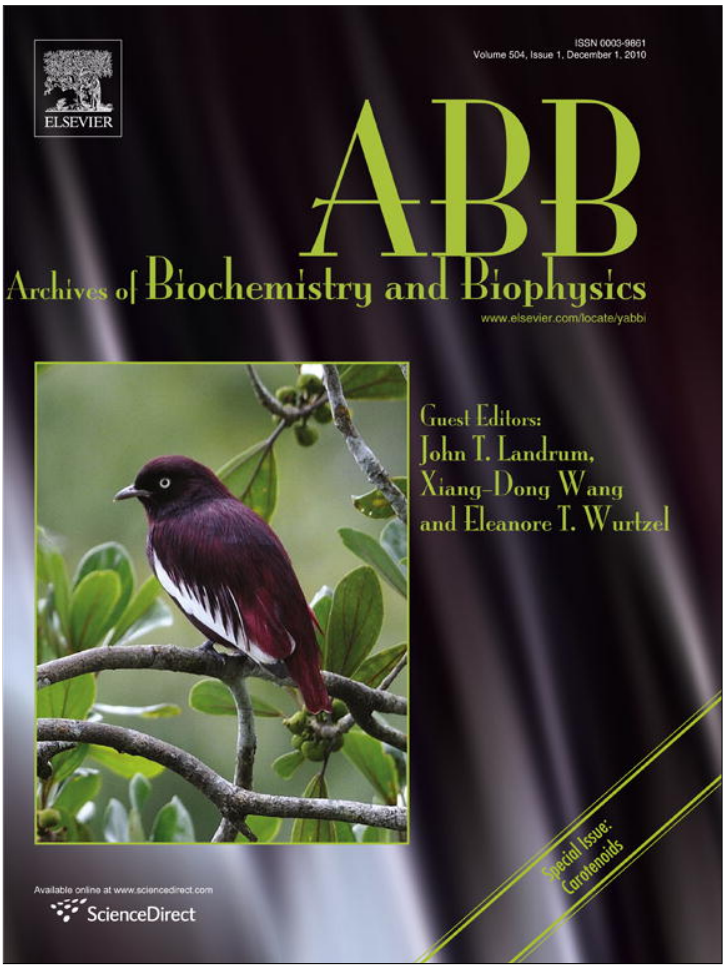

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Invited Article

From epoxycarotenoids to ABA: The role of ABA 8′-hydroxylases in drought-stressed maize roots

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ABSTRACT

The ability of plants to withstand drought, a potentially major constraint to yield and production, is influenced by abscisic acid (ABA). ABA is synthesized in the cytosol from plastid carotenoid pathway derived precursors, and later inactivated by the action of ABA hydroxylases. Endogenous accumulation of ABA is controlled by both its synthesis and catabolism. Enzymatic activity of ABA 8′-hydroxylase (ABA8Ox), also referred to as CYP707A, is considered one of the key steps in modulating ABA levels that control numerous physiological processes. To investigate the role of this enzyme, maize *ABA8Ox* gene family members were identified. *ABA8Ox* gene expression was then analyzed in different tissues and roots during the drought-stress response in maize. These genes were found to be expressed in all tissues, with a high degree of specificity to each tissue and some degree of overlap. Maize *ABA8Ox1a* and *ABA8Ox1b* were shown to be the major transcript components for regulating ABA catabolism in drought-stressed roots. Phylogenetic and gene-structure analyses were performed to extend the implications and infer the cause of ABA catabolism in other cereal crops.

Introduction

Human survival depends on food production, and drought is a potentially major constraint to yield and production of food crops. In maize, for example, drought accounts for at least 15% of the crop loss in sub-Saharan Africa [1]. Abscisic acid (ABA)[1] is a plant hormone that influences the plant's ability to withstand abiotic stresses such as drought, cold, salt and wounding [2]. ABA has additional roles in regulating embryo and seed development, maintenance of seed dormancy and germination, seed desiccation tolerance, vegetative development, general seedling growth, and in mediating pathogen defense responses (reviewed in [3]). Transgenic plants overexpressing ABA biosynthetic genes showed improved drought tolerance [4], while overexpression of ABA 8′-hydroxylases reduced ABA levels and produced ABA-deficient phenotypes [5]. Thus, the amount of ABA is determined by the balance between biosynthesis and catabolism [6], and one strategy for achieving maximum drought tolerance in crop plants, and thereby avoiding losses, is to increase the synthesis of endogenous ABA while controlling catabolism.

