In Vitro Cell. Dev. Biol.—Plant 36:488–491, November–December 2000

1054-5476/00 $10.00+0.00

PEROXIDASE ACTIVITY OF DESICCATION-TOLERANT LOBLOLLY PINE SOMATIC EMBRYOS

WEI TANG*

State Key Laboratory of Biochemical Engineering, Institute of Chemical Metallurgy, Chinese Academy of Sciences, Beijing 100080, P. R. China

(Received 29 May 2000; accepted 17 July 2000; editor S. A. Merkle)

Summary

Desiccation tolerance can be induced by culturing somatic embryos of loblolly pine (*Pinus taeda* L.) on medium supplemented with 50 μ*M* abscisic acid (ABA) and/or 8.5% polyethylene glycol (PEG_{6000}). Scanning electron microscopy of desiccated somatic embryos showed that the size and external morphology of the desiccation-tolerant somatic embryos recovered to the pre-desiccation state within 24–36 h, whereas the non-desiccation-tolerant somatic embryos did not recover and remained shriveled, after rehydration. Peroxidase activity of desiccated somatic embryos increased sharply after 1 d of desiccation treatment at 87% relative humidity (RH), and desiccation-tolerant somatic embryos had higher peroxidase activity compared to sensitive somatic embryos. Higher peroxidase activity of desiccation-tolerant somatic embryos may have allowed them to catalyze the reduction of H_2O_2 produced by drought stress, and protected them from oxidative damage.

Key words: Pinus taeda L; somatic embryogenesis; desiccation tolerance; peroxidase activity.

Introduction

Desiccation (partial drying) can improve the germination and plant regeneration response of conifer somatic embryos (Attree and Fowke, 1993). The successful induction of desiccation tolerance in somatic embryos has been reported for some angiosperm and conifer species (Bewley, 1979; Seneratna et al., 1990; Iida et al., 1992; Attree and Fowke, 1993; Brown et al., 1993; Florin et al., 1993). Partial drying has been described for Sitka spruce (Robertson et al., 1991), red spruce (Harry and Thorpe, 1991), black spruce (Tremblay and Tremblay, 1991), white spruce (Kong and Yeung, 1992), and Norway spruce (Bozhkov et al., 1992). Robertson et al. (1990) first observed that partial drying of interior spruce somatic embryos at high relative humidity (RH > 95%) increased germination frequency, decreased germination times, and improved the synchrony of root and shoot elongation compared to untreated somatic embryos. Of the various chemical and physical treatments that have been applied to induce desiccation tolerance, abscisic acid (ABA) and polyethylene glycol (PEG) have been frequently employed (Zeevart and Creelman, 1988; Attree and Fowke, 1993; Florin et al., 1993). ABA and PEG influence the accumulation of specific mRNAs, storage reserves, and storage lipid triacylglycerol (TAG) in conifer somatic embryos and zygotic embryos (Attree et al., 1992), some of which may be involved in seed protection. In some plant species, late embryogenesis abundant (LEA) proteins, whose accumulation increase dramatically at the beginning of zygotic embryo desiccation and which are regulated by ABA, are considered to be involved in protecting tissue from desiccation (Dure, 1993). Leal and Misra (1993) reported that late developmental stage white spruce zygotic embryos accumulated high levels of LEA transcripts. However, little is known about the precise mechanism by which ABA and PEG induce desiccation tolerance in conifer somatic embryos. Peroxidase catalyzes the reduction of H_2O_2 and protects embryos from oxidative damage (Creissen et al., 1994; Wakui et al., 1999). There is no report about peroxidase activity of desiccation-tolerant somatic embryos in conifers. In this investigation, I report the first evidence of increased peroxidase activity in desiccation-tolerant somatic embryos of loblolly pine.

Materials and Methods

Plant material. Loblolly pine seeds provided by Yingde Seed Orchard (Yingde, Guangdong Province, China) were disinfected by immersion in 70% v/v ethanol for 30 s and in 0.1% mercuric chloride for 20 min, followed by four or five rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the seeds and placed horizontally on a solidified callus induction medium in 100 ml Erlenmeyer flasks (40 ml medium in 100 ml flask, five to eight explants per flask).

