

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

Laura Hartmann,^a Lorenzo Pedrotti,^a Christoph Weiste,^a Agnes Fekete,^a Jasper Schierstaedt,^a Jasmin Göttler,^a Stefan Kempa,^b Markus Krischke,^a Katrin Dietrich,^{a,1} Martin J. Mueller,^a Jesus Vicente-Carbajosa,^c Johannes Hanson,^{d,e} and Wolfgang Dröge-Laser^{a,2}

^a Julius-von-Sachs-Institut, Pharmazeutische Biologie, Universität Würzburg, 97082 Würzburg, Germany

^b BSIO Berlin School of Integrative Oncology, Charité-Universitätsmedizin Berlin, D-13353 Berlin, Germany

^c Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Campus de Montegancedo, Universidad Politécnica de Madrid, 28223 Pozuelo de Alarcón, Madrid, Spain

^d Department of Molecular Plant Physiology, Utrecht University, 3584 CH Utrecht, The Netherlands

^e Umeå Plant Science Center, Department of Plant Physiology, Umeå University, SE-901 87 Umeå, Sweden

ORCID IDs: 0000-0003-3058-7570 (A.F.); 0000-0002-7910-9118 (J.S.); 0000-0001-8122-5460 (M.J.M.); 0000-0002-6332-1712 (J.V.-C.); 0000-0002-5605-7984 (J.H.)

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 efficient mechanisms to avoid or adapt to salt stress conditions (reviewed in Huang et al., 2012; Krasensky and Jonak, 2012; Deinlein et al., 2014; Gollack 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 (Verslues 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

¹ Current address: Metanomics GmbH, Tegeler Weg 33, D-10589 Berlin, Germany.

² Address correspondence to wolfgang.droege-laser@uni-wuerzburg.de. The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Wolfgang Dröge-Laser (wolfgang.droege-laser@uni-wuerzburg.de).
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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 (Para 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 (HXK1) functions as a major glucose sensor (Moore et al., 2003). Interestingly, bZIP1 transcription in seedlings is repressed by glucose and depends on HXK1 (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 of 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) (Msanne 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

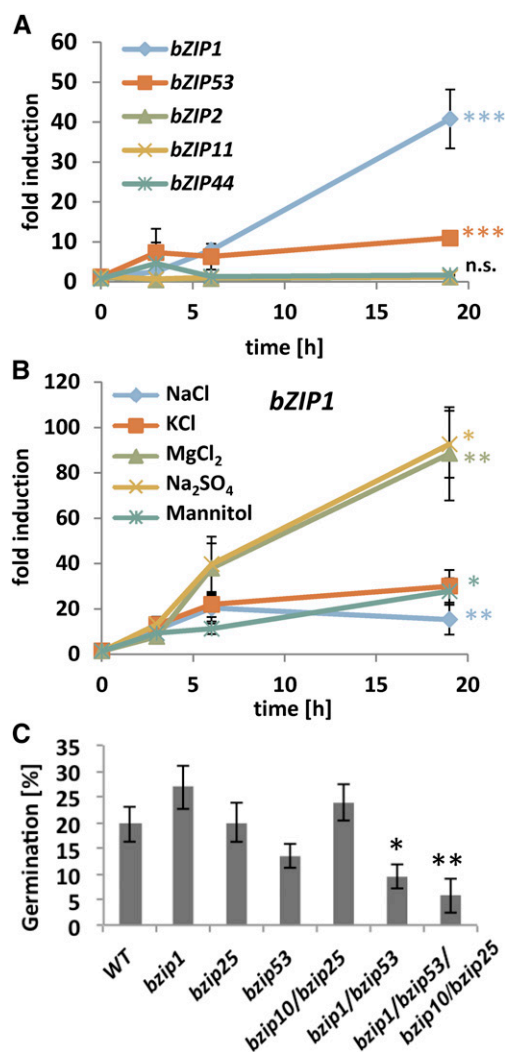


Figure 1. Stress-Induced Transcription of *bZIP1* and *bZIP53* and Their Functional Impact on Germination.

(A) Transcript abundance of the indicated group S1-bZIP genes was analyzed by RT-qPCR in roots of hydroponically grown Col-0 plants treated with 150 mM NaCl. Given are mean values (\pm se) of two to three biological and three technical replicates.

(B) Induction of *bZIP1* by equiosmotic concentrations of salt (NaCl, KCl, MgCl₂, and Na₂SO₄) or mannitol solutions. Osmolarity was adjusted to 0.25 mosM/L. Given are mean values (\pm se) of three biological and three technical replicates. The wild type and respective mutants 19 h after treatment (A) and untreated and treated wild type after 19 h (B) are compared by Student's *t* test.

(C) Germination rate of the wild type and the indicated bZIP single and multiple T-DNA insertion mutants grown on MS medium supplemented with 175 mM NaCl. Germination rates of stressed mutants are calculated in percentage of the respective untreated line. Given are mean values ($n = 170$ to 350; \pm sd); **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

minor transcriptional alterations (Obertello et al., 2010), we chose the *bzip1 bzip53* double mutant for further studies to disclose significant changes in salt-induced gene expression. It needs to be noted that the mutants did not show any visible growth phenotypes compared to the wild type.

In our experimental setup, we compared transcriptome 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* \leq 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 \geq 0.7-fold, *P* \leq 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 down-regulated 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), *EARL11* (Zhang and Schläppli, 2007), and *RD29B* (Msanne 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).

