

Heat shock responses of bean plants: involvement of free radicals, antioxidants and free radical/active oxygen scavenging systems

A. EDREVA*, I. YORDANOV**, R. KARDJIEVA*** and E. GESHEVA*

*D. Kostoff Institute of Genetics**, *M. Popoff Institute of Plant Physiology***, and *Institute of Catalysis****, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Abstract

In non-acclimated bean plants heat shock induced oxidative damage (increase of free radical concentration and drop of bound thiols, indicating aggregation of proteins) which was regulated by the enhanced activities of peroxidase and superoxide dismutase, as well as by the accumulation of polyphenols and especially of polyamines. In the plants acclimated to high temperature no oxidative damage occurred following heat shock.

Additional key words: catalase, peroxidase, *Phaseolus vulgaris*, polyamines, polyphenols, thiols, superoxide dismutase.

Introduction

Heat shock (HS) induces a network of responses in plants. Research on HS responses concentrates on heat shock proteins (HSP) and membranes as main sensors of HS (Süss and Yordanov 1986, Vierling 1991, Vigh *et al.* 1994, Boston *et al.* 1996). We supposed that free radicals (FR) and active oxygen (AO) species, and their control by antioxidative and antiradical systems could also be of importance, as it was demonstrated at other stresses (Ferrari-Iliou *et al.* 1992, Foyer *et al.* 1994, Low and Merida 1996). However, as mentioned by Nover (1994), scarce information is available relative to HS.

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Abbreviations: AO - active oxygen; CAT - catalase; d.m. - dry mass; EDTA - ethylenediaminetetraacetic acid; EPR - electron paramagnetic resonance; f.m. - fresh mass; FR - free radicals; HS - heat shock; HSP - heat shock proteins; HT - high temperature; PA - polyamines; PO - peroxidase; SOD - superoxide dismutase; TLC - thin layer chromatography.

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* fax: (+359-2) 75-70-87; e-mail: ttsonev@obzor.bio21.acad.bg

For this reason we studied: 1) FR generation; 2) the levels of cell antioxidants - polyamines (PA), polyphenols, bound thiols; 3) the activities of peroxidase (PO), catalase (CAT) and superoxide dismutase (SOD), scavenging H_2O_2 and superoxide FR. HS treated *Phaseolus vulgaris* plants were used as a model system. Before HS treatment, a part of plants was acclimated to increased temperature. This allows to distinguish between two types of HS responses - those of acclimated and non-acclimated plants.

Materials and methods

Plants: Bean (*Phaseolus vulgaris* L. cv. Cheren Starozagorsky) plants were grown in nutrient solution in a growth chamber (temperature of 23 - 25 °C, relative humidity of 60 - 70 %, photon flux density of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, 12-h photoperiod). At the age of 7 - 8 d plants were divided into 2 groups. The plants of the first group were acclimated to increased temperature [1st day 45 °C (5 h) and 25 °C (19 h); 2nd day 47.5 °C (5 h) and 25 °C (19 h)]; plants of the second group were not acclimated.

Heat shock: On the 3rd day acclimated and non-acclimated plants were subjected to HS (50 °C, 5 h); this resulted in wilting of leaves of non-acclimated plants, whereas leaves of acclimated ones exhibited no damage symptoms. Immediately after HS leaves were used for analyses. Plants not subjected to HS were used as controls.

Electron paramagnetic resonance (EPR): EPR analysis was carried out according to Michalov (1985), using lyophilized leaf material.

Content of thiols: A colorimetric technique (Edreva and Hadjiiska 1984) was applied, based on the interaction of thiol groups with the Ellman reagent (1959). Fraction of protein-bound thiols were determined.

Enzyme activities: Activities of peroxidase (PO; E.C.1.11.1.7), catalase (CAT; E.C.1.11.1.6) and superoxide dismutase (SOD; E.C.1.15.1.1) were measured. Material was homogenized with 0.05 M Tris-glycine buffer, pH 8.3, containing 17 % sucrose (m/v) (for extraction of PO), and with 0.05 M sodium phosphate buffer, pH 7.8, containing 1 mM EDTA- Na_2 (for extraction of SOD and CAT, respectively). PO activity was assayed by the method of Herzog and Fahimi (1973), CAT activity according to Bergmeyer (1970), and SOD activity according to Beauchamp and Fridovich (1971). One unit of PO and CAT catalyzes the decomposition of $1 \mu\text{M}$ (H_2O_2) $\text{mg}^{-1}(\text{protein}) \text{min}^{-1}$. One unit of SOD is that amount of enzyme inhibiting the reduction of substrate by 50 % per mg protein.

