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Resolving the Role of Plant NAD-Glutamate Dehydrogenase: III. Overexpressing Individually or Simultaneously the Two Enzyme Subunits Under Salt Stress Induces Changes in the Leaf Metabolic Profile and Increases Plant Biomass Production

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**Resolving the Role of Plant NAD-Glutamate Dehydrogenase: III.
Overexpressing Individually or Simultaneously the Two Enzyme Subunits
Increases Plant Biomass Accumulation Under Salt Stress**

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Abstract

Higher plants NAD-dependent glutamate dehydrogenase has a central position at the interface between carbon and nitrogen metabolism due to its ability to carry out the deamination of glutamate. In order to obtain a better understanding of the physiological function of NAD-GDH under salt stress conditions, transgenic tobacco (*Nicotiana tabacum* L.) plants that overexpress two genes from *Nicotiana plumbaginifolia* individually (*GDHA* and *GDHB*) or simultaneously (*GDHA/B*) were grown in the presence of 50mM NaCl. In the different *GDH* overexpressors, the NaCl treatment induced an additional increase in GDH enzyme activity indicating that a post-transcriptional mechanism regulates the final enzyme activity under salt stress conditions. A greater shoot and root biomass production was observed in the three types of *GDH* overexpressors following growth in 50mM NaCl, when compared to the untransformed plants subjected to the same salinity stress. Changes in metabolites representative of the plant carbon and nitrogen status were also observed. They were mainly characterized by an increased amount of starch present in the leaves of the *GDH*-overexpressors as compared to the WT, when plants were grown in 50mM NaCl. Metabolomic analysis revealed that overexpressing the two genes *GDHA* and *GDHB* individually or simultaneously, induced a differential accumulation of several carbon- and nitrogen- containing molecules involved in a variety of metabolic, developmental and stress-responsive processes. An accumulation of compounds such as digalactosylglycerol, erythronate and porphyrin was found in the *GDHA*, *GDHB* and *GDHA/B* overexpressors, suggesting that these molecules contribute to the improved performance of the transgenic plants under salinity stress conditions.

Key words: Glutamate dehydrogenase. Metabolome. Overexpression. Salt Stress. Subunit. Tobacco.

Abbreviations:

C, carbon ; CaMV 35S, cauliflower mosaic virus 35S promoter; DGG, Digalactosylglycerol; DW, dry weight ; FW, fresh weight ; N, nitrogen ; OE, overexpressors ; PAGE, polyacrylamide gel electrophoresis ; WT, wild type .

Introduction

Over the past five years, significant progress have been made towards obtaining a better understanding of the physiological function in plants of glutamate dehydrogenase (NAD-GDH; EC 1.4.1.2), an enzyme that catalyses the deamination of glutamate using NAD as a coenzyme. The enzyme operates at the interface of carbon (C) and nitrogen (N) metabolism, producing 2-oxoglutarate and ammonia (Hirel and Lea 2011). In several reports, it was proposed that GDH could operate in the direction of ammonia assimilation (Yamaya and Oaks 1987, Oaks 1995, Melo-Oliveira et al. 1996, Skopelitis et al. 2007). However, in recent studies using either transgenic plants (Laboun et al. 2009) or mutants (Miyashita and Good 2008, Fontaine et al. 2012), it has been clearly demonstrated that NAD-GDH deaminates glutamate, thus releasing 2-oxogutarate and ammonia, notably when there is a shortage of C. These three later studies have strengthened the previous findings of Fox et al. (1995), Stewart et al (1995) and Purnell and Botella (2007) who demonstrated that NAD-GDH deaminates glutamate *in vivo*, thus confirming that the GS/GOGAT assimilatory pathway is the major route for incorporating ammonia into organic molecules, irrespective of its metabolic origin (Lea and Miflin 2011, Yamaya and Kusano 2014, Zanin et al. 2015).

In the shoots and roots of higher plants, the GDH enzyme protein was originally found to exist as homo- or hetero-hexamers (Turano et al. 1996, Fontaine et al. 2006), almost exclusively located in the mitochondria of the phloem companion cells (Tercé-Laforgue et al. 2004a). The occurrence of seven isoenzymic forms of GDH, distinguishable following native

polyacrylamide gel electrophoresis (PAGE) prompted a number of groups to investigate their physiological role both in vegetative and reproductive organs during plant growth and development (Cammaerts and Jacobs 1985, Loulakakis and Roubelakis-Angelakis 1996, Fontaine et al. 2013, Marchi et al. 2013). It has also been shown that each of two distinct nuclear genes named *GDH1* and *GDH2* in *Arabidopsis thaliana* and named *GDHA* and *GDHB* in *Nicotiana plumbaginifolia* (Restivo 2004) encode a different subunit (α and β) composing the enzyme. These two genes and their corresponding translation products are differentially expressed according to the plant developmental stage, physiological status with respect to N nutrition and the organ examined (Melo-Oliveira et al. 1996, Purnell et al. 2005, Miyashita and Good, 2008). More recently, it has been found that in *Arabidopsis* there is another gene (*GDH3*) encoding a third active NAD-GDH isoenzyme preferentially expressed in the roots (Fontaine et al. 2012, 2013) and regulated by cytokinin (Marchi et al. 2013).

Thus, both the unique regulation of the GDH subunit composition and the fact that glutamate is a key molecule both in terms of metabolic function and signaling (Forde and Lea 2007), have rekindled an interest in elucidating further the role of the enzyme with respect to plant performance. Since, it has been shown that GDH plays a key role in controlling glutamate homeostasis (Labboun et al. 2009) and in replenishing a lack of carbon under certain physiological conditions (Miyashita and Good, 2008, Fontaine et al. 2012), it has become important to establish if increasing GDH activity will trigger changes both in root and leaf physiology and thus in plant growth and development.

Tobacco plants overexpressing the *E. coli* gene *GDHA*, which encodes NADPH-GDH were constructed by Ameziane et al. (2000). Plants with high NADPH-GDH activity grown in the field or under controlled conditions had a higher biomass and exhibited an increase in tolerance to a herbicide that inhibited GS activity (Nolte et al. 2004). The tobacco plants with increased NADPH-GDH activity also had higher water potentials than the control plants

following five days of water deficit (Mungur et al. 2006). The biomass of the grain of transformed maize overexpressing the same *E. coli GDHA* gene was higher than the controls when the plants were grown in the field under drought conditions (Lightfoot et al. 2007). A patent released in the USA by Schmidt and Miller (1999) described the use of plants transformed with nucleotide sequences encoding the α and β subunits of *Chlorella sorokiniana NADPH-GDH*. These plants exhibited improved properties such as increased growth and enhanced osmotic stress tolerance. In line with these findings, a maize GDH-null mutant was shown to be cold sensitive (Pryor, 1990). Following these studies, it was proposed that ammonia assimilation *via* NADH-GDH could have an advantage over GS, since the reaction catalyzed by the former uses less ATP per ammonia assimilated. However, a comprehensive physiological characterization of the tobacco and corn plants overexpressing the *E. coli GDHA* gene was not performed. It would have been useful to have established whether the additional GDH activity, presumably operating in the direction of glutamate synthesis, was able to divert plant metabolism in a beneficial way.

