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Oxidative stress response in sugarcane

Luis Eduardo Soares Netto

Abstract

Oxidative stress response in plants is still poorly understood in comparison with the correspondent phenomenon in bacteria, yeast and mammals. For instance, nitric oxide is assumed to play various roles in plants although no nitric oxide synthase gene has yet been isolated. This research reports the results of a search of the sugarcane expressed sequence tag (SUCEST) database for homologous sequences involved in the oxidative stress response. I have not found any gene similar to nitric oxide synthase in the SUCEST database although an alternative pathway for nitric oxide synthesis was proposed. I have also found several genes involved in antioxidant defense, *e.g.* metal chelators, low molecular weight compounds, antioxidant enzymes and repair systems. Ascorbate (vitamin C) is a key antioxidant in plants because it reaches high concentrations in cells and is a substrate for ascorbate peroxidase, an enzyme that I found in different isoforms in the SUCEST database. I also found many enzymes involved in the biosynthesis of low molecular weight antioxidants, which may be potential targets for genetic manipulation. The engineering of plants for increased vitamin C and E production may lead to improvements in the nutritional value and stress tolerance of sugarcane. The components of the antioxidant defense system interact and their synthesis is probably closely regulated. Transcription factors involved in regulation of the oxidative stress response in bacteria, yeast and mammals differ considerably among themselves and when I used them to search the SUCEST database only genes with weak similarities were found, suggesting that these transcription regulators are not very conserved. The involvement of reactive oxygen species and antioxidants in plant defense against pathogens is also discussed.

INTRODUCTION

Hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\circ}$) and the hydroxyl radical (OH°) are collectively called reactive oxygen species (ROS) and participate in multiple processes in plants. They are formed as toxic byproducts of respiration and photosynthesis and participate in plant defense against pathogens and may have signaling roles (Inze and Montagu, 1995; Bolwell, 1999).

One of the most important sources for the production of reactive oxygen species is the leakage of electrons from the mitochondrial respiratory chain, especially at the ubiquinone site, where reduced ubiquinone can donate electrons to molecular oxygen and generate superoxide radicals which can then be converted to hydrogen peroxide by spontaneous or by catalytic dismutation (Halliwell and Gutteridge, 1999). Superoxide radicals and hydrogen peroxide are not very reactive but they can be converted to hydroxyl radicals (which are very strong oxidants) by the Haber-Weiss reaction ($H_2O_2 + O_2^{-\circ} \rightarrow OH^\circ + OH^- + O_2$). This reaction is catalyzed by transition metals such as iron and copper (Halliwell and Gutteridge, 1999).

In chloroplasts, hydrogen peroxide and superoxide radicals are formed by both the leakage of electrons from transport chains and light dependent processes. Hydroxyl radicals can then be formed in the chloroplast through Haber-Weiss reaction as described for mitochondria. Singlet oxygen is another reactive oxygen species produced by an input of energy (*e.g.* light) to molecular oxygen. As would be expected, singlet oxygen is formed in high amounts in chloroplasts because this organelle is exposed to high intensity light (Halliwell and Gutteridge, 1999). Besides reactive oxygen species, the formation of reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite has been extensively reported in plants in spite of the fact that their source has not been clearly defined (reviewed by Bolwell, 1999). Reactive oxygen and nitrogen species can promote lesions in biomolecules such as DNA, proteins and lipids.

Like most aerobic organisms, plants have developed different systems to cope with the toxic effects of reactive oxygen and nitrogen species. The first line of antioxidant defense involves preventing the formation of reactive oxygen species. Metal chelators can prevent the formation of reactive oxygen species by suppressing processes such as the Haber-Weiss reaction and consequently reducing the production of the very reactive hydroxyl radical. The second line of antioxidant defense is composed of antioxidant enzymes and low molecular weight compounds. If the first line of antioxidant defense fails to prevent the formation of reactive species, antioxidant components decompose reactive species avoiding the formation of oxidative lesions in biomolecules. If reactive species can not be eliminated, and succeed in attacking biomolecules, various systems are able to repair oxidative lesions produced. It is important to

Departamento de Biologia, IB, Universidade de São Paulo, Rua do Matão 277, 05508-900 São Paulo, SP, Brazil.
Send correspondence to Luis Eduardo Soares Netto. E-mail: nettoles@ib.usp.br.

understand that these components do not act individually and there are regulatory systems, which coordinate the oxidative stress response. The signaling processes involved in oxidative stress response are not as well understood in plants as in bacteria, yeast and mammals.

Reactive oxygen and nitrogen species are sometimes employed for useful processes, *e.g.* phagocytosis takes advantage of reactive species to kill pathogens that are present in human blood (Halliwell and Gutterdige, 1999) and, interestingly, plants have also developed systems to increase the generation of reactive species in order to kill pathogens (see reviews by Bolwell, 1999; Grant and Loake, 2000),

In the research reported in this paper a search was made of the sugarcane expressed sequence tag (SUCEST) database to check for the presence of oxidative stress response components described in other organisms and investigate possible systems for destroying pathogens by the generation of reactive oxygen and nitrogen species.

MATERIALS AND METHODS

The identification of putative genes involved in the oxidative stress response of sugarcane was performed using the `cluster by keyword' service provided by the bioinformatic group of the SUCEST project (http://sucest.lad.dcc.unicamp.br/en/Services/services.html). Genes were considered similar when their basic local alignment search tool (blastx) e-value was less then e^{-20}. When components of the oxidative stress response systems of other organisms were not found by this procedure a t-blast N search was performed using the program available at the SUCEST site. In this case genes from organisms such as bacteria, yeast, mammals and plants other than sugarcane were searched against the SUCEST database, again using a cut-off e-value of less than e^{-20}. The clusters analyzed were from the CAP3 system, as denominated by the bioinformatic SUCEST group. Supplementary information can be found at http://sucest.lad.dcc.unicamp.br/private/mining-reports/UI/UI-mining.htm.

