Arabidopsis TBP-ASSOCIATED FACTOR 12 ortholog NOBIRO6 controls root elongation with unfolded protein response cofactor activity

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Plant root growth is indeterminate but continuously responds to environmental changes. We previously reported on the severe root growth defect of a double mutant in bZIP17 and bZIP28 (bz1728) modulating the unfolded protein response (UPR). To elucidate the mechanism by which bz1728 seedlings develop a short root, we obtained a series of bz1728 suppressor mutants, named nobirol, for rescued root growth. We focused here on nobirol6, which is defective in the general transcription factor component TBP-ASSOCIATED FACTOR 12b (TAF12b). The expression of hundreds of genes, including the bZIP60-UPR regulon, was induced in the bz1728 mutant, but these inductions were markedly attenuated in the bz1728nobirol6 mutant. In view of this, we assigned transcriptional cofactor activity via physical interaction with bZIP60 to NOBIRO6/TAF12b. The single nobirol6/taf12b mutant also showed an altered sensitivity to endoplasmic reticulum stress for both UPR and root growth responses, demonstrating that NOBIRO6/TAF12b contributes to environment-responsive root growth control through UPR.

The endoplasmic reticulum (ER) is a eukaryotic organelle where translated peptides undergo protein folding and further modifications before they are secreted as functional proteins. These processes are frequently disturbed by a broad range of intra- and extracellular stimuli that result in the accumulation of malformed proteins inside the ER and cause cellular stress called ER stress. The ER is equipped with a multilayered machinery to cope with such ER stress (1–3). A series of ER-associated protein quality control (ERQC) processes assesses and reprocesses affected proteins to maintain ER homeostasis. In addition, the unfolded protein response (UPR), a dedicated gene-regulatory network, enforces ERQC by inducing the expression of the genes encoding ER-resident chaperones and enzymes in response to ER stress.

UPR is modulated by multiple ER-anchored transcription factors (TFs) and multiple activation pathways. In the model vascular plant Arabidopsis (Arabidopsis thaliana), the three basic leucine zipper (bZIP) TFs—bZIP17, bZIP28, and bZIP-P60—are reported to modulate the UPR across the eukaryotes through two different activation pathways (1, 4). bZIP17 and bZIP28 are activated by posttranslational activation (5–8); in contrast, bZIP60 is activated by alternative splicing (9, 10). Whereas studies in mammals have revealed a strong association between the UPR and Alzheimer’s disease and inflammatory diseases (2), plant UPR serves as a versatile stress sensing mechanism to respond to a wide range of both biotic and abiotic stimuli (1).

The UPR also influences plant growth and development, particularly root growth. We previously reported that an Arabidopsis double mutant lacking both bZIP17 and bZIP28 function (bz1728) displays a severe reduction in vertical root elongation (11). The fact that the loss-of-function of the activator of two bZIPs SITE-2 PROTEASE (S2P) conveyed the similar root growth defects (11, 12), whereas the bz17 and bzzip28 single mutants showed normal root growth (5, 6, 11), implies that the UPR controls primary root growth through these two functionally redundant bZIPs. Single bzip60 mutants or double mutants of bzip60 and other UPR bZIP genes did not show any growth defects (11). However, the two bZIP60 activator INOSITOL REQUIRING ENZYME 1A (IRE1A) and IRE1B are reported to modulate primary root growth redundantly with bZIP17 (13), suggesting that plants employ multiple UPR pathways to various unfolded protein response | stress-responsive growth regulation | general transcription factor | root growth

Significance

Living organisms continuously rebalance their growth and defense/tolerance machineries upon environmental perturbation and energy limitation, which appear as trade-offs. The unfolded protein response (UPR) is a supposed underlying machinery for these trade-offs, responding to a broad spectrum of stress categories and modulating the fundamental growth in both animal and plant systems. We here report the incorporation of general transcription factor NOBIRO6/TAF12b into the UPR-mediated plant root growth control. This indicates that the gene regulation by UPR itself is a key to elucidate the growth trade-offs. Given previously reported roles of NOBIRO6/TAF12b in the signaling of two phytohormones, cytokinin and ethylene, our report proposes how multichannel signals interactively shape plants to survive and thrive in the wild.

