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CHARACTERIZATION OF ELECTROPHORETIC VARIANTS FOR Cu/ZnSOD IN SUNFLOWER: RESPONSE TO OXIDATIVE STRESS

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SUMMARY

Superoxide dismutases (SODs) and abscisic acid (ABA) are implicated in the response to environmental stresses. We have evaluated the susceptibility of *w-1*, a mutant with ABA deficiency and a variant isoform of chloroplastic Cu/ZnSOD, to oxidative stress. The results indicate that *w-1* leaves were less damaged by paraquat (MV) and H_2O_2 treatments in respect to the control, while no statistical differences were detected when water stress was imposed by leaf dehydration.

Spectrophotometric assays did not evidence a higher level of SOD activity in *w-1* extracts than in the control, confirming that ABA concentration does not modify SOD actvity. The better response of *w-1* leaves to MV treatment could be attributed to a higher efficiency of the chloroplastic isoform variant of SOD to induce the dismutation of superoxide anion radicals to hydrogen peroxide and oxygen, under oxidative stress.

Key words: ***Helianthus annuus* L., ABA-deficient mutant, electrophoretic isozyme variant, oxidative stress, superoxide dismutase**

INTRODUCTION

Environmental stresses (chilling, drought, salinity, air pollutants, *etc.*) act directly or indirectly through the formation of activated oxygen species. Plants have evolved different enzymatic and non-enzymatic mechanisms that can reduce oxidative stress by detoxifying harmful oxygen species (Scandalios, 1993). Superoxide dismutases (SODs; EC 1.15.1.1) are enzymes strongly implicated in the response to oxidative stress. SODs catalyze the disproportion of superoxide radical anions to hydrogen peroxide and molecular oxygen (Bowler *et al.*, 1992). These enzymes, that

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have been found in all aerobic organisms, cooperate with other enzymes involved in hydrogen peroxide-detoxification systems and play an essential role in protecting living cells against the indirect deleterious effects of superoxide free radicals (Scandalios, 1993; Bowler *et al.*, 1994). In plants, based upon metal ions present in the active centre, three forms of the enzyme exist: copper/zinc (Cu/ZnSOD), manganese (MnSOD) and iron (FeSOD), which are localized in different cellular compartments. The FeSOD is a dimeric enzyme present in prokaryotes and within the chloroplast of several plant species. The MnSOD appears to consist of identical subunits in either dimeric or tetrameric arrangement. The enzyme is widely distributed among prokaryotic and eukaryotic organism. In plants, MnSOD has been found in the mitochondrial matrix (Arron *et al.*, 1976; Jackson *et al.*, 1978; Baum and Scandalios, 1981; Scandalios, 1993; Bowler *et al.*, 1994) and in glyoxisomes and peroxisomes of some species (Sandalio and del R'o, 1987; Sandalio *et al.*, 1987; Corpas *et al.*, 1994). Plant Cu/ZnSOD is a dimeric enzyme, always present in the cytosol, consisting of two identical subunits. In addition to the cytosolic $Cu/ZnSOD_{cyt}$, a more anodic isoform is located in the chloroplast stroma (Asada *et al.*, 1973; Bowler *et al.*, 1994). Cu/ZnSOD activity has also been reported in the matrix spaces of mitochondria (Arron *et al.*, 1976; Jackson *et al.*, 1978; Sandalio *et al.*, 1987) and in glyoxysomes of watermelon, cotton, cucumber and sunflower (Sandalio and del R'o, 1987; Corpas *et al.*, 1994).

Recently an ABA-deficient mutant (*w-1*) of sunflower (*Helianthus annuus* L.), characterized by abnormal stomatal behaviour (Pugliesi *et al.*, 1994; Fambrini *et al.*, 1994) and several inbred lines and varieties were electrophoretically analyzed for SODs isozymes (Fambrini *et al.*, 1996). A banding pattern (zymogram) was identified in *w-1* in which chloroplastic $Cu/ZnSOD_{chl}$ has decreased mobility differing from all other tested genotypes (Fambrini *et al.*, 1996); the variant was coded by a nuclear gene with two codominant alleles (Fambrini *et al.*, 1997). A description of response to oxidative stress of this electrophoretic variant, also in relation to the leaf ABA content, is presented in this paper. Moreover, since the isoform of Cu/ZnSOD variant is located in the chloroplast, we induced in this organelle an oxidative stress by means of the herbicide methyl viologen (MV, a redox-compound that transfers electrons from photosystem I to oxygen, forming the superoxide anions) to test its functionality. The effects of H_2O_2 and water stress on leaf disks of the *w-1* mutant and control line were also analyzed: the extent of cellular damage was quantified by solute leakage, as a measure of membrane disruption. The relationships between stresses and the anti-oxidative enzymes activity in the different genotypes are discussed.

