ACTA BIOLOGICA CRACOVIENSIA Series Botanica 45/1: 59–62, 2003

BIOCHEMISTRY OF ZYGOTIC AND SOMATIC EMBRYOGENESIS IN SILVER FIR (*ABIES ALBA* MILL.)

ANDREJ KORMUŤÁK*, TERÉZIA SALAJ, RADOSLAVA MATÚŠOVÁ, AND BOŽENA VOOKOVÁ

Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, P.O. Box 39A, SK-950 07 Nitra, Slovak Republic

Received July 22; revision accepted January 7, 2003

The paper reports a comparative study of storage protein synthesis and enzyme activity during zygotic and somatic embryogenesis of silver fir. The SDS-PAGE profiles of storage proteins in zygotic and somatic embryos were similar but not identical. Six storage protein fractions were detected in zygotic embryos, as compared with eleven fractions in somatic embryos. The principal storage protein of zygotic embryos was represented by the 43 kDa fraction, and in somatic embryos by the 53 kDa fraction. Peroxidase activity was lower in the precotyledonary and cotyledonary stages of somatic embryos than in the corresponding developmental stages of zygotic embryos. However, following desiccation, the mature somatic embryos possessed three times higher peroxidase activity than the mature zygotic embryos. The reverse was true of the specific activity of esterase, which was higher in zygotic embryos than in somatic embryos in all stages of development.

Key words: *Abies alba* Mill., silver fir, embryogenesis, storage proteins, enzymes.

INTRODUCTION

Molecular characterization of conifer embryogenesis can enhance our basic understanding of the developmental processes underlying the formation of somatic and zygotic embryos. It can also aid in the development of stage-specific molecular markers that can be used to optimize somatic embryogenesis protocols (Misra, 1994). Storage proteins were the first compounds used as markers in comparing the developmental programs of these two types of embryogenesis (Hakman et al., 1990; Hakman, 1993). The presence of homologous proteins in mature somatic embryos together with their triglyceride content was suggested to indicate embryo quality (Cyr et al., 1991). Still other data indicate the potential of some enzymes to function as stage-specific markers of somatic embryogenesis. Peroxidases and chitinases were shown to differ between developmental stages of *Picea abies* somatic embryogenesis (Egertsdotter, 1998). According to Bagnoli et al. (1998) the antioxidant enzymes superoxide dismutase and catalase could be convenient markers to define the developmental stages in *Aesculus hippocastanum* somatic and zygotic embryogenesis. The same role was also postulated for peroxidase and esterase, whose isoenzyme patterns were shown to reflect the embryogenic potential of *Medicago sativa* and *Dactylis glomerata* suspension cultures (Hrubcová et al., 1994). We used both the above enzyme systems in the present study, designed to compare the metabolic potentials of developing zygotic and somatic embryos of silver fir.

MATERIALS AND METHODS

Non-embryogenic and embryogenic calli of silver fir (*Abies alba* Mill.) were initiated from one immature embryo each. Somatic embryogenesis was induced using SH medium (Vooková et al., 1998).

