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Overexpression of *ALDH10A8* and *ALDH10A9* Genes Provides Insight into Their Role in Glycine Betaine Synthesis and Affects Primary Metabolism in *Arabidopsis thaliana*

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Betaine aldehyde dehydrogenases oxidize betaine aldehyde to glycine betaine in species that accumulate glycine betaine as a compatible solute under stress conditions. In contrast, the physiological function of betaine aldehyde dehydrogenase genes is at present unclear in species that do not accumulate glycine betaine, such as *Arabidopsis thaliana*. To address this question, we overexpressed the Arabidopsis *ALDH10A8* and *ALDH10A9* genes, which were identified to code for betaine aldehyde dehydrogenases, in wild-type *A. thaliana*. We analysed changes in metabolite contents of transgenic plants in comparison with the wild type. Using exogenous or endogenous choline, our results indicated that *ALDH10A8* and *ALDH10A9* are involved in the synthesis of glycine betaine in Arabidopsis. Choline availability seems to be a factor limiting glycine betaine synthesis. Moreover, the contents of diverse metabolites including sugars (glucose and fructose) and amino acids were altered in fully developed transgenic plants compared with the wild type. The plant metabolic response to salt and the salt stress tolerance were impaired only in young transgenic plants, which exhibited a delayed growth of the seedlings early after germination. Our results suggest that a balanced expression of the betaine aldehyde dehydrogenase genes is important for early growth of *A. thaliana* seedlings and for salt stress mitigation in young seedlings.

Keywords: Betaine aldehyde dehydrogenase • Carnitine • Glycine betaine • Polyamines • Salt stress.

Abbreviations: ALDH, aldehyde dehydrogenase; BADH, betaine aldehyde dehydrogenase; CaMV, *Cauliflower mosaic virus*; CMO, choline monooxygenase; GB, glycine betaine; MDA, malondialdehyde; MS, Murashige and Skoog; NMR, nuclear magnetic resonance; PCA, principal component analysis; RT–PCR, reverse transcription–PCR; WT, wild type.

Introduction

Betaine aldehyde dehydrogenases (BADHs) belong to family 10 of the aldehyde dehydrogenase superfamily and are initially classified as substrate-specific oxidoreductases (EC 1.2.1.8) (Sophos and Vasiliou 2003). BADH enzymes oxidize betaine aldehyde to glycine betaine (GB) in species that accumulate GB as a compatible solute under stress conditions. A number of recent studies have shown that some BADHs or BADH-homologous proteins possess affinity for a range of aminoaldehyde substrates (Trossat et al. 1997, Sebela et al. 2000, Fitzgerald et al. 2009, and references therein). Fitzgerald et al. (2009) proposed that plant enzymes classified as BADHs can be divided into two subfamilies: those with activity and high specificity for betaine aldehyde as substrate (true BADHs), and those that show a broader affinity for a range of aminoaldehydes [high BADH homology aminoaldehyde dehydrogenases (HBH-AMADHs)]. Despite this classification, BADHs are so far believed to function mainly in the biosynthesis of GB. The physiological function of the *BADH* genes is at present unclear in species that do not accumulate GB, such as *Arabidopsis thaliana* or rice. Two genes, *ALDH10A8* (*At1g74920*) and *ALDH10A9* (*At3g48170*), were identified to code for BADHs in *A. thaliana* (Sakamoto and Murata 2002, Kirch et al. 2004). In a previous study, we investigated the possible function of *ALDH10A8* and *ALDH10A9* by using mutant lines (Missihoun et al. 2011). Four-week-old plants of a T-DNA knockout mutant of *ALDH10A8* were more sensitive to dehydration and salt stress than wild-type (WT) plants, thus indicating a role for the *ALDH10A8* gene in the response to salt in Arabidopsis (Missihoun et al. 2011). Likewise, Niu et al. (2008) reported that rice transgenic RNA interference (RNAi) lines with reduced *BADH2* expression were more susceptible to salt stress than WT plants, as determined by measuring growth parameters. Rice genotypes which lack the functional BADH2 enzyme showed >99% inhibition of mature seed production, if exposed to an NaCl solution at as low a concentration as 22 mM (Fitzgerald et al. 2010). These results suggest that genes encoding BADH are functional in plants not accumulating GB, through either the synthesis of small amounts of GB or other unknown pathways. To investigate this hypothesis in *A. thaliana*, we overexpressed the Arabidopsis *ALDH10A8* and *ALDH10A9* genes

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in WT *A. thaliana* and analyzed changes in the metabolite contents of the transgenic plants in comparison with the WT. Our results indicated that the orthologs of BADH (ALDH10A8 and ALDH10A9) in Arabidopsis are involved in the synthesis of GB using exogenous or endogenous choline. Overexpression of these two genes altered the contents of diverse metabolites including sugars (glucose and fructose) and amino acids in the transgenic plants compared with the WT. The plant metabolic responses to salt and the salt stress tolerance were also altered in the transgenic plants, which exhibited a delayed growth of the seedlings early after germination. Altogether, our previous and current results suggest that a balanced expression of the BADH genes is important for the early growth of *A. thaliana* seedlings and for salt stress mitigation.

Results

A. thaliana synthesizes low amounts of glycine betaine

To learn about functions of the BADH-coding genes in species such as *A. thaliana*, which do not accumulate GB, we overexpressed the Arabidopsis BADH-encoding genes *ALDH10A8* and *ALDH10A9* in the WT and changes in metabolite contents were examined in the transgenic plants in comparison with the WT. We re-assessed the synthesis of GB in the transgenic plants because of the controversy in the current literature regarding the accumulation of GB in Arabidopsis (Rhodes and Hanson 1993, Xing and Rajashekar 2001). The controversies are mainly due to the barely detectable level of GB in Arabidopsis. We therefore reasoned that the GB measurements would be less prone to errors in gain-of-function plants than in loss-of-function plants. Plants overexpressing single genes were crossed to obtain plants that overexpress both *ALDH10A8* and *ALDH10A9* genes under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (**Fig. 1**). The transgenic plants overexpressing both genes were thereafter called OE lines. The level of GB and its metabolic precursor choline were determined in shoot tissues of 4-week-old transgenic OE and WT plants by using an optimized quantitative ^{1}H-nuclear magnetic resonance (NMR) procedure (see the Materials and Methods). Low levels of GB and choline were detected in both WT and OE plants under control conditions although their levels were marginally higher in OE plants than in the WT (**Fig. 2**). When plants were exposed to NaCl for 1 week, the levels of choline and GB became significantly higher in the OE plants than in the WT (**Fig. 2**). Only GB levels increased further in WT and OE plants after 2 weeks of salt stress, and about twice the amount of GB was measured in the OE plants (1.2 μmol GB g^{-1} FW) compared with WT plants (**Fig. 2**). To investigate the influence of choline availability on the biosynthesis of GB in the WT Arabidopsis and the OE plants exposed to salt, the plants were fed with choline, and endogenous choline and GB levels were measured. An increase of choline was observed in both the WT and the OE plants fed with choline compared with plants not fed with choline. Consistently, the level of GB increased in WT and OE plants compared with plants grown in medium without exogenous choline (**Fig. 2**). These data indicate that *A. thaliana* can synthesize GB using either endogenous or exogenous choline after exposure to salt and that the *ALDH10A8* and *ALDH10A9* genes code for functional enzymes boosting GB synthesis. The choline feeding experiment demonstrated that the choline level seems to be one limiting factor.

