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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<sub>2</sub>O<sub>2</sub>) 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<sup>-1</sup> 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
<i>diageotropica (dgt)</i>	Auxin	Low sensitivity. Defect in a cyclophilin biosynthesis gene (a putative signal transduction component)	Oh et al. 2006
<i>Never ripe (Nr)</i>	Ethylene	Low sensitivity. Defective for the LeETR3 ethylene receptor	Wilkinson et al. 1995
<i>epinastic (epi)</i>	Ethylene	Overproduction. Unknown gene function	Fujino et al. 1988
<i>sitiens (sit)</i>	Abscisic acid	Deficiency. Defective for ABA-aldehyde oxidase gene	Harrison et al. 2011
<i>notabilis (not)</i>	Abscisic acid	Deficiency. Defective for NCED (carotenoid cleavage enzyme).	Burbidge et al. 1999
<i>aure (au)</i>	Photomorphogenesis	Low phytochrome content leading to decreased response to light. Defective for the phytochromobilin synthase gene	Muramoto et al. 2005
<i>high pigment (hp1)</i>	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
<i>green flesh (gf)</i>	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
<i>lutescent (l)</i>	Senescence	Early senescence. Unknown gene function	Jen 1974

4 g l<sup>-1</sup> lime (MgCO<sub>3</sub> + CaCO<sub>3</sub>), 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<sup>-2</sup> s<sup>-1</sup> 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 polyvinylpyrrolidone (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 nm and the concentration calculated using an extinction coefficient of 1.55 x 10<sup>-5</sup> mol<sup>-1</sup> cm<sup>-1</sup>. MDA content was expressed as nmol MDA g<sup>-1</sup> FW.

#### HYDROGEN PEROXIDE CONTENT

The content of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> content for all samples was determined using known H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> (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<sub>2</sub>O<sub>2</sub> as changes in absorbance at 240 nm over 1 min. CAT total activity was expressed as μmol min<sup>-1</sup> mg<sup>-1</sup> 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<sub>2</sub>O<sub>2</sub>. APX total activity was calculated using the extinction coefficient of 2.8 mM<sup>-1</sup> cm<sup>-1</sup> for ascorbate, expressed as ηmol ascorbate min<sup>-1</sup> mg<sup>-1</sup> 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 1 mM 5,5'-dithiozobis(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<sup>-1</sup> mg<sup>-1</sup> 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 destained 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-hydroxyphenyl)-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'-tetramethylethylenediamine (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<sub>2</sub> 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<sub>2</sub>, 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 ( $\pm$ SEM) of three independent replicates for lipid peroxidation (MDA content), H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> content, since these mutants showed enhanced and reduced H<sub>2</sub>O<sub>2</sub>, 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<sub>2</sub>O<sub>2</sub> content indicating a natural more strong regulation of the peroxidation levels. Nevertheless, the *Nr* mutant only exhibited a slight reduction in H<sub>2</sub>O<sub>2</sub> 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).

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, GPOX 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.

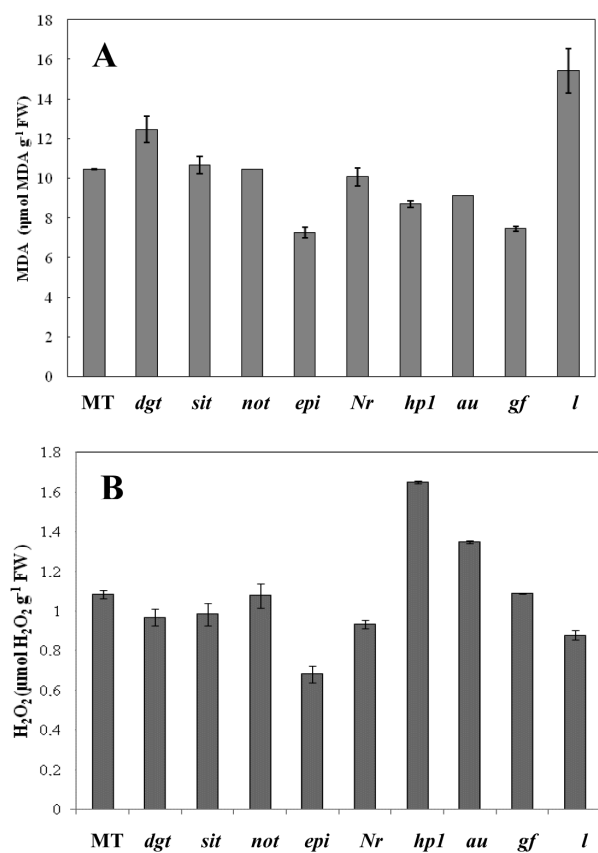


Fig. 1 – MDA (A) and  $H_2O_2$  (B) contents in extracts of leaves isolated from tomato mutants grown over 40 days after anthesis. MT (Micro-Tom); *dgt* (*diageotropica*); *sit* (*sitiensis*); *not* (*notabilis*); *epi* (*epinastic*); *Nr* (*Never ripe*); *hp1* (*high pigment 1*); *au* (*aurea*); *gf*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

