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Changes in Hydrogen Peroxide Levels and Catalase Isoforms Expression are Induced with Freezing Tolerance by Abscisic Acid in Potato Microplants

Martha E. Mora-Herrera[1,2], Humberto López-Delgado[1], Ernestina Valadez-Moctezuma[3] and Ian M. Scott[4]

[1]Programa Nacional de Papa, Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, (INIFAP), Metepec
[2]Centro Universitario Tenancingo, Universidad Autónoma del Estado de México, Carr. Tenancingo-Villa Guerrero Km 1.5 Tenancingo,
[3]Departamento de Fitotecnia, Universidad Autónoma Chapingo, Chapingo,
[4]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Ceredigion,
[1,2,3]México
[4]UK

1. Introduction

There is evidence that abscisic acid (ABA) has a protective signaling role in freezing stress in plants (Kobayashi et al., 2008), including mosses (Minami et al., 2003). ABA signaling networks and their actions are not totally understood, but H_2O_2 has been implicated as an intermediary in several ABA responses, where its roles include induction of the antioxidant system (Cho et al., 2009). Mora-Herrera & López-Delgado (2007), using *in vitro* microplants as employed in potato production programs, found freezing tolerance was enhanced by culture on ABA-containing medium. This ABA treatment tripled survival of a -6°C incubation in the cold-sensitive cv. Atlantic, while in the more cold-tolerant cv. Alpha, survival improved by two-thirds. In the ABA-treated microplants, they found the H_2O_2-scavenging enzyme ascorbate peroxidase increased in activity.

Stress tolerance in potato is growing in importance, as increases in potato production by developing countries greatly exceed other major crops (FAO, 2008). The present study used the microplant system to investigate effects of prolonged ABA treatment on catalase, another enzyme important in controlling cellular H_2O_2. Catalases are tetrameric, heme-containing oxidoreductases that dismutate H_2O_2 to water and oxygen. In plants, their peroxisomal location coincides with the cellular site of H_2O_2 generation by photorespiration or fatty acid β-oxidation (Feierabend, 2005)(Scheme 1). Evidence for catalase involvement in

these processes includes susceptibility of catalase mutants to photorespiration-promoting conditions (Queval et al., 2007), and catalase induction in nutrient stress conditions promoting fatty acid catabolism (Contento & Bassham, 2010). Catalases respond to a wide range of stresses (Du et al., 2008) and, most relevantly here, have been functionally implicated in low-temperature tolerance by transgenic experiments on rice (Matsumura et al., 2002). Moreover, there is evidence that catalase is an integral component of ABA-activated stress protection mechanisms (Xing et al., 2008).

Plant catalases occur in small gene families, whose differential expression reflects different roles (Feierabend, 2005). In *Arabidopsis*, *CAT2*, expressed in photosynthetic tissues (Du et al., 2008), is needed to cope with photorespiration (Queval et al., 2007). *Arabidopsis CAT1* is induced by treatments including cold and ABA (Du et al., 2008). Pharmacological and mutant studies have revealed that *CAT1* induction by ABA involves mitogen-activated protein kinase (MAPK) cascades, in which H_2O_2 is involved (Xing et al., 2008). Among maize catalases, *CAT3* is a chilling-acclimation responsive gene in seedlings, and a long-standing example of regulation by H_2O_2(Prasad et al., 1994). Maize *CAT1* is highly expressed as seeds dehydrate in late embryogenesis, and its promoter has an ABRE (ABA-responsive) element, while H_2O_2 was also implicated as a signal by Guan et al. (2000) and Zhang et al. (2006) showed *CAT1* induction by ABA in maize leaves involved MAPK cascades and H_2O_2.

In potato, previous studies have identified two, differentially expressed catalase genes (Santos et al., 2006). In photosynthesizing tissues, where photorespiration occurs, the principal one expressed was *CAT1*. Phylogenetic comparisons by Santos et al. (Santos et al., 2006) found potato *CAT1* was less similar to potato *CAT2* than to *Nicotiana CAT1* genes. Potato *CAT2* shares high identity with *N. plumbaginifolia CAT2*, characteristics of which include inducibility by stressful exposure to ultraviolet light, ozone or SO_2(Willekens et al., 1994). Consistent with an analogous role in stress conditions, potato *CAT2* was induced in plants suffering nematode or bacterial infection (Niebel et al., 1995). More recently, *CAT2* was found to be induced in potato leaves treated with H_2O_2, while *CAT1* was not (Almeida et al., 2005).

This study was undertaken with the hypotheses of catalase and H_2O_2 involvement in ABA-induced freezing tolerance in potato microplants. Moreover, catalase isoforms were predicted to show differential patterns of expression and activity in this process.

2. Materials and methods

2.1 Microplant material

Virus-free microplants of *Solanum tuberosum* L. cv. Alpha and cv. Atlantic, from the Germplasm Bank of the National Potato Program of the National Institute for Forestry Agriculture and Livestock Research (INIFAP), Toluca, México, were micropropagated as nodal cuttings*in vitro* at 20 °C, following previous protocols (Mora-Herrera et al., 2005). In every experiment, 24 microplants were cultured per treatment, and pooled into samples to achieve the weight required for the particular measurement.

2.2 Freezing treatments

Microplants cultivated 28 d on medium with 10µM (±)-*cis, trans*-ABA (Sigma, USA), or as controls without ABA, were transferred to peat moss (in 3 × 5 cm pots) pre-sterilized for 1 h

at 120 °C. These transplanted microplants were kept for 24 h at 20 °C under a 16 h photoperiod (fluorescent lights, 35 µmol m^2 s^{-1}, 400-700 nm), to allow recovery from the stress of transplantation, prior to exposure to -6 ±1 °C in darkness for 4 h, as previously (Mora-Herrera et al., 2005). H_2O_2 and catalase measurements were performed immediately after this freezing incubation.