RESULTS AND DISCUSSION

Possible sources of reactive oxygen and nitrogen species in sugarcane

Besides the generation of reactive oxygen species, mammalian cells can also produce reactive nitrogen species because they have nitric oxide synthase (NOS), a family of enzymes, which catalyzes the oxidation of arginine with the subsequent production of nitric oxide, a relatively stable free radical that participates in various signaling pathways. A growing body of evidence suggests that nitric oxide plays a key role in activating disease resistance in plants by acting as a signaling molecule and, possibly, as a direct antimicrobial agent (Grant and Loake, 2000; Bolwell, 1999). It is generally accepted that nitric oxide is produced in plants but no nitric oxide synthase gene has yet been cloned from plants. In my search of the SUCEST database I found no gene homologous to nitric oxide synthase, nor did I find PIN (Protein Inhibitor of Neuronal Nitric Oxide Synthase), although a gene similar to PIN has been found in *Arabidopsis thaliana* (Jaffrey and Snyder, 1996).

Nitric oxide (NO) can also be generated as a byproduct of denitrification, nitrogen fixation and/or respiration, *e.g.* nitric oxide can be produced from nitrite (NO_2^-) by the action of NADH nitrate reductases (Yamasaki and Sakihama, 2000). Nitric oxide can then be further converted to the extremely toxic peroxynitrite ($ONOO^-$) by a reaction involving the superoxide radical (Halliwell and Gutteridge, 1999). A putative NADPH nitrate reductase has been found in the SUCEST database involving clusters SCSBFL 4010b11.g and SCRLRZ3116c08.g, whose e-values were $5e^{-91}$ and $1e^{-85}$ respectively in relation to genes gi128191 and spP27968.

Another potential source of reactive species in plants is the β-oxidation of fatty acids in peroxisomes, where dehydrogenases use oxygen to oxidize fatty acid and generate hydrogen peroxide. I detected one of the enzymes involved in this process, glycolate oxidase, in the SUCEST database at clusters SCCCLR1c02a06.g and SCEQRT 2026e04.g, which possess e-values of e^{-171} and $1e^{-166}$, respectively, in relation to gene gi7431428.

NADPH oxidases are integral membrane proteins that promote superoxide formation by the oxidation of NADPH. In mammalian neutrophils, these proteins are involved in the host defense against pathogens (reviewed by Grant and Loake, 2000). The Rboha protein from *Arabidopsis thaliana* and from rice are similar to the mammalian $gp91^{phox}$, a subunit of neutrophil NADPH oxidase (Groom *et al.*, 1996; Keller *et al.*, 1998). I found one SUCEST cluster (scagrt2037g05.g) similar to the $gp91^{phox}$ subunit, the cluster having an e-value of $2e^{-50}$ in relation to the gi8131889 gene, but I found no clusters similar to other neutrophil NADPH oxidase subunits (*e.g.* $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$) in the SUCEST database nor in any other plant databases. This enzyme is discussed again later in this paper in the section on plant-pathogen interactions.

Metal chelators in sugarcane

Metallothioneins are low molecular weight, cysteine-rich proteins responsible for regulating the intracellular supply of biologically essential zinc and copper ions and phytochelatins are glutathione (GSH) polymers capable of binding transition metals, both types being found in sugarcane (see the companion paper by Figueira *et al.* (2001) in this volume).

Ferritin is an intracellular molecule that stores iron in a soluble, nontoxic, readily available form. The functional molecule is composed of 24 chains and is roughly spherical, containing a central cavity in which the polymeric fer-

ric iron core is deposited (Halliwell and Gutteridge, 1999). In contrast to the synthesis of ferritin in mammals, in plants the synthesis of this iron storage protein in response to iron is not regulated at the translational level but instead part of the response is mediated through a transduction pathway involving the plant hormone abscisic acid (Fobis-Loisy *et al.*, 1995). In maize there are two genes coding for ferritin Fer 1 and Fer2 (Fobis-Loisy *et al.*, 1995), and I found homologues for both these genes in the SUCEST database (Table I).

Low molecular weight antioxidants

Because they are well-characterized antioxidants, I will focus on discussing the biosynthetic pathways of glutathione (GSH), ascorbate (vitamin C) and alpha-tocopherol (vitamin E), although many other low molecular weight compounds may act as antioxidants but their protective effects and/or biosynthetic pathways are not clearly understood.

Ascorbate is the quantitatively predominant antioxidant in plant cells and is probably the most important antioxidant in plants, being found in all subcellular compartments (including the apoplast) and having an average concentration of 2-25 mM or more in the chloroplast stroma (reviewed by Smirnoff, 2000). Ascorbate is also the substrate for many of proteins described later in this paper in the section on antioxidant enzymes.

The reaction of ascorbate with active species such as singlet oxygen and hydroxyl radicals leads to the formation of a stable free radical (monodehydroascorbate radical). One of the fastest reactions of the monodehydroascorbate radical is its own dismutation, leading to the termination of free radical reactions (Halliwell and Gutteridge, 1999). The role of ascorbate in protecting plants against oxidative stress is also highlighted by the hypersensitivity of *Arabidopsis thaliana* mutant for vitamin C synthesis (reviewed by Smirnoff, 2000). Total ascorbate contents are generally higher in leaves, with decreasing amounts in stems and roots and relatively constant levels in all stages of fruits.