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degrees for equivalent root growth control. Clearly, the genetic machinery downstream of the UPR that connects ER stress to root growth is only partially understood.

Here, we describe *Arabidopsis* TATA-BINDING PROTEIN (TBP)-ASSOCIATED FACTOR 12b (TAF12b) as a transcription co-factor mediating UPR-associated root growth control. Eukaryotic transcription initiation starts with the assembly of the transcription preinitiation complex (PIC) onto promoters. The PIC comprises multiple general TF subcomplexes and RNA polymerase II (Pol II). One of these general TFs, TFIIID, is a core subcomplex for transcription initiation that consists of TBP and 13 or 14 TAFs, including TAF12. Studies in yeast (*Saccharomyces cerevisiae*) and metazoans revealed that TAF12 functions as a transcription cofactor by physically interacting with other TFs, such as human MYB (14) and activating transcription factor 7 (ATF7) (15), yeast INOsitol requiring 2 kinase (NOBIRO6) (16), and repressor/activator site binding protein 1 (Rap1p) (17).

The *Arabidopsis* genome encodes two orthologous copies of TAF12, TAF12a, and TAF12b. A molecular and physiological role was reported for TAF12b from two independent forward genetic studies. TAF12b was first identified as ENHANCED ETHYLENE RESPONSE 4 (EER4) during a screen for mutants exhibiting hypersensitive growth responses to the phytohormone ethylene (18). EER4 influenced the expression levels of ethylene-responsive genes via its transcriptional cofactor activity and physical interaction with the major ethylene signaling TF ETHYLENE INSENSITIVE 3 (EIN3) (18). TAF12b was independently identified as CYTOKININ-HYPERSENSITIVE 1 (CKH1) during a search for mutant callus showing altered responses to another phytohormone, cytokinin (19, 20). The *ckh1* mutant calli were characterized by greening and cellular proliferation due to the deregulation of the cytokinin transcriptional response.

In this study, we aimed to elucidate the mechanism behind the short root phenotype of the *bz1728* double mutant. From a series of reverse and forward genetics approaches, we isolated the *bz1728* suppressor mutant *nobiro6*, which was responsible for partial rescue of root growth. We identified the causal gene, NOBIRO6, as encoding TAF12b. We explored the transcriptome landscape of the *nobiro6* single mutant and the *bz1728 nobiro6* triple mutant, which revealed that TAF12b acts as a transcriptional cofactor for BZIP60-mediated induction of the UPR. By analyzing the physiological and genetic responses of *nobiro6* single mutants to ER stress, we determined that NOBIRO6/TAF12b participates in UPR-associated root growth control and elucidated the molecular mechanism by which TAF12b acts as an important coactivator regulating root growth through the UPR.

### Results

As reported previously (11), the 12-d-old *bz1728* seedlings showed a 93.7% reduction in their primary root growth compared to wild-type (WT) seedlings under normal growth conditions (Fig. 1A and B). To better understand the morphological basis of the short root seen in the *bz1728* mutant, we measured several cell-level growth indices. *Arabidopsis* vertical root growth relies on two major cues: cell division at the root apical meristem (RAM) and longitudinal cell expansion in the elongation zone (EZ). Consistent with the decreased root growth, we measured the longitudinal length of cortical cells in the differentiation zone (DZ), where fully expanded cells undergo terminal differentiation after the EZ. Cells from the *bz1728* mutant elongated 73.2% less than the corresponding WT cells (Fig. 1 C and D). However, we did not observe any visible defect in meristematic cell organization or cell layer differentiation in the root of *bz1728* seedlings. Our cell-level measurements therefore indicated that the short root seen in the *bz1728* mutant is due to compromised growth via combined defects from both RAM activity and longitudinal cell expansion.

