Characteristics and Expression Patterns of the Aldehyde Dehydrogenase (ALDH) Gene Superfamily of Foxtail Millet (*Setaria italica L.*)

Chen Zhu1,2,*1, Chen Ming2,3, Xu Zhao-shi2, Li Lian-cheng2, Chen Xue-ping1,*, Ma You-zhi2*

1 Department of Chemistry, University of Science and Technology of China, Hefei, Anhui, China, 2 Institute of Crop Science, Chinese Academy of Agricultural Sciences, National Key Facility for Crop Gene Resources and Genetic Improvement, Key Laboratory of Biology and Genetic Improvement of Triticeae Crops, Ministry of Agriculture, Beijing, China

Abstract

Recent genomic sequencing of the foxtail millet, an abiotic, stress-tolerant crop, has provided a great opportunity for novel gene discovery and functional analysis of this popularly-grown grass. However, few stress-mediated gene families have been studied. Aldehyde dehydrogenases (ALDHs) comprise a gene superfamily encoding NAD (P)-dependent enzymes that play the role of “aldehyde scavengers”, which indirectly detoxify cellular ROS and reduce the effect of lipid peroxidation mediated cellular toxicity under various environmental stresses. In the current paper, we identified a total of 20 ALDH genes in the foxtail millet genome using a homology search and a phylogenetic analysis and grouped them into ten distinct families based on their amino acid sequence identity. Furthermore, evolutionary analysis of foxtail millet reveals that both tandem and segmental duplication contributed significantly to the expansion of its ALDH genes. The exon-intron structures of members of the same family in foxtail millet or the orthologous genes in rice display highly diverse distributions of their exonic and intronic regions. Also, synteny analysis shows that the majority of foxtail millet and rice ALDH gene homologs exist in the syntenic blocks between the two, implying that these ALDH genes arose before the divergence of cereals. Semi-quantitative and real-time quantitative PCR data reveals that a few *SialDH* genes are expressed in an organ-specific manner and that the expression of a number of foxtail millet ALDH genes, such as, *SialDH7B1*, *SialDH12A1* and *SialDH1B* are up-regulated by osmotic stress, cold, H2O2, and phytohormone abscisic acid (ABA). Furthermore, the transformation of *SialDH2B*, *SialDH10A2*, *SialDH5F1*, *SialDH22A1*, and *SialDH3E2* into Escherichia coli (E.coli) was able to improve their salt tolerance. Taken together, our results show that genome-wide identification characteristics and expression analyses provide unique opportunities for assessing the functional roles of foxtail millet ALDH genes in stress responses.

Introduction

As they grow, plants often encounter a wide spectrum of environmental stresses [1], such as drought, salinity, extreme temperatures and oxidative stress. To assure the survival and prosperity of their offspring, plants regulate the expression of a wide range of stress-responsive genes capable of coping with various abiotic stresses, which negatively affect plants by eliciting rapid and excessive accumulation of reactive oxygen species (ROS) that leads to cellular injury (e.g., lipid peroxidation or protein and nucleic acid modification) [2,3]. Moreover, ROS reacting with lipids and proteins are known to cause an accumulation of toxic products (i.e. aldehydes), which in turn amplify ROS-induced damage. As such, a better understanding of the mechanisms involved in the evolutionary survival of plants to environmental stresses would be of great value.

Aldehyde dehydrogenases (ALDHs) represent an evolutionary conserved gene superfamily encoding NAD (P)-dependent enzymes that catalyze the irreversible oxidation of a wide range of endogenous and exogenous aromatic and aliphatic aldehydes into corresponding carboxylic acids [4]. Several studies show that many ALDHs protect against various environmental stressors by indirectly detoxifying cellular ROS and/or reducing lipid peroxidation [5]. For instance, overexpression of AtALDH3 reduces lipid peroxidation and increases resistance to osmotic stress, metal toxicity, H2O2 and paraquat treatment [6]. Furthermore, ectopic expression of ALDH7 in both Arabidopsis and tobacco enhances their protection against various osmotic stressors, such as dehydration and high salinity [7]. In addition, overexpression of ALDH2A1 in transgenic tobacco plants increases its stress tolerance and leads to decreased levels of malondialdehyde (MDA) [8]. *ALDH* genes also play vital roles in many fundamental metabolic pathways under normal conditions. For example, they facilitate in the synthesis and catabolism of a wide range of biomolecules, such as amino acids, lipids, and vitamins. As such, the ability of ALDHs to facilitate stress responses in plants has

Citation: Zhu C, Ming C, Zhao-shi X, Lian-cheng L, Xue-ping C, et al. (2014) Characteristics and Expression Patterns of the Aldehyde Dehydrogenase (ALDH) Gene Superfamily of Foxtail Millet (*Setaria italica L.*) PLoS ONE 9(7): e101136. doi:10.1371/journal.pone.0101136

Editor: Manoj Prasad, National Institute of Plant Genome Research, India

Received January 17, 2014; Accepted June 4, 2014; Published July 2, 2014

Copyright: © 2014 Zhu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Key Project for Research on Transgenic Biology (2013ZX08002-002) and the National 863 High-tech Project (2012AA10A309). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: mayouzhi@caas.cn (MYZ); chenxp08@ustc.edu.cn (CXP)

† These authors contributed equally to this work.
made these enzymes the focus of numerous studies on developing stress-resistant crops.

Aldehyde dehydrogenases constitute a diverse protein family found in various organisms. In 1999, the ALDH Gene Nomenclature Committee (AGNC) established criteria for cataloguing deduced ALDH protein sequences [9]. Sequences with more than 40% identity to a previously identified ALDH sequence represent a family and sequences with more than 60% identity within the ALDH family represent a protein subfamily. To date, ALDHs have been classified into 24 distinct families: ALDH10, ALDH12, ALDH21, ALDH22, ALDH23, and ALDH24 are unique to plants [10–13]. Completion of the genomic sequencing of various plants has allowed more and more aldehyde dehydrogenase genes to be identified and classified from lower to higher plants, such as Physcomitrella patens, Chlamydomonas reinhardtii, Arabidopsis thaliana, Vitis vinifera, Zea mays, Sorghum bicolor and Glycine max [10,12–15]. However, there have been any systematic investigations of aldehyde dehydrogenase families from the foxtail millet published.