The oxidized forms of ascorbate (monodehydroascorbate radical and dehydroascorbate) are regenerated through enzymatic reactions. I found sequences encoding both of these ascorbate-regenerating enzymes in the SUCEST database (Table II).

Given the importance of ascorbate in plant cells, it is surprising that its biosynthetic pathway is still not completely understood. Initially, ascorbate synthesis in plants was considered to be similar to the pathway described in animals such as the rat, with D-galacturonate and L-galactono-1.4-lactone as two key intermediates. Conklin *et al.* (1999) and Smirnoff (2000) favor another biosynthetic pathway with D-mannose and L-galactose as the key intermediates (Diagram 1):

Diagram 1

glucose-6-phosphate $\xrightarrow{1}$ fructose-6-phosphate $\xrightarrow{2}$

mannose-6-phosphate $\xrightarrow{3}$ mannose-1-phosphate $\xrightarrow{4}$

GDP-mannose $\xrightarrow{5}$ GDP-L - galactose $\xrightarrow{6}$ L-galactose $\xrightarrow{7}$

L-galactone-1.4- lactone $\xrightarrow{8}$ ascorbate

This pathway for ascorbate synthesis shares steps with the synthesis of cell wall polysaccharide. The genes involved in steps 5, 6 and 7 of Diagram 1 have not so far been cloned, although the respective enzymatic activities have been detected (reviewed by Smirnoff, 2000). Elucidation of the ascorbate biosynthetic pathway should allow the engineering of plants for increased ascorbate production, which will, supposedly, increase their nutritional value and stress tolerance. Homologues for the other steps shown in Diagram 1 are described in Table III.

Another important antioxidant, glutathione, is a tripeptide composed of glutamate, cysteine and glycine. The antioxidant properties of glutathione being due to the thiol group of its cysteine residue. Glutathione also binds transition metals, especially copper, and acts in the first line of antioxidant defense by preventing the formation of active species (reviewed by Halliwell and Gutteridge, 1999). Two genes (*GSH1* and *GSH2*) are responsible for the synthesis of glutathione and in *de novo* GSH biosynthesis the first step is rate-limiting (reviewed by Halliwell and Gutteridge, 1999). In plants, the product of the *GSH1* gene (γ-glutamy-

Table I - Ferritin homologues found in the SUCEST database.

Homologous genes from *Zea mays*	SUCEST cluster	E-value[a]	Matches
ferritin *ZmFer1*	scrflr1012h03.g	e-113	gi | 2130127 |
	scjlrz1020d12.g	4e-73	
	scezrz1014g05.g	9e-98	
	scezrz3049f12.g	2e-46	
ferritin *ZmFer2*	scbghr1061g09.g	1e-71	gi | 120510 |
	scqshr1020d10.g	2e-58	sp | P29390 |

[a]Calculated with the basic local alignment search tool (blastx) program.

Table II - Homologues of enzymes required for ascorbate regeneration.

Homologous genes from *Oryza sativa*	SUCEST cluster	E-values[a]	Matches
Cytosolic	sccclr1079d10.g	0.0	gi | 4666287 |
monodehydroascorbate	scepcl6020c09.g	0.0	
reductase (ascorbate free	scjlrt1014h01.g	0.0	
radical reductase (AFR))	scrllr1059h02.g	0.0	
GSH-dependent	scccl3002a02.b	e-100	gi | 6939839 |
dehydroascorbate	scccl4006g01.g	e-100	
reductase	scrffl4007d04.g	e-100	

[a]Calculated with the basic local alignment search tool (blastx) program.

Table III - Genes involved in ascorbate biosynthesis in sugarcane.

Homologous gene	SUCREST cluster	E-value[a]	Matches
G6PI_ glucose-6-phosphate isomerase, cytosolic (GPI, EC 5.3.1.9) from *Zea mays*. Diagram 1, step 1	scqslr1061e07.g scmcrt2087g05.g scqsrt2033c10.g	0.0	gi|1346073| sp|P49105|
Putative mannose-6-phosphate isomerase from *Arabidopsis thaliana*. Diagram 1, step 2	Scacsb1037g01.g sccccl4012b05.g scrlcl6033h03.g	2e-44 3e-62	gi|6957720| gi|9755456|
Phosphomanno mutase homologue from *Arabidopsis thaliana*. Diagram 1, step 3	scjlfl3019c04.g scachr1040e04.g sccccl4013c08.g	e-114 3e-66 2e-43	gi|7444149| pir||T02468
GDP-mannose pyrophosphorylase from *Arabidopsis thaliana*. Diagram 1, step 4	scccrt1002h04.g scccrz1001b11.g	0.0	gi|6646773| gi|7448154|
L-galactono-1.4-lactone dehydrogenase from *Brassica oleracea.* Diagram 1, step 8	scbglr1119f12.g	e-131	gi|7488598| pir||T14463

[a]Calculated with the basic local alignment search tool (blastx) program.

lcysteine synthetase) can be found in chloroplasts or in the cytosol, although I found no cytosolic isoform in sugarcane (Table IV).

Alpha-tocopherol (vitamin E) is the most important lipophilic radical-chain-breaking antioxidant in living tissues. It also stabilizes biological membranes. The absence of alpha-tocopherol in membranes can make them highly permeable and vulnerable to degradation (reviewed by Halliwell and Gutteridge, 1999). The oxidized form of alpha-tocopherol (the alpha-tocopheryl radical) can be reduced by ascorbate (reviewed by Halliwell and Gutteridge, 1999).

Tocopherols are synthesized from precursors in two principal steps, step 1 provides the hydrophobic isoprenoid tail while step 2 provides the homogentisic acid head group (Diagram 2).