Other studies have also been conducted to establish the impact of enhanced GDH activity on a range of higher plants, using various sources of genes encoding the enzyme. A positive impact of fungal or bacterial NADPH-GDH overexpression on growth and physiological traits was generally observed (Mungur et al. 2005, Abiko et al. 2010, Egami et al. 2012, Zhou et al. 2014). For example, ^{15}N -labelling experiments showed that the introduced *GDHA* gene from *Aspergillus niger*, together with the endogenous GS directly assimilated NH_4^+ , thus promoting an increase in dry weight, N content and yield in transgenic rice plants (Abiko et al. 2010). In potato plants overexpressing the fungal enzyme, the C and N content of the tubers was increased compared to the wild type (Egami et al. 2012). In contrast, in a recent study it was shown that biomass production of tobacco plants overexpressing genes encoding *N. plumbaginifolia* NAD-GDH was reduced by at least 30%, depending on the GDH subunit

composition used for the genetic manipulation. Variations in the subunit composition of the *GDH* overexpressors, also induced differences in the accumulation of C and N metabolites (Tercé-laforge et al. 2013). The ability of the NADPH-dependent fungal GDH to assimilate ammonium (Abiko et al. 2010), may account for the differences in growth and metabolism in transgenic plants that express the NAD-dependent plant enzyme, which catalyses ammonium formation. These contrasting results clearly indicate that the physiological impact of *GDH* overexpression largely depends on the type of GDH enzyme used for the genetic manipulation, as well as the source of reductant used for the enzymatic reaction.

To further investigate the function of the two NAD-GDH subunits, transgenic tobacco plants overexpressing the α and β subunits of *N. plumbaginifolia* NAD-GDH, individually or simultaneously (Tercé-Laforge et al. 2013), have been subjected to salt stress. Phenotypic analysis of the transgenic plants was carried out to evaluate the impact of increased GDH activity on plant biomass production. In addition to measuring the content of the main metabolites used as markers of plant growth and development, a metabolomic study was conducted to determine if each of the two enzyme subunits had a specific physiological function when the plants were subjected to salt stress conditions.

Results

Changes in the level of GDH overexpression under salinity stress

NADH-dependent aminating GDH activity was measured in both the roots and shoots of tobacco plants containing elevated amounts of the α and β subunits of *N. plumbaginifolia* GDH. For each construct, *35S-NpGDHA* and *35S-NpGDHB*, two independent transformants were selected (lines A1, A2 and B1, B2 respectively), which exhibited the highest amount of leaf GDH activity. Two transgenic lines expressing both genes (*GDHA/B*), lines A2b1 and

B1a2, were obtained by reciprocal crossing between the *GDHA* and *GDHB* overexpressors as described previously by Labboun et al. (2009).

In a fully expanded leaf of the transgenic lines A1, A2 (*GDHA* overexpressors), B1, B2 (*GDHB* overexpressors) and A2b1 and B1a2 (*GDHA/B* overexpressors), grown on a standard nutrient solution containing 10mM NO_3^- and 2mM NH_4^+ , both NADH-GDH aminating and NAD-GDH deaminating activities measured *in vitro*, were increased by at least five-fold and up to eleven-fold compared to the wild type (WT) control plants (Fig. 1). When the plants were grown on the same growth medium containing 50 mM NaCl, the increase in the NADH-GDH aminating activity was dependent on the transgenic line examined, ranging from 112% to 225% in the *GDHA* (lines A1, A2), *GDHB* (lines B1 and B2) and *GDHA/B* (lines A2b1 and B1a2) transformants in comparison to the unstressed transgenic plants. In the presence of NaCl, an increase in GDH activity of 183% was also observed both in the WT plants (Fig. 1A). In the three types of GDH overexpressors, the deaminating activity, which was considerably lower compared to the aminating activity, was increased by 160% in the *GDHA* plants and from 173 to 231% in the *GDHA* and *GDHA/B* plants in the leaves of the NaCl treated plants compared to the same transformants grown on a standard growth medium. A similar increase in the deaminating activity (179%) was also observed in the WT plants grown in the presence of 50mM NaCl (Fig. 1B). In the roots of NaCl-treated plants, the GDH aminating and deaminating activities were reduced in the six different transgenic lines, on average by 43% of the activity in the plants that were not subjected to salt stress. The GDH aminating and deaminating activities in the roots of all the transgenic plants treated with NaCl were comparable to that of the WT plants (Fig. 1C, D).

In a previous study, enzyme activity staining following PAGE showed that in the leaves of the two *GDHA* transformants (A1 and A2), only the most anodal isoenzyme (α -subunit) was induced compared to the WT, whereas in the two *GDHB* transformants (B1 and B2), a

considerable increase in the most cathodal isoenzyme (β -subunit) was visible. In the two *GDHA/B* double transformant lines (A2b1 and B1a2), the GDH isoenzyme pattern was typical of an active heterohexameric enzyme composed of an assembly of the two subunits α and β found in the leaves of *N. plumbaginifolia* (Labboun et al. 2009). Following growth in 50mM NaCl, a marked increase in the activity of the α and β subunits was visible after electrophoresis of leaf extracts of the *GDHA* A1 and *GDHB* B1 transformants respectively, thus confirming the results obtained by measuring the activity of the enzyme *in vitro*. The presence of NaCl in the growth medium also induced an extensive increase in the activity of the heterohexameric forms of NAD-GDH, that were detectable following electrophoresis of extracts of the double *GDHA/B* overexpressor A2b1. The increase in enzyme activity was to such an extent that it was not possible to identify the individual bands of NAD-GDH enzyme activity, that were visible following electrophoresis of extracts of plants not subjected to NaCl stress (Fig. 2). Similar results were obtained with the transgenic lines A2, B2 and B1a2 corresponding to the *GDHA*, *GDHB* and *GDHA/B* overexpressors respectively (data not shown). Following electrophoresis of WT extracts, NAD-GDH activity staining was very faint, this was maintained to avoid oversaturation of the staining due to the very high NAD-GDH enzyme activity present in all three types of *GDH* overexpressors. However, there was a slight increase in the intensity of the WT activity staining following NaCl treatment, which did not allow the identification of the different isoenzymes, due to difficulties in the separation of the GDH isoenzymes from extracts of mature tobacco leaves by PAGE (Labboun et al. 2009). Confirmation of the validity of the electrophoretic separation and GDH activity staining procedures was obtained by the clear visibility of seven NAD-GDH isoenzyme staining bands, following electrophoresis of extracts of Arabidopsis leaves (Fig. 2; far right lane).

