SHORT COMMUNICATION

Induction of peroxidases and superoxide dismutases in transformed embryogenic calli of alfalfa (*Medicago sativa* L.)

Jelena Platiša, Sonja Veljović-Jovanović, Biljana Kukavica, Branka Vinterhalter, Ann Smigocki, Slavica Ninković

*Center for Multidisciplinary Studies, University of Belgrade, Kneza Višeslava 1a, 11030 Belgrade, Serbia
Institute for Biological Research "S. Stanković", Despota Stefana 142, 11060 Belgrade, Serbia
USDA-ARS, Molecular Plant Pathology Laboratory, Beltsville, MD 20705, USA

Received 4 May 2007; received in revised form 6 July 2007; accepted 9 July 2007

**KEYWORDS**
Alfalfa; Embryogenic culture; Peroxidase; Superoxide dismutase; Transformation

**Summary**
Peroxidase (POD) and superoxide dismutase (SOD) enzyme activities were analyzed in non-regenerative transformed embryogenic lines of alfalfa (*Medicago sativa* L.) carrying wound-inducible oryzacystatin I (OC-I), wound-inducible oryzacystatin I antisense (OC-Ias), or hygromycin phosphotransferase (*hpt*) genes. All of the transformed lines analyzed had elevated levels of all POD isoforms. Three POD isoforms with pI values of approximately 4.5, 4.8, and 8.4, and one additional pair of isoforms with a pI value of approximately 8.8 were separated from tissue extracts of all transgenic lines. Isoelectrofocusing patterns revealed the induction of one isoform of SOD with a pI of about 5.6 in all transgenic lines compared with non-transformed embryogenic tissue. These results indicate that the process of transformation may disrupt redox homeostasis in alfalfa tissues.

**Introduction**

Development of new genetically modified plant varieties by means of transformation techniques often generates unintended effects not related to target traits (Filipecki and Malepszy, 2006). The occurrence of mutations and epigenetic changes in transgenic plant populations has been reported previously (Labra et al., 2001; Van der Vyver et al., 2003; Filipecki and Malepszy, 2006). It is also well known that changes in tissue cultures most likely occur by stress-response mechanisms related to tissue handling, regeneration, and clonal propagation (Phillips et al., 1994). If the transformation
process itself is assumed to be a stress, then it could be expected that it might also lead to a disturbance of redox homeostasis and changes in antioxidative metabolism. There are numerous published reports that have shown that varied suboptimal environmental conditions induce increased activity in antioxidative enzymes such as peroxidase (POD) and superoxide dismutase (SOD) (Imberty et al., 1985; Polle et al., 1994; Bestwick et al., 1998; Alscher et al., 2002). SOD (E.C. 1.15.1.1) catalyzes the dismutation of superoxide to hydrogen peroxide, which is then scavenged by PODs (E.C. 1.11.1.7) using a wide range of reducing substrates. PODs are encoded by a number of genes and are represented by numerous isoforms. Their physiological role is determined by sub-cellular localization and substrate availability (Heggie et al., 2005). In addition to their role in antioxidative defense, PODs are implicated in several physiological processes including senescence (Del Rio et al., 1998), cell growth and expansion (Schopfer et al., 2002; Kawano, 2003), development (Gazarian et al., 1998), reactive oxygen species generation (Schopfer et al., 2002), lignification (Goldberg et al., 1983), and suberization (Bernards et al., 1999).

To date, few articles have reported on changes in antioxidative enzymes due to the transformation process (Csiszár et al., 2005).

The aim of this article is to elucidate the transformation-induced changes in antioxidative metabolism in transformed embryogenic lines of alfalfa (*Medicago sativa* L.) carrying wound-inducible oryzacystatin I (OC-I) antisense (OC-I-as), or hygromycin phosphotransferase (hpt) genes. In this study, we determined activities and levels of two antioxidant enzymes, POD and SOD.

## Material and methods

**Plant material and culture conditions**

Initial embryogenic cultures of alfalfa (*M. sativa* L. cv. Zaječarska 83) were obtained from immature zygotic embryos (Ninković et al., 1998) and propagated by recurrent embryogenesis. This embryogenic culture was designated as an untransformed culture. Embryogenic cultures obtained after Agrobacterium tumefaciens transformation of single somatic embryos at the cotyledonal stage with the *OC-I* gene in either the sense or antisense orientation fused to a *pinII* promoter (Samac and Smigocki, 2003) were designated as OC-I sense and OC-I antisense lines. The green fluorescent protein reporter gene fused to a *CaMV35S* promoter and a selectable marker gene *nptII* fused to a nopaline synthase gene promoter were also expressed in these transfectants. Embryogenic culture transformed with the superbinary plasmid pToK233 that encodes for the *nptII*, *hpt* (hygromycin phosphotransferase), and *uidA* (GUS) genes (Ninković et al., 2004) was designated as an antibiotic resistant (AR) line. The transgenic nature of transformed embryogenic lines was confirmed by PCR amplification of *nptII* gene (data not shown). All cultures were maintained on MS medium (Murashige and Skoog, 1962) containing B5 vitamins and no hormones, under a 8/16-h photoperiod, irradiance of 47 μmol m⁻² s⁻¹, and temperature of 25 ± 2 °C.