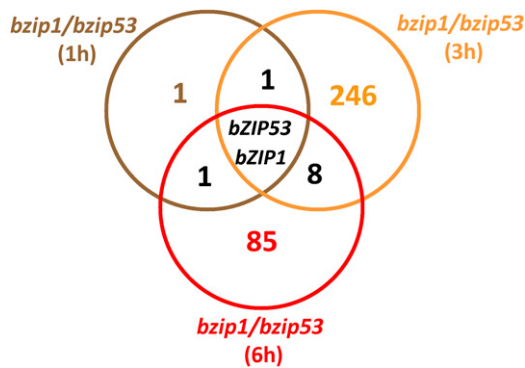


Figure 2. Venn Diagram Presenting the Number of Downregulated Genes Comparing Hydroponically Grown Wild-Type and *bzip1 bzip53* Mutant Plants 1, 3, and 6 h after Treatment with 150 mM NaCl ($\log_2 \geq 0.7$; $P < 0.01$).

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, bZIP1 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 under low oxygen stress (Baroja-Fernández et al., 2012; Pucciariello et al., 2012). Validation of these results by RT-qPCR studies revealed that *SUS4* expression is partially impaired in the *bzip1 bzip53* mutant (Figure 3C).

Carbohydrate measurements showed that 6 h after onset of salt stress, Glc, Fru, and Suc levels stayed constant or slowly decreased, whereas the concentrations of these sugars were more rapidly reduced in the mutant (Figure 3B; Supplemental Data Set 3). Interestingly, this correlated with a reduced induction of gluconeogenesis-associated genes that were partially impaired in the *bzip* double mutant. In particular, *PYRUVATE ORTHOPHOSPHATE DIKINASE (PPDK)* and *FRUCTOSE-1,6-BISPHOSPHATASE (FBP)* encode key enzymes in this pathway, which is important under metabolic situations where lipids are remobilized via the glyoxylate cycle to shuttle the C-backbones into carbohydrates (Hsu et al., 2011). In agreement with this finding, *MALATE ENZYME*, which is associated with the glyoxylate cycle, was also downregulated (Supplemental Data Set 1). Finally, *SWEET4* was found as one of the strongest misregulated genes, belonging to a gene family encoding sugar transporters (Chen et al., 2012; Xuan et al., 2013) (Table 1). Salt-induced expression profiles of selected genes (*PPDK* and *FBP*) were confirmed by RT-qPCR (Figures 3D and 3E). As previous work demonstrated that heterodimerization with group C bZIPs significantly enhances target gene activation (Weltmeier et al., 2006), we also tested the quadruple mutant (*bzip1 bzip53 bzip10 bzip25*), including potential group C heterodimerization partners. Indeed, salt-induced transcription of *PPDK* was reduced in *bzip1 bzip53* and completely abolished in the quadruple mutant, supporting the importance of group C heterodimers, at least for this target gene.

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 bZIPs 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). 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 bZIPs 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 [BCAT2]*, *METHYLCROTONYL-COA-CARBOXYLASE [MCCA]*, *HOMOGENTISATE 1,2-DIOXYGENASE [HGO]*, and *METHIONINE- γ -LYASE [MGL]*) (Mentzen 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 decrease, presumably due to reduced synthesis and/or degradation. However, the effect of bZIP1 and bZIP53 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 mechanism besides gene regulation may apply.

Table 1. List of Selected Genes Downregulated in Salt-Treated *bzip1 bzip53* Roots Compared to the Wild Type