The amounts of GDH mRNA in the leaves of the three types of *GDH* overexpressors, transcripts were similar, irrespective of the salt treatment, indicating that their high expression driven by the 35SCaMV promoter was not modified in the presence of NaCl. In the WT, a low mRNA signal was observed, only when the plants were grown in the presence of 50mM NaCl (Supplementary Fig. 1).

Phenotypic and physiological characterization of GDH overexpressors subjected to salt stress

As reported previously, for plants grown under standard nutrient conditions in the absence of NaCl, biomass production expressed as the total dry weight (DW) of shoots of the *GDHA* (A1 and A2) *GDHB* (B1 and B2) or *GDHA/B* (A2b1 and B1a2) overexpressors was significantly lower than the WT. In agreement with previously published results (Tercé-Laforgue et al. 2013), this reduction in shoot biomass production was more pronounced in the *GDHB* plants B1 and B2 (Fig. 3A). Under these standard growth conditions, compared to the WT, root biomass was also significantly reduced in the *GDHB* overexpressors B1 and B2 (Fig. 3B).

When WT plants were grown in the presence of 50mM NaCl, there was a reduction in root and shoot biomass production of 30% and 32% respectively. When compared to the WT treated with 50mM NaCl, the different lines of *GDHA*, *GDHB* and *GDHA/B* overexpressors subjected to the same salinity stress exhibited a 12 to 28% increase in shoot and a 44 to 98% increase in root biomass production. Only in the *GDHA* overexpressor (lineA2) the 12% increase observed in shoots was not significant (Fig. 3). For the three types of *GDH* overexpressors this increase in biomass was on average 22% for the shoots and 62% for the roots (see column OE in Fig. 3A, B). The increase in root and shoot biomass in comparison to the WT, in *GDH* overexpressing plants treated with 50mM NaCl, can be clearly seen in the phenotype shown in Fig. 4. When the three types of *GDH* overexpressors were grown under

normal conditions, the starch content of the leaves was considerably lower than the WT, with reductions in the range of 58-81% (Tercé-Laforgue et al. 2013). When the WT plants were grown in the presence of 50mM NaCl, the starch content of the leaves was 9 times lower when compared to the untreated plants, whereas for the *GDH* overexpressors the starch content was reduced to a lesser extent, by values ranging from 113 to 251% of the untreated plants (Fig. 3C).

When the plants were grown under standard nutrient conditions, the chlorophyll content was on average 165% higher in the *GDH* overexpressors when compared to the WT, without any obvious preferential accumulation in the *GDHA* (A1 and A2), *GDHB* (B1 and B2) or *GDHA/B* (A2b1 and B1a2) plants. However, when the plants were grown on 50mM NaCl, the chlorophyll content of the NAD-GDH overexpressors was greatly reduced from 3 to 5 times and no differences were observed with the WT (Fig. 3D). The leaf ammonium content increased by two fold when the WT and *GDH* overexpressing plants were treated with NaCl, this increase was less marked in the *GDHA/B* transgenic line A2b1 (Supplementary Figure 2). Preliminary soluble amino acid analyses revealed that in the leaves of NaCl treated WT plants and the three types of GDH overexpressors, a four fold increase in the proline content was detected in comparison to the WT plants grown in the absence of NaCl. Asparagine, glutamate and glutamine were reduced in the WT and GDH overexpressing leaves following treatment with 50mM NaCl, whilst aspartate was reduced by 50% in the NaCl treated WT and on average a further 47% in the GDH overexpressing leaves (Supplementary Table 1).

A more detailed GC/MS metabolic profile analysis was then performed on the NaCl-treated plants allowing the identification of 127 metabolites (Supplementary Table 2). Metabolites exhibiting significant changes in their concentration in the leaves, between the WT and the *GDHA*, *GDHB* and *GDHA/B* overexpressors (P value < 0.05) are presented in Table 1. In the leaves of the three *GDH* overexpressors, digalactosylglycerol (DGG), erythronate and

porphyrin were increased, whereas aspartate was detected in lower concentrations. Only in the *GDHA* and *GDHA/B* overexpressors was an increase in the amount of fumarate, citramalate, GABA and a decrease in the amount of inositol observed. This indicates that the modification of the concentration of these metabolites was the results of *GDHA* overexpression and that of *GDHB* did not have any impact on their accumulation. Three different groups of metabolites were identified as being present in a higher amount only in *GDHA* and *GDHA/B* overexpressors, or in lower amounts only in *GDHB* overexpressors (Table1).

In the roots of the different *GDH* overexpressors grown in the presence of NaCl, the content of the various metabolites measured in Supplementary Table 2, was not significantly modified in the three types of *GDHA*, *GDHB* and *GDHA/B* transgenic plants in comparison to the WT (data not shown).

Discussion

An increase in tolerance to drought stress was previously reported in two papers describing a preliminary phenotypic characterization of transgenic tobacco (Mungur et al. 2005, 2006) and maize plants (Lightfoot et al. 2007) overexpressing an *E. coli* NADPH-GDH. Both transgenic species displayed improved tolerance to water deficit with notably an increase in yield for the maize plants expressing the bacterial *GDH* constitutively. However, a more detailed physiological and biochemical characterization of these bacterial *GDH* overexpressors would have been necessary, in order to provide a possible explanation for their improved performance. In the present investigation, the three types of tobacco transgenic plants overexpressing the two GDH enzyme subunits individually or simultaneously were grown in the presence of 50mM NaCl, a moderate salinity stress situation similar to that used to assess the role of GS in proline production (Brugière et al. 1999) and close to the conditions occurring naturally in saline soils (Munns and Tester 2008) . We then investigated if

following growth under the salinity stress conditions of 50mM NaCl, *GDH* overexpression had any impact on biomass production and on the physiology of the plants, notably by conducting a detailed metabolomic analysis.