**Known UPR-Growth Pathways Are Inactive in *bz1728***

The signaling cascade for the phytohormone brassinosteroids (BR) has been reported to be regulated by UPR (12), and several *ems*-mutagenized *bri1* suppressor (ebs) mutations have been shown to suppress the negative UPR effect of BR-mediated vegetative growth (21–23). Accordingly, we generated triple mutants between the *bz1728* double mutant and each of the three *ebs* mutants *ebs1*, *ebs2*, and *ebs7*. Root growth was, however, comparable between the *bz1728* mutant and the *bz1728 ebs* triple mutants (Fig. 2C). We had previously observed that many defense-related genes are spontaneously up-regulated in *bz1728* roots (11). We ectopically expressed the *Pseudomonas putida* gene *NahG* in the *bz1728* mutant background, as *NahG* encodes an enzyme that hydrolizes the plant defense-signaling phytohormone salicylic acid, thereby weakening defense-related gene expression (24). The resulting transgenic lines displayed noticeably improved shoot growth, but still showed indifferent root growth to *bz1728* (Fig. 2A and B).
Identification of the nobiro6 Causal Mutation. To identify the causal mutation in nobiro6, we generated a buckcross population derived from a cross between bz1728 and bz1728nobiro6 (SI Appendix, Fig. S3). In 10-d-old seedlings, the resulting F1 progeny had the same root length as the bz1728 double mutant, while F2 progeny segregated long roots in a 1:3 ratio (χ² test, P > 0.89), indicating that nobiro6 relies on a single recessive mutation. To identify the causal locus, we sequenced the genome of the pooled genomic DNA from 30 F2 progeny with the bz1728nobiro6-type long root, as well as that of three F3 progeny with a bz1728-like short root. By comparison to the Arabidopsis reference genome, we identified more than 3,500 single nucleotide mutations for each sequenced sample. We then selected only those mutations that were fixed in the mutant pool and segregating in seedlings with a shoot root, resulting in three nonsynonymous mutations located on the short arm of chromosome 1 (SI Appendix, Fig. S3). Of those, a mutation introduced a premature stop codon at residue 431 (Q431X) in a protein-coding gene At1g17440 (Fig. 5C). This gene became our leading candidate for NBR6.

To validate At1g17440 as NBR6, we generated a genome-edited allele of this gene by CRISPR/Cas9 editing (nbr6-c1) in the bz1728 double-mutant background. Sanger sequencing of the At1g17440 genomic region of the transgenic plant revealed that nbr6-c1 resulted in a single-nucleotide insertion disrupting the first exon of the gene (Fig. 3 G and H). The bz1728nbr6-c1 line showed the same extent of root growth rescue as bz1728nobiro6 (Fig. 3 I and J). We also produced another triple mutant by crossing a T-DNA insertion line interrupting At1g17440 (nbr6-t1) with bz1728; the resulting bz1728nbr6-t1 seedlings showed a rescue of root growth similar to that seen in bz1728nobiro6 seedlings (Fig. 3 G, I, and J). Together, these results confirmed that At1g17440 is NBR6 and that all three mutant alleles (nobiro6, nbr6-c1, and nbr6-t1) are equivalently strong loss-of-function alleles that partially rescue the root growth defect characteristic of the bz1728 double mutant.

The nbr6 Mutation Decreased the Transcription of bz1728-Induced Genes. NBR6 encodes TAF12b, one of the two Arabidopsis orthologs of eukaryotic TAF12 and a subunit of the general TF TFID. Since previous studies identified NBR6 as a novel transcription cofactor in phytohormone signaling (18, 20), we anticipated that the nbr6 mutation might modulate the transcription of the bz1728 mutation to exert its visible rescue of root growth. To explore this possibility, we performed transcriptome deep sequencing (RNA-seq) on root samples collected from 12-d-old WT, bz1728, bz1728nobiro6, and the single nbr6-t1 mutant seedlings. We identified differentially expressed genes (DEGs) in each mutant in comparison to the WT with a |fold-change (FC)| ≥ 2 and a false-discovery rate < 0.05. We obtained more than 2,000 DEGs in the bz1728 double mutant; they presented roughly equal distribution between down-regulated and up-regulated genes (Fig. 4A). Although we identified the same number of DEGs in the bz1728nobiro6 triple mutant, more DEGs were down-regulated and fewer DEGs were up-regulated relative to the double mutant. Indeed, the mean FC of down-regulated DEGs decreased from 9.48 to 6.50 (Fig. 4A). Of the top 200 up-regulated DEGs in the bz1728 double mutant, only 46 were differentially expressed in the bz1728nobiro6 mutant; in contrast, of the top 200 down-regulated DEGs in the bz1728 double mutant, 166 remained differentially expressed in the bz1728nobiro6 triple mutant (Fig. 4B). The transcriptional effects of the single nbr6-t1 mutant were much...
more subtle, supporting a subsidiary cofactor role for NBR6 in global gene regulation (Fig. 4D). Previous studies on NBR6/TAF12b reported dozens of genes whose expression were affected in its single mutants with names of eer4 (18) or ckh1 (20). We therefore assessed these gene-expression patterns in the single nbr6-t1 mutant and observed changes in line with those described for eer4 but not for ckh1 (SI Appendix, Table S1). The apparent discrepancy concerning ckh1 might reflect the different tissues analyzed: differentiated tissues (eer4 and nbr6-t1) and dedifferentiated calli (ckh1). Our RNA-seq analysis thus revealed that the rescue of the limited root growth seen in the bz1728nobi6 mutant is accompanied by a suppression of transcriptional up-regulation dynamics in the bz1728 short root.