ABA-biosynthesis-related genes [7,8] and their regulatory mechanisms [3,9,10] have been identified in a wide range of plants. The first committed step in ABA biosynthesis is the oxidative cleavage of a 9-*cis*-epoxycarotenoid (C_{40}) to form xanthoxin (C_{15}). Xanthoxin is oxidized to form abscisic aldehyde, and then further oxidized to ABA [7,11]. The major route to inactivation of ABA is mediated by the cytochrome P450 monooxygenase ABA 8′-hydroxylase (ABA8Ox, also referred to as CYP707A) [6]. ABAOx hydroxylates ABA to yield 8′-hydroxy ABA thus depleting the active ABA pool (Fig. 1).

Extensive studies have been conducted on the small gene families that encode the ABA8Ox catabolic enzymes from *Arabidopsis* (*CYP707A1-CYP707A4*) [6,12], barley (*HvABA8Ox1* and *HvABA8Ox2*) [13], and beans (*Pv*CYP707A1-*Pv*CYP707A3) [14]. These studies showed that ABA catabolism under stress and recovery is mainly regulated at the transcriptional level.

Although there is a wealth of knowledge on ABA catabolism in model species, ABA catabolism in staple food crops is not as well understood. Cereal crops of global importance, such as maize, sorghum, wheat and rice, are evolutionarily related as members of the Poaceae (grasses) family. The aim of this study was to characterize the *ABA8Ox* gene family in maize and to elucidate the expression of ABA-catabolism-related genes in unstressed maize tissues and in maize roots affected by drought stress. Using comparative

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[1] *Abbreviations used:* ABA, abscisic acid; CT, threshold cycle; Os, *Oryza sativa*; Sb, *Sorghum bicolor*.

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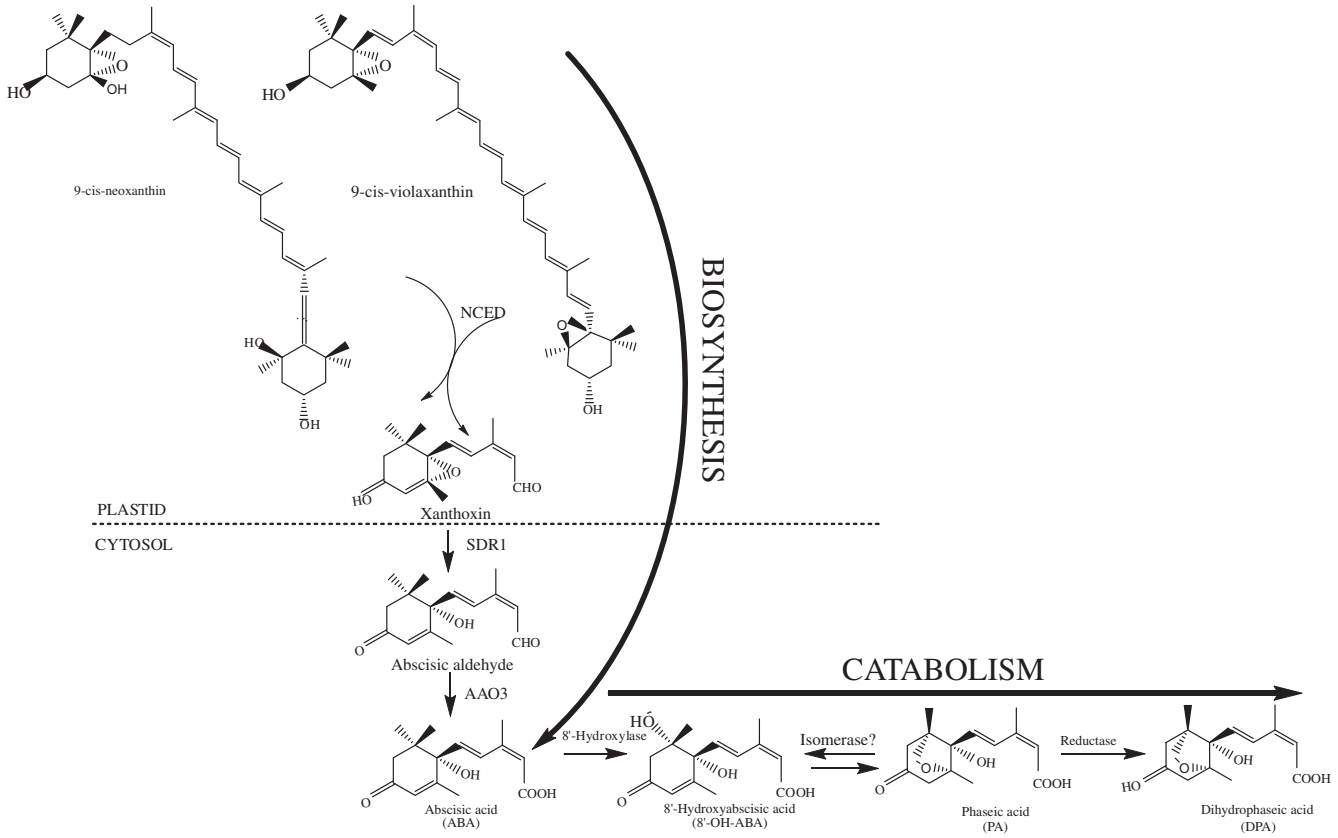