2.3 Determination of H_2O_2 content

H_2O_2 was measured by luminol-dependent chemiluminescence, as in Mora-Herrera et al. (2005), in 3 experiments, with 3 samples per treatment, and each assay replicated 6 times.

2.4 Quantification of catalase activity

Frozen shoot tissue (0.5 g) was powdered under liquid N_2, and extracted in 2 mL 50 mM potassium phosphate buffer (pH 7.2) containing 5 mM dithiothreitol, 1 mM ethylenediamine tetraacetic acid, and 1% polyvinylpyrrolidone. After clarification by centrifugation (11,000 *g*, 15 min, 4 °C), catalase activity (EC 1.11.1.6) was determined according to Aebi (1984). The total reaction mixture (3 mL) contained 20 µL extract (100 µg protein) and 30 mM H_2O_2 in 50 mM sodium/potassium phosphate buffer (pH 7.0). The reaction was initiated by H_2O_2 addition and followed by absorbance decrease at 240 nm (extinction coefficient 39.4 mM^{-1} cm^{-1}) every 20 s for 3 min, at 26 °C. Protein was determined using Bradford reagent. Catalase was measured in 3 experiments, each with 3 samples (assayed in triplicate) per treatment.

2.5 Catalase zymograms

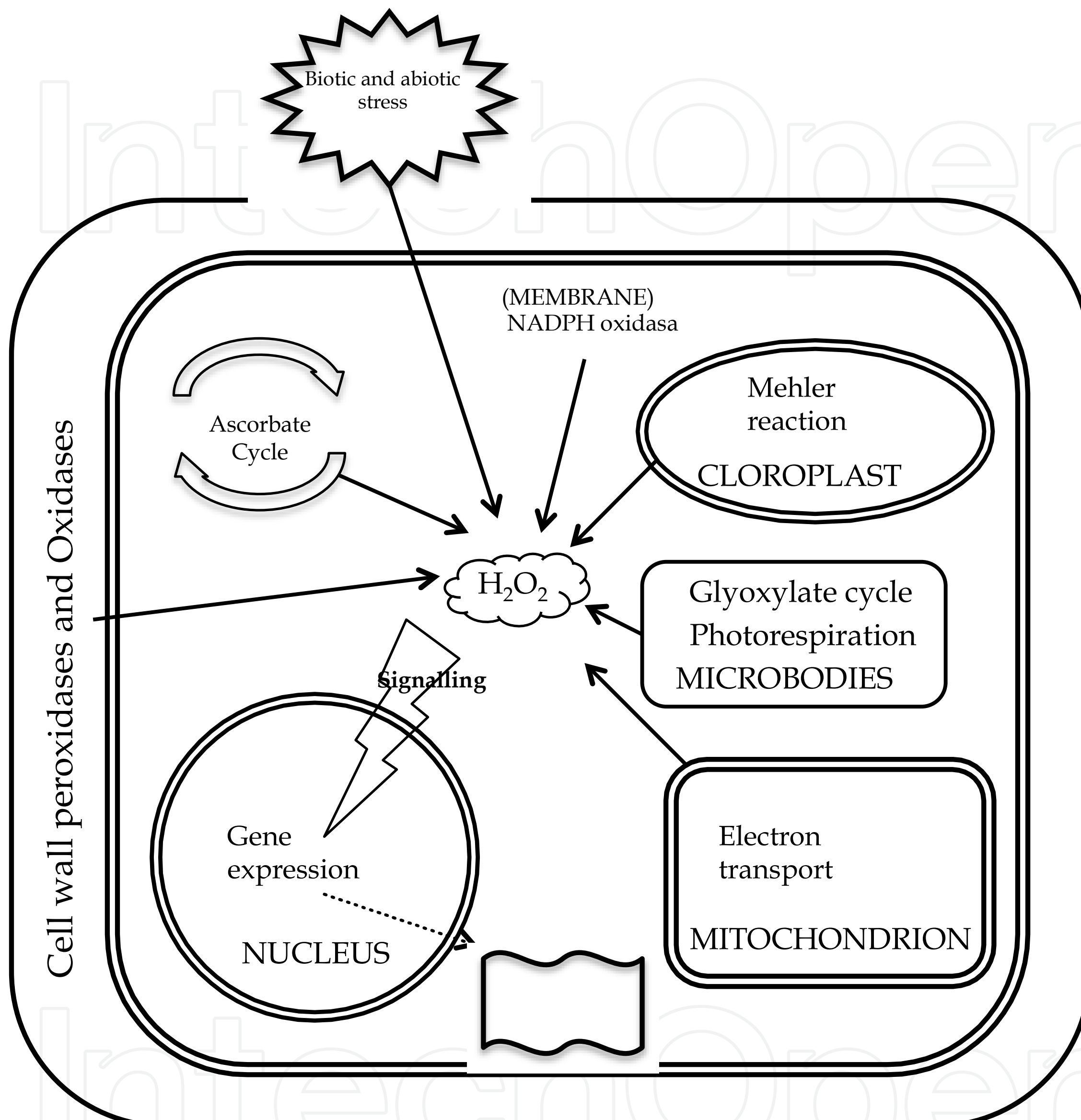
Enzymes were extracted by a similar method to Cruz-Ortega et al. (2002). Frozen tissue (0.1 g) was powdered in liquid N_2, then extracted in 100 µL potassium phosphate buffer (pH 7.8, 1 mM ethylenediaminetetraacetate, 1 mM phenylmethanesulfonyl fluoride, 10 mM dithiothreitol, 2% polyvinylpyrrolidone). The extracts were clarified at 11,000 *g* (10 min, 4 °C). Non-denaturing polyacrylamide gel electrophoresis, as described by Ougham (1987), was performed for 18-20 h at 4 °C. Catalase activity staining used the ferricyanide method of Woodbury et al., 1971. The GE Healthcare Life Sciences HMW Native Marker Kit containing bovine liver catalase (232 kDa) was used as a gel marker. Results shown are representative of 6 experiments.