**NBR6 Physically Interacts with the UPR TF bZIP60.** bZIP60 is another transcriptional activator that modulates the UPR together with bZIP17 and bZIP28; we previously reported its another transcriptional activator that modulates the UPR

NBR6 is a 684-aa protein with a single histone-fold domain (HFD) at its C terminus and multiple unannotated glutamine-rich repeats in its N-terminal long tail. Indeed, the expression of most genes from the bZIP60 regulon was highly induced in the bz1728 mutant relative to WT, but diminished in the bz1728nobi6 triple mutant (SI Appendix, Table S2). We confirmed the relative transcript levels of bZIP60 and two of its representative target genes, SECRETORY 31A (SEC31A) and BINDING PROTEIN 3 (BIP3), in bz1728 and bz1728nobi6 seedlings by qRT-PCR (Fig. 4C). We therefore hypothesized that NBR6 functions as a transcriptional coactivator of the bZIP60 regulon.

A role in transcriptional regulation would require that NBR6 localize to the nucleus, and it has been revealed by previous reports (18, 20). For further molecular details, we tested the subcellular localization of NBR6 and various truncated versions as fusion proteins with superfolder green fluorescent protein (sGFP). NBR6 is a 684-aa protein with a single histone-fold domain (HFD) at its C terminus and multiple unannotated glutamine-rich repeats in its N-terminal long tail. We transiently transfected Arabidopsis protoplasts with constructs encoding full-length sGFP-NBR6f, the N terminus of NBR6 without the HFD (amino acids 496 to 684; sGFP-NBR6c) (Fig. 4D). Different letters indicate significant differences (ANOVA post hoc Tukey’s HSD test, P < 0.01).

We then assessed the physical association of NBR6 and its derivatives with bZIP60 by bimolecular fluorescence complementation (BiFC) assay by transient transfection of Arabidopsis protoplasts (27). A C-terminally truncated form of bZIP60 (bZIP60n) employed for its spontaneous nuclear localization (28). We observed reconstitution of yellow fluorescent protein (YFP) for all combinations of transfected constructs expressing bZIP60n-YFP and NBR6f-YFP, NBR6n-YFP, or NBR6c-YFP, with a strong overlap between YFP fluorescence and that obtained for the nucleus marker histone H2B fused to cyan fluorescent protein (CFP) (Fig. 4F). As an independent confirmation, we conducted a split-luciferase complementation (split-LUC) assay by transient infiltration of Nicotiana benthamiana leaves (29). Consistent with the BiFC results, we detected LUC activity for all combinations of constructs, but not for individual constructs (Fig. 4G). These results supported the physical interaction of NBR6 and bZIP60 in the nucleus. However, both NBR6c and NBR6n can functionally interact with bZIP60 only via a single domain, whereas NBR6n can indirectly interact with bZIP60 through the larger TFIID complex. To test this
hypothesis, we performed a pull-down assay using protein extracts and partially purified recombinant proteins from TFIIH-free Escherichia coli. Accordingly, we purified recombinant glutathione S-transferase (GST)-bZIP60n and mixed the protein with lysates from cells induced to accumulate maltose-nant glutathione S-transferase (GST)-bZIP60n and mixed the TFIIID-free extracts and partially purified recombinant proteins from hypothesis, we performed a pull-down assay using protein interactions with bZIP60 via physical interaction through its HFD in the nucleus. Accordingly, we purified recombinant proteins from constructs encoding C-terminally truncated bZIP60 (bZ60n) and NBR6 derivatives. Constructs were transiently transfected in Arabidopsis mesophyll protoplasts. We used the BIP3 promoter (pBIP3) driving the transcription of the emerald firefly luciferase (eLUC) reporter gene as the target of bZIP60, as previously reported (28). Transient transfection of bZIP60n alone induced eLUC activity from the BIP3 promoter and failed to enhance the transactivation mediated by bZIP60n (4, Fig. 4 K). Together, these results demonstrate that NBR6 functions as a transcriptional cofactor to bZIP60 via physical interaction through its HFD in the nucleus.