GDH enzyme activity measured in both the aminating and deaminating directions *in vitro*, was higher in the leaves and roots of the transgenic plants overexpressing the *GDHA* (A1 and A2) *GDHB* (B1 and B2) and *GDHA/B* (A2b1 and B1a2) genes grown under standard nutrient conditions, when compared to the WT (Fig.1). When the three types of *GDH* overexpressors were grown in 50mM NaCl, the leaf GDH enzyme activity measured in the aminating or deaminating direction was increased from 112% to 225% in comparison to none salt-treated plants. Overall, the NADH-GDH aminating activity in the leaves of the overexpressing plants grown under salt stress conditions increased to a level that was on average 7 times higher compared to that measured in the WT (Fig. 1A, B). Such a remarkable increase in transgenic enzyme activity is rather unusual as compared to that obtained with other enzymes involved in N metabolism such as glutamine synthetase or alanine aminotransferase, when increases in enzyme activity were not greater than 3- to 4-fold (McAllister et al. 2012). Staining for NAD-GDH activity following PAGE, confirmed that, compared to plants grown under standard nutrient conditions, there was an increase in the enzyme deaminating activity in all three types of overexpressors grown in the presence of salt. This increase in NAD-GDH activity was due to an accumulation of *GDHA* or *GDHB* subunits either individually or concurrently (Fig. 2). A slight increase in both aminating and deaminating enzyme activity was also detected in extracts of WT plants grown in the presence of 50mM NaCl, thus confirming the stress-inducible responsiveness of the enzyme even when expressed at a much lower level (Debouba et al. 2006, Wang et al. 2007).

It is likely that the salt-induced increase in GDH enzyme activity in the leaves probably occurred at the post-transcriptional level, since the accumulation of *GDHA*, *GDHB*

and *GDHA/B* mRNA transcripts in the plants overexpressing the three types of transgene were similar in the control and in the NaCl treated plants (Supplementary Fig. 1). In other studies it has been observed that the expression of a transgene under the control of the CaMV promoter was not modified under various stress conditions (Kasuga et al. 1999), a finding that further supports our conclusion. It is also worth noting that the salt treatment decreased both the aminating and deaminating GDH activities in the roots of the three types of NAD-GDH overexpressors on average by 44%, to rates that were similar to the WT, which is the opposite of what occurred in the leaves (Fig. 1). This finding suggests that the post-transcriptional regulation of GDH activity when it is overexpressed, is organ-specific. There are a number of other examples of post-transcriptional regulation of GDH activity described in the literature. When tobacco plants were grown on ammonium, although the amount of *GDH* mRNA transcripts was similar to that of nitrate fed plants, both aminating and deaminating activities of the enzyme were considerably higher in the ammonium grown plants (Tercé-Laforgue et al. 2004b). In *Arabidopsis* the NADH-GDH activity in both the roots and leaves varied considerably following growth on a range of different nitrogen sources. It was difficult to establish whether there was a correlation with mRNA expression or the presence of α and β proteins, suggesting that there might have been both post-transcriptional and post-translational regulation (Turano et al. 1997). The physiological significance of such post-transcriptional regulatory mechanisms, which are known to play a major role for example in sucrose (Koch 2004), or proline metabolism (Peng et al. 1996, Kishor et al. 2014) will be worth further investigation. This will be particularly important if the amount of GDH protein accumulation is an important component, when producing or selecting plants with higher GDH activity that are more resistant to abiotic stress.

We observed that, following growth in 50 mM NaCl, there was a relatively small but significant increase in both root and shoot biomass production in the three types of *GDH*

overexpressors in comparison to the WT. On average, for all the transgenic lines examined, this increase was 22% for the shoots and 62% for the roots. The highest biomass accumulation in plants grown on 50mM NaCl was detected in the two transgenic lines that were *GDHB* overexpressors (B1 and B2, Fig. 3A and B). Thus, it can be concluded that *GDH* overexpression allows the transgenic plants to partly compensate for the 30% decrease in plant biomass production occurring when the WT control plants are subjected to salt stress. Moreover, we found that the detrimental effect of *GDH* overexpression on plant biomass production, previously observed under standard nutrient growth conditions (Tercé-Laforgue et al. 2013), was also attenuated in the presence of NaCl. This compensation mechanism was also apparent when examining the leaf starch content of around 600 nmol mg⁻¹ DW that was considerably decreased in the WT (around nine times) following NaCl treatment, but was on average only reduced around two times in the *GDH* overexpressors (Fig. 3C). It is possible that the increase in GDH activity limits the decrease in starch accumulation generally observed under osmotic stress conditions (Krasensky and Jonak, 2012). In a previous study we found that on a dry weight basis, there is more chlorophyll in the leaves of the transgenic lines grown on a standard complete nutrient solution, indicating that in addition to central C and N metabolism, the production of the photosynthetic machinery was also altered (Tercé-Laforgue et al. 2013). Following growth in the presence of NaCl, there was a considerable decrease in the leaf chlorophyll content, which was on average reduced 4 fold in the GDH overexpressors, but only 2 fold in the WT. A decrease in the chlorophyll content has generally been observed in salt treated plants, but this has depended on both the plant species examined and the duration of the salinity stress (Debouba et al. 2006, Wu et al. 2013). This suggests that although the *GDH* overexpressors lost much more chlorophyll in comparison to the WT, they were able to adapt their photosynthetic machinery to a moderate salinity stress in order to

maintain sufficient photosynthetic efficiency (Morant-Manceau et al. 2004, Shabala and Munns 2012).

Proline is known to play an important role as an osmoregulatory solute in plants subjected to drought and salt stress (Kishor et al. 2005, 2014) and in stabilizing cellular structures as well as scavenging free radicals (Hayat et al. 2012, Signorelli et al. 2014). Thus, a preliminary soluble amino acid analysis was carried out in order to check if the leaf proline content was increased following continuous salinity stress. As expected, we observed that in comparison to the WT plants grown without NaCl, a four-fold increase in proline was the most important change resulting from the presence of NaCl (Supplementary Table 1). This finding indicates that the plants responded to a continuous and moderate salinity stress in agreement with the data reported in the literature (Kishor et al. 2005, 2014). However, there was no major difference between the WT and *GDH* overexpressors, indicating that increased *GDH* activity did not have an impact on proline accumulation and therefore presumably proline does not directly contribute to the enhanced shoot and root biomass of the transgenic plants. A general decrease in most of the amino acids was also observed in the NaCl treated plants, which is a typical response of plants when subjected to a salinity stress. This decrease, suggests that under our experimental conditions, there was no evidence of protein breakdown that can occur, depending on the species, or the salt tolerance of a particular genotype (Widodo et al. 2009, Wu et al. 2013). Among the soluble amino acids, only aspartate was present in lower amounts in the three types of *GDH* overexpressors in comparison to the WT. The root amino acid profile was not significantly modified in the NAD-*GDH* overexpressors, confirming that the similar NAD(H)-*GDH* activity measured in the roots of the WT and transgenic plants, did not induce any detectable changes in metabolite accumulation in this organ.