*e-mail: nrgrkorm@savba.sk

PL ISSN 0001-5296

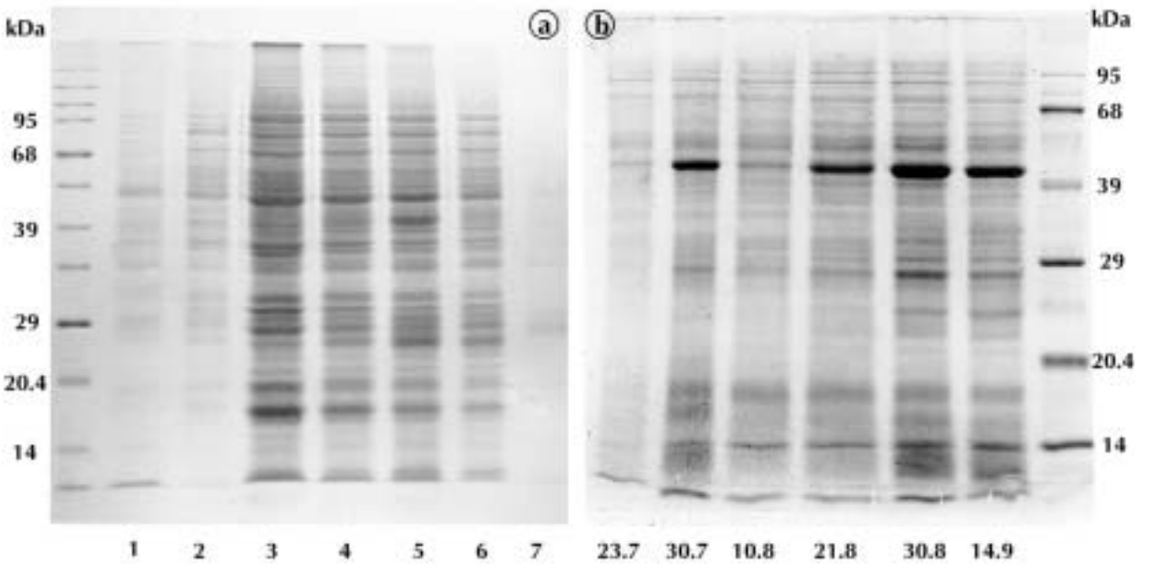

Fig. 1. SDS-PAGE profiles of storage proteins in somatic (**a**) and zygotic (**b**) embryos. Numbers 1 to 7 beneath the lanes in (a) represent developmental stages of somatic embryogenesis, while the numbers beneath the lanes in (b) refer to the days and months when developing zygotic embryos were analyzed. 1 – non-embryogenic callus; 2 – embryogenic callus; 3 – globular stage; 4 – torpedo-like stage; 5 – cotyledonary stage; 6 – desiccated mature embryos; 7 – regenerated seedlings.

Zygotic embryogenesis of silver fir was followed in a natural stand of the species in Jedľové Kostolany, west Slovakia, during July–September 2001. Following collection of cones from open pollination, the developing embryos were excised from the female gametophyte and extracted in 1.5 ml 100 mM TRIS-HCl buffer, pH 8.3, containing 5 mM cysteine-HCl, 5 mM Na_2EDTA, 500 mM sucrose and 0.5% (w/v) polyvinylpyrrolidone. The weight of excised embryos ranged between 0.1 and 0.4 g. The crude homogenate was centrifuged for 15 min at 20,000 g and 4°C. The supernatant was dialyzed through a Sephadex G 25 coarse column and then used as a source of enzymes. Proteins were quantified according to Bradford's method (Bradford, 1976). Peroxidase activity was measured spectroscopically at 470 nm by the guaiacol-H_2O_2 method of Erdelský and Frič (1979). Esterase activity was measured at 405 nm using 100 mM phosphate buffer, pH 7.4, p-nitrophenylacetate substrate dissolved in ethanol (5 mg/ml) and enzyme solution. The specific activity of the enzymes was expressed as the change in optical density per mg of proteins. For SDS-electrophoresis the embryos were homogenized in 0.125 M TRIS-HCl extraction buffer, pH 6.8, containing 22.5% (v/v) mercaptoethanol, 22.5% (v/v) glycerol and 9% (w/v) SDS. The crude homogenate was heated for 3 min at 95°C, clarified by centrifugation at 15,000 g for 10 min and subjected to SDS-PAGE (Laemmli, 1970).

RESULTS

The composition and developmental pattern of storage proteins in somatic and zygotic embryos of silver fir are similar but not identical. At least six protein fractions of zygotic embryos with approximate molecular masses of 56, 43, 28, 24, 16 and 14 kDa are the major storage reserves in seeds of conifers (Fig. 1b). The first two fractions were detected in the early phase of embryogenesis, on July 23, when developing embryos were still in the precotyledonary stage. Synthesis of the rest of the major storage proteins began at the early cotyledonary stage at the end of July, reaching their maximum concentrations in morphologically differentiated embryos on August 30. The only exception in this respect was the 24 kDa protein fraction, appearing in embryos in the second half of August only. Physiological maturation of zygotic embryos seems to be accompanied by depletion of some storage proteins, as evidenced by the decreased con-