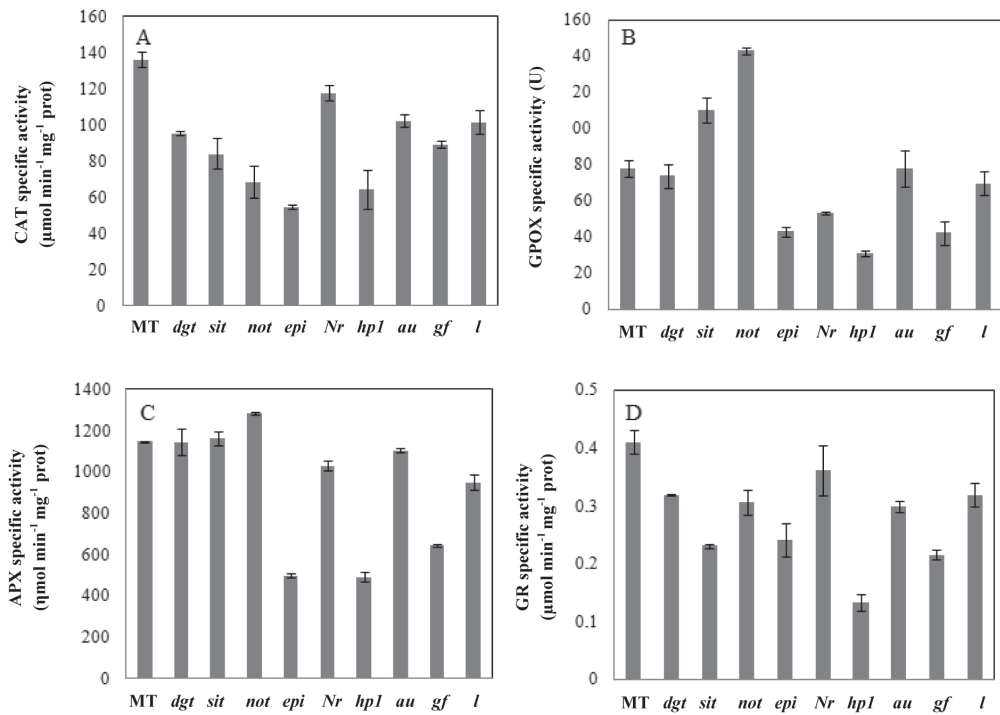


Fig. 2 - Catalase (CAT) (A), guaiacol peroxidase (GPOX) (B), ascorbate peroxidase (APX) (C) and glutathione reductase (GR) (D) total activities in extracts of leaves isolated from tomato mutants grown over 40 days after anthesis. MT (Micro-Tom); *dgt* (*diageotropica*); *sit* (*sitiens*); *not* (*notabilis*); *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.

