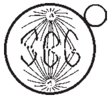
Genetics and Molecular Biology, 28, 3 (suppl), 548-554 (2005)
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Research Article

Eucalyptus ESTs corresponding to the protoporphyrinogen IX oxidase enzyme related to the synthesis of heme, chlorophyll, and to the action of herbicides

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Abstract

This work was aimed at locating *Eucalyptus* ESTs corresponding to the PROTOX or PPO enzyme (Protoporphyrinogen IX oxidase, E.C. 1.3.3.4) directly related to resistance to herbicides that promote oxidative stress, changing the functionality of this enzyme. PROTOX, which is the site of action of diphenyl-ether (oxyfluorfen, lactofen, fomesafen), oxadiazole (oxadiazon and oxadiargyl), and aryl triazolinone (sulfentrazone and carfentrazone) herbicides, acts on the synthesis route of porphyrins which is associated with the production of chlorophyll a, catalases, and peroxidases. One cluster and one single read were located, with e-values better than e-70, associated to PROTOX. The alignment results between amino acid sequences indicated that this enzyme is adequately represented in the ESTs database of the FORESTs project.

Key words: Eucalyptus, protoporphyrinogen IX oxidase, catalase, peroxidase, herbicide.

Received: Mau 28, 2004; Accepted: June 6, 2005.

Introduction

The protoporphyrinogen IX oxidase enzyme, known as Protox or PPO, corresponds to the site of action of several herbicides from different chemical groups. The herbicide action is implicated in promoting oxidative stress by producing free radicals. There exist three other mechanisms of action also related to oxidative stress: interference with the electron flow in the Photosystem I (diquat and paraquat), interference with the electron flow in the Photosystem II, more specifically with the D1QB complex (triazines, triazinones, chloroanilides, ureas and uracils), and glutamine synthetase inhibition (glufosinate).

Variability in the levels of action of the herbicides that act upon these sites primarily depends on characteristics of the site itself (amino acids sequence in the proteins and expression levels), as well as the plants ability to inacti-

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#Supported by CNPq.

vate free radicals produced, which actually generate plant intoxication. Variability in absorption and translocation capacity (even at small distances) is also an important factor that conditions the effectiveness of any compound with herbicidal action.

Tetrapyrrole biosynthesis is important in plants because it provides to many essential molecules involved in light harvesting, energy transfer, signal transduction, detoxification, and systemic acquired resistance (Wettstein *et al.*, 1995; Grimm, 1998; Molina *et al.*, 1999). The most abundant tetrapyrroles are chlorophyll and heme, which are important compounds for photosynthesis and respiration. Protoporphyrinogen IX oxidase (Protox, EC 1.3.3.4) is the last enzyme in the common pathway of chlorophyll and heme biosynthesis (Beale and Weinstein, 1990). Protox catalyzes the oxidative O_2-dependent aromatization of the colorless protoporphyrinogen IX to the highly conjugated protoporphyrin IX.

In this work, only ESTs related to the Protox enzyme will be addressed. Protox is a key enzyme for the production of porphyrins and chlorophylls in plants, and a site of action for several compounds with herbicidal activity (Figure 1). The study of ESTs related to detoxifying systems, which are important to define the levels of action of herbicides that cause oxidative stress, was already carried out by Alves *et al.* (2005).

This mechanism of action of herbicides and the compounds that act upon it have been presented in detail by Dodge (1992), Hess (1993), and Weller (2002). Inhibition of protoporphyrin IX synthesis by herbicides generates an intriguing accumulation of this pigment in plants. Protoporphyrin IX is an extremely reactive molecule which, in the presence of light and Oxygen, produces singlet Oxygen, a free radicals with a high lipid oxidation capacity (Figure 1). This fluorescent pigment is intensely produced in the chloroplasts by the action of Protox, from protoporphyrinogen IX. Since route regulation depends on the production of protoporphyrin IX inside organelles, inhibiting the activity of this enzyme in chloroplasts generates great protoporphyrinogen IX accumulation, which is released into the cytosol. Once in the cytosol, the protoporphyrinogen IX is non-enzymatically converted into protoporphyrin IX, which is accumulated at high concentrations (hundreds of times higher than normal). In contact with light, this pigment produces a great amount of free radicals which quickly burst the cell membranes, causing the death of tissues and plants.