Callus and somatic embryo induction. Callus and somatic embryo induction were carried out as described previously by using TE medium (Tang et al., 1998) as basic medium. Briefly, the procedure of somatic embryo production and associated media are: (1) Culture initiation: TE + 36.2 μ*M* 2,4-dichlorophenoxyacetic acid (2, 4-D) + 17.8 μ*M* 6-benzylaminopurine (BA) + 18.6 μ*M* kinetin (KT) (9 wk). (2) Callus proliferation: TE + 7.2 μ*M* 2, 4-D + 3.6 μ*M* BA + 3.7 μ*M* KT (9 wk). (3) Somatic embryo development: TE + 9.0 μ*M* 2, 4-D + 2.2 μ*M* BA + 2.5 μ*M* indole-butyric acid (IBA) + 2.3 μ*M* KT (6 wk). (4) Somatic embryo maturation: TE + 15.1 μ*M* ABA + 75 $g l^{-1}$ PEG_{6000} + 5 $g l^{-1}$ activated charcoal (9 wk). Cultures and embryos were subcultured every 3 wk. Then, embryos (derived from one embryogenic culture line) were used for the desiccation-tolerance treatments.

*Author to whom correspondence should be addressed (present address): Forest Biotechnology Group, Department of Forestry, North Carolina State University, Raleigh, NC 27695-7247, USA, Email wei_tang@ncsu.edu

