AtWRKY15 perturbation abolishes the mitochondrial stress response that steers osmotic stress tolerance in Arabidopsis

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Environmental stresses adversely affect plant growth and development. A common theme within these adverse conditions is the perturbation of reactive oxygen species (ROS) homeostasis. Here, we demonstrate that the ROS-inducible Arabidopsis thaliana WRKY15 transcription factor (AtWRKY15) modulates plant growth and salt/osmotic stress responses. By transcriptome profiling, a divergent stress response was identified in transgenic WRKY15-overexpressing plants that linked a stimulated endoplasmic reticulum-to-nucleus communication to a disrupted mitochondrial stress response under salt-stress conditions. We show that mitochondrial calcium-flux sensing might be important for regulating an active mitochondrial retrograde signaling and launching an appropriate defense response to confer salt-stress tolerance.

Results and Discussion

WRKY15 Is Induced by Oxidative and Salt Stresses. The group IId WRKY15 transcription factor is an early H2O2-responsive gene (At2g23320) (9, 26, 27). Gene-expression analysis using Genevestigator revealed that, besides H2O2 treatments, low CO2 availability, and pathogen infections, WRKY15 expression was also induced by salt stress (28). Salt- and oxidative-stress responsiveness of the WRKY15 transcript was confirmed by quantitative RT-PCR analysis (Fig. S1 A and B). The WRKY15 protein is predominantly located in the nucleus (29). To determine spatial and developmental expression patterns of WRKY15 in Arabidopsis, the WRKY15 promoter:β-glucuronidase (GUS) reporter gene fusion constructs were examined. In 3-d-old seedlings, GUS staining was primarily strong in the hypocotyl-to-root transition zone and in the root tip (Fig. S1C). In 9-d-old and mature seedlings, WRKY15 was expressed in the shoot apical meristem, trichomes, and trichome socket cells of young leaves (Fig. S1 D–G). In mature seedlings, GUS staining was also observed in the outer epidermal cell layer of leaf petioles (Fig. S1E). In roots, the WRKY15 promoter activity was detected mainly in the vascular cylinder, at lateral root initials, and mitochondrial signals to regulate the expression of ALTERNATIVE OXIDASE1a (AOX1a) (24). Because ABI4 is also a regulator of plastid retrograde signaling to repress photosynthetic gene expression (13, 16) and of ABSCISIC ACID (ABA) signaling (25), it might act as a molecular interface between retrograde and anterograde regulatory signals.

Here, we show that the hydrogen peroxide (H2O2)-responsive transcription factor WRKY15 functions as a negative regulator of salt- and osmotic-stress tolerance in Arabidopsis thaliana. Molecular phenotyping of WRKY15-overexpressing plants under salt stress revealed a pivotal role for MRR in mediating salt-stress tolerance. Furthermore, our results indicate that mitochondrial calcium-flux sensing is important for the activation of the mitochondrial stress response.
in the root tip (Fig. S1 H–M). Thus, the WRKY15 gene is expressed mainly in young, growing, and vascular tissues.

**WRKY15 Overexpression Promotes Leaf Growth and Plant-Biomass Production Through a Stimulated Cell-Expansion Rate.** We generated transgenic plants that constitutively and ectopically expressed WRKY15 under control of the cauliflower mosaic virus 35S promoter in both a Columbia-4 wild-type (Col4WT) and a catalase-deficient background (CAT2HP1) in which WRKY15 was originally identified as an H$_2$O$_2$-responsive gene (9, 26, 30). Three independent WRKY15 overexpression (designated WRKY15$^{OE}$) lines with high transgene expression were selected for further analysis (Fig. 1A). Although similar in germination rates and early development as control plants, fully grown WRKY15$^{OE}$ plants exhibited an increased leaf area (Fig. 1B and C), resulting in a 15–25% increased plant biomass (Fig. 1D).