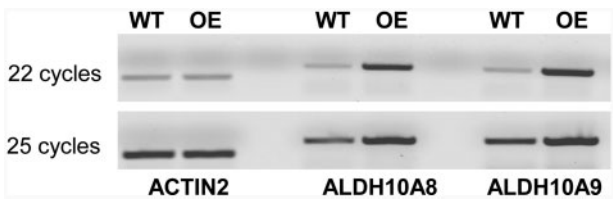

Fig. 1 RT–PCR-based gene expression analysis in plants. The level of expression of the *ALDH10A8* and *ALDH10A9* genes was determined in 1-week old wild-type (WT) and transgenic lines overexpressing both genes (OE). The PCR was run for 22 and 25 cycles.

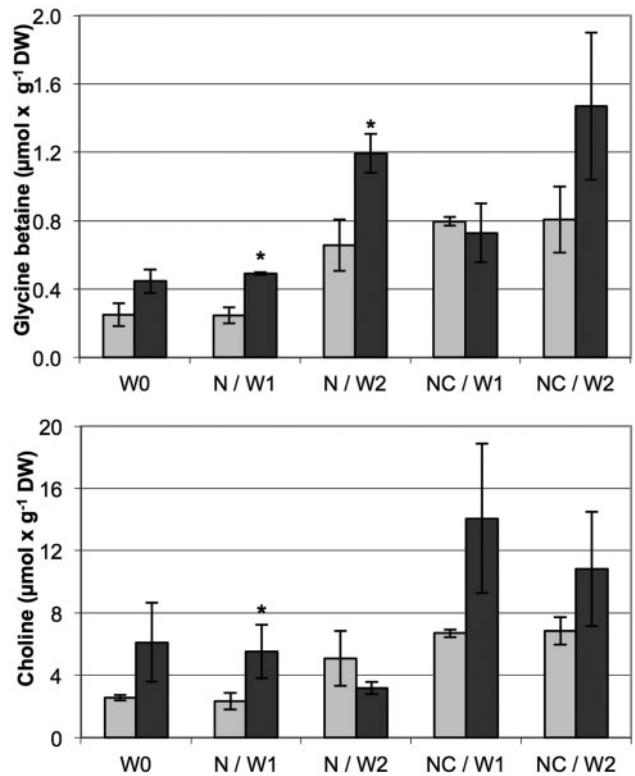

Fig. 2 Levels of choline and glycine betaine in plants. WT and OE represent the wild type and transgenic *A. thaliana* plants, and are indicated by light gray- and dark gray-shaded bars, respectively. OE plants overexpress both the *ALDH10A8* and *ALDH10A9* genes that code for proteins homologous to betaine aldehyde dehydrogenases in *A. thaliana*. W0, W1 and W2 denote unstressed plants harvested before the start of the treatment (W0), and 1 week (W1) and 2 weeks (W2) after the onset of salt stress, respectively. Four-week-old plants were treated with 200 mM NaCl (N) only or in combination with 5 mM choline (NC). Asterisks indicate a statistically significant difference between the WT and OE plants (*$P < 0.05$, Student's *t*-test).

Free carnitine increases under salt stress and is slightly enhanced in ALDH10A8- and ALDH10A9-overexpressing lines

Carnitine is a quaternary ammonium compound and a structural analog of GB. The biosynthesis of carnitine involves the oxidation of 4-*N*-trimethylaminobutyraldehyde to 4-*N*-trimethylaminobutyrate (γ-butyrobetaine), the direct precursor of L-carnitine. This reaction would be catalyzed by aminoaldehyde dehydrogenases, which could be ALDH10A8 and ALDH10A9. Therefore, the level of carnitine was measured in WT and OE leaf tissues. The level of carnitine was similar in WT and OE plants under control conditions but the OE plants accumulated marginally higher levels of carnitine than WT plants after 2 weeks of growth in hypersaline conditions (**Fig. 3**). Consistent with the difference in the biosynthesis routes of carnitine and GB, the addition of choline to the growth medium did not affect the accumulation of carnitine. These results suggest that the biosynthesis of carnitine appears to be regulated by salt, and that the effect of ALDH10A8 and ALDH10A9 overexpression on carnitine accumulation was very low.

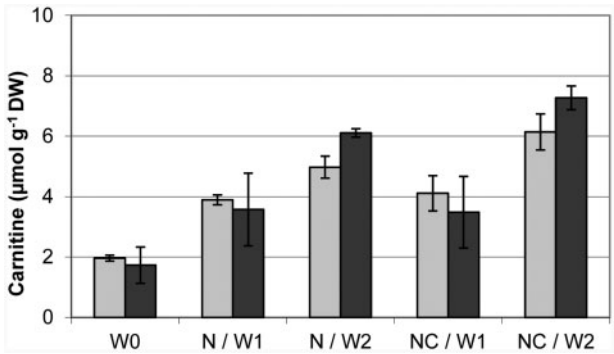

Fig. 3 Levels of carnitine in plants. WT and OE represent the wild type and transgenic *A. thaliana* plants, and are indicated by light gray- and dark gray-shaded bars, respectively. OE plants overexpress both the *ALDH10A8* and *ALDH10A9* genes that code for proteins homologous to betaine aldehyde dehydrogenases in *A. thaliana*. W0, W1 and W2 denote unstressed plants harvested before the start of the treatment (W0), and 1 week (W1) and 2 weeks (W2) after the onset of salt stress, respectively. Four-week-old plants were treated with 200 mM NaCl (N) only or in combination with 5 mM choline (NC). Asterisks indicate a statistically significant difference between the WT and OE plants (*$P < 0.05$, Student's *t*-test).