Foxtail millet (Setaria italica L.) is well known for its drought tolerance and is one of the oldest cultivated millet crops and an important food and fodder grain crop in arid and semi-arid regions of Asia and Africa. It is a diploid C4 panicoid crop with a small genome of ~515 Mb, a short life cycle, and a highly conserved genome structure relative to ancestral grass lineages [16]. As such, foxtail millet has been proposed as an ideal model crop for genetic and molecular studies. Recently, the US Department of Energy Joint Genomic Institute [17] and Beijing Genomics Institute (BGI), China [16] was able to fully sequence its genome, paving the way to additional genome-wide identification studies and the analysis of gene families in foxtail millet.

The SiNAC [18] and SiWD40 [19] gene families have recently been systematically analyzed. However, to date there are no reports on the analysis of the SiALDH gene family. In the current study, we identified 20 ALDH genes from foxtail millet, classified them into 10 different gene families, and investigated their expansion and evolutionary history by examining their duplication, chromosomal distribution, exon-intron structure and synteny map with their rice orthologs. Subsequently, we analyzed the expression profiles of these SiALDH genes in different tissues and under various abiotic stresses and identified the function of ten SiALDH genes expressed in transgenic E.coli in response to salinity-induced stress.

Results and Discussion

The foxtail millet ALDH gene family: nomenclature and phylogenetic analysis

Keywords, HMM profile, and BLAST searches uncovered that approximately twenty ALDH proteins exist in the S.italica genome. Furthermore, based on criteria established by the AGNC, 20 putative ALDH genes were also identified in S.italica genome and grouped into ten families (Table 1). Four families contained multiple members (ALDH2, six members; ALDH3, four members; ALDH10 and ALDH18, two members each), each of the other six families was represented by a single gene (ALDH5, ALDH6, ALDH7, ALDH11, ALDH12, and ALDH22) and each gene was assigned to different subfamilies (Table 1). A unique identifier was assigned to each of the S.italica ALDH proteins. A number, based on the present location of the ALDH gene on the chromosome, following the subfamily name was used to distinguish multiple members contained in a single family. Subcellular localization predictions revealed that most of S.italica ALDH proteins are located in the cytoplasm and mitochondrion. Moreover, ten S.italica ALDH proteins had splice variant and only primary transcripts were selected for phylogenetic and comparative analyses. Among the 20 foxtail millet ALDH genes identified, eighteen genes were supported by full length protein sequences and cDNA sequences (their corresponding GeneBank Accession are listed in Table 1); the other two genes were not found in GeneBank, but the full length sequences are shown in the Text S1.

In the present study, we listed the numbers of gene family members for each individual ALDH family in S.italica and ten other plant species (Arabidopsis thaliana, Oryza sativa, Sorghum bicolor, Zea mays, Vitis vinifera, Populus trichocarpa, Physcomitrella patens, Chlamydomonas reinhardtii, Volvox carteri, and Glycine max) and Homo sapiens (Table S1). Of note, there is a lack of ALDH1 and ALDH4 gene family members found in plants due to nomenclature errors made when the genes were originally identified [12]. Plants have 13 various ALDH gene families: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, ALDH21, ALDH22, ALDH23, and ALDH24; in addition, ALDH19 has only been identified within tomato genome [20]. ALDH21, ALDH23 and ALDH24 are unique to lower plants. Like other monocot/dicot plants, S.italica contain ten common core ALDH families: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, and ALDH22. Unfortunately, the evolutionary relationships of the ALDH protein in foxtail millet and other plants had never been performed.

In recent years, the ALDH protein in multiple plant species has been identified. As such, we chose several representational plants for analysis, including Arabidopsis thaliana, Oryza sativa, Sorghum bicolor, and Zea mays and constructed a phylogenetic tree of the ALDH genes in these species, in addition to foxtail millet. The tree was classified into ten major families (Fig 1 & Table 1) and ALDH proteins from the same families were clustered together (Fig 1). The phylogenetic tree also revealed that these plant ALDHs split into three clades and share the common core plant ALDH families, listed above. In addition, we found that the majority of foxtail millet ALDH families are more closely related to those in grass species (O. sativa, Z. Mays, and S. Bicolor). These results are consistent with the present understanding of plant evolutionary history [21].

Segmental and tandem duplication contribute to superfamily expansion

Gene duplication plays an important role in the expansion of gene families. It was recently reported that the foxtail millet genome underwent whole-genome duplication, similar to other grasses, approximately 70 MYA (million year ago) and most of the duplications were generated in the whole genome duplication (WGD) event shared by all grasses [16]. In the present analysis, we found that the 20 foxtail millet ALDH genes are randomly distributed on nine foxtail millet chromosomes (Fig 2A). Among them, eight genes (SiALDH3E1/SiALDH3E2, SiALDH5H1/SiALDH5H2, SiALDH2B1/SiALDH2B2 and SiALDH18B1/SiALDH18B2) are located in four pairs of segmental duplicated genome regions, likely caused by whole genome duplication. In addition, SiALDH2C2 and SiALDH2C3 genes are identified as a tandem duplication in foxtail millet ALDH family. In summary, four foxtail millet multi-member ALDH gene families are linked with either segmental or tandem duplication events, suggesting that segmental and tandem duplication events play significant roles in the expansion of SiALDH genes. Moreover, the contribution of selection on coding sequences can be quantified by measuring the ratio of nonsynonymous to synonymous substitutions (Ka/Ks). A pair of sequences with Ka/Ks<1 indicates that one sequence has undergone purifying selection while the other has been drifting neutrally. Alternatively, if Ka/Ks>1 indicates that one sequence has undergone positive selection.

Department of Energy Joint Genomic Institute [17] and Beijing Genomics Institute [18] have allowed more and more aldehyde dehydrogenase genes to be identified and classified from lower to higher plants, such as Physcomitrella patens, Chlamydomonas reinhardtii, Arabidopsis thaliana, Vitis vinifera, Zea mays, Sorghum bicolor and Glycine max [10,12–15].
Ks = 1 then both sequences have been drifting neutrally. And finally, in the rare event that Ka/Ks>1 specific sites in that sequence have been under positive selection [22]. Our results indicate that the Ka/Ks ratios for five duplicated pairs varied from 0.0024 to 0.1726 with an average of 0.06998 (Table S2), strongly indicating that the SiALDH family underwent strong purifying selection pressure. All five paralogous pairs showed Ks values > 1.1, signifying that these duplications might have occurred > 85.48 MYA (Table S2). This result suggests that duplications of these 5 paralogous pairs occurred before the whole genome duplication (WGD) of grass. However, we are cautious about asserting that these SiALDH duplicated before the WGD, considering that the pseudogenization or frame-shift mutation have the potential to inflate Ks value [23].