Because the final leaf size is determined by cell-division and cell-expansion rates, we assessed the cell numbers and cell size of abaxial epidermis cells in 21-d-old plants. In WRKY15$^{OE}$ plants, cell numbers did not change, but the average cell size increased (Fig. 1E), indicative of increased cell expansion. A common mechanism by which plants control cell size is the repeated replication of their DNA, resulting in cellular polyploidy, a process termed endoreduplication (31, 32). DNA ploidy-level analysis revealed that the number of cells with an 8C and 16C content was significantly higher in WRKY15$^{OE}$ plants than that of control plants (Fig. 1F). Moreover, from day 11 on, WRKY15$^{OE}$ plants displayed an increased endoreduplication index, which correlates with the mean nuclear

![Fig. 1.](https://www.pnas.org/content/109/49/20114/F1.large.jpg)
DNA content per cell (33), indicating that endoreduplication was stimulated (Fig. 1G). Taken together, in WRKY15OE and WT plants, the transition from the mitotic cycle to the endocycle is equivalent, as evidenced by a similar cell number, but in WRKY15OE plants, endoreduplication is intensified, correlating with the increased cell size.

Because no true loss-of-function T-DNA insertion mutants are available, transgenic plants containing artificial microRNA (amiR) constructs targeting WRKY15 were generated (34). These WRKY15-amiR plants showed a strong reduction in WRKY15 levels (below 20% of WT levels), were smaller than WT plants, and displayed a decreased average leaf cell area (Fig. S2 A and B). High WRKY15 transcript abundance in young and growing tissues (Fig. S1), together with the altered cell expansion upon WRKY15 perturbation (see Fig. 1 E–G for WRKY15OE plants and Fig. S2 A and B for WRKY15-amiR plants), support its involvement in plant growth and possibly endoreduplication, either directly or indirectly as a result of enhanced growth processes of which the relative contribution is not known.

**Elevated WRKY15 Expression Increases Sensitivity to Osmotic and Oxidative Stresses.** Transgenic Arabidopsis plants with perturbed WRKY15 expression were assessed for altered phenotypes when exposed to abiotic stress conditions. For oxidative stress, we used a bioassay in which photorespiration is induced by restricting gas exchange within Petri plates. Chlorophyll fluorescence was measured and the maximum quantum efficiency of photosystem (PS)II ($F_{v}/F_{m}$) was determined, which is an effective measure of plant stress (35). In CAT2HP1-WRKY15 plants, the decrease in $F_{v}/F_{m}$ was stronger than in control CAT2HP1 plants, hinting at an increased sensitivity to oxidative stress (Fig. 2A). In the Col4WT background, the catalase activity inhibitor 3-amino triazole (3-AT) was used to impose H$_2$O$_2$ stress and mimic catalase deficiency. Again, WRKY15OE plants were more susceptible to oxidative stress (Fig. 2B). WRKY15-amiR plants did not perform differently from WT plants when subjected to oxidative stress (Fig. S2C).

To examine salt-stress responses, control, WRKY15OE, and WRKY15-amiR plants were germinated and grown on medium containing increased salt concentrations. On 100 mM NaCl, control plants could still grow and remained green, whereas the growth of WRKY15OE plants was inhibited and chlorosis was initiated (Fig. S2 and S1), together with the altered cell expansion (see Fig. 1 E–G for WRKY15OE plants and Fig. S2 A and B for WRKY15-amiR plants), support its involvement in plant growth and possibly endoreduplication, either directly or indirectly as a result of enhanced growth processes of which the relative contribution is not known.

To assess whether overexpression of WRKY15 also altered the responsiveness to osmotic stresses, plants were germinated and grown in the presence of 25 mM mannitol, 100 mM D-sorbitol, or 75 mM NaCl. On salt or sorbitol, again the rosette area of WRKY15OE plants was significantly reduced, whereas mannitol stress only affected growth in the strongest overexpression line (WRKY15-9H) (Fig. 2E).

To determine the effect of salt-stress sensitivity at the cellular level, we examined the first leaf pairs of 2-wk-old WRKY15OE plants grown under control and salt-stress conditions (50 mM NaCl) by transmission-electron microscopy. This mild salt stress already initiated cellular degeneration in WRKY15OE leaves, as evidenced by the presence of deteriorated cells and large intercellular spaces (Fig. S3 A–D). This cellular phenotype was not observed under nonstressed conditions (Fig. S3 E–H).

