Crosstalk between Two bZIP Signaling Pathways Orchestrates Salt-Induced Metabolic Reprogramming in Arabidopsis Roots

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Soil salinity increasingly causes crop losses worldwide. Although roots are the primary targets of salt stress, the signaling networks that facilitate metabolic reprogramming to induce stress tolerance are less understood than those in leaves. Here, a combination of transcriptomic and metabolic approaches was performed in salt-treated Arabidopsis thaliana roots, which revealed that the group S1 basic leucine zipper transcription factors bZIP1 and bZIP53 reprogram primary C- and N-metabolism. In particular, gluconeogenesis and amino acid catabolism are affected by these transcription factors. Importantly, bZIP1 expression reflects cellular stress and energy status in roots. In addition to the well-described abiotic stress response pathway initiated by the hormone abscisic acid (ABA) and executed by SnRK2 (Snf1-RELATED-PROTEIN-KINASE2) and AREB-like bZIP factors, we identify a structurally related ABA-independent signaling module consisting of SnRK1s and S1 bZIPs. Crosstalk between these signaling pathways recruits particular bZIP factor combinations to establish at least four distinct gene expression patterns. Understanding this signaling network provides a framework for securing future crop productivity.

INTRODUCTION

Salt (NaCl) stress is a serious threat to food production, affecting around 30% of the agricultural land worldwide (Kronzucker and Britto, 2011). However, plants have established mechanisms to avoid or adapt to salt stress conditions (reviewed in Huang et al., 2012; Krasensky and Jonak, 2012; Deinlein et al., 2014; Golldack et al., 2014). Gaining insight into salt stress resistance mechanisms will be essential for developing strategies to enhance tolerance and, consequently, crop yield (Schroeder et al., 2013).

Salt stress is intrinsically complex since it implies both ion toxicity and an osmotic component (versusle et al., 2006; Huang et al., 2012). Although sensing of these cues is believed to take place at the membrane (Christmann et al., 2013), the respective sensors are not yet well defined (Kumar et al., 2013; Osakabe et al., 2013). After stress perception, a burst of reactive oxygen species mediated by NADPH oxidases (Chung et al., 2008) triggers an increase in cytosolic Ca2+ levels (Laohavisit et al., 2013) and the synthesis of the phytohormone abscisic acid (ABA) (Fujita et al., 2006, 2009; Umezawa et al., 2010; Huang et al., 2012).

Recent discoveries provide a detailed view on ABA-mediated stress signaling pathways (Fujii et al., 2009) sensed by the PYR/PYL/RCAR (PYRABACTIN RESISTANCE1/PYR1-like/REGULATORY COMPONENT OF ABA RECEPTOR1) coreceptors (Ma et al., 2009; Park et al., 2009). ABA bound to the receptor recruits members of the redundant PP2C (PROTEIN PHOSPHATASE 2C) family (Hao et al., 2011), thereby impeding their inhibitory action over crucial regulatory kinases belonging to the SnRK2 (SUCROSE-NON-FERMENTING1-RELATED PROTEIN KINASE2) family (Fujita et al., 2009). The active SnRK2 kinases phosphorylate different cellular targets such as AREB1 (ABA-RESPONSE-ELEMENT BINDING1) (Furihata et al., 2006), a member of the group A bZIP transcription factor (TF) family (Jakoby et al., 2002). Three related bZIPs, namely, AREB1, AREB2, and ABF3, cooperate as master regulators of ABA-dependent transcription through their binding to ABA-RESPONSIVE ELEMENT promoter cis-elements (Yoshida et al., 2010).

Transcriptome studies provided a valuable overview on the massive transcriptional reprogramming in response to abiotic stresses (Kilian et al., 2007). Osmotic stress imposed by salt or drought share common signaling networks that are in part ABA-dependent and ABA-independent (Huang et al., 2012). Whereas ABA-dependent transcriptional changes are regulated by AREB1-like...
bZIPs, ABA-independent responses are mediated by other TFs, e.g., DROUGHT-RESPONSIVE ELEMENT BINDING2 (Lata and Prasad, 2011).

Salt-induced defenses are energy dependent. As photosynthesis is strongly impaired under these conditions, a metabolic change is required to serve the plant’s energy demands. Nevertheless, the impact of salt stress on respiration is not fully understood and remains controversial (reviewed in Jacoby et al., 2011). In Arabidopsis thaliana leaves, dramatic salt-induced metabolic changes were discovered with respect to carbohydrate and amino acid metabolism (Kempa et al., 2008). Although roots are the primary targets of salt stress, little is known about metabolomic changes in salt-treated Arabidopsis roots and the regulatory signaling networks, particularly within the root.

TFs involved in metabolic reprogramming in salt-treated roots have yet to be characterized. Arabidopsis bZIP1 was found to be transcriptionally induced by salt treatment (Weltmeier et al., 2009), leading to enhanced or reduced tolerance to salt and drought stress when overexpressed or knocked out, respectively (Sun et al., 2012). However, the precise mechanism of action remains elusive. bZIP1 belongs to the group S1 bZIP factors (bZIP1, -2, -11, -44, and -53), which preferentially form heterodimers with group C (bZIP9, -10, -25, and -63) (Ehlert et al., 2006; Weltmeier et al., 2006). This so-called C/S1 network of bZIP TFs has been shown to regulate metabolic reprogramming under low energy stress (Hanson et al., 2008; Dietrich et al., 2011; Ma et al., 2011). In particular, bZIP1 and its closest homolog bZIP53 display a partially redundant function. Under starvation induced by extended nighttime, bZIP1 directly targets genes involved in amino acid metabolism, such as ASPARAGINE SYNTHETASE1 (ASN1) and PROLINE DEHYDROGENASE1 (Dietrich et al., 2011). Likewise, genome-wide binding studies in protoplasts revealed bZIP1 as a major regulator of N-related genes (Paro et al., 2014).

In Arabidopsis, the kinases SnRK1.1 (AKIN10) and SnRK1.2 (AKIN11), belonging to the SnRK1 family of protein kinases, have been shown to function as central integrators of plant stress and low energy signaling (Baena-González et al., 2007). It was proposed that SnRK1 responses were mediated by C and/or S1 bZIPs, but direct phosphorylation of any of these TFs remains to be demonstrated. Moreover, the impact of SnRK1s on salt stress responses is currently unclear.