Free polyamine levels fluctuate under salt stress independently of ALDH10A8 and ALDH10A9 overexpression

In previous studies, we found that ALDH10A9 in Arabidopsis can oxidize betaine aldehyde and other aminoaldehyde substrates, although the activity was lower than that of the orthologous enzymes in rice, barley and spinach (Missihoun et al. 2011). It was suggested that ALDH10A9 would be involved in the terminal catabolism of polyamines in the plants (Stiti et al. 2011). To test this hypothesis, the content of the polyamines was measured in OE and WT plants. The levels of agmatine, putrescine, cadaverine, tyramine and spermine were similar in WT and OE plants under control conditions (**Fig. 4**). Spermidine was higher in untreated OE plants than in the untreated WT. Salt treatment in both genotypes led to increased levels of free agmatine, cadaverine and tyramine, whereas putrescine and spermidine amounts were lower. Spermidine and cadaverine were significantly lower in the OE plants than in the WT after 1 and 2 weeks of salt treatment, respectively.

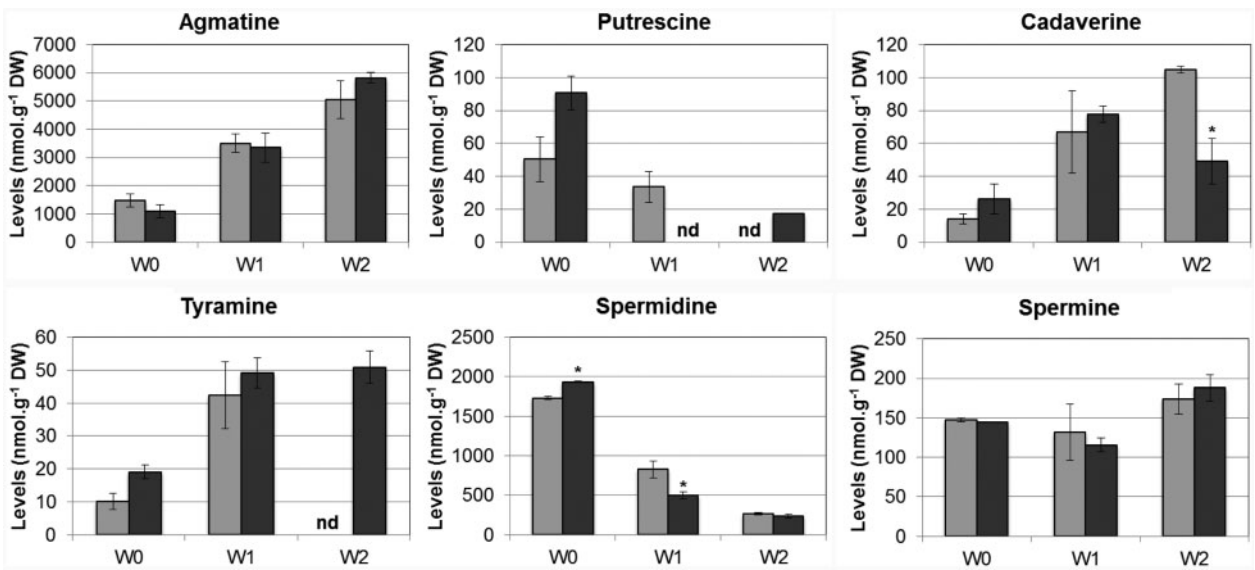

Fig. 4 Levels of selected polyamines. WT and OE represent the wild type and transgenic *A. thaliana* plants, and are indicated by light gray- and dark gray-shaded bars, respectively. OE plants overexpress both the *ALDH10A8* and *ALDH10A9* genes that code for proteins homologous to betaine aldehyde dehydrogenases in *A. thalana*. W0, W1 and W2 denote unstressed plants harvested before the start of the treatment (W0), and 1 week (W1) and 2 weeks (W2) after the onset of salt stress, respectively. Four-week-old plants were treated with 200 mM NaCl (N). nd denotes 'not detected'.

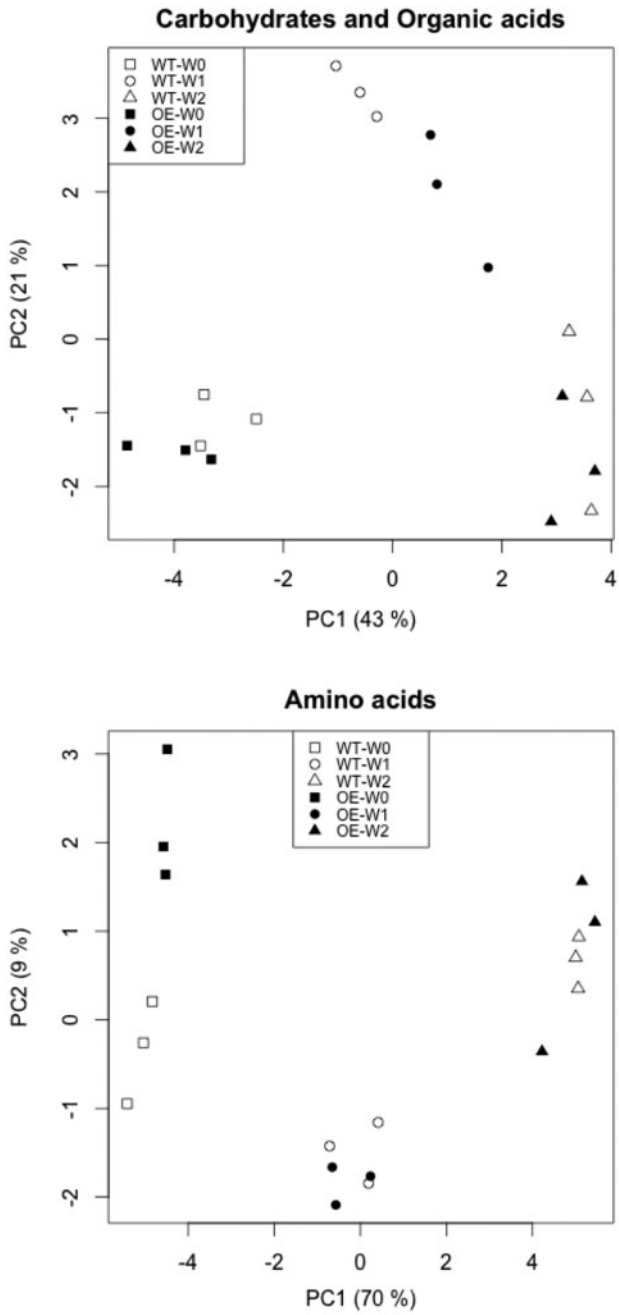

Fig. 5 Principal component analysis of the steady-state levels of metabolites in plants. WT and OE represent the wild-type and transgenic *A. thaliana* plants, respectively. OE plants overexpress both the *ALDH10A8* and *ALDH10A9* genes that code for proteins homologous to betaine aldehyde dehydrogenases in *A. thaliana*. The first two principal components (PCs) are shown. Open and filled symbols denote WT and OE plants, respectively. Squares, circles and triangles denote unstressed plants harvested before the start of the treatment (W0), or at 1 week (W1) and 2 weeks (W2) after the onset of salt stress (200 mM NaCl), respectively.