2.6 Analysis of transcripts by RT-PCR

Total RNA extractions used TRIzol isolation reagent, and treatment with DNase I (Invitrogen, USA). cDNAs were synthesized with Oligo$(dT)_{12-18}$ primer and SuperScript II reverse transcriptase (Invitrogen). PCR amplications of potato *CAT1* and *CAT2* used the primers of Santos et al. (2006). As an internal quantitative control, potato actin (NCBI accession X55751) primers (forward, 5'-AGACGCCTATGTGGGAGATG-3'; reverse, 5'-GCGAGCTTTTCTTTCACGTC-3') were used. After 40 cycles at 52 °C, PCR products were electrophoresed in 1% agarose and visualized with ethidium bromide. Images were acquired by a gel documentation system (UVItec, UK), and relative transcipt levels estimated with Quantity One v.4.6.5 software (Bio-Rad, USA). Results shown are representative of 4 experiments.

2.7 Statistical analysis

Statgraphics Plus v.5.0 (StatPoint Technologies, USA) was used for *t*-tests, and ANOVA with Tukey *post-hoc* tests ($P < 0.05$).



Scheme 1. H_2O_2 is produced in chloroplasts via the Mehler reaction, photorespiration in peroxisomes , glyoxylate cycle, and via electron transport in mitochondria. Cell wall peroxidases and NADPH oxidases in the plasma membrane also can increase the H_2O_2 production when the plant is under biotic or abiotic stress. The signaling role of H_2O_2 is mediated by enzymatic antioxidants one of them is catalase.

3. Results

3.1 Effects of ABA on H_2O_2 content of potato microplants

In vitro microplants were cultured for 28 d on MS medium supplemented with 10 µM ABA. In the study of Mora-Herrera & López-Delgado (2007), 10 µM was the highest ABA

concentration used, which gave greater improvements in freezing tolerance than lower concentrations. It also caused growth inhibition, but this did not detract from eventual growth and tuber yield of microplants transplanted to compost and glasshouse conditions (Mora-Herrera & López-Delgado, 2007). We investigated H_2O_2 and catalase in cv. Alpha and Atlantic microplants at three stages: (a) after 28 d of culture in the presence (or absence) of ABA, (b) 24 h after transplanting from *in vitro* culture to compost, and (c) after 4 h of freezing (-6 °C) in microplants transplanted 24 h previously to compost.

Shoot H_2O_2 contents were on average 24% higher in microplants (of either cv.) that had been cultured for 28 d on ABA-containing medium (Fig. 1). This ABA-induced elevation of H_2O_2 contents persisted in microplants transplanted for 24 h to compost, and also after these transplanted microplants had been subjected to freezing (Fig. 1). It was also notable that freezing treatment also increased H_2O_2, by 23% on average in the transplanted microplants (Fig. 1).