Diagram 2

Geranylgeranyl-diphosphate (GDDP) $\xrightarrow{1}$ phytyl-dyphosphate

phytyl-dyphosphate $\xrightarrow{2}$ 4 - hydroxyphenylpyruvic acid → homogentisic acid (HGA)

Diagram 3 shows the union of the head and the tail groups and the subsequent steps needed for tocopherol biosynthesis. The intermediate 2,3 - dimethyl-5- phytylquinol has been identified by tracer experiments with radio-labeled compounds but so far the enzymes required for catalysis of steps 4 and 5 have not been isolated in any plant (Arango and Heise, 1998; Hirschberg, J., 1999). The genes which I detected in the SUCEST database encoding enzymes involved in vitamin E synthesis are described in Table V. The great interest in increasing vitamin E content by engineering is highlighted by the fact that the sequence of phytyl/prenyl transferase gene (step 3) has been patented (Table V).

Diagram 3

phytyl dyphosphate + HGA $\xrightarrow{3}$ 2 -methyl-6-phytylquinol $\xrightarrow{4}$ 2,3 - dimethyl-5-phytylquinol $\xrightarrow{5}$ gama-tocopherol $\xrightarrow{6}$ alpha tocopherol

Antioxidant enzymes

Superoxide dismutase (SOD) was the first enzyme reported as being able to decompose a free radical. This en-

Table IV - Genes involved in glutathione biosynthesis in sugarcane.

Homologous gene	SUCRESTcluster	E-value[a]	Matches/Hits
Chloroplasmatic GSH1 precursor (gamma-glutamylcysteine synthetase) from *Lycopersicon esculentum* First step in glutathione synthesis	scvprz2041g04.g scjlrt1018b02.g scqglr2025d03.g scjlrt1018g11.g	e-109 2e-71 2e-71 2e-15	sp|O22493 gi|6651029|
GSH2 Glutathione synthetase from *Brassica juncea* Second step in glutathione synthesis	scqsrt1034d10.g scbglr1096a08.g scjllb2077f06.g	2e-77 5e-68 4e-31	gi|4808537|

[a]Calculated with the basic local alignment search tool (blastx) program.

Table V - Homologues of the sugarcane alpha tocopherol biosynthetic pathway.

Homologous gene	SUCEST cluster	E-value[a]	Matches/hits
Geranylgeranyl reductase from *Nicotiana tabacum*. Diagram 2, first step	scqglr2025h03.g scrufl1024e08.g scsgfl4035g02.g	0.0 2e-36 2e-77	gi|4733939| gb|AAD28640.1| gi|6815059|
(4HPPD) *PDS1* 4hydroxyphenylpyruvate dioxygenase from *Hordeum vulgare.* Diagram 2, second step	scjllr1103a12.g	3e-56	gi|3334222| sp|O48604|
Phytyl/prenyltransferase PDS2 from *Arabidopsis thaliana*. Diagram 3, third step	scjflr1013f09.g	e-102	emb/AX046714[c]
Gamma-tocopherol methyltransferase G-TMT from *Arabidopsis thaliana*. Diagram 3, sixtieth step	scvprz2039h03.g scsgst1071e12.g scsblb2037f06.g	e-118 3e-74 1e-67	gi|4106538|g

[a]Calculated with the basic local alignment search tool (blastx) program.
[b]Calculated with the tblastN program.

zyme decomposes the superoxide anion radical by catalyzing the reaction $O_2^{-\circ} + O_2^{-\circ} + 2H^+ \rightarrow O_2 + H_2O_2$. In maize, at least nine superoxide dismutase isoenzymes are coded by nine non-allelic nuclear genes: *Sod1, Sod2, Sod 3.1* (previously referred as *Sod3*), *Sod 3.2, Sod 3.3, Sod 3.4, Sod4, Sod4A* and *Sod5* (Zhu and Scandalios, 1993). The cytosolic isozymes SOD-2, SOD-4, SOD-4A and SOD-5, and the chloroplast associated SOD-1 are copper and zinc-containing homodimeric enzymes. The superoxide dismutase 3.x enzymes are manganese-containing proteins and belong to a differentially expressed multigenic family, with the SOD 3.Xs all sharing the same first nine amino acids which seem to be required to direct these proteins to mitochondria. SOD 3.1 is located in mitochondria and complement the hypersensitivity to oxidative stress of yeast cells which lack mitochondrial SOD (Zhu and Scandalios, 1995). I found five superoxide dismutase isoforms in the SUCEST database (Table VI). The high number of these isoforms indicate that the superoxide radical is very toxic to plants, although this free radical is not very reactive. One possible explanation for the toxicity of the superoxide radical is that it can react with nitric oxide generating peroxynitrite, which is a very strong oxidant.

Catalase protects cells from hydrogen peroxide which can be generated from superoxide dismutase catalyzed reaction, by the β-oxidation of fatty acids in peroxisomes or by other processes. This hemeprotein catalyses the reaction $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$. Plant catalases derive from a common ancestral gene and can be divided into three distinct groups (Inzé and Montagu, 1995). The first, and major, group includes maize Cat1, barley Cat1, rice CatB and most of the dicotyledon catalases, while the second group is an apparently dicotyledon-specific set of catalases which include tobacco Cat2 and tomato catalase. The maize Cat2 gene is loosely related to this group. The third group is a monocotyledon-specific set of catalases which include the maize Cat3, barley Cat2 and rice CatA catalseas. I found clusters with similarities to all the three maize isoforms (CAT 1, CAT 2 and CAT 3) in the SUCEST database (Table VII).

Table VI - Superoxide dismutase (SOD) homologues in sugarcane.