Polyamines: The fraction of free PA was analyzed. The method of Delétang (1977) was used which is based on a combination of ion exchange column chromatography, thin layer chromatography (TLC) and colorimetry.

Polyphenols: TLC on cellulose plates in the systems *n*-butanol:ethanol:water [4:1:2 (v/v) - I direction] and acetic acid:water [15:85 (v/v) - II direction] was applied (Edreva and Hadjiiska 1980). Polyphenols were observed as fluorescing spots in UV + NH₃. A 1 % AlCl₃ (m/v) in 96 % ethanol (v/v) was used to specify flavonoids; the corresponding spots were eluted and determined according to Graham (1992). The total polyphenols were quantified in 80 % (v/v) ethanolic extracts using the same method.

In all experiments (except EPR spectrometry) fresh leaf material was used.

Statistical analysis: Experiments were repeated three or five times, with four replicates per experiment, using leaves of about 10 plants per replicate. Significance of differences was given by the Student's test at $P \leq 5\%$ or 1% .

Results

Electron paramagnetic resonance spectra: EPR signal was generated in leaves of bean plants. It appears to be due to a stable organic free radicals (FR); according to Leprince *et al.* (1994) organic FR may derive from interaction of primarily produced short-lived oxygen FR with stabilizing trap organic molecules, such as polyphenols. Values of *g* factor (Table 1) are proximal to the value of free electron ($g = 0.0023$), this proving the existence of FR in the system studied. The EPR signal exhibited similar intensities in both acclimated and non-acclimated control plants. A tendency to decline was noticed in HS-treated acclimated plants. A moderate but persisting increase was induced by HS in non-acclimated plants (Table 1).

Table 1. Concentration of free radicals [spin $g^{-1}(\text{d.m.})$] and values of *g*-factor (ratio of the mechanical and magnetic movements of the electron) in leaves of acclimated bean plants (AP) and non-acclimated plants (NAP) subjected to heat shock. Data are means from five experiments each with four replicates. Standard deviations are less than 10 % of the means. Values in the same column followed by different letters are significantly different at $P \leq 5\%$ (Student's *t*-test).

Variants		FR concentration	<i>g</i> - factor
NAP	control	9.4×10^{15} a	2.0022
	HS	9.9×10^{15} b	2.0025
AP	control	9.4×10^{15} a	2.0022
	HS	9.2×10^{15} c	2.0021

Enzyme activities: The high ratio of CN⁻-resistant SOD (95 - 97 % of total activity) pointed that Mn-SOD is the predominant enzyme form in bean leaves. Almost equal activity was found in controls and acclimated HS-treated plants, whereas an increase was observed in the non-acclimated plants after HS (Table 2).

HS treatment provoked an increase of PO activity in both acclimated and non-acclimated bean plants, more prominent in the latter; controls showed almost equal PO activity (Table 2).

HS induced a decline in CAT activity of acclimated and particularly in non-acclimated plants; controls appear quite similar (Table 2). This is consistent with data summarized by Willekens *et al.* (1995).

Table 2. Activity of superoxide dismutase, peroxidase and catalase and content of bound thiols in leaves of acclimated bean plants (AP) and non-acclimated plants (NAP) subjected to heat shock. Enzyme activities are expressed as enzyme units and % of the corresponding controls. Content of bound thiols is presented as $\mu\text{mol g}^{-1}(\text{d.m.})$ and % of the corresponding controls. Data are means from three experiments each with four replicates. Standard deviations are less than 10 % of the means. Values in the same column followed by different letters are significantly different at $P \leq 5\%$.

Variants		Superoxide dismutase		Peroxidase		Catalase		Bound thiols	
		[U]	[%]	[U]	[%]	[U]	[%]	$\mu\text{mol g}^{-1}(\text{d.m.})$	[%]
NAP	control	38 a		0.82 a		8.96 a		18.67 a	
	HS	53 b	139	1.87 b	228	4.40 b	49	2.83 b	15
AP	control	36 a		0.85 a		8.84 a		18.33 a	
	HS	37 a	103	1.14 c	134	6.62 c	74	30.33 c	165

Thiols: Control plants contained similar amounts of bound thiols. Following HS, the acclimated plants exhibited increased levels of thiols whereas in the non-acclimated ones a marked decrease was observed (Table 2).