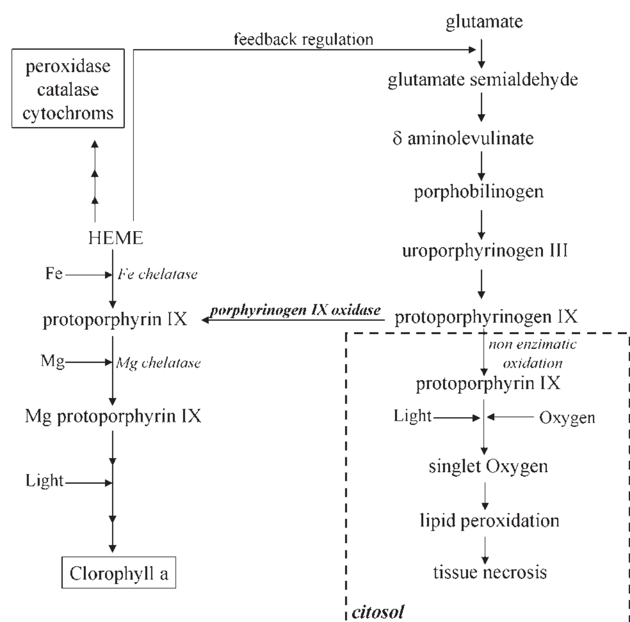

Figure 1 - Synthesis route of porphyrins in plants (adapted from Dodge, 1991; Hess, 1993; Nelson and Leningher, 2000; Weller, 2002).

Watanabe *et al.* (2001) discuss in detail the action and codification of Protox, crucial for heme and chlorophyll production. This enzyme is present and acts on the chloroplasts (producing chlorophyll) and mitochondria (producing heme), but is entirely codified in the nucleus. The authors also studied and demonstrated the existence of intracellular translocation of Protoporphyrinogen and Protoporphyrin IX. The results indicated that the excess of these compounds present in the chloroplasts and cytosol, as a consequence of the sub-lethal action of herbicides, could be used in the mitochondria to produce heme groups, which are essential for the formation of catalases and peroxidases.

Catalases and peroxidases are important in the reduction of stresses of different origins, because they are important free radical inactivators (Chaudière and Ferrari-Iliou, 1999). Thus, raising the amount or activity of Protox could be an important tool to increase photosynthetic efficiency (related to the chlorophyll a content), or to increase tolerance to herbicides and stresses of an environmental (especially nutritional, thermal, and hydric) or biological nature (associated with pests, diseases, and weeds).

It must be highlighted that the toxic action of herbicides is exclusively associated with the interaction with Protox from chloroplasts. Oxyfluorfen is the main selective herbicide in eucalyptus, and is largely used in conventional planting areas, being overtop applied after seedling transplantation. Sulfentrazone, which has been recently registered in Brazil, is applied on the same way and shows great potential for use in *Eucalyptus* because of its broad control spectrum, selectivity to several clones, long residual effect and high solubility, witch makes possible the transposition through the litterfall with rain water and allows sulfentrazone to be used in reduced tillage areas. Rodrigues and Almeida (1998) presented the conditions of use of these herbicides in foresting areas.

Both oxyfluorfen and sulfentrazone can intoxicate *Eucalyptus*, mainly when the herbicides are not applied immediately after transplanting. The young leaves that are formed between planting and the application show greater

sensitivity to the compounds, and can present quite pronounced injuries.

Considering the information here presented, the synthesis route of protoporphyrin could be altered with two objectives: a) tolerance to herbicides and production of chlorophylls, related to chloroplast forms of prototoporphyrinogen IX osidase; b) production of heme in association with the synthesis of cytochrome a, cytochrome c, catalase and peroxidase enzymes, related to mitochondria forms of Protox.

