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Biochemical and histological characterization of tomato mutants

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ABSTRACT

Biochemical responses inherent to antioxidant systems as well morphological and anatomical properties of photomorphogenic, hormonal and developmental tomato mutants were investigated. Compared to the non-mutant Micro-Tom (MT), we observed that the malondialdehyde (MDA) content was enhanced in the *diageotropica* (*dgt*) and *lutescent* (*l*) mutants, whilst the highest levels of hydrogen peroxide (H_2O_2) were observed in *high pigment 1* (*hp1*) and *aurea* (*au*) mutants. The analyses of antioxidant enzymes revealed that all mutants exhibited reduced catalase (CAT) activity when compared to MT. Guaiacol peroxidase (GPOX) was enhanced in both *sitiens* (*sit*) and *notabilis* (*not*) mutants, whereas in *not* mutant there was an increase in ascorbate peroxidase (APX). Based on PAGE analysis, the activities of glutathione reductase (GR) isoforms III, IV, V and VI were increased in *l* leaves, while the activity of superoxide dismutase (SOD) isoform III was reduced in leaves of *sit*, *epi*, *Never ripe* (*Nr*) and *green flesh* (*gf*) mutants. Microscopic analyses revealed that *hp1* and *au* showed an increase in leaf intercellular spaces, whereas *sit* exhibited a decrease. The *au* and *hp1* mutants also exhibited a decreased in the number of leaf trichomes. The characterization of these mutants is essential for their future use in plant development and ecophysiology studies, such as abiotic and biotic stresses on the oxidative metabolism.

Key words: antioxidant enzymes, leaf and root anatomy, oxidative stress, *Solanum lycopersicum.*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural crops of the world and has been widely used as a model in several fields of plant research. This has significantly contributed to the understanding of the relationship between tomato plants and environmental stress, such as high-light (Wang et al. 2010), salt (Monteiro et al. 2011), drought (Fischer et al. 2011), temperature (Page et al. 2010) and metal (Gratão et al. 2008, Cartes et al. 2010) stress as well as pathogens and diseases (Song et al. 2011). Due to a relatively compact genome (950 Mb)

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combined with a marker-saturated genetic linkage map (Sol Genomics Network), rich germoplasm collections (Tomato Genetics Resource Center) and highly efficient transformation (Pino et al. 2010), a lot of information is available for this plant species, especially at the molecular level.

The plethora of mutants available in tomato is the most advantageous characteristic of this model plant, through which the genetic and biochemical network of many responses is getting better understood (Emmanuel and Levy 2002, Thompson et al. 2004, Calvenzani et al. 2010). Additionally, an advantageous tool is the tomato mutant collection introgressed into the Micro-Tom (MT) (Carvalho et al. 2011a), available at “HCPD-Lab Micro-Tom Mutants”, a small size and rapid life cycle tomato cultivar (Meissner et al. 1997), which has been and will be a successful material for exploitation of several aspects of plant development, such as hormonal and photomorphogenic responses (Gratão et al. 2009, Carvalho et al. 2010, Campos et al. 2009).

New insights and major advances in several field of plant biology can be obtained by the use of mutants, however, it is essential that such mutants are characterized at all levels. Thus, in this work, we characterized biochemical responses inherent to antioxidant system as well anatomical properties of nine tomato mutants (Table I) on the basis of some specific anatomical aspects and key antioxidant enzymes that can be directly related to the mutants responses to environmental stresses.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of the tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom (MT) and its introgressed mutants exhibiting hormonal and photomorphogenic alterations (Table I) were from the “HCPD-Lab Micro-Tom Mutants” collection maintained at the Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ), Universidade de São Paulo (USP), Brazil. The seeds were sown in trays containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, Brazil) and expanded vermiculite, supplemented with 1 g l^{-1} 10:10:10 NPK and

TABLE I
Tomato hormonal, photomorphogenic and developmental mutants introgressed into cv Micro-Tom.

Mutant	Mutation class	Effect/Gene function	Reference
diageotropica (*dgt*)	Auxin	Low sensitivity. Defect in a cyclophilin biosynthesis gene (a putative signal transduction component)	Oh et al. 2006
Never ripe (*Nr*)	Ethylene	Low sensitivity. Defective for the LeETR3 ethylene receptor	Wilkinson et al. 1995
epinastic (*epi*)	Ethylene	Overproduction. Unknown gene function	Fujino et al. 1988
sitiens (*sit*)	Abscisic acid	Deficiency. Defective for ABA-aldehyde oxidase gene	Harrison et al. 2011
notabilis (*not*)	Abscisic acid	Deficiency. Defective for NCED (carotenoid cleavage enzyme).	Burbidge et al. 1999
aure (*au*)	Photomorphogenesis	Low phytochrome content leading to decreased response to light. Defective for the phytochromobilin synthase gene	Muramoto et al. 2005
high pigment (*hp1*)	Photomorphogenesis	Increased response to light. Defective for a gene homologous to DDB1A of Arabidopsis, which codes for a protein interacting with DET1 (HP2), a repressor of photomorphogenesis	Liu et al. 2004
green flesh (*gf*)	Senescence	Thylakoid grana and light-harvesting chlorophyll-binding proteins (LHCP) persist during senescence. STAY-GREEN (SGR) protein necessary for chlorophyll degradation	Barry et al. 2008
lutescent (*l*)	Senescence	Early senescence. Unknown gene function	Jen 1974

4 g l^{-1} lime ($MgCO_3$ + $CaCO_3$), maintained in a greenhouse under automatic irrigation (four times a day), average mean temperature of 28°C, 11.5 h / 13 h (winter/summer) photoperiod, and 250–350 µmol m^{-2} s^{-1} PAR irradiance (natural radiation reduced with a reflecting mesh (Aluminet–Polysack Industrias Ltda, Leme, Brazil). Fifteen days after germination, plants were transferred to 150 mL pots containing the described soil mix and fertilizer. At 40 days after anthesis, leaves were collected, washed in distilled–deionized water and stored at –80°C for later biochemical analysis.

Enzyme Extraction and Protein Determination

The following steps were carried out at 4°C unless stated otherwise. Leaves were homogenized (2:1, buffer volume : fresh weight) with liquid nitrogen in a mortar with a pestle in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol (DTT) and 5% (w/v) insoluble polyvinylpolypyrrolidone (Barbosa et al. 2012). The homogenate was centrifuged at 10,000 x *g* for 30 min, and the supernatant was stored in separate aliquots at -80°C prior to enzymatic analysis. Protein concentration for all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Lipid Peroxidation Determination

Lipid peroxidation was determined by estimating the content of thiobarbituric acid reactive substance (TBARS). Malondialdehyde (MDA) was monitored by measuring at 535 and 600 ηm and the concentration calculated using an extinction coefficient of 1.55 x $10^{-5}mol^{-1}$ cm^{-1}. MDA content was expressed as nmol MDA g^{-1} FW.