**Transcript Profiling Reveals Induced Unfolded Protein Response and Impaired Mitochondrial Stress Response in WRKY15OE Plants.** RNA of three independent replicates of WT and WRKY15OE seedlings grown in the absence and presence of 50 mM NaCl was hybridized to Affymetrix GeneChip Arabidopsis 1.0R arrays. A two-factor ANOVA revealed 598 up-regulated and 750 down-regulated transcripts in WRKY15OE plants, independently from salt-stress treatment (Table S1). Among the up-regulated transcripts, genes involved in the endoplasmic reticulum (ER) stress response were significantly enriched (36–38) (Fig. S4A and Tables S1 and S2). This response, also known as the unfolded protein response (UPR), is an evolutionarily conserved transcriptional response that is triggered by the accumulation of unfolded or misfolded proteins in the ER lumen and is essential to maintain ER homeostasis (37). The UPR triggers (i) enhancement of protein-
folding by induction of ER-resident molecular chaperones, foldases, and high-capacity Ca\(^{2+}\)-binding proteins; (ii) increase in protein degradation capacity to remove improperly folded proteins; and (iii) in mammals, also attenuation of translation to limit the entry of nascent polypeptides when conditions are unsuitable for proper folding (36, 39, 40). Besides the induction of core UPR genes (37), WRKY15 overexpression also significantly repressed transcript levels of proteins involved in protein synthesis (Fig. S4A and Tables S1 and S2), indicative of a complete ER stress response in WRKY15\(^{OE}\) plants.

Growth on more severe salt-stress conditions intensified the induction of core UPR genes in WRKY15\(^{OE}\) plants (Fig. S3 C and D), which correlated with their increased salt-stress-sensitivity phenotype (Fig. 2D). Integration of salt-adaptation responses and ER stress signaling was observed before in mutants defective in protein N-glycosylation (41, 42). Dissimilar to WRKY15\(^{OE}\) plants, salt-/osmotic-stress sensitivity in these mutants was associated with root-tip swelling and enhanced lateral-root development that salt-/osmotic-stress sensitivity in these mutants was associated with root-tip swelling and enhanced lateral-root development. However, the role of N-glycosylation in determining the response to other stresses such as salt stress is less clear.

In WRKY15\(^{OE}\) mutants, the rosette area was 20% larger than that in WT plants (Fig. S4C), indicative for a potential link between Ca\(^{2+}\) signaling, mitochondria rapidly take up Ca\(^{2+}\) from the ER Ca\(^{2+}\)\(\text{SERCA}\) inhibitor, and, thereby, increases the cytosolic Ca\(^{2+}\) concentration (44). In mammalian cells, a tight communication exists between the ER and mitochondria with Ca\(^{2+}\) as the potential mediator signal (45, 46). By analogy, a significant enrichment for Ca\(^{2+}\)-induced genes was found among the WRKY15-regulated genes (47) (Fig. S5B and Tables S1 and S3). To assess the involvement of Ca\(^{2+}\)-mediated interorganellar signaling in WRKY15\(^{OE}\) plants, we evaluated the effect of cyclopiazonic acid (CPA) (which is a specific SERCA inhibitor and, thereby, increases the cytosolic Ca\(^{2+}\) concentration) (48) on MDR gene expression. During salt stress, CPA clearly intensified the MDR gene expression in WT plants (see Fig. 3A for \(AOX1a\) and Fig. S3C for three additional MDR genes), indicating that increased cytosolic Ca\(^{2+}\) concentrations promoted MDR. Furthermore, CPA alleviated significantly the salt-stress-induced growth reduction (Fig. 3B), indicative for a potential link between Ca\(^{2+}\)-mediated MDR activation and salt-stress tolerance. In contrast, in WRKY15\(^{OE}\) plants, CPA only mildly enhanced MDR gene expression and growth performance under salt stress, suggesting that the potential increase in cytosolic Ca\(^{2+}\) provoked by CPA addition was not sufficient to overcome the WRKY15-dependent inhibitory effect on MDR gene expression (Fig. 3A and B and Fig. S5C). To assess whether mitochondrial Ca\(^{2+}\)-flux sensing was deregulated in WRKY15\(^{OE}\) plants, we determined the effect of ruthenium red (RR) on the salt-stress-induced MDR gene expression. RR abolishes the uptake of cytosolic Ca\(^{2+}\) by mitochondria through inhibition of the Ca\(^{2+}\)-unipporter channel located in the inner mitochondrial membrane (49). During salt stress, RR reduced MDR gene expression in WT plants to levels similar to those in salt-treated WRKY15\(^{OE}\) plants (see Fig. 3C for \(AOX1a\) and Fig. S3D for three additional MDR genes) and mimicked the stress-sensitivity phenotype of WRKY15\(^{OE}\) plants (Fig. 3D). These results indicate that mitochondrial Ca\(^{2+}\)-flux sensing might be necessary to launch a salt-stress response, involving MDR gene expression. Whether this cascade of events is solely necessary for a proficient defense response enabling plants to withstand moderate salt-stress conditions remains to be elucidated.