Considering tolerance to herbicides, locating different configurations of the enzyme or promoters that would condition different levels of expression could allow genotypes more tolerant to the mentioned herbicides to be obtained. Their evaluation can be done through the application of normal rates of herbicides followed by injury evaluation, or by applying low rates and monitoring the concentrations of intermediate and final products of the route.

With respect to the production of cytochromes, catalases and peroxidases, the lack of some information about the enzyme systems that operate in mitochondria makes the most suitable procedure to consist in the identification of genotypes with the highest concentrations or levels of activity of these enzymes. Evaluating the concentrations of intermediate products is essential to determine which points in the route (enzymes) will be surely critical for genotype discrimination. The greatest information gap corresponds to the lack of sequencings of the mitochondrial form of the enzyme. The paper by Watanabe *et al.* (2001) is an exception. The authors isolated and sequenced spinach Protox cDNA which encodes a homologue of tobacco mitochondrial Protox II. Immunoblot analysis of spinach leaf extract detected two proteins with apparent molecular masses of 57 an 55 kDa in chloroplasts and mitochondria, respectively. In vitro translation experiments indicated that two translation products (59 and 55 kDa) are produced from Protox II mRNA, using two in-frame initiation codons. Transport experiments using green fluorescent protein-fused Protox II suggested that the larger and smaller translation products target exclusively to chloroplasts and mitochondria, respectively.

This work was aimed at locating *Eucalyptus* ESTs corresponding to the protoporphyrinogen IX oxidase enzyme (PROTOX or PPO; E.C. 1.3.3.4) directly related to the action of several herbicides and involved in the production of chlorophyll and heme.

Material and Methods

This work resulted from an analysis of the information bank produced in the first stage of the Eucalyptus Genome Project (Projeto Genoma do *Eucalyptus* - FORESTs), jointly developed by FAPESP and a consortium of four companies in the forestry industry (Duratex, Ripasa, Suzano, and VCP) and executed with the participation of 20 laboratories from the State of São Paulo associated with the AEG network (https:forests.esalq.usp.br). In all, 123,889 reads constructed from expressed sequence tags (ESTs) of cDNA libraries, mainly derived from *Eucalyptus grandis* tissues, were obtained. The tissues were removed from different organs of plants submitted to different growing conditions. The makeup and coding of the libraries are described in Table 1.

The search for enzyme sequences corresponding to PROTOX was performed using the BLAST tool (Altschud *et al.*, 1997). The amino acid sequences for those enzymes, described for different plant species, were compared with the information from the FORESTs project database using the "tBLASTn" option, allowing the identification of Clusters associated with them. Only Clusters adequately aligned with the amino acid sequences were selected, using an e-value < e-70 as a selection criterion. The Clusters that best aligned with sequences obtained from the literature were chosen for the next step.

The nucleotide sequences of the selected Clusters were compared with the NCBI (National Center Biotechnology Information) and the geneBank amino acid sequence databases after translation in all possible frames. The procedure allowed to confirm the alignment with sequences of the enzyme, to find the translation frame for the

Table 1 - Codes and source tissues of cDNA libraries approved by the FORESTS project.

Code	Tissues / growing condition
BK1	Bark, sapwood, heartwood, and pith of 8-year old *E. grandis* trees
CL1	*E. grandis* calluses formed in the dark
CL2	*E. grandis* calluses formed in the light
FB1	Buds, flowers and fruits
LV1	Seedling leaves
LV2	Leaves from trees efficient and poorly efficient in phosphorus and boron utilization
LV3	Leaves colonized with the caterpillar *Thyrinteina* sp. for 7 days
RT3	Nursery seedling roots
RT6	Roots of trees resistant and susceptible to frost
SL1	*E. grandis* seedlings grown in the dark and exposed to light for 3 h prior to RNA extraction
SL4	*E. globulus* seedlings grown in the dark
SL5	*E. saligna* seedlings grown in the dark
SL6	*E. urophylla* seedlings grown in the dark
SL7	*E. grandis* seedlings grown in the dark
SL8	*E. camaldulensis* seedlings grown in the dark
ST2	Stems of six-month old seedlings susceptible to water deficit
ST6	Stems of seedlings susceptible to water deficit
ST7	Stems of trees resistant and susceptible to frost
WD2	*E. grandis* wood

cluster and to obtain values of identity percents and similarity probability values (e-value) for sequences from different plant species.

