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Short Communication

Eucalyptus ESTs involved in the production of 9-*cis* epoxycarotenoid dioxygenase, a regulatory enzyme of abscisic acid production

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Abstract

Abscisic acid (ABA) regulates stress responses in plants, and genomic tools can help us to understand the mechanisms involved in that process. FAPESP, a Brazilian research foundation, in association with four private forestry companies, has established the FORESTs database (https://forests.esalq.usp.br). A search was carried out in the *Eucalyptus* expressed sequence tag database to find ESTs involved with 9-*cis* epoxycarotenoid dioxygenase (NCED), the regulatory enzyme for ABA biosynthesis, using the basic local BLAST alignment tool. We found four clusters (EGEZLV2206B11.g, EGJMWD2252H08.g, EGBFRT3107F10.g, and EGEQFB1200H10.g), which represent similar sequences of the gene that produces NCED. Data showed that the EGBFRT3107F10.g cluster was similar to the maize (*Zea mays*) NCED enzyme, while EGEZLV2206B11.g and EGJMWD2252H08.g clusters were similar to the avocado (*Persea americana*) NCED enzyme. All *Eucalyptus* clusters were expressed in several tissues, especially in flower buds, where ABA has a special participation during the floral development process.

Key words: Eucalyptus EST, abscisic acid, water deficit, drought resistance, NCED.

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Plant tolerance to drought involves several biochemical processes, whose understanding of is essential for the development of individuals with greater water stress withstanding capacity, thus enabling agricultural frontiers to be expanded into regions where water availability is a limiting factor.

The mechanisms of resistance to water deficit act separately or in combination. In general, these mechanisms involve physiological and morphological alterations that have a molecular/genetic basis. For this reason, genotypes differing in water deficit tolerance must present qualitative and quantitative differences in genic expression. A specific physiological response to water deficit represents, in reality, the combination of previous molecular events, which were activated by the perception of a stress signal. Understanding how these events are activated/inactivated and how they interact among themselves is essential for the development of new varieties tolerant to drought periods (Nepomuceno *et al.*, 2001).

One of the best-known mechanisms of tolerance to environmental stress is the production of Abscisic Acid (ABA). In higher plants, ABA plays important roles in embryo development and seed dormancy, in addition to being involved in plant adaptation to several stresses (drought, salinity, cold). When ABA is applied to plants, it causes the stomata to close quickly, thus reducing water loss by transpiration. In addition, water stress promotes a rapid increase in ABA content in the plants (Qin and Zeevaart, 1999). Evidence indicates that this increase in ABA is attributed to *de novo* synthesis (Zeevaart and Creelman, 1988).

Since the discovery of ABA in the beginning of the 60s, a great deal of effort has been dedicated to understanding its biosynthesis. By means of genetic and biochemical studies, the biosynthesis pathway of ABA in higher plants is now understood in detail. Recently, all major genes that code for enzymes in the ABA biosynthesis pathway were

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identified (Schwartz *et al.*, 2003). The new challenge is now to understand how these biosynthesis genes and the biosynthesis pathway are regulated as a whole (Xiong and Zhu, 2003).

In order to determine which step in ABA biosynthesis is stimulated by water stress, it is essential to first establish its biosynthesis pathway. ABA is a sesquiterpenoid (C_{15}), and two routes have been proposed for its biosynthesis. The first is the straight pathway from isopentenylpyrophosphate (C_5), via farnesyl pyrophosphate (C_{15}), into ABA. Current evidence indicates that this pathway operates in fungi (Zeevaart, 1999). And the second is the indirect pathway in which ABA is a product of carotenoid cleavage (C_{40}). This "indirect route" step remained unknown for many years due to the difficulty in promoting these reactions *in vitro*. With the identification and characterization of the ABA-deficient mutant gene, this problem was eventually solved.

