Journal of Plant Physiology 171 (2014) 78–84

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Involvement of peroxidase activity in developing somatic embryos of *Medicago arborea* L. Identification of an isozyme peroxidase as biochemical marker of somatic embryogenesis

Piedad Gallego[a], Luisa Martin[c], Antonio Blazquez[b], Hilario Guerra[a], Nieves Villalobos[a,*]

[a] Departamento de Fisiología Vegetal, Facultad de Biología, Universidad de Salamanca, Plaza de los Doctores de la Reina s/n, E-37007 Salamanca, Spain
[b] Departamento de Estadística, Facultad de Economía y Empresa, Universidad de Salamanca, Plaza de los Doctores de la Reina s/n, E-37007 Salamanca, Spain
[c] Departamento de Fisiología Vegetal, Facultades de Biología, Universidad Complutense de Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 6 July 2013
Received in revised form 8 September 2013
Accepted 24 September 2013
Available online 18 November 2013

Keywords:
Embryogenic markers
Isoenzymes
Medicago arborea L.
Peroxidase activity
Somatic embryogenesis

ABSTRACT

The legume *Medicago arborea* L. is very interesting as regards the regeneration of marginal arid soils. The problem is that it does not have a good germinative yield. It was therefore decided to regenerate *via* somatic embryogenesis and find a marker of embryogenic potential. In this study, peroxidase activity was evaluated in non-embryogenic and embryogenic calli from *M. arborea* L. A decrease in soluble peroxidase activity is observed in its embryonic calli at the time at which the somatic embryos begin to appear. This activity is always lower in embryonic calli than in non-embryonic ones (unlike what happens in the case of wall-bound peroxidases). These results suggest that peroxidases can be considered to be enzymes involved in somatic embryogenesis in *M. arborea*. In addition, isozyme analyses were carried out on protein extracts using polyacrylamide gel electrophoresis. The band called P5 was detected only in embryogenic cultures at very early stages of development. This band was digested with trypsin and analyzed using linear ion trap (LTQ) mass spectrometer. In P5 isoform a peroxidase-L-ascorbate peroxidase was identified. It can be used as a marker that allows the identification of embryological potential.

Introduction

One of the main environmental problems is the global increase in marginal soils, which are easily eroded owing to the lack of plant cover. The use of leguminous species would be a good solution (Rejili et al., 2007) for improving soils that show alterations since in arid and semiarid zones nitrogen is a limiting factor and part of that nitrogen could come from the *Rhizobium*–Legume symbiosis (Zahran, 1999). Within the legume family, most commercially important annual seeds – soya, beans, peas, chick-peas *etc.* – are from species that show a decrease in production when grown on arid or altered soils (Zahran, 1999). The characteristics of the legume *Medicago arborea* L., its capacity to adapt to extreme conditions and its lack of a dormancy period means that this species is very interesting as regards the regeneration of marginal arid soils. Additionally, it could offer an alternative to soya with respect to the production of biomass and grain protein in arid zones, where it could be cultivated and used as forage under conditions that would not be tolerated by other species. The problem of using this species is that it does not undergo a synchronized germination under natural conditions and neither does it have a good germinative yield. It was therefore decided to study its behavior when cultured *in vitro*.

From the physiological point of view, *M. arborea* has been little studied and there are only sporadic references in the literature to its behavior in *in vitro* cultures. The regeneration of this species *via* somatic embryogenesis was described by Gallego et al. (2001), who used cotyledons, petioles, hypocotyls and leaves as explants. These authors showed that in general the embryonic calli were yellowish-white in color, friable and slow-growing, whereas non-embryogenic calli were green, hard and faster-growing.

The morphological characteristics of calli give an idea of their embryogenic potential, but this visual appreciation is subjective and can often only be applied after prolonged culture times. The use

Abbreviations: BA, benzyladenine; IEF, Isoelectric focusing; KIN, kinetin; LTQ, linear ion trap; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; 4-MN, 4-metoxy-α-naphtol; MSMurashige and Skoog (1962), médium; MSI, MS medium supplemented with 2 mg l^{-1} of 2,4-D; MSI + BA, MS medium supplemented with 9 μM 2,4-D and 9 μM benzyladenine; MSI + KIN, MS medium supplemented with 9 μM 2,4-D and 9 μM kinetin; MS/MS, tandem mass spectrometry; MSI + TDZ, MS medium supplemented with 9 μM 2,4-D and 1 μM thidiazuron; MSII, MS medium supplemented with 2.25 mM 2,4-D without cytokinins; TDZ, thidiazuron.

* Corresponding author at: Departamento de Fisiología Vegetal, Facultad de Biología, Universidad de Salamanca, Plaza de los Doctores de la Reina s/n, E-37007 Salamanca, Spain. Tel.: +34 923294471; fax: +34 923294682.
E-mail address: crta@usal.es (N. Villalobos).

0176-1617/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.jplph.2013.09.017

of biochemical or molecular markers that are able to show up the embryogenic potential of such calli would offer a good tool to carry out a follow-up of the embryogenic status of calli (Tchorbadjieva and Odjakova, 2001; Nieves et al., 2003; Balen et al., 2009).

These biochemical or molecular markers of embryogenic potential have been identified in several plant species (Wilde et al., 1988; Feirer and Simon, 1991), but they are not sufficiently precise or rapid to offer a real advance in the detection of embryogenesis. Because the developmental program of cells involves the appearance or disappearance of specific proteins at specific times, an alternative for a simple, rapid identification of embryogenic calli could be the analysis of enzyme and isoenzyme activities.