Based on the translation frame that produced the best alignments and using the software GENERUNR, the nucleotide sequence corresponding to the clusters was translated into amino acids for the identification and analysis of Open Read Frames. The amino acid sequences corresponding to ORFs were aligned with the amino acid sequences of different plant species (with e-value < e-70 as previously described). The software CLUSTAL was used to align the sequences and to estimate the phylogenetic distances represented in consensus phylogenetic trees obtained from a total of 1,000 bootstrap trials. The trees were built up using the neighbor-joining method and were plotted with TreeView.

Results and Discussion

The PROTOX enzyme (Protoporphyrinogen IX oxidase) is effectively represented in the FORESTs Sequence Database. Using the "tBLASTn" option in the BLAST tool, it was possible to identify two clusters with similarity indices (e-value) better than e-70 (Table 2).

The results of the alignments using nucleotide sequences of the clusters, translated into amino acid sequences in the different translation frames, with amino acid sequences available at the NCBI and the geneBank, are presented in Tables 3 and 4.

The cluster "EGCBSL4281G09.g" corresponds to a single read with 784 nucleotides. Frame +2 was used to translate the base sequences into amino acids. The start codon was located at positions 26, and the nucleotide sequence was truncated by an end codon at the position 641. Coding was obtained for a total of 205 amino acids. The alignment of the amino acid sequences from different species is represented in Figure 2. The sequence corresponding to the cluster "EGCBSL4281G09.g" aligned to the start region of the sequences from literature. If the same amino acid was observed in a certain position for all studied species, the column was marked with an asterisk enabling the identification of several conserved regions of the protoporphyrinogen IX oxidase with lengths ranging from one to thirteen amino acids.

The phylogenetic distance matrix obtained from the amino acid sequences is represented in Figure 3. The least phylogenetic distances in relation to cluster "EGCBSL 4281G09.g" were 0,272 and 0,274 verified for the species *Oryza sativa* and *Zea mays*, repectively. These species were the only two grasses considered in this study and formed a specific branch of the tree. The phylogenetic distance from *Oryza sativa* to *Zea mays* was only "0,105". A second branch with short phylogenetic distance (0,093) was produced by the two Solanaceae species (*Solanum tuberosum* and *Nicotiana tabacum*). A third branch was formed by *Spinacea oleracea* (Amaranthacea) and *Arabidopsis thaliana* (Brassicacea) with a phylogenetic distance of 0,253 between the two species.

Table 2 - FORESTs clusters, identified through the tBlastn tool, which showed similarity with the PROTOX enzyme (protoporphyrinogen IX oxidase, E.C. 1.3.3.4).

Species	Accession	Cluster	e-value
Zea mays	CAD10611	EGEQST1002B11.g	3.00E-75
		EGCBSL4281G09.g	1.00E-70
Sorghum bicolor	CAD10608	EGEQST1002B11.g	3.00E-73
Oryza sativa	CAD10607	EGEQST1002B11.g	3.00E-75
Brassica napus	CAD10606	EGCBSL4281G09.g	5.00E-78
		EGEQST1002B11.g	5.00E-77
Beta vulgaris	CAD10605	EGEQST1002B11.g	8.00E-70
Gossypium hirsutum	CAD10604	EGCBSL4281G09.g	2.00E-87
		EGEQST1002B11.g	2.00E-85
Glycine Max	CAD10603	EGEQST1002B11.g	7.00E-81
		EGCBSL4281G09.g	7.00E-74
Triticum aestivum	CAD10602	EGEQST1002B11.g	6.00E-74
Arabidopsis thaliana	CAD10598	EGEQST1002B11.g	1.00E-79
		EGCBSL4281G09.g	6.00E-77
Triticum aestivum	CAC34012	EGEQST1002B11.g	6.00E-74
Spinacia oleracea	BAA96808	EGEQST1002B11.g	2.00E-70

