ascorbate peroxidase · *L. leptolepis*

Abbreviations

CAT	Catalase
SOD	Superoxide dismutase
APX	Ascorbate peroxidase
AOS	Active oxygen species
H_2O_2	Hydrogen peroxide
BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
KT	Kinetin
CH	Casein hydrolysate
PEG	Polyethylene glycol
MS	Murashige and Skoog basal medium

Introduction

Somatic embryogenesis has been induced in several conifer species (Haggman et al. 2005); however, this has not yet been reported in *L. leptolepis*.

Abscisic acid (ABA) regulates many important aspects of plant development, including the synthesis of seed

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storage proteins and lipids, the promotion of seed desiccation tolerance and dormancy, and the inhibition of the phase transitions from embryonic to germinative growth and from vegetative to reproductive growth (Bellaire et al. 2000). ABA plays an important role in embryo development as well as growth and development throughout the plant life cycle. It has been shown that early stage somatic embryos of most conifers require the addition of 20–50 μM exogenous ABA in order to develop into cotyledonary stage (Stasolla et al. 2002). In several angiosperm and coniferous species, endogenous ABA was found to increase during the formation of mature zygotic embryos (Carrier et al. 1999; Kapik et al. 1995; Kong et al. 1997). In addition, ABA mediates some aspects of physiological responses to tolerance of water, salt, hypoxic and cold stress, and high osmoticum (Shinozaki and Yamaguchi-Shinozaki 2000).

Recently, there have been many experiments carried out on several plant species with the objective of explaining the role of oxidative stress in plant morphogenesis (de Marco and Roubelakis-Angelakis 1996; Cui et al. 1999; Papadakis et al. 2001; Papadakis and Roubelakis-Angelakis 2002). Oxidative stress is inevitably present in somatic embryo development as a particular cell differentiation. Active oxygen species (AOS) could be one possible link between oxidative stress and plant regeneration in tissue culture (Gupta and Datta 2003/2004). H_2O_2 is a form of AOS. H_2O_2 is generated via superoxide, presumably in a non-controlled manner, during electron transport processes such as photosynthesis and mitochondrial respiration. H_2O_2 generation is also induced in plants following exposure to a wide variety of abiotic and biotic stimuli. These include extremes of temperature, UV irradiation, ozone exposure, phytohormones, such as ABA, dehydration, wounding and pathogen challenge (Neill et al. 2002). When plants are exposed to stressful conditions, such as drought, low temperature, high salt, high light intensity or in vitro culture, H_2O_2 and other AOS can be produced in excess and accumulate in vitro. This phenomenon is commonly known as oxidative stress (Cassells and Curry 2001; Konieczny et al. 2008). Overproduction or accumulation of AOS in cells can disturb the redox system of the cell and influence the main metabolic pathways by direct changes in enzyme activity, membrane properties, lipids, DNA structure and other biomolecules (Cassells and Curry 2001). Plant cells possess well-developed systems of antioxidant enzymes such as SOD, CAT, POD, APX to regulate the level of AOS and the concentration of AOS to effectively reduce oxidative damage (Dat et al. 2000; Mittler 2002). SOD is the first enzyme in the detoxifying process, which converts superoxide anion to H_2O_2 (Gupta and Datta 2003/2004). The principal H_2O_2 scavenging enzyme in plants is CAT, which is located in peroxisomes, glyoxysomes and in mitochondria. Differences in the activity of the antioxidant enzymes during organogenesis and somatic embryogenesis in several plant species cultured in vitro were reported (Chen and Murata 2002). APX is a key H_2O_2 scavenging enzyme in the cytosol, chloroplast, and mitochondria of higher plants (Libik et al. 2005). Additionally, H_2O_2 is a weak oxidizing agent of relatively long lifetime and has the role of a secondary messenger involved in signal transduction via its influence on the expression of some genes (Neill et al. 2002; Vranov et al. 2002; Libik et al. 2005; Hung et al. 2005). H_2O_2 can induce the expression of genes encoding APX in germinating rice embryos and in *Arabidopsis* leaves (Karpinski et al. 1999), and wounding induced the expression of gene encoding a catalase via H_2O_2 in embryos and leaves of maize (Guan et al. 2000). H_2O_2 is also involved in the regulation of stomatal closure in response to ABA and elicitors (Pei et al. 2000).

