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ДИНАМИКА НА АНТИОКИСЛИТЕЛНИЯ ОТГОВОР НА *IN VITRO* РАСТЕНИЯ, ПОДЛОЖЕНИ НА СВЕТЛИНЕН СТРЕС
DIFFERENT TIMINGS OF ANTIOXIDATIVE RESPONSE OF *IN VITRO* PROPAGATED PLANTS UNDER LIGHT STRESS

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Резюме

Растенията от три вида икономически важни култури **-** *Vitis vinifera* L., *Solanum lycopersicon* Mill. и *Nicotiana benthamiana* L., бяха отглеждани *in vitro* при слаба светлина (50 μmol $m^{-2}s^{-1}$) и впоследствие прехвърлени на силна светлина (200 μmol $m^{-2}s^{-1}$) в продължение на 7 дни. За идентифициране на симптомите на окислителния стрес и възстановяване след стреса беше отчетена активността на ензимите, свързани с аскорбат-глутатионовия цикъл, както и експресията на гени, кодиращи тези ензими. Активността на SOD, CAT и APX беше по-висока на 2-3-и и 6-ти ден в *S. lycopersicon* и на 1-2-и и 5-7-ми ден в *N. benthamiana* и *V. vinifera.* Експресията на съответните транскрипти показват значително увеличаване през първия ден в *S. lycopersicon,* докато в *N. benthamiana* и *V. vinifera* беше установена двувълнова зависимост с пикове в първия и седмия ден. Тези резултати показват, че двата вида *Solanaceae* показват различни стратегии, когато са подложени на светлинен стрес, доказващи уникалността на отговора на всеки вид. Освен това поведението на *N. benthamiana* кореспондира тясно с реакцията на видове като *V. vinifera.*

Abstract

Three plant species, the worldwide economically relevant crops *Vitis vinifera* L.and *Solanum lycopersicon* Mill. and the model tobacco *Nicotiana benthamiana* L. were propagated *in vitro* under low light (50 μmol $m^{-2}s^{-1}$) and transferred to HL (200 μmol $m^{-2}s^{-1}$) for 7 days. To identify oxidative stress symptoms and recovery we monitored the activity of enzymes related with the ascorbate-glutathione cycle and the expression of the genes that code for these enzymes. SOD, CAT and APX activities were higher on d2-3 and d6 in *S. lycopersicon* and on d1-2 and d5-7 in *N. benthamiana* and *V. vinifera*. The expression of the respective transcripts showed a significant increase on d1 in *S. lycopersicon* while in *N. benthamiana* and *V. vinifera* a bimodal pattern was found, with peaks on d2 and d7. These results indicate that the two *Solanaceae* display different strategies when responding to light stress, evidencing further the uniqueness of the response of each species. Moreover, the behaviour of *N. benthamiana* falls closely into the pattern of a woody species such as *V. vinifera*.

Ключови думи: аскорбат-глутатионов цикъл, ex vitro, фотоокислителен стрес, real-time PCR.
Key words: asc-glut cycle, *ex vitro* growth, photooxidative stress, real-time PCR.

INTRODUCTION

In plants, photooxidative stress caused by exposure to high light can occur in structures emerging from tissues under dark or low light conditions. For example, leaves in shade in the canopy can experience large fluctuations in irradiance (Demmig-Adams and Adams, 1992). In these situations excitation energy in excess of that required for photosynthetic metabolism creates the potential for the photoinhibition of photosynthetic electron transport (Niyogi, 1999; Mullineaux et al., 2006; Ort and Baker, 2002). As a consequence, the excessive production of reactive oxygen species (ROS) can cause oxidative damage to cellular components. The involvement of ROS in several biotic and abiotic stresses is well documented (for review see Mittler et al., 2004), but aside from oxidative damage, they play an important role in the control and regulation of biological processes, such as growth and development, cell cycle, programmed cell death and

hormone signalling. The role of ROS as signalling molecules suggests that, during the course of evolution, plants achieved a high degree of control over ROS toxicity. This control appears to require a large gene network, whose functioning is beginning to be unveiled.

