CCCH protein-PvCCCH69 acted as a repressor for leaf senescence through suppressing ABA-signaling pathway

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Abstract
CCCH is a subfamily of zinc finger proteins involved in plant growth, development, and stresses response. The function of CCCH in regulating leaf senescence, especially its roles in abscisic acid (ABA)-mediated leaf senescence is largely unknown. The objective of this study was to determine functions and mechanisms of CCCH gene in regulating leaf senescence in switchgrass (Panicum virgatum). A CCCH gene, PvCCCH69 (PvC3H69), was cloned from switchgrass. Overexpressing PvC3H69 in rice suppressed both natural senescence with leaf aging and dark-induced leaf senescence. Endogenous ABA content, ABA biosynthesis genes (NCED3, NCED5, and AAO3), and ABA signaling-related genes (SnRKs, ABI5, and ABF2/3/4) exhibited significantly lower levels in senescencing leaves of PvC3H69-OE plants than those in WT plants. PvC3H69-suppression of leaf senescence was associated with transcriptional upregulation of genes mainly involved in the light-dependent process of photosynthesis, including light-harvesting complex proteins, PSI proteins, and PSII proteins and downregulation of ABA biosynthesis and signaling genes and senescence-associated genes. PvC3H69 could act as a repressor for leaf senescence via upregulating photosynthetic proteins and repressing ABA synthesis and ABA signaling pathways.

Introduction
Natural or stress-induced leaf senescence adversely affects photosynthetic capacity and plant productivity1–3. Leaf senescence development is regulated at multiple levels, involving molecular, transcriptional, post-transcriptional, and metabolic processes3–5. At the transcriptional level, CCCH zinc finger proteins with three Cys and one His residues as the conserved motif that function as RNA-binding proteins and regulate RNA metabolism have been found to act as key regulators of leaf senescence in Arabidopsis thaliana and rice (Oryza Sativa). CCCH genes, AtKHZ1 and AtKHZ2, can accelerate leaf senescence when overexpressed in Arabidopsis6. OsDOS and OsTZF1 were found to be negative regulators of leaf senescence in rice7–9. Despite the knowledge of the involvement of CCCH genes in leaf senescence, the upstream and downstream regulatory mechanisms of CCCHs controlling leaf senescence remain largely unknown.

Several CCCH genes have been found to interact with abscisic acid (ABA), a well-known stress hormone, in the regulation of seed germination, plant growth, and stress responses. A CCCH gene cloned from Arabidopsis, AtTZF1, was reported to upregulate the expression of ABA-response genes, RD29A and COR15A, for regulating plant growth and stress responses4. Other CCCH genes in Arabidopsis, including AtC3H49 (AtTZF3) and AtC3H20 (AtTZF2) was inducible by ABA and also feed forward to upregulate ABA-response genes, such as RD29B during drought stress10. AtTZF4/5/6 genes are found to be upregulated under ABA treatment and in turn enhance ABA biosynthesis and signaling by activating NCED9 and...
**Results**

**PvC3H69 as a nuclear-localized protein without transactivation function**

The subcellular localization of PvC3H69 was studied by fusing it with a GFP tag. From Fig. 1a, it clearly showed that GFP signal of PvC3H69-GFP was merged with the DAPI stained nucleus, while the GFP control was dispersed in the nucleus and cytosol, indicating that PvC3H69 was exclusively localized in the nucleus.

To test whether PvC3H69 had transcriptional activity, we performed yeast-based transactivation assay. As shown in Fig. 1b, PvC3H69 and the negative control (GUS) fused with the GAL4 DNA-binding domain (GAL4-DB) did not activate the reporter gene in the yeast system, while the positive control of a known transcription factor, PvC3H72, had transactivity. Furthermore, we carried out in planta transcriptional activity assay by transient co-expressing 35S::PvC3H69-GAL4-DB (effector), GAL4(4x):GUS (reporter), and 35S::LUC (internal control) in Arabidopsis protoplasts, with 35S::PvC3H72-GAL4-DB and 35S::GAL4-DB as the positive and empty vector controls, respectively (Fig. 1c). The positive control of PvC3H72 activated the expression of the GUS reporter gene, while PvC3H69 did not activate or suppress the expression of the GUS reporter gene (Fig. 1d). Taken together, PvC3H69 showed no transcriptional activity in neither yeast-based nor in planta assay.