The metabolic response of both the WT and the transgenic plants to the NaCl treatment, prompted us to analyze in more detail their leaf metabolite composition. Based on the amino acid and carbohydrate profiles, which were specific for the leaves of the *GDHA*, *GDHB* and *GDHA/B* overexpressing plants, it appears that under salt stress conditions there is a specific pattern of metabolite accumulation when the two subunits (α or β) are overexpressed individually or simultaneously. This finding strengthens the hypothesis that the physiological role of the individual α and β subunits and their assembly is different, depending on their relative abundance when the enzyme is in the form of homohexamers or heterohexamers (Tercé-Laforgue et al. 2013). More strikingly, we found that in comparison to plants grown under standard nutrient conditions, the pattern of soluble amino acid accumulation was different in the three types of *GDH* overexpressors subjected to salinity stress. However, in the presence of NaCl, we did not observe the differences in the glutamate content or in the glutamate to glutamine ratio in the *GDHA*, *GDHB* and *GDHA/B* overexpressors like we did in the absence of NaCl (Tercé-Laforgue et al. 2013). Interestingly, we observed that in the presence of NaCl there was a reduction in the amount of aspartate in all the *GDH* overexpressors as compared to the WT.

A more detailed analysis of the leaf metabolite composition of salt stressed plants performed by GC/MS analysis confirmed that for a number of the metabolites, the pattern of accumulation was specific for each *GDH* overexpressor (Table 1). For example, the increase in malate, spermidine and palmitic acid was specific to *GDHA* overexpressors. Only in the *GDHB* overexpressors was the group of metabolites *myo*-inositol, threonate, glycerol-3-P, hydroxyproline, threonate-lactone and nicotinate, present in lower amounts. In the *GDHA/B* overexpressors, higher amounts of metabolites involved mostly in C and N metabolism were found. The fumarate, citramalate, GABA and inositol content were increased in the *GDHA* and *GDHA/B* double expressors, suggesting that in the transgenic plants it is the α subunit

that is controlling their level of accumulation. Due to the complexity of the metabolites and cognate metabolic pathways involved, we will not go into details into the interpretation of their physiological function in relation to increased GDH activity. However, these alterations in the metabolic profiles specific for the three types of *GDH* overexpressors fit well with our previous hypothesis that a single GDH subunit or a heteromeric assembly is able to differentially regulate part of a metabolic pathway (Tercé-Laforgue et al. 2013). The metabolic pathways that are involved play a major role in N assimilation, or are at the interface between C and N metabolism, but they are also involved in a variety of other biological processes such as stress/defence and cell wall metabolism. Conspicuously, it seems that these alterations in metabolism are different when the plant is subjected to a continuous salt stress, suggesting a key role for the two GDH subunits in the adaptation of the plant to various environmental conditions.

One of the main goals of our metabolic profiling study was to determine if the increase or decrease in certain metabolites could explain the improved biomass production, observed only when the three types of *GDH* overexpressors were grown in 50mM NaCl. Interestingly, we found that the content of a core set of four metabolites, DGG, erythronate, porphyrin and aspartate was modified in all three GDH overexpressors: increased for the first three and decreased for the latter. DGG is a galactolipid that forms part of the headgroup of digalactosyldiacylglycerol (DGD) a major component of the thylakoid lamellae. An increase in its content was observed during the acclimation of freeze dehydration of spruce (Angelcheva et al. 2014). Characterization of galactolipid-deficient *Arabidopsis* mutants revealed that a decrease in the proportion of DGD results in a reduction of chlorophyll content and photosynthetic activity, alterations in chloroplast ultrastructure, and an impairment of growth (Dörmann et al. 1995). The increase in DGG content in the GDH overexpressors

could be a way to circumvent the deleterious effect of the salt stress by increasing glycolipid abundance and thus improving membrane stability in the chloroplasts.

Erythronate is precursor of a number of C containing acid molecules present in both humans and plants (<http://www.ebi.ac.uk/chebi/chebiOntology.do?jsessionid=7FA46C0F40B372D4255ABCD00F2B7C5B?chebiId=37654>).

This molecule was found to increase in plants subjected to salt (Kazachkovka et al. 2013) and temperature stress (Kaplan et al. 2004). Intriguingly, the amount of erythronate accumulation was much higher in resurrection plants compared to other species that are not tolerant to desiccation (Gechev et al. 2014). It is therefore possible that erythronate may participate in the improved salt stress tolerance of the *GDH* overexpressors.

The decrease in aspartate identified in the *GDHA*, *GDHB* and *GDHA/B* overexpressors is much more difficult to interpret. In tobacco, the concentration of this amino acid was found to be modulated depending on the intensity and duration of the salt stress (Zhang et al. 2011). These authors emphasized that there is probably a diversion of aspartate synthesis toward the synthesis of other amino acids such as proline, which could explain why the *GDH* overexpressors are able to compensate for a shortage of other amino acids. In line with this hypothesis, Miyashita and Good (2008) and Fontaine et al. (2012) found an increase in aspartate in NAD-GDH-deficient mutants of *Arabidopsis* placed in the dark. Aspartate could be a source of organic acids *via* the deaminating activity of NAD-GDH to compensate for the lack of C. It may well be that in the three types of *GDH* overexpressors, more C is diverted from aspartate to limit the detrimental effect of the salt stress on carbohydrate availability, fitting in with the limited decrease in starch found in the three types of *GDH* overexpressors (Fig. 3).

Porphyry intermediates in the leaves of higher plants are crucial tetrapyrrole molecules for alleviating reactive oxygen species-induced stress (Phung and Phung, 2015a). An increase in the leaf porphyry content in the transgenic plants overexpressing the two *GDH* subunits

individually or simultaneously, suggests that the metabolite participates in their improved resistance to the salinity stress. Overexpression of protoporphyrinogen oxidase (PPO) protected transgenic rice from drought-induced cytotoxicity, demonstrating that manipulation of porphyrin biosynthesis can produce drought-tolerant plants (Phung et al. 2011, 2015b). Our results on the *GDH* overexpressors support a possible role for tetrapyrroles in signaling their metabolic state and in plant protection under drought stress conditions.