In addition to their function as energy supply, sugars are also important signaling molecules (Hanson and Smeekens, 2009). In Arabidopsis, HEXOKINASE1 (HXX1) functions as a major glucose sensor (Moore et al., 2003). Interestingly, bZIP1 transcription in seedlings is repressed by glucose and depends on HXX1 (Kang et al., 2010; Dietrich et al., 2011). These findings support the view that bZIP1 transcription responds to the glucose status of the cell. Along this line, all group S1 members are translationally repressed by sucrose due to a conserved upstream open reading frame (Wiese et al., 2004; Weltmeier et al., 2009). Although plant’s energy resources are assumed to have an impact on efficient stress responses, the nature of relevant metabolic parameters, their sensing, and a functional connection to group S1 TFs have not yet been clarified.

Here, we provide several lines of evidence demonstrating the function of bZIP1 and its interlinking C/S1 bZIP partners in salt-stressed Arabidopsis roots. (1) We demonstrate that bZIP1 transcription integrates signals about metabolic and/or energy status of stressed cells. (2) Using combined transcriptome and metabolic approaches, we define the function of bZIP1 in reprogramming carbohydrate and amino acid metabolism. (3) In addition to the well-described SnRK2/AREB signaling module, we identify a second, structurally related SnRK1/group S1 bZIP signaling module functioning in salt-treated roots. (4) Crosstalk among these bZIP factors allows a regulatory circuit to build up, providing a means to integrate information about the metabolic situation of the cell with salt stress response programs.

RESULTS

bZIP1 Transcription in Roots Is Induced by Ionic or Osmotic Stimuli

To gain insight into the function of group C and S1 TFs in plant stress responses, RT-qPCR experiments were performed on salt-treated, hydroponically grown Arabidopsis Col-0 plants. The results reproduced public transcriptome data (Weltmeier et al., 2009), demonstrating that specifically bZIP1 and, to a minor extent, bZIP53 were transcriptionally induced by NaCl treatment (Figure 1A). In comparison to well described markers of abiotic stress responses, such as RESPONSIVE TO DESSICATION29B (RD29B) (Msnanne et al., 2011), a relatively slow transcriptional activation kinetic was observed, showing the strongest increase later than 6 h after treatment (Supplemental Figure 1A). Transcription of all group C members showed only minor salt-induced changes (Supplemental Figure 1B). Importantly, the response was found only in roots, but not in leaves (Supplemental Figure 1C). We therefore focused our study on bZIP1 and bZIP53 in the Arabidopsis root system.

To further characterize conditions inducing bZIP1 transcription, we treated roots with several salts (NaCl, KCl, Na2SO4, and MgCl2) and the osmotically active sugar mannitol using identical osmotic strength in all experiments. All treatments induced bZIP1 transcription (Figure 1B). Whereas NaCl and KCl led to similar induction patterns, stronger activation was triggered by multivalent ions, suggesting that besides osmotic cues, ionic stimuli are sensed in a specific manner.

bZIP1 bZIP53 Mutants Show Reduced Salt Tolerance

To address a putative function of C/S1 bZIPs in salt responses, seeds of bzip1 and bzip53 single and double mutants (Dietrich et al., 2011; Sun et al., 2012) were germinated on 175 mM NaCl (Figure 1C). Whereas bzip1 and bzip53 showed no significantly reduced germination rates, the correspondent double mutant was clearly impaired. Although group C bZIPs are known heterodimerization partners of S1 bZIPs in abiotic stress responses (Weltmeier et al., 2006; Alonso et al., 2009), the bzip10 bzip25 double mutant was not affected in germination. Nevertheless, a quadruple bzip1 bzip53 bzip10 bzip25 line showed a tendency to be less salt tolerant than the bzip1 bzip53 double mutant. We therefore concluded that bZIP1 and bZIP53 are important, potentially redundant players in salt stress responses, which are functionally supported by bZIP10 and bZIP25.

Transcriptomic Analyses Reveal Functions of bZIP1 and bZIP53 in Metabolic Reprogramming under Salt Stress

In order to gain mechanistic insights into bZIP1 and bZIP53 function in salt-stressed roots, genome-wide transcriptome analyses were performed. As bzip1 single mutants showed only
In our experimental setup, we compared transcript profiles of hydroponically grown, 6-week-old wild-type and mutant roots, salt treated for 0, 1, 3, and 6 h. To minimize the input of the circadian clock, plant material was harvested simultaneously 1 h before the end of the light period. In a parallel approach, roots were harvested for metabolite analyses. The complete data are provided in Supplemental Data Sets 1 to 4.

The wild-type plants showed substantial transcriptional upregulation upon salt stress (1 h, 851 genes; 3 h, 1415 genes; 6 h, 2016; log ≥2-fold, P ≤ 0.01), which is in agreement with previously published data sets (Kilian et al., 2007). Hence, this system is suitable for performing the proposed study. In contrast to this high number of differentially expressed genes (DEGs), only five or four genes were down- or upregulated 1 h after salt stress when bzip1 bzip53 and the wild type were compared (Figure 2; Supplemental Data Set 1). In particular, bZIP1 and bZIP53 transcription was reduced, as is expected for the double mutant. Nevertheless, more DEGs could be observed after 3 and 6 h of salt treatment, as displayed by the Venn diagram shown in Figure 2 (log2 ≥ 0.7-fold, P ≤ 0.01). This kinetic is concurrent with the relatively slow induction of bZIP1 and bZIP53, which increases significantly only after 3 to 6 h of salt treatment. It has to be noted that almost no overlap within the set of DEGs was observed at the 3- and 6-h time points, indicating a sequential regulatory activity of the TFs involved.

In order to define the functional impact of bZIP1 and bZIP53, Gene Ontology annotation and MapMan (Thimm et al., 2004) analyses were performed. As pointed out in Table 1, the most significantly downregulated genes 3 h after salt treatment correspond to fermentation, response to low oxygen stress, and carbohydrate metabolism. After 6 h of salt treatment, expression of several known stress-related marker genes, such as SENESCENCE-ASSOCIATED1 (SEN1) (Oh et al., 1996), DARK-INDUCED2 (DIN2) (Fujiki et al., 2005), EARLI1 (Zhang and Schläppi, 2007), and RD29B (Mamane et al., 2011), were found to be downregulated in the bzip1 bzip53 mutant (Table 1; Supplemental Data Set 1). Strikingly, a set of genes involved in catabolism of specific amino acids was identified, supporting a function of these bZIPs in reprogramming the root metabolism to respond adequately to the applied stress.