Fig. 1. ABA biosynthesis and catabolism in higher plants. Carotenoid precursors of ABA are synthesized in the plastid. The *cis*-xanthophylls are cleaved by a family of 9-*cis*-epoxycarotenoid dioxygenases (NCED) to form the first 15-carbon precursor, xanthoxin. Xanthoxin moves to the cytosol and is converted to abscisic aldehyde by a short-chain dehydrogenase reductase (SDR1), which is then oxidized to ABA by an abscisic aldehyde oxidase (AAO3). ABA is hydroxylated to 8′-hydroxy ABA in the presence of 8′-hydroxylase (ABA8Ox), and subsequently converted to phaseic acid and dihydrophaseic acid. The genes involved in these downstream steps are still unknown.

genomics, orthologs were also identified in other grass genomes, creating the possibility of breeding these plants for drought tolerance.

Materials and methods

Plant materials and stress treatments

Maize (*Zea mays* L.) inbred line B73 plants were field grown in the Bronx, New York, sibling-pollinated, and endosperm and embryo tissues were dissected 20 days after pollination (DAP). Non-stressed leaf and root tissues and drought-stressed root samples were collected at the six-leaf stage. B73 was grown in a greenhouse as described previously [8]. Dissected tissues were stored at −80 °C until analysis.

Sequence analyses

The genomic DNA sequences of ABA hydroxylases of *Arabidopsis thaliana*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays* were obtained from NCBI GenBank, Gramene, Phytozome and PlantGDB, respectively (Table 1). The sequences were analyzed using Vector NTI Suite, Version 9.0 (InforMax, North Bethesda, MD), and processed for gene-structure analysis, while deducing cDNA and protein se-

Table 1
Summary of ABA8Ox enzymes and genes in grasses (maize, rice, and sorghum) and Arabidopsis. CYP, cytochrome P450.

Enzyme	*Arabidopsis thaliana*	*Oryza sativa*	*Zea mays*		*Sorghum bicolor*
		Gramene	BAC clones/ESTs	MAP	Phytozome
ABA8Ox1	At4g19230 (CYP707A1)	LOC_Os02g47470	AC194862 (*ABA8Ox1a)* DR806072; EC902187; DY688015; CO520018; EB706067; DR803102; EE157729	5.06	Sb04g030660
	At5g45340 (CYP707A3)		AC182107 (*ABA8Ox1b)* CD433445; EE190691; EE169703; EE036846; EE023371; EC902781; EE036847; EC894886; EE169704; EC884504; EC898571; EC884505	4.06	
ABA8Ox2	At2g29090 (CYP707A2) At3g19270 (CYP707A4)	LOC_Os08g36860	AC212409 (*ABA8Ox2)* CO460095; CO456959; CO459318; DV494257; EB701816; AI670285; EE040561; CO456726; DV529214; CA398898; DY623142; DY239249; EB701815	4.04	Sb07g022990
ABA8Ox3		LOC_Os09g28390	AC190490 (*ABA8Ox3a)* CD941324; CD941122; CD955590	2.06	Sb02g026600
			AC195926 (*ABA8Ox3b)* EC904849; DV527897; EE043845; DR785156; EE175998; EE036989 DV514713; DR962771; DV519081	7.02	

quences. Sequence comparisons for maize ABAOx proteins are shown in Table 2. Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with 500 bootstrap replication support using MEGA3 software [14]. Chromosomal mapping was performed using the WebAGCoL package [15] or MaizeGDB.