Time	ID	Gene Name/Function ^a	FC (log ₂) ^b	P Value
3 h		Fermentation/hypoxia		
	AT2G34390	<i>NIP2;1 (NOD26-LIKE INTRINSIC PROTEIN2;1)</i>	−2.64	5E-04
	AT3G43190	<i>SUS4 (SUCROSE SYNTHASE4)</i>	−1.73	4E-05
	AT1G43800	<i>FTM1 (FLORAL TRANSITION AT THE MERISTEM1)</i>	−1.64	5E-05
	AT5G39890	<i>HUP43</i>	−1.59	4E-04
	AT5G10040	Unknown protein	−1.55	6E-03
	AT5G15120	Unknown protein	−1.28	2E-04
	AT3G02550	<i>LBD41 (LOB DOMAIN-CONTAINING PROTEIN41)</i>	−1.28	2E-03
	AT2G16060	<i>AHB1 (CLASS I HEMOGLOBIN)</i>	−1.18	2E-05
	AT4G33070	<i>PDC1 (PYRUVATE DECARBOXYLASE1)</i>	−1.12	3E-03
	AT5G50590	<i>HSD4 (HYDROXYSTEROID DEHYDROGENASE4)</i>	−0.99	5E-07
	AT1G77120	<i>ADH1 (ALCOHOL DEHYDROGENASE1)</i>	−0.92	2E-03
6 h		Amino acid metabolism		
		BCAA		
	AT5G34780	<i>BCDH (Branched-Chain α-Keto Acid Dehydrogenase E1 α-subunit, putative)</i>	−1.52	1.5E-05
	AT1G10070	<i>BCAT-2 (ARABIDOPSIS THALIANA BRANCHED-CHAIN AMINO ACID TRANSAMINASE2)</i>	−1.39	2.4E-06
	AT1G03090	<i>MCCA (METHYLCROTONYL-COA CARBOXYLASE)</i>	−1.28	4.6E-07
	AT3G06850	<i>BCE2 (DIHYDROLIPOAMIDE BRANCHED-CHAIN ACYLTRANSFERASE), DIN3 (DARK INDUCIBLE3)</i>	−1.13	2.1E-05
	AT3G13450	<i>Branched-chain α-keto acid dehydrogenase E1 β, DIN4 (DARK INDUCIBLE4)</i>	−0.84	1.3E-05
	AT4G34030	<i>MCCB (3-METHYLCROTONYL-COA CARBOXYLASE)</i>	−0.81	1.2E-07
	AT3G45300	<i>IVD (ISOVALERYL-COA-DEHYDROGENASE)</i>	−0.79	1.9E-04
	AT1G55510	<i>BCDH BETA1 (BRANCHED-CHAIN α-KETO ACID DECARBOXYLASE E1 β-SUBUNIT)</i>	−0.70	4.0E-05
		Tyr		
	AT5G54080	<i>HGO (HOMOGENISATE 1,2-DIOXYGENASE)</i>	−1.26	1.4E-08
	AT5G53970	<i>TAT7 (TYROSINE AMINOTRANSFERASE7)</i>	−1.03	1.5E-05
	AT4G23590	<i>TYROSINE AMINOTRANSFERASE</i>	−0.74	1.2E-03
	AT4G28410	<i>RSA1 (ROOT SYSTEM ARCHITECTURE1)</i>	−0.70	1.8E-02
		Gln/Asn		
	AT3G47340	<i>ASN1 (GLUTAMINE-DEPENDENT ASPARAGINE SYNTHETASE1)</i>	−1.19	1.4E-03
	AT5G07440	<i>GDH2 (GLUTAMATE DEHYDROGENASE2)</i>	−0.73	1.1E-05
	AT3G16150	<i>ASPARAGINASE B1</i>	−0.70	7.7E-04
		Met		
	AT1G64660	<i>MGL (METHIONINE GAMMA-LYASE)</i>	−1.21	4.3E-04
		Lys		
	AT4G33150	<i>LKR (LYSINE-KETOGLUTARATE REDUCTASE), SDR (SACCHAROPINE DEHYDROGENASE), bifunc.</i>	−0.84	2.8E-06
		Pro		
	AT3G30775	<i>PROD1 (PROLINE DEHYDOGENASE1), ERD5 (EARLY RESPONSIVE TO DEHYDRATION5)</i>	−0.81	1.3E-05
		Thr		
	AT1G08630	<i>THA1 (THREONINE ALDOLASE1)</i>	−0.78	4.6E-04
		Ala		
	AT2G13360	<i>AGT1 (ALANINE:GLYOXYLATE AMINOTRANSFERASE1)</i>	−0.75	1.6E-03
	AT4G39660	<i>AGT2 (ALANINE:GLYOXYLATE AMINOTRANSFERASE2)</i>	−0.72	2.3E-05
6 h		Carbohydrate metabolism		
	AT3G28007	<i>SWEET4</i> , putative sugar transporter, MtN3 family	−1.90	6E-07
	AT4G15530	<i>PPDK (PYRUVATE ORTHOPHOSPHATE DIKINASE)</i>	−1.29	8E-04
	AT1G43670	<i>FBP (FRUCTOSE-1,6-BISPHOSPHATASE)</i>	−0.95	7E-04
	AT5G23660	<i>SWEET2</i> , putative sugar transporter, MtN3 family	−0.93	5E-04
	AT1G58180	<i>BCA6 (BETA CARBONIC ANHYDRASE 6)</i>	−0.91	2E-06
	AT5G57655	<i>XYLOSE ISOMERASE</i> family protein	−0.90	2E-06
	AT4G33110	S-adenosyl-L-methionine-dependent <i>METHYLTRANSFERASES</i> superfamily protein	−0.90	5E-07
	AT1G12240	<i>ATBETAFRUCT4 (FRUCTOSIDASE4)</i>	−0.78	9E-03
	AT5G18670	<i>BMV3, BAM9 (BETA-AMYLASE9)</i>	−0.77	8E-05
	AT5G20250	<i>RS6 (RAFFINOSE SYNTHASE6)</i>	−0.76	4E-04
	AT1G63180	<i>UGE3 (UDP-D-GLUCOSE/UDP- D-GALACTOSE 4-EPIMERASE3)</i>	−0.75	4E-04

(Continued)

Table 1. (continued).

Time	ID	Gene Name/Function ^a	FC (log ₂) ^b	P Value
6 h		Stress-related genes		
	AT3G60140	<i>DIN2</i> (DARK INDUCIBLE 2); β -GLUCOSIDASE30, function unknown	−1.80	6E-06
	AT4G35770	<i>SEN1</i> (SENESCENCE ASSOCIATED1), unknown function	−1.60	2E-04
	AT4G12480	<i>EARLI 1</i> (EARLY ARABIDOPSIS ALUMINUM INDUCED1)	−1.43	7E-06
	AT5G52300	<i>RD29B</i> (RESPONSIVE TO DESSICATION 29B)	−1.16	7E-04
	AT3G17520	LEA (LATE EMBRYOGENESIS ABUNDANT protein)	−1.15	6E-04
	AT3G59930	DEFL (DEFENSIN-LIKE family protein)	−1.04	1E-03
	AT4G37220	Cold acclimation protein <i>WCOR413</i> family	−1.04	4E-05
	AT1G52690	<i>LEA7</i> (LATE EMBRYOGENESIS ABUNDANT7)	−0.85	8E-02
	AT2G35300	<i>LEA18</i> (LATE EMBRYOGENESIS ABUNDANT18)	−0.77	4E-02
	AT5G13170	<i>SAG29</i> (SENESCENCE-ASSOCIATED PROTEIN 29)	−0.75	4E-04

^aGenes selected from the transcriptome data, ordered by the given functional aspects.