Homologous gene	SUCEST cluster	E-value[a]	Matches/hits
SOD3.4 (mitochondrial Mn-SOD) from *Zea mays*	sccclr1048a06.g scjfrt1009a12.g sccccl7001h12.g sccccl4006f07.g	e-123 e-124 6e-97 7e-71	gi|82728| gi|1174391| sp|P41980|
SOD4A (cytoplasmic) from *Zea mays*	scrflr2034d01.g sccclr2c03d05.g sceqlb1063b04.g	4e-84 5e-85 5e-85	gi|134597| sp|P23345|
SODCC.2 or SOD2 (cytoplasmic) from *Mesembryanthemum crystallinum*	scjlrt1019c03.g	2e-50	gi|3334333 |sp|O49044|
SOD2 or *SODCC.1* (cytoplasmic) from *Zea mays*	sccclr1024e11.g sceqrt2099a01.g scqgfl3056a03.g	2e-83 2e-83 2e-83	gi|134613 |sp|P11428|
SODCP (chloroplasmatic) from *Oryza sativa*	sccclr1068h03.g scutst3092a07.g	6e-82 2e-69	gi|3915008| sp|P93407|

[a]Calculated with the basic local alignment search tool (blastx) program.

Table VII - Catalase homologues in sugarcane.

Homologous genes from *Zea mays*	SUCEST cluster	E-value[a]	Matches/hits
CAT1 (peroxisomal)	sccccl3080h11.g scjfrz2029b02.g scqslb1051h04.g	0.0 0.0 e-113	gi|1084476|
CAT3 (mitochondrial)	scqslr1018e07.g scvplr1028a03.g sceprt2044h02.g scqglb1029h01.g	0.0 0.0 e-155 e-152	gi|1345683| sp|P18123|
CAT2 (peroxisomal or cytoplasmic)	sccccl3120d10.g	4e-72	gi|231689| sp|P12365|

[a]Calculated with the basic local alignment search tool (blastx) program.

Ascorbate peroxidase (APX) is another key enzyme for controlling hydrogen peroxide concentration, due to its

ability to catalyze the decomposition of hydrogen peroxide at the expense of ascorbate (Smirnoff, 2000). The sequence of this heme-containing protein is distinct from other peroxidases and different forms of ascorbate peroxidase occur in chloroplasts, cytosol, mitochondria, peroxisomes and glyoxysomes. In the SUCEST database I found cytoplasmic isoforms, such as clusters sceqlr1093e10.g and scvpcl6062d09.g with e-values of e^{-133} and $3e^{-85}$, respectively, in relation to gene gi7489542 and chloroplasmatic isoforms at clusters sceqrt2099g01.g and scrlfl4026h03.g with e-values of e^{-114} and e^{-103}, respectively, in relation to gene gi7484622.

Ascorbate oxidase (AO), which also uses ascorbate as substrate, is a cell wall localized glycoprotein belonging to the family of blue copper oxidases. Its role in plants has not been well defined but its enzymatic activity is already known i.e. ascorbate + $O_2 \rightarrow$ 2 dehydroascorbate + 2 H_2O. The expression of ascorbate oxidase is closely correlated with rapid cell expansion and is induced by auxin (Horemans *et al.*, 2000). I found ascorbate oxidase isoforms in the SUCEST database at clusters sceqam2039a03.g and scepam1021h07.g, with e-values of $5e^{-121}$ and $1e^{-87}$, respectively, in relation to the genes gi114268 and spQ00624.

Glutathione peroxidase is well-characterized in mammals which for many years was thought to occur only in mammalian cells, although later it was also found in other organisms (Halliwell and Gutterdige, 1999). Mammalian glutathione peroxidase possesses a selenium cysteine at its active site, but it is not known if this is the case in other organisms. I found genes similar to phospholipid isoform of glutathione peroxidase in the SUCEST database, with clusters scjlam1062a05.g, scccrz2c01f01.g and sccccclr2001f02.g having e-values of $6e^{-80}$, $3e^{-80}$ and $3e^{-80}$, respectively, in relation to the gene giQ06652.

Glutathione reductase is the enzyme responsible for regeneration of glutathione for new redox cycles. I found two glutathione reductases isoforms in the SUCEST database, one chloroplasmatic isoform at clusters scbfrz2050c06.g and scjfrt1062b07.g with e-values of e^{-108} and $2e^{-82}$, respectively, in relation to the gene gi 7431851 and the other cytosolic isoform at clusters scjfrt1009a01.g, scmcrt2089h02.g and scmcrt2085h09.g with e-values of 0.0, e^{-140} and e^{-107}, respectively, in relation to gene gi4106694.

Peroxiredoxins are a large family of thiol-dependent peroxidases, which use thioredoxin to reduce peroxide. Like glutathione peroxidases, these proteins are able to reduce hydrogen peroxide and alkyl hydroperoxides in bacteria, yeast, mammals (including humans) and plants (Rhee *et al.*, 1999). The active site is a cysteine residue located at the N-terminal part of the molecule, all members of this family possessing this residue, although some contain another cysteine residue at the C-terminal portion (2-Cys-Prx). Peroxiredoxins with only one conserved cysteine residue are called 1-Cys-Prx, but I found none of these in the SUCEST database. The C-terminal cysteine residue seems to be involved in the interaction with thioredoxin. Type 2 peroxiredoxins and Peroxiredoxin Q (PrxQ) possess only weak sequence similarity with 1-cys and 2-cys peroxiredoxins, although their enzymatic mechanism is similar (Rhee *et al.*, 1999). Peroxiredoxins are generally abundant proteins that are present in various cell compartments and in various isoforms (Table VIII).