The nrb6 Mutation Alleviates the ER Stress-Induced Root Growth Reduction. We next wished to determine to what extent the loss of NBR6 alone might affect plant responses to ER stress. In double genotypes, expression of NBR6 increased eLUC activity from the BIP3 promoter (Fig. 4 K). Together, these results demonstrate that NBR6 functions as a transcriptional cofactor to bZIP60 via physical interaction through its HFD in the nucleus.

The nrb6 Mutation Alleviates the ER Stress-Induced Root Growth Reduction. We next wished to determine to what extent the loss of NBR6 alone might affect plant responses to ER stress.
Under normal growth conditions, the nbr6-t1 single mutant showed a mild reduction in the growth of both roots and shoots. The length of the primary root from 12-d-old nbr6-t1 seedlings was 87.3% that of WT (Fig. 5A and B). Similarly, rosette diameter of 28-d-old nbr6-t1 plants was 11.5% smaller than that in WT plants of the same age (Fig. 5C and D).

To further characterize the phenotypes of nbr6 single mutants, we measured root growth upon activation of ER stress. Accordingly, we transferred 2-d-old seedlings grown on normal growth medium to fresh medium containing various concentrations of the two ER stress agents, tunicamycin (Tm) and dithiothreitol (DTT). After 10 d on the new medium, primary root elongation in WT seedlings had decreased in a dose-dependent manner for both Tm and DTT, as previously reported (26, 30, 31) (Fig. 5E and G). Although primary root growth also slowed down in the nbr6-t1 single mutant as an effect of the ER stress agent, the mutant response was less pronounced than that of WT seedlings, as nbr6-t1 roots were already shorter than WT under control conditions and failed to respond to higher concentrations of Tm and DTT (Fig. 5E–H). We interpret these results as indicating the possible participation of NBR6 to ER stress-responsive control of root growth.

The nbr6 Mutation Alters UPR Activity. To investigate stress-responsive UPR activity in the nbr6-t1 mutant, we collected root tissues from seedlings grown on 30 ng L⁻¹ Tm (Fig. 5F) to measure relative transcript levels of the representative UPR target genes BIP3 (a shared target of bZIP28 and bZIP60), SEC31A (a bZIP60 target), and CALRETICULIN 2 (CRT2, a bZIP28 target) by qRT-PCR (11, 32, 33). Tm induced the expression of all three genes in WT, as expected (Fig. 6A). Importantly, relative transcript levels reached lower levels in the nbr6-t1 mutant. In addition, all three genes were slightly induced even under control conditions in the mutant, resulting in a 40 to 50% reduction in their FC between control and Tm treatment (Fig. 6B). This result was consistent with the alleviation of root growth reduction upon ER stress in the nbr6-t1 mutant (Fig. 5E–H).

To obtain a global view of the apparent induction of the UPR by the nbr6-t1 mutant under control conditions, we looked at the transcript levels of other UPR-related genes in our RNA-seq dataset (Fig. 4A). In addition to BIP3, SEC31A, and CRT2, we determined transcript levels for 20 known UPR target genes and three UPR-bZIP genes in WT and nbr6-t1. We grouped UPR target genes into three clades based on the bZIP regulating their transcription, bZIP28 targets, bZIP60 targets, and bZIP28 and bZIP60 shared targets, as reported previously (11). The transcript levels of all three bZIP genes and most of their target genes increased in the nbr6-t1 mutant (Fig. 6C). Notably, the transcription of bZIP28 and bZIP60 shared target genes was more weakly induced than that of the other clades.
In conclusion, the nrb6-1l single mutant moderately induced UPR globally, likely explaining its shorter root under control conditions (Figs. 5A and 6C). Overall, the genotypes WT, bz1728, bz1728/STOP, and nrb6-1l exhibit varied UPR activity levels and varied primary root growth in an inversely associated relationship (SI Appendix, Fig. S5).