^bFold change (log₂) of differentially expressed genes.

ASN1 is the only upregulated gene involved in amino acid biosynthesis, which is in line with the hypothesis that Asn functions in C/N transport under stress (Lam et al., 1994). However, in the *bzip1 bzip53* mutant, *ASN1* transcription was only slightly reduced. Importantly, the quadruple mutant has a much stronger effect, indicating differences in gene regulation when compared with that of genes involved in amino acid degradation. Overall, based on these results, bZIP1 and bZIP53 are major regulators of stress-induced metabolic reprogramming of amino acid degradation, presumably to support metabolism with an alternative energy resource (Ishizaki et al., 2006; Araújo et al., 2010).

The ABA-SnRK2-AREB Pathway Is Dispensable for Salt-Induced bZIP1 Expression

The phytohormone ABA is an important signaling molecule in abiotic stress responses. Thus, we studied *bZIP1* transcription in salt-treated roots of mutants that are affected in stress-induced ABA biosynthesis (*aba2*) (Cheng et al., 2002) or ABA signaling (*snrk2.2 snrk3 snrk6* and *areb1 areb2 abf3*) (Supplemental Figure 5). The latter have been demonstrated to block the ABA response on the level of SnRK2 kinases (Fujita et al., 2009) or group A bZIP TFs (Yoshida et al., 2010), respectively. Although at early time points a minor impact on *bZIP1* expression cannot be excluded, later, at around 19 h after salt treatment, no significant difference between the wild type and mutants could be observed. Hence, the ABA-SnRK2-AREB pathway has no major impact on salt-induced *bZIP1* activation (Figure 5A). Nevertheless, as demonstrated in Figure 5B, *bZIP53* transcription partially depends on the SnRK2/AREB pathway, demonstrating a unique regulatory mechanism.

Mutant Analyses Position bZIP1 in Both ABA-Dependent and -Independent Abiotic Stress Signaling Networks

Although the ABA-SnRK2-AREB pathway is dispensable for salt-induced *bZIP1* transcription, the transcriptome studies 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 *EARLI*) 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 *MCCA* 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 *TYROSINE 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