Because the ER acts as a dynamic Ca\(^{2+}\) reservoir and UPR proteins are important in regulating the activity of the SERCA pump (44, 50), constitutive UPR activation in WRKY15\(^{OE}\) plants might possibly disturb the cellular Ca\(^{2+}\) homeostasis. During intracellular Ca\(^{2+}\) signaling, mitochondria rapidly take up Ca\(^{2+}\) from the cytosol via the Ca\(^{2+}\)-selective unipporter channel located at the inner membrane (49). Mitochondrial Ca\(^{2+}\) uptake has a biphasic...
dependence on cytosolic Ca\textsuperscript{2+} because it is facilitated by Ca\textsuperscript{2+}/calmodulin (CaM) (Ca\textsuperscript{2+}-activated CaM) and inactivated by sustained cytosolic Ca\textsuperscript{2+} levels (51). Therefore, uncontrolled endoplasmic Ca\textsuperscript{2+} release in WRKY15\textsuperscript{OE} plants might facilitate an unceasing mitochondrial Ca\textsuperscript{2+} uptake, potentially causing mitochondrial desensitization. Hence, during salt stress, signal-induced Ca\textsuperscript{2+} fluxes might not be sensed and no MDR gene expression is activated in WRKY15\textsuperscript{OE} plants, possibly causing salt-stress sensitivity (Fig. 4).

**Is WRKY15 a Transcriptional Regulator of MRR?**  The failure to activate MDR gene expression during salt stress in WRKY15\textsuperscript{OE} plants suggests that WRKY15 might function as a repressor of MRR. Substantial evidence indicates that many genes are repressed by WRKY transcription factors bound to their promoters (52), and particular insights into repressor functions of WRKY proteins were obtained with PeWRKY1, OsWRKY71, and HvWRKY1 and HvWRKY2 (53, 54). In addition, chromatin immunoprecipitation studies revealed that W-box sequences in the promoters of patho-
gen-defense genes are constitutively occupied by WRKY tran-
scription factors, even in the absence of pathogen infection or elicitor treatment (55). Upon a specific stimulus, allosteric inter-
actions might cause the release of WRKY factors from their cog-
nate W-box elements and its possible replacement by other WRKY
proteins. Therefore, WRKY proteins are thought to act in a net-
work of mutually competing participants with temporal displace-
ment (55). Interestingly, active repression of basal expression
has already been reported for AOX1a, which is widely used as
a model to study MRR (14, 19, 56). In a deletion study of the
AOX1a promoter, a strong repressor element had been identified
that relieves the repression of AOX1a upon stress application (57).
Furthermore, three W-box motifs were found within the AOX1a
promoter region, of which one is located within the 93-bp MRR
region that is important for full AOX1a induction upon treatment
with antimycin A (AA) or monofluorooacetate (MFA) that chemi-
cally perturb mitochondrial function (18). A mutant lacking this W-
box motif (mut1) had a strongly reduced response to AA and MFA,
suggesting a potential role for WRKY proteins in the regulation of
AOX1a gene expression (18). Besides in AOX1a, W-box motifs
were significantly overrepresented in the promoters of WRKY15-
repressed genes (Tables S1 and S3). Therefore, it is possible that, in
the absence of stress, MDR gene expression is repressed by
WRKY15, which is either (i) inactive and constitutively occupies the W boxes or (ii) actively represses the basal gene expression.
Upon salt stress, the WRKY15 activity might be modified, thereby
derpressing or activating MDR gene expression.