Repair of oxidative damage

If reactive oxidative species have escaped from the previous defense systems they can reach biomolecules provoking oxidative lesions. These lesions can be repaired by various systems, most of them acting on DNA, but some on proteins and lipids. For more details about DNA repair see the companion paper by Costa *et al.* (2001) also in this volume.

Phospholipid hydroperoxide glutathione peroxidase (PHGPX), an isoform of glutathione peroxidase, is able to repair oxidative lesions in lipids by decomposition of lipid peroxides and organic hydroperoxide at the expense of glutathione (GSH). I found phospholipid hydroperoxide glutathione peroxidase isoforms in the SUCEST database at clusters SCJLAM1062a05.g, SCCCRZ2c01f01.g and SCCCCLR2001F02.G with tblast N e-values of $4e^{-81}$, $2e^{-82}$ and $2e^{-82}$, respectively, in relation to gene spO48646.

In plant proteins, oxidative lesions such as methionine sulfoxide and disulfide bridges are also repairable,

Table VIII - Peroxiredoxin homologues in sugarcane.

Homologous genes	SUCEST cluster	E-value[a]	Matches/hits
2-Cys-Prx, 2-cys peroxiredoxin (thioredoxin peroxidase) from *Arabidopsis thaliana* *c*hloroplasts	scjllr1033f07.g	e-105	gi|9758409
	scsbhr1050a06.g	2e-49	
	scrllr1016f09.g	e-104	gb|AAC78473.1
PrxQ, peroxiredoxin Q (bacterioferritin co-migratory protein (BCP)) from *Sedum lineare*	scbglr1096c09.g	2e-73	gi|6899842
CPrxII type 2 (new isoform) from *Brassica rapa*	scutsb1031f05.g	4e-71	gi|4928472|
	scepfl3084d08.g	8e-71	
Peroxiredoxin type 2 (new isoform) from *Arabidopsis thaliana*	scezrz1014f10.g	1e-75	gi|5441879|

[a]Calculated with the basic local alignment search tool (blastx) program.

with methionine sulfoxide reductase reducing methionine sulfoxide back to methionine at the expense of thioredoxin (Moskovits *et al.*, 2000) while protein disulfide isomerase acts as a chaperone due to its ability to rearrange disulfide bonds (Li and Larkins,1996). I found both methionine sulfoxide reductase and protein disulfide isomerase in the SUCEST database (Table IX). Thioredoxin is a protein that participates in various redox reactions through the reversible oxidation of its active center, dithiol, to a disulfide. The thioredoxin disulfide thus formed is then reduced back to the dithiol form by the action of thioredoxin reductase which catalyzes the reaction NADPH + oxidized thioredoxin → $NADP^+$ + reduced thioredoxin. In plants there are three thioredoxin isoforms and I found all of them in the SUCEST database (Table IX). Glutaredoxins, like the thioredoxin isoforms, are small proteins (12-13 kDa) with two vicinal cysteine residues at their active sites. Glutaredoxin active site is CPFC, while the thioredoxin active site sequence is CGPC. I also found glutaredoxin isoforms in the SUCEST database (Table IX).

In summary, except for the nucleus, each sugarcane cell compartment possess at least one antioxidant (Figure 1). The occurence of a particular antioxidant depends also on the tissue concerned, for example the concentration of ascorbate is higher in the upper parts of plants (reviewed by Smirnoff, 2000). The great number of redundant antioxidant components can be explained by the fact that each one may have a particular importance for a specific location and/or in a specific situation. These facts indicate that oxidative stress is an important phenomenon in plants because their sugarcane cells spend a considerable amount of energy expressing genes related to this process.

Table IX - Repair systems of proteins in sugarcane.

Homologous genes	SUCEST cluster	E-value[a]	Matches/hits
Peptide methionine sulfoxide reductase from *Lactuca sativa*	sccclr1c01b03.g scmcrt2089a06.g	1e-90 3e-64	gi|6635341|
PDI-like protein (endoplasmic reticulum lumen) from *Zea mays*	scaglb1070d02.g scjlrt1006b12.g scrlfl4026e12.g	0.0 0.0 0.0	gi|1709619| sp|P52588|
Thioredoxin H-type (TRX-H, cytoplasmic) from *Oryza sativa*	scccfl5056d02.g sceqlr1007c04.g	6e-43 6e-43	gi|3915131| sp|Q42443|
Thioredoxin F precursor (chloroplasmatic) from *Mesembryanthemum crystallinum*	scepsd2070d12.g	2e-23	gi|7430844| pir||T12261
Thioredoxin M-type (TRX-M) from *Zea mays* chloroplasts	sccclr1048a01.g scrflr1012g03.g	1e-86 5e-65	gi|3334376| sp|Q41864|
Thioredoxin reductase (NADPH, cytoplasmic from *Arabidopsis thaliana*	scbglr1120d04.g	e-137	gi|7488370| pir||T41743
Glutaredoxin from *Oryza sativa*	scccfl1097c06.g scvpam1055f03.g	1e-47 4e-47	gi|7430859| pir||JC5445

[a]Calculated with the basic local alignment search tool (blastx) program.