Hydrogen Peroxide Content

The content of H_2O_2 was determined as described by Alexieva et al. (2001). Plant tissues were homogenized in 0.1% (m/v) trichloroacetic acid. The homogenate was centrifuged at 12,100 x *g* for 15 min at 4°C and 200 µL of supernatant was added to 200 µL of 100 mM potassium phosphate buffer (pH 7.0) and 800 µL of 1 M KI. The absorbance was read at 390 nm. H_2O_2 content for all samples was determined using known H_2O_2 concentration curve as a standard.

Catalase Total Activity Determination

CAT total activity was assayed as described previously by Gratão et al. (2008) at 25°C in a reaction mixture containing 1 mL 100 mM potassium phosphate buffer (pH 7.5) and 2.5 µL H_2O_2 (30% solution). The reaction was initiated by the addition of 25 µL of protein extract and the activity determined by following the decomposition of H_2O_2 as changes in absorbance at 240 nm over 1 min. CAT total activity was expressed as µmol min^{-1} mg^{-1} protein.

Guaiacol Peroxidase Total Activity Determination

Guaiacol peroxidase (GPOX) total activity was determined following the reaction medium containing 250 µL phosphate–citrate buffer (sodium phosphate dibasic 0.2 M: citric acid 0.1 M, pH 5.0), 150 µL protein extract and 25 µL 0.5% guaiacol, which was vortexed and incubated at 30°C for 15 min and the reaction was stopped by quickly cooling in an ice water bath, followed by the addition of 25 µL of 2% sodium metabisulphide solution (Gomes-Junior et al. 2006). One enzyme activity unit (U) of GPOX corresponds to an increase of 0.001 in absorbance per min per mg protein. The GPOX activity was evaluated by monitoring the absorbance at 450 nm. One enzyme activity (U) of GPOX corresponds to an increase of 0.001 in absorbance per min per mg protein.

Ascorbate Peroxidase Total Activity Determination

Ascorbate peroxidase (APX) total activity was determined by monitoring the rate of ascorbate

oxidation at 290 nm at 30°C. The reaction was initiated by the addition of 40 μL protein extract to 1 mL of a medium containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2. APX total activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate, expressed as ηmol ascorbate min^{-1} mg^{-1} protein.

Glutathione Reductase Total Activity Determination

GR activity was assayed spectrophotometrically at 30°C in a mixture consisting of 1.7 mL, 100 mM potassium phosphate buffer (pH 7.5) containing 1mM 5,5´-dithiozbis(2-nitrobenzoic acid) (DTNB), 1 mM oxidized glutathione (GSSG) and 0.1 mM NADPH. The reaction was started by the addition of 50 μL of protein extract. The rate of reduction of oxidized glutathione was followed in a spectrophotometer by monitoring the change in absorbance at 412 nm over 1 min (Monteiro et al. 2011). GR total activity was expressed as μmol min^{-1} mg^{-1} protein.

Polyacrylamide Gel Electrophoresis (PAGE)

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out as described by Martins et al. (2011). For denaturing SDS-PAGE, the gels were rinsed in distilled-deionized water and incubated overnight in 0.05% Coomassie blue R-250 in methanol:acetic water 40:7:53 (v/v/v) solution and distained with successive washes in methanol: acetic acid: water 40:7:53 (v/v/v) solution. Equal amounts of protein were loaded onto each lane. Electrophoretic analysis was carried out under non-denaturing condition in 10% polyacrylamide gels, followed by SOD, CAT and GR activities staining as described by Gomes-Junior et al. (2006), with equal amounts of protein being loaded onto each gel lane. Electrophoresis buffers and gels were prepared as described by Gomes-Junior et al. (2006), except that SDS was excluded.

Glutathione Reductase Activity Staining

GR activity following non-denaturing PAGE was determined as described by Gomes-Junior et al. (2007). The gels were rinsed in distilled-deionized water and incubated in the dark for 30 min at room temperature in a reaction mixture containing 250 mM Tris (pH 7.5), 0.5 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*- tetrazolium bromide (MTT), 0.7 mM 2,6-dichloro-*N*-(4-hydroxylphenyl)-1,4-benzoquinoneimine sodium salt (DPIP), 3.4 mM GSSG and 0.5 mM NADPH. One unit of bovine liver GR (Sigma, St. Louis, USA) was used as a positive control of activity.

Superoxide Dismutase Activity Staining

SOD activity staining was carried out as described by Monteiro et al. (2011). After non-denaturing-PAGE separation, the gel was rinsed in distilled-deionized water and incubated in the dark, at room temperature, in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium (NBT) and 0.3% N,N,N´,N´-tetramethylethyllenediamine (TEMED). After 30 min the gels were rinsed with distilled–deionized water and then illuminated in water until the development of achromatic bands of SOD activity on a purple-stained gel. One unit of bovine liver SOD (Sigma, St. Louis, USA) was used as a positive control of activity.

Light Microscopy (LM)

Leaf and root tissues of tomato plants were fixed in a modified Karnovsky solution (2% glutaraldehyde; 2% paraformaldehyde and 5mM $CaCl_2$ in 0.05 M cacodylate buffer at pH 7.2) for 48 h. Afterwards, the samples were rinsed in 0.1 M cacodylate buffer and post fixed for 1 h at room temperature with 1.0% osmium tetroxide in the same buffer. After washing with a 0.9% NaCl solution, these samples were

contrasted *in loco* with 2,5% uranyl acetate at 4°C (12 hours), dehydrated in a graded acetone series (25%-100%) and embedded in Spurr resin for 48 h. Semithin sections (120–200nm) were collected in glass slides, stained with 2% toluidine blue (water solution) for 5 minutes, rinsed in distilled water and air-dried (Gratão et al. 2009). The sections were permanently mounted in Entellan resin, observed and documented using an upright light microscope (Axioscop 2, Zeiss, Jena, Germany).

Scanning Electron Microscopy (SEM)

Leaf fragments of tomato plants were fixed using the same fixative solution as described before, dehydrated in an ethanol series (30%-100%), followed by critical point dried through liquid CO_2, mounted in metal stubs, sputter coated with gold and examined under a Zeiss LEO 435VP scanning electron microscope at 20 KV.