TABLE 1. Changes in peroxidase activity during zygotic and somatic embryogenesis of silver fir

Date	Zygotic embryogenesis		Somatic embryogenesis	
	Developmental stage	Specific activity	Developmental stage	Specific activity
July 30	Precotyledonary	0	Non-embryogenic callus	3.15 ± 0.050
Aug. 10	Early cotyledonary	0.25 ± 0.00	Embryogenic callus	0.93 ± 0.015
Aug. 21	Advanced cotyledonary	0.20 ± 0.00	Precotyledonary	0.11 ± 0.005
Aug. 30	Morphologically differentiated embryos	0.16 ± 0.00	Cotyledonary	0.11 ± 0.005
Sept. 14	Physiologically mature embryos	0.08 ± 0.00	Mature embryos	0.24 ± 0.005
			Regenerated seedlings	1.83 ± 0.080

TABLE 2. Changes in esterase activity during zygotic and somatic embryogenesis of silver fir

Date	Zygotic embryogenesis		Somatic embryogenesis	
	Developmental stage	Specific activity	Developmental stage	Specific activity
July 30	Precotyledonary	0.98 ± 0.140	Non-embryogenic callus	5.78 ± 0.310
Aug. 10	Early cotyledonary	2.29 ± 0.135	Embryogenic callus	1.74 ± 0.035
Aug. 21	Advanced cotyledonary	2.54 ± 0.150	Precotyledonary	1.25 ± 0.050
Aug. 30	Morphologically differentiated embryos	2.33 ± 0.076	Cotyledonary	1.23 ± 0.066
Sept. 14	Physiologically mature embryos	1.99 ± 0.120	Mature embryos	1.43 ± 0.090
			Regenerated seedlings	4.74 ± 0.240

centrations of 56, 43 and 28 kDa fractions between August 30 and September 9 (Fig. 1b).

Compared to zygotic embryos, the storage protein pattern of somatic embryos involved additional protein fractions of 36, 35, 32, 31 and 19 kDa size. In contrast to zygotic embryos, the 14 kDa fraction was rather faint in somatic embryos (Fig. 1a). The main difference between the two types of embryos, however, is related to the principal 43 kDa storage protein fraction in zygotic embryos and the 53 kDa fraction in their somatic counterparts. The 43 kDa protein fraction becomes distinct only at the cotyledonary stage of somatic embryos and disappears in desiccated mature embryos. Numerous protein fractions characteristic of mature somatic embryos may be detected in non-embryogenic callus already. Maximum synthesis of them was observed at either the globular or cotyledonary stages of somatic embryo development. A no less conspicuous feature of the storage protein pattern is the nearly complete depletion of storage proteins during germination of somatic embryos. As shown in Figure 1a, only a few faint fractions of storage proteins (53, 34 and 28 kDa) were detected in regenerated seedlings (lane 7).

Considerable differences between zygotic and somatic embryos of silver fir were also revealed at the enzyme level. The peroxidase activity of mature somatic embryos was triple the corresponding enzyme activity of dormant zygotic embryos (Tab. 1). Starting with the early cotyledonary stage, a decline in peroxidase activity was registered throughout zygotic embryogenesis, and the situation was similar during somatic embryogenesis. However, peroxidase activity changed abruptly during two stages of somatic embryogenesis. The first stage was the transition of non-embryogenic to embryogenic callus, accompanied by a conspicuous decline in specific enzyme activity. The second stage was that of regenerated seedlings, which had seven times higher peroxidase activity than mature somatic embryos.

Esterase activity in dormant zygotic embryos and mature somatic embryos was comparable, slightly higher in the former (Tab. 2). The activity of this enzyme could also be detected at the precotyledonary stage of zygotic embryos. As with peroxidase, the transition of non-embryogenic callus to embryogenic tissue was marked by a decrease in esterase activity. Similarly, regenerated seedlings had higher enzyme activity than mature somatic embryos.