Evidence indicates that this pathway works in green plants and was obtained with carotenoid-deficient mutants in research using marked $^{18}O_2$, where it was observed that in roots under water stress and in etiolated plant parts there was a 1:1 stoichiometric ratio between the disappearance of violaxanthin and neoxanthin and the formation of ABA and its catabolites, phaseic and dihydrophaseic acid (Zeevaart, 1999).

The direct proof of the pathway where ABA biosynthesis occurs by carotenoid cleavage came from the VP14 ABA-deficient corn mutant and its corresponding cloned VP14 gene (Tan *et al.*, 1997). In enzyme assays, the recombinant VP14 protein cleaves 9-*cis*-epoxycarotenoid producing 2-*cis*,4-*trans*-xanthoxin and C25-apocarotenoid (Schwartz *et al.*, 1997a). Due to the fact that no isomerization reaction occurs at the C_{15} precursor level, the precursor carotenoids must be in the 9-*cis* configuration, and converted into ABA, which is, by definition, 2-*cis*,4-*trans* (Qin and Zeevaart, 1999). Evidence indicates that the conversion of xanthoxin into ABA is not a limiting factor in ABA biosynthesis, and this last step in the pathway is not regulated by water deficit, since the carotenoid substrate is abundantly available in photosynthetic tissues (Parry *et al.*,1990). By elimination, 9-*cis*-epoxycarotenoid cleavage is the limiting step in the ABA biosynthesis pathway.

Qin and Zeevaart (1999) obtained a bean gene, PvNCED1, through cloning, which codes for the 9-*cis*-epoxycarotenoid cleavage enzyme, and analyzed its enzymatic activity and location. Its water stress response was characterized in the form of mRNA and protein levels in leaves and roots. The results demonstrated that the cleavage reaction is the key step in abscisic acid biosynthesis regulation, and was induced by water stress.

The ABA-deficient mutants served as tools to reveal the ABA biosynthesis pathway. Because of early seed germination and the appearance of plant wilting, these faulty mutants were isolated from a number of plant species, including *Zea mays, Lycopersicon esculentum, Nicotiana tabacum, Solanum tuberosum, Hordeum vulgare* and *Arabidopsis thaliana*. Before the molecular identities of the affected genes were known, a principal ABA biosynthesis route was revealed by modeling ABA biosynthetic intermediate products in combination with nutritional assays involving those mutants. These studies suggested that ABA in higher plants is synthesized through an indirect pathway by cleavage of a C_{40} precursor carotenoid, followed by a two-step conversion of intermediate xanthoxin into ABA via ABA-aldehyde (Taylor *et al.*, 2000; Finkelstein and Rock, 2002; Seo and Koshiba, 2002; Schwartz *et al.*, 2003). So far, the main genes and enzymes from ABA-deficient mutants have been characterized in *Arabidopsis* (Schwartz *et al.*, 2003). The *Arabidopsis* information can be applied to other plant species since the pathway and its respective genes are highly conserved in angiosperms (Xiong and Zhu, 2003).

The first specific step in the ABA biosynthesis pathway is the epoxidation of zeaxanthin and antheraxanthin into violaxanthin, which takes place within the plastids. This step is catalyzed by the enzyme zeaxanthin epoxidase (ZEP), which had its molecular identity first revealed in tobacco (Marin *et al.*, 1996). After a series of structural modifications, violaxanthin is converted into 9-*cis*-epoxycarotenoid. The oxidative cleavage of the main 9-*cis*-neoxanthin epoxycarotenoid by 9-*cis* epoxycarotenoid dioxygenase (NCED) generates an intermediate C_{15} xanthoxin (Schwartz *et al.*, 1997b). This was considered the first step in the ABA biosynthesis pathway. The xanthoxin product is then exported to the cytosol, where it is converted into ABA by a two-step reaction via ABA-aldehyde. A short-chain dehydrogenase/reductase (SDR) alcohol, coded by the AtABA2 gene (Rook *et al.*, 2001; Cheng *et al.*, 2002; González-Guzman *et al.*, 2002), catalyzes the first step of this reaction and generates ABA-aldehyde. The ABA-aldehyde oxidase enzyme (AAO) then catalyzes the last step in the biosynthesis pathway (Xiong and Zhu, 2003), as presented in Figure 1.