Here, we measured the H_2O_2 content and compared the transcript level of three antioxidant enzymes genes (*SOD*, *CAT* and *APX*) during somatic embryo development of *L. leptolepis* cultured on different media. We have also discussed the relationship between the development of somatic embryos of *L. leptolepis* and the H_2O_2 content.

Materials and methods

Plant material

Immature seeds were collected from Dagujia Seed Orchard in Liaoning Province, China. These were disinfested with 0.1% HgCl and rinsed three times with distilled water. Seeds were cultured on MS-basal medium containing 30 g l^{-1} sucrose, 3 g l^{-1} Phytagel, 1.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg l^{-1} benzyladenine (BA), 2 mg l^{-1} kinetin (KT), 500 mg l^{-1} glutamine, and 500 mg l^{-1} caseine hydrosylate (CH) to induce embryogenic callus. The pH of the medium was adjusted to 5.8.

Embryogenic callus was transferred to somatic embryo maturation medium consisting of MS basal medium, and containing 30 g l^{-1} sucrose, 3 g l^{-1} Phytagel, 29 mg l^{-1} abscisic acid (ABA), 106 g l^{-1} polyethylene glycol (PEG), 250 mg l^{-1} glutamine, and 250 mg l^{-1} CH. The pH of the medium was adjusted to 5.8. Embryo callus induction and propagation of pine were incubated in the darkness at 25°C.

Somatic embryos were collected at days 0, 1, 2, 3, 5, 7, 10, 14, 16, 21, 24, 35, and 45 following transfer to embryo maturation medium (Fig. 1). All samples were frozen in liquid Nitrogen, and stored at −70°C until RNA extraction.