Polyamines: In the fraction of free PA, the diamines putrescine and cadaverine and the triamine spermidine were identified (Table 3). The occurrence of cadaverine in bean which is uncommon to plants appears to be characteristic for *Leguminosae* family as also stated by Flores (1990). Approximately equal contents of PA were registered in controls. HS induced a moderate increase in the acclimated plants and a massive accumulation (about 10 times higher than the corresponding controls) in the non-acclimated ones (Table 3).

Table 3. Content of free polyamines, flavonoids and total polyphenols [$\text{mg g}^{-1}(\text{d.m.})$] in leaves of acclimated bean plants (AP) and non-acclimated plants (NAP) subjected to heat shock. Absolute values and % of the corresponding controls are presented. Data are means from three experiments each with four replicates. Standard deviations are less than 10 % of the means. Values in the same column followed by different letters are significantly different at $P \leq 1\%$ and 5% .

Variants		Putrescine		Cadaverine		Spermidine		Flavonoids	Polyphenols
		[%]		[%]		[%]		[%]	[%]
NAP.	control	1.46 a		1.04 a		1.25 a		0.92 a	3.00 a
	HS	16.03 b*	1099	12.50 b*	1200	15.71 b*	1256	1.75 b	191
AP	control	1.38 a		1.01 a		1.18 a		1.00 a	2.83 a
	HS	1.89 c	137	1.67 c	165	1.58 c	134	1.25 a	125
									3.83 c
									135

Polyphenols: The TLC pattern of polyphenols in both controls consisted of eight components, four of them being determined as flavonoids. In both acclimated and non-acclimated plants, four new polyphenols appeared after HS, and the amount of flavonoids increased as well as the content of total polyphenols. The response was more strongly expressed in the non-acclimated plants (Table 3).

Discussion

The finding that no differences between acclimated and non-acclimated control plants occurred points that the acclimation *per se* is not related to the parameters studied.

However, important metabolic shifts occurred after HS. Differential HS responses in acclimated and non-acclimated plants was demonstrated. In the latter, an oxidative damage may be judged from the drop of bound thiols, indicating aggregation of protein molecules. Correspondingly, a rise of FR was registered, characteristic for an excited state of cell. The moderate extent of FR "burst" suggested that the event is regulated, as can be deduced from the enhanced activities of H_2O_2 and $O_2^{\bullet-}$ scavenging enzymes (PO, SOD) and the accumulation of antioxidants (polyphenols, PA). These results are consistent with an increased release of superoxide FR from leaves of rice plants following HS (Averyanov *et al.* 1993); a rise of PO activity in wheat (Kongjika and Shameti 1996) and tomato (Lurie *et al.* 1997), and of SOD activity in *Nicotiana plumbaginifolia* (Tsang *et al.* 1991), as well as an accumulation of PA in rice plants (Roy and Ghosh 1996).

Protective function could be ascribed to the dramatic increase of PA content in the non-acclimated HS-treated plants. Besides being antioxidants, PA regulate pH, osmotic and ion cell homeostasis, as well as the structural integrity and functionality of macromolecules, membranes and cell walls (Yordanov *et al.* 1989, Schuber 1989, Yordanov 1995, Edreva 1996, Kumar *et al.* 1997). Moreover, formation of PA conjugates - phenylamides, was recently reported in HS-treated bean leaves (Edreva *et al.* 1995), as well as an increase of flavonoids and total polyphenols (see Table 3). Phenylamides (Bors *et al.* 1989) and flavonoids (Bors *et al.* 1990) should contribute to FR control during HS of non-acclimated beans.

In acclimated plants, it appears that no oxidative damage developed following HS: bound thiols increased, this implying unfolded, reactive (but not aggregated) state of protein molecules. No "burst" of FR/AO was observed, and factors involved in its control (PA, SOD, PO, polyphenols) were not markedly enhanced. Phenylamide formation was not observed (Edreva and Yordanov, unpublished). Hence, mechanisms different from those triggered in non-acclimated plants underlie the HS-responses of acclimated plants. According Cherry *et al.* (1994) during thermo-adaptation a novel set of genes, *non-hsp* genes, are expressed which are not regulated in the same way as *hsp* genes. Their products, non-HSP, may have important role in sustained thermotolerance. Recently, the expression of a unique plastid-localized HSP was reported to be genetically linked to acquired thermotolerance (Joshi *et al.* 1997).

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