Quantitative real-time PCR

RNA extraction and quantitative real-time PCR were performed using gene-specific primers (Table 3) and normalized to actin, as previously described [16]. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen Sciences, Maryland), and DNase I-treated (Invitrogen, Carlsbad, CA) prior to first strand cDNA synthesis using oligo (dT) as a primer and Superscript™ III RT (Invitrogen, Carlsbad, CA). One microliter of 50 μM oligo $(dT)_{20}$ and 1 μl of 10 mM dNTP mix were mixed with 8 μl of DNase I treated total RNA (~1 μg) and incubated at 65 °C for 5 min, and left on ice for at least 1 min. Ten microliters of cDNA synthesis mix (2 μl of 10× RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT™ (40 U/μl), 1 μl of Superscript™ III RT (200 U/μl) were added and incubated for 50 min at 50 °C and reactions terminated at 85 °C for 5 min. Samples were collected by brief centrifugation and 1 μl of RNase H added and incubated for 20 min at 37 °C. cDNA samples were amplified on the MyIQ Single-Color Real-Time PCR detection system (Bio-Rad, Hercules, CA), using iQTM SYBR Green Supermix (Bio-Rad, Hercules, CA). Two microliters (5 ng/μl) of cDNA; 15 μl of 2× iQTM SYBR Green Supermix; 11 μl of nuclease-free water; 1 μl (20 ρm/μl) of each gene-specific primer were used in a 30 μl reaction volume. Thermal cycling conditions included an initial incubation at 94 °C for 10 s, followed by 35 cycles of 95 °C for 10 s, 58 °C for 35 s, and 72 °C 10 s. Melt curve analysis was performed to verify primer specificity, and PCR products were confirmed by sequencing. The relative quantity of the transcripts was calculated by using the comparative threshold cycle (CT) method. Actin mRNA was amplified simultaneously for normalization between samples. Values were expressed as the mean of three RT-PCR replicates ± standard deviation.

Table 2
Comparison of sequence similarity (top) and identity (bottom) between Zea *mays* ABA8Ox proteins.

	ABA8Ox1a (%)	ABA8Ox1b (%)	ABA8Ox2 (%)	ABA8Ox3a (%)
ABA8Ox1b	90.4			
	88.1			
ABA8Ox2	63.1	60.4		
	49.9	48.3		
ABA8Ox3a	66.5	64.7	70.2	
	54.4	53.0	61.7	
ABA8Ox3b	66.2	64.7	72.8	82.6
	54.0	52.7	63.0	78.3

Results and discussion

Identification and characterization of ABA 8′-hydroxylases in maize

Five putative *ABA8Ox* genes were found in maize *(ABA8Ox1a, ABA8Ox1b, ABA8Ox2, ABA8Ox3a, ABA8Ox3b)*. These genes are homologs of the four known genes in *Arabidopsis* [6,12] and three genes in rice [17]. The ABA8Ox proteins are predicted to localize to the cytosol, the predominant site for ABA catabolism to phaseic acid and dihydrophaseic acid (Fig. 1). Table 1 summarizes the putative genes encoding ABA8Ox enzymes and their chromosomal positions in maize along with homologs in *Arabidopsis*, rice, and sorghum. The sequence similarity and identity between the *Zea mays* ABA8Ox proteins are presented in Table 2 and alignments of the grass ABA8Ox proteins are shown in Supplementary Fig. S1.

The exon–intron boundaries were deduced and confirmed through selected expressed sequence tags (ESTs) where available (Table 1). All members of the *ABA8Ox* gene family from all three grass species contain multiple introns (Fig. 2). To better understand the evolutionary origin of the various gene copies, phylogenetic analysis was conducted using the deduced protein sequences. The structures of the genes are shown with their orthologs grouped into one of three clades, *ABA8Ox1* (*Ox1*), *ABA8Ox2* (*Ox2*), and *ABA8Ox3* (*Ox3*). Orthologs in the three species shared exon–intron organization that distinguished each paralog, except Sorghum which has fewer exons compared to its orthologous genes in the *Ox3* clade. The *Ox1* clade had the fewest number of exons (five) whereas the *Ox2* clade had the most (nine) (Fig. 2). Classification of clades based on gene structure was similar to the protein phylogenetic organization.

