Somatic Embryogenesis in Saffron *(Crocus sativus* L.): Morphological Differentiation and the Role of the Antioxidant Enzymatic System

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Abstract

The ontogenic developmental stages of saffron somatic embryogenesis have been studied and characterized using light and electron microscopy. The embryogenic callus underwent internal segmented divisions with the formation of globular embryos that were attached to the callus surface by a broad multicelular structure. Further development of the embryoids was characterized by the emergence of a shoot apical meristem and cotyledon (monopolar stage) and subsequent differentiation of a minicorm in the basal part of the embryo (dipolar stage). During the morphological differentiation changes in the antioxidant enzymatic system of the somatic embryos were detected with increased SOD and catalase activities during the initial stages of the process. The fact that MDHAR activity was markedly higher than DHAR could point towards a regeneration of ascorbic acid mostly dependent of NADH. The isoforms of SOD were studied being detected 2Mn-SODs and 4Cu,Zn-SODs. Although all the isoforms were expressed during the successive stages of somatic embryogensis an increase in the levels of Mn-SODs and a decrease in Cu,Zn-SODs during the last two stages was observed.

INTRODUCTION

Recently, a link between Reactive Oxygen Species (ROS), such as peroxide radicals, hydrogen peroxide and superoxide, and plant developmental physiology has been suggested (Van Breusegem et al., 2001). Regenerative pathways in plant tissue cultures, and among them somatic embryogenesis, comprised a series of developmental processes in which hydrogen peroxide has been shown to be involved (Earnshaw and Johnson, 1987; Kairong et al., 1999; Tian et al., 2003). It has been demonstrated that hydrogen peroxide combined to the ascorbate-glutathione cycle is involved in the maintenance of cell wall plasticity and the stimulation of organized cell division (De Gara et al., 1997) these two processes are required during the initial stages of somatic embryogenesis. In this context, changes in the isoforms of several antioxidant enzymatic systems have been considered as markers for the different stages of somatic embryogensis (Bagnoli et al., 1998).

The object of this work is to characterize the differentiation at early stages of somatic embryogenesis in saffron, coupled to the study of the antioxidant enzymatic system and its relation to the ascorbate-glutahatione cycle as a mean to improve the regenerative potential of somatic embryogenesis in saffron.

MATERIALS AND METHODS

Plant Material

Nodular embryogenic calli were developed from cultured meristematic tissue of saffron corms as described by Piqueras et al. (1999). Healthy resting corms were surface sterilized and dissected under aseptic conditions to obtain the initial explants composed of meristematic tissue (a rectangular section of the central meristematic region of each corm. The explants were incubated in the dark on solid MS (Murashige and Skoog 1962)

medium supplemented with 2,4 D (0.1 mg /ml) and BA (0.5 mg /ml). Under these conditions a nodular embryogenic calli could be observed after 5 weeks. The emergent calli have been subcultures each four weeks during the last six years remaining embryogenic so far.

Transmission Electron Microscopy

Samples were fixed, post fixed, dehydrated and embebed in Spurr's resin (Piqueras et al., 1994). Blocks were sectioned on a Reichter ultramicrotome. Semithin sections were stained with toluidine blue and observed with a Leica optical microscopy Thin sections for transmission electron microscopy were picked up on copper girds and stained with uranyl acetate followed by lead citrate. The ultrastructure of the tissues was observed with a Philips technical electron microscope.

Determination of Antioxidant Enzymatic Activities and Lipid Peroxidation

Catalase, superoxide dismutase and the ascorbate-glutathione cycle enzymes were measured as described by Hernández et al (2001). Superoxide dismutase isoforms were separated by PAGE and stained in situ using the technique of Beauchamp and Fridovich (1971) The extent of lipid peroxidation in the tissues was estimated by determining the concentration of substances reacting with thiobarbituric acid (Cakmak and Horst, 1991).

RESULTS AND DISCUSSION

The ultrastructural study of the embryogenic nodular calli revealed a structure composed of cell clumps of meristematic morphology located at the surface of the embryogenic callus or surrounded by parenchymatic tissue (Figure 1). The meristematic cells presented a dense cytoplasm and thickened cell walls situated at the periphery of the embryogenic callus and underwent internal segmented divisions (Figure 2). Continued divisions formed globular embryos that were attached to the callus surface by a broad multicelular structure (stage 1) (Figure 3). Further development of the embryoids was characterized by the emergence of a shoot apical meristem and cotyledon (monopolar stage 2) and subsequent differentiation of a minicorm in the basal part of the embryo (dipolar stage 3). Fully developed somatic embryos were separated from the embryogenic calli and showed a well-developed shoot meristem with leaves at one end and a small rooted corm at the opposite end (stage 4).