MATERIALS AND METHODS

Plant materials

The inbred lines and varieties of sunflower (*Helianthus annuus* L.) used in this study were obtained from the Agricultural Plant Biology Department, Pisa (Italy). Achenes were germinated in Petri dishes, on filter papers moistened with distilled water, in growth chamber at 25±1°C in the dark. After 3-4 days, germinated seeds were transferred to 8 cm diameter pots containing a mixture of soil and sand plus an initial dose of complete fertilizer (Osmocote 14-14-14, Sierra UK, England). Plants were grown in a growth chamber at 21±1°C, 1.0-1,1 kPa *VPD*. A 16^h photoperiod was provided by fluorescent lighting (Sylvania Day Light F36W/56) with light intensity of 180 µmol quanta m^{-2} s^{-1}. Flower buds of C1 (normal type) and *w-1* (mobility variant) were hand emasculated and crossed; F_2 populations were obtained by selfing F_1 plants. Experiments were started with 3-week-old plants at the four-leaf stage.

ABA extraction and determination

Quantitative analysis of ABA was performed on crude aqueous extracts by a solid-phase radioimmunoassay (RIA) based on the use of a monoclonal antibody (DBPA1) raised against free (S)-ABA, as previously described (Fambrini *et al.*, 1994). Leaf disks (20-30 mg *FW*) were weighed, immediately frozen in liquid nitrogen and extracted with distilled water (water:tissue ratio 50:1, v/w) for 16^h at 4°C in the dark. Experiments to validate RIA results with sunflower crude extracts have previously been reported (Pugliesi *et al.*, 1994).

MV, H_2O_2 and dehydration treatments: solute leakage measurement

MV (methyl viologen: 1,1' -dimethyl-4,4' -bipyridinium dichloride) damage was analyzed as described by Bowler *et al.* (1991) with modifications. Leaf disks (ϕ=1.34 cm) collected from *w-1* mutant and C1 control line, were incubated in Petri dishes containing distilled water or MV solution at 21°C in the dark. After 16^h, they were illuminated for 3^h (35 µmol quanta $m^{-2}s^{-1}$) and transferred to the darkness for additional 16^h. For H_2O_2 treatments, leaf disks for both genotypes were incubated at 21°C for 4^h under fluorescent lamps (35 µmol $m^{-2}s^{-1}$). Leaf dehydration was conducted as previously described (Fambrini *et al.*, 1994). The samples were then placed in flasks (150 ml) with 8 ml distilled water and after 24^h the conductivity of the solutions obtained was measured; a second measurement was performed after autoclaving (121°C for 15 min) to release all solutes. Finally, according to Sen Gupta *et al.*, (1993), the relative leakage ratio was determined by dividing the first value by the conductivity of the sample after autoclaving (100% of leakage).

Superoxide dismutase activity assay

The SOD activity assay, based on the method of Beuchamp and Fridovich (1971) as modified by Dhindsa and Matowe (1981), is based on measuring the inhibition by SOD of the photochemical reduction of nitroblue tetrazolium. The reaction mixture contained 50 mM phosphate buffer pH 7.8, 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium, 2 μM riboflavin, and the enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes under fluorescent lamps. The reaction was terminated after 10 min. by switching off the light. Unilluminated samples served as blanks. The tubes were stirred and the blue color was measured at 560 nm. The volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

Statistical analysis

Statistical significance between mean values was assessed using a Student's *t*-test. The data in Table 2 were analyzed by multifactorial analysis of variance, and differences among means were determined by LSD, at $P=0.01$.

RESULTS AND DISCUSSION

The inbred lines and varieties of sunflower showed two distinct superoxide dismutase isozymes resolved by polyacrylamide gel electrophoresis, conduced in non-denaturating conditions on proteins extracted from cotyledons (Figure 1).