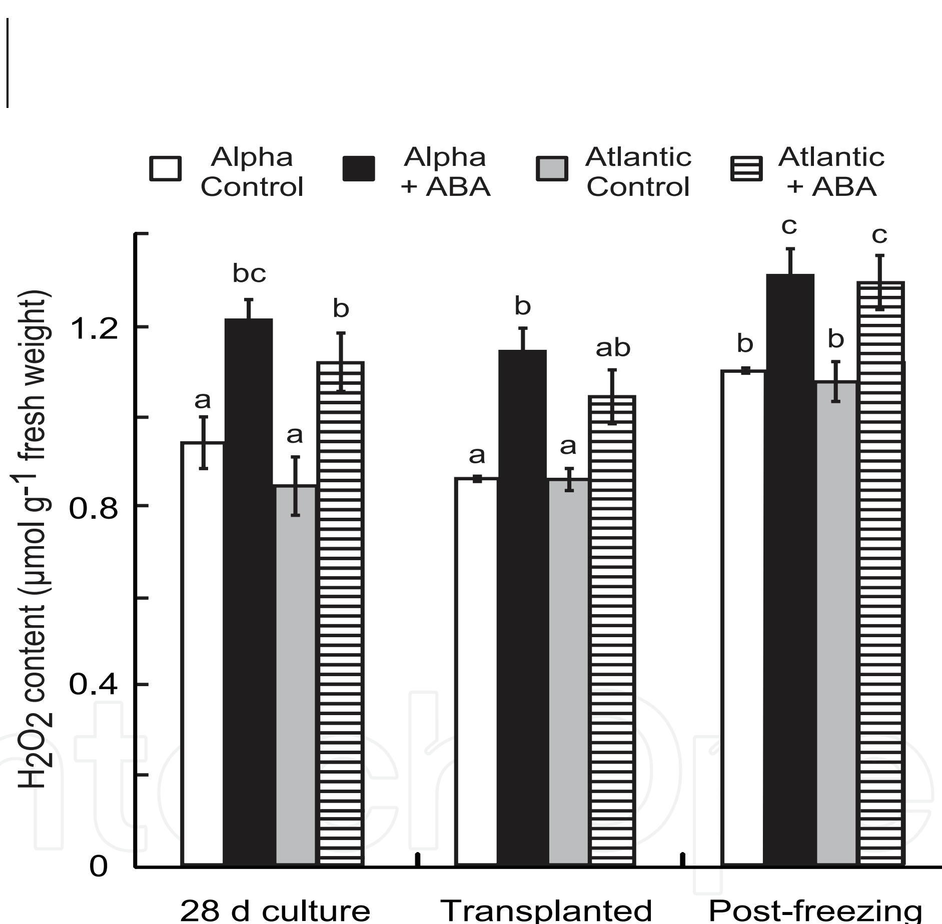


Fig. 1. H_2O_2 content of microplants (cvs. Alpha and Atlantic) grown in the presence of ABA (10 µM), or its absence (controls), assayed at three stages. '28 d culture', after 28 days of *in vitro* culture. 'Transplanted', 24 h after transfer to compost. 'Post-freezing', immediately after 4 h of freezing (-6 °C). Bars show means (n = 3) ± SE, those with different letters differing significantly (ANOVA, P< 0.05).

3.2 Effects of ABA on CAT1 and CAT2 transcripts

RT-PCR with primers specific to *CAT1* or *CAT2* was used to compare the abundance of their transcripts in response to ABA treatment (Fig. 2). The results showed*CAT1* and *CAT2* were differentially regulated by ABA, in both cvs. Relative abundance of *CAT1* transcripts was lower by 25% on average, while *CAT2* transcripts increased up to 4-fold, in ABA-treated microplants (Fig. 2). In consequence, *CAT2* was the gene predominantly expressed in ABA-treated microplants.

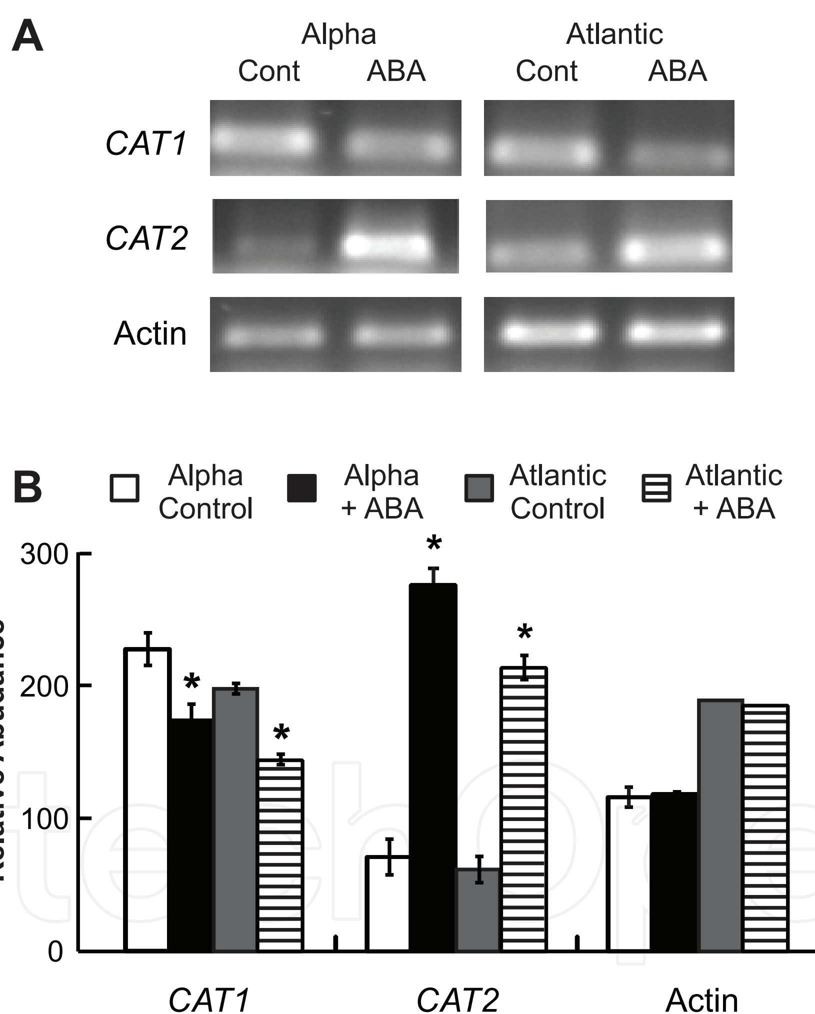


Fig. 2. Effects of ABA (10 µM) on *CAT1* and *CAT2* transcripts in microplants (cvs. Alpha and Atlantic) grown *in vitro* for 28 d. (A) RT-PCR products in agarose gels typical of 4 experiments. (B) Mean relative abundance (± SE) of RT-PCR products in 4 experiments. Actin was the internal control. *ABA treatments significantly different to controls (*t*-tests, $P < 0.05$).

3.3 Effects of ABA on catalase activities

Native gels stained for enzyme activity ('zymograms') confirmed the occurrence of catalase isozymes (Fig. 3), as would be expected from the expression of more than one gene. The faster-migrating native isozyme was greatly increased in ABA-treated microplants of both cvs (Fig. 3), and was attributed to the CAT2 protein, based on the similar effects of ABA on *CAT2* transcripts (Fig. 2) and the immunological evidence of Santos et al. (2006). This isozyme showed similar migration to a 232-kDa standard of bovine liver catalase (Fig. 3). Less expected was the occurrence of more than one slower-migrating isozyme (Fig. 3), since Santos et al. (2006) reported only one, which they assigned as CAT1. The two slower-migrating bands were apparently absent in zymograms of ABA treatments, which represented a more dramatic difference in CAT1 activity than the 25% reduction in *CAT1* transcripts seen in RT-PCR.