**Suppression of dark-induced leaf senescence in switchgrass and rice by overexpression of PvC3H69**

To understand the functions of PvC3H69 regulating leaf senescence, we overexpressed the gene under driven of maize ubiquitin promoter in switchgrass and rice by Agrobacterium-mediated transformation, with transformation being confirmed by PCR and GUS staining (Fig. S1a–d). Overexpressing PvC3H69 (abbreviated as C3H69-OE hereafter) resulted in transgenic plants of both switchgrass and rice retained more green leaves in each tiller and lower level of leaf senescence compared to their respective wild-type (WT) plants (Fig. 2a, b). Leaf photosynthetic efficiency (Fv/Fm) and net photosynthetic rate (Pn) of C3H69-OE lines were significantly higher than that of WT. Chlorophyll (Chl) content of the second to fourth leaves showed significantly higher levels in C3H69-OE lines than that in WT plants (Fig. 2c, d). Under dark conditions, leaves of WT plants exhibited severe leaf senescence with 90% loss of Chl content, whereas Chl content of transgenic lines maintained at 50% of the control level; Fv/Fm of transgenic lines also were significantly higher than that of WT (Fig. 3a–h). Those results demonstrated that PvC3H69 played a positive role in suppressing dark-induced leaf senescence.

**Suppression of ABA synthesis and signaling by overexpression of PvC3H69**

Exogenous application of 5 or 20 µM ABA significantly accelerated leaf senescence in rice WT plants, as shown in Fig. 4a, b. In contrast, C3H69-OE-Oz lines of rice were less sensitive to ABA treatment. Leaf Chl content and Fv/Fm of overexpression lines were significantly higher than those of WT plants. Expression levels of three ABA-responsive genes (OsLIP9, OsLEA3, and OsRAB16A) increased 120 to 5000 times in WT plants, but only increased ~4 to 25 times in C3H69-OE-Oz transgenic lines of rice when treated with 20 µM ABA (Fig. 4c). These results demonstrated that PvC3H69 suppressed ABA-accelerated leaf senescence in rice by downplaying ABA responses.

To understand whether PvC3H69 suppress leaf senescence may involve downregulating ABA signaling, key
genes in the PP2C-SnRKs-ABF signaling pathway were further examined. Without ABA treatment (mock), only one SnRK family gene, OsSAPK1, showed significantly lower expression level in C3H69-OE-Oz transgenic lines compared to WT. With 5 or 20 µM ABA treatment, three (OsSAPK1, OsSAPK6, and OsSAPK10) out of 10 SnRK genes (OsSAPK1 to OsSAPK10) (Fig. S2) and four transcription factor genes (OsABI5, OsABF2, OsABF3, and OsABF4) showed significantly lower expression levels in C3H69-OE-Oz transgenic lines compared to WT (Fig. 5).

We further measured expression levels of four ABA biosynthesis genes (OsABA2, OsZEP, OsNCED3, OsNCED5) and ABA content in leaves of WT and C3H69-OE-Oz lines. As shown in Fig. 6, significantly lower expression levels of OsNCED3 and OsNCED5 were detected in C3H69-OE-Oz lines than in WT, and ABA content in C3H69-OE-Oz lines was also significantly lower than that in WT (Fig. 6a, b), indicating that PvC3H69 suppressed ABA biosynthesis.