Statistical Analysis

The experimental design was randomized with three replicates for each flask/treatment/time interval and the results were expressed as mean and standard error of mean (±SEM) of three independent replicates for lipid peroxidation (MDA content), H_2O_2 content, CAT, GPOX, APX and GR total activities.

RESULTS AND DISCUSSION

It is known for years the importance of genetic variability (Kozak et al. 2011). The use of mutants has been shown to be an extremely powerful tool for general use in biology (Azevedo et al. 1990, 2004a, b, Brennecke et al. 1996). Our group has consistently produced mutants or introgressed mutations into the tomato MT cultivar (Carvalho et al. 2011a). We have also concentrated efforts on the study of plant development and the responses of plants to environmental stresses employing several distinct techniques and experimental designs (Arruda and Azevedo 2009, Campos et al. 2009, Arruda et al. 2011, Ghelfi et al. 2011). The tomato mutants analyzed in this study exhibited clear biochemical and histological alterations. Lipid peroxidation measured as MDA indicated that the mutants *sit*, *not* and *Nr* exhibited similar values to MT, whereas *epi*, *hp1*, *au* and *gf* mutants exhibited lower MDA contents (Fig. 1A). On the other hand, increase in MDA content was observed for *dgt* and *l* mutants, the latter exhibiting a 47.42% higher MDA content when compared to MT (Fig. 1A). The reduced and enhanced lipid peroxidation in *gf* and *l* mutant, respectively, are probably due to its inherent characteristics of delayed (Akhtar et al. 1999) and early (Jen 1974) leaf senescence, respectively. However, the distinct MDA contents observed in *gf* and *l* (Fig. 1A) cannot be explained by changes in H_2O_2 content, since these mutants showed enhanced and reduced H_2O_2, respectively (Fig. 1B), indicating that other ROS are involved in the control of lipid peroxidation in these genotypes. But overall, it is clear that, for the majority of the mutants tested, changes in MDA were detected when compared to MT, suggesting that they might respond differently to an oxidative stress situation.

It has been previously reported that besides the characteristics of retention of chlorophyll during maturation, the *gf* mutant delayed leaf senescence in air and ethylene treatments (Akhtar et al. 1999). Although a wide range of reports have shown the control of ethylene on membrane lipid peroxidation (Hodges and Forney 2000, Flors et al. 2007), the effect of ethylene on this response appears to be complex, since the *epi* mutant, which overproduces ethylene, exhibited a reduced MDA content, whilst for the *Nr* mutant, which confers ethylene insensitivity, the MDA content was identical to the control MT (Fig. 1A). The mutant *epi* also exhibited reduced H_2O_2 content indicating a natural more strong regulation of the peroxidation levels. Nevertheless, the *Nr* mutant only exhibited a slight reduction in H_2O_2 production when compared to MT (Fig. 1B).

It appears that other hormones are involved in the lipid peroxidation, such as auxin, since *dgt*, which confers auxin insensitivity, exhibited enhanced MDA production (Fig. 1A) and H_2O_2 (Fig. 1B) content, as well as abscisic acid, since exaggerated H_2O_2 was observed in *sit* and *not* mutants (Fig 1B). However, ABA mutants did not exhibit altered MDA contents (Fig. 1A), indicating that, in fact, a complex network may be built to explaining the biochemical mechanisms controlled by hormones during membrane alteration. Moreover, it is not surprising that light signalling can be involved in this network. Both photomorphogenic mutants, *au* and *hp1*, which show light-perception deficiency and amplification, respectively, reduced similarly MDA content compared to the MT control, whereas *hp1* exhibited increased H_2O_2 production (Fig. 1B).

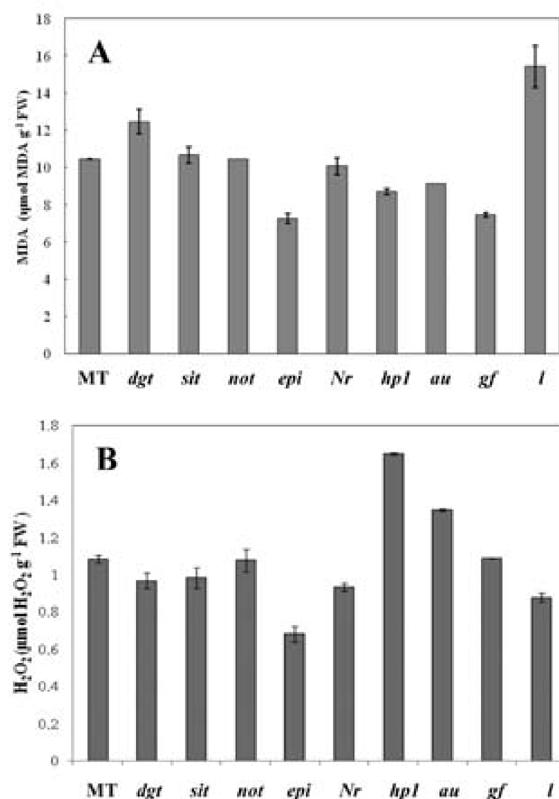

Fig. 1 – MDA (A) and H_2O_2 (B) contents in extracts of leaves isolated from tomato mutants grown over 40 days after anthesis. MT (Micro-Tom); *dgt* (*diageotropica*); *sit* (*sitiens*); *not* (*notabilis*); *epi* (*epinastic*); *Nr* (*Never ripe*); *hp1* (*high pigment 1*); *au* (*aurea*); *gf* (*green flesh*) and *l* (*lutescent*). Values are the means of three replicates ±SEM.

The analysis of key important antioxidant enzymes of all mutants indicated an important hormonal and photomorphogenic control of the antioxidant system. The reduced CAT activity of *dgt* (Fig. 2A) mutant can explain the increase in H_2O_2 content (Fig. 1B). Moreover, it also explains the increased MDA in *dgt* (Fig. 1A), since the activities of the other peroxidases analyzed, GPOX (Fig. 2B) and APX (Fig. 2C), did not differ from MT, whereas GR activity was reduced (Fig. 2D). Although these results indicate an important role of auxin in the antioxidant signalling, others hormones seem to regulate enzyme activity. CAT activity was reduced in *sit* and *not* mutants (Fig. 2A), a result that might explain the enhanced H_2O_2 production observed for this mutant (Fig. 1B), but in contrast, GPOX activity of *sit* and *not* (Fig. 2B) and APX activity of *not* (Fig. 2C) were highly increased indicating that although there is a close relationship between peroxidases and ABA, this can involve multiple pathways, pointing towards a complex multifaceted hormonal response. For example, GOPX and APX were severely reduced in *epi* mutant, but were not enhanced in *Nr* when compared to MT (Fig. 2B and 2C). Besides this, SOD (at least isoform II) is decreased in *epi* and *Nr* leaves (Fig. 3B). When GR is concerned (Fig. 2D), a wide range of activity levels is observed among the mutants; for instance, only *Nr* exhibits activity at the same range of MT, while all other mutants exhibit reduction in total GR activity. When the GR isoenzyme profile is concerned (Fig. 3A), up to six distinct isoforms were observed identified with some specific changes among the mutants which were not only related to increase or decrease in GR activity, but to presence or absence of GR isoforms, indicating that the ascorbate-glutathione cycle may be affected differently by the distinct mutations, which may also result in distinct responses of these mutants to stressful situations.