## Isoform analysis

Tissue culture samples for POD and SOD analysis (0.5g) were collected after 2, 4, 6, and 8 weeks in culture and subsequently ground in liquid nitrogen. The tissue powder was transferred to an extraction buffer (100 mM K-phosphate buffer, pH 6.5, containing 1 mM phenylmethylsulfonyl fluoride and 1% (w/v) polyvinylpyrrolidone), mixed and allowed to thaw slowly at 4 °C. The homogenate was then centrifuged for 15 min at 10,000g at 4 °C. Soluble protein fraction was quantified by Bradford’s method (1976), which was modified for Micro Plate Reader LKB 5060-00 (GDV, Roma, Italy). Absorbance was measured at 620 nm and BSA was used as a standard.

To determine POD activity, native electrophoresis was performed on 5% stacking and 10% running gels, with a reservoir buffer consisting of 0.025 M Tris and 0.192 M Gly (pH 8.3), at 24 mA for 120 min. Isoelectric focusing (IEF) for POD and SOD analysis was carried out in a 7.5% polyacrylamide gel (PAGE) with 3% ampholite with pH gradient from 3 to 9. The gel was pre-focused for 30 min and then a constant voltage of 2000 V and initial current of 50 mA were applied. The amount of total protein applied to each well was 20 μg for native electrophoresis and 25 μg for IEF. After electrophoresis, the gels were stained for POD and SOD activity. PODs were visualized by immersing the gels in 10% 4-chloro-1-naphthol and 3% H₂O₂ in 100 mM sodium acetate buffer (pH 4.0) for 15 min in order to visualize the bands. In addition, POD activity was assayed in the crude extract (10 μL) in an incubation mixture containing 20 mM pyrogallol (A₄₅₀ = 2.47 mM⁻¹ cm⁻¹), 4 mM H₂O₂, 100 mM K-phosphate buffer (pH 6.5) at 30 °C. Enzyme activity was determined spectrophotometrically at 430 nm (Shimadzu UV-160, Kyoto, Japan). The quantification of total SOD activity was estimated from IEF gels by densitometric analysis using ImageMaster TotalLab version 1.11 software (Nonlinear Dynamics Ltd., Durham, USA). The values are given as a sum for each band density in the line on the gel.

## Results and discussion

POD and SOD enzyme activities were analyzed in non-regenerative transformed embryogenic lines of
alfalfa \((M. \text{ sativa} \ L.)\) carrying wound-inducible \(OC-I\), wound-inducible \(OC-Ias\), or \(hpt\) genes and compared with a regenerative untransformed control.

The analysis of POD activity by native PAGE showed the existence of three isoforms \((P \ 1, \ 2, \ \text{and} \ 3)\) in the wounded untransformed and all transformed lines (Figure 1). The POD level was increased in all transformed clones, and the highest intensity of bands was obtained in the \(OC-I\) antisense line. Wounding of the tissue that mimics an insect attack caused additional induction of POD activity in the untransformed and \(OC-I\) antisense line (Figure 1). Induction of POD activity often accompanies a disturbance of redox homeostasis and is used to indicate oxidative stress. The differences observed in plant tissue response to wounding may be explained by the effects of uncontrolled gene insertion and its position during the transformation process (Filipecki and Malepszy, 2006). Csiszar et al. (2005) also found that some transgenic wheat calli bearing the \(MsALR\) (alfalfa aldose/aldehyde reductase) gene showed elevated antioxidant enzyme activity (SOD, catalase, and/or POD) compared with the control.

To assess whether POD level in the embryogenic calli of alfalfa was affected by the developmental stage or availability of nutrients, we analyzed POD and SOD isoform patterns during 8 weeks of culture growth. Protein content, measured in newly developed tissue during the 2 months of biomass multiplication, showed an increasing trend in all transformed clones throughout the entire period of analysis (data not shown). However, throughout the entire period, a relatively constant level of specific POD activity in all transformed clones was determined by IEF (Figure 2). In the three transgenic lines, the two POD isoforms with a \(pI\) value of 8.8 (POD 1 and 2), and the similar pair of cationic isoforms with a \(pI\) approximately 8.6 (POD 3 and 4), also found in the control line, were separated. Preliminary results of cytometric flow analysis showed that all transformed lines were triploid, compared with the diploid control (Ninković, unpublished results). This could open a new approach in the investigation of these phenomena if these two results are shown to be related.