Table 3 - Translation frames for the nucleotide sequence of read EGCBSL4281G09.g, e-values, amino acid sequence lengths corresponding to the enzyme protoporphyrinogen IX oxidase (E.C. 1.3.3.4), and identity percentages in relation to the cluster, for different higher plant species.

Species	Frame	g.i.	e-value	Length of sequences[(1)]	Identity (%)
Arabidopsis thaliana	+2	56550711	3e-84	537	71
Nicotiana tabacum	+2	4105186	5e-72	548	64
Sichorium intybus	+2	6002912	6e-78	555	70
Solanum tuberosum	+2	3093410	2e-70	557	61
Spinacea oleracea	+2	8648059	3e-75	562	70
Oryza sativa	+2	34902564	8e-71	563	62
Zea mays	+2	6715441	4e-78	535	71

[(1)]Number of coded amino acids.

The cluster “EGEQST1002B11.g” is a sequence of 804 nucleotides that is consensual in three reads. Frame +3 was used to translate the nucleotide sequences into amino acids. Coding was obtained for a total of 179 amino acids. The alignment of the amino acid sequences is represented in Figure 4. The sequence corresponding to the cluster “EGEQST1002B11.g” aligned to the end region of the sequences from literature. It was possible to identify several conserved regions in the final part of Protoporphyrinogen IX oxidase. The lengths of the conserved regions ranged from one to seven amino acids.

Table 4 - Translation frames for the nucleotide sequence of cluster EGEQST1002B11.g, e-values, amino acid sequence lengths corresponding to the enzyme protoporphyrinogen IX oxidase (E.C. 1.3.3.4), and identity percentages in relation to the cluster, for different higher plant species.

Species	Frame	g.i.	e-value	Length of sequences[1]	Identity (%)
Arabidopsis thaliana	+3	56550711	3e-78	537	89
Nicotiana tabacum	+3	4105186	6e-79	548	80
Sichorium intybus	+3	6002912	5e-78	555	80
Solanum tuberosum	+3	3093410	7e-79	557	81
Spinacea oleracea	+3	8648059	1e-74	562	78
Oryza sativa	+3	34902564	3e-79	563	80
Zea mays	+3	6715441	1e-78	535	81

[1]Number of amino acids.