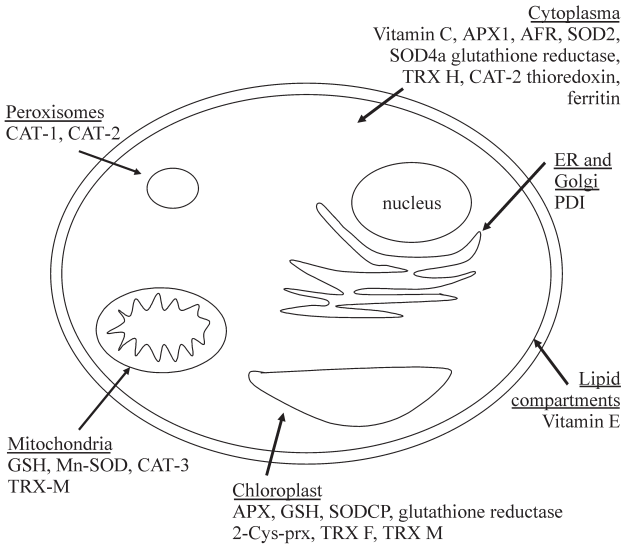

Figure 1 - Overview of the antioxidants found in sugarcane. The location of each antioxidant was based only on sequence similarities and not on experimental data. More details can be found in the corresponding table or in the supplementary data at http://sucest.lad.dcc.unicamp.br/private/mining-reports/UI/UI-mining.htm. Antioxidant components, whose cellular location is not known in other organisms, are not represented in this scheme.

Oxidative stress response regulators

All the antioxidant components described above do not act independently. Their synthesis is very tightly regulated in both time and space. For example, if a superoxide dismutase isoform is induced without the concomitant induction of a hydrogen peroxide-removing enzyme an increase of the concentration of hydrogen peroxide will occur. Because of this I conducted a search for oxidative stress response regulators in the SUCEST database but found no homologues for oxidative stress regulator from mammals (NF-kappaB, AP1). Only two homologues (one from bacteria and the other from yeast) with similarity to SUCEST clusters were found (Table X), but the overlap between them was narrow. It seems that most of the transcriptional regulators involved in the oxidative stress response (Oxy R, SoxR, SoxS, MarA, ArcA, Fnr and Fur from bacteria and YAP1, YAP2, MAC1, SKN7, ACE1, ACE2, MSN2, MSN4, HAP1, HAP2, HAP3 and HAP4 from yeast) do not have homologues in sugarcane, although I did find a plant homologue for a transcription factor involved in the regulation of superoxide dismutase (Table X). A possible explanation for this observation is that in spite of the fact that genes for antioxidant enzymes are very similar their transcriptional regulators are only poorly conserved among plants, animals, yeast and bacteria. It is, in fact, known that oxidative stress regulators from bacteria, yeast

Table X - Oxidative stress regulators.

Homologous genes	SUCEST cluster	E-value[a]	Matches/hits
RpoS (sigma-38) from *Escherichia coli*	scbfsd1038h02.g	2e-22[b]	sp|p13445
HAP5 from *Saccharomyces cerevisiae*	sccclr1022h01.g	6e-33[b]	Q02516
Zinc-finger protein	scjllr1033g12.g	2e-47	gi|7488436|
Lsd1 from *Arabidopsis thaliana*	sccccl3120b10.g	2e-45	pir||T10580

[a]Calculated with the basic local alignment search tool (blastx) program.
[b]Calculated with the tblastN program.

and mammals differ considerably (Storz and Polla, 1996). However, it is possible that homologues of these transcription factors are present in sugarcane but that their mRNAs are rare.

Participation of reactive species in plant-pathogen interactions

Reactive oxygen species are well known for their deleterious properties during oxidative processes such as ischemia/reperfusion, aging, cancer and cell death. However, an increasing number of reports describe the possible role of reactive oxygen species (especially of hydrogen peroxide) as cell messenger in signal transduction pathways, particularly in signaling in the response of plants to pathogens has been extensively reported (see rviews by Bolwell, 1999; Bowler and Fluhr, 2000; Grant and Loake, 2000).

A great number of biochemical pathways are involved in plant response to pathogen attack, but in this section I will focus on the role of reactive oxygen species and antioxidants. In summary, the plant response starts with the recognition of the pathogen elicitor by a receptor in the plant cell membrane, which activates local processes such as the release of reactive oxygen species (the oxidative burst) to the extra-cellular medium and formation of cell wall appositions. This often results in a hypersensitive response (HR), a type of programmed cell death located at the site at which the pathogen attempts to enter the plant. A delayed response occurs by a long-range signaling mechanism which later provokes a systemic acquired resistance (SAR) in which localized exposure to pathogen gives rise to whole-plant resistance to unrelated pathogens, this type of resistance lasting up to several months (reviewed by Bowler and Fuhr, 2000).

The proteins involved in the generation of reactive oxygen species during the oxidative burst remain to be established, but it has been assumed that a membrane localized NADPH oxidase is responsible for the occurrence of the oxidative burst (see reviews by Bowler and Fluhr, 2000; Grant and Loake, 2000). The $gp91^{phox}$ protein is a subunit of neutrophil NADPH oxidase, involved in the oxidative burst of these phagocytes. In the SUCEST database, I found one gene (cluster SCAGRT2037G05.g) homologous to the mammalian $gp91^{phox}$ gene, but no other genes similar to neutrophil NADPH oxidase subunits such as $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ were not found either in the SUCEST or in other plant databases. The $p47^{phox}$ and $p67^{phox}$ proteins are regulatory subunits of mammalian NADPH oxidase, and it may be that plant and animal regulatory complexes are, at least in part, controled by different mechanisms (reviewed by Grant and Loake, 2000).

Other enzymes which may be involved in the production of reactive oxygen species during plant hypersensitivity reactions are cell wall peroxidase isoforms that operate at alkaline pH and apoplastic amine, diamine and polyamine oxidases (Grant and Loake, 2000), but I found no homologues for these in the SUCEST database.