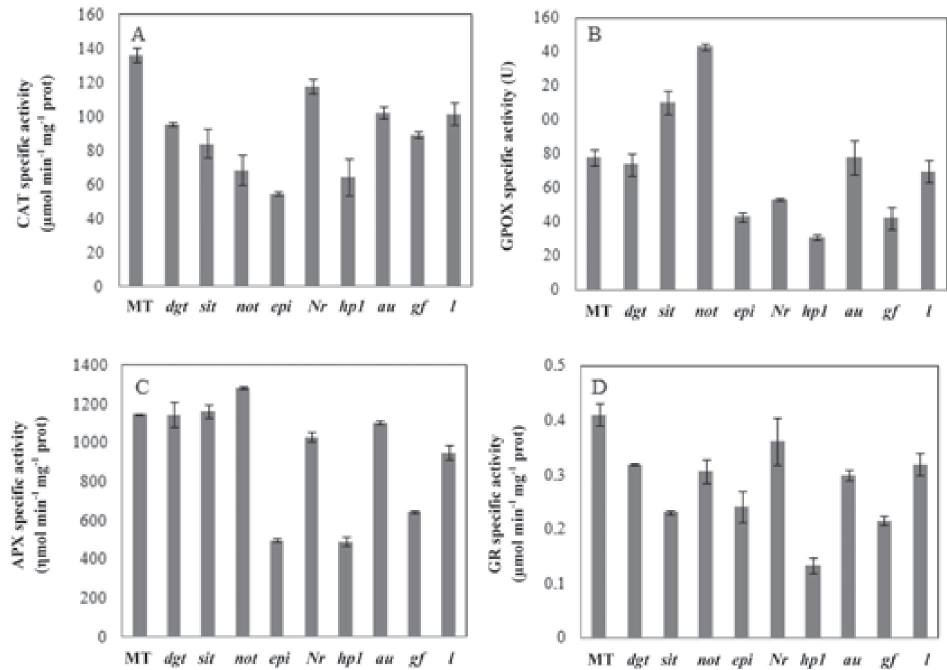

Fig. 2 - Catalase (CAT) (A), guaiacol peroxidase (GPOX) (B), ascorbate peroxidase (APX) (C) and glutathione reductase (GR) (D) total activities in extracts of leaves isolated from tomato mutants grown over 40 days after anthesis. MT (Micro-Tom); *dgt* (*diageotropica*); *sit* (*sitiens*); *not* (*notabilis*); *epi* (*epinastic*); *Nr* (*Never ripe*); *hp1* (*high pigment 1*); *au* (*aurea*); *gf* (*green flesh*) and *l* (*lutescent*). Values are the means of three replicates ±SEM.

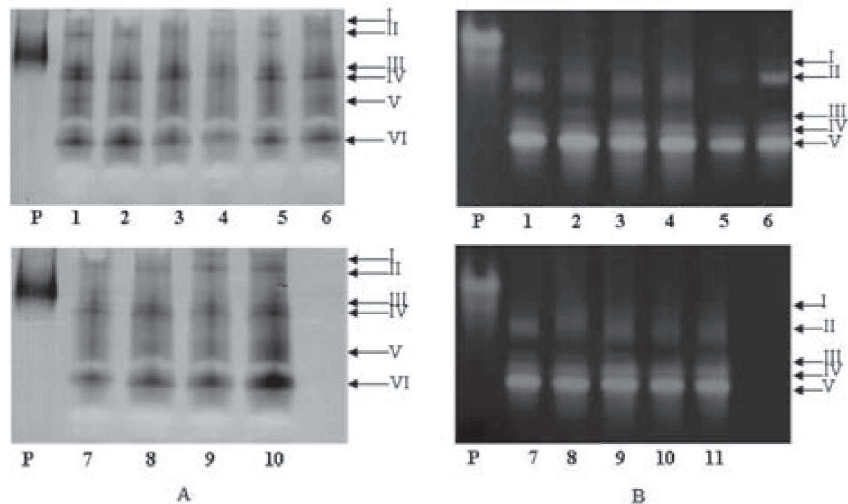

Fig. 3 - Glutathione reductase activity staining (A) and superoxide dismutase activity staining (B) following non-denaturing polyacrylamide gel electrophoresis of leaf extracts isolated from tomato mutants grown over 40 days after anthesis. The lanes listed in (A) are: (P) Saccharomyce cerevisiae GR standard; (1) MT; (2) *dgt*; (3) *sit*; (4) *not*; (5) *epi*; (6) *Nr*; (7) *hp1*; (8) *au*; (9) *gf* and (10) *l*., and in (B) are: (P) bovine SOD standard; (1) MT; (2) *dgt*; (3) *sit*; (4) *not*; (5) *epi*; (6) *Nr*; (7) MT; (8) *hp1*; (9) *au*; (10) *gf* and (11) *l*. MT (Micro-Tom); *dgt* (*diageotropica*); *sit* (*sitiens*); *not* (*notabilis*); *epi* (*epinastic*); *Nr* (*Never ripe*); *hp1* (*high pigment 1*); *au* (*aurea*); *gf* (*green flesh*) and *l* (*lutescent*).