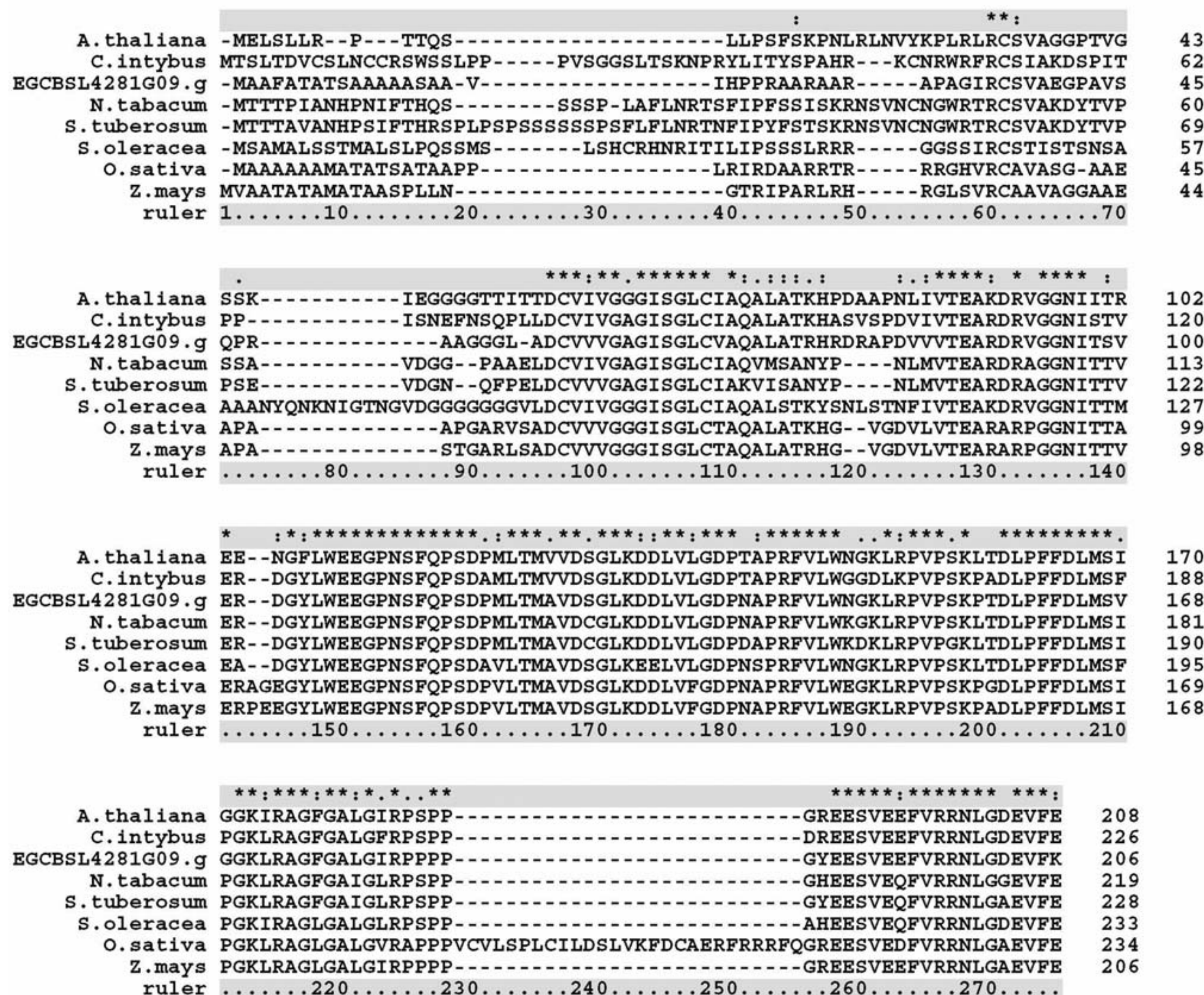

Figure 2 - Fragments of sequences corresponding to the start region of the protoporphyrinogen IX oxidase enzyme from different plant species.

The phylogenetic distance matrix obtained from the amino acid sequences from literature and the cluster "EGE QST1002B11.g" is represented in Figure 5. The maximum phylogenetic distance between the various species was 0.218. Considering the sequences for the final part of the enzyme protoporphyrinogen IX oxidase, the phylogenetic distances from *Eucalyptus* to the other species ranged from 0.184 (for *Zea mays*) to 0.218 (for *Spinacea oleracea*). Two branches formed by Poaceae and Solanaceae species were observed. A third branch enclosed *Arabidopsis thaliana* and *Spinacea oleracea*.