Discussion

Trade-offs between plant defense and growth are now widely accepted concepts (34, 35), whereas growth trade-offs in response to other stress categories are seldom considered. In this study, we discovered an inverse association between plant UPR activity and primary root growth (SI Appendix, Fig. S5). Using chemical agents and genetic manipulation, we observed UPR activity and primary root growth (∼30% of so many genes in Arabidopsis that are regulated by the nucleus), as well as loss-of-function allele of the TFIID component TAF12b indicated that transcriptome reprogramming is likely to provide a key to understanding the root growth defect by bz1728. As plant UPR responds to a broad spectrum of both biotic and abiotic stimuli (1, 4), and as the bz1728 short root is due to composite defects of multiple root growth determinants (Figs. 1 and 3), we propose that the plant UPR acts as a key node between trade-offs, by integrating multiple stressful signals into a single regulatory module to control root growth as a function of the sum of all intracellular stress. Given that ethylene and cytokinin modulate both root growth and development (36, 37), the UPR may integrate these two phytohormone signaling pathways with NBR6/TAF12b at growth and development (36, 37), the UPR may integrate these two phytohormone signaling pathways with NBR6/TAF12b at their crossroads (SI Appendix, Fig. S5). Future studies aimed at identifying other nobi6 mutants will provide more players to integrate phytohormone homeostasis, stress signaling, and cell growth control in a model of the center stress and UPR.

The transcriptome upheaval observed in the bz1728 double mutant (Fig. 4D) masked whether the mutant short root was due to a defect in a growth-promoting signal or an excessive growth-limiting stress signal. The discovery and characterization of nobi6 answered this question, insofar as the induction of so many genes in bz1728 is likely to be highly detrimental to root growth (Figs. 3 and 6). This hypothesis is also supported by the observation that many up-regulated genes in bz1728 are stress-responsive (11). We note that the rescue of root growth by nobi6 was partial, and the UPR downstream genes responsible for the root growth defect are still unknown. The bZIP60 regulon is a possible candidate, although the overexpression of bZIP60 did not affect root growth (38). However, the triple mutant of three UPR-bZIPs is embryo-lethal (11), and the ire1a ire1b double mutant lacking activity for the bZIP60 activators had modest but significant root growth defects that were enhanced when it was combined with bzip17 in the ire1a ire1b bzip17 triple mutant (13). These results indicate that the short root phenotype of the bz1728 double mutant and the associated transcriptive tumors are induced by multiple redundantly functioning ER stress-responsive transcription modulators, including bZIP60, and that NBR6 is a shared cofactor for their full activity.

We demonstrated that NBR6 functions as a transcriptional cofactor of the bZIP60 regulon, which requires physical interaction between bZIP60 and the C-terminal HFD of NBR6 (Fig. 4). In parallel, another transcriptional cofactor was reported for UPR, the nuclear factor Y (NF-Y) trimeric complex. A previous study found that a specific NF-Y complex physically interacts with bZIP28 through its HFD and assists the target promoter recognition, which are analogous to that of mammalian UPR-TF ATF6 (39). Given that NF-Y complex also interacts with TFIID (40), these suggest how plant UPR-bZIPs form dimers to each other (11, 39) and how they are incorporated into the regulation of UPR downstream genes with other global TFs.