It remains now to determine what are the regulatory control mechanisms that trigger the accumulation of the stress-protective molecules such as DGG, erythronate and porphyrin when GDH activity is increased in plants submitted to a continuous salt stress. Nevertheless, our findings strengthen the current thinking that GDH plays a key role not only in regulating the C status of the plant (Miyashita and Good 2008, Fontaine et al. 2012) but also in metabolic signaling (Dubois et al. 2003, Tercé-Laforgue et al. 2004a, Skopelitis et al. 2006), irrespective of whether plants are subjected to abiotic stress conditions.

Material and Methods

Plant Material and growth

Tobacco (*N. tabacum*, cv. Xanthi XHFD8; INRA, Versailles, France) was grown on coarse (diameter = 1-2.5 mm) sand (Bellanger-Sopromat, Courbevoie, France) throughout plant development. Starting at the bottom of the seedlings, each emerging leaf was numbered and tagged. From a batch of 8-week old plants, twelve plants of uniform development and numbering 7 leaves each were selected. Plants were grown in a controlled environment growth chamber (16h light, 350-400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 26°C; 8h dark, 18°C) and watered with a standard complete nutrient solution containing 10mM NO_3^- and 2mM NH_4^+ as nitrogen sources (Coïc and Lesaint, 1971). Plants were automatically watered for 1 min (flow rate for each plant: 50 ml min^{-1}) every 2 h. Three plants were used for each experiment. Four weeks

later, leaves were numbered 8, 9, 11, 13, 15, 20 (from bottom to top). To apply a salinity stress, the plants were grown under the same conditions but the nutrient solution contained 50mM NaCl. Following separation into shoots and roots, the entire root system was frozen in liquid nitrogen and immediately reduced to a homogenous powder, which was stored at -80°C and used for all the further experiments. Leaf 10, corresponding to a fully expanded leaf (photosynthetic activity of approximately $5.8 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), was used for all physiological analyses. From this leaf, 1cm^2 sections of leaf blade tissue without the main midrib were randomly collected and pooled into two groups. One was weighed and then lyophilised to determine the fresh and dry weights. The other was weighed, frozen and used to determine the quantity of chlorophyll per fresh weight. The remaining leaf tissue was frozen in liquid N_2 and immediately reduced to a homogenous powder which was stored at -80°C and used for all the further experiments. All the harvesting of fresh material was carried out between 1-5 p.m.

Production of tobacco plants overexpressing GDH

The production of GDH overexpressors was performed essentially as described by Tercé-Laforgue et al. (2013). Briefly, DNA fragments containing the *GDHA* (*NpGDHA*) cDNA and the *GDHB* (*NpGDHB*) cDNA from *N. plumbaginifolia* (Restivo 2004) were subcloned in the sense orientation into the binary vector pBI121 to obtain *35S-NpGDHA* and *35S-NpGDHB* constructs. Seeds from the primary transformants were collected and the resulting transgenic plants were screened for kanamycin resistance. Homozygous T3 progeny of *GDHA*, *GDHB* and *GDHA/B* overexpressors were used for further studies.

RNA gel blot analysis

Northern blot analysis was performed as described previously (Tercé-Laforgue et al. 2004b). The probe used for *N. plumbaginifolia* GDH mRNA detection corresponded to the conserved coding region of the cDNA corresponding to the two genes *GDHA* and *GDHB* encoding subunit β and α respectively (Restivo 2004). Total RNAs were extracted from a pool of three transgenic and three control plants. Relative mRNA amounts were determined by using a phosphoimager (BAS-1500, Fuji, Japan).

Metabolite extraction and analyses

Fresh plant material was used for metabolite extraction. For leaf metabolome analysis, all steps were adapted from the original protocol described by Fiehn (2006), following the procedure described in Amieur et al. (2012). The ground frozen leaf samples (25mg fresh weight) were resuspended in 1 ml of frozen (-20°C) water:chloroform:methanol (1:1:2.5) and extracted for 10 min at 4°C with shaking at 1400 rpm in an Eppendorf Thermomixer. Insoluble material was removed by centrifugation and 900 μ l of the supernatant were mixed with 20 μ l of 200 μ g/ml ribitol in methanol. Water (360 μ l) was then added and after mixing and centrifugation, 50 μ l of the upper polar phase were collected and dried for 3h in a Speed-Vac and stored at -80°C. For derivatisation, samples were removed from -80°C storage, warmed for 15 min before opening and Speed-Vac dried for 1 h before the addition of 10 μ l of 20 mg/ml methoxyamine in pyridine. The reactions with the individual samples, blanks and amino acid standards were performed for 90 min at 28°C with continuous shaking. 90 μ l of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) were then added and the reaction continued for 30 min at 37°C. After cooling, 50 μ l of the reaction mixture were transferred to an Agilent vial for injection. For the analysis, 3 h and 20 min after derivatisation, 1 μ l of the derivatised sample was injected in the Splitless mode onto an Agilent 7890A gas chromatograph (GC) coupled to an Agilent 5975C mass spectrometer (MS). The column used

was a Restek Rxi-5SilMS (30 m with 10 m Integra-Guard column). The oven temperature ramp was 70 °C for 7 min, then 10 °C/min up to 325 °C, which was maintained for 4 min. For data processing, raw Agilent datafiles were converted into the NetCDF format and analyzed with AMDIS (<http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis>). Peak areas were then determined using the quanlynx software (Waters) after conversion of the NetCDF file into the masslynx format. Statistical analyses were carried out using TMEV <http://www.tm4.org/mev.html>. Univariate analyses by permutation (1-way ANOVA and 2-way ANOVA) were first used to select the metabolites exhibiting significant changes in their concentration. Free ammonium was extracted from leaves with 2% (w/v) 5-sulfosalicylic acid (1 mL per 10 mg DW) as described by Ferrario-Méry et al. (1998) and the content determined by the phenol/hypochlorite assay (Berthelot reaction). The starch content of the leaves was determined as described by Ferrario-Méry et al. (1998). Chlorophyll was estimated in crude leaf extracts according to Arnon (1949). Soluble protein was determined using a commercially available kit (Coomassie Protein assay reagent, Biorad, München, Germany) using bovine serum albumin as a standard.

Statistics

For measurement of enzyme activities and metabolite analyses, results are presented as mean values for three plants with standard errors ($SE = SD/\sqrt{n-1}$, where SD is the standard deviation and n the number of replicates). For the metabolome study on NaCl treated plants, statistical analyses were performed between the three replicates corresponding to the WT plants and between the six individual plants corresponding to the *GDHA* (three plants A1 and three plants A2), *GDHB* (three plants B1 and three plants B2) and *GDHA/B* (three plants A2b1 and three plants B1a2) overexpressors. All the statistical analyses were performed using the Student t-test functions of the XLStat-Pro 7.5 (Addinsoft, New York, USA) software.