A possible mechanism by which WRKY15 might regulate gene expression is through an interaction with CaM. Ca\textsuperscript{2+}/CaM medi-
ted transcriptional regulation has been reported for several
transcription factors, modulating both their DNA-binding ability and
transcriptional activity (58). WRKY15 contains a conserved Cu\textsuperscript{2+}-
dependent CaM-binding domain (CaMBD) and has CaM-binding
ability (59). To evaluate the involvement of Ca\textsuperscript{2+}/CaM-
mediated regulation, we generated transgenic plants that overex-
pressed a mutant form of WRKY15 with amino-acid substitutions
at two of the six conserved hydrophobic CaMBD residues (WRKY15-F79RL86R\textsuperscript{OE} ). Similar amino-acid substitutions in the
CaMBD of WRKY7, also member of the WRKYIIId subfamily,
completely abolished CaM binding (59). WRKY15-F79RL86R\textsuperscript{OE} plants with similar transgene expression levels as WRKY15\textsuperscript{OE}
plants did not respond differently from WRKY15\textsuperscript{OE} plants (Fig. S5 E–G). This observation might indicate that the transcriptional control is either (i) independent from Ca\textsuperscript{2+}/CaM-mediated signals and regulated by a different mechanism [such as Cu\textsuperscript{2+}-dependent (de)phosphorylation], or (ii) dependent on the Ca\textsuperscript{2+}/CaM threshold because the effect of WRKY15 overexpression might be much more profound than any potential effect of WRKY15 on MDR gene expression during normal salt-stress signaling, or (iii) mediated by an upstream (CaM-dependent) regulator.

**Materials and Methods**

**Plant Material and Growth Conditions.** Transgenic WRKY15 plants of A. thaliana (L.) Heynh. were obtained as described in **SI Materials and Methods**. Plants were grown in vitro on Murashige and Skoog (MS)-containing agar medium at 21 °C and 65–80 μmol·m\textsuperscript{-2}·s\textsuperscript{-1} in a 16-h light/8-h dark regime.

**Stress Treatments.** For the photorespiration-promoting conditions, plants were grown on MS agar medium for 2.5 wk. Then, the plates were transferred to a continuous light regime after replacing the surgical tape (Micropore; 3M) to restrict gas exchange. Treatments were done in the presence or absence of the catalase inhibitor 3-AT (3 μM). The maximum efficiency of the PSI photochemistry (F\textsubscript{v}/F\textsubscript{m}) was determined using a PAM-2000 chlorophyll fluorometer and Imag-
ingWin software application (Walz). For salt stress, plants were germinated and grown on MS agar medium containing 50, 75, or 100 mM NaCl. For mannitol and sorbitol stress, the MS agar medium was supplemented with 25 mM mannitol or 100 mM a-sorbitol, respectively.

**Site-Directed Mutagenesis of the WRKY15 CaM-Binding Domain.** The two hydrophobic amino acids, F\textsubscript{79} and L\textsubscript{86}, important in CaM binding of WRKY15 (59) were substituted with Arg (denoted as F\textsubscript{79}R and L\textsubscript{86}R) with the Gene-
Tailor Site-Directed Mutagenesis System (Invitrogen). For primer sequences, see Table S4. Generation of transgenic plants overexpressing the WRKY15-
F\textsubscript{79}R-L\textsubscript{86}R ORF was as described for overexpression plants.