Carbohydrate, organic acid and amino acid profiles are affected by overexpressing *BADH*-homologous genes *ALDH10A8* and *ALDH10A9*

After considering targeted metabolic reactions of *ALDH10A8* and *ALDH10A9*, their roles in metabolism were further examined by comparing the steady-state content of 46 primary metabolites between WT and OE plants. The metabolites included amino acids, carbohydrates (sugars) and organic acids. For clarity and to show subtle changes between metabolites that were often masked when we combined all compounds together in the analyses, the measured compounds were placed into two groups (i.e. carbohydrates and organic acids together in group 1 and amino acids in group 2) and subjected to principal component analysis (PCA). The global analysis indicated a shift of the metabolite profiles after 1 and 2 weeks of salt treatment in both WT and OE plants. The changes caused by the salt treatment, explained by the first component of the PCA (PC1), account for 43% in the group of carbohydrates and organic acids, and 70% of the total variation between the samples in the group of amino acids (**Fig. 5**). The WT and the transgenic OE plants differed slightly in their amino acid, carbohydrate and organic acid contents under control conditions and 1 week after salt treatment, respectively, when considering the PC2 axis (**Fig. 5**). Examination of the PC1 and PC2 loadings suggested that the major difference between WT and OE plants involved proline and mannose for PC1, and glycine and fumarate for PC2 (**Supplementary Fig. S1**).

Substantial changes occurred in the relative content of metabolites when plants were subjected to salt stress. To identify the metabolites affected concerned by those changes, the steady-state level of each compound was compared between the WT and the OE plants. Glucose, fructose and raffinose contents were found to be low in untreated OE plants compared with the WT (**Fig. 6**). In contrast, the untreated OE plants contained higher amounts of trehalose and maltose than the WT. Glucose and fructose contents remained significantly lower in the OE plants than in the WT after 1 week of exposure to salt. In contrast, sucrose, galactinol, sorbitol and the organic acids citrate and malate were higher in the OE plants than in the WT after 1 week of salt treatment. Raffinose, myo-inositol, succinate and fumarate were low in the OE plants compared with the WT after 2 weeks of salt treatment. The levels of the other sugars and organic acids were similar between both genotypes after 2 weeks of salt treatment even if differences were noticed at the previous time points. The levels of sugars and organic acids were differentially altered in the OE plants depending on the growth conditions (untreated vs. salt treated) and the duration of the treatments.

Serine, glutamine, ammonium and phenylalanine were lower, whereas lysine was higher in the OE plants than in the WT under control conditions (**Fig. 7**). No difference was seen for the other amino acids in OE plants and the WT in control conditions. After 1 week of exposure to salt, serine increased in both the OE plants and the WT, and the level of serine was significantly higher in the OE plants than in WT plants (**Fig. 7**). The glycine content was lower in the OE plants than in the WT in these conditions, whereas the levels of the other amino acids were similar in both genotypes. The level of serine remained significantly higher in the transgenic OE plants than in the wild-type plants after the second week of salt stress. In addition, we found that the threonine level was higher whereas asparagine and arginine levels were lower in the OE plants than in the WT