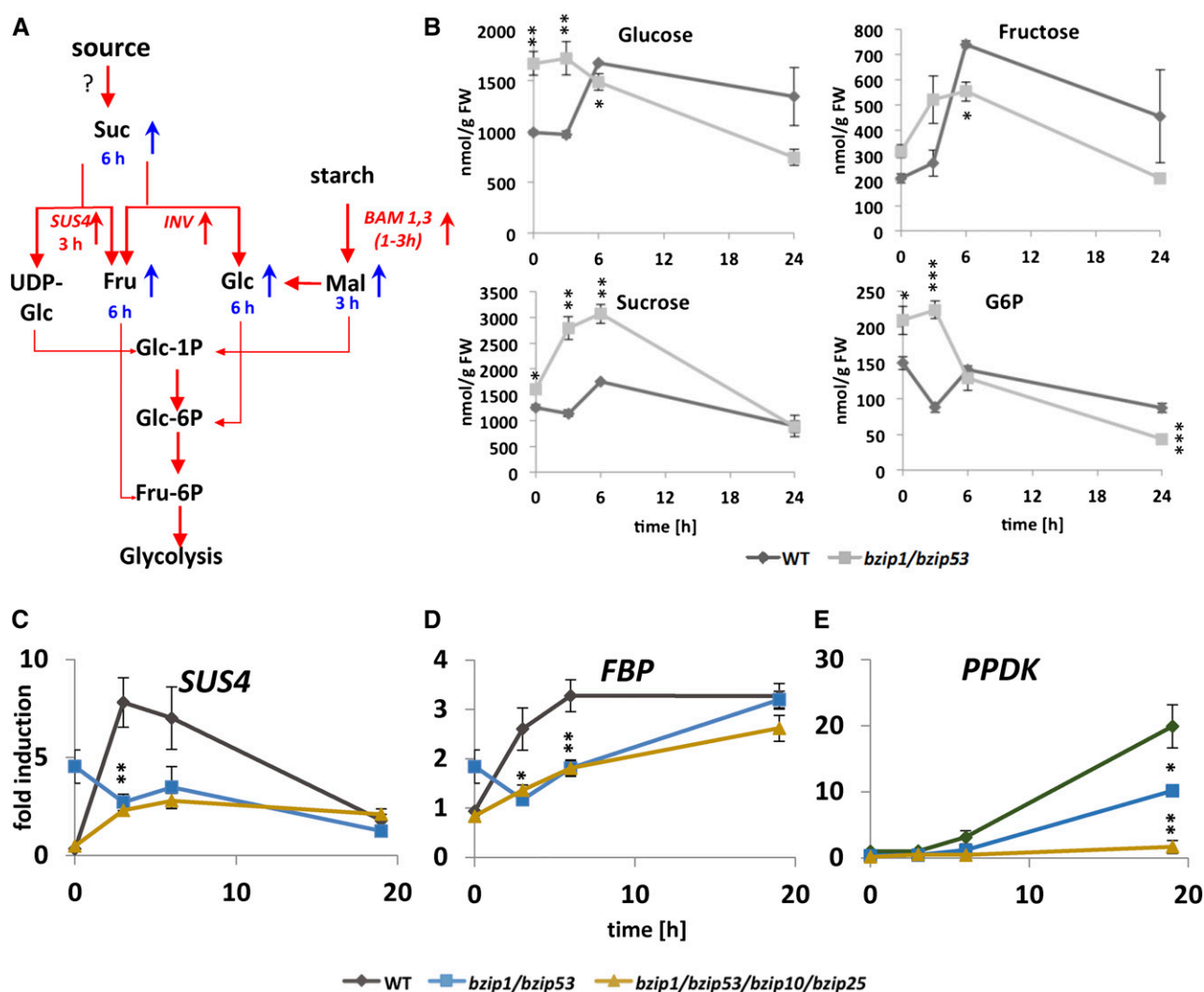


Figure 3. Changes in Carbohydrate Metabolism Comparing NaCl-Treated Hydroponically Grown Roots of Wild-Type and *bzip1 bzip53* Mutants.

(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 (\pm 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 (\pm 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.

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 PPK 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 phenotypic 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.

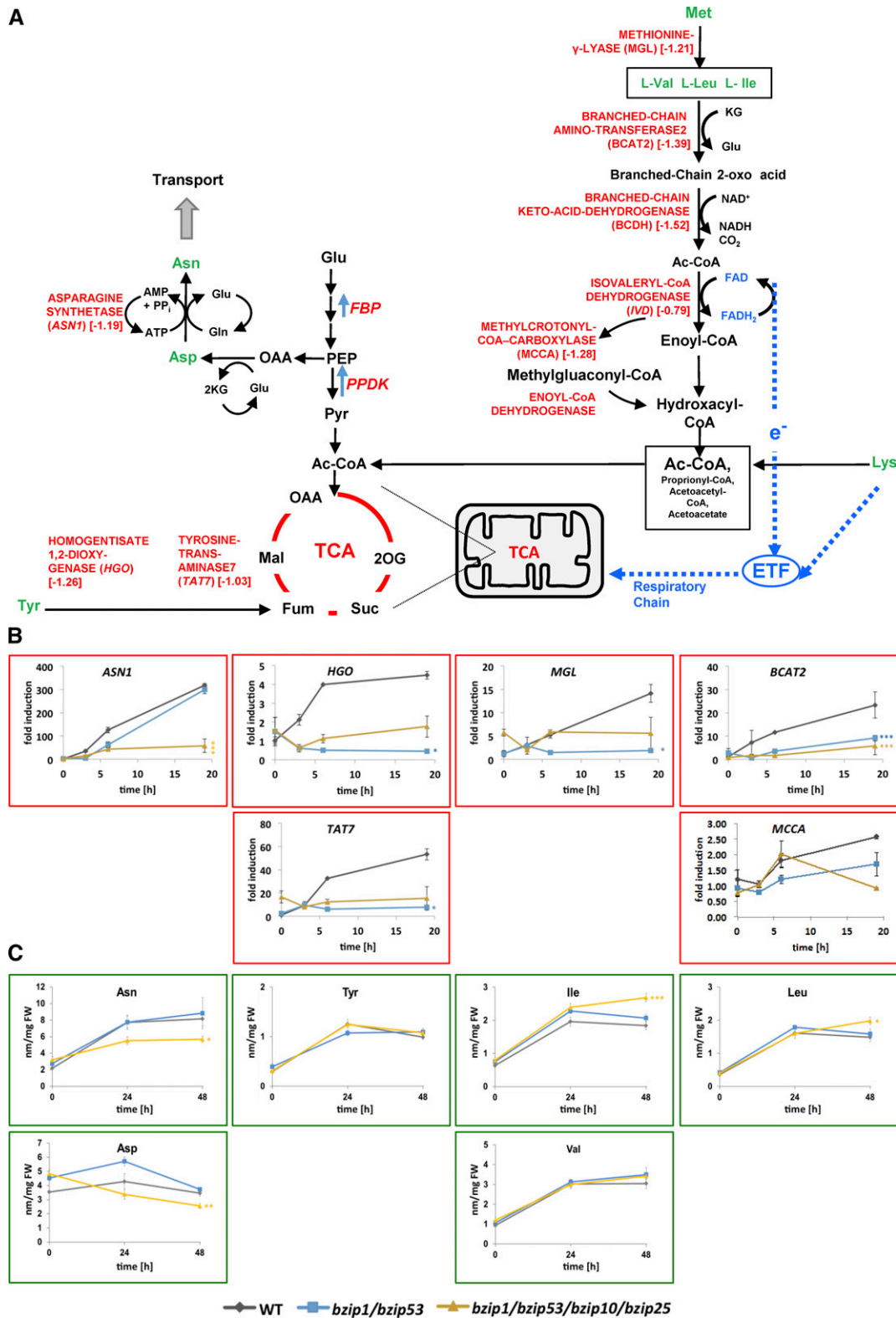


Figure 4. Impact of C and S1 bZIP Factors on Amino Acid Metabolism of Salt-Treated Roots.