The asynchronous mode of development observed in the embryogenic callus of saffron has been previously described in different monocots during somatic embryogenesis (Wang et al., 1999; Fereol et al., 2002). In embryogenic calli of saffron developed in this work, the ontogeny of somatic embryos studied by optical and electron microscopy proceeded through various states of development, which agree with the sequence of embryogenic differentiation reported in several examples of monocots (Ho and Vasil, 1983; Fransz and Schell, 1990; Samaj et al. 2003).

The level of lipid peroxidation (biochemical indicator of oxidative stress) was measured for each stage of the developmental process, showing a positive correlation of this parameter with the progression of somatic embryogenesis and confirming the implication of oxidative stress in the process (Figure 4**).** As we have observed in the embryogenic callus of saffron, other authors have reported increased levels of lipid peroxidation during somatic embryogenesis (Adams et al., 1999) being related to embryogenic competence in carrot (Deighton et al., 1997). It is possible that the free radical mediated lipid products generated by plant tissue cultures cause both cytotoxic and cytoactive activities, conditioned by other factors such as developmental status and the level of activation of the antioxidant system.

The antioxidant enzymatic activities increased with the differentiation of the somatic embryos although a slight decrease was observed during the last stage of the process when the embryo was completely developed (Figures 5 and 6). In saffron embryogenic cultures a p-hydroxy mercury benzoic acid sensitive APX activity (class III peroxidase) has been detected. The fact that MDHAR activity was markedly higher than

DHAR could point towards a regeneration of ascorbic acid mostly dependent of NADH. The isoforms of SOD were studied and 2Mn-SODs and 4Cu,Zn-SODs were detected. Although all the isoforms were expressed during the successive stages of somatic embryogensis, an increase in the levels of Mn-SODs and a decrease in Cu,Zn-SODs during the last two stages was observed (Figure 7). As our results have shown, the ontogenic development of somatic embryos in saffron appears to be regulated by some components of the antioxidant enzymatic system and more specifically by SOD activity, which has been shown to express a characteristic quantitative pattern of expression for each embryogenic stage. Similar changes in the expression of SODs isoforms have been reported by Bagnoli et al. (1998) in somatic embryos of horse chesnut.

In agreement with our results, it has been reported (Kairong et al., 1999; Belmonte et al., 2003) that the onset and differentiation of somatic embryogenesis is modulated among other factors by the redox balance of the embryogenic tissues. To some extend a certain level of oxidative stress is required to promote the formation of embryogenic cells and to trigger its specific morphogenic pathway (Earnshaw and Johnson, 1987; Benson et al., 1992; Kairong et al., 1999). In this context, hydrogen peroxide could function as a component of the complex signal transduction chain required to regulate somatic embryogenesis as well as other developmental processes in plant cells (Van Breusegem et al., 2001).

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Figures

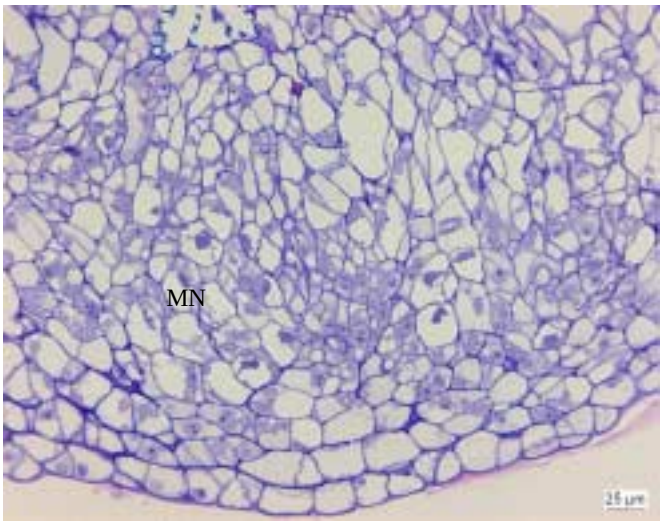

Fig. 1. (A) Histological section of nodular cormogenic calli showing meristematic nodules (MN) precursors of globular somatic embryos.