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Disclosure

The authors have no conflict of interest to declare.

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Table 1 Changes in metabolite content in leaves of the three types of *GDH* overexpressors submitted to a salt stress.

Metabolite	Category	<i>GDHA</i>	<i>GDHB</i>	<i>GDHA/B</i>
		FC/WT	FC/WT	FC/WT
Digalactosylglycerol	Lipid metabolism	2.9	2.4	2.2
Erythronate	C metabolism	1.7	1.7	1.7
Porphyrin	Chl metabolism	1.4	1.3	1.7
Aspartate	N metabolism	0.8	0.5	0.6
Fumarate	C metabolism	4.4		2.3
Citramalate	C metabolism	2.9		1.9
GABA	N metabolism	2.4		1.9
Inositol	Stress defence	0.7		0.7
Malate	C metabolism	2.2		
Spermidine	Polyamine	1.9		
Palmitic acid	CW metabolism	1.3		
<i>Myo</i> -Inositol	Metabolism		0.7	
Threonate	CW metabolism		0.7	
Glycerol-3-P	C metabolism		0.5	
Hydroxyproline	N metabolism		0.6	
Threonate-lactone	CW metabolism		0.7	
Nicotinate	Secondary meta		0.6	
Glycine	N metabolism			2.4
Valine	N metabolism			2.3
Phenylalanine	N metabolism			2.4
Leucine	N metabolism			2.4
γ -Tocopherol	Stress defence			2.4
O-Acetyl-Serine	N metabolism			1.9
Xylulose	C metabolism			1.5
4-Cafeoylquininate	CW metabolism			1.4
Glucopyranose	C metabolism			1.3
Threitol	Stress defence			1.3

FC/WT corresponds to the fold change in the three types of *GDH* overexpressors compared to wild type (WT) plants grown on 50mM NaCl for metabolites exhibiting significant variations in their amount. The fold changes (FC) are the mean of the two lines overexpressing *GDHA* (lines A1 and A2), *GDHB* (lines B1 and B2) and *GDHA/B* (lines A2b1 and B1a2). For the metabolome analysis, a Student t-test (P value ≤ 0.05) was performed using the data obtained on three individual plants for the WT and the *GDHA* (lines A1 and A2), *GDHB* (Lines B1 and B2) and *GDHA/B* (lines A2b1 and B1a2) overexpressors grown in the presence of 50mM NaCl. C = carbon; CW = cell wall; N = nitrogen.

Figure legends

Fig. 1 GDH activity in WT and transgenic tobacco plants overexpressing two *NAD-GDH* genes from *N. plumbaginifolia*. (A) NADH-GDH aminating activity in leaves. (B) NAD-GDH deaminating activity in leaves. (C) NADH-GDH aminating activity in roots. (D) NAD-GDH deaminating activity in roots. The enzyme activity was measured in plants grown on a standard complete nutrient solution (black columns alone) and on the same nutrient solution containing 50mM NaCl (white columns) for 12 weeks. When the enzyme activity is higher in the presence of NaCl the white column is on top of the black column. When the enzyme activity is lower in the presence of NaCl the white column is below the black column. For comparison the activity was measured in the wild type (WT) and in transgenic lines overexpressing *GDHA* (two independent lines A1, A2), *GDHB* (two independent lines B1, B2), and the two genes simultaneously *GDHA/B* (two independent lines A2b1, B1a2). The mean for the six transgenic lines is indicated by OE (overexpressors). GDH activity was measured on three individual plants for each line. Values are the mean \pm SE. Asterisks (*) and circles (°) indicate a t-test with a *P*-value <0.05 when differences for the NADH- or the NAD-GDH activity were obtained between WT plants and GDH overexpressors treated and not treated with 50 mM NaCl respectively. Significant differences (*P*-value <0.05) between plants treated and not treated with NaCl are indicated by plus (+).

Fig. 2 NAD-GDH isoenzyme pattern of tobacco leaves from WT and transgenic plants overexpressing the two genes encoding GDH. The soluble protein extracts of leaves were subjected to native PAGE followed by NAD-GDH in-gel deaminating activity staining. The amount of protein loaded onto each lane was calculated on a similar DW basis for each leaf sample (0.5 mg). The position of the homohexameric forms of GDH from *N. plumbaginifolia* expressed in the A1 and B1 transgenic lines of *N. tabacum* is indicated by the symbols α and

β. (C): Plants grown on the standard complete nutrient solution. (NaCl): Plants grown on the standard complete nutrient solution containing 50mM NaCl. On the right hand side of the gel, the NAD-GDH isoenzyme pattern of a leaf extract from Arabidopsis was used as a marker to check that the different GDH isoenzymes (7 detected in Arabidopsis, Fontaine et al. 2006) were correctly separated in the transgenic tobacco plants. In-gel activity staining was performed on a pool of leaf samples from three individual plants.

Fig. 3 Biomass production, starch and chlorophyll contents of WT and transgenic plants overexpressing GDH. Shoot (A) and root (B) plant biomass production, leaf starch (C) and chlorophyll (D) contents of the WT and *GDHA* (lines A1 and A2), *GDHB* (lines B1 and B2) and *GDHA/B* (lines A2b1 and B1a2) overexpressors subjected to salt stress. The mean for the six transgenic lines is indicated by OE (overexpressors). Plants were grown on a standard complete nutrient solution (black columns) and on the same nutrient solution containing 50mM NaCl (white columns) for 12 weeks. When biomass, chlorophyll or starch is higher in the presence of NaCl the white column is on top of the black column. When biomass, chlorophyll or starch is lower in the presence of NaCl the white column is below the black column. Measurements were performed on three individual plants. Values are the mean \pm SE. Asterisks (*) and circles (°) indicate a t-test with a *P*-value <0.05 when there are differences between WT plants and GDH overexpressors treated and not treated with 50mM NaCl respectively. Significant differences (*P*-value <0.05) between plants treated and not treated with NaCl are indicated by plus (+).

Fig. 4 Phenotype of WT and GDH overexpressors subjected to salinity stress. The WT and *GDHA* (line A1), *GDHB* (line B1) and *GDHA/B* (line A2b1) overexpressors with 20 leaves,

following growth for 12 weeks on a standard complete nutrient solution supplemented with 50mM NaCl. Shoots (A) and roots (B).