**The bzip1 bzip53 Mutant Is Affected in Primary Carbohydrate Metabolism**

As highlighted in Table 1, most of the genes found to be downregulated in bzip1 bzip53 roots at 3 h of salt treatment are connected to fermentation and low oxygen response. In particular, this can be observed for the whole set of anaerobic core genes as defined by Pucciariello et al. (2012) (e.g., NODULIN 26 INTRINSIC PROTEIN2.1, ALCOHOL DEHYDROGENASE1, PYRUVATE DECARBOXYLASE, SUCROSE SYNTHASE4 [SUS4], HYPOXIA-RESPONSIVE UNKNOWN PROTEIN43 [HUP43], and LOB-DOMAIN-CONTAINING PROTEIN41). Altogether, these data support the view that a major shift to nonoxidative energy metabolism occurs in salt-treated roots, which is partially impaired in the bzip1 bzip53 double mutant.

To further support this hypothesis, transcriptome data were correlated with detailed carbohydrate measurements. Indeed, salt treatment led to major quantitative and qualitative alteration in carbohydrate composition. Most prominent within 3 to 6 h after stress, Glc, Fru, and Suc levels rose significantly (Figures 3A and 3B).
Strikingly, the ratio between mono- and disaccharides differed between bzip1 bzip53 and the wild type (Figure 3B). For example, whereas the Glc levels of the wild type strongly increased 3 to 6 h after salt stress, similar levels were already present in the mutant under unstressed conditions. Moreover, although the initial Suc level in the mutant was slightly higher in comparison to the wild type, a further significant increase was observed within the first 6 h of treatment. Hence, bZIPI and bZIP53 have a major impact on carbohydrate homeostasis during the onset of the salt stress response program.

One of the most significantly DEGs in primary carbohydrate metabolism in the array data set is SUS4, encoding a SUCROSE SYNTHASE that participates in Suc breakdown, in particular metabolism in the array data set is SUS4, encoding a SUCROSE SYNTHASE that participates in Suc breakdown, in particular

homeostasis during the onset of the salt stress response program. Indeed, all measured intermediates accumulated immediately after salt stress, peaking within 6 and 24 h (Supplemental Figure 3 and Supplemental Data Set 4). Whereas transcription of TCA cycle genes was not significantly altered in the bzip1 bzip53 mutant (Supplemental Data Set 1), TCA cycle intermediates citrate, succinate, 2-ketoglutarate, and fumarate accumulated in the bzip1 bzip53 mutant to higher levels than in the corresponding wild type. The amounts of these metabolites eventually returned to wild-type levels within the first 24 h. In comparison to the wild type, the malate concentrations remained at high levels within the 48-h period of the performed measurements. Taken together, although the TCA cycle is affected in the bzip1 bzip53 mutant, this is not due to bZIP-specific gene regulation.

The bzip1 bzip53 Mutant Is Affected in the Catabolism of a Specific Subset of Amino Acids

As pointed out in Table 1, bzip1 bzip53 mainly affect genes involved in amino acid catabolism. Overall eight genes coordinating degradation of branched-chain amino acids (BCAAs; Val, Leu, and Ile) (Binder, 2010), as well as Met and Tyr, were partially impaired in the mutant. This indicates a major function of these bZIPIs in breakdown of a particular set of amino acids (Figure 4A). The array results of DEGs were confirmed by independent RT-qPCR experiments (Figure 4B). Interestingly, for all analyzed genes (BRANCHED-CHAIN AMINO ACID TRANSFERASE2 [BCT2], METHYLcrotonyl-CoA-CARBOXYLASE [MCCA], HOMOGENTISATE 1,2-DIOXYGENASE [HGO], and METHIONINE-γ-LYASE [MGL] (Mertzen et al., 2008; Binder, 2010), the use of a quadruple bZIP mutant (bzip1 bzip53 bzip10 bzip25) did not further reduce the expression found in the bzip1 bzip53 double mutant. We therefore assume that regulation of the genes involved in BCAA degradation differs from that of PPDK.

Salt treatment led to a transient increase in the overall amino acid levels during the first 6 h. At later time points, concentrations of many amino acids decreased, presumably due to reduced synthesis and/or degradation. However, the effect of bZIPI1 and bZIIP53 was restricted to particular amino acids. In the wild type, BCAA (Val, Leu, and Ile) concentrations decreased after 24 and 48 h of salt stress, which correlated with the activation of the metabolic genes encoding enzymes in amino acid degradation. This process was partially blocked in the bzip mutants. Downregulation of gene expression levels could clearly be correlated with amino acid levels (Figure 4C; Supplemental Figure 4). However, only minor differences between the wild type and mutants are seen for Tyr or Lys. It needs to be taken into account that steady state amino acid measurements do not differentiate between biosynthesis, degradation, and flux into other pathways. Moreover, other regulatory mechanisms besides gene regulation may apply.

Finally, acting as compatible solutes, sugars such as raffinose are implicated in establishing salt stress tolerance (Krasensky and Jonak, 2012). Whereas the raffinose concentration increased steadily in the wild type, this increase was impaired 6 h after salt treatment in the bzip1 bzip53 mutant (Supplemental Figure 1). These findings corresponded with a reduction in RAFFINOSE SYNTHASE (DIN10) expression, though the transcriptional changes were minor. Altogether, the bZIPIs under investigation have an important impact on the salt-induced metabolic shift in carbohydrate metabolism.

A metabolic switch to fermentation should be reflected on the level of the tricarboxylic acid (TCA) cycle. Indeed, all measured intermediates accumulated immediately after salt stress, peaking within 6 and 24 h (Supplemental Figure 3 and Supplemental Data Set 4).
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(Continued)
how bZIP1 interacts with the ABA-SnRK2-AREB pathway, we revealed that several well described ABA-responsive genes are downregulated in the bzip1 bzip53 mutant, such as EARLY ARABIDOPSIS ALUMINUM INDUCED1 (EARLI1), RD29B, and LATE EMBRYOGENESIS ABUNDANT PROTEIN76 (LEA76). To assess how bZIP1 interacts with the ABA-SnRK2-AREB pathway, we studied the expression of these genes in aba2, snrk2.2 snrk3 snrk6, and areb1 areb2 abf3 as well as bzip1 bzip53 and bzip1 bzip53 bzip10 bzip25 mutants. These mutant-based expression studies allowed us to group the array-derived genes into four classes.

Class 1 genes (e.g., LEA76 and EARLI1) are group A bZIP targets that depend on all components of the ABA pathway (Figure 5C; Supplemental Figure 6). As these genes were transcriptionally induced more rapidly than bZIP1 and as activation was unchanged in the C/S1 quadruple mutants (Figure 5D), they are probably not direct C/S1 target genes. Hence, minor differences in expression observed in the array data set are most likely due to indirect crosstalk during the salt stress response.