Phylogenetic analysis of ABA8Ox protein sequences from the grasses and selected dicots are shown in Fig. 3. For the grass clades, ABA8Ox2 and ABA8Ox3 appear to form sister clades that are evolutionarily diverged from a common ancestor of the ABA8Ox1 clade. During the speciation events among monocots, it appears that some grasses lost some of their *ABA8Ox* genes. This assumption is partially supported by the absence of *ABA8Ox2* in barley (data not shown). The ABA8Ox2 (labeled as such) from barley is actually a true ABA8Ox3 based on its sequence similarity with other grass genes (Supplementary Fig. S1). The absence of an *ABA8Ox2* gene in barley could be due to either incomplete genome sequencing or, at least in part, evolutionary mechanisms.

Spatial analysis of ABA 8′-hydroxylase transcripts

Among the sampled tissues, maize paralogs of *ABA8Ox* varied in relative expression, suggesting that in each tissue, different combinations of ABA8Ox are responsible for ABA catabolism. *ABA8Ox3a*

Table 3
Primers used in this study of maize *ABA8Ox* genes.

Gene	No.	Primer sequence	Orientation	Accession No.
ABA8Ox1a	1774	ATGCTCGTGCTCTTCCACCACCT	Forward	AC194862
	1769	TATACCGCCATACCATATCCATCCGCC	Reverse	
ABA8Ox1b	1774	ATGCTCGTGCTCTTCCACCACCT	Forward	AC182107
	1772	GGAAGCGGTTTTTCGCGTTCCTGG	Reverse	
ABA8Ox2	1785	AGCCTACGAGGAGAACGATG	Forward	AC212409
	1786	TCAGGAACCCTTGGTAGTGC	Reverse	
ABA8Ox3a	1783	ACAGAAAGGGGCGTGAGACCGA	Forward	AC190490
	1778	AGGCGAGCAAAGAAGAATTTCAA	Reverse	
ABA8Ox3b	1784	ACAGAAATGGCCGCCATGAGACCGA	Forward	AC195926
	1781	ATTTCTTCTCCCCCTCAAGGTAAT	Reverse	
Actin	1134	CGATTGAGCATGGCATTGTCA	Forward	J01238
	1135	CCCACTAGCGTACAACGAA	Reverse	

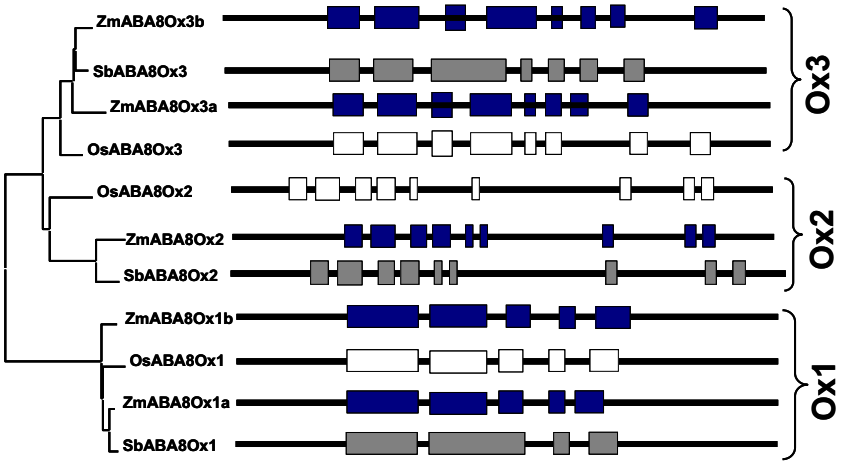

Fig. 2. *ABA8Ox* gene family. Gene structures (with boxes indicating exons and thin lines representing introns) for paralogs and orthologs of the *ABA8Ox* gene family in *Zea mays* (Zm), *Oryza sativa* (Os) and *Sorghum bicolor* (Sb) (accession numbers are presented in Table 1). Gene structures are compared to protein phylogenetic tree on left which was produced based on sequences shown in Supplementary Fig. S1.