Structural divergences are prevalent in duplicate genes and, in most cases, have led to the generation of functionally distinct paralogs [24]. Investigation into the gene structures of multiple gene families (Fig. S1) shows that duplicate genes of SiALDH have a highly diverse distribution of exons and introns in the foxtail millet genome. In addition, nonduplicate genes of SiALDH also have different gene structures. For example, ALDH10 genes from rice [25] and grape [12] had the same numbers of exons while exhibiting nearly identical lengths, whereas the first exon of SiALDH10A1 and SiALDH10A2 was clearly different in the foxtail millet genome. We hypothesize that this difference is attributed to a rapid evolution of these genes through gene duplications or via their integration into the genome following reverse transcription [26–20].

Evolutionary relationship of ALDH gene families between foxtail millet and rice

Researchers can gain a better understanding of the genomic structure/evolution of a lesser-studied taxon via comparative genomic analyses [29]. As such, we were able to infer the probable function of the orthologous genes by comparing the genomes of foxtail millet and rice. Large-scale synteny analysis identified 90% (18/20) of SiALDH to have shared synteny with their orthologs of the rice genome. Among them, 10 pairs (SiALDH2C3/OsALDH2C1, SiALDH2C3/OsALDH2C3, SiALDH5F1/OsALDH5F1, SiALDH6B1/OsALDH6B1, SiALDH7B1/OsALDH7B1, SiALDH10A2/OsALDH10A5, and SiALDH11A1/OsALDH11A3) unambiguously existed in both foxtail millet and rice genomes. There were some much stronger cases for a gene/regional duplication where two separate genes of foxtail millet were related to one or two genes in the rice genome, such as SiALDH2C1/SiALDH2B5, SiALDH3E1/SiALDH3E2, SiALDH6B1/SiALDH10A2, and SiALDH18B1/SiALDH18B2. These results suggest that the majority of SiALDH genes share a common ancestor with OsALDH genes counterparts. Certainly, some SiALDH genes were not mapped to any syntenic blocks with rice, such as SiALDH2C4, SiALDH2A1, and SiALDH22A1 (Figure 2B), but it does not mean that these ALDH genes from foxtail millet and rice do not share a common ancestor. Rather, this can be explained by the fact that foxtail millet and rice chromosomes have undergone extensive rearrangements and fusions that possibly lead to selective gene loss [16].

We next want to determine if functional conservations have been maintained between orthologous genes in the two related species. As such, we calculated the ratios of nonsynonymous (Ka) to synonymous (Ks) substitution rate (Ka/Ks) for orthologous gene pairs of foxtail millet SiALDHs with those of rice (22 pairs). We found that the ratios of Ka/Ks for all 22 orthologous gene pairs were less than 1, indicating that functional conservation is likely maintained between these orthologous pairs. We further estimated
the approximate divergence time of those orthologous gene pairs. Eight orthologous gene pairs (SiALDH2B2-OsALDH2B1, SiALDH5F1-OsALDH5F1, SiALDH7B1-OsALDH7B1, SiALDH7B6-OsALDH7B6, SiALDH10A1-OsALDH10A1, SiALDH10A9-OsALDH10A9, and SiALDH18B2-OsALDH18B2) have calculated Ks values that vary from 0.3481 to 0.5655 with an average of 0.4467, indicating that the divergence time of these genes was somewhere between 26.77 and 43.50 MYA (Table S2) at a period of time following the divergence between Potidaea and Panicoideae. Additionally, five of the orthologous pairs (SiALDH2C1-OsALDH2C3, SiALDH2B1-OsALDH2B5, SiALDH3H1-OsALDH3H1, SiALDH3H2-OsALDH3H2, and SiALDH18B2-OsALDH18B2) had Ks values from 0.6273 to 0.8862 with an average of 0.7487 (Table S2), signifying that their divergence occurred at some point between 48.25 and 68.17 MYA, after the whole genome duplication (WGD) of grass. Taken together, these results are comparable to evolutionary studies of protein-coding genes annotated from a recently released draft genome sequence of foxtail millet. Alternatively, the other nine orthologous pairs showed Ks values larger than 1 (Table S2), demonstrating that these orthologous pairs occurred before the whole genome duplication (WGD) of grass, but we are cautious about these results and believe that further research is required to be certain of this data.

Orthologous genes conceivably had identical functions, but tended to diverge in regulatory and coding regions which led them...
to altered the expression patterns and acquire new functions, respectively [24]. In this study, we compared the exon-intron structures of ALDH genes identified in the foxtail millet genome with those found in rice. Our results revealed that a number of exonic losses and gains occurred during the evolution of ALDH genes in both species at the 5' or 3' terminal and even in the middle of their sequences, such as OsALDH18B1/SiALDH18B1, OsALDH2B5/SiALDH2B1 (Fig. 3). Furthermore, SiALDH18B1 have acquired an additional exon between the first and second exon of OsALDH18B1 (Fig. 3), OsALDH2B5 and SiALDH2B1 have the same number of exons but exhibit different exons lengths (Fig. 3), suggesting that these foxtail millet ALDH genes might possess different functions. On the other hand, compared to rice, several SiALDH genes have identical exon/intron structure.
Expression profiles and promoter analysis of foxtail millet ALDH genes

We ran semi-quantitative RT-PCRs to observe the organ-specificity of SiALDH genes using three tissues: young root, stem, and leaf. We found that the majority of SiALDH genes were expressed in the stem and leaf and had significantly lower expression levels (and in some cases were even non-detectable) in the root, such as SiALDH7B1, SiALDH2C1, SiALDH5E1, and SiALDH22A1. SiALDH12B1 and SiALDH15B1 were mainly expressed in the stem and SiALDH3E1 was expressed in the root, stem and leaf, unlike its sister gene, SiALDH3E2 (Fig. 3), indicating that a portion of foxtail millet ALDH genes have similar gene functions to ALDH genes in rice. However, the insertion and deletion of amino acids in the proteins with identical gene structures should not be disregarded. As such, further investigations are essential in order to illustrate the specifics of any functional divergence between SiALDH genes.