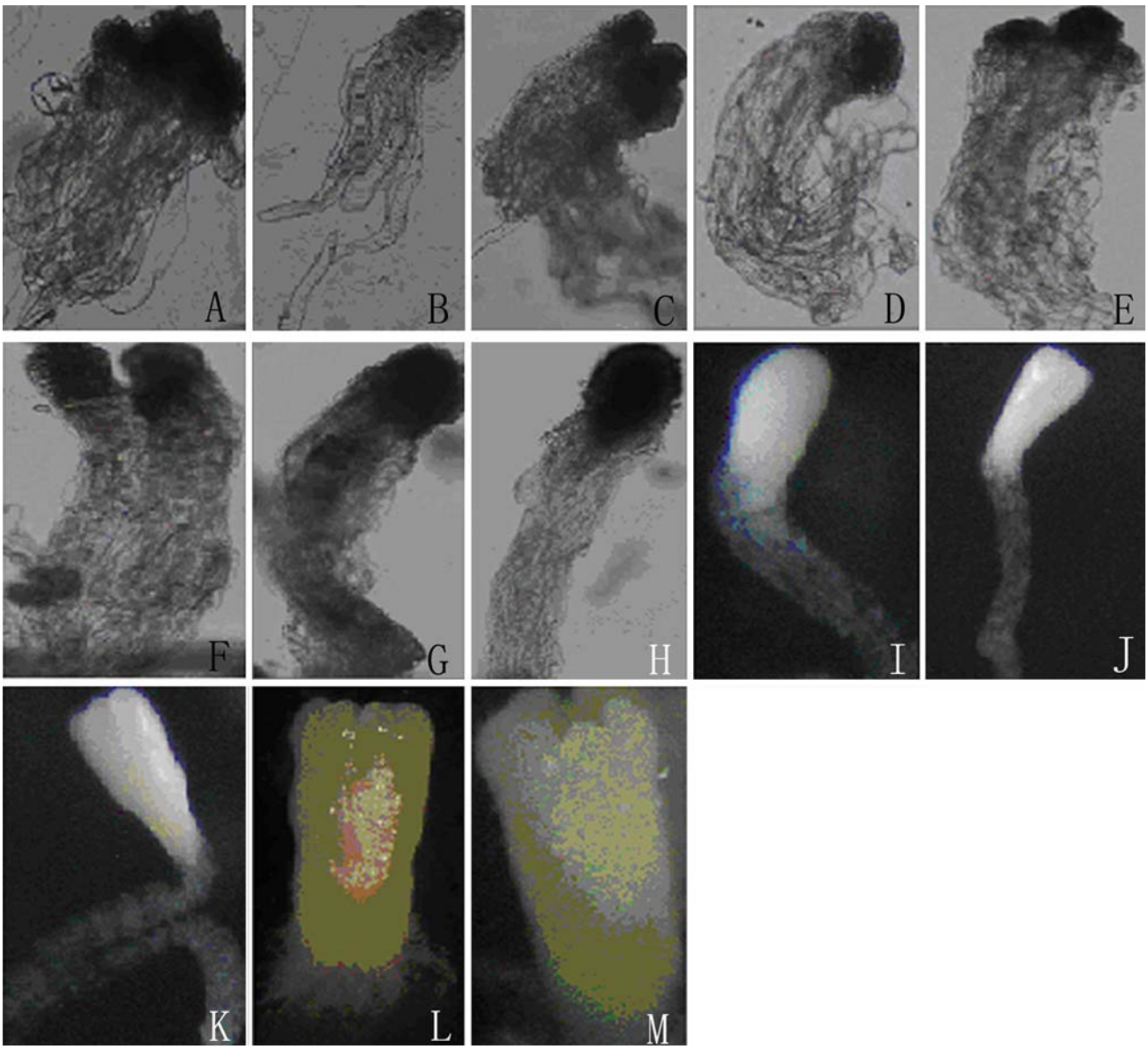

Fig. 1 Morphology of somatic embryogenesis of *L. leptolepis* at 13 time points. **a** day 1, **b** day 2, **c** day 3, **d** day 5, **e** day 7, **f** day 10, **g** day 14, **h** day 16, **i** day 21, **j** day 24, **k** day 35, and **l** day 45

Measurement of H_2O_2

H_2O_2 was measured using the method of Ferguson et al. with slight modifications (Ferguson et al. 1983). Four replicates of somatic embryos at 13 time points (~0.5 g fresh weight) were homogenized in 1.5 ml cold acetone. The extract and washings were centrifuged for 10 min at 3,000 rpm. To 1 ml supernatant, 0.1 ml 5% Ti_2SO_4 and 0.2 ml NH_4OH were added dropwise and mixed thoroughly. The mix was then centrifuged for 10 min at 3,000 rpm, and the precipitates were washed repeatedly with 2 ml acetone until the supernatant was colorless. The precipitates were solubilized in 5 ml 2 N H_2SO_4, made up to H_2SO_4 and filtered prior to measurement of A at 415 nm against a blank which had been carried through the same procedure. Standards in the range of 0–100 μmol l^{-1} H_2O_2 were also reacted with Ti_2SO_4 and carried through the procedure.

Quantitative RT–PCR assay of SOD, CAT and PAX transcript levels

Relative quantification of *SOD*, *CAT* and *PAX* expression in somatic embryogenesis was achieved by real-time RT–PCR. Total RNA was extracted from somatic embryo tissue at different developmental stages using the CTAB

method. First-strand cDNA was synthesized using MMLV (Toyobo, Japan) according to the manufacturer's instructions. Real-time RT–PCR was performed using SYBR Green (TOYOBO, Japan). Primers of genes used in real-time RT–PCR were designed according to the sequence of NCBI accessions. Forward (F) and reverse (R) as follows: SODF: 5′-CAGGAGACAACGGTCCCACA-3′, SODR: 5′-AACTCCATCAGAACCCGCAAC-3′, according to the accession number X58579.1, which amplifies a 216 bp fragment; CATF: 5′-GATCGTGCGTTTCTCAACTGT-3′, CATR: 5′-AAGGGATTCCAAGATCATCAA-3′, according to the accession number AJ29945.1, which amplifies a 293 bp fragment; APXF: 5′-GTGAAGAGCAAGACGGGAGG-3′, APXR: 5′-TTAGTGGCATCAGGCAAACG-3′, according to the accession number AF326783, which amplifies a 263 bp fragment. In addition to the primers for the endogenous control, β-actin (forward, 5′-GTTCTCA GTGGTGGTTCTAC-3′, and reverse, 5′-GACCCTGACTC CTCATACTC-3′) reactions were run in duplicate on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the following thermal cycling profile: 94°C for 2 min, then at 94°C for 30 s, 55°C 30 s, and 72°C for 30 s for 35 cycles. After 35 cycles, samples were run using the dissociation protocol. This assay was performed twice to minimize variations due to sample handling. In order to compensate for differences in loading and RT efficiency (based on our previous studies, which indicated that the analyzed β-actin mRNA levels are quite constant in tissues, regardless of the developmental or physiological conditions) this β-actin was used as an endogenous control. Thus the reported *SOD* and *CAT* transcript levels are normalized relative to β-actin.