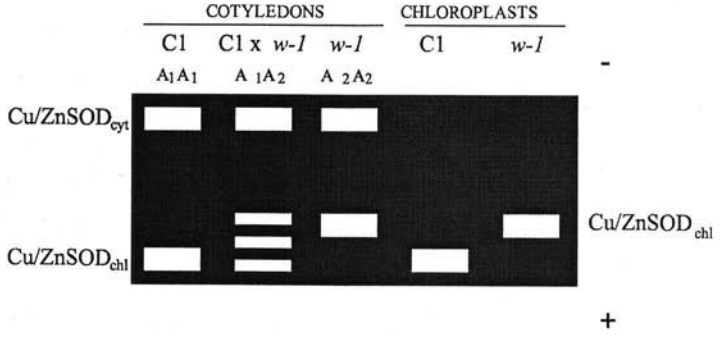

Figure 1: Cu/ZnSOD isozyme patterns of protein extracts from cotyledons or isolated chloroplasts of sunflower homozygous parental types A_1A_1 (inbred lines: C1), A_2A_2 (ABA-deficient mutant: w-1) and F_1 hybrids A_1A_2 (C1 x w-1). The more anodic isozymes are the chloroplast-located Cu/Zn superoxide dismutase ($Cu/ZnSOD_{chl}$).
(Schematic zimogram redrawn from Fambrini et al., 1996; 1997).

Samples of leaf chloroplast extracts revealed that the more anodic isozyme is the chloroplast-associated superoxide dismutase ($Cu/ZnSOD_{chl}$), while the cathodic one detected in cotyledons could be a cytosolic or glyoxysomal Cu/Zn superoxide

dismutase ($Cu/ZnSOD_{cyt}$) (Fambrini *et al.*, 1996; 1997). The *w-1* is an ABA-deficient mutant with altered stomatal behaviour (Pugliesi *et al.*, 1994; Fambrini *et al.*, 1994), and it showed a different electrophoretic pattern in which the $Cu/ZnSOD_{chl}$ has decreased mobility (Figure 1). The $Cu/ZnSOD_{cyt}$ showed in *w-1* the same mobility displayed by the normal type (Fambrini *et al.*, 1997). The genetic analysis suggests that the mobility variant for $Cu/ZnSOD_{chl}$ is coded by a single locus with codominant alleles (Fambrini *et al.*, 1997). Moreover, the results of heterozygous plants that exhibit the heterodimeric isozyme with electrophoretic mobility intermediate between those of their respective homodimeric forms also indicate that the $Cu/ZnSOD_{chl}$ exists as a functional dimer (Figure 1).

To evaluate the association between the electrophoretic variant of $Cu/ZnSOD_{chl}$ displayed by the *w-1* genotype and its ABA-deficiency, an experiment of water stress was performed. Detached leaves of a small F_2 population were allowed to dehydrate and leaf ABA contents were measured after a 40-50% fresh weight was lost. Preliminary data (Table 1) indicate a minor increase in ABA level in A_2A_2 genotype with respect to the A_1A_1 and A_1A_2 genotypes. This result could suggest the association between the two traits, but analysis of larger F_2 progenies is required.

Table 1: ABA content in detached leaves of the F_2 population (A_1A_1, A_1A_2 and A_2A_2 genotypes) placed on a bench at 21°C, under light (180 μmol quanta m^{-2} s^{-1}), with the abaxial surfaces uppermost, and allowed to dehydrate (40-50% FW loss).

Genotype	ABA (ng g^{-1} initial FW)	
	Non stressed	Stressed
A_1A_1 and A_1A_2	42.1 a	411 a
A_2A_2	42.3 a	180 b

Values in the same column, followed by different letters, are significantly different ($P \leq 0.01$) according to Student's *t*-test.

SODs and ABA have been reported to be involved in tolerance to active oxygen species (AOS) produced in plants during common environmental stresses. Here, we evaluate the susceptibility of *w-1*, a mutant with ABA deficiency and a variant isoform of chloroplastic Cu/ZnSOD, to oxidative stress. The results (Table 2) indicate that *w-1* leaves are less damaged by paraquat (MV) and H_2O_2 treatments with respect to the control, while no statistical differences were detected when water stress was imposed by leaf dehydration.

Table 2: The oxidative stress on leaf disks of two sunflower genotypes (*w-1* and C1): effect of methyl viologen (MV), H_2O_2 and leaf dehydration on relative leakage ratio.