The wide network of antioxidants providing protection against ROS consists mostly of enzymes such as superoxide dismutase (SOD), that scavenges the superoxide radical ($O_2^{\cdot-}$), and ascorbate peroxidase (APX) and catalase (CAT), that detoxify H_2O_2 (Niyogi, 1999; Panchuk et al., 2002). In addition, lipid soluble, membrane-associated antioxidants (*eg* tocopherols, в-carotene and ubiquinone) and water soluble antioxidants (*eg* glutathione and ascorbate) play a role in preventing lipid oxidation and accumulation of ROS (Niyogi, 1999). L-ascorbic acid (AsA) is an abundant metabolite that plays important roles both in stress physiology and in growth and development. In the detoxification of ROS, AsA has the capacity to directly eliminate several different ROS including singlet oxygen, superoxide and hydroxyl radicals (Padh, 1990). Indirectly it eliminates H_2O_2 through the activity of APX. Glutathione (r-L-Glu-L-Cys-Gly, GSH), aside from being a major reservoir of non-protein reduced sulphur, has crucial functions in cellular defence and protection, preventing the denaturation of proteins caused by stress imposed oxidation of thiol groups. Glutathione reacts chemically with a range of ROS, while enzyme-catalysed reactions link GSH to the detoxification of H_2O_2 in the ascorbate-glutathione cycle (asc-glut, Noctor et al., 2002) which keeps the cellular pools of AsA and GSH in their reduced state by a set of enzymes using NAD(P)H such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). The asc-glut cycle besides impairing oxidative stress also regulates photosynthesis in response to light conditions through the dissipation of excess excitation energy (Asada, 1999). The response to photooxidative stress varies between species and ontogenic phases. Apparently, different genes coding for antioxidative enzymes have evolved, although the regulatory signalling systems could still be similar (Karpinski et al., 1997).

Usually, investigations focused on the response to excess light use short-term, artificial high irradiances (Karpinski et al., 1997; Chang et al., 2004) instead of a more physiological continuous stress-inducing intensity. Plants cultured *in vitro* develop in heterotrophic conditions under very low light intensities. I*n vitro* propagated plants are potentially capable of attaining measurable photosynthetic rates (van Huylenbroeck et al., 2000; Carvalho et al*.,* 2001), however this capacity does not prevent the symptoms of photoinhibition that appear when plants are subjected to the higher light intensities applied upon transfer to *ex vitro* (Carvalho et al., 2001). Furthermore, *in vitro* conditions predispose plants to a down-regulation of photosynthesis, either resulting from a lack of CO_2 in the culture vessels, or from the feedback inhibition of Calvin cycle enzymes by the sucrose supply in the media. Thus, the transition phase can be used to comprehensively study the response to high light stress and the recovery period when plants adjust to the new conditions. We have studied *in vitro* propagated plants of grapevine (*Vitis vinifera* L.), tobacco (*Nicotiana benthamiana* L.) and tomato (*Solanum lycopersicon* Mill.) and monitored the activity of enzymes related with the ascorbate-glutathione cycle and the expression of the genes that code for these enzymes.

MATERIAL AND METHODS

Plant material and sampling

Nodal buds of *Vitis vinifera* L., var. Touriga Nacional were used as explants for *in vitro* multiplication. Seeds of *Nicotiana benthamiana* L. and *Solanum lycopersicon* Mill. were germinated *in vitro* for 3 weeks in Murashige and Skoog (1962), MS (Duchefa Biochemie, Haarlem, NL) basal medium. *In vitro* shoots were sub-cultured every four weeks into MS basal medium supplemented with 0.5 µM б-naphthaleneacetic acid (NAA) and 2.0 µM benzylaminopurine (BAP) for *N. benthamiana* and *V. vinifera* and 4.5 µM BAP for *S. lycopersicon*. Shoots were elongated in MS with BAP at 1.67 µM for two weeks. For root induction, explants received a supplement of 2 µM NAA for 5 days. Cultures were kept in a growth chamber at a photosynthetic photon flux density (PPFD) of 45 ± 5 µmol $m^{-2}s^{-1}$ and a photoperiod of 16/8h. Temperature was 25 ± 1єC during the light and 22 ± 1єC during the dark.