Results

Changes in H_2O_2 content during somatic embryo development

The H_2O_2 content in somatic embryos during somatic embryo development was measured. The results showed that H_2O_2 content ($\sim$0.1145 μmol/g) was the lowest on day 0. The H_2O_2 content began to increase dramatically when tissues were transferred on ABA and PEG induction medium, and reached a maximum on day 5 (0.7766 μmol/g). The H_2O_2 content of tissues in ABA and PEG induction medium increased more than 5.78 times within the first 5 days from day 0. A reduction in H_2O_2 accumulation was observed from day 7 to day 10, which peaked again on day 21, and then H_2O_2 content started to decrease again from day 24 to day 35 followed by a dramatic increase from day 45 when somatic embryos have completely matured (Fig. 2).

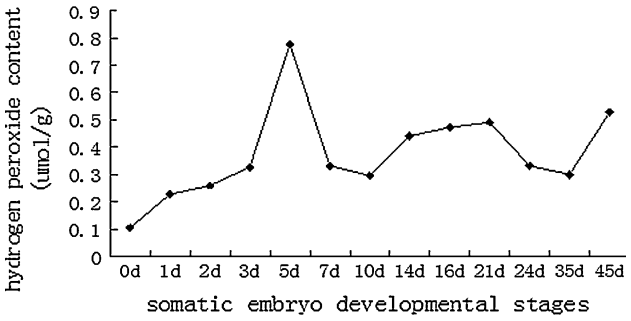

Fig. 2 Changes in H_2O_2 content during somatic embryo development in callus cultures of *L. leptolepis*

Analysis of SOD transcript levels during somatic embryo development by real-time RT–PCR

The mRNAs from 13 samples were detected with the same efficiency. Therefore, the analyzed transcript levels correspond with the sum of the relative quantities. Analysis of the dissociation curves of *SOD* and β-actin control samples showed a single melting peak (Fig. 3), which indicated a specific signal corresponding to the *SOD* target sequences and the endogenous control, respectively. The fluorescent signal was not detected in all negative-control samples, proving that the RNA extraction procedure, including the DNase treatment, effectively removed genomic DNA from the RNA samples. No contamination appeared during PCR procedure. A fluorescence threshold value (Ct) was calculated for each sample. For each standard curve (Fig. 4), the correlation coefficients ranged from 0.9978 to 0.9995 indicating a high degree of confidence in the measurement of copy number of molecules in the samples.

The relative abundance of the *SOD* transcript at 13 developmental stages was demonstrated during somatic embryo development of *L. leptolepi* by RT–PCR. The transcript levels of the *SOD* were lowest on day 0 (Fig. 5) tissues cultured on non-ABA and PEG induction medium. But which became high when tissues were transferred to ABA and PEG induction medium, and the expression quantity reached a maximum on d3, and then dropped from day 5 to day 45.

Analysis of CAT transcript levels during somatic embryo development by real-time RT–PCR

The expression pattern of *CAT* were analyzed and summarized during somatic embryogenesis of *L. leptolepis* by real-time RT–PCR. The mRNAs from 13 samples were detected with the same efficiency. Analysis of the dissociation curves of the *CAT* in 13 samples showed a single melting peak (Fig. 3). The fluorescent signal was detected in the negative-control sample, and the dissociation curves indicate that there was a low melting peak in the negative-