**Global gene expression analysis of ABA- and senescence-related genes in PvC3H69-overexpression transgenic plants**

Comparative analysis of transcriptome of C3H69-OE-Oz and WT leaves exposed to dark for 10 d to induce leaf senescence and those leaves collected prior to dark exposure (0 d) were performed by using Illumina-HiSeq™ 4000. Principle component analysis (PCA) of transcriptomic data showed that differentially expressed genes (DEGs) in WT leaves at 0 d (WT-0), C3H69-OE-Oz leaves at 0 d (C3H69-OE-Oz -0), WT leaves at 10 d of dark treatment (WT-10), and C3H69-OE leaves at 10 d of dark treatment (C3H69-OE-Oz -10) were clustered in separate groups, with DEGs in C3H69-OE-Oz -0 and
WT-0 closely related and DEGs in WT-10 and C3H69-OE-Oz-10 were well separated (Fig. S3a). Compared with WT-0, 754 DEGs were upregulated and 580 downregulated in C3H69-OE-Oz-0. A total of 3522 upregulated and 2586 downregulated DEGs in C3H69-OE-Oz-10 compared to WT-10 (Fig. S3b).
Fig. 3 Phenotype and physiological parameters for dark-induced senescence in switchgrass and rice. a Detached leaves of switchgrass WT and C3H69-OE lines exposed to dark treatment. b Relative expression level of PvC3H69 in switchgrass WT and C3H69-OE-Pv lines under dark treatment. c, d Fv/Fm and chlorophyll content in switchgrass WT and C3H69-OE-Pv lines under dark treatment. e Detached leaves of rice WT and C3H69-OE-Oz lines under dark treatment. f Relative expression level of PvC3H69 in rice WT and C3H69-OE-Oz lines. g, h Fv/Fm and chlorophyll content in rice WT and C3H69-OE-Oz lines under dark treatment. Letters above bars indicate significant difference at $P < 0.05$. Bar = 0.5 cm.
Fig. 4 Phenotypic and physiological effects of ABA on dark-induced leaf senescence in transgenic lines (C3H69-OE-Oz) of rice. a Phenotype of rice WT and C3H69-OE-Oz lines under exogenous ABA treatment. Excised leaves from rice transgenic lines and WT were treated with 0, 5, 20 μM ABA under darkness. b Chlorophyll content and Fv/Fm of rice WT and C3H69-OE-Oz lines under exogenous ABA treatment. c Relative expression level of ABA specific response genes in rice WT and C3H69-OE-Oz lines under exogenous ABA treatment. Letters above bars indicate significant difference at P < 0.05.
Gene Set Enrichment Analysis (GSEA) of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were carried out to understand biological functions of DEGs related to dark-induced leaf senescence due to overexpressing \(PvC3H69\). Comparative analysis of DEGs between WT-10 and \(C3H69\)-OE-Oz -10 found \(PvC3H69\)-regulated genes and their functions related to the suppression of dark-induced leaf senescence. The most enriched GO term pathways of \(PvC3H69\)-regulated genes were "photosynthesis", "thylakoid", "signaling", "macromolecular complex", "response to hormone", "structural molecule activity", and "signal transducer activity" (Fig. S4a and Table S2). In particular, the most enriched KEGG pathways were related to hormones and photosynthetic metabolism including "photosynthesis", "carbon fixation in photosynthetic organisms", "alpha-Linolenic acid metabolism", "diterpenoid biosynthesis", "flavonoid biosynthesis", "tryptophan metabolism", "carotenoid biosynthesis", "Linoleic acid metabolism", and "plant hormone signaling pathway" (Fig. S4b and Table S3). The results indicated that \(PvC3H69\) could mainly regulate photosystems and hormone metabolism and signaling to delay dark-induced senescence.

Further analysis of genes in "hormones regulation overview" found DEGs related to ABA, indoleacetic acid (IAA), jasmonic acid (JA), ethylene, salicylic acid (SA), and gibberellic acid (GA). Most DEGs in ABA pathway were downregulated under darkness in plants overexpressing \(PvC3H69\) (Fig. 7a).