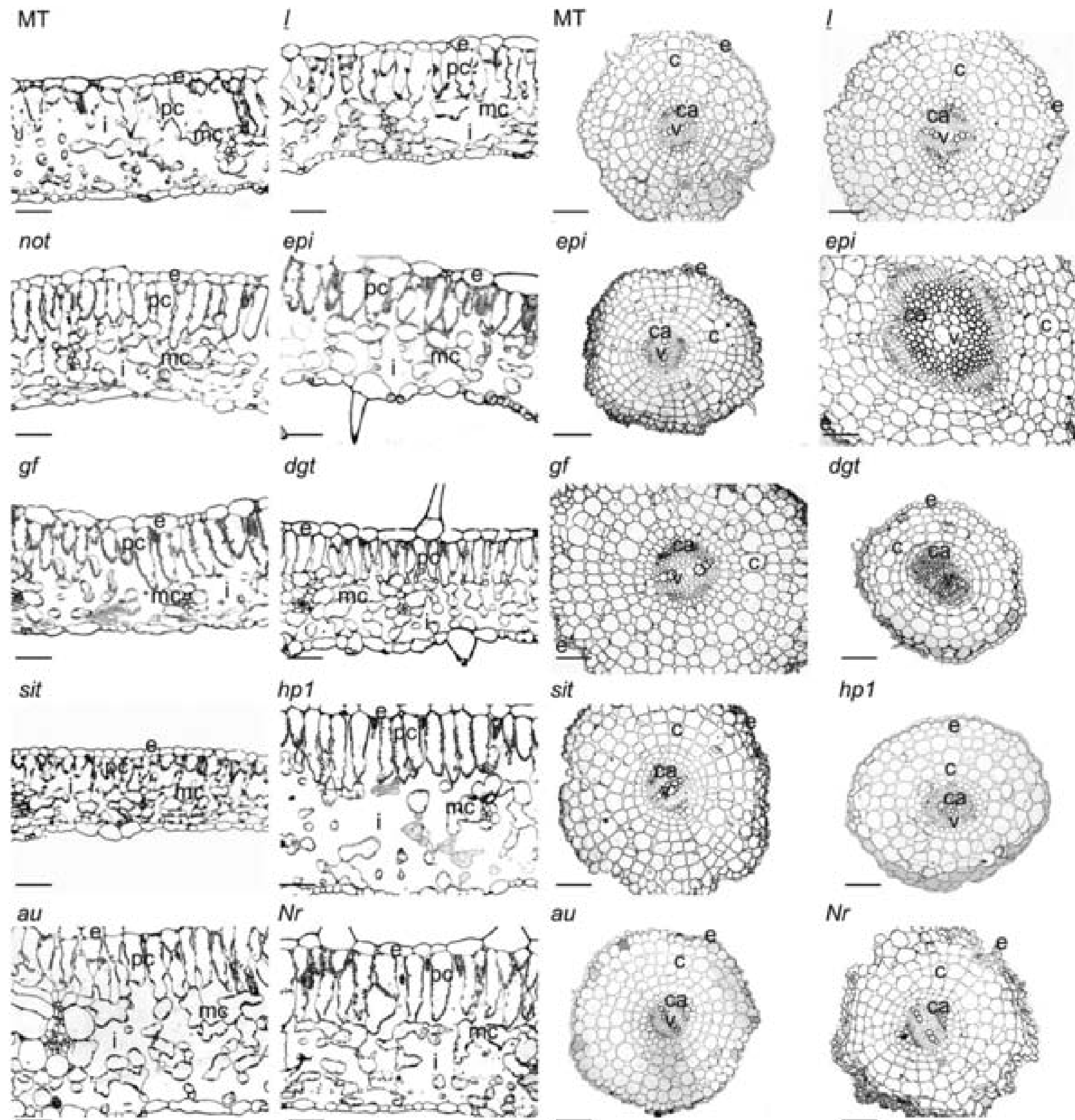

Fig. 4 - Leaf and root cross-sections of tomato mutants grown in nutrient solution observed by light microscopy. Leaves (A); Roots (B); c = cortex; ca = cambium; e = epidermis; i = intercellular spaces; sc = spongy PC = palisade mesophyll cells; v = vascular cylinder. Bars = (A) = 50μm; (B) 3-4 = 100 μm. MT (Micro-Tom); *dgt* (*diageotropica*); *sit* (*sitiens*); *not* (*notabilis*); *epi* (*epinastic*); *Nr* (*Never ripe*); *hp1* (*high pigment 1*); *au* (*aurea*); *gf* (*green flesh*) and *l* (*lutescent*).

In fact, there are many faces of interaction between ethylene and compounds of oxidative stress. This hormone appears to modulate the biochemical pathways of oxidative stress in a tissue and stressor dependent manner as previously suggested by Monteiro et al. (2011) when working with tomato hormonal mutants subjected to salt and Cd-induced oxidative stress. Moreover, as pointed out by Liu et al. (2008), it is still difficult to *understand* whether the ethylene produced during stress is a causal agent or a consequence.

Another intriguing question comes from the results obtained with the *hp1* mutant. *hp* mutants are known for their exaggerated light responses, such

as increased content of carotenoids, flavonoids and vitamin C (Jarret et al. 1984), which are likely to be antioxidants recruited by *hp* plants to deal with light stress (Bino et al. 2005). Nevertheless, the *hp1* mutant did not show any significant change in activity for the enzymes tested; on the contrary, the only change observed was a lower activity when compared to the control MT (Fig. 2). Although it appears that that the lower activity of the enzymes is probably due to reduced lipid peroxidation in *hp1*, we were also surprised by the exaggerated H_2O_2 production in this mutant, further suggesting that light signalling is complex. In fact, *au*, which is light perception deficient mutant, also exhibited reduced levels of lipid peroxidation and H_2O_2 compared to MT (Fig. 1). Thus, a molecular detailed analysis of the biochemical observations for both photomorphogenic mutants needs to be further examined.

Since an additional characteristic displayed by *hp* mutations is a disturbance in water loss (Galpaz et al. 2008, Carvalho et al. 2011b), we are currently investigating whether *hp1* display an exaggerated induction of antioxidant system under stress condition, such as salt and metal stress.

Previous observations for some of the mutants used in this study revealed anatomical or structural alterations in roots, leaves or fruits. For example, Gratão et al. (2009) noticed that *Nr* and *dgt* exhibited a disorganization of the chloroplasts in the mesophyll cells, such as enhanced and diminished intercellular spaces in *Nr* and *dgt* leaves, respectively, which we also have observed for these mutants (Fig. 4A). Additionally, *not* and *sit* have a decrease in the size of mesophyll cells, especially in the palisade parenchyma (Fig. 4A) and an increase in root diameter and more large external cortical cells layers (Fig. 4B). Although this characteristic is probably due to ABA deficiency, producing wilted plants, excessive water loss in *hp1* (Carvalho et al. 2011b) does not result in such characteristics.