Class 2 genes such as the BCAA catabolic genes BCAT2 and MCC2 clearly depend on bZIP1 and bZIP53, as demonstrated by the respective mutant analyses. Interestingly, group C bZIPs appear to have no impact on the respective gene regulation. In accordance with the induction profile of bZIP1, these genes were less rapidly induced than class 1 genes. Moreover, class 2 genes depend on both AREB1/AREB2/ABF3 TFs and SnRK2s. These data indicate that both group A and group S1 bZIP signaling pathways merge to regulate class 2 transcription.

Class 3 genes such as TYROSI NE AMINOTRANSFERASE7 (TAT7) and HGO do not depend on the ABA-AREB-SnRK2 pathway, instead depending on group S1 bZIPs. Again, group C has no or only a minor impact.

Finally, mutant analyses studying class 4 gene expression, such as PPDK, DIN2, or ASN1, clearly showed no dependency on group A bZIPs but regulation by group S1, which was further enhanced in the C/S1 quadruple mutant (Figure 5D; Supplemental Figure 6). Interestingly, kinases of the SnRK2 family interfered with class 4 gene expression in a not yet well defined manner.

Chromatin Immunoprecipitation Analysis Reveals Binding of bZIP1 to the BCAT2 and TAT7 Target Promoter

In agreement with previous data, we could detect strong binding of HA-tagged bZIP1 to the BCAT2 promoter using chromatin immunoprecipitation (ChIP) coupled to PCR. Promoter scanning revealed a strong binding to a G-box-rich region close to the TATA-box (designated ProBCAT2-3) (Figure 5E), whereas promoter regions more upstream were hardly or not at all bound by bZIP1. As salt treatment did

### Table 1. (continued).

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*Genes selected from the transcriptome data, ordered by the given functional aspects.

*Fold change (log2) of differentially expressed genes.
not lead to enhanced ChiP signals, binding is constitutive. In contrast, salt-induced ChiP signals were detected for all tested TAT7 promoter primers, supporting a stimulus-induced bZIP1 binding mechanism. The LEA76 and PPDK promoters showed limited background binding, indicating that these are no- or low-affinity targets.

SnRK1 Signaling Is Required for Salt-Induced bZIP1 Transcription

Due to its proposed function in metabolic reprogramming, we focused on potential upstream signaling compounds to deduce which metabolic inputs are mediated via bZIP1 activity. SnRK1.1 and SnRK1.2 kinases are known to mediate responses upon energy deprivation (Baena-González et al., 2007). Due to redundancy, single snrk1.1 knockout lines show only limited phenotypical and molecular alterations, whereas double knockout mutants are lethal (Baena-González et al., 2007). We therefore established a β-estradiol-inducible snrk1.2 artificial microRNA approach in an snrk1.1 background, designated snrk1. Immunoblot analyses confirmed the loss-of-function approach (Supplemental Figure 7). In comparison to salt-treated wild-type roots, bZIP1 transcription was strongly reduced in snrk1 (Figure 6A). These data indicate that SnRK1 signaling is required for salt-induced bZIP1 transcription.

Figure 3. Changes in Carbohydrate Metabolism Comparing NaCl-Treated Hydroponically Grown Roots of Wild-Type and bzip1 bzip53 Mutants.

(A) Simplified overview of carbohydrate metabolism. Changes in sugar concentrations or gene expression are indicated by blue or red arrows, respectively (see Supplemental Data Set 3 and [B]). Times (in h) indicate maxima of metabolites (blue) or transcript abundance (red).

(B) Changes in carbohydrate concentration (nmol/g fresh weight [FW]) after salt treatment in the wild type (black) and bzip1 bzip53 (gray). Given are mean values (±sd) of three biological replicates.

(C) to (E) RT-qPCR analysis of SUS4 (C), FBP (D), and PPDK (E) expression in wild-type (black), bzip1 bzip53 (blue), and bzip1 bzip53 bzip10 bzip25 (brown) plants. Given are mean expression values (±sd) of three biological and three technical replicates. Significant differences between the wild type and mutants have been defined by Student’s t test for each time point: *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 4. Impact of C and S1 bZIP Factors on Amino Acid Metabolism of Salt-Treated Roots.

(A) Simplified overview of the catabolism of BCCAs (Ile, Leu, and Val), Met, Lys, and Tyr and biosynthesis of Asn. Relevant enzymes are indicated in red and array data of the correspondent genes differentially regulated in \textit{bzip1 bzip53} are given in brackets. ETF, ELECTRON-TRANSFER-FLAVOPROTEIN.
Ca²⁺ Signaling Induces bZIP1 Transcription

Ca²⁺ signaling is important in regulating various stress responses. Indeed, salt-induced bZIP1 transcription was strongly impaired by the Ca²⁺ blocker LaCl₃ (Figure 6B). In contrast to LaCl₃, the calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthelenesulfonamidohydrochloride (W7) has been shown to generate cytosolic Ca²⁺ transients in Arabidopsis (Kaplan et al., 2006). Indeed, W7 treatment partially substituted for the salt stress to induce bZIP1. Altogether, stress and metabolic signaling events are integrated into bZIP1 transcription, which is further relayed into salt-specific gene regulation.

DISCUSSION

In order to cope with salt stress, plants produce protective compounds, adjust their ion homeostasis, and remodel primary metabolism to serve the energy demand under stress (Deinlein et al., 2014; Müller et al., 2014). Focusing on Arabidopsis roots, which are the primary targets of salt stress, this study revealed a rapid and substantial reprogramming of the transcriptome leading to metabolism to promote the plant’s survival under stress conditions, and is integrated in abiotic stress signaling networks.

bZIP1 Transcription Integrates Cues from Stress- and Energy-Dependent Signaling Pathways

Salt or mannitol treatment led to an activation of the bZIP1 promoter specifically in Arabidopsis roots. As transcriptional responses to mannitol and several ionic sources of equal osmolarity differ considerably, it is tempting to speculate that both ionic and osmotic cues are sensed and transmitted into bZIP1 transcription. In comparison to classical salt response marker genes, bZIP1 is induced relatively slowly, showing the strongest induction later than 6 h after salt treatment. In contrast, the other members of the C/S1 network of bZIPs are not regulated by salt in roots, indicating a specific function of bZIP1. Nevertheless, the closest homolog bZIP53 is also transcriptionally induced, but only to a minor extent. Mutant analysis demonstrates that germination of bzip53 and bzip1 bzip53 is increasingly impaired by salt treatment. Although in our assay system, bzip1 mutants displayed comparable germination rates to those of the wild-type plants, Sun et al. (2012) demonstrated in several phenotypical assays that two independent bzip1 T-DNA insertion mutants are less tolerant to salt and drought treatments. Taken together, these data support the view that both TFs share partially redundant functions in salt-treated roots, as has been previously described in the dark-induced starvation response (Dietrich et al., 2011).