Further analysis of DEGs involved in ABA biosynthesis and signaling pathways found nine genes in ABA biosynthesis pathway were downregulated in \(C3H69\)-OE-Oz when compared to those in WT by comparing between \(C3H69\)-OE-Oz -0 and WT-0 and between \(C3H69\)-OE-Oz -10 and WT-10. As for DEGs in ABA signaling-related genes, 22 out of 34 were downregulated in \(C3H69\)-OE-Oz -0 and all 34 genes were downregulated in \(C3H69\)-OE-Oz -10 when compared to WT-0 and WT-10, respectively (Fig. 7b). In addition to ABA-related genes, DEGs encoding for NAC, MYB, and WRKY family transcriptional factor genes known...
as regulators in leaf senescence were found mostly down-regulated in C3H69-OE-Oz plants at 0 d of dark treatment and all of them were downregulated in C3H69-OE-Oz plants at 10 d of dark treatment in comparison to their respective WT plants (Fig. 7c). As for DEGs involved in photosynthesis system, five light-harvesting complexity proteins (Lhca2, Lhcb1, Lhcb4, Lhcb5, and Lhcb6) and seven photosynthesis proteins, including 4 PSI proteins (PsaH, PsaK, PsaN, PsaO) and three PSII proteins (PsbO, PsbQ, and Psb27), were upregulated in C3H69-OE-Oz -0 compared to those in WT-0. DEGs encoding for PSI (PsaB, PsaE, PsaK, PsaL, and PsaO) and PSII components (PsbB, PsbO, PsbP, PsbQ, PsbR, PsbW, PsbY, and Psb27) were upregulated in C3H69-OE-Oz -10 compared to those in WT-10 (Fig. 8a, b).

The expression of selected DEGs was confirmed with qRT–PCR analysis, including four ABA signaling-related genes (SnRK2, ABI5, NAC103, and NAC58), three other hormonal signaling pathways (ARR, ERF103, and ORE1), and two chlorophyll degradation genes (SGR and NOL). The relative expression of these nine genes in qRT–PCR analysis corresponded to those generated from the RNA-Seq, supporting the reliability of RNA-Seq results (Fig. S5).

**Discussion**

The CCCH-type zinc finger family includes multiple genes, and PvC3H69 cloned from switchgrass in our study was found to be a homology of ZmC3H38 and OsTZF124, but exhibited unique characteristics from the homologs in maize and rice. PvC3H69 was located in nucleus, but had no transcriptional activity (Fig. 1a–d), suggesting it is not a transcriptional factor. Other CCCH proteins in rice and Arabidopsis with functions in DNA binding, RNA binding, and mRNA turnover or silencing can shuttle between the nucleus and cytoplasm foci under different stresses9,26. TZF1 proteins have been found to directly interact with stress regulators, such as RD21A and PR5 or the mRNA of Ank-p9,16. However, whether PvC3H69 has mRNA or protein binding function deserves further investigation.

Although CCCH genes are known to regulate leaf senescence, different genes in the CCCH family were found to have distinct functions with AtKHZ1 and AtKHZ2 being characterized as a positive regulator inducing leaf senescence in Arabidopsis6 and OsDOS and OsTZF1 as a negative regulator of leaf senescence in rice7–9. In our study, PvC3H69 acted as a negative regulator, which suppressed both natural senescence associated with leaf aging and dark-induced leaf senescence, as manifested by the maintenance of greater chlorophyll content, photochemical efficiency, and net photosynthetic rate in plants overexpressing PvC3H69 (Figs. 2 and 3). Furthermore, transcriptomic analysis of plants overexpressing PvC3H69 found that a large number of genes involved in the light-dependent process of photosynthesis, including light-harvesting complex proteins (Lhca2, Lhcb1, Lhcb4, Lhcb5, Lhcb), PSI proteins (PsaH, PsaK, PsaN, PsaO) and...
PSII proteins (PsbO, PsbQ, Psb27) were upregulated in natural senescent leaves and those in PSI systems (PsaB, PsaF, PsaK, PsaL, PsaO) and PSII components (PsbB, PsbO, PsbP, PsbQ, PsbR, PsbW, PsbY, Psb27) were upregulated in dark-induced senescent leaves (Fig. 8). In contrast, DEGs encoding NAC (NAC58, NAC103 et al.), MYB-like, and WRKY (WRKY45 et al.) family transcriptional factor genes which are known regulators in leaf senescence were downregulated in C3H69-OE plants in comparison to WT plants (Fig. 7c). The combined physiological and transcriptomic data provided strong evidence that PvC3H69 acted as a repressor for leaf senescence, which helped to sustain or maintain photosynthesis by enhancing light-harvesting and photochemical capacity in both PS I and PS II of photosynthesis.