(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 *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-aminoethyl)-5-chloro-1-naphthalenesulfonamid-hydrochloride (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 metabolic adaptation. Here, we demonstrate that the group S1 *bZIP* TFs *bZIP1* and *bZIP53* play an important role in the root-specific response to salt. As summarized in the model in Figure 7, this study provides a mechanistic view of how *bZIP1* signaling is regulated by stress and metabolic cues, reprograms C- and N-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 knockdown 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 4. (continued).

(B) RT-qPCR analysis of the salt-induced gene expression comparing wild-type (black), *bzip1 bzip53* (blue), and *bzip1 bzip53 bzip10 bzip25* (brown) plants. Given are mean fold (\pm SE) induction values of two to three biological and three technical replicates. Student's *t* test compares the wild type and mutant at the respective time point: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

(C) Quantification of selected amino acid concentrations observed in the mutants indicated in **(B)**. Given are mean values (\pm SD) of *n* = 6 to 10 biological replicates. Student's *t* test: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001

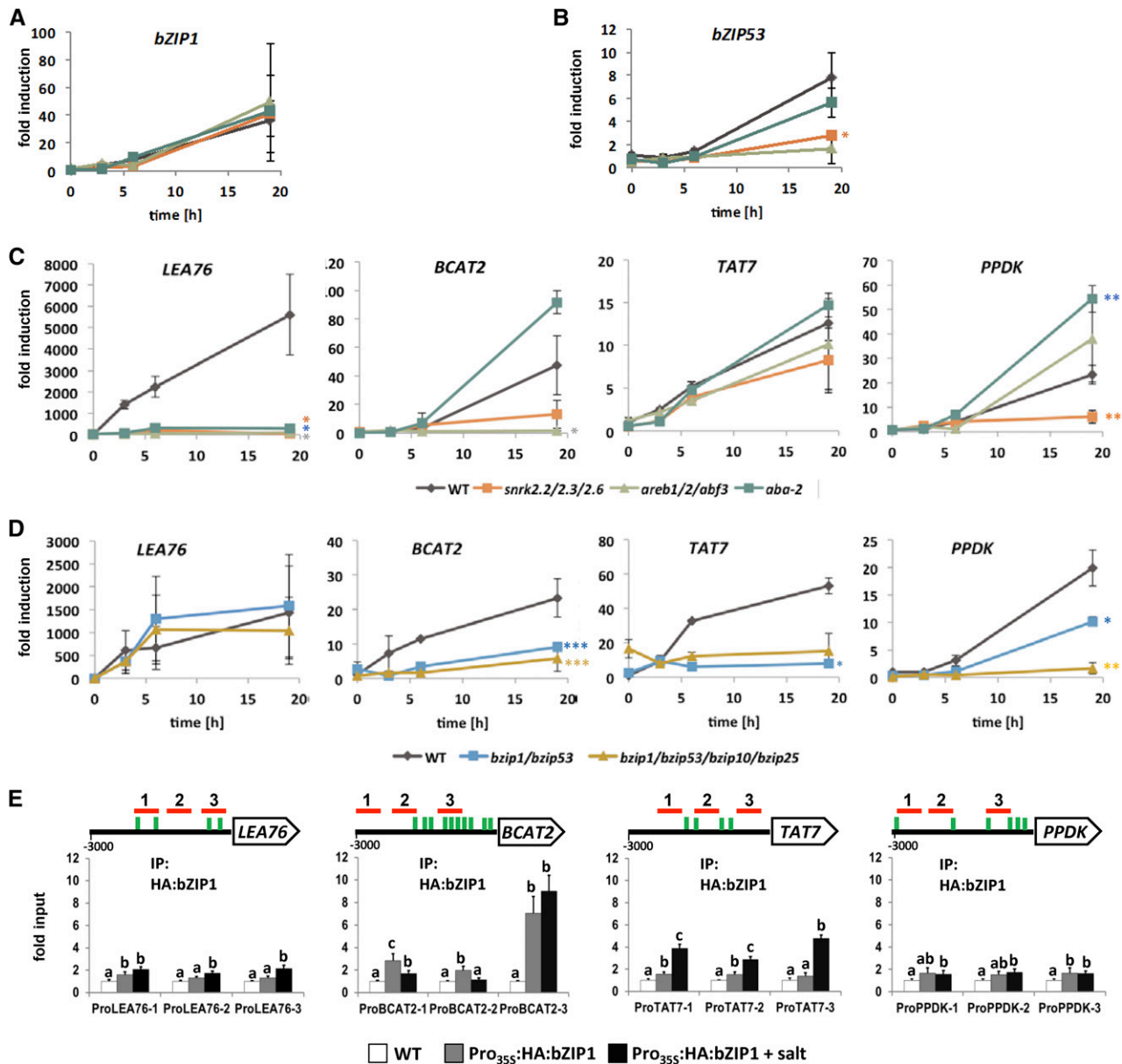


Figure 5. Mutant and ChIP-PCR Analysis to Study Regulation of *bZIP1*, *bZIP53*, and Potential Target Genes.

(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 (\pm SE) 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 *Pro_{35S}: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.