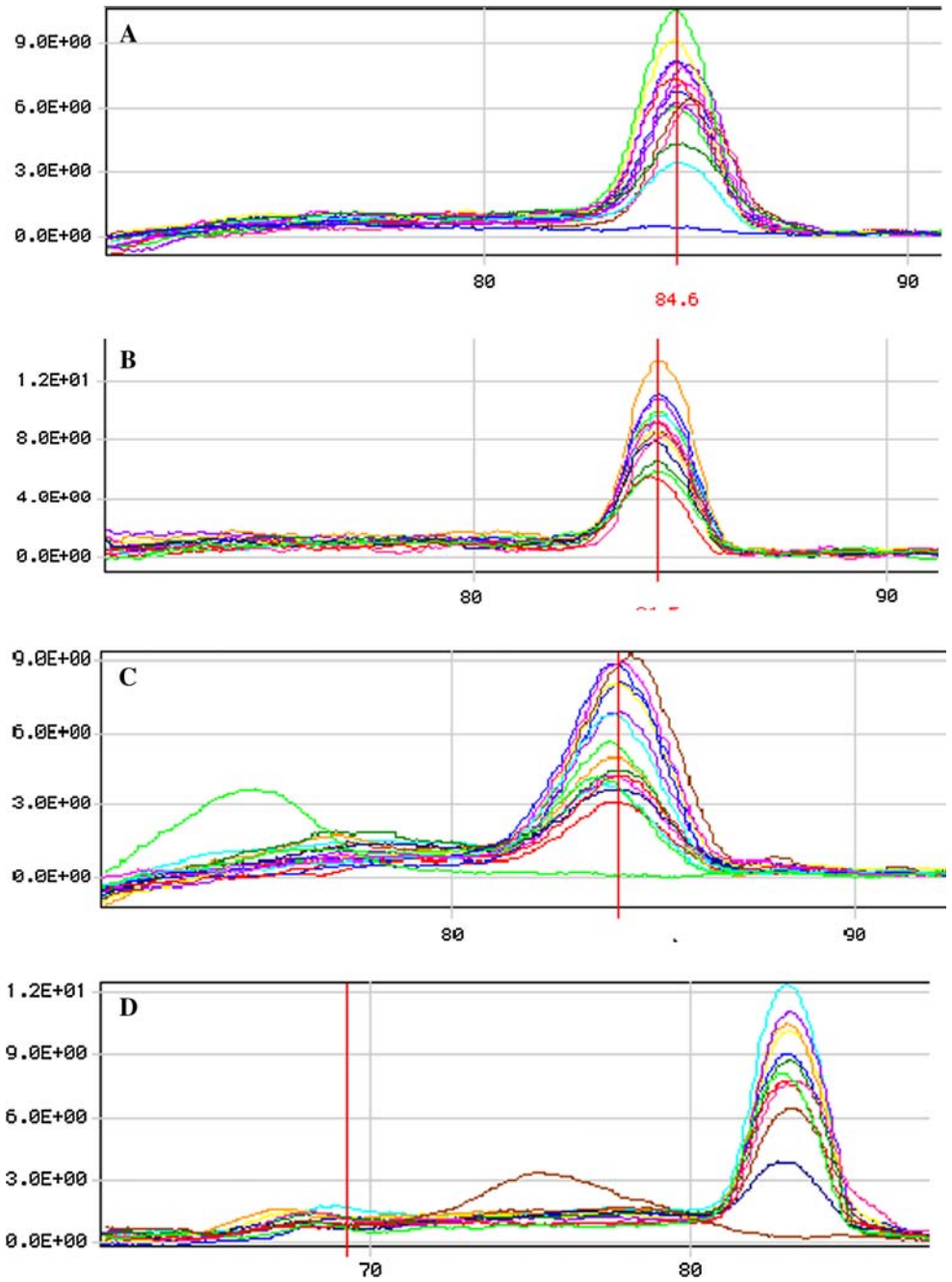

Fig. 3 Dissociation curves of expression patterns of genes encoding antioxidant enzymes. **a** β-actin (control), **b** SOD, **c** CAT, and **d** APX

control sample (Fig. 3). However, the peak of the negative-control was different from that of the samples, and the negative-control did not show any amplification when the PCR product was detected by electrophoresis on an agarose gel. A fluorescence threshold value (Ct) was calculated for each sample. For the *CAT* standard curve (Fig. 4) the correlation coefficient was 0.999 which indicates confidence in the measurement of the copy number of molecules in the sample.