The underlying molecular mechanisms and key regulatory pathways for CCCH suppressing leaf senescence are yet to be fully understood. The delay in stress-induced leaf senescence by OsTZF1 has been associated with regulating stress-related genes (i.e., AK112082, JAZ1, Ferritin, MT-type1, ChaC-like)8,9. In our study, DEGs by PvC3H69 were highly enriched in hormone metabolism.

Fig. 7 MapMan analysis of “hormone regulation overview” and heatmap of downregulated genes in rice C3H69-OE-Oz lines relative to rice WT plants exposed to 0 and 10 d of dark treatment in ABA pathway and senescence-associated genes through transcriptome analysis. a MapMan analysis of “hormones regulation overview”. b Fold change heatmap of ABA synthesis genes and ABA-signaling genes related to leaf senescence in C3H69-OE-Oz -0 (C3H69-OE-Oz transgenic lines prior to dark treatment) vs WT-0 (WT prior to dark treatment) and C3H69-OE-Oz -10 (C3H69-OE-Oz transgenic lines under 10-day dark treatment) vs WT-10 (WT under 10-day dark treatment) transcriptome analysis. c Fold change heatmap of senescence-associated genes (SAGs) in C3H69-OE-Oz -0 vs WT-0 and C3H69-OE-Oz -10 vs WT-10 transcriptome analysis.
Fig. 8 Photosynthetic proteins upregulated in rice C3H69-OE-Oz lines relative to WT plants exposed to 0 and 10 d of dark treatment in transcriptome analysis. 

a MapMan overview DEGs in C3H69-OE-Oz (overexpression plants of PvC3H69) vs WT under 0 and 10 d of dark treatment. 

b Upregulated photosynthetic proteins in C3H69-OE-Oz -0 vs WT-0 and C3H69-OE-Oz -10 vs WT-10 transcriptome analysis.
and signaling pathways, with most genes in ABA biosynthesis and signaling being downregulated significantly by overexpressing \( Pvc3H69 \) (Fig. 7b). Moreover, transgenic rice plants overexpressing \( Pvc3H69 \) exhibited lower sensitivity of leaf senescence to ABA treatment compared with WT plants (Fig. 4a, b). These results indicated that function of \( Pvc3H69 \) is involved in ABA.

ABA-induced leaf senescence involves activation of ABA biosynthesis genes, such as \( NCEDs, ABA2, AAO3, \) and ABA signaling genes, such as \( ABIS, ABF2, \) and \( ABF3^{18–22}. OsNECD3 \) and \( OsNECD5 \) are involved in xanthophyll cleavage for ABA biosynthesis\(^ {27,28}. \) The relatively lower content of ABA in \( C3H69-OE \) plants could be resulted from the downregulated transcript level of \( OsNECD3 \) and \( OsNECD5. \) Consistently, global genes expression analysis showed that nine genes in ABA biosynthesis, including \( OsNCED3 \) and \( OsAAO3 \) were downregulated, indicating that \( Pvc3H69 \) could be a negative regulator for ABA biosynthesis (Fig. 6a, b). In ABA signaling, \( PP2C-SnRK-ABF \) regulatory model is considered as the core pathway that is required for ABA-triggered Chl degradation\(^ {25,22}. \) In this model, PYL as a receptor can accept ABA signal and form PYL-ABA bound complexity, which leads to \( PP2C \) inactivated and released the repressed SnRKs (mainly \( SnRK2s; \) the activated SnRKs can phosphorylate ABA-response binding factors (ABFs)\(^ {29,30}. \) \( SnRK2 \) genes are plant-specific serine/threonine kinases involving in plant responses to abiotic stresses and ABA-dependent plant development\(^ {31,32}. \) In rice, there are 10 SnRK proteins in \( SnRK2.0 \) family and are designated as SAPKs (stress-activated protein kinase). Among them, \( OsSAPK8, OsSAPK9, \) and \( OsSAPK10, \) were also activated by ABA\(^ {33}. \) \( OsSAPK1 \) can be upregulated by osmotic stressors and it has been reported that \( OsSAPK1 \) can be directly regulated by \( OsNAC2 \) through an ABA-dependent pathway\(^ {34}. \) Ectopic expression of \( SAPK6 (OSRK1) \) in tobacco confers reducing ABA sensitivity\(^ {35,36}. \) In our study, three \( OsSAPKs, OsSAPK1, OsSAPK6, \) and \( OsSAPK10 \) were downregulated by overexpressing \( Pvc3H69. \) All these results suggested that \( Pvc3H69 \) could interrupt both ABA biosynthesis and signaling, thereby suppressing leaf senescence in \( C3H69-OE \) plants.