ALDH and Setaria italica L.

(0aALDH3F1/SaALDH3F1, 0aALDH11A5/SaALDH11A1, 0aALDH16A5/SaALDH16A2, 0aALDH2B1/SaALDH2B2, and 0aALDH3H1/SaALDH3H1) (Fig. 3), indicating that a portion of foxtail millet ALDH genes have similar gene functions to ALDH genes in rice. However, the insertion and deletion of amino acids in the proteins with identical gene structures should not to be disregarded. As such, further investigations are essential in order to illustrate the specifics of any functional divergence between SiALDH genes.

Expression profiles and promoter analysis of foxtail millet ALDH genes

We ran semi-quantitative RT-PCRs to observe the organ-specificity of SiALDH genes using three tissues: young root, stem, and leaf. We found that the majority of SiALDH genes were expressed in the stem and leaf and had significantly lower expression levels (and in some cases were even non-detectable) in the root, such as SiALDH7B1, SiALDH2C1, SiALDH5E1, and SiALDH22A1. SiALDH12B1 and SiALDH15B1 were mainly expressed in the stem and SiALDH3E1 was expressed in the root, stem and leaf, unlike its sister gene, SiALDH3E2 (Fig. 3). Similar results were observed in other duplicated pairs, such as SiALDH18B1/SiALDH18B2 and SiALDH2C2/SiALDH2C3 (Fig. 4).

A comprehensive promoter analysis is capable of providing a reference for functional predictions of the 20 stress-related foxtail millet ALDH genes. For this purpose, we identified regulatory elements that were identified in the DNA sequences (~1 kb region upstream of the predicted SiALDH genes) using plantCARE [30]. A number of regulatory elements, including MBS, ARE, LTR and ABRE, known to be involved in responses to either abiotic stresses or hormone levels were found to be overrepresented in the 1kB region upstream of the SiALDH gene (Table S3). The 1 kb region upstream of all SiALDH genes have light or ABA regulatory elements (Table S3). Taken together, this data may indicate that the predicted SiALDH genes have a crucial role in stress-mitigation in the foxtail millet.

We next subjected the 20 SiALDH genes to quantitative expression analysis in order to decipher the role of SiALDH genes during diverse environmental situations: dehydration (20% PEG-6000), salinity, high temperature, cold, ABA, and H2O2 stress (0, 1, 6, 12, 24, 48 h treatment durations) (Fig. 5 and Table S4). ALDH2 family members required for male plant fertility were first regulated following PEG-6000, NaCl, H2O2, and heat treatment, the transcript levels of SaALDH12 were down-regulated (Fig. 5A-F), suggesting potential roles for this gene in mediating cell damage during heat shock.

In plants, ALDH2 family members play a key role in proline (Pro) degradation and their expression levels have been shown to be up-regulated via various stresses [46]. For example, both endogenous proline and salinity can induce the expression levels of Arabidopsis ALDH2A1. Moreover, OsALDH12 gene is induced by drought and salt stresses. Our data (Fig. 5A-F) reveal that SaALDH12 was upregulated under all abiotic stress conditions and ABA treatment, suggesting a possible role for it in foxtail millet during periods of environmental hardship.

The SaALDH18 family, encoding a Δ1-pyrroline-5-carboxylate synthetases (PCSs; EC 2.1.2.1 and EC 2.7.2.11), is a bifunctional protein that contains an N-terminal amino-acid kinase domain and a C-terminal dehydrogenase domain. These enzymes play an important role in the biosynthesis of proline [47]. Furthermore, the expression levels of ALDH18 in a number of plants have been shown to be up-regulated in response to osmotic stress [40,49]. Similarly, we also showed that SiALDH18 was up-regulated following PEG-6000, NaCl, H2O2, low temperature, and ABA (Fig. 5A-E).
Figure 3. Phylogenetic analysis and exon-intron structures of foxtail millet and rice ALDH genes. Numbers above or below branches of the tree indicate bootstrap values. Coding exons, represented by ashy, were drawn to scale. Dashed lines connecting two exons represent introns. Members of respective ALDH families are depicted in a specific background color.

doi:10.1371/journal.pone.0101136.g003
The \textbf{ALDH5} gene family is comprised of succinic semialdehyde dehydrogenases (SSADH; EC 1.2.1.24), which participates in GABA ‘shunt’ pathway in bacteria, plants and animals. In plants, \textit{ALDH5} mutations cause enhanced accumulation of reactive oxygen intermediates and cell death in response to light and heat stress [50]. Our results (Fig. 5A–F) indicate an upregulation of \textit{SiALDH5} by all of the investigated stressors except for low temperature.

The \textbf{ALDH6} gene family is composed of methylmalonyl semialdehyde dehydrogenases (EC 1.2.1.27). Thus far, the function of these genes in plants is not clear. Here we found an upregulation in the expression of \textit{SiALDH6} under PEG-6000, NaCl, and ABA treatment (Fig. 5B, C and E). However, we believe their exact functions should be elucidated further.

Moreover, \textit{ZmALDH22A1} was recently found to be up-regulated in response to a variety of stressors such as dehydration, high salinity, and ABA treatment [51]; the same consequence in foxtail millet is displayed in Fig. 5A–F. Nonetheless, \textit{ALDH22} is not known to be induced by osmotic stress in rice and \textit{Arabidopsis} [25,52]. As such, the role of \textit{ALDH22} in plants still needs further study.

In summary, the overall variability in gene expression patterns implies that \textit{SiALDHs} participate in a complex network of pathways in order to perform different physiological functions in response to different challenges. This comprehensive expression profile provides a clue to the role of \textit{SiALDHs} in imparting stress tolerance.