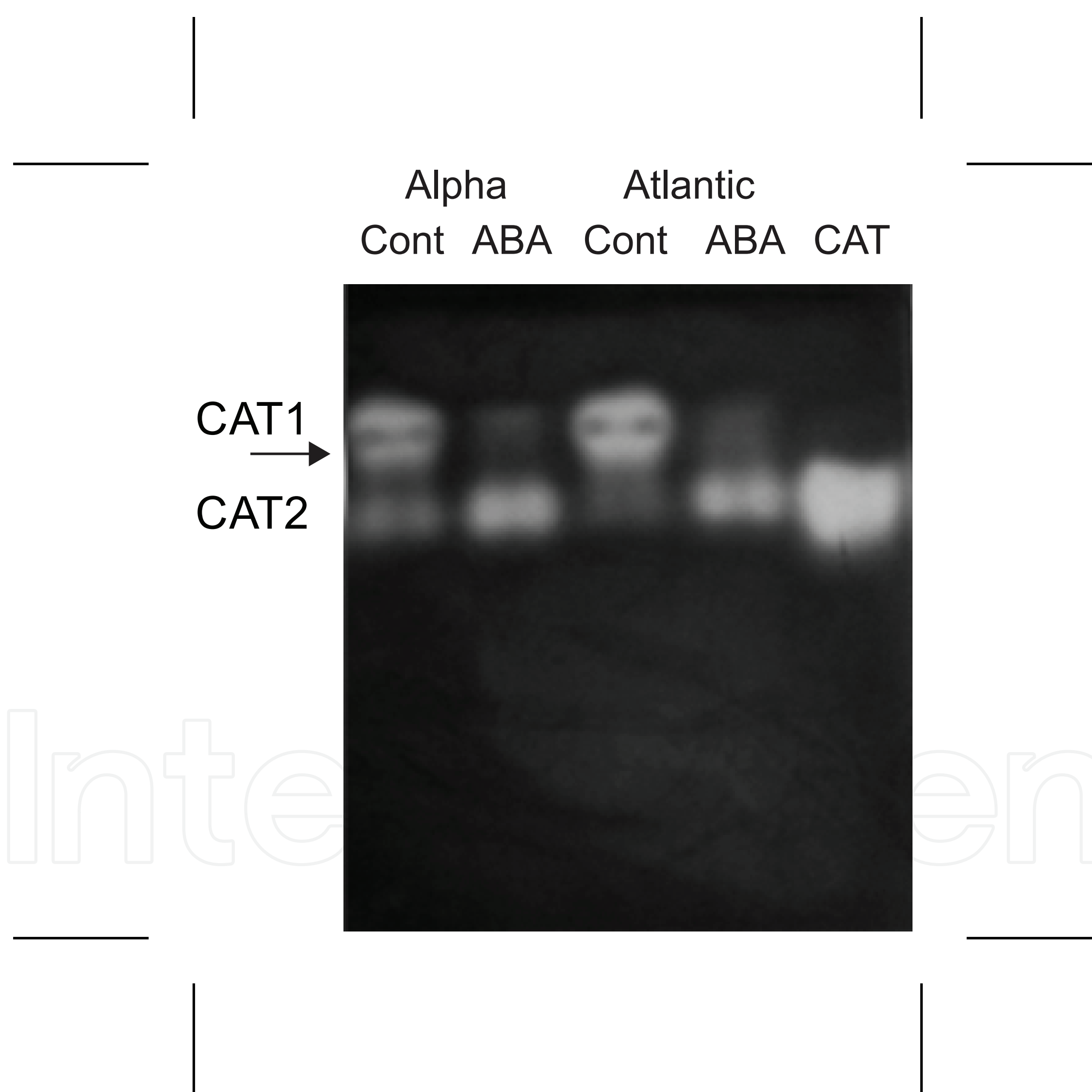


Fig. 3. Catalase zymograms of microplants (cvs. Alpha and Atlantic) grown in vitro in the presence of ABA (10 µM), or its absence (controls) for 28 d. 'CAT' lane: bovine liver catalase (232 kDa). Labels on left: attribution of bands to CAT1 or CAT2 isoforms. Arrow indicates possible heterotetrameric form.

The isozymes had different distributions in microplant shoot tissues. In zymograms of leaves, the two slower-migrating bands dominated, though a faint CAT2 band was visible (Fig. 4). Stem zymograms, in contrast, showed the CAT2 band only (Fig. 4).