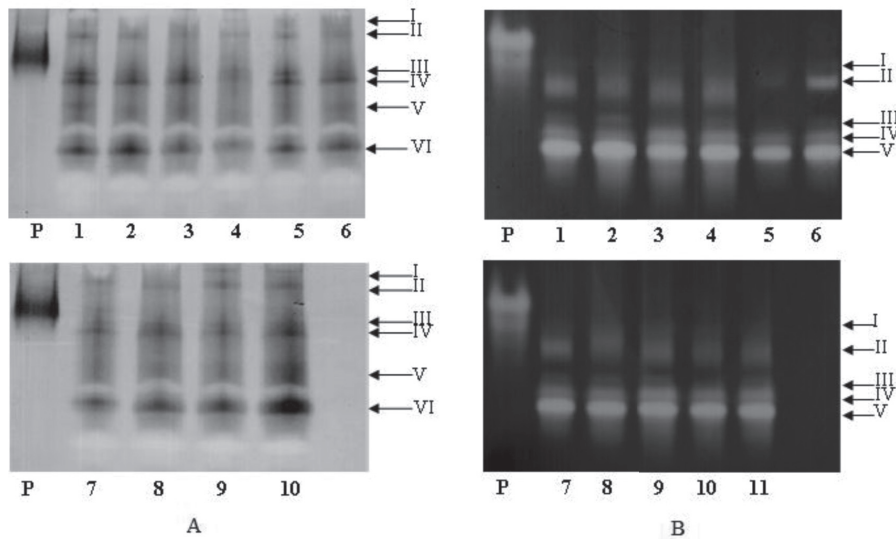


Fig. 3 - Glutathione reductase activity staining (A) and superoxide dismutase activity staining (B) following non-denaturing polyacrylamide gel electrophoresis of leaf extracts isolated from tomato mutants grown over 40 days after anthesis. The lanes listed in (A) are: (P) *Saccharomyce cerevisiae* GR 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*).