Relative leakage ratio

Genotype	Treatment								
	MV solution (M)			H_2O_2 solution (M)			Leaf dehydration (%)		
	0	10^{-5}	10^{-4}	0	10^{-1}	1	0	30	50
w-1 (A_2A_2)	9.4 a	11.9 a	62.2 a	10.9 a	18.4 a	34.4 a	13.1 a	15.8 a	16.9 a
C1 (A_1A_1)	11.2 a	40.8b	64.2 a	13.4 a	24.9 b	8.6 b	12.4 a	12.3 a	17.7 a

Values in the same column, followed by different letters, are significantly different ($P \leq 0.01$) according to LSD test.

Resistance to MV has been found in genotypes with constitutively enhanced SOD activity. In our case, spectrophotometric assays do not evidence higher level of SOD activity in *w-1* extracts than in the control (also after herbicide treatment: data not shown). We could speculate that better response of *w-1* leaves to MV treatment can be attributed to a higher efficiency of the chloroplastic isoform variant of SOD to induce the dismutation of superoxide anion radicals to hydrogen peroxide and oxygen. Additional experiments are necessary to test this hypothesis, because the mutant showed also a tolerance to H_2O_2 which strongly reduces the activity of Cu/ZnSOD (Casano *et al.*, 1997).

CONCLUSIONS

Many unresolved questions remain about the regulation of the expression of *sod* genes and a role of hormones. It has been postulated that abscisic acid is involved in a differential transcriptional activation of some of the SOD genes (Zhu and Scandalios, 1994; Sakamoto *et al.*, 1995; Kurepa *et al.*, 1997); however, in all cases studied total enzymatic activity remained constant (Zhu and Scandalios, 1994; Kurepa *et al.*, 1997). Our results confirm that ABA concentration in plant tissue does not modify SOD activity. In fact, endogenous ABA level in *w-1* is substantially lower than in the control but no statistical differences in SOD activity were observed.

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CARACTERIZACION DE VARIANTES ELECTROFORETICAS PARA Cu/ZnSOD EN EL GIRASOL: REACCION AL ESTRES OXIDANTE

RESUMEN

La dismutacion de superoxido (SOD) y el acido abscisico (ABA) son incluidos en las reacciones vegetales al estres del medio ambiente. Hemos evaluado la sensibilidad de *w-1*, mudante careciente del acido abscisico y de una variante isoforme cloroplastica Cu/Zn de la dismutacion de superoxido, al estres oxidante. Los resultados han mostrado que las hojas fueron menos dañadas por paracuato y los tratamientos con H_20_2 al respecto del control, mientras las diferencias estadisticas no se manifestaron cuando el estres fue causado por la dehidratacion de hojas.

El analisis espectrofotometrico no mostro mas alto nivel de la actividad de la dismutacion de superoxido en los extractos del mudante *w-1* que ese en el control. Eso confirma que la concentracion del acido abscisico no provoca las modificaciones de la actividad de la dismutacion de superoxido. Mejor reaccion de hojas *w-1* al tratamiento por paracuato puede atribuirse a la mas grande capacidad de la variante isoforme cloroplastica de la dismutacion de superoxido de provocar la dismutacion de los radicales de superoxido anion al peroxido hidrogeno y oxigeno, durante el estres oxidante.

CARACTÉRISATION DE VARIANTES ÉLECTROPHORÉTIQUES OUR LE Cu/ZnSOD DANS LE TOURNESOL, RÉACTION AU STRESS OXYDANT

RÉSUMÉ

Les dismutations superoxydes (SOD) et l'acide abscissique (ABA) sont impliqués dans les réactions au stress environnemental. Nous avons évalué la sensibilité de *w-1*, un mutant ayant une carence en ABA et des variantes isomorphes de chloroplastique Cu/ZnSOD à un stress oxydant. Les résultats indiquent que les feuilles *w-1* avaient été moins endommagées par le paraquat (MV) et par les traitements H_2O_2 en comparaison avec le groupe contrôle, alors qu'aucune différence statistique n'avait été détectée quand le stress dû au manque d'eau avait été imposé par déshydradation des feuilles.

L'analyse spectrophotométrique n'a pas montré un niveau plus élevé de l'activité SOD dans les extraits *w-1* que dans le groupe de contrôle, confirmant que la concentration ABA ne modifie pas l'activité SOD. La meilleure réaction des feuilles *w-1* au traitement MV peut être attribuée à une plus grande capacité de la variante SOD isomorphe chloroplastique de provoquer une dismutation de radical anion superoxyde au peroxyde d'hydrogène et à l'oxygène dans des situations de stress oxydant.