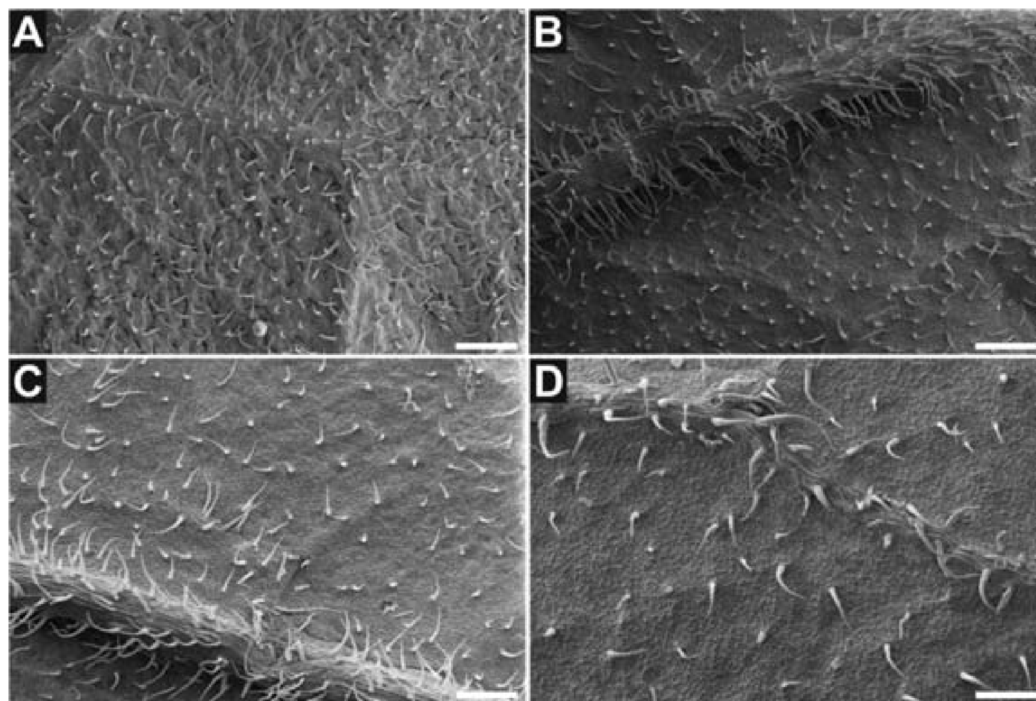

Fig. 5 - Trichome distribution in the abaxial epidermis of the leaf in tomato mutants observed by scanning electron microscopy. (A) *dgt* (*diageotropica*); (B) MT (micro-tom); (C) *au* (*aurea*); (D) *hp1* (*high pigment 1*). Bars = 500 µm.

On the contrary, *hp1* increases intercellular spaces of mesophyll (Fig. 4A) and in accordance to this result, the *hp1* mutation was shown to dramatically increase the periclinal elongation of leaf palisade mesophyll cells (Cookson et al. 2003) as well as to increase plastid number of fruit pericarp chloroplasts (Cookson et al. 2003, Liu et al. 2004). Additionally, we verified that this mutant exhibits a reduced diameter and more large external cortical cell layers of the roots (Fig. 4B). However, the altered mechanisms which result in aspects of *hp1* need to be further elucidated, although, so far, it is known that *hp1* display exaggerated light response, and that *hp* alleles represent mutations in a tomato *UV-DAMAGED DNA-BINDING PROTEIN 1* (*DDB1*) homolog (Liu et al. 2004). The relationship between exaggerated light response and structural alteration is still very complex because it was also observed an increase in the intercellular spaces in *au* (Fig. 4A), which is light perception-deficient.

Scanning electron micrographs of the abaxial epidermis leaf surface in tomato mutants showed different trichomes distribution (Fig. 5). While an increase was observed in trichomes distribution in *dgt* leaves, the abaxial epidermis leaf surface of *au* and *hp1* showed decreased trichomes distribution compared with MT leaves. Although it is evident that light controls the leaf morphology and anatomy in tomato, the similarity of the responses observed in this study between *au* and *hp1* needs to be dissected more thoroughly. In a similar manner, opposite mutations converging into the same response can also be observed in *l* and *gf* mutants, which exhibited an increase in root diameter and larger external cortical cell layers (Fig. 4B).

The characterization of ultrastructural alterations in these mutants is very important since these mutants are being tested under a range of biotic stresses in our laboratories. Previous studies have shown that plants subjected to environmental stresses show a wide range of ultrastructural changes (Vitória et al. 2003, 2006) that can be further intensified or result in different alterations by the combination of the changes induced by the mutations with the changes induced by the stress applied.

In this paper we have reported biochemical and histological alterations in tomato mutants. The results provide new insight into the base of the photomorphogenic, hormonal and developmental mutations. Although now the use of new molecular and genomics tools have provided useful information about the developmental control, our results through the use of simple mutants have raised interesting questions about the factors that control the oxidant and antioxidant signalling as well as roots and leaves anatomy. However, while the factors that we have approached might shed light on the regulation of biochemical and anatomical mechanisms, we are now exploring other tomato mutants through the molecular and physiological approaches involving jasmonic acid insensitive, brassinosteroid insensitive or defective, gibberellin defective or constitutive gebberellin response and cryptochrome deficient. Moreover, doubles and multiples mutants, besides those mutants in this current work, will provide for the elucidation of a wide range of complex responses

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RESUMO

Neste trabalho, analisamos as respostas bioquímicas inerentes ao sistema antioxidante, assim como propriedades morfológicas e anatômicas de mutantes

fotomorfogenéticos e hormonais de tomateiro. Comparados ao não mutante Micro-Tom (MT), observamos que o conteúdo de malondialdeído (MDA) aumentou nos mutantes *diageotropica* (*dgt*) e *lutescent* (*l*), enquanto os maiores níveis de H_2O_2 foram encontrados nos mutantes *high pigment 1* (*hp1*) e *aurea* (*au*). Análises de enzimas antioxidantes mostraram que todos os mutantes reduziram a atividade de catalase (CAT) quando comparado a MT. A guaiacol peroxidase (GPOX) aumentou em ambos os mutantes *sitiens* (*sit*) e *notabilis* (*not*), enquanto no mutante *not* houve aumento da atividade de ascorbato peroxidase (APX). Baseado nas análises de PAGE, as isoformas III, IV, V e VI de glutationa redutase (GR) aumentaram nas folhas do mutante *l*, enquanto a isoforma III de superóxido dismutase (SOD) reduziu nas folhas dos mutantes *sit*, *epi*, *Never ripe* (*Nr*) e *green flesh* (*gf*). Análises por microscopia revelaram que os mutantes *hp1* e *au* tiveram um aumento nos espaços intercelulares de folhas, enquanto o mutante *sit* mostrou um decréscimo. Micrografias eletrônicas revelaram que a superfície foliar dos mutantes *au* e *hp1* apresenta reduzida distribuição de tricomas. A caracterização destes mutantes é essencial para que possam ser utilizados em estudos de desenvolvimento e ecofisiologia, tais como estresses abióticos e bióticos no metabolismo oxidativo.