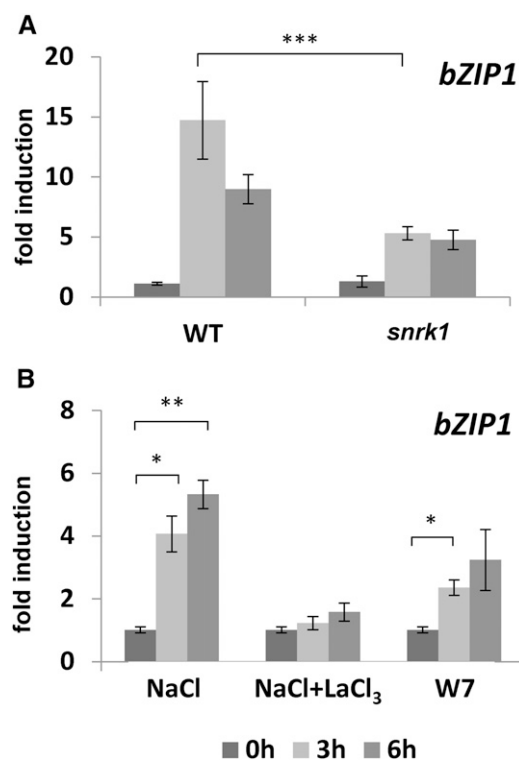


Figure 6. Transcription of *bZIP1* in Roots Depends on Various Signaling Pathways.

(A) RT-qPCR analysis of wild-type and inducible *snrk1* loss-of-function plants. Given are mean fold (\pm sd) induction values of four to six biological replicates

(B) Ca^{2+} signaling was manipulated by the inhibitor La^{3+} or the Ca^{2+} agonist W7, respectively. Three-week-old plants were grown on MS medium and treated with NaCl for 0, 3, or 6 h. Duration of treatments is indicated by a color code. Given are mean fold (\pm se) induction values of three to four biological replicates. All experiments are repeated at least three times. Student's *t* test: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

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

3 to 6 h after the onset of stress. In the same time span, Suc levels highly increase in the *bzip1 bzip53* mutant, presumably because Suc is not degraded to hexoses, as occurs in the wild type. In leaves of salt-treated plants, the shift in sugar levels is fed by starch breakdown (Kempa et al., 2008). Accordingly, the transcriptome data set shows β -AMYLASE gene activation in roots. Moreover, sink-driven transport processes to the root may occur (Ludewig and Flügge, 2013). *SWEET4* and *SWEET2* are examples of genes misregulated in *bzip1 bzip53* that encode putative sugar transporters (Chen et al., 2012; Xuan et al., 2013). In a second phase, the transient increase in Glc, Fru, and Suc returns to initial levels around 24 h after onset of stress. Taken together, the bZIPs under investigation significantly alter carbohydrate metabolism, both under stressed and unstressed conditions. As a complex regulatory network modulates carbohydrate metabolism, the mechanisms underlying how the TFs interfere are difficult to access.

Transcriptome data 6 h after salt stress support a metabolic shift to gluconeogenesis. Key genes, such as *PPDK* and *FBP*, are transcriptionally downregulated in the *bzip1 bzip53* mutant. Again, both genes are typically expressed under anaerobic conditions (Hsu et al., 2011; Pucciariello et al., 2012). As carbohydrate-driven fermentation provides only limited ATP production under nonoxidative conditions, noncarbohydrate substrates, such as lipids or proteins, are used for energy metabolism (Mentzen et al., 2008; Araújo et al., 2011). Here, we demonstrate that breakdown of specific amino acids is regulated by bZIPs under investigation in order to provide C-skeletons to the gluconeogenesis pathway. Moreover, recently, FBP has been proposed to function as a Fru sensor (Cho and Yoo, 2011). Hence, via altered sugar sensing, reduced FBP expression in the bZIP mutant might lead to a perturbed 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). Moreover, Tyr degradation is also regulated by the bZIPs under study and feeds into the TCA cycle via fumarate (Dixon and Edwards, 2006). The TCA cycle often operates in a modular fashion in plants, meaning that parts of the cycle are involved in distinct metabolic pathways. 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