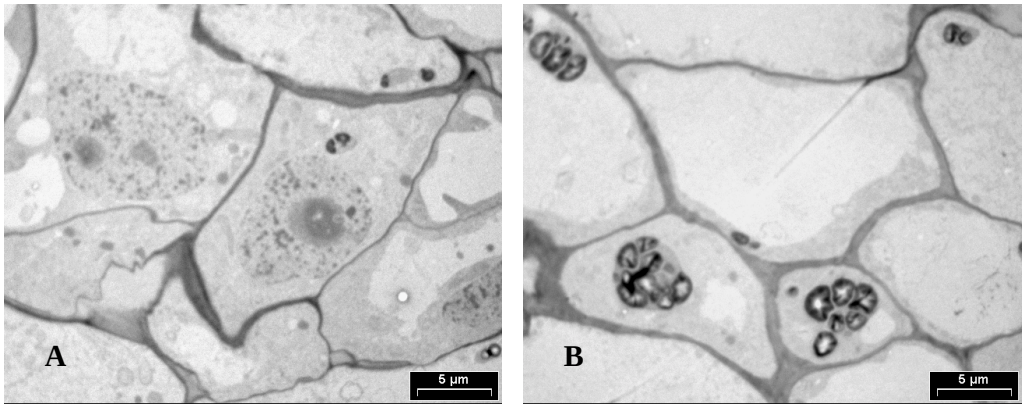

Fig. 2. (A) Meristematic cells from the central part of an embriogenic meristematic nodule.(B) Parenchimatic cells at the periphery of the embryogenic meristematic nodules.

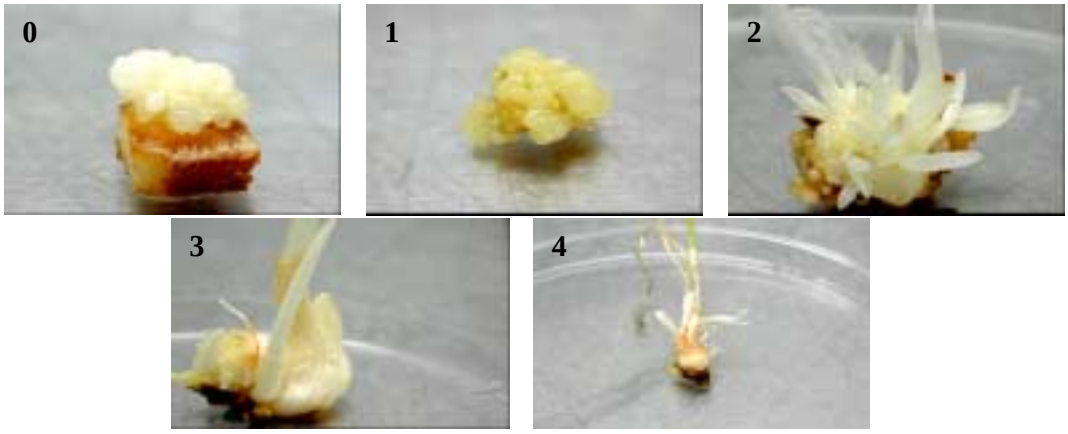

Fig. 3. Developmental stages during somatic embryogenesis in nodular calli of saffron. (0) initial callus formation from meristematic tissue, (1) nodular callus with globular embryos, (2) somatic embryos showing monopolarity, (3) somatic embryo showing bipolarity, (4) somatic embryo with leaves and a basal corm independent from the callus.

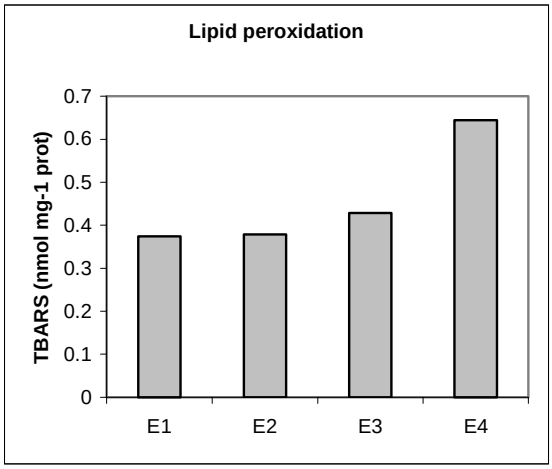

Fig. 4. Levels of lipid peroxidation measured in different stages of saffron somatic embryogenesis.