According to the research results obtained for ABA biosynthesis, it can be seen that the identification and isolation of genes involved in its production is extremely important, especially with respect to the gene that codes for the NCED enzyme, which is a step of the pathway whose efficiency could determine a higher or lower degree of plant resistance to environmental stresses. Based on this information, this work was aimed at identifying, in the FORESTs database, sequences that are homologous to the gene under consideration, already identified in other species.

Sequences representative of the NCED genes (involved in the production of abscisic acid) were selected from the NCBI (National Center for Biotechnology Information) or geneBank database. These peptide sequences were used in a search within the database generated by FORESTs (https:forest.esalq.usp.br), using the BLAST

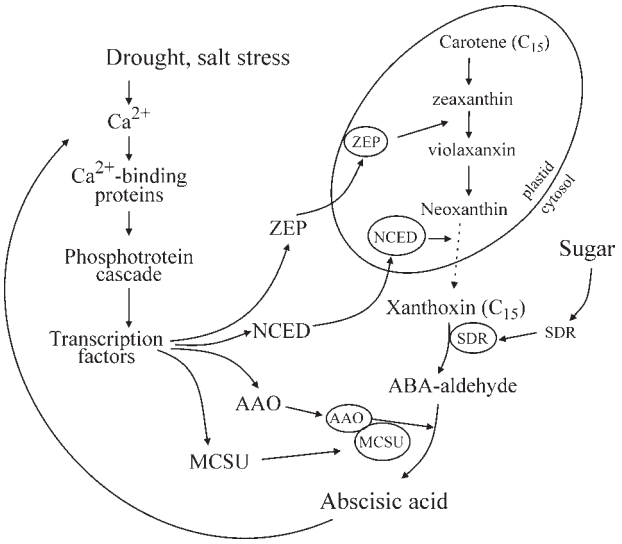

Figure 1 - ABA biosynthesis regulation. The NCED step (indicated by a dashed line) probably limits ABA biosynthesis in leaves. ZEP, zeaxanthin epoxidase; NCED, 9-*cis* epoxycarotenoid dioxygenase; AAO, ABA-aldehyde oxidase; MCSU, MoCo sulfurase; SDR, short-chain alcohol dehydrogenase/reductase. Adapted from Xiong and Zhu (2003).

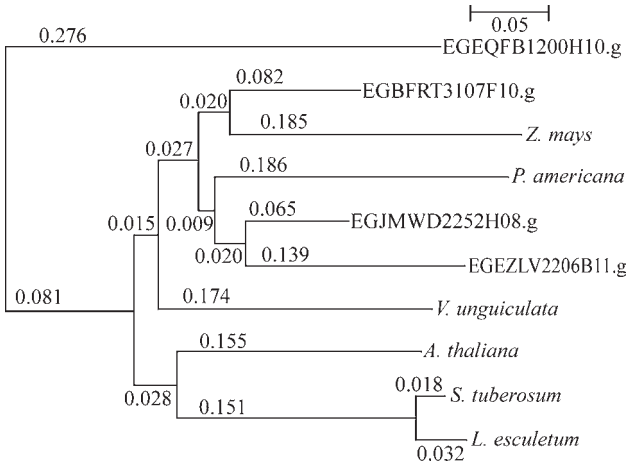

Figure 2 - Dendrogram showing the distance relations between the amino acid sequences of 9-*cis*-epoxycarotenoid dioxygenase for *Eucalyptus* clusters EGEZLV2206B11.g, EGJMWD2252H08.g, EGBFRT3107F10.g and EGEQFB1200H10.g, and the 9-*cis*-epoxycarotenoid dioxygenase of *A. thaliana*, *L. esculetum*, *S. tuberosum*, *V. unguiculata*, *Z. mays* and *P. americana*. The phylogenetic distance between sequences in the tree was calculated using the neighbor-joining algorithm.