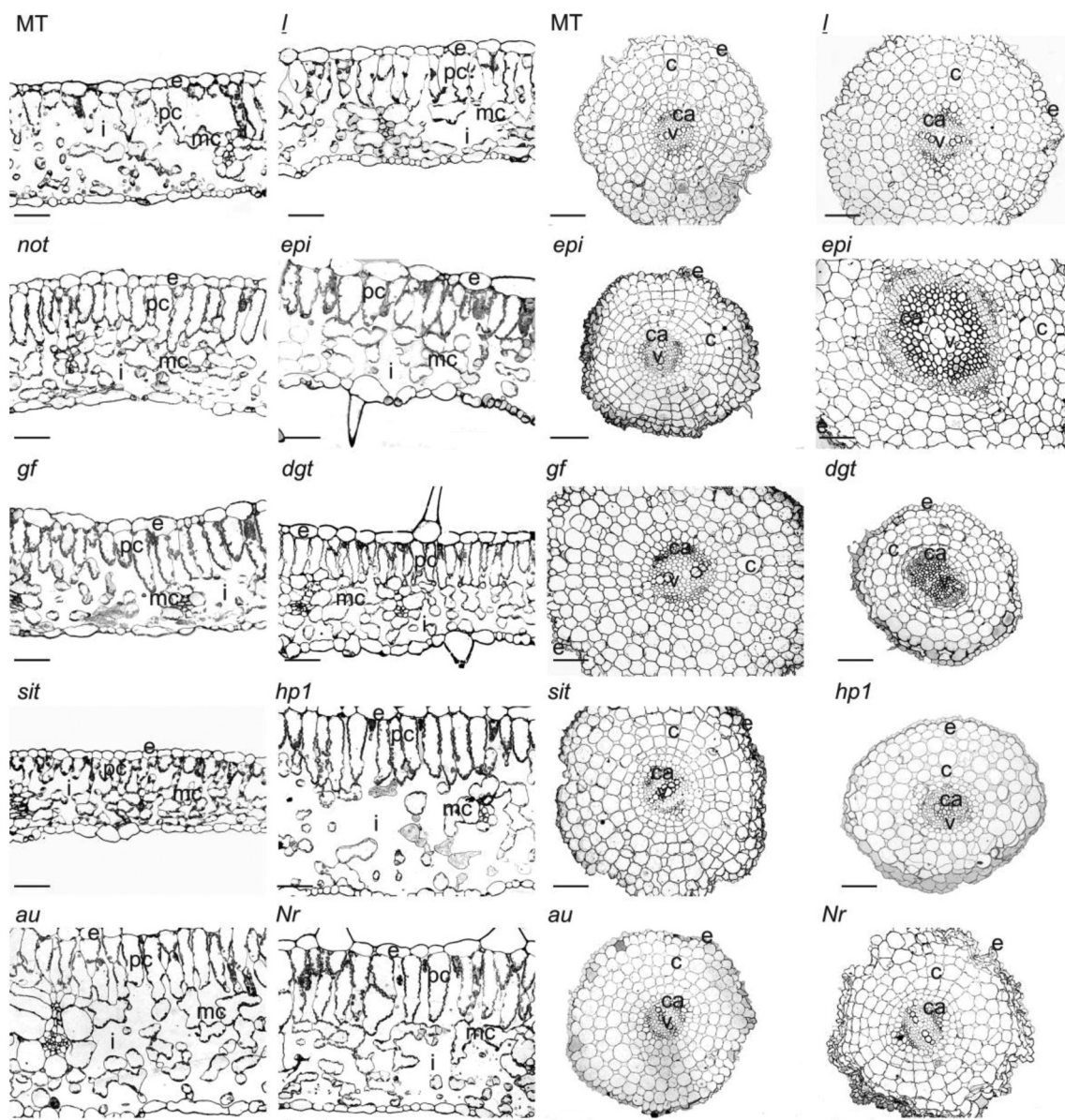


Fig. 4 - Leaf and root cross-sections of tomato mutants grown in nutrient solution observed by light microscopy. Leaves (A); Roots (B); c = cortex; ca = cambium; e = epidermis; i = intercellular spaces; sc = spongy PC = palisade mesophyll cells; v = vascular cylinder. Bars = (A) = 50µm; (B) 3-4 = 100 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* (*lutescens*).

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 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.

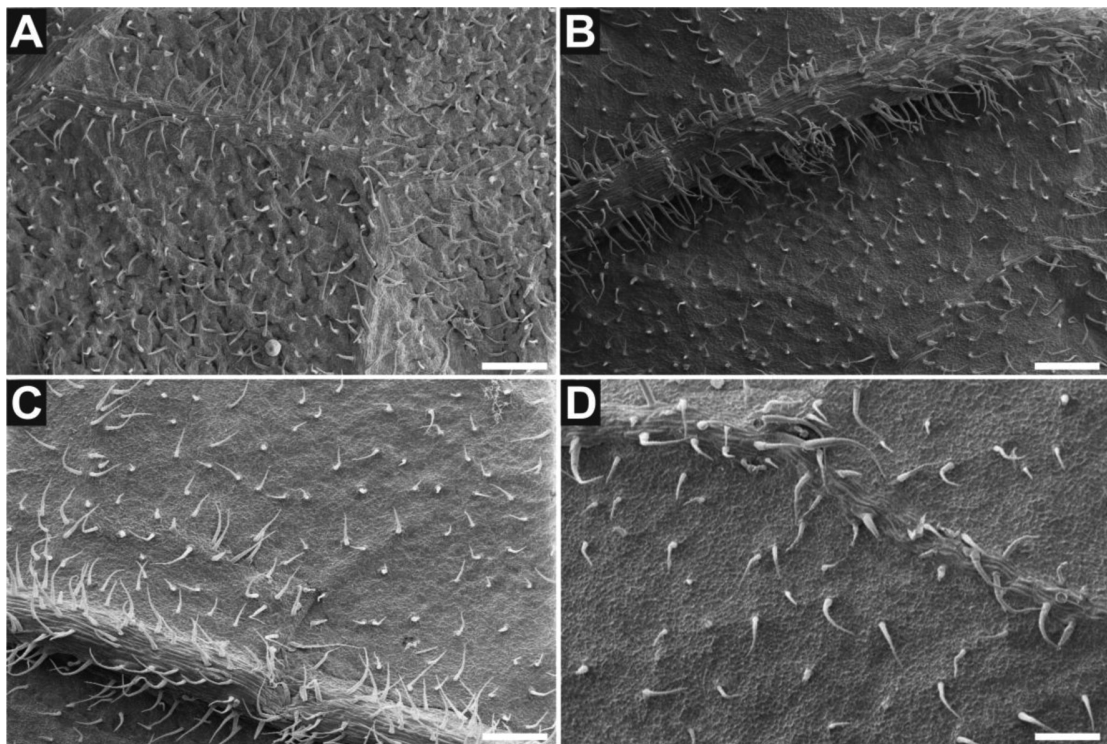


Fig. 5 - Trichome distribution in the abaxial epidermis of the leaf in tomato mutants observed by scanning electron microscopy. (A) *dgt* (*diageotropica*); (B) MT (*micro-tom*); (C) *au* (*aurea*); (D) *hp1* (*high pigment 1*). Bars = 500  $\mu$ m.

On the contrary, *hpl* increases intercellular spaces of mesophyll (Fig. 4A) and in accordance to this result, the *hpl* 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 *hpl* need to be further elucidated, although, so far, it is known that *hpl* display exaggerated light response, and that *hp* alleles represent mutations in a tomato *UV-DAMAGED DNA-BINDING PROTEIN 1* (*DDBI*) 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 *hpl* 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 *hpl* 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 gibberellin 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<sub>2</sub>O<sub>2</sub> foram encontrados nos mutantes *high pigment 1* (*hpl*) 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 glutatona 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 *hpl* 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 *hpl* 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*.

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