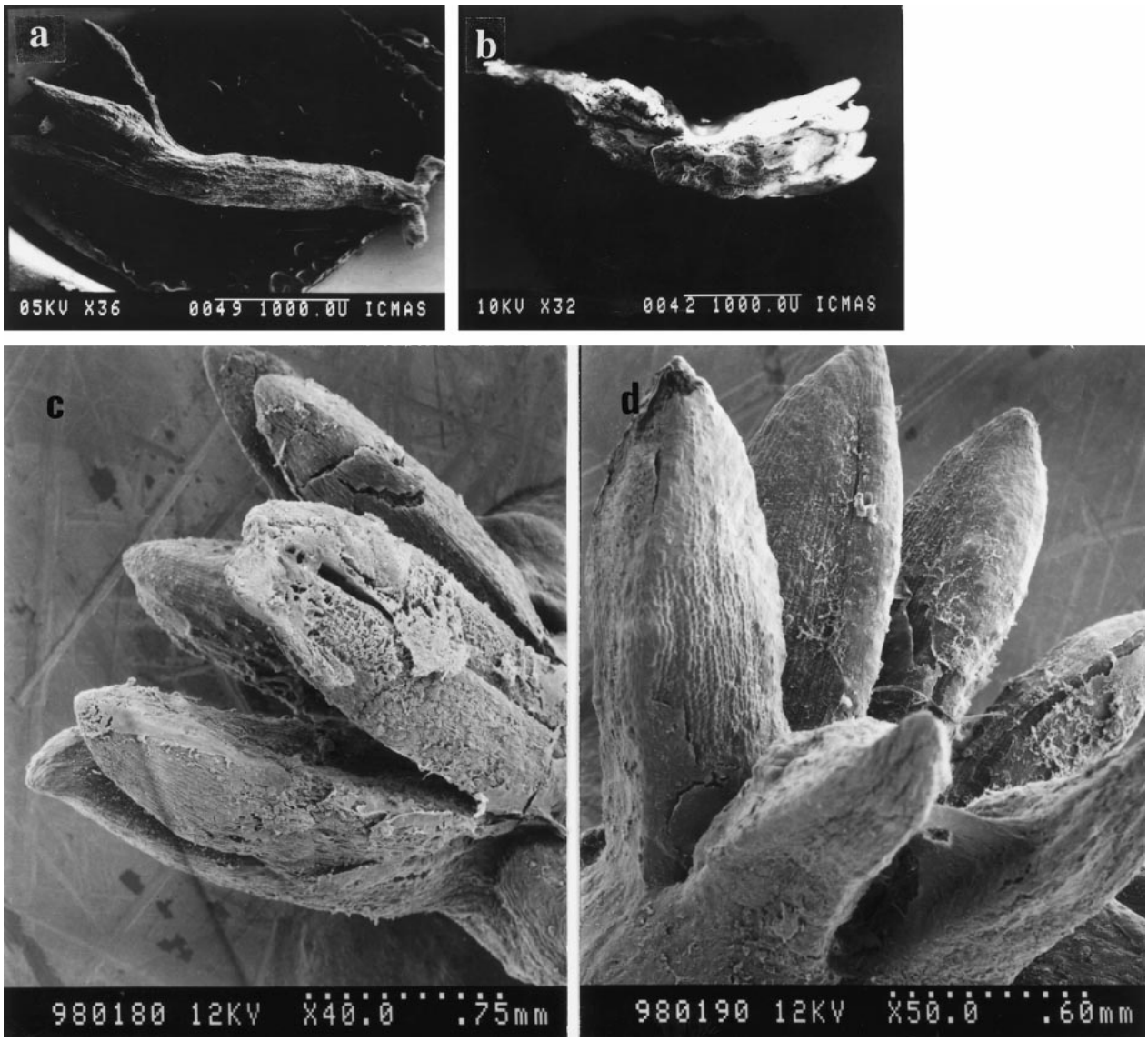

FIG. 1. Scanning electron microscopy of desiccation-tolerant embryos and desiccation-sensitive embryos in loblolly pine. (a), The surface of desiccation-tolerant embryos was covered with a mesh-like structure due to wrinkling of the cells. (b), The surface of desiccation-sensitive embryos appeared to be torn and less wrinkled. (c), Desiccation-sensitive somatic embryo cotyledons with an irregular cell surface. d, Desiccation-tolerant somatic embryo cotyledons with regularly arranged epidermal cells.

ABA and PEG treatment, desiccation, and rehydration. After embryos matured on TE medium (Tang et al., 1998) with 15.1 μ*M* ABA, 75 g l^{-1} PEG_{6000}, and 5 g l^{-1} activated charcoal, mature somatic embryos were randomly selected and treated with higher concentrations of ABA and PEG_{6000} (compared to that used in somatic embryo maturation) to induce desiccation tolerance. The culture medium was replaced by TE supplemented with: (1) 50 μ*M* ABA; (2) 8.5% PEG_{6000}; or (3) 50 μ*M* ABA + 8.5% PEG_{6000}. As a control, medium without ABA or PEG_{6000} was used. After 7 d of incubation in darkness at 25°C, cotyledon-stage embryos were selected by sieving through a 200 μm nylon mesh and then washed with sterile deionized water. Embryos were transferred to sterile filter paper, which was moistened with a few drops of sterile deionized water, in a 60 × 15 mm plastic Petri dish. Somatic embryo desiccation was performed according to the method of Takahata et al. (1993). Embryos in a Petri dish were dried through a series of desiccators in which the relative humidity (RH) was kept constant using a saturated solution of K_2SO_4 (RH 87%), Na_2CO_3 (RH 80%), NaCl (RH 70%), NH_4NO_3 (RH 61%), $Ca(NO_3)_2{\cdot}4H_2O$ (RH 50%) or $K_2CO_3{\cdot}1.5H_2O$ (RH 40%). They were transferred each day from a desiccator at a higher RH to one at a lower RH. After the desiccation treatment, the filter paper bearing the embryos was transferred to TE agar (0.7%)-solidified medium containing 2% sucrose and incubated at 25°C under a 16-h photoperiod with light provided by cool white fluorescent bulbs (75 μmol m^{-2} s^{-1}). The germination frequency of embryos was determined after desiccation-tolerant somatic embryos were transferred to plant growth regulator-free TE medium (Tang et al., 1998) for 1 wk. Each treatment was replicated three times, and each replicate consisted of 30–50 somatic embryos.