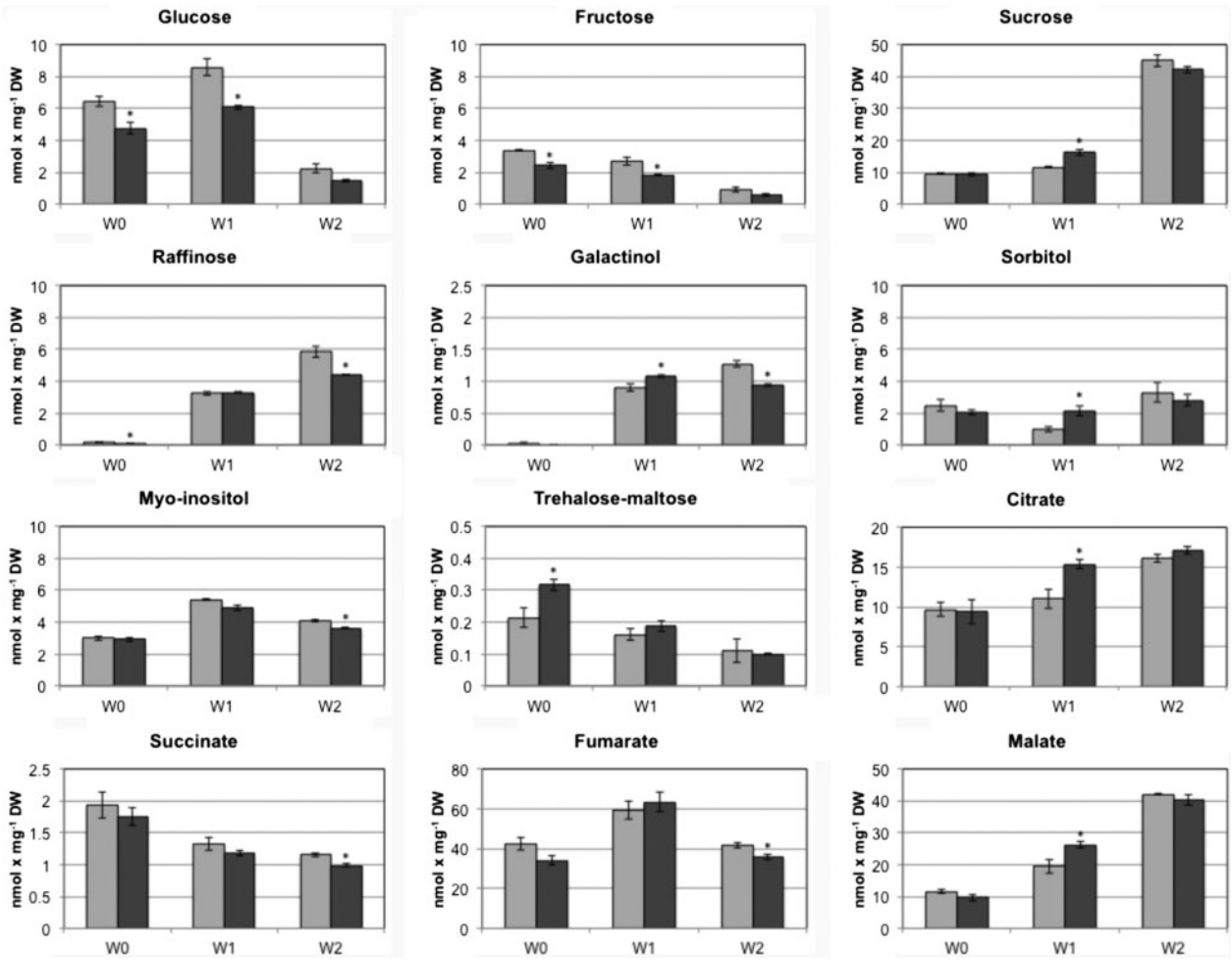

Fig. 6 Comparison of the steady-state levels of sugars and carboxylic acids. WT and OE represent the wild-type and transgenic *A. thaliana* plants, and are indicated by light gray- and dark gray-shaded bars, respectively. OE plants overexpress both the *ALDH10A8* and *ALDH10A9* genes that code for proteins homologous to betaine aldehyde dehydrogenases in *A. thaliana*. W0, W1 and W2 denote unstressed plants harvested before the start of the treatment (W0), and 1 week (W1) and 2 weeks (W2) after the onset of salt stress, respectively. Asterisks indicate a statistically significant difference between WT and OE plants (*$P < 0.05$, Bonferroni test).

after 2 weeks of salt treatment. As was the case for sugars, the levels of the amino acids were differentially impaired in OE plants depending on the growth conditions and the duration of salt stress. The accumulation of serine and its increase in response to salt were altered in the OE plants under all conditions tested.

In addition to analyzing the steady-state level of compounds, the percentage decrease or increase after 1 or 2 weeks treatment relative to the metabolite levels in untreated plants or after 1 week treatment, respectively, were calculated for each metabolite to examine further the alterations caused by the salt treatment. Sugar and organic acid contents increased in both WT and OE plants in response to the salt treatment. Fifteen of the 21 sugars and organic acids showed similar accumulation patterns in WT and OE plants, whereas the patterns were different for six other compounds (**Table 1**). Those six compounds were: mannose, sucrose, raffinose, galactinol, fumarate and malate. The increase in the levels of sucrose and malate was more pronounced in the OE plants than in the WT after 1 week of exposure to salt. Two weeks after exposure to salt, the increase in mannose and raffinose contents was less pronounced in OE plants than in the WT. A similar pattern was seen for fumarate, which decreased more in the OE plants than in the WT. Galactinol decreased in the OE plants whereas its level increased in WT plants after 2 weeks of salt stress.

Like sugars and organic acids, the levels of the majority of amino acids increased in both WT and OE plants in response to the salt treatment. However, nine amino acids were differentially altered in WT and OE plants (**Table 2**). In contrast to sugars and organic acids, most changes that differed between WT and the OE plants occurred during the first week of exposure to salt rather than the second week. Serine and phenylalanine significantly increased in OE plants after 1 week of salt stress. In contrast, the increase in lysine, leucine and arginine contents was less pronounced in the OE plants than in the WT after the same period. Similar observations were made for glutamate and methionine, which behaved differently in WT and OE plants.

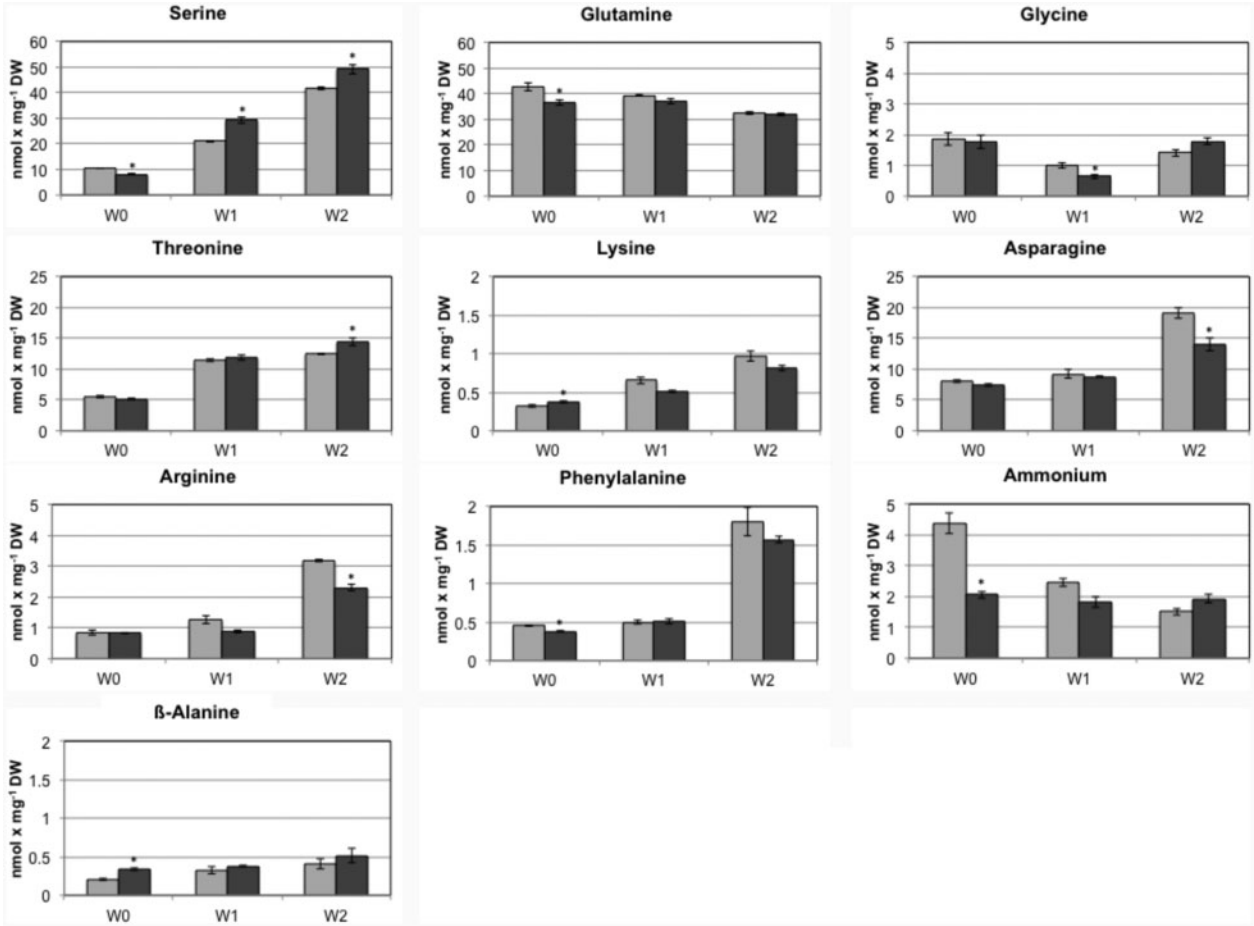

Fig. 7 Comparison of the steady-state levels of amino acids. WT and OE represent the wild-type and transgenic *A. thaliana* plants, and are indicated by light gray- and dark gray-shaded bars, respectively. OE plants overexpress both the *ALDH10A8* and *ALDH10A9* genes that code for proteins homologous to betaine aldehyde dehydrogenases in *A. thaliana*. W0, W1 and W2 denote unstressed plants harvested before the start of the treatment (W0), and at 1 week (W1) and 2 weeks (W2) after the onset of salt stress, respectively. Asterisks indicate a statistically significant difference between WT and OE plants (*$P < 0.05$, Bonferroni test).