Pharmacological evidence supports the view that cytosolic Ca²⁺ bursts observed in response to salt are sufficient to induce bZIP1. In contrast, an active ABA/SnRK2/AREB signaling pathway is not required for bZIP1 induction. However, it has to be stressed that genetic approaches do not eliminate indirect effects, which might explain minor changes in bZIP1 expression. Interestingly, mutations in the ABA signaling pathway partially impair salt-induced bZIP53 transcription, indicating TF-specific differences. Further studies are needed to unravel the mechanistic differences in regulation of these genes.

Generally, stresses such as salt treatment are believed to interfere with plant energy homeostasis (Baena-González and Sheen, 2008). The Arabidopsis SnRK1s are evolutionary conserved kinases that facilitate metabolic adaption to stress and energy starvation (Baena-González et al., 2007). Applying an inducible knockout approach, SnRK1s were found to be crucial for full-level bZIP1 transcription. Although the C/S1 bZIP TFs have been implicated as likely targets of these kinases, experimental proof for direct phosphorylation is still missing. The metabolic cues related to energy starvation and the upstream components regulating SnRK1 activity are not yet well defined (Crozet et al., 2014). As we detected substantial sugar resources within the salt-stressed roots, further studies are needed to unravel whether rapid changes in carbohydrate concentrations or other metabolic cues are relayed into SnRK1 activity and ultimately bZIP1 transcription.

In summary, metabolic and stress-related signaling pathways merge to regulate the bZIP1 promoter. Whether the promoter itself acts as a signal integration platform or whether crosstalk occurs further upstream remains elusive.

bZIP1 and bZIP53 Reprogram Primary Carbohydrate and Amino Acid Metabolism to Help Roots Adapt to Salt Stress Conditions

Bioinformatic and systems biology approaches implicate several group S1 bZIP factors as crucial regulators of metabolic reprogramming (Gutiérrez et al., 2008; Usadel et al., 2008). As recently described in dark-treated leaves (Dietrich et al., 2011), bZIP1 shows strong transcriptional responses in salt-treated roots, whereas activation of bZIP53 is marginal under both stress conditions. We therefore assume that bZIP1 serves as the main transcriptionally regulated “driver” of these responses, although bZIP53 may partially substitute for a loss of bZIP1 in the mutant plant. Here, we demonstrate that these TFs regulate carbohydrate energy metabolism (fermentation and gluconeogenesis) and catabolism of specific amino acids, providing the enzymatic framework to remobilize carbon skeletons of proteins to satisfy stress-related energy demands.
Figure 5. Mutant and ChIP-PCR Analysis to Study Regulation of bZIP1, bZIP53, and Potential Target Genes.

(A) to (C) RT-qPCR analysis of salt-treated mutants impaired in defined components of the ABA-SnRK2-AREB pathway studying bZIP1 (A), bZIP53 (B), and the potential targets PPDK, TAT7, BCAT2, and LEA76 (C). Wild type (black), aba2 (blue), snrk2.2 snrk3 snrk6 (orange), and areb1 areb2 abf3 (light green) (see Figure 7 for details).

(D) RT-qPCR analysis of the same target genes in wild-type (black), bzip1 bzip53 (blue), and bzip1 bzip53 bzip10 bzip25 (brown) mutant plants. Given are mean fold (± st) induction values of two to three biological and three to four technical replicates. Student’s t test compares the wild type and mutants at the respective time point: *P < 0.05, **P < 0.01, and ***P < 0.001.

(E) ChIP-PCR analyses for the indicated promoters using an α-HA-antibody to detect binding of HA:bZIP1. Upper panel shows the promoter scanning experiments indicating localization of the primer derived PCR products (top, red) and putative G-box-related binding sites (green). Lower panel shows a comparison of fold input levels calculated relative to wild-type (white; set to 1) and Pro35S:HA:bZIP1 plants untreated (gray) and induced with salt (black). Results were obtained from two plant pools per line and two to four independent ChIP experiments. Significant differences have been determined by one-way ANOVA followed by a Bonferroni post-hoc test and are labeled with individual letters.
Interestingly, both transcriptome and metabolic studies propose two-phase transitions in primary C-metabolism in salt-treated roots. Within the first 3 h, wild-type roots show hypoxia-/fermentation-related gene expression, which is partially impaired in the bzip1 bzip53 mutant. Although the functional impact of these genes in salt stress response has not been demonstrated, these findings propose a major shift to nonoxidative energy metabolism. Interestingly, recent studies demonstrated that HRE2, an important TF in hypoxia-related transcription, is transcriptionally induced by salt and osmotic stress and is required for growth on salt-containing medium (Park et al., 2011). Taken together, a crucial function of low oxygen-responsive genes in abiotic stress defense may be postulated; however, further studies are needed to evaluate the biological impact of this crosstalk.

A reduction in oxidative energy metabolism is in line with the proposed function of the SnRK1-bZIP1 pathway in starvation response (Baena-González et al., 2007; Dietrich et al., 2011). In particular, regulation of the hypoxia marker gene SUS4, encoding a Suc-degrading enzyme (Baroja-Fernández et al., 2012), depends on bZIP1/bZIP53, which might explain the increase in monosaccharides upon stress and/or protein degradation (Ndimba et al., 2005). Nevertheless, the transcriptome data do not support a direct impact of bZIPs on protein biosynthesis. After 6 h, amino acid concentration decreases again, which is correlated with the degradation of a specific set of amino acids. Importantly, transcriptional upregulation of the cognate bZIP TFs precedes the onset of the expression of amino acid catabolic genes. In particular, a set of genes involved in degrading BCAAs (Leu, Val, and Ile), Met, and Lys is activated, which feeds into the acetyl-CoA pool (AcCoA pool) (Binder, 2010; Araújo et al., 2011). AcCoA can provide intermediates for the TCA cycle, which is perturbed during stress. More strikingly, recent studies disclosed that BCAA breakdown can provide electrons both directly to the electron transport chain via the electron transfer flavoprotein complex as well as indirectly feeding the TCA cycle with metabolic intermediates (Ishizaki et al., 2005; Araújo et al., 2010, 2011). After salt treatment, a transient increase in the concentration of most amino acid is observed. This might be due to reduced protein biosynthesis upon stress and/or protein degradation (Ndimba et al., 2005). Nevertheless, the transcriptome data do not support a direct impact of bZIPs on protein biosynthesis. After 6 h, amino acid concentration decreases again, which is correlated with the degradation of a specific set of amino acids. Importantly, transcriptional upregulation of the cognate bZIP TFs precedes the onset of the expression of amino acid catabolic genes. In particular, a set of genes involved in degrading BCAAs (Leu, Val, and Ile), Met, and Lys is activated, which feeds into the acetyl-CoA pool (AcCoA pool) (Binder, 2010; Araújo et al., 2011). AcCoA can provide intermediates for the TCA cycle, which is perturbed during stress. More strikingly, recent studies disclosed that BCAA breakdown can provide electrons both directly to the electron transport chain via the electron transfer flavoprotein complex as well as indirectly feeding the TCA cycle with metabolic intermediates (Ishizaki et al., 2005; Araújo et al., 2010). Moreover, Tyr degradation is also regulated by the bZIPs under investigation (Araújo et al., 2010). However, it remains to be determined whether these effects are specific to bZIP1/bZIP53 mutants or are part of a broader regulatory network modulating carbohydrate metabolism.