Scanning electron microscopy. Somatic embryos were prepared for scanning electron microscopy according to Fowke et al. (1994) with some modification. Tissues were fixed overnight in 4% glutaraldehyde and 100 m*M* phosphate buffer (pH 7.0), washed one time in 100 m*M* phosphate buffer (pH 7.0) for 30 min, followed by dehydration in successive ethanol

solutions of 85, 95, and 100%, each repeated twice for 5 min. Specimens were dried in a critical-point-drier with CO_2 for 2 h, mounted on Cu stubs and gold-coated. The samples were examined and photographed using a Hitachi S-800 scanning electron microscope.

Peroxidase activity. The enzyme activity was assayed according to the procedure reported by Kim and Yoo (1996). Briefly, 0.1 g FW somatic embryos (30–50 somatic embryos) was homogenized in 1 ml phosphate buffer (pH 6.0, 0.1 *M*) for 5 min. Homogenates were then centrifuged at 12,000 rpm for 5 min at 4°C and the supernatant was used for peroxidase activity assay. Peroxidase activity was determined at 30°C with a spectrophotometer (470 nm) following the formation of tetraguaiacol in a 3 ml reaction mixture containing 1 ml of 0.1 *M* phosphate buffer (pH 6.0), 1 ml of 15 m*M* 2-methoxyphenol (guaiacol), 1 ml of 3 m*M* H_2O_2, and 5 μl of enzyme extract. One unit of peroxidase activity (U) represented the amount of enzyme catalyzing the oxidation of 1 mol of guaiacol in 1 min. Data were analyzed by an Analysis of Variance, and mean comparisons were made with the Least Significant Difference test at 5% level of probability.

Results and Discussion

Scanning electron microscopy of desiccation-tolerant somatic embryos. This investigation showed that no visual differences in morphological appearance were observed between the somatic embryos treated and untreated with ABA and PEG_{6000}, except that the addition of ABA and PEG_{6000} reduced embryo growth slightly. Scanning electron microscopy observation of non-desiccated embryos revealed that their epidermal cells were arranged regularly and that their cell surfaces were smooth, similar to the appearance reported by Nitta et al. (1997) and Wakui et al. (1999). Scanning electron microscopy of desiccated somatic embryos revealed that the external morphology of the desiccation-tolerant and -sensitive embryos differed. The surface of the desiccation-tolerant embryos was covered with a mesh-like structure due to wrinkling of the cells (Fig. 1a), whereas the surface of desiccation-sensitive embryos appeared to be torn and less wrinkled than those of the desiccation-tolerant embryos (Fig. 1b). A similar morphology was reported in desiccation-tolerant somatic embryos of white spruce (Fowke et al., 1994) and *Brassica napus* L. (Wakui et al., 1999). After transfer to TE germination medium (Tang et al., 1998), desiccation-sensitive embryos did not show recovery of their morphology to that before desiccation, and remained shriveled with an irregular cell surface after 7–10 d of rehydration (Fig. 1c). Desiccation-tolerant somatic embryos imbibed water rapidly and within 24–36 h had regained the same size and appearance as they had before desiccation. Their epidermal cells appeared turgid and were arranged regularly (Fig. 1d). Most of these somatic embryos developed roots within 7–10 d.

Peroxidase activity of desiccation-tolerant somatic embryos. My results indicate that desiccation-tolerant somatic embryos induced by ABA and/or PEG_{6000} have higher peroxidase activity, improved tissue organization after desiccation, and improved survival. H_2O_2 levels increase in plant cells after exposure to many environmental stresses such as radiation, drought, wounding, extreme temperature, and pathogen attack (Baker and Orlandi, 1995; Yahraus et al., 1995; Gadea et al., 1999). Prolonged accumulation of toxic amounts of H_2O_2 within the cell is eventually lethal. To avoid these deleterious effects, all organisms express peroxidase, which functions as a detoxifying enzyme by catalyzing the reduction of H_2O_2 (Creissen et al., 1994). Peroxidase activity of desiccation-tolerant somatic embryos increased sharply after 1 d of desiccation treatment at 87% RH (Table 1). Compared to controls, the germination frequency of desiccation-tolerant embryos was improved after desiccation treatment (Table 2). It has been shown that ABA affects the stabilization of the cell membrane system (Pustovoitova, 1987). This investigation suggests that ABA and/or PEG_{6000} also probably promoted the production of peroxidase in desiccation-tolerant somatic embryos, which catalyzed the reduction of H_2O_2, and protected the embryos from oxidative damage.