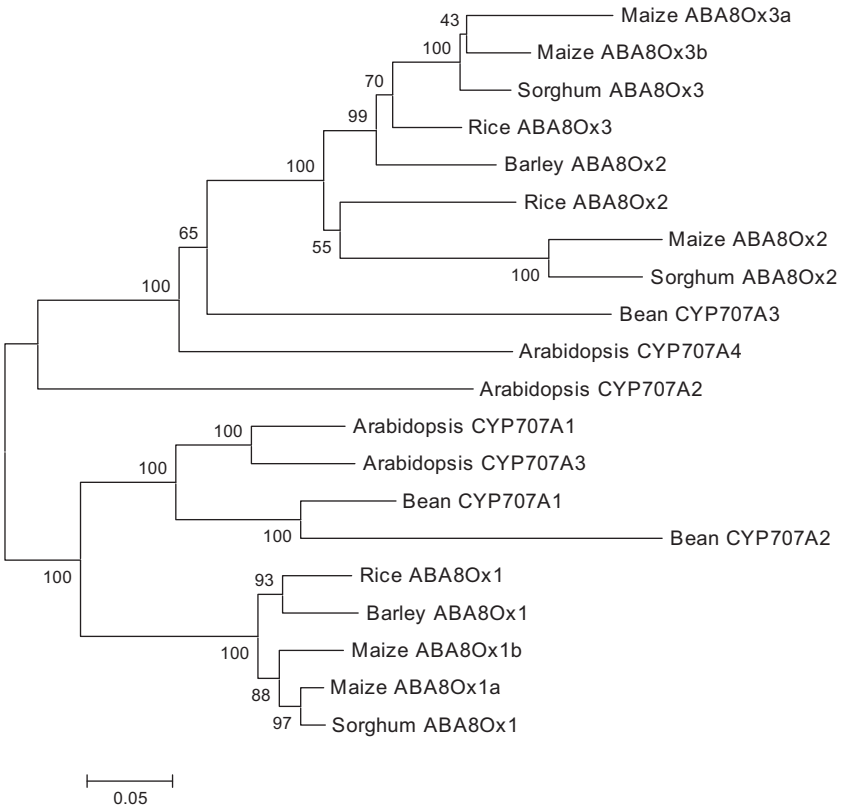

Fig. 3. Phylogenetic analysis of proteins encoded by members of the *CYP707A* gene subfamily for representative monocots and dicots. A neighbor-joining tree was constructed using the following protein sequences (given as accession numbers): *Zea mays* (ABA8Ox1a, DR806072; ABA8Ox1b, CD433445; ABA8Ox2, CO460095; ABA8Ox3a, EC904849; ABA8Ox3b, CD941324), *Oryza sativa* (ABA8Ox1, LOC_Os02g47470; ABA8Ox2, LOC_Os08g36860; ABA8Ox3, LOC_Os09g28390), *Sorghum bicolor* (ABA8Ox1, Sb04g030660; ABA8Ox2, Sb07g022990; ABA8Ox3, Sb02g026600), *Hordeum vulgare* (ABA8Ox1, DQ145930; ABA8Ox2, DQ145931), *Arabidopsis thaliana* (CYP707A1, At4g19230; CYP707A2, At2g29090; CYP707A3, At5g45340; CYP707A4, At3g19270), *Phaseolus vulgaris* (CYP707A1, DQ352541; CYP707A2, DQ352542; CYP707A3, DQ352543). Amino acid distances were subjected to Poisson correction. Numbers indicate bootstrap support for individual nodes. Bootstrap support higher than 40% is indicated at respective nodes ($n = 500$).

was found to be predominantly expressed in leaves, *ABA8Ox1a* in roots, *ABAOx2* and *ABAOx1a* in the endosperm, and *ABA8Ox2*, *ABA8Ox1a* and *ABA8Ox1b* in the embryo (Fig. 4). The abundance of maize *ABA8Ox1a* in the roots suggests a prominent role in root ABA catabolism. In this study, *ABA8Ox3a* exhibited over 1000-fold higher expression in leaves than the other paralogs, a much higher difference than that exhibited by paralogs expressed in other sampled tissues. Therefore, maize *ABA8Ox3a* could be an important target in the control of maize leaf ABA catabolism, given that ABA can be mobilized from any tissue. Tissue specific patterns of mRNA accumulation have also been observed for *ABA8Ox* gene family members in other species. In *Arabidopsis*, CYP707A3 (an Ox1 homo-