**Microarray Analysis.** Triplicate batches of shoot material of 20 Col4WT and
WRKY15-9.6 plants (growth stage 1.05) (60) germinated and grown on half-
strength MS agar medium supplemented with 0 or 50 mM NaCl were har-
vested for total RNA isolation. Details on RNA preparation, microarray hy-
bridization, data processing, and statistical analysis are provided in **SI Materials and Methods**. The microarray data are available at the GEO da-
Quantitative RT-PCR Analysis. RNA isolation, cDNA synthesis, and quantitative RT-PCR analyses were performed as described (61) using SYBR Green (Invitrogen) and gene-specific primers (Table S4). Actin-related protein 7 (deregulated in only 7 of the 1,685 conditions in Genevestigator) (28) was used for the normalization of relative transcript levels.

Microscopy and Flow-Cytometric Analyses. Size and number of abaxial pavement cells in leaves and the nuclear DNA content distribution were determined as described (26).


Supporting Information

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SI Materials and Methods

Stress-Responsiveness Experiments. For the oxidative stress-induction experiment, control and catalase-deficient CAT2HPI plants were grown in soil and subjected to high-light (HL) irradiation as described (1). MIDDLE-aged leaves were harvested after 0, 20, and 40 min and 2, 3, and 8 h of HL exposure. For the salt-stress induction experiment, Col0WT plants were grown in LifeRaft (Osmotek) in vitro cultivation systems and treated with 150 mM NaCl as described (2). Root material was harvested after 0, 3, 6, and 12 h of salt treatment.

Generation of Transgenic Arabidopsis Plants. For overexpression plants, the full-length ORF of WRKY15 was cloned by homology cloning into the binary vector pDONR221 (Invitrogen) and the pCaMV35SS overexpression vector pK7WG2D (3). The construct was transformed by floral dip (4) into both Arabidopsis WT (Col4WT) and catalase-deficient (CAT2HPI) plants (5). Homozygous lines with a single T-DNA locus were selected via segregation analysis, RNA gel-blot analysis, and quantitative RT-PCR.

For loss-of-function plants, homozygous plants from the GABI-Kat T-DNA insertion line GABI(97)A12 (6) were selected by genomic PCR. Transgene expression was monitored via quantitative RT-PCR.

To generate amiR plants, WRKY15-specific sequences were identified with the WMD Web MicroRNA Designer (www.weigeworld.org). The miRNA precursors were constructed as described (7), cloned into pK7WG2D, and transformed by floral dip into Col0WT Arabidopsis plants. Homozygous amiR plants at all two independent events were identified as described for the overexpression lines. The PCR primer sequences used for the construction of transgenic plants are presented in Table S4.

Microarray Analysis. In three independent experiments, RNA was isolated from shoot material of 20 WT (Col4WT) and WRKY15OE (WRKY15-9.6) plants (developmental stage 1.05) (8) with TRIzol Reagent (Invitrogen). Concentration and quality of the RNA were determined as described (9). Each of the different pools of WT and transgenic plants, germinated and grown in absence or presence of 50 mM NaCl, were hybridized to 12 GeneChip Arabidopsis Tiling 1.0R arrays (Affymetrix; www.affymetrix.com). For hybridization, 7 μg of total RNA was directly reverse-transcribed to double-stranded cDNA in a CDNA reverse-transcription reaction (without amplification) according to the manufacturer’s protocol [Whole Transcript (WT) Double-Stranded Target Assay; Affymetrix]. Subsequently, the sample was fragmented and labeled with biotin according to the manufacturer’s protocol (WT Double-Stranded DNA Terminal Labeling Kit; Affymetrix). Hybridization and scanning (GeneChip scanner 3000; Affymetrix) were done according to the manufacturer’s instructions at the Nucleomics Core Facility (Leuven, Belgium; www.nucleomics.be). Raw data were processed all together with the RMA algorithm (10) using the Tiling 1.0R array chip description file for quantitative mRNA expression analysis (11) and subsequently subjected to a two-factor ANOVA with the MultiExperiment Viewer of TM4 (12). The P values of the F statistics were corrected for multiple testing to assess the false-discovery rate with the publicly available software QVALUE (http://genomine.org/qvalue) (13), with λ ranging from 0.0 to 0.95 by 0.05. Genes with P values of < 0.001 and Q values of ≤ 0.005, 0.01, and 0.04 for, respectively, treatment, genetypie, and interaction significant effects were retained for further analysis.