Ex vitro root expression took place for seven days as in Carvalho et al. (2001), with the sole exception of PPFD at plant level, which was 200 ± 10 µmol $m^{-2}s^{-1}$. The analyses were performed in samples of leaves at time zero of *ex vitro* growth (d0) and on d1, d2, d4 and d7, collected in the middle of the light period.

Native PAGE, activity staining and quantification

Leaf material (0.5 g) was ground in liquid N_2 in the presence of 50% (w/w) polyvinylpolypyrrolidone. For extraction, 5 mL of ice-cold 350 mM Tris-HCl buffer pH 8.0 supplemented with 0.2 mM phenylmethylsulphonylfluoride, 20 mM sodium ethylenediaminetetraacetate, 11 mM sodium diethyldithiocarbamate and 15 mM cysteine were used. Homogenates were centrifuged at 27 000 *g* for 10 min at 4 єC. The supernatants were desalted through PD-10 columns (Amersham Pharmacia Biotech, Little Chalfont, UK). Protein concentration was determined according to Bradford (1976), using a commercial kit (Bio Rad, Hercules, CA). Isoforms of SOD, CAT, GR and APX were separated using nondenaturing polyacrylamide gels by the procedure of Laemmli (1970). Equal amounts of protein extracts (25 µg) were loaded on 7% (CAT) or 10% (GR, SOD and APX) polyacrylamide gels.

For SOD, the gel was stained according to Rao et al. (1996) and visualization was performed according to Donahue et al. (1997). To identify KCN- and H_2O_2-sensitive isoforms, the incubation solution contained 3 mM KCN or 5 mM H_2O_2, respectively. KCN inhibits CuZnSOD while both CuZnSOD and ISOD are sensitive to H_2O_2, allowing the discrimination of MnSOD. To visualize CAT activity, gels were stained by the procedure of Anderson et al. (1995) and isoforms of APX and GR were visualized according to Carvalho et al. (2006). Relative quantification of all isoenzyme activities was determined using the software Quantity One (Bio-Rad, Hercules, CA) in comparison to control (d0=100%).

RNA isolation, cDNA preparation and Real-Time PCR

Total RNA from leaves was extracted by adapting the method described by Gevaudant et al. (1999). RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and reverse transcribed using random hexamers and Superscript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

The real-time PCR was performed using 0.5 µM gene-specific primers and master mix iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in an iQ5 Real Time PCR (Bio-Rad, Hercules, CA). In order to compare data from different PCR runs or cDNA samples, C_T values were normalized to the C_T value of *Act2*, a housekeeping gene expressed at a relatively high and constant level. Gene expression was calculated using the $\Delta\Delta C_T$ method. Results are presented as fold variation in relation to control (d0). Three independent measures were made for each time point (n=3). The results were statistically evaluated through variance analysis comparing the days. Significant values were discriminated with Tukey's post test, $p<0.01$, using GraphPad InStat (GraphPad Software, CA).

RESULTS AND DISCUSSION

To analyze the effects of the transfer of micropropagated plants to an autotrophic environment under high light, the functioning of the antioxidative network was assessed during the first week of *ex vitro* growth. We quantified the mRNA levels of nine genes encoding ROS scavenging and antioxidant enzymes using quantitative real time RT-PCR. In parallel, the activities of their isoforms by activity staining in non-denaturing polyacrylamide gels were also determined. The timing of up-regulation of antioxidative genes after transfer to *ex vitro* differed between the three species. In tomato some transcripts increased up to 250 fold immediately after the onset of high light (d1), declining thereafter, while tobacco and grapevine showed a bimodal pattern of transcript expression with peaks measured between 10- to 30-fold variation and 5- to 16-fold, respectively, at d2 and d7 (Fig 1).