Further evaluation of the relative transcript changes of ABA-biosynthesis and ABA-signaling genes in leaves in \( C3H69-OE \) lines and WT exposed to dark found that \( Pvc3H69 \) caused downregulation of all genes in the ABA signaling pathway in a greater magnitude compared to those genes for ABA biosynthesis, suggesting ABA signaling could be more sensitive to negative transcriptional control of \( Pvc3H69. \) In addition, \( ABIS \) and \( ABF2/3/4 \) are key phosphorylating substrates for SnRKs which belong to basic leucine zipper (bZIP) TFs and AREB binding TFs, respectively, and positively affect leaf senescence by directly anchoring the promoter of \( SAGs \) including \( SGR, NYC1, \) and \( ABR^{5,22}. \) The reduced transcript level of \( SnRKs, ABIS, \) and \( ABF2/3/4 \) in \( C3H69-OE \) plants treated with ABA (Fig. 5) indicated that \( Pvc3H69 \) could negatively regulate ABA-induced leaf senescence mainly through \( PP2C-SnRK-ABF \) signaling pathway.

In summary, overexpression of \( Pvc3H69 \) in rice or its native plants resulted in a stay-green phenotype, strongly suggesting that \( Pvc3H69 \) was a negative regulator in leaf senescence. \( Pvc3H69 \) could facilitate the stay-green phenotype or delayed leaf senescence mainly by upregulating light-dependent process of photosynthesis, including light-harvesting complex proteins, PSI proteins, and PSII proteins and repressing ABA biosynthesis and signaling genes and senescence-associated genes such as \( NCED3/5, AAO3, SnRK1/6/10, ABF2/3/4, ABIS, SGR, \) and \( NYC1 \) (Fig. 9). \( Pvc3H69 \) suppression of ABA-mediated leaf senescence with leaf aging or induced by darkness was mainly through regulating \( PP2C-SnRK-ABF \) signaling.

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**Fig. 9 Proposed pathways of \( Pvc3H69 \) regulating ABA-mediated leaf senescence: \( Pvc3H69 \) could repress the expression of \( NCED3/5 \) and \( AAO3, \) and then repressed ABA synthesis. In ABA transduction signaling, \( Pvc3H69 \) could also repress the expression of \( SnRK1/6/10 \) and further decrease the phosphorylation of \( SnRK1/6/10 \) on \( ABF2/3/4 \) and \( ABIS, \) and then further repressed the expression of \( SGR1/NYC1 \) and PSI/PSII protein degradation, then delay the leaf senescence.**
pathway. Future research could identify its upstream regulatory factors and further confirm the functions of PvC3H69 in leaf senescence induced by other abiotic stress, such as heat, drought, and salinity in order to improve plant tolerance to diverse environmental stresses.