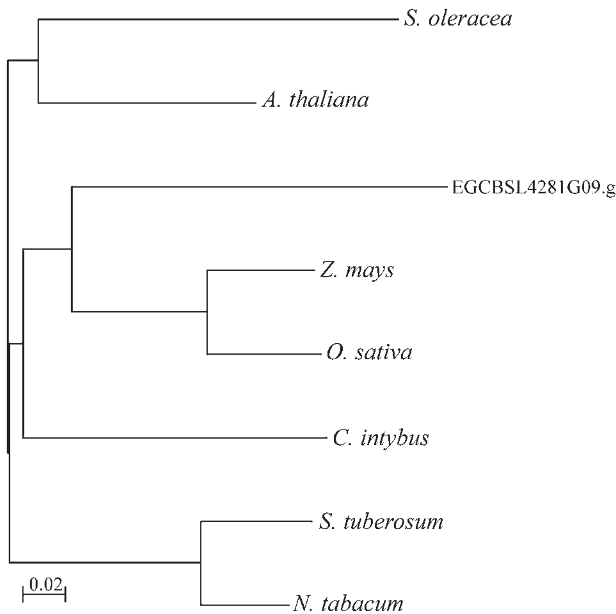

Figure 3 - Graphical representation of the phylogenetic distances obtained from amino acid sequences corresponding to the start region of the enzyme protoporphyrinogen IX oxidase of the studied species.

The phylogenetic study indicated that the grass species (*Zea mays* and *Oryza sativa*) sequences showed higher identity levels, forming an independent branch. These results are important since they are consistent with the available information on the sensitivity of species from different botanical groups to herbicides that act on Protox. Several compounds act preferentially on dicotyledon species. Thus, the existence of a branch in the phylogenetic tree consisting exclusively of grasses could indicate the presence of functional variability in the enzyme and that such variability could be related to a higher or lower sensitivity to herbicides under field conditions. Locating the possible regions and sequences associated with the differential reaction to herbicides could be of great value in establishing breeding programs assisted by genomic tools in the future.

This observation has an even greater relevance when we consider that the level of knowledge about this site of action of herbicides is much lower than that available for nearly all the others. In effect, no information was found in the literature concerning to sequences corresponding to