Table 1 Changes in the levels of sugars and organic acids in the wild-type and transgenic Arabidopsis plants upon salt treatment

	Mannose		Sucrose		Raffinose		Galactinol		Fumarate		Malate	
	WT	OE	WT	OE	WT	OE	WT	OE	WT	OE	WT	OE
W1/W0	57	274	121	175	1840	3,268	>5E-04	>1E-05	142	185	170	276
W2/W1	388	159	392	263	181	134	142	87	71	57	223	153

WT and OE denote the wild type and the transgenic plants, respectively. Four-week-old plants were used in the experiments. W0, W1 and W2 refer to unstressed plants before the treatment (W0), salt-treated plants after 1 week (W1) and salt-treated plants after 2 weeks (W2), respectively. The values in the table indicate the ratios of the metabolite contents after 1 week (W1/W0) or 2 weeks (W2/W1) salt treatment.

Significant changes in the pattern of metabolite accumulation between WT and OE plants are gray-shaded (Bonferroni test, $P < 0.05$, $n = 3$ replicates).

Only glycine and ammonium were found to increase significantly in the OE plants compared with the WT plants after 2 weeks of salt stress.

Overexpression of *ALDH10A8* and *ALDH10A9* genes impaired growth after germination and tolerance to the salt stress in seedlings

To examine further the effects of the overexpression of ALDH10A8 and ALDH10A9 on growth and stress responses, Arabidopsis seedlings were grown *in vitro* in Murashige and Skoog (MS) medium supplemented with different amounts of NaCl or mannitol. We observed that untreated OE seedlings remained smaller with short cotyledons than WT seedlings for 1 week after germination (**Fig. 8A**). There was no difference in the germination rate of WT and OE seedlings. The short stature of the OE seedlings disappeared by the end of the second week of growth after germination, at which stage both WT and OE seedlings were similar in size (**Fig. 8A**). One-week-old transgenic seedlings looked very similar to the 1-week-old WT seedlings on medium with either 50 mM NaCl or 100 mM mannitol. Most

Table 2 Changes in the levels of amino acids in the wild type and transgenic Arabidopsis plants upon salt treatment

	Serine		Glycine		Glutamate		Lysine		Leucine		Arginine		Methionine		Phenylalanine		Ammonium	
	WT	OE	WT	OE	WT	OE	WT	OE	WT	OE	WT	OE	WT	OE	WT	OE	WT	OE
W1/W0	203	359	56	37	87	83	206	137	227	171	150	105	79	91	110	135	83	88
W2/W1	199	169	143	283	97	95	152	159	176	179	256	265	95	80	370	308	49	107

WT and OE denote the wild type and the transgenic plants, respectively. Four-week-old plants were used in the experiments. W0, W1 and W2 refer to unstressed plants before the treatment (W0), salt-treated plants after 1 week (W1) and salt-treated plants after 2 weeks (W2), respectively. The values in the table indicate the ratios of the metabolite contents after 1 week (W1/W0) or 2 weeks (W2/W1) salt treatment.
Significant changes in the pattern of metabolite accumulation between WT and OE plants are gray-shaded (Bonferroni test, $P < 0.05$, $n = 3$ replicates).

OE seedlings grown on 100 mM NaCl died or lost their Chl compared with the WT seedlings, which showed fewer stress symptoms than transgenic seedlings (Fig. 8B). As seen under control conditions, the OE seedlings were smaller than the WT seedlings grown on 200 mM mannitol. Malondialdehyde (MDA) is commonly used as marker of membrane lipid peroxidation that occurs during stress. Plants tolerant to stress often accumulate less MDA than sensitive plants. The MDA content was significantly higher in the OE seedlings than in the WT, when they were grown on MS medium containing 100 mM NaCl (Fig. 8C). No difference was seen between both genotypes on MS medium or MS medium with 50 mM NaCl, 100 and 200 mM mannitol. We also measured the proline content in the seedlings. A sharp increase of the proline level was observed in both WT and OE seedlings. No difference was seen in the proline content of both genotypes in 50 and 100 mM salt. However, the OE seedlings contained higher amounts of proline than the WT when grown on medium with mannitol added (Fig. 8C).

Discussion

To examine the function of the two *BADH*-homologous genes *ALDH10A8* and *ALDH10A9* in *A. thaliana*, we overexpressed them in WT Arabidopsis plants and compared changes in metabolite contents of the transgenic plants with those in the WT. Our results showed that GB is synthesized at a low level in Arabidopsis, and overexpression of Arabidopsis *BADH* genes or salt treatment enhanced GB. This result is in agreement with other studies, wherein high expression of heterologous *BADH* genes in Arabidopsis did not increase the GB amount to the level often measured in plants which accumulate GB as a compatible solute (Fitzgerald et al. 2009, Chen and Murata 2011). The level of GB measured in the OE plants is about half of the amount of GB measured in *A. thaliana* plants upon cold acclimation (Xing and Rajashekar 2001), thus suggesting that the amount of GB synthesized in *A. thaliana*, although low, may be stress-dependent.

Although still low, the level of GB increased upon choline addition (Fig. 2). This indicates that *A. thaliana* can synthesize GB from both endogenous and exogenous choline sources and that the ALDH10A8 and ALDH10A9 proteins are involved in the biosynthesis. The absence of a functional choline monooxygenase (CMO) protein in non-GB-accumulating plants thus appears not to be the only constraint for GB biosynthesis in *A. thaliana*. A high expression level of the BADH proteins is also important. Indeed, the lack of GB accumulation in near-isogenic lines of maize and sorghum, which differ in their GB contents, was previously found to be unrelated to the *CMO* gene or CMO protein (Peel et al. 2010). It is more likely that the biosynthesis of GB in GB non-accumulating plants is under tight regulation of CMO and BADH, the two enzymes constituting the pathway. For instance, the overexpression of the *OsCMO* gene from rice cv. Nipponbare (*Oryza sativa* L. ssp. japonica) in tobacco plants increased GB levels and elevated tolerance to salt stress in the transgenic plants (Luo et al. 2012). Protein analysis demonstrated that a functional OsCMO protein with the correct size was present in transgenic tobacco but hardly accumulated in WT rice plants. Instead, a large amount of truncated OsCMO protein was detected in WT rice seedlings in response to salt stress (Luo et al. 2012).