All transgenic lines had an additional isoform with a \(pI\) of about 8.4 (POD 5) (Figure 2). The untransformed line showed one anionic isoform with \(pI\) 4.5 (POD 11) in 2-week-old samples, which disappeared in the following weeks. The same isoform (POD 11) and an additional isoform with a \(pI\) of 4.8 (POD 10) were present throughout the entire period in all transgenic lines. These anionic POD isoforms (POD 10 and 11) were the most abundant in the \(OC-I\) antisense line. The AR line was the only line showing an isoform with a \(pI\) around 5.1 (POD 9). IEF analysis showed that the POD activity of the control line decreased in the 8th week. This effect was not observed in any of the transgenic lines. When total POD activity was measured and expressed in terms of fresh weight, the \(OC-I\) antisense clone showed values two times higher than all other clones (Table 1). Both the untransformed and \(OC-I\) lines showed a decrease in total POD activity starting with the 4th week, while a similar decrease was observed in the AR line in the 8th week. Results showed that the transformation of embryogenic tissue led to elevated activity in all POD isoforms throughout the entire growth period, as well as induction of new isoforms.

SOD catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen.

![Figure 1. Native PAGE of POD isoforms in OC-I, OC-I antisense, and AR (antibiotic resistant) embryogenic lines of alfalfa, before (C) and after wounding (W). Total protein (20 \(\mu\)g) was placed in each well. Arrows indicate different POD isoforms.](image-url)
Isoelectrofocusing patterns revealed the existence of a neutral isoform of SOD with a pI of 6.8 (SOD 1), which was the most abundant in the OC-I antisense line (Figure 3). Two isoforms with pIs of 5.1 (SOD 5) and 5.9 (SOD 2) were present in all lines, with one or two additional isoforms with pIs 5.8 (SOD 3) and 5.6 (SOD 4), respectively, present only in the transformed lines. The estimation of total SOD activity performed by densitometry analysis using Total Lab showed the highest activity in the OC-I antisense line in the 2nd week, and this was two times higher than all of the others (Table 2). The decrease of SOD activity in the 8th week, present in all of the lines, was the most pronounced in the OC-I antisense transgenic line.

Few studies have investigated the effects of constitutive OC-I, a cysteine proteinase inhibitor from rice, expression on whole plant physiology. The results have shown fluctuations in a broad range, from evidence that the presence of high levels of OC-I inhibitor does not interfere with normal development processes in tobacco plants (Masoud et al., 1993) to claims that the constitutive expression of the OC-I genes induces pleiotropic effects in transgenic tobacco plants (Gutierrez-Campos et al., 2001). One study showed that OC-I expression has an environment-dependent effect on the plant phenotype by enhancing the chilling tolerance (Van der Vyver et al., 2003).

Our results show elevated levels of all POD isoforms, increased levels of the neutral SOD isoforms with pI 6.8 (SOD 1), and induction of SOD isoforms with pI 5.6 (SOD 4) in all transformed clones. The results indicate that the transformation itself, not the protease inhibitor expression, disturbs the redox homeostasis during alfalfa somatic embryo transformation. The embryogenic clones of alfalfa (M. sativa L.) transformed with different constructs carrying OC-I, OC-I antisense, or hpt genes were not able to regenerate the whole plant compared with the untransformed line (Ninković, unpublished results). Elucidation of this phenomenon requires further analysis.

Unintended and heritable genomic changes detected in the transgenic plant population should be considered as a part of the plant program to induce controlled variability following the stress of the transformation procedure (Labra et al., 2001). Since most of the plant transformation, such as this in our study, is performed using A. tumefaciens, some of the effects on the plant genome and enzyme activities may be the result of an infection, as has been suggested by Filipecki and Malepszy.
Also, findings of the polyploidy in transformed lines (Ninković, unpublished results) indicate that the mutagenic stress response is due to a programmed loss of the cellular control. Our results on overexpression of POD/SOD may be used as an indicator of undesired consequences of the transformation process. Further study of the mechanisms underlying the induction of new isoforms of SOD and POD and total POD activity in the transformed lines of alfalfa would contribute to the understanding of the regulation of antioxidant metabolism. Using the POD profile changes as the indicator of undesired effects of the transformation process may improve transformation technique selection towards one that yields the lowest number of disruptions as well as increased regenerability.

### Acknowledgments

This research was supported by the Ministry of Science and Environmental Protection of Republic of Serbia (Projects no. 143020B and 143026B).

### References