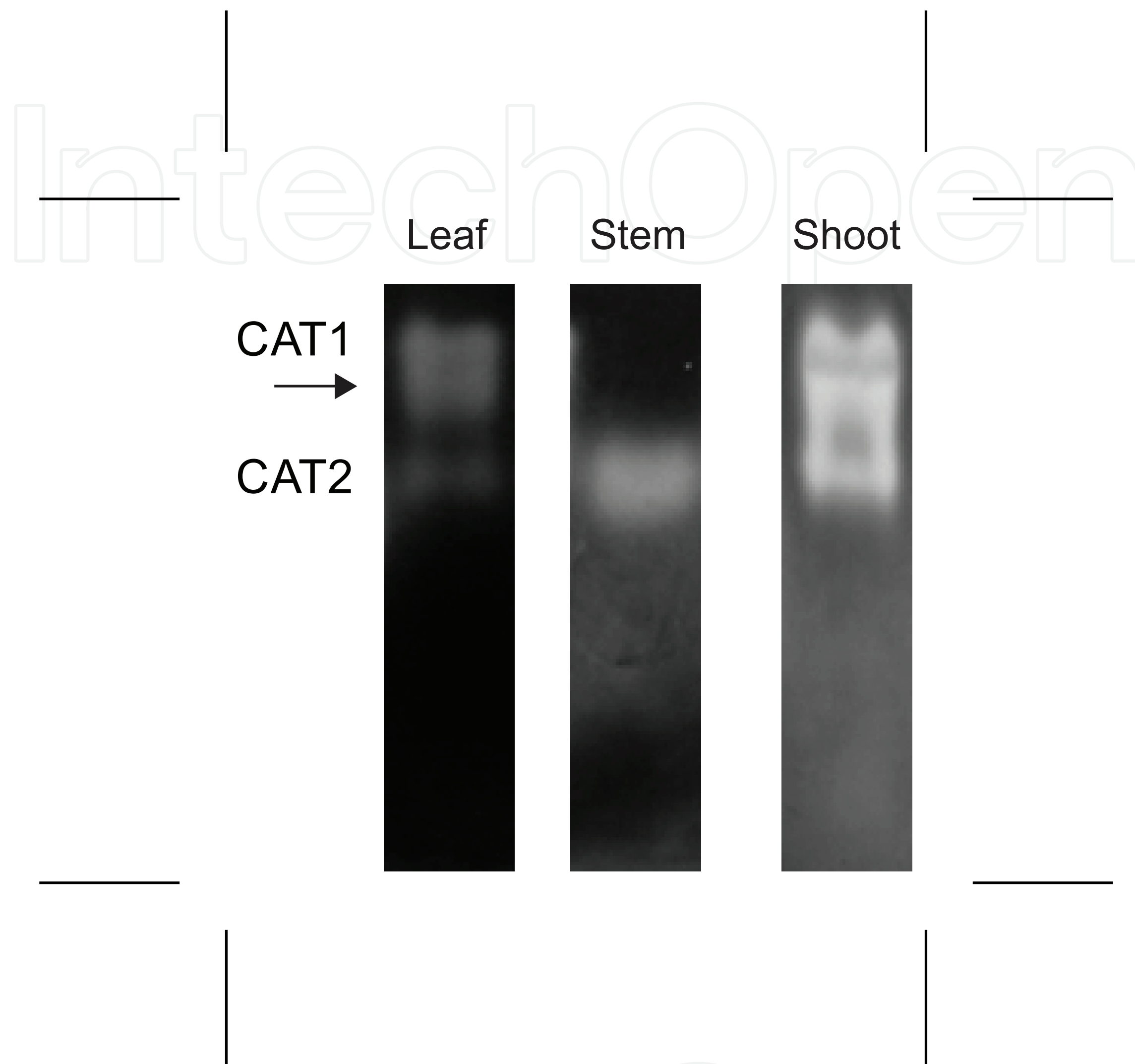


Fig. 4. Catalase zymograms of leaves, stems or whole-shoots of microplants (cv. Alpha) cultured *in vitro* (without ABA). Labels on left: attribution of bands to CAT1 or CAT2 isoforms. Arrow indicates possible heterotetrameric form.

Quantifications of catalase activity indicated the changed isozyme profiles induced by growth on ABA medium resulted in a net decrease, at least in the enzymic assay conditions used. Significant reductions (of 22% on average) were observed in ABA-treated microplants of either cv., relative to untreated controls, both before and after transplantation from *in vitro* culture to compost (Fig. 5).

Catalase activities in ABA-treated and control microplants showed differential responses to freezing. Post-freezing catalase activities in ABA-treated microplants were not significantly different to pre-freezing levels (Fig. 5). In controls, by contrast, catalase activities were lower after freezing, by 33% on average. The net result was that post-freezing catalase activity was not significantly different in ABA-treated and control microplants (Fig. 5).