Besides betaine aldehyde, BADHs can also oxidize 4-*N*-trimethylaminobutyraldehyde and 3-aminopropanal, which are intermediate compounds in the synthesis of L-carnitine and the catabolism of polyamines, respectively (Charrier et al. 2012, Rippa et al. 2012). Our results suggest that ALDH10A8 and ALDH10A9 may not be involved in carnitine synthesis. Alternatively, their expression level in the WT might be sufficient for carnitine synthesis. The use of knockout mutants of both genes will help to verify these hypotheses. The level of β-alanine was higher in the OE plants than in the WT, particularly under control conditions. This indicates that 3-aminopropanal is a substrate for ALDH10A8 and ALDH10A9 in Arabidopsis cells. The substrate 3-aminopropanal comes from the oxidation of spermine by a polyamine oxidase. The accumulation pattern of spermine was similar between WT and OE plants, but the high level of β-alanine in the OE plants indicates that ALDH10A8 and ALDH10A9 are involved in the terminal catabolism of this polyamine.

The overexpression of ALDH10A8 and ALDH10A9 has an impact on several other metabolites in addition to their putative direct substrates or reaction products. The primary metabolism of sugars and amino acids is altered in the OE plants. We explain these changes as an indirect effect of the overexpression of the BADH enzymes. BADHs and other ALDHs use one molecule of NAD(P) to generate one reducing equivalent during the oxidation of the aldehyde substrates. BADH overexpression might thus cause an excess of reducing equivalents, which in turn altered the metabolism of sugars and amino acids. NAD(P) and NAD(P)H are shared by several metabolic reactions in the different cell compartments and therefore influence the redox potential in the cell. Disequilibrium in their ratio was reported

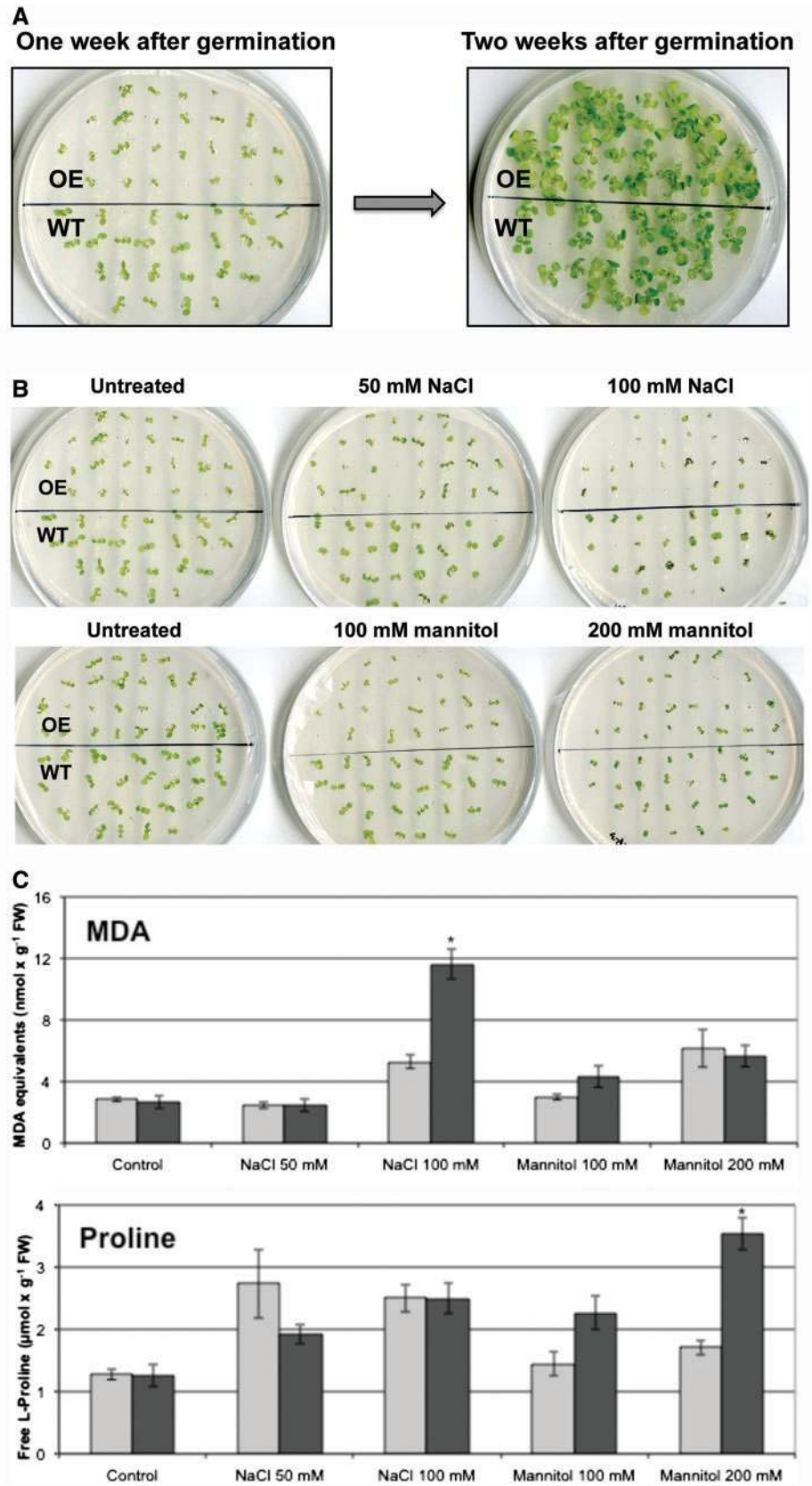

Fig. 8 Comparison of growth and responses to stress early after germination. WT and OE represent the wild-type and transgenic *A. thaliana* plants. OE plants overexpress both the *ALDH10A8* and *ALDH10A9* genes that code for proteins homologous to betaine aldehyde dehydrogenases in *A. thaliana*. Photographs of the plates in (A) were taken 1 and 2 weeks after germination of seeds.(B) One-week-old seedlings on MS medium, containing 50 or 100 mM NaCl or 100 and 200 mM mannitol. (C) MDA and proline contents of 1-week-old seedlings grown on different media; asterisks in (C) indicate a statistically significant difference between WT and OE plants (*$P < 0.05$, Student's *t*-test).

to influence other metabolic reactions in which they are involved (Oliver and McIntosh 1995).