TABLE 1

PEROXIDASE ACTIVITY OF DESICCATION-TOLERANT MATURE SOMATIC EMBRYOS OF LOBLOLLY PINE UNDER DIFFERENT RELATIVE HUMIDITIES (87% TO 40%)

Treatment	Peroxidase activity (U g^{-1} FW)*					
	87%	80%	70%	61%	50%	40%
ABA	24.3 b	25.9 b	30.5 b	42.1 b	49.7 b	50.6 b
PEG_{6000}	21.5 b	23.1 b	27.2 b	38.5 b	45.2 b	47.3 b
ABA + PEG_{6000}	31.7 a	33.8 a	36.2 a	47.9 a	59.8 a	61.2 a
Control	11.8 c	12.7 c	13.5 c	15.6 c	16.2 c	16.9 c

* Peroxidase activity of somatic embryos was determined after 5–8 h of desiccation treatment. Each treatment was replicated three times, and each replicate consisted of 30–50 somatic embryos (0.1 g FW). Values represent the means. Means followed by the same letter within columns are not significantly different at the 0.05 level of confidence.

TABLE 2

THE GERMINATION FREQUENCY (%) OF DESICCATION-TOLERANT MATURE SOMATIC EMBRYOS OF LOBLOLLY PINE UNDER DIFFERENT RELATIVE HUMIDITIES (87% TO 40%)

Treatment	Germination frequency (%)*					
	87%	80%	70%	61%	50%	40%
ABA	72.3 b	74.9 b	79.5 b	82.1 b	87.7 b	90.6 b
PEG_{6000}	71.5 b	73.1 b	77.2 b	81.5 b	85.2 b	87.3 b
ABA + PEG_{6000}	75.3 a	78.3 a	83.2 a	89.9 a	91.8 a	96.2 a
Control	67.8 c	68.7 c	70.5 c	71.8 c	71.9 c	72.9 c

* The germination frequency of somatic embryos was determined after one week of desiccation treatment. Each treatment was replicated three times, and each replicate consisted of 30–50 somatic embryos. Values represent the means. Means followed by the same letter within columns are not significantly different at the 0.05 level of confidence.

Acknowledgments

This research was supported by China Postdoctoral Science Foundation (97-11). I wish to acknowledge Professor Ron Sederoff for his critical reading and useful suggestion and Professor Fan Ouyang for his guidance and encouragement.

References

Attree, S.M.; Fowke, L.C. Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tiss. Organ Cult. 35:1–35; 1993.

Attree, S.M.; Pomeroy, M.K.; Fowke, L.C. Manipulation of conditions for improved triacylglycerol biosynthesis and desiccation tolerance. Planta 187:395–404; 1992.

Baker, C.J.; Orlandi, E.W. Active oxygen species in plant pathogenesis. Annu. Rev. Phytopathol. 33:299–321; 1995.

Bewley, J.D. Physiological aspects of desiccation tolerance. Annu. Rev. Plant Physiol. 30:195–238; 1979.

Bozhkov, P.V.; Ledebenka, L.A.; Shiryaeva, G.A. A pronounced synergistic effect of abscisic acid and 6-benzyladenine on Norway spruce (*Picea abies* L.) somatic embryo maturation. Plant Cell Rep. 11:386–389; 1992.

Brown, D.C.W.; Watson, E.M.; Pechan, P.M. Induction of desiccation tolerance in microspore-derived embryos of *Brassica napus*. In Vitro Cell Dev. Biol. Plant 29:113–118; 1993.

Creissen, C.P.; Edwards, E.A.; Mullineaux, P.M. Glutathione reductase and ascorbate peroxidase. In: Foyer, C.H.; Mullineaux, P.M., eds. Cause of photoxidative stress and amelioration of defense systems in plants. Boca Raton: CRC Press; 1994:344–364.