Enhancement of salt stress tolerance of recombinant \textit{E.coli} harboring foxtail millet \textit{ALDH} genes

The expression of foreign plant genes can directly contribute to increasing stress tolerance in bacteria host cells [53,54]. In plants, abiotic stress can trigger the generation of reactive oxygen species (ROS) that disrupts cellular homeostasis and induce the expression of genes involved in defense mechanisms [55]. Moreover, aldehyde dehydrogenases play a key role in the detoxification of various aldehyde molecules produced in response to abiotic stress. Thus, we expressed ten \textit{SiALDH} proteins (\textit{SiALDH2C2}, \textit{SiALDH2C1}, \textit{SiALDH2B2}, \textit{SiALDH10A2}, \textit{SiALDH5F1}, \textit{SiALDH22A1}, \textit{SiALDH3E1}, \textit{SiALDH3E2}, \textit{SiALDH11A1}, and \textit{SiALDH12A1}) in \textit{E.coli} in an attempt to determine the function of foxtail millet \textit{ALDH} proteins to salt stress condition, SDS-PAGE analysis results demonstrated that the molecular weights of the ten recombinant proteins agreed with the predicted molecular weights (Fig. S2). Subsequently, we spotted aliquots of recombinant strains harboring the empty vector pET-28a (Control) and 10 recombinant vectors (pET-SiALDH2C2, pET-SiALDH2C1, pET-SiALDH2B2, pET-SiALDH10A2, pET-SiALDH5F1, pET-SiALDH22A1, pET-SiALDH3E1, pET-SiALDH3E2, pET-SiALDH11A1, and pET-SiALDH12A1) onto LB plates and supplemented them with either 500 mmol/L or 800 mmol/L of...
NaCl. The growth status of Rosetta cells (transformed with Control and recombinant vectors) indicated that the recombinants and control cells showed similar growth on LB medium, suggesting that exogenous SiALDHs do not restrain the cell growth.

Results show that high salt severely inhibits the growth of the control strain. Amazingly, the five recombinants (pET-SiALDH2B2, pET-SiALDH10A2, pET-SiALDH5F1, pET-SiALDH22A1, and pET-SiALDH3E2) were able to grow normally at different dilution gradients under 500 mmol/L NaCl (Fig. 6). Similarly, under 800 mmol/L NaCl, we can clearly see that Rosetta cells harboring SiALDH10A1, SiALDH22A1, and SiALDH5F1 were able to still grow normally at high bacterial concentrations (Fig. 6), revealing that these five SiALDH genes (SiALDH2B2, SiALDH10A2, SiALDH5F1, SiALDH22A1, and SiALDH3E2) are associated with high salt tolerance. This enhancement of salt tolerance further indicates that the expression of these foxtail millet ALDH proteins in host cells is able to confer their protective function against protein damage, cellular membrane disruption, and cellular apoptosis; their exact function in plants needs to be further examined.

**Summary**

Aldehyde dehydrogenases (ALDHs) are a set of NAD (P)\(^+\)-dependent enzymes that oxidize a wide range of aldehydes into their corresponding carboxylic acids. The identification and characterization of ALDH gene families has been well studied in different plant species. However, currently there are no reports investigating ALDH gene families in the foxtail millet. In the current study, we identified 20 ALDH genes in the foxtail millet genome, grouped them into ten families, and then assigned each a unique identifier to each of the predicted *S. italica* ALDH proteins based on the criteria established by the ALDH Gene Nomenclature Committee (AGNC). Importantly, evolutionary analysis reveals that both tandem and segmental duplications have contributed significantly to the expansion of the foxtail millet ALDH genes. Our gene structure analysis shows that ALDHs from the same family in foxtail millet or the orthologous genes in rice display highly diverse distributions of their exonic and intronic regions. Comparative synteny analysis between foxtail millet and rice genomes show that the majority of SiALDH and OsALDH gene homologs are located in syntenic regions between the two, indicating that these ALDH genes share common ancestors. In addition, we found that analysis of the Ka/Ks ratios of paralogous and orthologous gene pairs facilitates in a deeper understanding of
the evolutionary significance of gene-duplications and divergence. Furthermore, we show for the first time that a few SiALDH genes extremely organ-specific and that the expression pattern of a number of SiALDH genes is affected by several stressors, including dehydration, salinity, cold, ABA treatment, and heat stress. Finally, we find that the transformation of SiALDH2B2, SiALDH10A2, SiALDH5F1, SiALDH22A1, and SiALDH3E1 into E.coli was able to improve its salt tolerance. Taken together, this work lays a solid foundation for future studies on the function of ALDH genes in foxtail millet and many other grasses, as well.

Methods and Materials

Identification and annotation of foxtail millet ALDH genes

Rice [15] and A. thaliana [10] ALDH amino acid sequences were retrieved from the PHYTOZOME v9.1 database (http://www.phytozome.org/) and utilized to identify homologous peptides from foxtail millet by BLASTP search using default settings. The keywords “ALDH”, “Aldehyde dehydrogenases”, and the HMM profiles of the ALDH domain PF00171, KOG2450 (aldehyde dehydrogenase), KOG2451 (aldehyde dehydrogenase), KOG2453 (aldehyde dehydrogenase) and KOG2456 (aldehyde dehydrogenase) were all used as queries for searching the foxtail millet genomic database to identify ALDH and ALDH-like sequences [56]. Similarity searches were also performed through BLASTP in GeneBank non-redundant protein database to eliminate possible exclusions of any additional ALDH member. All hits with the excepted values <1.0 were retrieved and all redundant sequences were removed using the decrease redundancy tool (http://web.expasy.org/decrease_redundancy/). Each non-redundant sequence was monitored for the presence of the conserved ALDH domain (PF00171) by SMART (http://smart.embl-heidelberg.de/) [57], Pfam (http://pfam.sanger.ac.uk/) and CDD (Conserved Domain Database) [58] searches. Likewise, the two active sites (PS00070 (cysteine active site) and PS00687 (glutamic acid active site)) were checked by PROSITE (http://prosite.expasy.org/). The deduced ALDH polypeptides were annotated using criteria established by the ALDH Gene Nomenclature Committee (AGNC) [3,9]. In brief, deduced amino acid sequences greater than 40% identical to other previously identified ALDH sequences were defined as a family, while sequences with greater than 60% identity were defined as a protein subfamily. Deduced amino acid sequences with less than 40% identity described a new ALDH protein family. YLoc [59,60] was used to predict the foxtail millet ALDH gene subcellular localization.