Palavras-chave: enzimas antioxidantes, anatomia foliar e radicular, estresse oxidativo, *Solanum lycopersicum*.

REFERENCES

AKHTAR MS, GOLDSCHMIDT EE, JOHN I, RODONI S, MATILE P AND GRIERSON D. 1999. Altered patterns of senescence and ripening in gf, a stay-green mutant of tomato *Lycopersicon esculentum* Mill.). J Exp Bot 50: 1115-1122.

ALEXIEVA V, SERGIEV I, MAPELLI S AND KARANOV E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24: 1337–1344.

ARRUDA MAZ AND AZEVEDO RA. 2009. Metallomics and chemical speciation: towards a better understanding of metal-induced stress in plants. Ann Appl Biol 155: 301-307.

ARRUDA SCC, BARBOSA HS, AZEVEDO RA AND ARRUDA MAZ. 2011. Two-dimensional difference gel electrophoresis applied for analytical proteomics: fundamentals and applications to the study of plant proteomics. Analyst 136: 4119-4126.

AZEVEDO RA, ARANA JL AND ARRUDA P. 1990. Biochemical genetics of the interaction of the lysine plus threonine resistant mutant *Ltr*1* with opaque-2 maize mutant. Plant Sci 70: 81-90.

AZEVEDO RA ET AL. 2004a. Genetic control of lysine metabolism in maize endosperm mutants. Funct Plant Biol 31: 339-348.

AZEVEDO RA ET AL. 2004b. Regulation of lysine metabolism and endosperm protein synthesis by the opaque-5 and opaque-7 maize mutations. J Agr Food Chem 52: 4865-4871.

BARBOSA HS, ARRUDA SCC, AZEVEDO RA AND ARRUDA MAZ. 2012. New insights on proteomics of transgenic soybean seeds: evaluation of differential expressions of enzymes and proteins. Anal Bioanal Chem 402: 299-314.

BARRY CS, MCQUINN RP, CHUNG MY, BESUDEN A AND GIOVANNONI JJ. 2008. Amino acid substitutions in homologs of the STAY-GREEN protein are responsible for the green-flesh and chlorophyll retainer mutations of tomato and pepper. Plant Physiol 147: 179-187.

BINO RJ, RIC DE VOS CH, LIEBERMAN M, HALL RD, BOVY A, JONKER HH, TIKUNOV Y, LOMMEN A, MOCO S AND LEVIN I. 2005. The light-hyperresponsive *high pigment-2^{dg}* mutation of **tomato**: alterations in the fruit m**et**abolome. New Phytol 166: 427-438.

BRADFORD MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72: 248–259.

BRENNECKE K, NETO AJS, LUGLI J, LEA PJ AND AZEVEDO RA. 1996. Aspartate kinase in the maize mutants ASK1-LT19 and opaque-2. Phytochemistry 41: 707-712.

BURBIDGE A, GRIEVE TM, JACKSON A, THOMPSON A, MCCARTY DR AND TAYLOR IB. 1999. Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14*. Plant J 17: 427-431.

CALVENZANI V, MARTINELLI M, LAZZERI V, GIUNTINI D, DALL'ASTA C, GALAVERNA G, TONELLI C, RANIERI A AND PETRONI K. 2010. Response of wild-type and high pigment-1 tomato fruit to UV-B depletion: flavonoid profiling and gene expression. Planta 231: 755-765.

CAMPOS ML, DE ALMEIDA M, ROSSI ML, MARTINELLI AP, LITHOLDO JUNIOR CG, FIGUEIRA A, RAMPELOTTI-FERREIRA FT, VENDRAMIM JD, BENEDITO VA AND PERES LEP. 2009. Brassinosteroids interact negatively with jasmonates in the formation of anti-herbivory traits in tomato. J Exp Bot 60: 4347-4361.

CARTES P, JARA AA, PINILLA L, ROSAS A AND MORA ML. 2010. Selenium improves the antioxidant ability against aluminium-induced oxidative stress in ryegrass roots. Ann Appl Biol 156: 297-307.

CARVALHO RF, AIDAR ST, AZEVEDO RA, DODD IC AND PERES LEP. 2011b. Enhanced transpiration rate in the *high pigment 1* tomato mutant and its physiological significance. Plant Biol 13: 546-550.

CARVALHO RF, CAMPOS M, PINO L, CRESTANA S, ZSOGON A, LIMA J, BENEDITO V AND PERES L. 2011a. Convergence of developmental mutants into a single tomato model system: 'Micro-Tom' as an effective toolkit for plant development research. Plant Methods 7: 18.

CARVALHO RF, QUECINI V AND PERES LEP. 2010. Hormonal modulation of photomorphogenesis-controlled anthocyanin accumulation in tomato (*Solanum lycopersicum* L. cv Micro-Tom) hypocotyls: physiological and genetic studies. Plant Sci 178: 258-264.

COOKSON PJ, KIANO JW, SHIPTON CA, FRASER PD, ROMER S, SCHUCH W, BRAMLEY PM AND PYKE KA. 2003. Increases in cell elongation, plastid compartment size and phytoene synthase activity underlie the phenotype of the *high pigment-1* mutant of tomato. Planta 217: 896-903.

EMMANUEL E AND LEVY AA. 2002. Tomato mutants as tools for functional genomics. Curr Opin Plant Biol 5: 112-117.

FISCHER I, CAMUS-KULANDAIVELU L, ALLAL F AND STEPHAN W. 2011. Adaptation to drought in two wild tomato species: the evolution of the Asr gene family. New Phytol 190: 1032-1044.

FLORS V, PARADÍS M, GARCÍA-ANDRADE J, CEREZO M, GONZÁLEZ-BOSCH C AND GARCÍA-AGUSTÍN P. 2007. A tolerant behavior in salt-sensitive tomato plants can be mimicked by chemical stimuli. Plant Signal Behav 2: 50-57.

FUJINO DW, BURGER DW, YANG SF AND BRADFORD KJ. 1988. Characterization of an ethylene overproducing mutant of tomato (*Lycopersicon esculentum* Mill Cultivar VFN8). Plant Physiol 88: 774-779.

GALPAZ N, WANG Q, MENDA N, ZAMIR D AND HIRSCHBERG J. 2008. Abscisic acid deficiency in the tomato mutant *high-pigment 3* leading to increased plastid number and higher fruit lycopene content. Plant J 53: 717-730.