**Experimental procedures**

**Gene cloning and vector construction**

The full-length gene of PvC3H69 (Phytozome accession no.: Pavir.J04795.1), with 394 amino acids without intron was amplified from the gDNA of a lowland ecotype ‘Alamo’ switchgrass. The gene was firstly cloned into the Gateway entry vector pENTR/D (Invitrogen). It was subcloned into p2GWF7.0, pGBK7 (Invitrogen), and pVT1629通过LR reaction (Invitrogen). The primers used for PvC3H69 cloning are listed in Table S1.

**Observation of subcellular localization of PvC3H69-GFP**

The PvC3H69 was subcloned into a modified gateway-compatible P2GWF7.0 vector to put PvC3H69 in fusion with GFP. By polyethylene glycol (PEG)–mediated Arabidopsis protoplast transformation, the PvC3H69-GFP fusion gene was overexpressed in Arabidopsis protoplasts. DAPI was used to stain the nucleus, and the GFP signals were detected under a Zeiss LSM 780 laser scanning confocal microscope (Carl Zeiss SAS, Jena, Germany).

**Transactivation assay**

PvC3H69 was subcloned into the BD vector pGBK7 to fuse PvC3H69 with the DNA-binding domain of GAL4. The pGBK7-PvC3H69 and the control vector pGBK7-GUS (UidA gene) were then transformed into the yeast strain Y2HGold (Clonetech), separately. The pGBK7-PvC3H69 was used as a positive control. The transformed positive clones grown well on SD/-Trp were then grown on plates containing SD/-Trp-Ade-His and SD/-Trp-Ade-His + 25 mM 3-AT for auto-transactivation assay.

For the transcriptional activity assay of PvC3H69 in plant cells, the C3H69 was cloned into the 35S promoter-driven pZB370 vector to fuse with the yeast GAL4 DNA-binding domain (GAL4BD) as effector (pZB369-PvC3H69), while the vector without the target gene was used as the negative control. As a positive control, PvC3H72 has been reported to be a transcriptional activator.

**Plant transformation and verification**

Switchgrass genetic transformation followed the protocol described in Xu et al. Embryogenic calluses of a selected line ‘HR8’ from switchgrass lowland ecotype ‘Alamo’ were infected with Agrobacterium tumefaciens strain ‘AGL1’ harboring the binary vector pVT1629-PvC3H69 with the target gene under driven of the maize ubiquitin promoter, and selected the putative transgenic lines on 50 mg L\(^{-1}\) hygromycin (Sigma). GUS staining and regular PCR for the presence of the T-DNA fragment of transgenic lines were the same as reported previously.

Nipponbare (Oryza sativa japonica cv Nipponbare) was used in this study. The transformation system was referred to Toki et al. Leaves of transgenic lines were stained with GUS solution. DNA was extracted from the C3H69-OE plants for PCR detection. RNA was extracted from the leaves of transgenic lines as a template for determining the transcriptional level of PvC3H69.

**Plant growth conditions and dark treatment**

Switchgrass transgenic lines and WT were grown in the green house with temperatures set at 28 °C/22 °C, day/night with a 14-h light/10-h darkness. The plants were watered twice a week. In order to induce leaf senescence, the middle 1/3 part of whole detached full-expand leaves from 3-month-old seedlings were cut into 3-cm segments and placed in a dark room with air temperature controlled at 28 °C. Leaf samples were collected at the time points of 0, 10, and 15 day for phenotypic and physiological analysis. Five-month-old plants were used for phenotypic and physiological analysis of natural senescence.

T3 seeds of WT and transgenic rice ‘Nipponbare’ were sterilized and germinated on 1/2 Murashige and Skoog (MS) medium, which were transferred to bucket filled with IRRI (International Rice Research Institute) nutrient solution in a growth chamber controlled at 30 °C during the day and 25 °C at night with 16-h light/8-h of darkness. The middle 1/3 part of whole detached full-expand leaves of 6-week-old plants were cut into about 3-cm fragments for dark treatment with air temperature controlled at 25 °C. Leaf fragments were collected at the time points of 0 d (day), 10 d, and 13 d for further phenotype and physiological index measurement. For ABA treatment, leaf fragments (same cut with the dark treatment) of WT and transgenic rice were soaked in different concentration ABA solutions (0, 5, and 20 µM) under darkness. Leaf samples were collected at the time points of 0, 7, and 10 d for further phenotype and physiological analysis. Samples at 7 day were used for qRT–PCR analysis.