Promoter-GUS Analysis. The upstream WRKY15 promoter region was amplified by PCR from the Col0WT genomic DNA with primers (Table S4) and cloned into pKGWFS7 (3), generating an in-frame GFP-GUS fusion. The construct was transformed into the Col0WT plants. Homozygous plants were assayed for Gus staining (14). Samples were photographed with a stereomicroscope (Stemi SV11; Zeiss) or with a Nomarski differential interference contrast microscope (BX51; Olympus).

Transmission-Electron Microscopy. The first leaf pairs of 14-d-old seedlings grown on MS agar medium in the absence or presence of 50 mM NaCl were used for transmission-electron microscopic analysis as described (15).

Protein Analysis. ConcanaVALin A staining was done as described (16).

Fig. S1. Spatiotemporal expression patterns of WRKY15. (A) Relative WRKY15-transcript abundance in middle-aged leaves of high-light (HL)-treated catalase-deficient (CAT2HP1) plants. (B) WRKY15-transcript abundance in root tissue of salt-treated Col0WT plants. Error bars show SEM (n = 3). (C–M) Spatial and developmental expression patterns of WRKY15. Promoter activity was visualized by histochemical GUS staining. (C) Three-d-old seedling. (D) Nine-d-old seedling. (E) Seedling at developmental stage 1.06. (F) Epidermis of a young leaf with trichomes of the seedling shown in E. (G) Detail of trichome on the primary leaf of the seedling shown in I. (H) Roots (arrowheads indicate lateral root initials). (I) Root tip. (J) Primary root stem. (K) Lateral root formation of the seedling shown in E. (L and M) Transverse section through the primary root stem (L) and the root apical meristem (M). (Scale bars: D and E, 2 mm; C and H, 0.5 mm; F and G, 100 μm; I–K, 50 μm; L and M, 10 μm.)
**Fig. S2.** Plant growth and stress performance of WRKY15-amiR plants. (A) WRKY15-transcript accumulation in shoot and root tissues of 3-wk-old WT (Col0WT) and WRKY15-amiR plants. (B) Cell size and number in the first leaves of 3-wk-old seedlings. Leaf-blade area is shown at the top of the frame. Error bars show SEM (n = 8–10). (C) Maximum quantum efficiency of PSII (Fv/Fm) in leaves of Col0WT and WRKY15-amiR plants after exposure to photorespiration-promoting conditions in the presence of the catalase inhibitor 3-AT (3 μM). Error bars show SEM (n = 18). (D) Three-wk-old WT and WRKY15-amiR plants germinated and grown on 0 and 100 mM NaCl. (E) Rosette area of 3-wk-old Col0WT and WRKY15-amiR plants grown under control and salt-stress conditions (100 mM NaCl). Error bars show SEM (n = 15–45). *P < 0.05; **P < 0.001; ***P < 0.0001 (Student t test).
Fig. S3. Transmission-electron microscopic analysis of WRKY15^{OE} plants. (A–D) Transmission-electron micrographs of the first leaves of WT (A and B) and WRKY15^{OE} (C and D) plants grown under mild salt stress (50 mM NaCl). Arrows and arrowheads indicate large intercellular spaces and deteriorated cells, respectively. (E–H) Transmission-electron micrographs of the first leaves of WT (E and F) and WRKY15^{OE} (G and H) plants grown under controlled conditions.
Fig. S4. Activated UPR, altered protein glycosylation, and ER stress sensitivity in WRKY15\textsuperscript{OE} plants. (A) Enrichment of ER stress response within constitutively WRKY15\textsuperscript{OE}-expressed genes. Bars indicate the absolute number of transcripts constitutively induced (gray) or repressed (dark gray) in WRKY15\textsuperscript{OE} plants overlapping with ER-stress–responsive transcripts identified in three different studies. The total number of differentially expressed genes upon the different treatments is indicated in parentheses. Numbers within bars give fold enrichment of the ER-stress–responsive genes within the