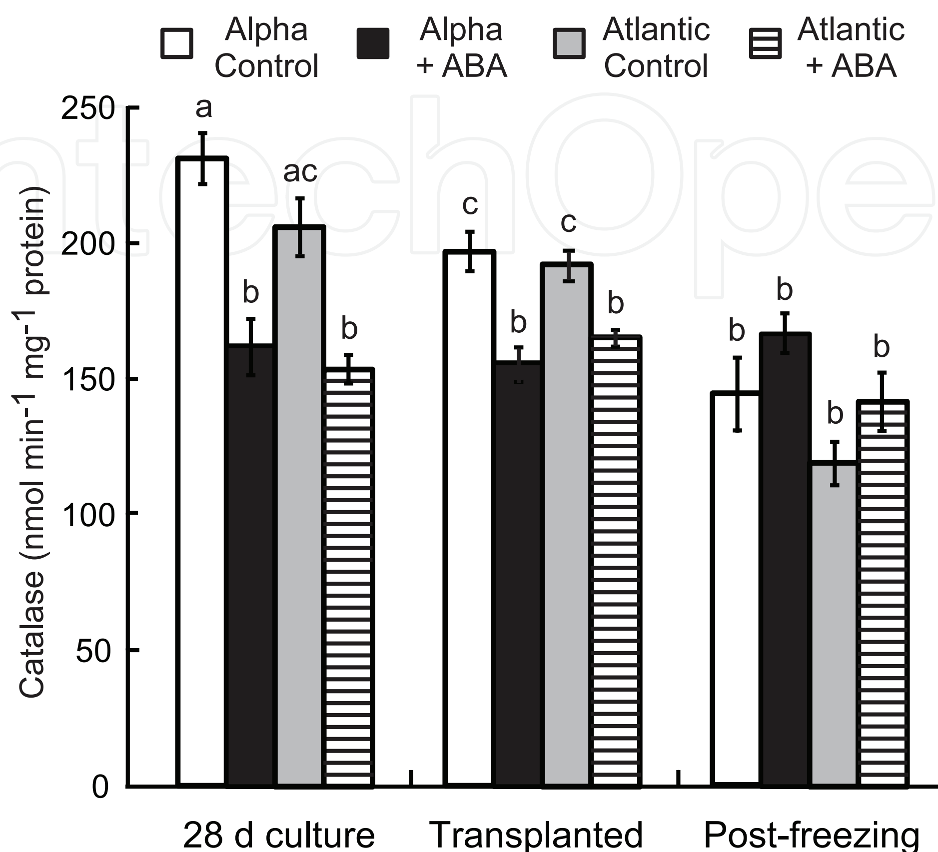


Fig. 5. Catalase activity of microplants (cvs. Alpha and Atlantic), grown in the presence of ABA (10 µM), or its absence (controls), assayed at three stages. '28 d culture', after 28 days of *in vitro* culture. 'Transplanted', 24 h after transfer to compost. 'Post-freezing', immediately after 4 h of freezing (-6 °C). Bars are means (n = 5 - 6) ± SE, those with different letters differing significantly (ANOVA, $P< 0.05$).

4. Discussion

This paper belongs to a series on protection by growth regulators against freezing stress in potato microplants (Mora-Herrera et al., 2005, Mora-Herrera & López-Delgado 2007). One finding was that freezing treatment increased H_2O_2 levels. Despite recognition that abiotic stress is likely to promote formation of reactive oxygen species (Jaspers & Kangasjärvi, 2010), direct studies of the effects of sub-zero temperatures on tissue H_2O_2 are surprisingly sparse. It is therefore worth aligning our results with the only comparable recent study (Yang et al., 2007), especially since concerns have been expressed about variability of H_2O_2 literature data (Queval et al., 2008).

Yang et al. (2007) subjected wheat plants to -6 ºC for 6 h, the temperature being changed from, and back to, 20 ºC over 6 h periods. H_2O_2 (measured spectrophotometrically after reaction with KI)increased from ca. 1.2 to 2.1 μmol g^{-1} in this treatment (Yang et al., 2007). These values are comparable to H_2O_2 in potato microplants in this and previous papers (López-Delgado et al., 1998; Mora-Herrera et al., 2005). The increase from ca. 0.87 to 1.1 μmol $g^{-1}H_2O_2$ in our (ABA-untreated) microplants resulted from a treatment of similar severity (-6 ºC for 4 h), but was measured without any post-freezing period.

The present study was prompted by the finding that 28 d culture with ABA protected microplants in freezing (Mora-Herrera & López-Delgado, 2007). In these prolonged exposures to ABA, H_2O_2 levels were higher by an average (± SD) of 24 ± 7.3% across cvs. and experimental stages (*n* = 6). This was also seen in treatments with another class of protective growth regulators, the salicylates (López-Delgado et al., 1998; Mora-Herrera et al., 2005). The H_2O_2 increment in culture with these growth regulators was notably consistent. H_2O_2 was 27% higher on 100 μM salicylate (Mora-Herrera et al., 2005), and 24% on 1 μM acetylsalicylate (López-Delgado et al., 1998). This may reflect a tight control of maximal H_2O_2 in healthy tissues to avoid toxic concentrations (Queval et al., 2008).

Despite the increase in H_2O_2 induced by freezing treatment, the difference between ABA-treated and untreated microplants was maintained. Therefore, cellular mechanisms for H_2O_2 generation were not saturated by either treatment. The origin of H_2O_2 induced by ABA has been identified as superoxide generation by plasma membrane NADPH oxidases, encoded by *Rboh* (*r*espiratory *b*urst *o*xidase *h*omolog) genes (Cho et al., 2009). Recent work in maize indicates that the ABA-induced expression and activity of NADPH oxidases is further stimulated by the resultant H_2O_2 in a MAPK-regulated positive feedback (Lin et al., 2009).

The *Arabidopsis* RbohD NADPH oxidase was recently also implicated in a systemic reactive oxygen signal in plants subjected to stresses including ice-water cooling (Miller et al., 2009). This class of enzymes, which have now been characterized in potato tubers (Kobayashi et al., 2007), are therefore candidates for H_2O_2 production in both ABA and freezing treatments of the microplants. While it is obviously probable that freezing resulted in H_2O_2 generation by cellular processes under stress (Jaspers & Kangasjärvi, 2010), cellular signaling may also have been involved.

The redox state adjustment indicated by higher H_2O_2 levels may have been a factor in the growth retardation that was another shared effect of ABA (Mora-Herrera & López-Delgado, 2007) and acetylsalicylate (López-Delgado et al., 1998), since a direct pre-treatment with H_2O_2 can itself inhibit microplant growth in culture (López-Delgado et al., 1998). If NADPH oxidases were responsible for the ABA-induced H_2O_2, it could be relevant that certain *Arabidopsisatrboh* mutants are defective in ABA inhibition of root growth (Kwak et al., 2003).

We investigated catalase, as a principal H_2O_2 scavenger, in ABA-treated microplants. RT-PCR and zymogram analyses revealed contrasting ABA responses for different catalase forms. *CAT2* transcripts and the relevant isozyme were strongly ABA-inducible. Given the increased H_2O_2 levels in ABA-treated microplants, and the H_2O_2-inducibility of potato *CAT2* (Almeida et al., 2005), this gene may have an ABA-induction mechanism like *Arabidopsis CAT1* (Xing et al., 2008) and maize *CAT1*(Lin et al., 2009). Our data are consistent with potato *CAT2* as the ortholog of the stress-inducible *N. plumbaginifolia CAT2* (Willekens et al., 1994; Santos et al., 2006).