Supplementary data

Supplementary Fig. 1 Quantification of *NAD-GDH* mRNA in leaves of the WT, *GDHA*, *GDHB*, *GDHA/B* overexpressors treated with NaCl. Changes in the steady state level of *NAD-GDH* mRNA transcripts. (A) RNA gel blot showing the steady state level of *NAD-GDH* mRNA transcripts which were detected by hybridization with the conserved coding region of *GDHA* and *GDHB* cDNAs from *N. plumbaginifolia* (Restivo et al., 2004) ³²P-labelled probes. Each sample contains (10mg) total RNA. Hybridization was performed on total leaf RNA isolated from the WT plants and the *GDHA* (line A1), *GDHB* (line B1) and *GDHA/B* (line A2b1) transformants grown on standard nutrient medium, with or without 50mM NaCl (NaCl). (B) An ethidium bromide stained gel is presented to show that similar amounts of total RNA were loaded onto each lane. The experiments were repeated using three different plants with similar results.

Supplementary Fig. 2. Leaf ammonium content of WT and transgenic plants overexpressing GDH treated with NaCl. The WT and the *GDHA* (lines A1 and A2), *GDHB* (lines B1 and B2) and *GDHA/B* (lines A2b1 and B1a2) overexpressors were subjected to salt stress. Plants were grown on a standard complete nutrient solution (black columns) and on the same nutrient solution containing 50mM NaCl (white columns). Measurements were performed on three individual plants. Values are the mean \pm SE. Asterisks * indicate a t-test with a *P*-value <0.05 when there are differences between the WT plants and GDH overexpressors treated with NaCl. The mean for the six transgenic lines is indicated by OE (overexpressors).

Supplementary Table 1. Concentration and proportion of soluble amino acids in leaves of WT tobacco plants and the three types of *GDH* overexpressors subjected to salt stress.

Amino acids were separated and quantified in a fully expanded leaf of the wild type control plants (WT), *GDHA* (lines A1 and A2), *GDHB* (lines B1 and B2) and *GDHA/B* (lines A2b1 and B1a2) overexpressors following growth in 50mM NaCl. The amino acid concentrations are expressed as nmol g⁻¹ DW. Their relative proportions is indicated in brackets. Values are mean \pm SE. For each line three individual plants were analyzed. * Significant difference between the WT and different transgenic lines grown in the presence of 50mM NaCl with a t-test *P* value <0.05. ° Significant difference for the WT plants grown in the presence or in the absence of 50mM NaCl with a t-test *P* value <0.05.

Supplementary Table 2. Metabolome analysis of the WT and of the three types of *GDH* overexpressors grown on 50mM NaCl. Normalized values correspond to the peak area, standard (ribitol)⁻¹. leaf DW⁻¹. SD = Standard deviation. nd = not detected. A t test at *P*<0.05 was performed between the WT plants and the two transgenic lines corresponding to the three types of *GDH* overexpressors to identify metabolites exhibiting an increase or a decrease in their concentration. Leaf samples were collected from three different plants for the WT and for each transgenic plant grown in the presence of 50mM NaCl. For transgenic lines A1 and A2 (*GDHA* overexpressors), transgenic lines B1, B2 (*GHDB* overexpressors) and transgenic lines A2b1, B1a2 (*GDHA/B* overexpressors) values are the mean of the data obtained from three individual plants.

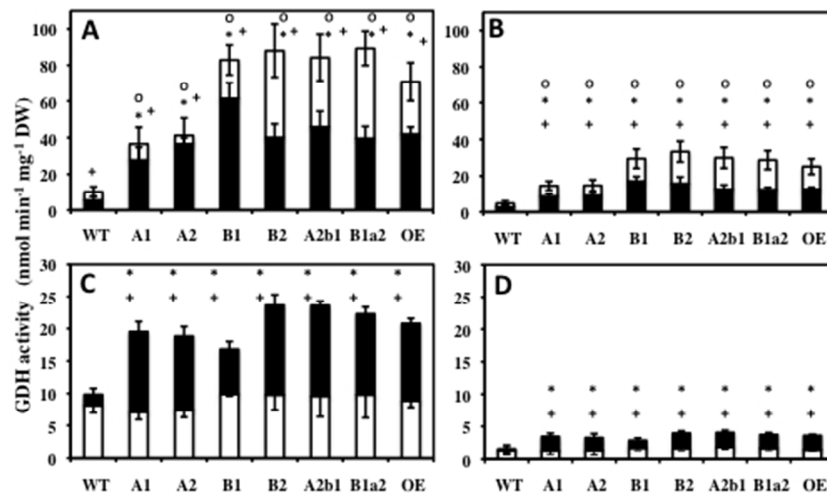


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Significant differences (P-value <0.05) between plants treated and not treated with NaCl are indicated by plus (+).
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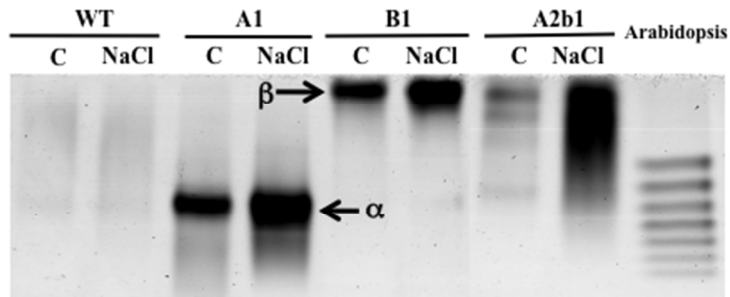


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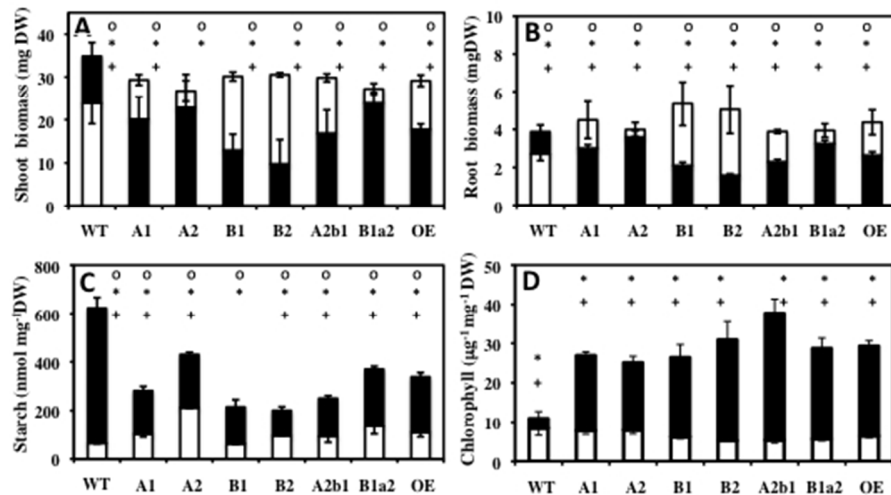


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