DISCUSSION

Against the general assumption that soluble storage proteins are similar in zygotic and somatic embryos of conifers (Hakman et al., 1990; Hakman, 1993), our data indicate the presence of a higher number of storage proteins in somatic embryos of silver fir than

in their zygotic counterparts. Among six supernumerary fractions revealed in somatic embryos, the most conspicuous was the 53 kDa fraction, marking the divergent nature of somatic embryos. The 16 and 19 kDa fractions were detected in the embryogenic callus only; probably these fractions can be used to distinguish between non-embryogenic and embryogenic calli. The transition between these tissues represents the initial stage of somatic embryogenesis in conifers. Pâques et al. (1993) detected both non-embryogenic callus-specific and embryogenic callus-specific polypeptides to distinguish the two types of calli of *Picea abies*. In *Azadirachta indica* this transition is accompanied by changes in the isoperoxidase pattern (Preetha et al., 1995). Our data demonstrate the potential of peroxidase and esterase for use in tracking not only that transition but also the germination of *Abies alba* somatic embryos. The higher peroxidase activity in non-embryogenic callus is due to increased levels of phenolic substances in this tissue, some of which serve as substrates in peroxidase-catalyzed reactions (Hrubcová et al., 1994). Peroxidase and esterase activity during zygotic and somatic embryogenesis are relatively constant in both embryogenesis types, with a higher level of activity expressed in zygotic embryos. The opposite was observed at maturation, however. There was a pronounced decline in enzyme activity in zygotic embryos reaching dormancy, in contrast to the situation in mature somatic embryos, which exhibited increased activity of both enzymes.

ACKNOWLEDGEMENT

This study was financially supported by the VEGA grant agency, project no. 2/7250/20.

REFERENCES

BAGNOLI F, CAPUANA M, and RACCHI ML. 1998. Developmental changes of catalase and superoxide dismutase isoenzymes in zygotic and somatic embryos of horse chestnut. *Australian Journal of Plant Physiology* 25: 909–913.

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.

CYR DR, WEBSTER FB, and ROBERT DR. 1991. Biochemical events during germination and early growth of somatic embryos and seed of interior spruce (*Picea glauca engelmanni* complex). *Seed Science Research* 1: 91–97.

ERDELSKÝ K, and FRIČ F. 1979. *Practical and analytical methods in plant physiology*. SPN, Bratislava.

EGERTSDOTTER U. 1998. Somatic embryogenesis in forest trees: the regulation of somatic embryo development in conifers. *Development of integrated systems for large-scale propagation of elite plants using in vitro techniques. COST 822*, Report activities, 1996, European Commission 1998, 276–227. Luxembourg.

HAKMAN I. 1993: Embryology in Norway spruce (*Picea abies*). An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. *Physiologia Plantarum* 87: 148–159.

HAKMAN I, STABEL P, ENGSTRÖM P, and ERIKSSON T. 1990. Storage protein accumulation during zygotic and somatic embryo development in *Picea abies* (Norway spruce). *Physiologia Plantarum* 80: 441–445.

HRUBCOVÁ M, CVIKROVÁ M, and EDER J. 1994. Peroxidase activities and content of phenolic acids in embryogenic and non-embryogenic alfalfa cell suspension cultures. *Biologia Plantarum* (Praha) 39: 175–182.

LAEMMLI UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

MISRA S. 1994. Conifer zygotic embryogenesis, somatic embryogenesis, and seed germination: Biochemical and molecular advances. *Seed Science Research* 4: 357–384.

PÂQUES M, DU JARDIN P, HAND P, and HAKMAN I. 1993. Markers of somatic embryogenesis in conifers (*Picea abies* as model). *Biotechnology of Trees. Abstracts of 5th International Workshop of the IUFRO Working Party S2.04-07*, 18–22 October 1993. Balsain (Segovia), Spain.

PREETHA N, YASODHA R, and GURUMURTHI K. 1995. Peroxidase isoenzymes as markers of organogenesis in *Azadirachta indica* Juss. *Indian Journal of Plant Physiology* 38: 92–93.

VOOKOVÁ B, GAJDOŠOVÁ A, and KORMUŤÁK A. 1998. Somatic embryogenesis in hybrid firs: Induction, characteristics of ESM and development of somatic embryo. *Folia Oecologica* 24: 137–142.