tool (Altschud *et al.*, 1990), where the probability value (e-value) was used to validate the sequences. The FORESTs database was formed consisting of 123,889 reads obtained from expressed sequence tags (ESTs) acquired from cDNA libraries of tissues from different organs of *E. grandis*, *E. globulus, E. saligna, E. urophylla* and *E. camaldulensis.* The libraries consisted of woods (WD), bark (BK), flower buds (FB), leaves (LV), seedlings (SL), stem (ST), root (RT), and callus (CL).

Four *Eucalyptus* clusters (EGEZLV2206B11.g, EGJ MWD2252H08.g, EGBFRT3107F10.g, EGEQFB1200H 10.g) found in the FORESTs database (Table 1) represent similar sequences to the sequence that codes for the gene responsible for producing the 9-*cis* epoxycarotenoid dioxygenase enzyme (NCED). This gene is extremely important in controlling the production of abscisic acid.

According to the dendrogram in Figure 2, generated from the amino acid sequences for the NCED enzyme of different organisms and *Eucalyptus* clusters, it can be seen that the *Eucalyptus* cluster EGBFRT3107F10.g showed more similarity to the corn (*Zea* mays) NCED enzyme, while EGEZLV2206B11.g and EGJMWD2252H08.g clusters were similar to the avocado (*Persea americana*) NCED. Chernys and Zeevaart (2000) found that ABA biosynthesis in avocado is regulated at the level of carotenoid cleavage, which is catalyzed by NCED.

The abscisic acid biosynthesis occurs in fruits, seeds, roots, leaves and branches, even though its synthesis is widely distributed throughout the plant. The highest production of that hormone, as a response to drought, occurs in leaves, followed by a small production in branches and roots, while fruits do not change their ABA contents as a drought response (Coll *et al.*, 1992). This situation can be observed in the sources of reads that made up the clusters under consideration. The EGEZLV2206B11.g cluster is from only two types of tissues, leaves (LV) and stem (ST),

Table 1 - Similar clusters to the NCDE gene, found in the FORESTs database.

Organism	Accession number	GI	*Eucalyptus* cluster							
			EGEZLV2206B11.g		EGJMWD2252H08.g		EGBFRT3107F10.g		EGEQFB1200H10.g	
			E-value	Ident.	E-value	Ident.	E-value	Ident.	E-value	Ident.
Phaseolus vulgaris	AAF26356	6715257	0.0	78%	e-131	80%	5e-94	74%	4e-90	36%
Arabidopsis thaliana	NM_117945	18415070	0.0	58%	3e-99	63%	3e-83	71%	4e-76	35%
Lycopersicon esculentum	AJ439079	28974076	0.0	70%	e-131	77%	2e-98	38%	3e-94	75%
Persea americana	AF224672	12655868	0.0	72%	e-114	69%	8e-88	69%	3e-84	36%
Vigna unguiculata	AB030293	9857289	0.0	69%	e-132	80%	2e-93	74%	1e-89	36%
Solanum tuberosum	AJ276244	7209268	0.0	69%	e-133	78%	1e-96	39%	2e-94	74%
Zea mays	U95953	2232016	0.0	69%	e-133	68%	2e-97	40%	3e-90	72%

and the highest number of reads came from stem tissues. The EGJMWD2252H08.g and EGBFRT3107F10.g clusters are from only one type of tissue, wood (WD) and root (RT), respectively. The EGEQFB1200H10.g cluster presents cDNAs from different tissues of plants, but showed a higher number of reads from flower bud tissues (FB) and leaves (LV3). The NCED gene expression in flower buds is expected due to special participation of ABA during the floral development process, as observed by Tan *et al.* (2003) in studies with AtNCED genes in *Arabidopsis thaliana*. These studies indicate that the developmental control of ABA synthesis involves localized patterns of AtNCED gene expression.

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