Sequence alignments, phylogenetic and promoter analysis

The protein sequences of ALDH genes in A. thaliana, O. sativa, S. Bicolor, Z. Mays [10] and S.italica were also retrieved from the PHYTOZOME v9.1 database (http://www.phytozome.org/). Subsequently, we performed multiple alignments of ALDH protein sequences using ClustalX2.0 [61]. The alignments results was adjusted using BioEdit V7.0.5.3 [62] and eliminated the portions of the sequences that could not be reliably aligned. Phylogenetic trees were constructed with the MEGA5.10 Beta4 [63] software utilizing the neighbor-joining (NJ) method and the bootstrap test was replicated 1000 times. The entire alignment has been provided as Text S2.

In order to investigate the promoter regions of the SiALDH gene family, the 1 kb upstream regions (based on the position of the genes provided by the S.italica annotation information) were selected and analyzed using plantCARE [30]. Known as stress-mediated regulatory elements are listed in Table S3.

Physical Mapping, Gene Duplication and Gene Structure Analysis

Specific chromosomal location and segment duplication of SiALDH genes were determined byFeatView (http://
genomevolution.org/CoGe/FeatView.pl) tool. The genes were plotted separately on to all nine foxtail millet chromosomes according to their ascending orders of physical position (bp) and then displayed using Adobe Illustrator CS6. Duplications in the foxtail millet genome and synteny between foxtail millet and rice were established utilizing the SynMap tool (detailed settings are as following: Blast Algorithm: Last, DAGChainer options and Merge syntenic Blocks use the recommended parameters, and the synonymous (Ks) and non-synonymous (Ka) substitution rates were calculated using the CODEML program [64] and confirmed with the GEno tool at the CoGe Web site (http://synteny.cnr.berkeley.edu/CoGe/). The formulas $T = Ks/2 \lambda (6.5 \times 10^{-5})$ [65,66] was used to calculate time of duplication and divergence of each SiALDH genes. Next, we identified tandem duplications of ALDH genes in the foxtail millet genome by checking their physical locations in individual chromosomes. Tandem duplicated genes were defined as adjacent homologous ALDH genes on the foxtail millet chromosomes, with no more than one intervening gene. All paralogous and orthologous gene pairs are listed in Table S5 and displayed using Adobe Illustrator CS6. Finally, the exon/intron structures of foxtail millet ALDH genes were determined from alignments of their coding sequences with corresponding genomic sequences using the est2genome program [67]. The diagrams of exon-intron structures were obtained using the online program FancyGene [68].

### Plant materials, growth conditions and treatments

The foxtail millet variety, Tie-8396, was used for all experiments. The seeds were germinated on wet filter paper at room temperature for 2 days and planted in pots with a mixture of peat/forest and vermiculite (1:1 v/v). Plants were grown in a greenhouse (28°C/day/20°Cnight, 16 h photoperiod, natural lighting, 70% relative humidity) and when the seedlings were five weeks old they were chronologically plunged into a 250 mM NaCl solution, a 100 μM ABA solution, a 20%PEG-6000 solution, and 100 μM H₂O₂ solutions for multiple periods of time (0, 1, 6, 12, 24, and 48 h). Moreover, we also subjugated the seedlings to 4 h of 42°C heat stress in an incubator followed recovery (0, 1, 6, 12, 24, and 48 h) at room temperature. Similarly, for cold stress tests, seedlings were placed in a 4°C freezer; also sampled at 0, 1, 6, 12, 24, and 48 h. Concurrently, the roots, stems, leaves of the untreated seedlings were also harvested for RNA extraction. These samples were put in liquid nitrogen and stored in −80°C. The above experiments were repeated three times to ensure precision and reproducibility.

### RNA extraction, Semi-quantitative and quantitative RT-PCR analysis

Total RNA was extracted using the RNAprep Pure Plant Kit (TIANGEN, Beijing) according to the manufacturer’s instructions. Prime Script RT reagent Kit (TaKaRa, Dalian) was used for reverse transcription. The condition for Semi-quantitative PCR amplification of cDNA was as follows: denature at 94°C for 10 min and 32 (actin) or 35(gene) cycles for 94°C 30 s; 58°C (gene) or 60°C (actin) 30 s; 72°C 30 s, followed by further incubation for 5 min at 72°C (1 cycle). Furthermore, densitometry and band quantization was performed utilizing Bio-Rad Quantity One software. Quantitative PCR was performed on the Applied Biosystems 7500 real-time PCR system using Super Real PreMix Plus (SYBR Green) (TIANGEN, Beijing), as per instructions. The PCR thermal cycle conditions were as follows: denature at 95°C for 15 min and 40 cycles for 95°C, 10 s; 60°C, 20 s; 72°C, 32 s. The specificity of the PCR reactions was determined by analyzing the melting curve. The constitutive actin gene (AF288226.1) was used as the endogenous control; quantification of the 20 SiALDH gene transcript was analyzed using the $2^{-\Delta \Delta Ct}$ method [69]. The primers used for real-time PCR analysis were designed with primique [70] and listed in Table S6. The each experiment repeat three times.

### Construction of expression vector pET- ALDH and the expression of foxtail millet ALDH genes in response to salinity

Primers in Table S6 were subsequently used for cloning SiALDH genes from cDNA. Amplification was performed as follows: 10 min at 95°C; 32 cycles of 40 s at 94°C, 2 min at 68°C; followed by a final extension for 10 min at 72°C. PET-28a (+) was linearized via BamHI and SacI restriction digestion. Then according to the In-Fusion HD Cloning System instructions, the cloned ALDH genes were sub-cloned into the PET-28a (+). For functional expression, E.coli Rosetta competent cells (CoWin Bioscience Co., Ltd. Beijing) were transformed with either the recombinant plasmid (PET-ALDH/Rosetta) or with PET-28a (+) vector lacking SiALDH genes (Control). The PET-ALDH/Rosetta and control were both allowed to grow overnight with shaking at 37°C. We next transferred 400 μl of each culture mixture into a fresh 40 ml liquid LB with kanamycin and shaken at 37°C for about 3 h. β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mmol/L in order to induce the expression of the inserted gene. After a 16 h induction (16°C), the samples (adjusting the original A600 value of all E.coli groups to the same value) were spotted onto the LB agar plates supplemented with NaCl (500 mmol/L, 800 mmol/L). The experiment was repeated three times with similar results.