The development of specific markers could provide a huge advance in the early determination of the presence of somatic embryos, thereby enabling their formation and use to be optimized.

In the literature it has been reported (Thibaud-Nissen et al., 2003; Belmonte et al., 2005) that, among other factors, the initiation and differentiation of somatic embryogenesis is regulated by the redox balance of embryonic tissues. For some authors, increasing a certain level of oxidizing stress is necessary for promoting the formation of embryogenic cells (Kairong et al., 1999; Kairong et al., 2002; Imin et al., 2005; Pasternak et al., 2005). In this context, changes in antioxidant enzyme systems could act as the signal required for the acquisition of embryonic competence, as proposed by Blázquez et al. (2009) for saffron. Additionally, an increased number of publications have linked reactive oxygen species (ROS) to somatic embryogenesis (Zavattieri et al., 2010; Rodríguez-Serrano et al., 2012; Gómez-Garay et al., 2013).

In light of the publications consulted, we consider that peroxidase isoenzyme patterns are appropriate for detecting somatic embryogenesis (Krsnik-Rasol, 1991; Bouazza et al., 1993). Accordingly, here we analyzed the peroxidase activity of different calli from *M. arborea*, the differences that take place when embryogenesis occurs, and the possibility of their use as markers.

Materials and methods

Plant material

We used seedlings of *Medicago arborea* L. that were in a developmental stage in which the first leaf with three folioles had already formed to obtain explants. Leaves, cotyledons, hypocotyls and petioles cut into 5-cm segments were used as the explants.

Culture was carried out over two months in Murashige/Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 2 mg l^{-1} of 2,4-D (MSI), MS medium supplemented with 9 μM 2,4-D and 9 μM kinetin (MSI + KIN), MS medium supplemented with 9 μM 2,4-D and 9 μM benzyladenine (MSI + BA) and MS medium supplemented with 9 μM 2,4-D and 1 μM thidiazuron (MSI + TDZ). After two months (subculturing in the same medium and the same conditions every four weeks) half of the explants were left under the same conditions whereas the other half were transferred to MS supplemented with 2.25 mM 2,4-D without cytokinins (MSII medium).

Fifty explants were used per treatment and each experiment was carried out in triplicate.

Data were acquired monthly over 5 months after the start of the incubation of the explants in MSI medium, and then each month after the calli had been transferred to MSII medium.

Protein extraction

For the protein extraction the method of Hrubcová et al. (1994) with some modifications was followed.

Samples of approximately 2.5 g of the different types of calli studied were homogenized in a mortar at 4 °C with a 50 mM phosphate extractant buffer (pH7.2) at a proportion of 1:2 (w:v) and 0.1 g of polyvinylpyrrolidone (PVP) per ml of buffer. The homogenates were centrifuged for 20 min at 18,000× *g* at 4 °C. The supernatants were collected and used for the determination of soluble proteins.

The precipitate was homogenized in the same amount of 1 M NaCl as that used previously in the buffer and was then centrifuged following the same procedure as above. The supernatant was collected and used to determine wall-bound protein.

Protein quantification

The protein content of the supernatants was determined according to Bradford (1976), measuring optical density at 595 nm with bovine serum albumin as standard.

Enzyme assays

Peroxidase activity was determined spectrophotometrically using guaiacol as substrate (Lobarzewski et al., 1990), measuring the increase in absorbance at 470 nm. The assay mixture was prepared according to the modified method of Hrubcová et al. (1994) and consisted of 3 ml of 60 mM phosphate buffer (pH 5.5), 50 μl of 20 mM guaiacol, 60 μl of 12.3 mM H_2O_2 and 10 μl of enzyme extract.

Peroxidase activity was expressed in units of peroxidase/gram fresh weight. One unit of peroxidase was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol (ε_{470} = 26.6 mM cm^{-1}) in 1 min under optimal conditions (Bergmeyer et al., 1974).

Gel electrophoresis

The extracts were analyzed electrophoretically under native conditions, using vertical gels of 10% polyacrylamide in Laemmli (1970) buffer without SDS. The samples contained 10 μg/ml of protein, grains of sucrose and 0.5 μl of bromophenol blue from a 1% solution in the electrode buffer. The gel buffer contained 0.192 M glycine and 0.025 M Tris at pH 8.5.

Pre-electrophoresis was performed over 15 min at 20 mA, after which an electric current of 25 mA/gel was applied until the bromophenol front reached the end of the gel. To visualize the bands, the gels were incubated for 10 min in a solution containing 50 mM phosphate buffer (pH 5.6), 1% guaiacol and 0.4% H_2O_2.

Identification of peroxidases

The protein stains were cut manually with a blade and transformed to Eppendorf tubes containing 100 μl of bidistilled deionized water (Mili Q).

Proteins selected for analysis were in-gel reduced, alkylated and digested with trypsin according to Shevchenko (Shevchenko et al., 1996). Briefly, bands were washed twice with water, shrunk 15 min with 100% acetonitrile and dried in a Savant SpeedVac for 30 min. Then the samples were reduced with 10 mM dithioerytritol in 25 mM ammonium bicarbonate for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 15 min in the dark. Finally, samples were digested with 12.5 ng/ml sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. Finally, the samples were dried in a Savant SpeedVac, and dissolved in 0.2% formic acid just before analysis.

Peptides were analyzed using a nanoliquid chromatography–MS/MS on an linear ion trap (LTQ) mass spectrometer (Thermo