In tomato three isoforms of APX were identified, two constitutive and up-regulated in the second half of the experiment (APX-A and APX-B) and one repressed (APX-C), from d2 onwards (Table 1). From the relative positions of the bands and by comparison with Arabidopsis isozymes (e.g. Panchuk et al., 2002), it was deduced that APX-C corresponds to the cytosolic APX1. In tobacco, on the other hand, only two isoforms (APX-A and APX-B) were present, both constitutive and without noticeable variation. Three different isoforms of APX were detected in grapevine, one constitutive (C) and two induced (A and B). The band APX-C corresponds to the cytosolic APX1 and was detected since d0, reaching a maximum on d1. After the minimum level on d4 a second peak was detected on d7. The induced isoforms APX-A and APX-B were detected on d1 and increased their activity on d2, after which they were no longer detected. The activation of the antioxidative response through the asc-glut cycle was further confirmed by the induction of mRNA expression (Fig. 1), tobacco *APX3* expression was significantly high on d2 and d7 and *APX1* showed a tenfold increase on d7 (Fig 1). In grapevine *APX1* was significantly upregulated from on d1 and d7; *APX3* expression increased from d2. In tomato, both the activity and gene expression of cytosolic APX1 increased at day 1, apparently in relation to the higher irradiance after transfer to *ex vitro*, for high light is necessary to overcome the stabilizing effect of ascorbate and to induce significant cytosolic *APX* expression (Karpinski et al., 1997). The enhanced activity of APX1 resulted in a higher proportion of enzyme activity in the cytosol what is critical for the control of the signalling role of H_2O_2, communicating the information to the nucleus (Shigeoka et al., 2002) and thus regulating the molecular mechanisms of tolerance against oxidative stress.

In gel analysis revealed one CAT isoform in grapevine, with a peak of activity on d2 (*ca* 150% increment), a decrease on d4 to values equivalent to d0 and a minimum on d7 (Table 1). In tobacco also one isoform was detected, with maximum activity on d1 while in tomato two constitutive isoforms (CAT-A and CAT-B) were detected, CAT-A decreasing in the second half of the experiment and CAT-B peaking on d2. In tomato, *CAT* showed a significant upregulation on d1, while in tobacco *CAT* showed the same pattern as *APXs* and *SODs*, with a peak on d2 (25-fold) and a smaller one on d7 (seven-fold) and in grapevine, it was steadily upregulated from d1 on (Fig 1).

Redox homeostasis and ROS pools and signalling are decisive for the acclimation to new light conditions (Mullineaux et al., 2006). The asc-glut cycle, present in practically every cellular compartment (Mittler et al., 2004) performs a crucial role in the control of ROS in each compartment. CAT, with a low affinity to H_2O_2 and present mainly in peroxisomes, is associated with the processing of H_2O_2 generated in photorespiration (Vandenabeele et

Table 1. Relative quantification of the in gel activities of APX, SOD, GR and CAT isoenzymes in relation to d0 (d0 = 100%). Total protein extracts of leaves of *V. vinifera*, *S. lycopersicon* and *N.benthamiana* plants at d0, 1, 2, 4 and 7 d of *ex vitro* growth were subjected to native PAGE, followed by activity staining for each of the four enzymes. €GR activity is only available for *V. vinifera*; [b]These two APX isoforms are not present in *V. vinifera* at d0, their activity can only be detected from d1, thus the value 100% on d1 is used for relative comparison

	Nicotiana benthamiana					*Solanum lycopersicon*					*Vitis vinifera*				
	0	**1**	**2**	**4**	**7**	**0**	**1**	**2**	**4**	**7**	**0**	**1**	**2**	**4**	**7**
GR-B											100[a]	109	84	63	67
GR-A											100[a]	138	87	92	77
APX-A	100	97	93	95	98	100	99	98	129	132		100[b]	135		
APX-B	100	104	103	100	91	100	105	106	123	121		100[b]	129		
APX-C						100	97	96			100	244	186	62	48
CAT-A	100	254	90	69	107	100	107	101	83	76	100	86	251	103	70
CAT-B						100	104	125	116	114					
SOD-A	100	108	115	128	145	100	93	86	83	81	100	132	133	117	
SOD-B	100	104	117	133	148	100	88	80	79	80	100	149	157	141	
SOD-C						100	93	85							