Proteins can function as alternative respiratory substrates under stress (Ishizaki et al., 2005; Araújo et al., 2010, 2011). After salt treatment, a transient increase in the concentration of most amino acid is observed. This might be due to reduced protein biosynthesis upon stress and/or protein degradation (Ndimba et al., 2005). Nevertheless, the transcriptome data do not support a direct impact of bZIPs on protein biosynthesis. After 6 h, amino acid concentration decreases again, which is correlated with the degradation of a specific set of amino acids. Importantly, transcriptional upregulation of the cognate bZIP TFs precedes the onset of the expression of amino acid catabolic genes. In particular, a set of genes involved in degrading BCAAs (Leu, Val, and Ile), Met, and Lys is activated, which feeds into the acetyl-CoA pool (AcCoA pool) (Binder, 2010; Araújo et al., 2011). AcCoA can provide intermediates for the TCA cycle, which is perturbed during stress. More strikingly, recent studies disclosed that BCAA breakdown can provide electrons both directly to the electron transport chain via the electron transfer flavoprotein complex as well as indirectly feeding the TCA cycle with metabolic intermediates (Ishizaki et al., 2005; Araújo et al., 2010). Moreowr, Tyr degradation is also regulated by the bZIPs under investigation (Araújo et al., 2010). However, it remains to be determined whether these effects are specific to bZIP1/bZIP53 mutants or are part of a broader regulatory network modulating carbohydrate metabolism. Consequently, not all reactions in the pathway carry the same flux (Sweetlove et al., 2010). Although TCA cycle gene expression in bzip1 bzip53 mutants is not affected, this issue is well documented on the level of several TCA cycle intermediates of the second half.
of the TCA cycle (succinate, malate, and fumarate), which significantly increase in the mutant after salt treatment. This fragmented TCA cycle has been demonstrated to take place in *Lotus japonicus* roots under anoxia, where alanine aminotransferase links glycolysis to the TCA cycle (Rocha et al., 2010).

In contrast to genes involved in amino acid catabolism, **ASN1** transcription leads to Asn biosynthesis, which is regulated by bZIP10 and bZIP25 (group C) and to a minor extent by bZIP1 and/or bZIP53. Accordingly, Asn levels increase both in dark-treated leaves and in salt-stressed roots. Asn has been proposed to function as a transported form of C and N (Lam et al., 1998, 2003). In darkened leaves, Asn has been shown to be derived from pyruvate by PPDK activity (Lin and Wu, 2004). Consistently, PPDK is also upregulated in roots and partially depends on C/S1 bZIPs. As bZIP1 and bZIP53 coordinate an alternative metabolic program that provides means to support survival under low-energy stress in leaves as well as salt stress in roots, it is tempting to speculate that these bZIPs may have a broad function in general stress management.

**Figure 7.** Model Summarizing the Findings on the Regulation of bZIP1 Transcription in Salt Stress Response in Arabidopsis Roots and the Crosstalk of the ABA-Independent SnRK1-bZIP1 Pathway and ABA-Dependent SnRK2-AREB Pathway.

Due to the mutant and ChIP-PCR analysis, putative bZIP1 target genes can be classified as dependent on the ABA/SnRK2/AREB pathway (class 1), the SnRK1/bZIP1 pathway (class 3), or both (class 2). Class 4 genes depend on group C, and S1 bZIPs and are independent of AREB-like TFs. A potential interaction between SnRK1 and SnRK2 kinases is proposed by Umezawa et al. (2013) and Rodrigues et al. (2013).
Importantly, only a limited number of these genes was found to be differentially expressed in the bzip1 bzip53 transcriptome data set (e.g., EARL1 and RD29B; depicted as class 1 genes). Indeed, almost no bZIP1 promoter binding was observed for the ABA response gene LEA76, and mutant analysis demonstrated that this “classical” ABA-dependent gene is not strongly dependent on C and S1 bZIPS.

In contrast, class 2 genes functioning in amino acid degradation (e.g., BCA72) are direct S1 targets in salt-stressed roots. Promoter scanning by ChiP-PCR reveals that HA-tagged bZIP1 directly and constitutively targets the BCA72 promoter in close vicinity to the transcriptional start site. Here, a number of G-box-related cis-elements are located, which are known to function as bZIP binding sites. Due to constitutive promoter occupancy of the 35S-driven bZIP1, a transcriptional and/or posttranslational salt-stimulated bZIP1 activation mechanism can be anticipated. Interestingly, class 2 transcription depends on group A and S1 bZIPS but not on group C signaling. Whether bZIP1 forms homo- or heterodimers with group A or a yet unknown heterodimerization partner needs to be studied. Nevertheless, salt-induced phosphorylation of group A might provide a possible activation mechanism. Alternatively, both bZIP signaling pathways may integrate their cues via independent G-box cis-elements.

Class 3 genes depend only on group S1 factors but not on group A or C bZIPS. More strikingly, bZIP1 binding is induced after salt treatment, indicating a regulatory mechanism, which is distinct from the constitutive binding of BCA72 (class 2). Although formation of bZIP1 homodimers has been described in vitro (Kang et al., 2010), it needs to be demonstrated whether homo- or heterodimers are formed in vivo. Interestingly, similar to class 2 genes, class 3 genes belong to the same functional context (Tyr degradation). This indicates that functionally related genes may share the same transcriptional regulation.