### Data Analysis

Statistical analyses were completed by One-way ANOVA followed by Tukey’s test utilizing Origin 9.1 software. $P$ values of less than 0.05 were considered statistically significant.

### Supporting Information

#### Figure S1 Phylogenetic analysis and exon-intron structures of foxtail millet ALDH from the same family.

Numbers above or below branches of the tree indicate bootstrap values. Coding exons, represented by ashy, were drawn to scale. Lines connecting two exons represent introns.

#### Figure S2 The SDS-PAGE analysis for recombinant PET-ALDH.

Total proteins from SiALDH2C1, SiALDH2C2, SiALDH2B2, SiALDH10A2, SiALDH5F1, SiALDH22A1, SiALDH3E1, SiALDH3E2, SiALDH11A1, and SiALDH12A1 were separated by SDS-PAGE. The differential protein bands of SiALDH11A1, and SiALDH12A1 were about 61 kDa, 64 kDa, 63 kDa, 60 kDa, 62 kDa, 62 kDa, 58 kDa, 57 kDa, 59 kDa, and 71 kDa, respectively. 

### Table S1 Numbers of ALDH family members identified in various organisms.

| Organism | Number of Members |
|----------|-------------------|
| Species  |                   |
| Setaria italica | 20 |

### Table S2 The Ka/Ks ratios and estimated divergence time for orthologous ALDH proteins between foxtail
millet and rice and paralogous ALDH proteins in foxtail millet.

(DOCX)

Table S3 The promoter analysis of the 20 foxtail millet ALDH genes.

(XLSX)

Table S4 Relative expression values of 20 foxtail millet ALDH genes in response to various stress and hormone treatments. One-way ANOVA analysis of variance showed significant differences between group means (P<0.05). Tukey’s multiple comparison test showed significant differences between the means of groups depicted by the different letters (P<0.05). (XLSX)

Table S5 Paralogous gene pairs within foxtail millet and orthologous gene pairs between foxtail millet and rice.

(DOCX)

Table S6 A list of Primers sequences of 20 foxtail millet ALDH genes for real-time PCR and gene clone.

(DOCX)

References

1. Knight H, Knight MR (2001) Abiotic stress signalling pathways: specificity and cross-talk. Trends in Plant Science 6: 262–267.
2. Bartels D (2001) Targeting detoxification pathways: an efficient approach to obtain plants with multiple stress tolerance? Trends in plant science 6: 284–286.
3. Gill SR, Gaba S, Tuteja N (2010) Reactive oxygen species and antioxidative machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48: 909–930.
4. Yoshida A, Rabetsky A, Hsu LC, Chang C (1998) Human aldehyde dehydrogenase gene family. European Journal of Biochemistry 251: 549–557.
5. Singh S, Brocker C, Koppaka V, Chen Y, Jackson BC, et al. (2013) Aldehyde dehydrogenases in cellular responses to oxidative/electrophilic stress. Free Radical Biology and Medicine 56: 89–101.
6. Sankur B, Bartels D, Kirch HH (2000) Overexpression of a stress-inducible aldehyde dehydrogenase gene from Arabidopsis thaliana in transgenic plants improves stress tolerance. The Plant Journal 35: 452–464.
7. Rodrigues SM, Andrade MO, Gomes AP, DaMatta FM, Baracat-Pereira MC, et al. (2006) Arabidopsis and tobacco plants ectopically expressing the soybean antiquitin-like ALDH gene display enhanced tolerance to drought, salinity, and oxidative stress. Journal of experimental botany 57: 1909–1918.
8. Huang W, Ma X, Wang Q, Gao Y, Xie Y, et al. (2008) Significant improvement of stress tolerance in tobacco plants by overexpressing a stress-responsive aldehyde dehydrogenase gene from maize (Zea mays). Plant Molecular Biology 68: 451–463.
9. Vasiliiou V, Bairoch A, Tipton KF, Nebert DW (1999) Eukaryotic aldehyde dehydrogenase (ALDH) genes: human polymorphisms, and recommended nomenclature based on divergent evolution and chromosomal mapping. Pharmacogenetics 9: 421–434.
10. Kirch HH, Bartels D, Wei YL, Schnable PS, Wood AJ (2004) The ALDH gene superfamily of Arabidopsis. Trends in Plant Science 9: 371–377.
11. Kotchoni SO, Jimenez-Lopez JC, Kayode SO, Gachomo EW, Bahia-Moussa L (2012) The soybean aldehyde dehydrogenase (ALDH) protein superfamily. Gene 495: 128–133.
12. Zhang YC, Mao LY, Wang H, Brocker G, Yin XJ, et al. (2012) Genome-Wide Identification and Analysis of Grape Aldehyde Dehydrogenase (ALDH) Gene Superfamily. PLoS One 7.
13. Brocker C, Vasiiliou M, Carpenter S, Carpenter G, Zhang YC, et al. (2013) Aldehyde dehydrogenase (ALDH) superfamily in plants: gene nomenclature and comparative genomics. Planta 237: 189–210.
14. Jimenez-Lopez JC, Gachomo EW, Seufferheld MJ, Kotchoni SO (2010) The maize ALDH protein superfamily: linking structural features to functional specificities. BMC Structural Biology 10.
15. Kotchoni SO, Jimenez-Lopez JC, Gao D, Edwards V, Gachomo EW, et al. (2010) Modeling-dependent protein characterization of the rice aldehyde dehydrogenase (ALDH) superfamily reveals distinct functional and structural features. PLoS One 5: e11516.
16. Zhang G, Liu X, Quan Z, Cheng S, Xu X, et al. (2012) Genome sequence of foxtail millet (Setaria italicica) provides insights into grass evolution and biofuel potential. Nature biotechnology 30: 549–554.
17. Bennetzen JL, Schmutz J, Wang H, Pericfeld R, Hawkins J, et al. (2012) Reference genome sequence of the model plant Setaria. Nature biotechnology 30: 555–561.

