Arabidopsis CPR5 plays a role in regulating nucleocytoplasmic transport of mRNAs in ethylene signaling pathway

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Abstract

Key message Arabidopsis CPR5 is involved in regulation of ethylene signaling via two different ways: interacting with the ETR1 N-terminal domains, and controlling nucleocytoplasmic transport of ethylene-related mRNAs.

Abstract The ETR1 receptor plays a predominant role in ethylene signaling in Arabidopsis thaliana. Previous studies showed that both RTE1 and CPR5 can directly bind to the ETR1 receptor and regulate ethylene signaling. RTE1 was suggested to promote the ETR1 receptor signaling by influencing its conformation, but little is known about the regulatory mechanism of CPR5 in ethylene signaling. In this study, we presented the data showing that both RTE1 and CPR5 bound to the N-terminal domains of ETR1, and regulated ethylene signaling via the ethylene receptor. On the other hand, the research provided evidence indicating that CPR5 could act as a nucleoporin to regulate the ethylene-related mRNAs export out of the nucleus, while RTE1 or its homolog (RTH) had no effect on the nucleocytoplasmic transport of mRNAs. Nuclear qRT-PCR analysis and poly(A)-mRNA in situ hybridization showed that defect of CPR5 restricted nucleocytoplasmic transport of mRNAs. These results advance our understanding of the regulatory mechanism of CPR5 in ethylene signaling.

Keywords CPR5 · Ethylene · Signaling · Nucleocytoplasmic transport · Arabidopsis

Introduction

Ethylene is the simplest gaseous plant hormone and widely distributed in plant tissues and cells. Previous studies showed that ethylene, as an important plant hormone, played important roles in regulating plant growth and development, such as seed germination, fruit maturity, flower development, sex determination (Kamachi et al. 1997; Yamasaki et al. 2000), and leaf development, senescence, and abscission (Bieleski and Reid 1992; Kieber and Ecker 1993; Guerrero et al. 1998). Ethylene is also involved in plant responses to biotic and abiotic stresses, including resistance to hypoxia (Fukao and Bailey-Serres 2008; Rzewuski and Sauter 2008; Magneschi and Perata 2009; Justin and Armstrong 2010), drought and salt responses, inhibition of hypocotyl elongation under dark condition, and relief of photooxidation stress (Roman and Ecker 1995; Bleecker and Kende 2000).

When ethylene or ethylene precursor ACC was added to the medium, the dark-grown Arabidopsis seedlings exhibited typical characteristics, termed the ethylene “triple response” with exaggerated apical hook, inhibited root and hypocotyl elongation, and swelled hypocotyl (Bleecker et al. 1988; Guzmán and Ecker 1990). Using the ethylene “triple response” assay, a large number of Arabidopsis mutants with altered ethylene sensitivity were isolated. Based on genetic studies of the ethylene responsive mutants, a linear ethylene signal transduction pathway emerged (Bleecker et al. 1988; Guzmán and Ecker 1990; Kieber et al. 1993; Roman and Ecker 1995; Guo and Ecker 2004).

Ethylene signaling pathway starts with the binding of ethylene to receptors, and then transmits through downstream factors, and finally reaches the nucleus and activates expression of the ethylene-responsive genes; thereby, resulting in various ethylene responses. So far, some of the key regulatory factors in the pathway have been isolated, including the ethylene receptors (ETR1, ETR2, ERS1, ERS2, EIN4) (Chang et al. 1993; Hua et al. 1998; Hua and Meyerowitz...
plants, such as K+ dynamic balance, ABA signal transduction, and CPR5 based on their regulatory functions in the ETR1 receptor, we and colleagues previously reported that RTE1 may promote ETR1 signaling by influencing the conformation of the ethylene binding domain and/or its equilibrium state (Resnick et al. 2008). The Arabidopsis RTE1 encodes a protein containing 250 amino acids, mainly localized in the ER and Golgi (Zhou et al. 2007; Dong et al. 2008). There are three homologues of RTE1 in tomato, one of them was reported to be involved in the regulation of ethylene responses (Barry and Giovannoni 2006; Klee 2006; Ma et al. 2012). The rice RTE1 homologue OsRTH1 and the carnation RTE1-like genes (DeRTE1 and DeRTH1) were shown to be involved in ethylene regulated functions in seedling growth and flower senescence (Yu et al. 2011; Zhang et al. 2012).

RTE1 is a positive regulator of the ETR1 receptor, and they can physically interact (Resnick et al. 2006; Dong et al. 2010). Genetic analyses showed that RTE1 is essential for ETR1 to function in Arabidopsis, but not for the other ethylene receptors (Resnick et al. 2006, 2008). It was suggested that RTE1 may promote ETR1 signaling by influencing the conformation of the ethylene binding domain and/or its equilibrium state (Resnick et al. 2008). The Arabidopsis RTE1-HOMOLOG (RTH), shared the similar gene expression pattern and protein subcellular localization with RTE1, was thought to regulate ethylene signaling via a physical interaction with its homologue (Zheng et al. 2017).