In contrast to TAF12b, no role has been reported for its ortholog TAF12a. Considering that TAF12 is typically encoded by a single and essential gene in yeast and metazoans, the viability of the nrb6-1l mutant and the absence of a clearly defined role for TAF12a suggest that the functional differentiation of the two Arabidopsis TAF12 proteins. In yeast and metazoans, TAF12 is a shared component of TFIIID and the PIC subcomplex Spt-Ada-Gcn5 acetyltransferase (SAGA). These two subcomplexes have different target promoter preferences and influence the transcription of different genes involved in housekeeping or stress responses (41). The two Arabidopsis TAF12 proteins were recently shown to exhibit different affinities toward TFIIID and SAGA complexes (42). The fungal pathogen Candida albicans also boasts two copies of TAF12 that participate in the growth of this unicellular organism nonredundantly, with each one showing selective interaction with the TFIIID or SAGA complexes (43). Therefore, plants may also exploit multiple TAF12 isoforms for different purposes based on their interacting PIC subcomplexes, which may improve transcriptome plasticity and contribute to the strong environmental adaptability of plants.

Materials and Methods

Plant Materials and Growth Conditions. A. thaliana accession Columbia (Col-0) was used in this study. The T-DNA insertion lines (listed in SI Appendix, Table S3) were obtained from ABRC (Arabidopsis Biological Resource Center). The CRISPR/Cas9-mediated nrb6-1l mutant was induced with the pKAMA-ITACHI system (44). Transgenic bz1728 lines expressing NahG were generated with Agrobacterium-mediated plant transformation as described previously (45). Detailed procedures for vector plasmid construction and transgenic plant generation are described in SI Appendix, Supplementary Materials and Methods. Seeds were surface-sterilized and stratified at 4°C for 3 d in the dark before being sown onto the modified germination agar medium or on soil (11). Plants were grown in a growth chamber with a 16-h light/8-h dark photoperiod with fluorescent lights at 80 ± 10 μmol m−2 s−1, 22°C and 65% relative humidity.

Root Microscopy Imaging. Roots from 7-d-old Arabidopsis seedlings were stained with 10 mg L−1 propidium iodide (Thermo Fisher Scientific). The imaging data were obtained using a Leica SPS confocal laser scanning microscope with a HC PL APO 20× 0.7 dry objective and a 488-nm argon-ion laser, or an Olympus FV1200 microscope with a UPlanSApo 20× 0.75 dry objective and a 559-nm LD laser line. The obtained images were exported with the software LAS X (version 3.7.2.22383; Leica) or FV10-ASW (version 4.02; Olympus). For cell length assays, each cell length was manually measured using imageJ (v1.53g) (46).

Micrografting. Root-shoot grafting of 3-d-old seedlings was performed as described previously (47). The grafted seedlings were grown vertically for other 7 d, and then each seedling was photographed and the primary root growth was measured manually using ImageJ.

EMS Mutagenesis. Details of the procedure followed a previous report (48). In brief, 0.5 g of bz1728 seeds were treated with 0.4% (vol/vol) EMS (M0880, Sigma-Aldrich) for 8 h at room temperature. After six washes with distilled water, the M0 seeds were immediately sown on soil and grown in the growth conditions described above. The harvested M0 seeds were vertically grown on the modified germination agar medium for 12 d, and the seedlings with longer roots than their neighbors were rescued for further validation of the phenotype in later generations.

Genome Resequencing. A total of 1 μg of Arabidopsis genomic DNA for each sample was subjected to DNA library construction for the Ion Proton system (Thermo Fisher Scientific) with the NEBNext Fast DNA library prep set (E6270, New England Biolabs). The sequencing resulted in an average of 6.2 million reads (151 nt in length on average) for each library. Read mapping and genetic variant calling were conducted as described in a previous report (45). Only biallelic single-nucleotide polymorphisms supported by more than five reads were retained for analysis.

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RNA Extractions, DNA Synthesis, and qPCR. Total RNA was extracted with an RNase-free mini kit (Qiagen) and RNA-free DNase set (Qiagen). Firststrand cDNA synthesis was conducted using the SuperScript VILO cDNA synthesis kit (Invitrogen). qPCR was performed on a 7500 Fast Real-Time PCR system and 7500 software (v2.0.6; Applied Biosystems). Fast SYBR Green master mix (Applied Biosystems) was used for amplification. Arabidopsis ACTIN2 was used as reference gene. Primer sequences used for qPCR are given in Table S4.