Fig.1. *Changes in the expression levels of genes of the antioxidative system in N. benthamiana (a), V. vinifera (b) and S. lycopersicon (c). Quantification of mRNA levels of those genes was performed on d0, 1, 2, 4, and 7 of ex vitro growth. mRNA was isolated from leaves, converted to cDNA, and subjected to real-time PCR. Relative amounts were calculated and normalized with respect to Act2 mRNA. Each time point is compared to day 0 leaves (n = 3). For clarity purposes, three different scales were used*

al., 2004). However, it is also important when excessive H_2O_2 is formed, as under oxidative stress (Mittler, 2002). In tobacco and grapevine, as expected, APX activity increased at the onset of photooxidative stress, while the strongest up-regulation of CAT activity and transcript abundance only occurred latter, resulting in a steady decrease in H_2O_2 (Carvalho et al., 2006). In tomato, however, *APX1* and *APX3* abundance remained close to basal levels, indicating that the enzyme present on d0 could sustain the increase in activity observed later on.

The three SOD isoforms present in tomato are MnSODs, the constitutive SOD-A and B were slightly down-regulated during the experiment while SOD-C disappeared after d2. In tobacco SOD-A corresponds to an ISOD while SOD-B is a MnSOD (Table 1). In grapevine three different SOD isoforms were detected, two constitutive MnSODs, SOD-A and SOD-B, and one induced, SOD-C, a copper-zinc isoform. Transcript abundance of tomato *MnSOD* and *ISOD* was high on d1, unlike *Cu-ZnSOD*, which increased only slightly (Fig 1). In tobacco, the three transcript isoforms presented the characteristic bimodal variation, *ISOD* showing the highest abundance. In grapevine only *ISOD* and *MnSOD* showed the bimodal pattern while *Cu-ZnSOD* was upregulated from d1.

SOD is crucial to dismutate O_2^- into the less reactive ROS H_2O_2 and its transcription, expression and activity is up-regulated in response to photooxidative stress (Kliebenstein et al., 1998). The results observed in the three species studied put in evidence the response of SOD to the changes in the light regime. The high levels of *MnSOD* and MnSOD activity can also be related to the high sugar content in previous heterotrophic growth conditions (Koch, 1996).

In grapevine two isoforms of GR were seen on gels, both constitutive, GR-A and GR-B (Table 1), corresponding, respectively to the plastid GOR1 and to the cytosol GOR2, as evidenced by molecular weight comparison. In *Arabidopsis* under excessive light *GOR2* expression was maintained constant, increasing slightly at the recovery period (Karpinski et al., 1997), whereas in grapevine, GR activity decreased slightly thus appearing to be suficient to maintain cellular GSH/GSSG.

Tomato *DHAR* and *MDHAR* transcripts followed the pattern of up-regulation on d1, while in tobacco the abundance of both transcripts was once more bimodal, with peaks on d2 and d7 as in grapevine where *DHAR* also presented a bimodal pattern while *MDHAR* was downregulated until d7 (Fig 1).

CONCLUSION

From this study it is possible to conclude that comprehensive analyses are necessary to draw a reliable picture of the overall response to stress conditions. Our results indicate that the two species from the *Solanaceae* family display different strategies when responding to light stress, evidencing further the uniqueness of the response of each species. Tobacco and grapevine displayed a typical bimodal pattern, with a peak of expression of key genes of antioxidative response on d2, correlating well with the generation of ROS (Carvalho et al., 2006) and a second peak on d7, indicating a cellular signalling for the simultaneous formation of new structures, roots and new leaves. In tomato, the peak of gene expression takes place earlier, on d1, and the absence of a second peak can be explained by the early protrusion of roots, not connected in time with leaf expansion.

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