constitutively WRKY15\textsuperscript{OE}-expressed genes compared with the Arabidopsis genome. Core UPR, core UPR genes; TM\textsuperscript{↑} and TM\textsuperscript{↓}, genes induced and repressed by tunicamycin, respectively; DTT\textsuperscript{↑} and DTT\textsuperscript{↓}, genes induced and repressed by dithiothreitol, respectively. *P ≤ 0.001; **P ≤ 1.0 \times 10^{-5}; ***P ≤ 1.0 \times 10^{-20} (Fisher’s exact test). (B) Accumulation of UPR marker genes (PDIL1, PDIL2, and unknown protein) in WRKY15\textsuperscript{OE} plants grown under control conditions. Expression data were obtained by quantitative real-time PCR on a biological repeat experiment and validate reproducibility of the microarray results. (C) Transcript accumulation of UPR marker genes in WT and WRKY15\textsuperscript{OE} plants grown without (control) or with 100 mM NaCl (salt). (D) Transcript accumulation of UPR marker genes in azygous control (WRKY15-9A) and transgenic WRKY15\textsuperscript{OE} (WRKY15-9H) plants grown without (control) or with 75 mM NaCl (salt) in an independent experiment, consolidating that UPR genes are superinduced in WRKY15\textsuperscript{OE} plants upon salt stress. (E) Affinodetection of glycoproteins containing high-mannose-type N-glucans with concanavalin A. Protein sizes are indicated on the left. Arrowheads mark differences in glycosylated protein composition. (F) Rosette area of 3-wk-old azygous (A) and transgenic WRKY15\textsuperscript{OE} (H) plants grown on 0.05 μg/mL tunicamycin. Error bars show SEM (n = 3 plates containing 60 plants). **P < 0.0001 (Student t test).
Fig. S5. Integration of MDR expression and calcium-mediated signaling. (A) Failure of WRKY15OE plants to induce a mitochondrial stress response upon salt treatment. Accumulation of MDR marker genes (AOX1a, steroid sulfotransferase, Multidrug and Toxic compound Extrusion (MATE) efflux family protein, and ANAC013) in Col4WT, CAT2HP1, and WRKY15OE plants grown under mild salt stress (50 mM NaCl). Expression data were obtained by quantitative real-time PCR on a biological repeat experiment and validate reproducibility of the microarray results. (B) Transcript abundance of MDR marker genes in azygous control (WRKY15-9A) and transgenic WRKY15OE (WRKY15-9H) plants grown without (control) or with 5 μM CPA, 50 mM NaCl (salt), or both (salt+CPA). (C) Transcript abundance of MDR marker genes in control (black bars) and WRKY15OE (white bars) plants grown without (control) or with 10 μM RR, 50 mM NaCl (salt), or both (salt+RR). (D) Enrichment of Ca^{2+}-induced genes among WRKY15OE differentially expressed genes. Bars indicate the absolute number of transcripts in the overlap, and numbers within bars give fold enrichment of the Ca^{2+}-induced genes within the WRKY15OE differentially expressed genes compared with the Arabidopsis genome. W15↑ and W15↓, genes constitutively induced and repressed in WRKY15OE plants, respectively; Salt-W15↓, salt-impaired genes in WRKY15OE plants. *P = 0.0015; **P = 2.08 × e^{-8}; ***P = 1.36 × e^{-12} (Fisher’s exact test). (E) Rosette area of 2-wk-old Col4WT, WRKY15OE, and WRKY15-F79RL86R OE plants grown under control and salt-stress conditions. Error bars show SEM (n = 25–60 plants). **P < 0.001; ***P < 0.0001 (Student t test). (F) Accumulation of the MDR marker gene (MATE efflux family protein) in Col4WT, WRKY15OE, and WRKY15-F79RL86R OE plants grown under control and salt-stress conditions. Error bars show SEM (n = 25–60 plants). **P < 0.001; ***P < 0.0001 (Student t test). (G) Accumulation of the UPR marker genes in Col4WT, WRKY15OE, and WRKY15-F79RL86R OE plants grown in the absence of stress.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)
Table S4 (DOC)