RNA-Seq. Arabidopsis samples were collected as four biological replicates. The roots from six 12-d-old seedlings were pooled as one replicate. Library construction for Illumina sequencing used the NEBNext Ultra II RNA library prep kit (E7770, New England Biolabs). Paired-end sequencing was performed on an Illumina HiSeq instrument, resulting in an average of 2.14 million reads (2 × 151 bp) per library. Clean reads were aligned to the Arabidopsis reference genome from Araport11 (49) with a Kallisto-Sleuth pipeline (v0.44.0) (50) for identifying DEGs. Detailed procedures are described in Supplemental Materials and Methods.

Transient Expression Assays with Arabidopsis Mesophyll Protoplasts. Isolation and transformation procedures of Arabidopsis mesophyll protoplasts, as well as microscopy imaging, were conducted as described previously (11). The pGK-sGFP vector (45) was employed to determine the subcellular localization and transactty of NBR6 derivatives. The pUC-VYCE(R) and pUC-VYNE(R) vectors were employed for BiFC analysis (27). Dual-luciferase reporter assays were performed to assess NBR6 transactivity. The pGK-ELUC vector (45) was used to drive expression of the emerald firefly luciferase reporter gene (elUC) by the BiP promoter. Cauliflower mosaic virus (CaMV) 35S promoter-driven Renilla luciferase (hRluc) was used as internal control. LUC and RlUC activities were measured with reagents from a Dual-Luciferase Reporter Assay System (E1910, Promega) on an EnSpire Multimode Plate Reader and EnSpire Workstation software (v4.13.3005.1482; Perkin-Elmer). Detailed procedures for vector plasmid construction are described in Supplemental Materials and Methods.

Split-Luciferase Assays. Agrobacterium-mediated transient infiltration in N. benthamiana leaves was conducted as previously reported (29) with minor adaptations. Leaf discs 7 mm in diameter cut from infiltrated leaves were applied to measure the LUC activity by EnSpire Multimode Plate Reader (Perkin-Elmer).

Chemical Treatments. Vertically grown 2-d-old Arabidopsis seedlings were transferred to new agar growth medium containing various concentrations of Tm (T7765; Sigma-Aldrich) or DTT (D1071; Tokyo Chemical Industry). The same volume of dimethyl sulfoxide (DMSO) was used as mock control. The seedlings were grown vertically for other 7 d, then primary roots were photographed and their growth was measured manually using ImageJ.

Pull-Down Assays, SDS/PAGE, and Immunoblotts. Tag-fused protein production and purification was achieved by employing the pCold system (TaKaRa Bio) and E. coli Rosetta(DE3) strain (Merck) according to the manufacturer’s instructions. Detailed procedures for vector plasmid construction are described in Supplemental Materials and Methods. The GST-tagged protein was extracted from cell pellets and purified on glutathione Sepharose 4B (GE Healthcare Life Sciences) beads, constituting the bait sample for pull-down assays. A total cell lysate obtained from E. coli cells producing the MBP-tagged protein was mixed with the loaded glutathione beads. The pull-down assay was carried out with Pierce GST protein interaction pull-down kit (Thermo Scientific). The resulting pulled-down proteins were run on 10% Bio-Tris NuPAGE Novex gel with MES running buffer (Thermo Scientific), after which these proteins were transferred to a 0.2-μm nitrocellulose membrane with the Transblot Turbo transfer system (Bio-Rad) and then subjected to immunoblot and chemiluminescent signal detection with the iBind Western blot system (Thermo Scientific), ECL Prime detection reagent (Thermo Scientific), and ChemiDoc MP basic imaging system (Bio-Rad). Anti-GST (ab3416, Abcam; dilution 1:500), anti-MBP (EB032, New England Biolabs; dilution 1: 10,000) and anti-mouse IgG (W4021, Promega; dilution 1:2,500) antibodies were used for immunoblotting.

Statistical Analyses. Welch’s t test and one-way ANOVA post hoc Tukey’s honestly significant difference (HSD) test were applied for two-sample and greater than two-sample comparisons, respectively.

Data Availability. The raw sequence data of Arabidopsis genome sequencing and RNA-seq were deposited at the National Center for Biotechnological Information Sequence Read Archive (BioProject IDs PRJNA688172 and PRJNA687636). In-house scripts used in this study are available via the GitHub repository at https://github.com/junesk9/. All other study data are included in the article and/or supporting information.

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