**Physiological analysis of transgenic switchgrass and rice**

Leaf net photosynthetic rate (Pn) was measured with a LI-6400 system (LI-COR Inc., Lincoln, NE) equipped with a standard 2 × 3 cm\(^{2}\) leaf chamber with light-emitting diodes
as a light source. The measurements were taken at the PAR of 800 μmol m⁻² s⁻¹ and flow rate of 500 μmol s⁻¹. The block temperature was set to 25 °C for optimal temperature. For leaf photochemical efficiency (Fv/Fm), leaves were put in a 30 min dark-adaptation period and measured using a fluorescence induction monitor (OPTI-Sciences, Hudson, USA) as the ratio of variable (Fv) to maximum (Fm) fluorescence. Chlorophyll content (Chl) was measured by extracting chlorophyll from 0.1 g fresh leaves in 10 ml dimethyl sulfoxide (DMSO) under dark for 4 d and measuring the absorbance at 663 and 645 nm. The blades were dried in an oven at 80 °C for dry weight. Chl content was calculated using the formula described in Arnon.

For endogenous ABA content analysis, leaves of 4-week-old rice seedlings were used to measure the endogenous ABA content. ABA was extracted from 500 mg frozen leaf powder according to the method reported by Krishnan et al. The samples were suspended with extraction buffer (methanol:water:acetic acid, 80:19:1, v/v/v) and shake for 12 h at 4 °C, then centrifuged at 14,000 rpm for 20 min. The supernatant was collected in a new tube, pellet were reextracted with 500 μl extraction buffer, shaken for 4 h at 4 °C under darkness, then centrifuged at 14,000 rpm for 20 min at 4 °C. Two tubes of supernatant were mixed and dried using centrifugal vacuum concentrator and then dissolved in 300 μL methanol. The resulted supernatant ABA concentration was determined by high-performance liquid chromatograph using SCIEX-6500Qtrap mass spectrometer (HPLC-MS/MS; Agilent1290, Agilent, USA).

Rice transcriptome analysis and qRT–PCR analysis

For transcriptome analysis, total RNA was extracted from the leaves of 4-week-old rice WT and overexpressing PvC3H69 (C3H69-OE) plants exposed to dark treatment for 10 d and prior to dark treatment (0 d) according to the manufacturer’s instructions using RNA extract kit (Invitrogen, Carlsbad, CA, USA). mRNA was enriched by Oligo (dT) beads. Then the enriched mRNA was fragmented into short fragments and reverse into transcribed into cDNA. cDNA were purified with QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands). Three independent biological replicates were conducted for the WT and C3H69-OE plants. The differently expressed genes in WT and C3H69-OE plants were classified functionally using the biological process category of Rice Gene Ontology (ftp://ftp.ensemblgenomes.org/pub/plants/release-39). Significant interactors were determined using a two-sample analysis (t test).

For qRT–PCR analysis, total RNA extraction, first-strand cDNA synthesis, PCR reaction, and data analysis were the same as our previously reported by Xie et al. Primers for qRT–PCR are listed in Table S1.

Statistical analysis

Leaf senescence (including dark-induced and age-induced) and ABA treatment effects and variations among WT and transgenic plants for physiological parameters and gene expression levels were analyzed using SAS v9.2 (SAS Institute, Cary, NC, USA). Mean data were separated Fisher’s protected LSD at the probability of 0.05.

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Author contributions

B.H. developed research ideas, organized results, and wrote the manuscript; B.X. developed research idea, designed experiments, and organized data analysis. Z.X. and G.Y. performed experiments, S.L. and C.Z. conducted the transcriptomic qRT–PCR verification; Z.X. analyzed all data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Supplementary information

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