Text S1 The information of 20 SiALDH genes. 20 SiALDH gene CDS and protein sequences were completely listed. (TXT)

Text S2 Multiple alignments of ALDH amino acid sequences from A. thaliana, O. sativa, S. Bicolor, Z. Mays and S. italic. (TXT)

Acknowledgments

Grateful thanks are due to Prof. Diao Xian-Min, Institute of Crop sciences, Chinese Academy of Agricultural Sciences, Beijing, China for providing the foxtail millet variety (Tei-8305). We are especially grateful to Prof. Shujin Hu, North Carolina State University, Raleigh, North Carolina, USA for helpful critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: CM CZ. Performed the experiments: CZ. Analyzed the data: CZ CM XXP MYZ. Contributed reagents/materials/analysis tools: MYZ CXP LL XZS. Wrote the paper: CZ CM.

PLOS ONE | www.plosone.org 12 July 2014 | Volume 9 | Issue 7 | e101136
50. Bouche´ N, Fait A, Bouchez D, Møller SG, Fromm H (2003) Mitochondrial
59. Briesemeister S, Rahnenfuhrer J, Kohlbacher O (2010) Going from where to
49. Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K
48. Razavizadeh R, Ehsanpour A (2009) Effects of salt stress on proline content,
47. Igarashi Y, Yoshiba Y, Sanada Y, Yamaguchi-Shinozaki K, Wada K, et al.
46. Miller G, Honig A, Stein H, Suzuki N, Mittler R, et al. (2009) Unraveling
45. Bartels D, Salamini F (2001) Desiccation Tolerance in the Resurrection
44. Gallardo K, Job C, Groot SP, Demol H, et al. (2001) Proteomic
43. Valverde F, Losada M, Serrano A (1999) Engineering a central metabolic
42. Fitzgerald TL, Waters DL, Henry RJ (2009) Betaine aldehyde dehydrogenase in
41. Nakamura T, Yokota S, Muramoto Y, Tsutsui K, Oguri Y, et al. (1997)
40. Rathinasabapathi B, Burnet M, Russell BL, Gage DA, Liao PC, et al. (1997)
39. Shin J-H, Kim S-R, An G (2009) Rice aldehyde dehydrogenase7 is needed for
38. Stiti N, Missihoun TD, Kotchoni SO, Kirch HH, Bartels D (2011) Aldehyde
dehydrogenases in Arabidopsis thaliana: biochemical requirements, metabolic
37. Kirch HH, Nair A, Bartels D (2001) Novel ABA-and dehydration-inducible
36. Wen Y, Wang X, Xiao S, Wang Y (2012) Ectopic expression of VpALDH2B4, a
35. Mohan Murali Archary V, Jena S, Panda KK, Panda BB (2008) Aluminium
34. 50. Bouche´ N, Fait A, Bouchez D, Møller SG, Fromm H (2003) Mitochondrial
33. 49. Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K
32. 48. Razavizadeh R, Ehsanpour A (2009) Effects of salt stress on proline content,
31. 47. Igarashi Y, Yoshiba Y, Sanada Y, Yamaguchi-Shinozaki K, Wada K, et al.
30. 46. Miller G, Honig A, Stein H, Suzuki N, Mittler R, et al. (2009) Unraveling
29. 45. Bartels D, Salamini F (2001) Desiccation Tolerance in the Resurrection
28. 44. Gallardo K, Job C, Groot SP, Demol H, et al. (2001) Proteomic
27. 43. Valverde F, Losada M, Serrano A (1999) Engineering a central metabolic
26. 42. Fitzgerald TL, Waters DL, Henry RJ (2009) Betaine aldehyde dehydrogenase in
25. 41. Nakamura T, Yokota S, Muramoto Y, Tsutsui K, Oguri Y, et al. (1997)
24. 40. Rathinasabapathi B, Burnet M, Russell BL, Gage DA, Liao PC, et al. (1997)
23. 39. Shin J-H, Kim S-R, An G (2009) Rice aldehyde dehydrogenase7 is needed for
22. 38. Stiti N, Missihoun TD, Kotchoni SO, Kirch HH, Bartels D (2011) Aldehyde
dehydrogenases in Arabidopsis thaliana: biochemical requirements, metabolic
21. 37. Kirch HH, Nair A, Bartels D (2001) Novel ABA-and dehydration-inducible
20. 36. Wen Y, Wang X, Xiao S, Wang Y (2012) Ectopic expression of VpALDH2B4, a
19. 35. Mohan Murali Archary V, Jena S, Panda KK, Panda BB (2008) Aluminium
18. 34. 50. Bouche´ N, Fait A, Bouchez D, Møller SG, Fromm H (2003) Mitochondrial
17. 33. 49. Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K
16. 32. 48. Razavizadeh R, Ehsanpour A (2009) Effects of salt stress on proline content,
15. 31. 47. Igarashi Y, Yoshiba Y, Sanada Y, Yamaguchi-Shinozaki K, Wada K, et al.
14. 30. 46. Miller G, Honig A, Stein H, Suzuki N, Mittler R, et al. (2009) Unraveling
13. 29. 45. Bartels D, Salamini F (2001) Desiccation Tolerance in the Resurrection
12. 28. 44. Gallardo K, Job C, Groot SP, Demol H, et al. (2001) Proteomic
11. 27. 43. Valverde F, Losada M, Serrano A (1999) Engineering a central metabolic
10. 26. 42. Fitzgerald TL, Waters DL, Henry RJ (2009) Betaine aldehyde dehydrogenase in
9. 25. 41. Nakamura T, Yokota S, Muramoto Y, Tsutsui K, Oguri Y, et al. (1997)
8. 24. 40. Rathinasabapathi B, Burnet M, Russell BL, Gage DA, Liao PC, et al. (1997)
7. 23. 39. Shin J-H, Kim S-R, An G (2009) Rice aldehyde dehydrogenase7 is needed for
6. 22. 38. Stiti N, Missihoun TD, Kotchoni SO, Kirch HH, Bartels D (2011) Aldehyde
dehydrogenases in Arabidopsis thaliana: biochemical requirements, metabolic
5. 21. 37. Kirch HH, Nair A, Bartels D (2001) Novel ABA-and dehydration-inducible
4. 20. 36. Wen Y, Wang X, Xiao S, Wang Y (2012) Ectopic expression of VpALDH2B4, a
3. 19. 35. Mohan Murali Archary V, Jena S, Panda KK, Panda BB (2008) Aluminium
2. 18. 34. 50. Bouche´ N, Fait A, Bouchez D, Møller SG, Fromm H (2003) Mitochondrial
1. 17. 33. 49. Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K