The OE plants were shorter early after germination and more sensitive to NaCl than the WT. Such phenotypes have never been reported for plants overexpressing stress-related ALDHs in the past. In contrast, transgenic plants overexpressing members of the ALDH families 3, 7 or 22 were reported to be more tolerant to abiotic stresses including high salinity, and they did not show any growth retardation (Sunkar et al. 2003, Kotchoni et al. 2006, Rodrigues et al. 2006, Huang et al. 2008). The phenotypes of the OE plants thus appear specific for ALDH10A8 and ALDH10A9, and distinguish them from the previously studied Arabidopsis stress-related ALDHs. We speculate that this could relate to the increase of GB in these plants and a regulatory role for GB. That implies that the role of GB might differ between plants that naturally accumulate GB (wherein GB functions as a compatible solute) and non-GB-accumulating plants, in which the low level of GB is unlikely to account for an osmotic effect.

Materials and Methods

Plant material, growth conditions and stress treatments

Arabidopsis thaliana (ecotype Col-0) was used throughout this work. WT and transgenic seeds were sown on MS agar plates (Murashige and Skoog 1962) or soil. Transgenic plants were selected on MS agar plates containing 50 mg l^{-1} kanamycin. Plants were allowed to grow under white light of approximately 120–150 µmol m^{-2} s^{-1} with a 16 h photoperiod at 22°C, unless stated otherwise. Tissues of 14-day-old and 6-week-old plants were used for gene expression analyses.

For chemical treatments, seeds were sown on MS agar plates (containing 50 mg l^{-1} kanamycin for selection of transgenic seeds) and transferred to soil 1 d after germination. Four-week-old plants were irrigated with 200 mM NaCl or 200 mM NaCl including 5 mM choline. Control plants received only water. Each soil pot received 40 ml of either of these solutions every 2 d for the duration of the experiments. Three time points were considered: W0, W1 and W2 corresponding to the start of the experiment (control), 1 week and 2 weeks of salt treatment, respectively.

The shoot was sampled from 12 plants then pooled in one sample replicate for each genotype per treatment. Three sample replicates representing 36 biological replicates were thus obtained in each case. The germination of seeds, the growth pattern and the stress response of seedlings were monitored from seeds plated on MS medium and kept in the growth chamber with the same conditions as above. The measurement of MDA and proline were performed as described before (Missihoun et al. 2011). Samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Molecular cloning and generation of transgenic plants

The coding sequence of the *ALDH10A8* gene was isolated from the clone pda07810 (RAFL07-07-L09) (RIKEN Institute) after digestion with *Sac*I. The fragment was subcloned into the binary pROK2 vector (Baulcombe et al. 1986). For *ALDH10A9*, a cDNA fragment was amplified from the clone pda01165 (RAFL05-07-N03) (RIKEN Institute) with the following primers Aldh8-Fwd, 5′-TCACCACCTCTAGTAGCAGAGAGAG-3′; and AF370333-RT-Rev, 5′- GAAGGGTCTCTTGCTTTATTGGT-3′. The expected 1,620 bp amplicon was subcloned into the pJET1.2 vector provided in the CloneJET™ PCR Cloning Kit (Fermentas). The recombinant plasmid was digested with *Bgl*II to isolate the ALDH10A9 coding sequence. The *Bgl*II fragment (1,066 bp) was then cloned into the *Bam*HI site of the binary pROK2 vector. Both recombinant binary vectors were introduced into *Agrobacterium tumefaciens* GV3103. One *A. tumefaciens* clone harboring either the *CaMV35S::ALDH10A8* or the *CaMV35S::ALDH10A9* fusion constructs were used to transform *A. thaliana* WT plants (ecotype Col-0) by the floral dip method (Clough and Bent 1998). Single *ALDH10A8*- or *ALDH10A9*-overexpressing plants were selected on kanamycin plates and the expression of the transgene was confirmed. Double-overexpressing plants were obtained by crossing plants overexpressing ALDH10A8 and ALDH10A9 and termed OE lines. The *ALDH10A9*-overexpressing plants were used as female plants and the *ALDH10A8*-overexpressing plants were used as male plants for pollination.

RNA isolation and reverse transcription–PCR (RT–PCR)

Isolation of total RNA and RT–PCR analyses were performed as described by Missihoun et al. (2011). Gene-specific primers used to amplify first-strand cDNAs were AY093071-RT-fwd (5′-GATCTTGCATGGTGGTTCCCGA-3′) and AY093071-RT-rev (5′-AAGCACAAAGATTTGAACAGACAGC-3′) for ALDH10A8; and AF370333-RT-fwd (5′-TGTTCTTTGTGGAGGAGTTCGTC-3′) and AF370333-RT-rev (see above) for ALDH10A9. Transcripts of the *A. thaliana* ACTIN2 (At3g18780) gene were used as reference (An et al. 1996) and amplified with the primers Ath_Actin 2_fwd (5′-GGAATCCACGAGACAACCTATAAC-3′) and Ath_Actin 2_rev (5′-AGGAATCGTTCACAGAAAATGTTTC-3′).

Metabolite analysis

Metabolites were determined as previously described (Renault et al. 2010). GB, carnitine and choline were detected and quantified in the plant extracts by using the ^{1}H-NMR technique. Approximately 50 mg of freeze-dried plant material were suspended in 1 ml of pure ethanol and thoroughly shaken. Suspensions were heated at 85°C until ethanol was completely evaporated. The residues, re-suspended with 1 ml of 99.9% D_2O containing 500 µM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as an internal standard, were shaken at 4°C for 1 h. Homogenates were clarified by centrifugation: 15,000×*g*, 4°C, 20 min. DSS was used as a reference both for determination of chemical shifts and for quantification of the signal of interest. ^{1}H-NMR spectra were recorded on a Bruker NMR spectrometer operating at a frequency of 400 MHz. The processing of the spectra was carried out using MestReNova software. Characteristic ^{1}H chemical shifts, used for quantification of choline, carnitine and GB in D_2O are 3.190, 3.220 and 3.252 p.p.m., respectively in reference to external calibration curves performed with pure reference standards.

Statistical analysis

PCA was performed on the data sets obtained for the measured metabolites in WT and OE plants by using the algorithms embedded in the R software package (www.r-project.org). Data weres log2-transformed and normalized to the mean of the entire sample set for each metabolite before the analysis. Zero values from signal below the detection limit were handled as described by Renault et al. (2010). The steady-state level of each compound was compared between the WT and the OE plants. In addition to analyzing the steady-state levels of metabolites, the decrease or increase of the metabolite level after 1 week relative to the level in untreated plants was calculated for each metabolite to examine the alterations due to the salt treatment. The normalized values of the relative metabolite contents were computed in SPSS Statistics 17.0.0 and subjected to analysis of variance (ANOVA) test. The Bonferroni test was used for post-hoc comparisons.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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