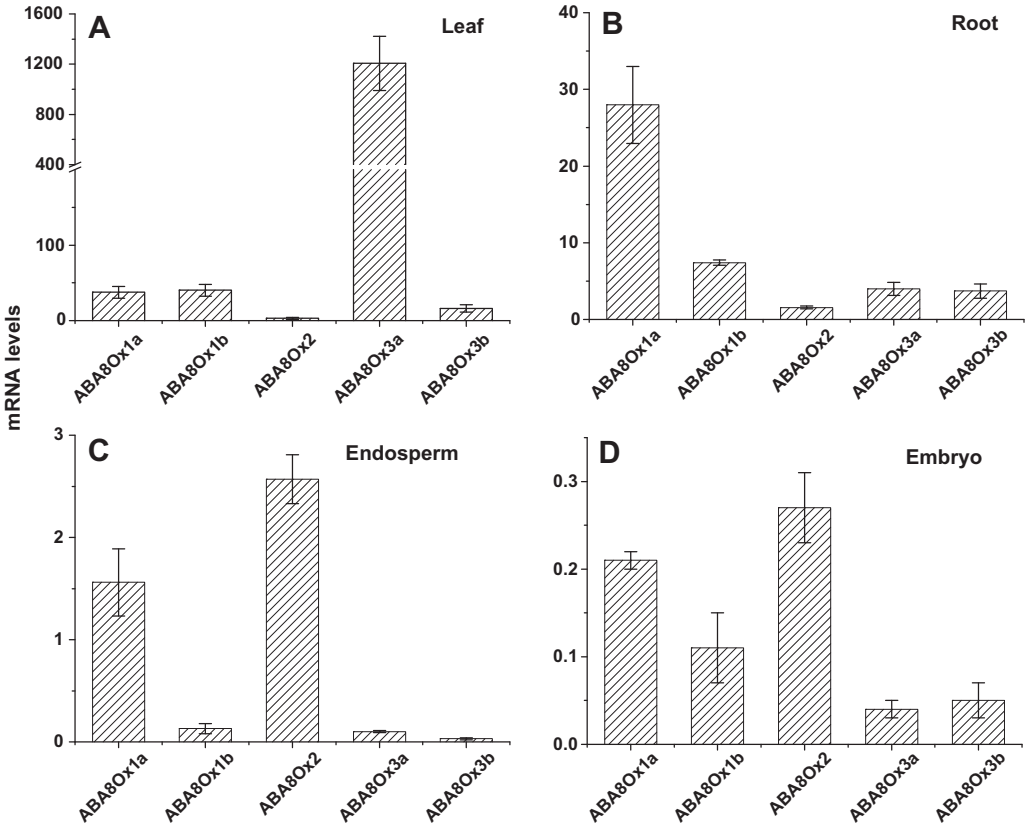

Fig. 4. Transcript profiles of maize inbred line B73 genes encoding ABA 8′-hydroxylases. Means of three replicates ± SD are presented. Embryo and endosperm were collected at 20 DAP from field-grown plants; leaf and root samples were collected from seedlings at the six-leaf stage.