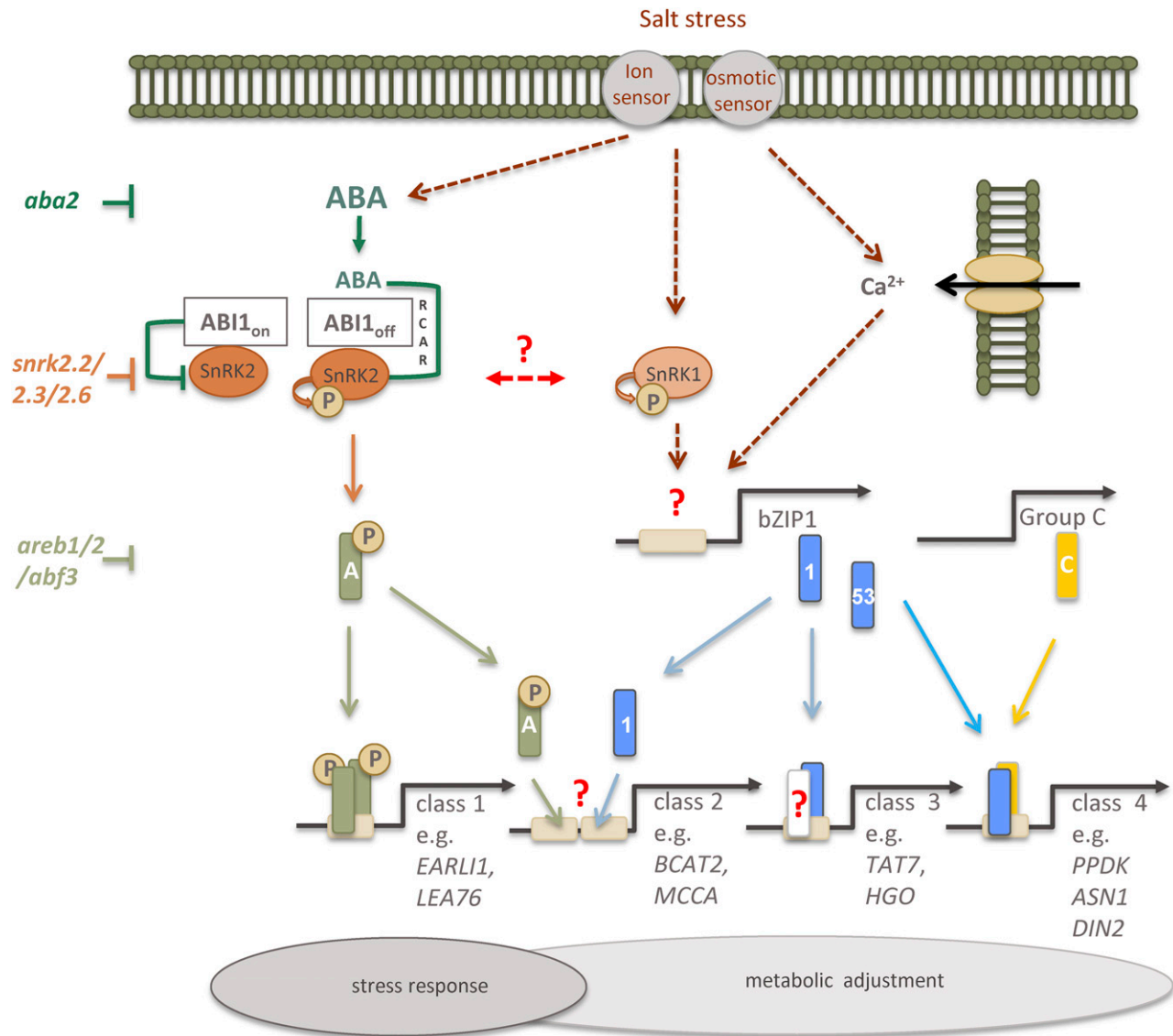


Figure 7. Model Summarizing the Findings on the Regulation of *bZIP1* Transcription in Salt Stress Response in Arabidopsis Roots and the Crosstalk of the ABA-Independent SnRK1-*bZIP1* Pathway and ABA-Dependent SnRK2-AREB Pathway.

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).

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 PPK activity

(Lin and Wu, 2004). Consistently, PPK 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.

ABA-Independent SnRK1/S1-bZIP- and ABA-Dependent SnRK2/AREB Signaling Pathways Regulate Specific and Overlapping Sets of Target Genes

Group A bZIPs have been demonstrated to regulate a substantial set of genes responsive to salt stress (Yoshida et al., 2010).

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., *BCAT2*) are direct S1 targets in salt-stressed roots. Promoter scanning by ChIP-PCR reveals that HA-tagged bZIP1 directly and constitutively targets the *BCAT2* 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 *BCAT2* (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 bZIP53 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 (Ehlert et al., 2006; Weltmeier 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_059343) and *bzip1-2* (SALK_069489) 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*, Pro_{35S}: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 aseptically grown plants cultured 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 μ M), LaCl₃ (300 μ M), and W7 (100 μ M) (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). 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 \times MS without sugars after a 6-h NaCl (400 mM) or mock treatment. Subsequently, samples were incubated with cross-linking buffer (50 mM

$\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 5.8, and 1% [v/v] formaldehyde) for 30 min under vacuum. Cross-linking was stopped by incubating the samples in glycine buffer (50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ 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 grinded. 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 MgCl_2 , 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 release 2.4 (Gentleman et al., 2004). Data were normalized using the robust multichip average expression measure in the Affy package (Gautier et al., 2004). Differentially expressed genes were identified using the LIMMA 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 $\sim 3 \times 10^{-3}$ 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* (At5g28770), *bZIP10* (At4g02640), *bZIP25* (At3g54620), *bZIP9* (At5g24800), *ASN1* (At3g47340), *BCAT2* (At1g10070), *LEA76* (At3g15670), *RD29B* (At5g52300), *PPDK* (At4g15530), *DIN2* (At3g60140), *SEN1* (At4g35770), *DIN10* (At5g20250), *TAT7* (At5g53970), *HGO* (At5g54080), *MCCA* (At1g03090), *MGL* (At1g64660), *SUS4* (At3g43190), *SWEET4* (At3g28007), *SWEET2* (At3g14770), *KIN10* (At3g01090), *KIN11* (At3g29160), *UBI5* (At3g62250), *ACTIN7* (At5g09810), and *ACTIN8* (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 abf3* 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.

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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.

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