GHELFI A, GAZIOLA SA, CIA MC, CHABREGAS SM, FALCO MC, KUSER-FALCÃO PR AND AZEVEDO RA. 2011. Cloning, expression, molecular modeling and docking analysis of glutathione transferase from *Saccharum officinarum*. Ann Appl Biol 159: 267-280.

GOMES-JUNIOR RA, GRATÃO PL, GAZIOLA SA, MAZZAFERA P, LEA PJ AND AZEVEDO RA. 2007. Selenium-induced oxidative stress in coffee cell suspension cultures. Funct Plant Biol 34: 449–456.

GOMES-JUNIOR RA, MOLDES C, DELITE FS, GRATÃO PL, MAZZAFERA P, LEA PJ AND AZEVEDO RA. 2006. Nickel elicits a fast antioxidant response in *Coffea arabica* cells. Plant Physiol Biochem 44: 420-429.

GRATÃO PL, MONTEIRO CC, ANTUNES AM, PERES LEP AND AZEVEDO RA. 2008. Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. Ann Appl Biol 153: 321-333.

GRATÃO PL, MONTEIRO CC, ROSSI ML, MARTINELLI AP, PERES LEP, MEDICI LO, LEA PJ AND AZEVEDO RA. 2009. Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. Environ Exp Bot 67: 387–394.

HARRISON E, BURBIDGE A, OKYERE P, THOMPSON J AND TAYLOR IB. 2011. Identification of the tomato ABA-deficient mutant *sitiens* as a member of the ABA-aldehyde oxidase gene family using genetic and genomic analysis. Plant Growth Regul 64: 301–309.

HCPD - LAB MICRO-TOM MUTANTS [www.esalq.usp.br/tomato].

HODGES DM AND FORNEY CF. 2000. The effect of ethylene, depressed oxygen and elevated carbon dioxide on antioxidant profiles of senescing spinach leaves. J Exp Bot 51: 645–655.

JARRET RL, SAYAMA H AND TIGCHELAAR EC. 1984. Pleiotropic effects associated with the chlorophyll intensifier mutations *high pigment* and *dark green* in tomato. *Amer Soc Hort Sci* 109: 873-878.

JEN JJ. 1974. Carotenoids of yellow and red lutescent tomatoes. J Agr Food Chem 22: 908-910.

KOZAK M, BOCIANOWSKI J, LIERSCH A, TARTANUS M, BARTKOWIAK-BRODA I, PIOTTO FA AND AZEVEDO RA. 2011. Genetic divergence is not the same as phenotypic divergence. Mol Breed 28: 277-280.

LIU K, SHEN L AND SHENG J. 2008. Improvement tolerance of tomato seedlings with an antisense DNA for 1-aminocyclopropane-1-carboxylate synthase. J Plant Nutr 31: 809-827.

LIU Y, ROOF S, YE Z, BARRY C, VAN TUINEN A, VREBALOV J, BOWLER C AND GIOVANNONI J. 2004. Manipulation of light signal transduction as a means of modifying fruit nutritional quality in **tomato**. Proc Natl Acad Sci USA 101: 9897-9902.

MARTINS PF, CARVALHO G, GRATÃO PL, DOURADO MN, PILEGGI MA, ARAUJO WL AND AZEVEDO RA. 2011. Effects of the herbicides acetochlor and metolachlor on antioxidant enzymes in soil bacteria. Process Biochem 46: 1186-1195.

MEISSNER R, JACOBSON Y, MELAMED S, LEVYATUV S, SHALEV G, ASHRI A, ELKIND Y AND LEVY A. 1997. A new model system for tomato genetics. Plant J 12: 1465-1472.

MONTEIRO CC, CARVALHO RF, GRATÃO PL, CARVALHO G, TEZOTTO T, MEDICI LO, PERES LEP AND AZEVEDO RA. 2011. Biochemical responses of the ethylene-insensitive *Never ripe* tomato mutant subjected to cadmium and sodium stresses. Environ Exp Bot 71: 306–320.

MURAMOTO T, KAMI C, KATAOKA H, IWATA N, LINLEY PJ, MUKOUGAWA K, YOKOTA A AND KOHCHI T. 2005. The tomato photomorphogenetic mutant, *aurea*, is deficient in phytochromobilin synthase for phytochrome chromophore biosynthesis. Plant Cell Physiol 46: 661-665.

OH K, IVANCHENKO MG, WHITE TJ AND LOMAX TL. 2006. The *diageotropica* gene of tomato encodes a cyclophilin: a novel player in auxin signalling. Planta 224: 133-144.

PAGE D, GOUBLE B, VALOT B, BOUCHET J, CALLOT C, KRETZSCHMAR A, CAUSSE M, RENARD C AND FAUROBERT M. 2010. Protective proteins are differentially expressed in tomato genotypes differing for their tolerance to low-temperature storage. Planta 232: 483-500.

PINO LE, LOMBARDI-CRESTANA S, AZEVEDO MS, SCOTTON DC, BORGO L, QUECINI V, FIGUEIRA A AND PERES LEP. 2010. The Rg1 allele as a valuable tool for genetic transformation of the tomato Micro-Tom model system. Plant Methods 6: 23.

SOL GENOMICS NETWORK. [http://solgenomics.net].

SONG W, MA X, TAN H AND ZHOU J. 2011. Abscisic acid enhances resistance to *Alternaria solani* in tomato seedlings. Plant Physiol Biochem 49: 693-700.

THE C. M RICK TOMATO GENETICS RESOURCE CENTER. [http://tgrc.ucdavis.edu].

THOMPSON AJ, THORNE ET, BURBIDGE A, JACKSON AC, SHARP RE AND TAYLOR IB. 2004. Complementation of notabilis, an abscisic acid-deficient mutant of tomato: Importance of sequence context and utility of partial complementation. Plant Cell Environ 27: 459-471.

VITÓRIA AP, DA CUNHA M AND AZEVEDO RA. 2006. Ultrastructural changes of radish leaf exposed to cadmium. Environ Exp Bot 58: 47-52.

VITÓRIA AP, RODRIGUEZ APM, CUNHA M, LEA PJ AND AZEVEDO RA. 2003. Structural changes in radish seedlings exposed to cadmium. Biol Plant 47: 561-568.

WANG M, JIANG W AND YU H. 2010. Effects of exogenous epibrassinolide on photosynthetic characteristics in tomato (*Lycopersicon esculentum* Mill) seedlings under weak light stress. J Agr Food Chem 58: 3642-3645.

WILKINSON JQ, LANAHAN MB, YEN HC, GIOVANNONI JJ AND KLEE HJ. 1995. An ethylene-inducible component of signal-transduction encoded by *Never-ripe*. Science 270: 1807-1809.