Finally, class 4 genes, such as PPDK or ASN1, are regulated by group S1 and C bZIPS, but not by AREB-like bZIPS. In addition to these well-defined genes in primary metabolism, stress-responsive genes, such as DIN2 and SEN1, belong to this class (Figure 5; Supplemental Figure 6). Focusing on PPDK, bZIP1 did not show substantial binding to this promoter at least at the time point analyzed. However, recent ChIP sequencing studies propose a transient promoter occupancy of bZIP1 that complicates the interpretation of ChIP data (Para et al., 2014). As bzip1 bzip53 show partially impaired PPDK transcription, the alternative explanation that bZIP3 is more important for regulating PPDK transcription should be taken into consideration. Previous results demonstrated that S1 bZIPS preferentially heterodimerize with group C bZIPS, thus potentiating target gene expression (Ehliert et al., 2006; Wettmeier et al., 2006). Along this line, the bzip1 bzip53 bzip10 bzip25 mutant is completely impaired in target gene expression, supporting the impact of C and S1 bZIPS on transcription of class 4 genes. Interestingly, although class 4 PPDK transcription is not dependent on AREB-like bZIPS, it is impaired in the SnRK2-triple mutant. Here, the limitations of mutant approaches become obvious, as indirect effects are difficult to evaluate. Observations such as phosphorylation of SnRK1 by SnRK2, which has been found in phosphoproteomic studies (Umezawa et al., 2013), or crosstalk via PP2C phosphatases (Rodrigues et al., 2013) might explain these findings.

Taken together, two structurally related SnRK-bZIP signaling modules orchestrate salt-responsive gene expression in roots (Figure 7). Whereas the SnRK2-AREB pathway responds to ABA and regulates general defense-related functions, the SnRK1-bZIP module is involved in metabolic reprogramming by integrating information on the plant’s energy and carbohydrate resources. Importantly, both pathways regulate specific sets of genes but display substantial crosstalk on the level of bZIP-type transcriptional regulators. Elaborated genetic, ChIP, and heterodimerization studies are required to address this additional layer of regulatory complexity. Importantly, unraveling the sophisticated network underlying the salt stress response will provide insights into how to precisely manipulate plants to engineer stress tolerant crops.

METHODS

Plants Lines and Culture

The two Arabidopsis thaliana Col-0 bzip1 T-DNA insertion lines bzip1-1 (Salk_069343) and bzip1-2 (SALK_088489) used in this study were characterized by Dietrich et al. (2011). These lines, which were used by Sun et al. (2012), exhibit highly related phenotypes after salt treatment. These findings indicate that the T-DNA insertion in the bzip1 gene causes the observed alterations. The following mutant lines were used in this study: bzip1 bzip53, bzip1 bzip53 bzip10 bzip25, Pro35S::HA::bZIP1 (Dietrich et al., 2011), aba2 (Cheng et al., 2002), snrk2.2 snrk3 snrk6 (Fujita et al., 2009), areb1 areb2 abf3 (Yoshida et al., 2010), and snrk1.1 (Baena-González et al., 2007). snrk1 lines were constructed by floral dip transformation (Weigel and Glazebrook, 2002) of the snrk1.1 mutant with an artificial microRNA targeting SnRK1.2 (http:// wmd3.weigelworld.org/cgi-bin/webapp.cgi), making use of the Gateway vector pMDC7 (Curtis and Grossniklaus, 2003).

Plants were cultured hydroponically (8/16 h day/night regime) according to Gibeaut et al. (1997). Roots of 6-week-old plants were treated with 150 mM NaCl or equimolar concentrations of salts (KCl, MgSO4, and Na2SO4) or mannitol, respectively (final osmolarity 0.25 mosM/L). To minimize the effect of circadian regulation, root material from salt-treated wild-type and mutant lines utilized for RT-qPCR and transcriptome studies was harvested simultaneously 1 h before the end of the light period. Alternatively, roots of 3-week-old aspecifically grown plants cultivated on MS medium (Murashige and Skoog, 1962) were treated with 450 mM NaCl (final concentration) for the time periods indicated. In this system, chemical compounds such as β-estradiol (10 μM), LaCl3 (300 μM), and W7 (100 μM) (Kaplan et al., 2006) can be applied easily.

Molecular Biology Methods

Immunoblot and RT-qPCR techniques (using SYBR Green) were performed as described by Dietrich et al. (2011). The following antibodies were used: AKIN10 (Agrisera Ab10919) and anti-HA tag (Abcam ab9110). Cy5-labeled secondary antibodies were used: AKIN10 (Agrisera Ab10919) and anti-HA tag (Abcam ab9110). Cycling conditions were as follows: 10 min at 95°C, 40 cycles of 20 s at 95°C, 10 s at 55°C, and 30 s at 72°C, linked to a default dissociation stage program to detect nonspecific amplification. The ubiquitin (UBI5) gene was used for sample normalization. PCR primers are given in Supplemental Table 1. If not stated differently, calculated values are derived from two to three biological and three to four technical replicates.

ChIP-PCR

Root material (~5 g) was harvested from 3-week-old plants grown on 1× MS without sugars after a 6-h NaCl (400 mM) or mock treatment. Subsequently, samples were incubated with cross-linking buffer (50 mM
KH₂PO₄/K₂HPO₄ buffer, pH 5.8, and 1% [v/v] formaldehyde) for 30 min under vacuum. Cross-linking was stopped by incubating the samples in glycin buffer (50 mM KH₂PO₄/K₂HPO₄ buffer, pH 5.8, and 0.3 M glycine) for 15 min under vacuum followed by further washing with ice-cold water. Samples were frozen in liquid nitrogen and subsequently grind. Nuclei extraction was performed in a cooling chamber at 4°C. Therefore, root material was resuspended in 24 mL ice-cold extraction buffer (1 M hexylenglycol, 50 mM PIPES-KOH, pH 7.2, 10 mM MgCl₂, 5 mM β-mercaptoethanol, and 1 tablet/10 mL complete protease inhibitor cocktail tablets [Roche]) and was cleared by filtration through two layers of Miracloth. One milliliter of 25% Triton X-100 was added dropwise to the extract. After incubation for 15 min, nuclei were isolated by density gradient centrifugation using a 35% Percoll cushion. The nuclei pellet was resuspended in sonication buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, pH 8.0, 0.25% SDS, and protease inhibitor) prior to sonification for 20 × 20 s. Chromatin was cleared by centrifugation for 15 min at 11,000g, 4°C, and frozen in aliquots. For each immunoprecipitation, 15 µg chromatin and 4 µg ChIP-grade α-HA (ab9110) antibody (Abcam) were used. Seventy microliters of protein A-coated magnetic beads [Invitrogen] dissolved in ice-cold extraction buffer, supplemented with protease inhibitor [Roche], was applied to each sample. Antibody-antigen binding was achieved during a 2-h incubation step at 4°C and slow rotation on an Intellimixer. To remove unspecifically bound proteins, beads were washed four times with washing buffer supplemented with protease inhibitor, before precipitated protein-DNA complexes were dissolved in elution buffer. Precipitated DNA was quantified by RT-qPCR using the oligonucleotide primers summarized in Supplemental Table 1. Data were normalized to DNA input, which was quantified by ACTIN8 transcript abundance. Results were obtained from two independent plant pools per line from which two to four independent ChIP experiments were performed.