The expression pattern of *CAT* was different to that of *SOD*. When somatic embryos (day 0) were cultivated on medium without ABA and PEG, expression levels of *CAT* was also the lowest during the whole somatic embryogenesis. Expression levels of *CAT* began to increase slightly when somatic embryos were transferred to ABA and PEG induction medium. But expression levels of *CAT* was lower in early somatic embryogenesis, and there was a very lower expression peak at day 5, and then expression

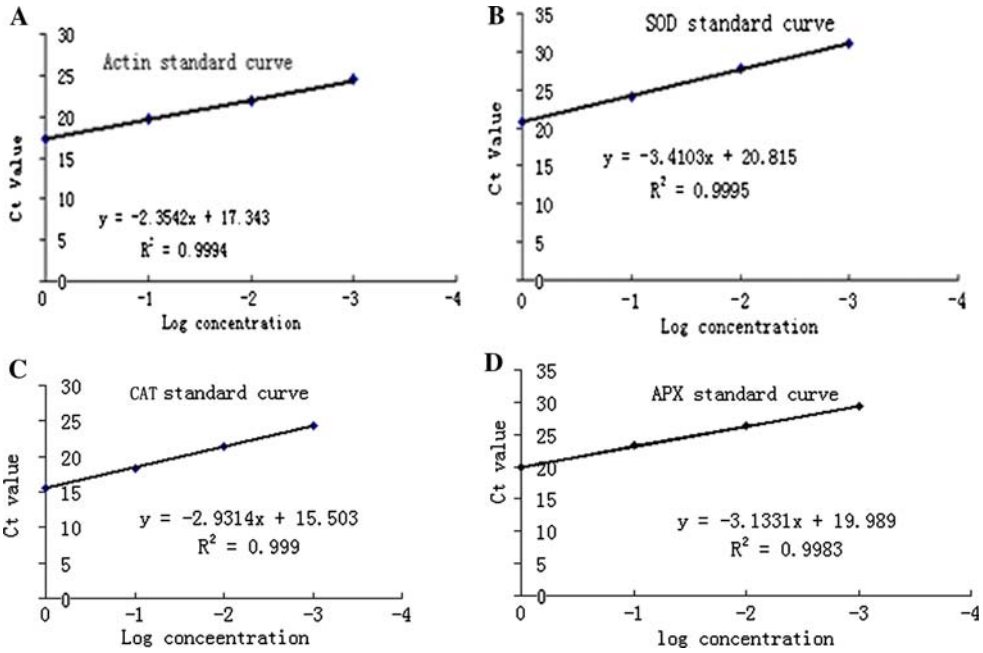

Fig. 4 Standard curves of expression patterns of genes encoding antioxidant enzymes. **a** β-actin (control), **b** SOD, **c** CAT, and **d** APX. Ct was plotted against the log of the initial level of transcript for each gene to generate standard curves

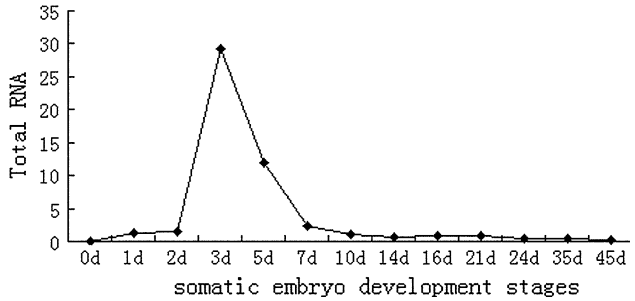

Fig. 5 Expression patterns of SOD during somatic embryo development of *L. leptolepis*

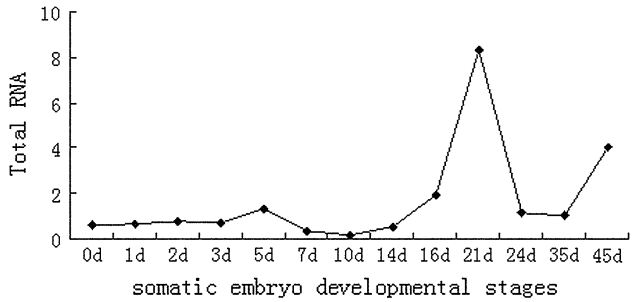

Fig. 6 Expression patterns of CAT during somatic embryo development of *L. leptolepis*

quantity began to drop from day 7 to day 10, followed by increasing from day 14, and reached a maximum at day 21, declined dramatically from day 24 to day 35, and then *CAT* transcript levels began to increase again when somatic embryos matured at day 45 (Fig. 6).