Various reports (reviewed by Grant and Loake, 2000) suggest that nitric oxide is formed during the oxidative burst and that it is important for plant resistance to pathogens, although, as discussed above, no gene encoding a nitric oxide synthase isoform has yet been isolated. I did not detect any gene similar to any of the mammalian nitric oxide synthase isoforms in the SUCEST database. In sugarcane, nitrite reductase (SUCEST clusters SCSBFL 4010b11.g and SCRLRZ3116c08.g) could be an alternative method for the generation of nitric oxide. During oxidative bursts both nitric oxide and the superoxide radical are produced, and this may result in the formation of the very powerful oxidant peroxynitrite which may be able to kill pathogens since it is known that both nitric oxide and peroxynitrite help to increase the destruction of pathogens during the oxidative burst (Grant and Loake, 2000). Peroxynitrite can also induce damage to host cells due to its high reactivity, and Bryk et. al. (2000) have reported that bacterial peroxiredoxin isoforms possess peroxynitrite reductase activity. It is possible that one of the sugarcane peroxiredoxin isoforms described in Table VIII could ameliorate toxicity of peroxynitrite to host cells.

Reactive oxygen species, especially hydrogen peroxide, have been proposed to have a signaling role in the systemic acquired resistance (see reviews by Bolwell, 1999; Bowler and Fluhr, 2000; Grant and Loake, 2000). Because oxidative burst precede the accumulation of salicylic acid it is reasonable to assume that reactive oxygen species could activate the synthesis of salicylic acid, and it has been shown that hydrogen peroxide activates the salicylic acid producing enzyme benzoic acid 2-hydroxylase (reviewed by Wojtaszek, 1997), although so far the gene for this enzyme has not been isolated. The isolation of this enzyme may contribute to the understanding of the role of reactive oxygen species in systemic acquired resistance signaling.

A further possible link between salicylic acid and reactive oxygen species is the fact that this acid binds to catalase (Chen *et al.*, 1993) and, moreover, salicylic acid has been shown to inhibit the activity of two enzymes involved in the hydrogen peroxide decomposition (catalase

and ascorbate peroxidase) but not to peroxidases involved in lignin formation. The inhibition of catalase and ascorbate peroxidase would result in an increased concentration of hydrogen peroxide which may activate pathogen-related genes. However, this pathway is controversial because some authors have shown that the elevated hydrogen peroxide levels resulting from the inhibition of catalase and ascorbate peroxidase are not required for induction of the systemic acquired resistance, while other authors have doubted that the expression of pathogenesis related genes is induced by hydrogen peroxide (reviewed by Wojtaszek, 1997). Other possible roles for reactive oxygen species signaling would be the activation of Mitogen-Activated Protein (MAP) kinases and/or alteration of the cellular redox status (reviewed by Grant and Loake, 2000). It has been shown that MAP kinase is encoded by the salicylic acid induced kinase (*SIPK*) gene (reviewed by Bowler and Fluhr, 2000) and I found that the SUCEST clusters SCEQRT 1030A09.g, SCAGRT2039G04.g and SCCCRZ2C04 G10.g present very high similarity to *SIPK* from *Zea mays*, the blastx e-values being 0.0, e-159, e-156, respectively, for genes gi|4239889| and gi|1362151.

The participation of reactive nitrogen species in the systemic acquired resistance response has also been postulated but the confirmation of this hypothesis awaits the identification of a nitric oxide generating system in plants. Moreover, the soluble isoform of guanylate cyclase, one of the most important targets for nitric oxide signaling, and cGMP- 3',5'- cyclic phosphodiesterase (responsible for cGMP degradation) were not identified in the SUCEST database nor in other plant database, so if nitric oxide is in fact a plant messenger its signaling pathways are likely to differ considerably from those described in mammals.

The role of reactive oxygen species in the destruction of pathogens and cell signaling during the hypersensitivity and systemic acquired resistance responses is still poorly characterized, and a deeper understanding of the mechanisms involved may be an important step for agriculture in order to select plants more resistant to different types of stress.

RESUMO

A resposta ao estresse oxidativo não é bem conhecida em plantas como em bactérias, leveduras e humanos. Por exemplo, assume-se que óxido nítrico tem várias funções em plantas apesar do gene que codificaria para óxido nítrico sintetase nunca ter sido isolado. Este trabalho descreve os resultados de uma busca no banco de dados de seqüências expressas de cana de açúcar (SUCEST) de genes envolvidos na resposta ao estresse oxidativo. Eu não encontrei genes similares a óxido nítrico no banco de dados do SUCEST, mas uma via alternativa para a produção deste radical livre pode ser proposta. Eu também encontrei vários genes envolvidos na defesa antioxidante, como quelantes de metais, antioxidantes de baixo peso molecular, enzimas antioxidantes e sistemas de reparo. Ascorbato (vitamina C) é um importante antioxidante em plantas porque é encontrado em altas concentrações em células vegetais e porque é substrato de ascorbato peroxidase, uma enzima que eu encontrei em diferentes isoformas no banco de dados do SUCEST. Eu também encontrei várias enzimas envolvidas na biossíntese de antioxidantes de baixo peso molecular que podem ser alvos para manipulação genética. A obtenção de plantas modificadas geneticamente que sintetizariam vitaminas C e E em altos níveis poderiam melhorar o valor nutricional e a tolerância a estresses de cana de açúcar. Os diversos componentes do sistema de defesa antioxidante interagem entre si e as suas sínteses devem ser muito bem reguladas. Fatores de transcrição envolvidos na regulação da resposta ao estresse oxidativo de bactérias, leveduras e de humanos diferem consideravelmente entre si e quando foram utilizados para buscas no banco de dados do SUCEST, somente genes com similaridades fracas foram encontrados, sugerindo que estas proteínas não são muito conservadas. O envolvimento de espécies reativas de oxigênio e nitrogênio na defesa de plantas contra patógenos também é discutido neste trabalho.

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