**Transcriptome Studies**

Transcriptome analysis was performed on root material from hydroponically grown Col-0 wild-type and bzip1 bzip53 plants 1, 3, and 6 h after treatment with 150 mM NaCl. RNA purity and integrity were confirmed using an RNA 6000 Nano Assay (Agilent) and gel electrophoresis. cRNA labeling, hybridization, washing, and scanning of Affymetrix Arabidopsis ATH1 GeneChips (Affymetrix) were performed according to Affymetrix OneCycle Lab protocols. Data were analyzed statistically using the R language environment for statistical computing (http://www.r-project.org) version 2.9 and Bioconductor package. The obtained P values were corrected for multiple testing errors using the BH procedure (Benjamini and Hochberg, 1995) and transferred to Microsoft Excel. The probe set sequences were aligned to the TAIR9 gene model database of transcripts (www.arabidopsis.org). Data analyses (triplicate) were performed using MapMan (Thimm et al., 2004).

**Metabolic Studies**

The metabolic profile from treated and untreated root material was determined 1, 3, 6, 24, and 48 h after treatment using gas chromatography-mass spectrometry (Kempa et al., 2008). Amino acid levels were determined by UPLC-ESI-qTOF-MS (Acquity UPLC, Synapt HDMS G2; Waters) prior to derivatization with the AccQ Ultra kit (Waters) as described (Salazar et al., 2012). Sugars were analyzed using a Waters Acquity ultra-high-performance liquid chromatograph coupled to a Waters Micromass Quattro Premier triple quadrupole mass spectrometer with an electrospray interface. Chromatographic separation was performed according to application note WA60126 with a modified flow rate of 0.2 mL/min. Sugars were detected in the negative electrospray mode (ESI) at a source temperature of 120°C and a capillary voltage of 3.25 kV. Nitrogen was used as desolvation and cone gas with flow rates of 800 liters h⁻¹ at 350°C and 25 liters h⁻¹. The mass spectrometer was operated in the multiple-reaction monitoring mode using Argon as collision gas at a pressure of ~3 × 10⁻³ bar.

Bioinformatic and statistical analyses were performed with GraphPad Prism, Origin, and XCELL software using the statistic tests indicated in the figure legend.

**Accession Numbers**

Arabidopsis Genome Initiative identifiers for the genes mentioned in this article are as follows: bZIP53 (At3g62420), bZIP1 (At5g49450), bZIP63 (At3g28770), bZIP10 (At4g02640), bZIP25 (At3g54620), bZIP9 (At5g24800), ASN1 (At3g47340), BCA72 (At1g10070), LEA76 (At3g15670), RD29B (At5g23000), PPDK (At4g15530), Dn12 (At3g60140), SEN1 (At4g35770), Dn10 (At5g20250), TAT7 (At5g3970), HGO (At5g54800), MCCA (At1g03090), MGL (At1g64660), SUS4 (At3g43190), SWEET4 (At3g28007), SWEET2 (At3g14770), KIN10 (At3g01090), KIN11 (At3g29160), UBI5 (At3g82250), ACTIN7 (At5g09810), and ACTIN6 (At1g49240).

**Supplemental Data**

Supplemental Figure 1. Transcription of group C and S1 bZIPs after salt treatment.

Supplemental Figure 2. Carbohydrate metabolism in salt-treated wild-type and bzip1 bzip53 roots.

Supplemental Figure 3. Metabolites of the TCA cycle but not transcription of the related genes is altered in the bzip1 bzip53 mutant.

Supplemental Figure 4. Impact of C and S1 bZIP factors on amino acid metabolism.

Supplemental Figure 5. RT-qPCR validation of salt-induced expression of SnRK2 and AREB genes in the wild type and the respective snrk2.2 snrk3 snrk6 and areb1 areb2 areb3 multiple T-DNA insertion mutants.

Supplemental Figure 6. RT-qPCR analysis of bZIP1 regulated class 1 to 4 genes.

Supplemental Figure 7. Characterization of snrk1 mutant plants.

Supplemental Table 1. List of primers used in this study.

Supplemental Data Set 1. Summary of transcriptome results comparing salt-treated wild-type and bzip1 bzip53 roots.

Supplemental Data Set 2. Summary of amino acid concentrations comparing salt-treated wild-type and bzip1 bzip53 and bzip1 bzip53 bzip10 bzip25 roots.

Supplemental Data Set 3. Summary of carbohydrate concentrations comparing salt-treated wild-type and bzip1 bzip53 roots.

Supplemental Data Set 4. Relative amount of selected metabolites comparing salt-treated wild-type and bzip1 bzip53 roots.

**ACKNOWLEDGMENTS**

We thank J.K. Zhu, K. Shinozaki, E. Baena-González, and N.H Chua for providing mutants, Sarah Frosch for technical assistance, and Susanne Gilling for proofreading. The research was supported by DFG DR273/11-2 and FP7 Marie Curie ITN MERIT (GA 264474).

**AUTHOR CONTRIBUTIONS**

L.H. performed most of the research, with the exception of snrk1 generation (L.P.), ChIP-PCR (C.W.), amino acid analyses (J.S., A.F., and C.W.).
carbohydrate analyses (M.K. and M.J.M.), metabolic profiling (S.K.), transcriptomics and data analysis (J.H.), a part of the RT-qPCR studies (J.G.), and characterization of particular bZIP mutants (J.V.-C.). The work was supervised and designed by K.D. and W.D.-L. W.D.-L. wrote the article.

Received February 20, 2015; revised July 2, 2015; accepted July 22, 2015; published August 14, 2015.

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Crosstalk between Two bZIP Signaling Pathways Orchestrates Salt-Induced Metabolic Reprogramming in Arabidopsis Roots
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Plant Cell 2015;27:2244-2260; originally published online August 14, 2015; DOI 10.1105/tpc.15.00163

This information is current as of June 29, 2017

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