Analysis of APX transcript levels during somatic embryogenesis by real-time RT–PCR

The mRNAs from 13 samples were detected with the same efficiency. Therefore, the analyzed transcript levels corresponded with the sum of the relative quantities. Analysis of the dissociation curves of *APX* from 13 samples showed a single melting peak (Fig. 3), which indicates a specific signal. However, the fluorescent signal was detected in negative-control samples. The dissociation curves show that there was a low melting peak in negative-control samples (Fig. 3), but the location of the peak was different from the samples, and negative-controls did not show any amplification when the PCR product was detected by electrophoresis on an agarose gel. A fluorescence threshold value (Ct) was calculated for each sample. For the standard curve, the correlation coefficient was 0.9983, indicating a high degree of confidence for the measurement of the copy number of molecules in the samples (Fig. 4).

The expression patterns of the *CAT* were analyzed and summarized during somatic embryo development of *L. leptolepis* using real-time RT-PCR, which was similar to the expression pattern of *CAT*. The expression quantity was lowest at day 0 tissue cultivated on the medium without ABA and PEG. Expression of the *APX* began to increase slowly when somatic embryos were transferred to ABA and PEG induction medium. With a peak at day 3 followed by decreasing *APX* expression quantity, the expression quantity started to increase significantly from day 14, and reached a maximum at day 21. Expression began to drop at

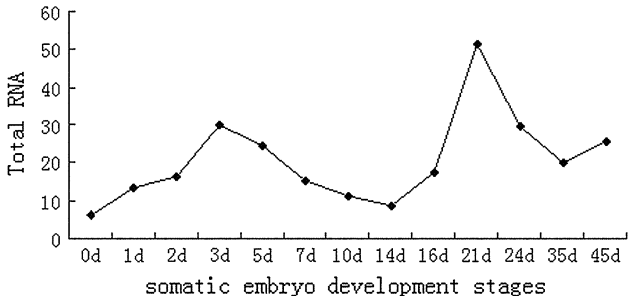

Fig. 7 Expression patterns of APX during somatic embryo development of *L. leptolepis*

day 24 and day 35, but increased slightly at day 45 when somatic embryos matured (Fig. 7).

Discussion

Changes in H_2O_2 content during somatic embryo development

H_2O_2 content was lowest when tissues were cultured in ABA and PEG free medium. As tissues were transferred to somatic embryogenic developmental medium, the H_2O_2 content began to increase dramatically and reach a maximum at day 5, suggesting that ABA and PEG might induce the production of H_2O_2. PEG can form non-osmosis water stress in the cell, and cause the water to reduce and enhance the density of cell content, to finally promote the growth of the somatic embryo. The level of ABA also increases in response to stress treatments in various plants, and is thought to act as a signal for the initiation of acclimation to these stresses (Shinozaki and Yamaguchi-Shinozaki 1997; Siddiqui et al. 1998; Hare et al. 1999; Kikuchi et al. 2006). Various stress substances or conditions might stimulate ABA. Different pieces of evidence link ABA to AOS. In guard cells, ABA induces the production of H_2O_2 and the activation of calcium channels by H_2O_2 mediates the induction of stomata closure (Staneloni et al. 2008). Another reason might be tissues and cells occupy a stage of rapid fission (Fig. 1, days 1–5). Higher concentration of H_2O_2 is a signal to promote rapid fission of tissues. A reduction of H_2O_2 accumulation due to a signal for fission increases development of the cells from day 7 to day 10 Fig. 1, days 7–10), then H_2O_2 content began to increase slowly and reach a subpeak at day 21, H_2O_2 content started to reduce again from day 24 to day 35 until the somatic embryo had matured at day 45 (Fig. 1). Sugars, proteins and esters accumulated from day 24 to day 35, and full accumulation of these compounds was essential for somatic embryo maturation and might be a reason for drop of adjustment H_2O_2 content. H_2O_2 content also had ascension when somatic embryo completely matures at day 45 (Fig. 1, day 45), this stage was somatic embryo entering the latter stage of ripening. This made the shape and the physiological biochemical characteristics of the somatic embryo more similar with the zygote embryo and normal germination.