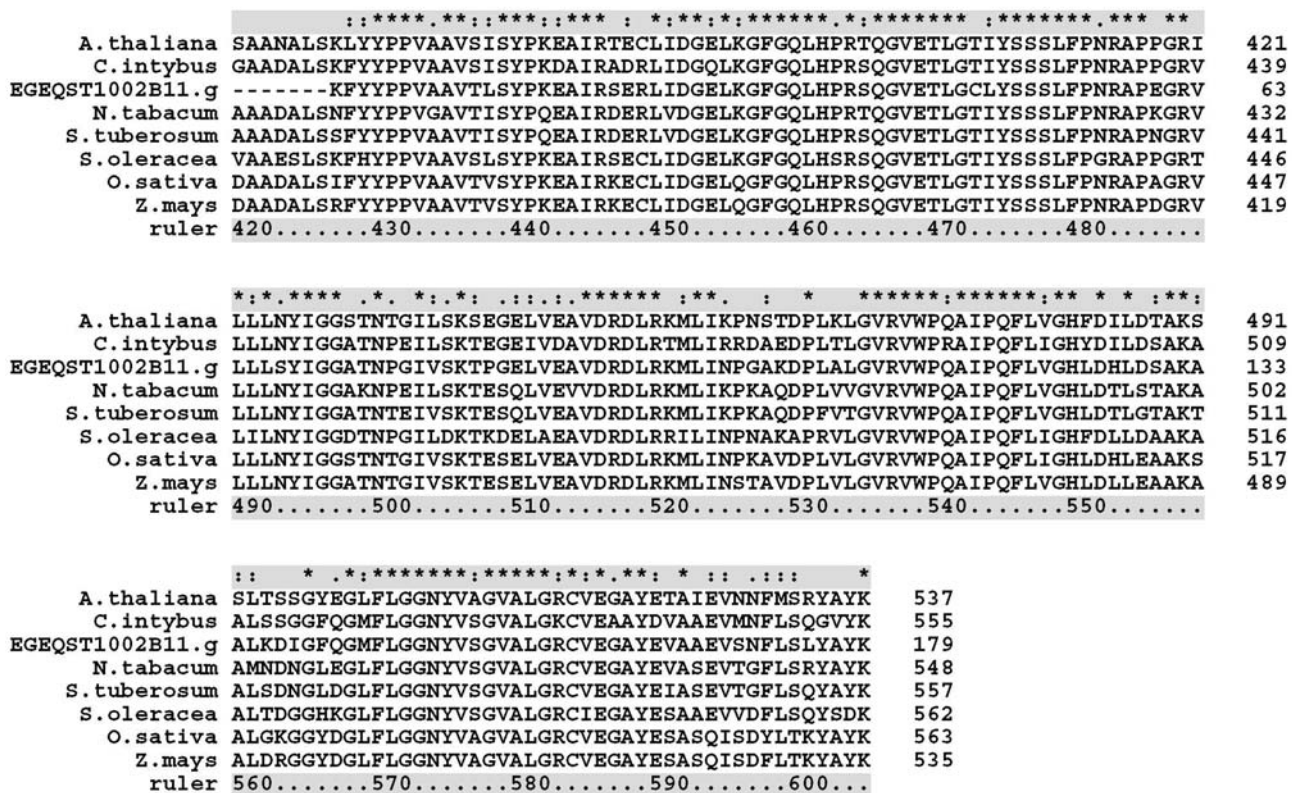

Figure 4 - Fragments of sequences corresponding to the end region of the protoporphyrinogen IX oxidase enzyme from different plant species.

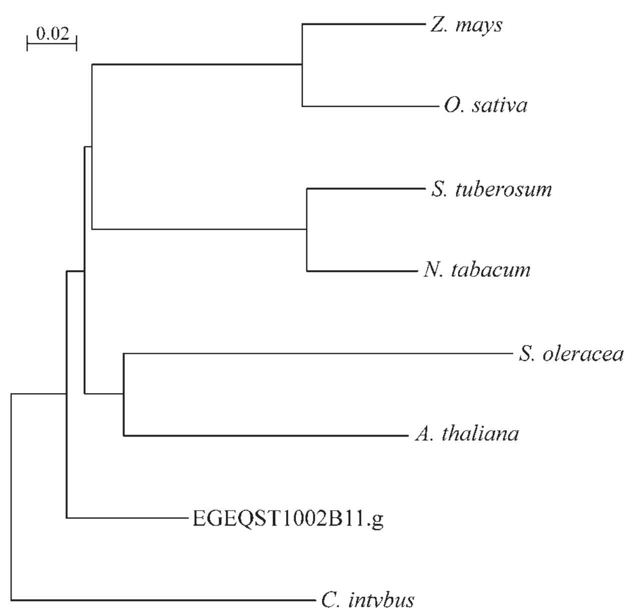

Figure 5 - Graphical representation of the phylogenetic distances obtained from amino acid sequences corresponding to the end region of the enzyme protoporphyrinogen IX oxidase of the studied species.

SNPs or promoters that could be associated with resistance to Protox-inhibiting herbicides.

Results indicated that it is viable to locate preserved regions in the nucleotide sequences corresponding to Protox for developing specific primers to study this enzyme. Such primers are essential to complete the identification of the gene considering that only the start and final regions of protoporphyrinogen IX oxidase from *Eucalyptus grandis* were already sequenced by FORESTs project. The study of the gene can allow to find promoters and SNPs to induce higher or lower levels of expression of the gene or activity of the enzyme. These, in turn, could be associated with different levels of sensitivity to herbicides, growth (related to chlorophyll accumulation) or tolerance to stresses (related to the accumulation and activity of catalase and peroxidase enzymes).

The potential use of such compounds as oxyfluorfen, sulfentrazone, and other herbicides that act on the Protox enzyme as tools for the development of chemical genetics studies must be pointed out. Such process, described by Blackwell and Zhao (2003), corresponds to the use of chemical compounds to regulate metabolic routes in plants and to predict results that could be obtained by means of genetic alterations to them.

Acknowledgments

We would to acknowledge RIPASA S.A. Celulose e Papel, DURATEX, Votorantim Celulose e Papel, Suzano/Bahia Sul and FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) by the financial support.

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Associate Editor: Helaine Carrer