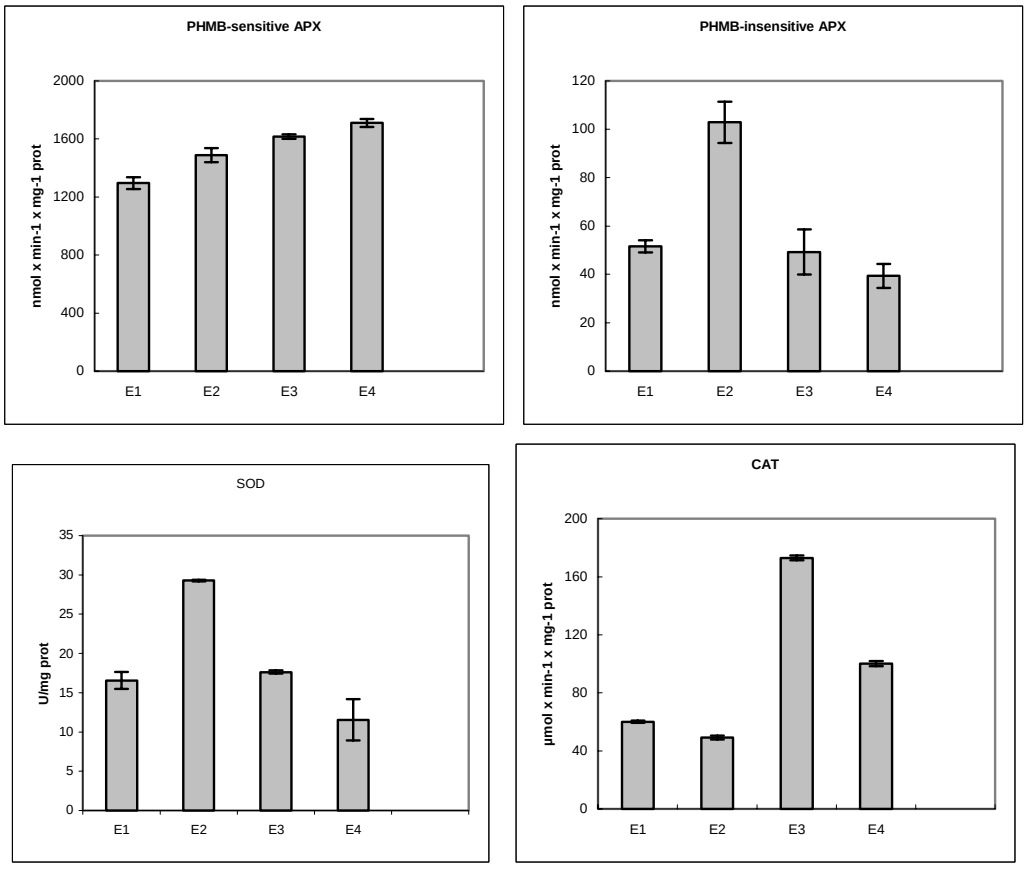

Fig. 5. PHMB-sensitive ascorbate peroxidase (APX), PHMB-insensitive APX , catalase (CAT) and superoxide dismutase (SOD) activities measured during different stages of saffron somatic embryogensis.

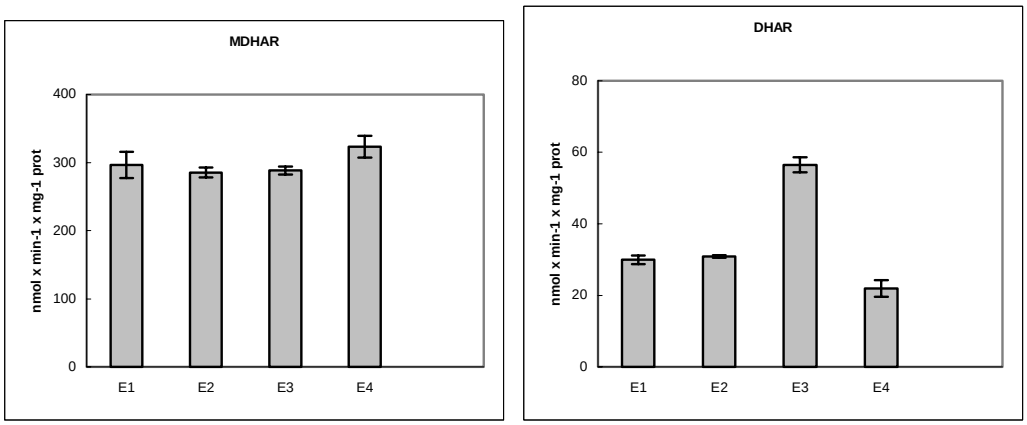

Fig. 6. Monodehydroascorbic acid reductase (MDHAR) and dehydroascobic acid reductase (DHAR) activities measured during the stages of saffron somatic embryogenesis.

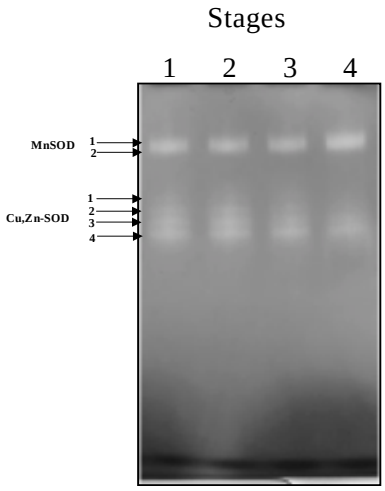

Fig. 7. SODs isoforms detected in the different stages of saffron somatic embryogenesis.

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