The expression of antioxidase genes during somatic embryo development

In the dark, or in nonphotosynthetic tissues, AOS is produced mainly by leakage of the electron transport chain in mitochondria or microsomes (Vanlerberghe and McIntosh 1997) and during fatty acid oxidation (Mittler 2002). Increased production of AOS may lead to oxidative stress and cellular damage resulting in seed deterioration (Bailly 2004; Kranner et al. 2006). To cope with oxidative stress, plants have evolved several enzymatic and non-enzymatic systems. The channels by which H_2O_2 mediates the induction of stomata closure, is by inducing the expression of antioxidant genes *SOD* (Sakamoto et al. 1995; Guan and Scandalios 1998a; Bueno et al. 1998), and *CAT* (Guan and Scandalios 1998b; Guan et al. 2000), and increase the activities of antioxidative enzymes such as SOD, CAT, guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) in plant tissues (Prasad et al. 1994; Gong et al. 1998; Bellaire et al. 2000). SOD reduces superoxide radicals (O_2^-) to H_2O_2, CAT reduces H_2O_2 to water and dioxygen, APX reduces H_2O_2 to water and monodehydroascorbate, thus preventing the formation of the highly reactive hydroxyl radical (OH) that can cause lipid peroxidation, protein denaturation, and DNA mutations. To date, the antioxidant gene/enzyme systems for CAT, SOD and APX have been well characterized in many plants (Willekens et al. 1995).

In this study, transcript levels of *SOD*, *CAT* and *APX* were the lowest when somatic embryos were cultured on callus-propagation medium without ABA and PEG. When they were transferred on mature somatic embryo induction differentiation medium, the expression patterns of *SOD*, *CAT* and *APX* were different.

The results indicated that the *CAT*, *SOD* and *APX* cooperate to adjust differentiation and development of somatic embryos. H_2O_2 generated at different times catalyze and scavenge different enzymes, and this mad oxidation system maintain suitable level during somatic embryogenesis. Transcript levels of *SOD* enhanced gradually at early somatic embryo development. The peak of *SOD* transcript levels appeared at day 3, when the cell fission formed the multi-cell proembryo. High expression of *SOD* may promote embryonic cell differentiation as well as the early somatic embryo development. SOD activities were high in the stem and root tip of meristematic tissues

(Gupta and Datta 2003/2004). With the multi-cell proembryo further fission and development, the transcript levels dropped gradually, and at day 45 the same level as day 0 was detected. The transcript levels of *SOD* dropped due to H_2O_2. Massive accumulation of H_2O_2 in vivo has suppressed transcription of *SOD* and destroyed the active oxygen elimination system. Therefore, embryonic cell differentiation might be regulated by the relationships between *SOD*, O_2 with H_2O_2.

Expression patterns of *CAT* and *APX* were different with that of *SOD*. Expression of *CAT* and *APX* began to increase slowly when calli were transferred on ABA and PEG induction medium. The first expression peak of *APX* appeared at day 3, but there was a very low expression peak of *CAT* at day 5. And transcript levels of the two genes began to drop gradually until day 14, the expression quantity started to increase significantly, and reached maximum at day 21. Then expression began to drop at day 24 and day 35, but increased slightly at day 35. The expression patterns of *CAT* and *APX* was similar to changes of H_2O_2 content during somatic embryo development. This indicated that H_2O_2 induced the transcription of *CAT* and *APX* during somatic embryogenesis.

Suitable density H_2O_2 has induction action to somatic embryo development, but mechanism is not actually clear (Cui et al. 1999). In this study, H_2O_2 obviously induced and promoted the somatic embryo formation of *L. leptolepis*. It is possible H_2O_2 influenced gene expression through the cell signal transduction system and thus induced somatic embryo formation. The embryonic cell formation is also a cell differentiation process, and the core of this process is the gene difference expression. Therefore, H_2O_2 might induce somatic embryogenesis at the molecular level.

In summary, ABA was essential for stimulation of somatic embryogenesis in *L. leptolepis*. ABA might induce H_2O_2 and AOS generation, H_2O_2 caused stresses and induced *CAT*, *SOD*, and *APX* expression during somatic embryogenesis of *L. leptolepis*. Exogenous H_2O_2 may improve generation frequency of somatic embryos of *L. leptolepis*.

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