CAT1 transcripts, in contrast, showed a 25% reduction in abundance in ABA-treated microplants. Zymograms showed more dramatic difference, with the putative CAT1 band absent in ABA treatments. Almeida et al. (Almeida et al., 2005) found H_2O_2 treatment reduced CAT1 in immunoblots and zymograms, whereas *CAT1* in RNA gel blots did not show the same decline. As in our study, therefore, there was a disparity between the RNA and protein findings, which suggested post-transcriptional effects of ABA and H_2O_2 on CAT1 expression. Spectrophotometric assays showed a consistent net reduction in catalase activity in ABA-treated microplants at standard temperature. This suggests the zymograms, where the decline in CAT1 appeared more dramatic, were better indicators of enzymic activity than the RT-PCR.

In zymograms of field-grown plants, Almeida et al. (Almeida et al., 2005) saw only one slower-migrating band, attributed to CAT1, whereas our *in vitro* microplants yielded two slower-migrating bands. An extra isozyme could reflect a third, uncharacterized catalase potato gene, since at least three occur in confamilial species (Santos et al., 2006). On the other hand, the coincidental expression patterns (Figs. 3-4) of the two slower-migrating bands suggested at least one (presumably the faster-migrating) may have been a heterotetramer of CAT1 and CAT2 proteins, analogous to those in other species (Feierabend, 2005).

Heterotetrameric isoforms probably depend on the different loci being co-expressed in a given cell type (Feierabend, 2005), and in some respect the distribution of *CAT1* and *CAT2* expression may have differed *in vitro* and in the field. In microplants under standard conditions, the isoforms did have different tissue distributions. In stem zymograms only the CAT2 band was visible, while leaf zymograms were dominated by the two bands that putatively included CAT1, consistent with an association of CAT1 with photorespiration (Santos et al., 2006). It is furthermore possible that catalase could be differentially distributed in different types of leaf cells, as has been observed for H_2O_2 and ascorbate peroxidase (Galvez-Valdivieso et al., 2009).

In theory, the reduced catalase activity seen in spectrophotometric assays could have facilitated a controlled H_2O_2 increase to adjust growth and prime defenses against abiotic stress. Our data suggest the leaf would be the critical site of these events, since it was the leaf-localized isoforms whose decline was evidenced by isozyme results.

Tsai & Kao (2004) also saw a decrease in catalase activity in ABA treatment, of rice roots. On the other hand, other studies have found increased activity in response to ABA (Agarwal et al., 2005, Zhang et al., 2006, Du et al., 2008, Kumar et al., 2008). Our model system was different in that the microplants experienced prolonged growth on ABA-containing medium. This may have brought a different physiological adjustment to those seen in single, brief treatments, whose effects are transient (Du et al., 2008). It may be more pertinent that, on exposure to freezing, catalase activity levels were maintained in ABA-treated microplants, but not in controls.

The potential value for food security of ABA and catalase lies in their association with coping mechanisms for stresses that challenge crop production (Cho et al., 2009). Crop species in which the effects of ABA on catalase had previously been investigated were cereals (Tsai & Kao 2004, Agarwal et al., 2005, Zhang et al., 2006) or legumes (Kumar et al., 2008). We have added potato to this list. Morever, we suggest that experimental systems like the cultured microplants may have particular biotechnological relevance, because

exploitation of stress tolerance mechanisms are likely to involve the stable changes in physiology seen in prolonged treatments.

5. Conclusion

Freezing tolerance-enhancing treatments with ABA caused differential changes in catalase isoforms and activities, in concert with changes in H_2O_2 levels. At least one isoform may have been a heterotetramer of CAT1 and CAT2 proteins. This may reflect a tight control of maximal H_2O_2 in healthy tissues to avoid toxic concentrations. Knowledge of stress tolerance mechanisms involve stable changes in physiology during prolonged treatments.

6. Acknowledgments

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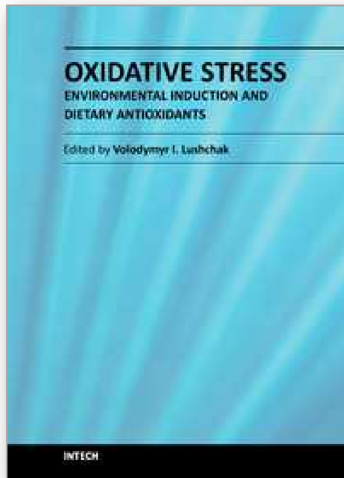

Oxidative Stress - Environmental Induction and Dietary Antioxidants

Edited by Dr. Volodymyr Lushchak

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This book focuses on the numerous applications of oxidative stress theory in effects of environmental factors on biological systems. The topics reviewed cover induction of oxidative stress by physical, chemical, and biological factors in humans, animals, plants and fungi. The physical factors include temperature, light and exercise. Chemical induction is related to metal ions and pesticides, whereas the biological one highlights host-pathogen interaction and stress effects on secretory systems. Antioxidants, represented by a large range of individual compounds and their mixtures of natural origin and those chemically synthesized to prevent or fix negative effects of reactive species are also described in the book. This volume will be a useful source of information on induction and effects of oxidative stress on living organisms for graduate and postgraduate students, researchers, physicians, and environmentalists.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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