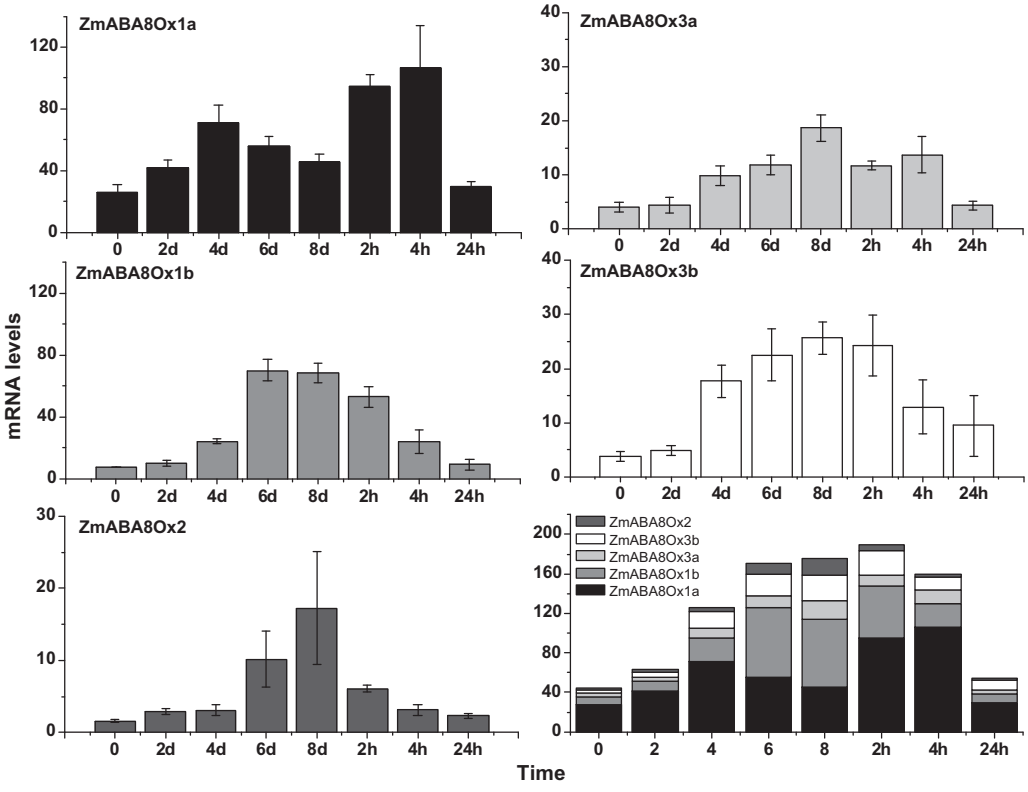

Fig. 5. Drought-stress-modulated levels of transcripts encoded by *Zea mays* (Zm) *ABA8Ox* genes controlling inactivation of ABA in maize roots. Means of three replicates ± SD are presented. See Table 1 for GenBank accession numbers. Bottom right panel shows the sum (stacked) of all transcripts controlling ABA catabolism. 0–8 days are days under drought and 2–24 h are hours under rehydration with water.

log, based on Fig. 3) plays a prominent role in ABA catabolism in the vegetative tissues while CYP707A2 was found to play a significant role in seed dormancy [6,18,19]. In *Phaseolus vulgaris*, high ABA8Ox activity in leaves during dehydration was found to be due solely to an increase in *CYP707A3* transcripts while no change was observed in transcript levels of *CYP707A1* and *CYP707A2* [20].

Modulation and contribution of ABA 8′-hydroxylases during water deficiency in roots

Because drought stress is first detected by the roots, it is likely that this organ can sense and respond to abiotic stress, leading to the mechanics of ABA accumulation. This suggests that ABA8Ox modulation in roots can determine drought tolerance in the entire plant. Data presented in Fig. 5 suggest that all five maize *ABA8Ox* genes are modulated by drought stress: their relative transcript levels paralleled dehydration and rehydration (Fig. 5) and modulation of ABA levels (measured in the same samples, as reported earlier in [8]). In roots, expression of *ABA8Ox1a*, followed temporally by that of *ABA8Ox1b*, suggests an important role for these two genes in modulating root ABA levels. *ABA8Ox1a* levels peaked at 4 days of drought stress, whereas *ABA8Ox1b* transcript levels peaked at 6 days. *ABA8Ox1a*, but not *ABA8Ox1b*, also showed further elevation in response to rehydration within 2 h of watering on day 8 (Fig. 5). These data also suggest that since all *ABA8Ox* genes are expressed in roots, it is reasonable to assume that each of them contributes to the modulation of ABA concentration in that organ. Pooled transcript levels suggested temporal, compensatory variations in expression of each of the paralogous genes, particularly between *ABA8Ox1a* and *ABA8Ox1b*. *ABA8Ox1a* was found to contribute the most to root *ABA8Ox* transcript levels in early responses to drought stress, followed by *ABA8Ox1b, ABA8Ox3b*, *ABA8Ox3a* and *ABA8Ox2* (Fig. 5).

Conclusions

Many factors contribute to whole-plant adaptation and tolerance to drought stress. This hypothesis is consistent with the existence of various quantitative trait loci reported in previous studies on drought stress [21,22]. Here, evidence is provided for control of ABA levels in roots mediated by ABA8Ox1a as the main early-response catabolic enzyme followed temporally by ABA8Ox1b. Drawing from the phylogenetic analysis, *ABA8Ox1* orthologs can be considered potential targets for the engineering of drought-tolerance across the grass family.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.07.005.

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