Dure, L. Structural motifs in Lea proteins. In: Close, T.J.; Bray, E.A., eds. Plant responses to cellular dehydration during environmental stress. Rockville: The American Society of Plant Physiologists; 1993:91–103.

Florin, B.; Tessereau, H.; Leocouteux, C.; Dicher, C.; Petiard, V. Long-term preservation of somatic embryos. In: Redenbaugh, K., ed. Synseeds. Boca Raton: CRC Press; 1993:133–162.

Fowke, L.C.; Attree, S.M.; Rennie, P.J. Scanning electron microscopy of hydrated and desiccated mature somatic embryos and zygotic embryos of white spruce (*Picea glauca* (Moench) Voss). Plant Cell Rep. 13:612–618; 1994.

Gadea, J.; Conejero, V.; Vera, P. Developmental regulation of a cytosolic ascorbate peroxidase gene from tomato plants. Mol. Gen. Genet. 262:212–219; 1999.

Harry, I.S.; Thorpe, T.A. Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. Bot. Gaz. 152:446–452; 1991.

Iida, Y.; Watabe, K.; Kamada, H.; Harada, H. Effect of abscisic acid on the induction of desiccation tolerance in carrot somatic embryos. J. Plant Physiol. 140:356–360; 1992.

Kim, Y.H.; Yoo, Y.J. Peroxidase production from carrot hairy root cell culture. Enzyme Microb. Technol. 18:531–536; 1996.

Kong, L.; Yeung, E.C. Development of white spruce somatic embryos: II. Continual shoot meristem development during germination. In Vitro Cell Dev. Biol. Plant 28:125–131; 1992.

Leal, I.; Misra, I. Developmental gene expression in conifer embryogenesis and germination. III. Analysis of crystalloid protein mRNAs and desiccation protein mRNAs in the developing embryo and megagametophyte of white spruce (*Picea glauca* (Moench) Voss). Plant Sci. 88:25–37; 1993.

Nitta, T.; Takahata, Y.; Kaizuma, N. Scanning electron microscopy of microspore embryogenesis in *Brassica* spp. Plant Cell Rep. 16:406–410; 1997.

Pustovoitova, T. Effect of abscisic acid on betacyanin leakage from plant tissues. Biol. Plant 29:338–341; 1987.

Robertson, D.R.; Lazaroff, W.R.; Webster, F.B. Interaction between maturation and high relative humidity treatments and their effects on germination of Sitka spruce somatic embryos. J. Plant Physiol. 138:1–6; 1991.

Robertson, D.R.; Sutton, B.C.S.; Flinn, B.S. Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at high relative humidity. Can. J. Bot. 68:1086–1090; 1990.

Seneratna, T.; McKersie, B.D.; Bowley, S.R. Artificial seed of alfalfa (*Medicago sativa* L.). Induction of desiccation tolerance in somatic embryos. In Vitro Cell Dev. Biol. 26:85–90; 1990.

Takahata, Y.; Brown, D.C.W.; Keller, W.A.; Kaizuma, N. Dry artificial seed and desiccation tolerance induction in microspore-derived embryos of broccoli. Plant Cell Tiss. Organ Cult. 35:121–129; 1993.

Tang, W.; Ouyang, F.; Guo, Z.C. Studies on embryogenic callus induction and plant regeneration in loblolly pine. Sci. Silvae Scinicae 34:115–119; 1998.

Tremblay, L.; Tremblay, F.M. Carbohydrate requirement for the development of black spruce (*Picea mariana* (Mill)B.S.P.) and red spruce (*P. rubens* Sarg.) somatic embryos. Plant Cell Tiss. Organ Cult. 27:95–103; 1991.

Wakui, K.; Takahata, Y.; Kaizuma, N. Scanning electron microscopy of desiccation-tolerant and sensitive microspore-derived embryos of *Brassica napus* L. Plant Cell Rep. 18:595–600; 1999.

Yahraus, T.; Chandra, S.; Legendre, L.; Lowe, P. Evidence for a mechanically induced oxidative burst. Plant Physiol. 109:1259–1266; 1995.

Zeevart, J.A.D.; Creelman, R.A. Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39:439–473; 1988.