CPR5 was initially isolated from the research on plant systemic acquired resistance (Bowling et al. 1997; Boch et al. 1998). The study showed that CPR5 participated in different physiological and pathological processes in plants, such as K+ dynamic balance, ABA signal transduction, redox balance, programmed cell death, and ROS status and signal transduction (Kirik et al. 2001; Jing et al. 2005, 2010; Jing and Dijkwel 2008). CPR5 was also shown to be involved in the regulation of gene replication, cell division, cell proliferation and spontaneous cell death (Brininstool et al. 2008; Perazza et al. 2011; Bao and Hua 2014). More recently, it was reported that CPR5 may act as a nucleoporin to play a role in controlling of triggering immunity and programmed cell death (Gu et al. 2016).

In the present study, we provided evidence showing that CPR5 can directly interact with the N-terminal transmembrane domains of the ETR1 receptor. CPR5 may mediate the interaction with the ETR1 receptor to regulate ethylene signaling. By using poly(A)-mRNA in situ hybridization and nuclear qRT-PCR analysis, we detected the effect of CPR5 on the nucleocytoplasmic transport of ethylene-related mRNAs, suggesting that CPR5 may regulate nucleocytoplasmic transport of mRNAs in ethylene signaling pathway. These observations significantly advance our understanding of the regulation mechanism of CPR5 in ethylene signaling.

### Results

**CPR5 can bind to the N-terminal domains of ETR1**

As both CPR5 and RTE1 can directly bind to the ETR1 receptor (Dong et al. 2010; Wang et al. 2017), we attempted to examine whether they bind to the same site on the receptor. At first, the ETR1 was divided into three segments with different domains: the ETR1 (1–368 aa) contains three transmembrane domains and the GAF domain, the ETR1 (308–596 aa) has the histidine kinase domain, and the ETR1 (604–739 aa) consists of the response regulatory domain (Fig. 1A). Yeast split-ubiquitin assay was performed and results indicated that both CPR5 and RTE1 can interact with the ETR1 (1–368 aa), but not the other two fragments (Fig. 1B, C).

We further divided the ETR1 (1–368 aa) into three segments: the ETR1 (1–78 aa) contains two transmembrane domains, the ETR1 (79–146 aa) contains one transmembrane domain, and the ETR1 (140–368 aa) has only the GAF domain (Fig. 1D). Yeast split-ubiquitin assay showed that CPR5 could not interact with any of the segments (Fig. 1E). In contrast, RTE1 did interact with the ETR1 (1–78 aa), but not the other two segments (Fig. 1F).

The interactions were further examined by a bimolecular fluorescence complementation (BiFC) assay in living plant cells. As shown in Fig. 2, the BiFC results are consistent with those of the yeast split-ubiquitin experiments. These observations indicated that both CPR5 and RTE1 could bind to the N-terminal domains of ETR1. CPR5 could bind to a larger area on the receptor, while RTE1 could bind to a smaller region.
Synergistic effect may not exist between CPR5 and RTE1 in regulating the ETR1 receptor signaling

The fact that both CPR5 and RTE1 could bind to the ETR1 N-terminal domains prompted us to investigate whether there exists a synergistic effect in their regulation of the ETR1 receptor signaling. To answer this question, we generated the double mutant *cpr5-T3 rte1-3* by a genetic cross between *cpr5-T3* (Wang et al. 2017) and *rte1-3* (Resnick et al. 2006). Ethylene “triple response” assay showed that all the etiolated seedlings of *cpr5-T3*, *rte1-3*, and *cpr5-T3 rte1-3* were ethylene hypersensitive (Fig. 3A, B). The hypocotyl growth of the double mutant *cpr5-T3 rte1-3* appeared similar to that of *cpr5-T3* or *rte1-3*,
suggesting that synergistic effect may not exist in their regulation of the ETR1 receptor signaling.

**CPR5 affects the nucleocytoplasmic transport of the ETR1 mRNA**

As CPR5 may act as a nucleoporin in controlling of triggering immunity and programmed cell death (Gu et al. 2016), we examined whether CPR5 could function in regulating the nucleocytoplasmic transport of the mRNAs in ethylene signaling pathway. By in situ poly(A)-mRNA hybridization, we detected strong nuclear poly(A) signals in cpr5-T3, but not in WT, rte1-3, or rth-1 (Fig. 4). This observation indicated that the nuclear export of mRNAs was defected in the cpr5 mutant.

We next examined the nuclear accumulation of the ETR1 receptor mRNA in the mutant plants. The total RNA and nuclear RNA were extracted from the 10-day-old seedlings of WT, cpr5-T3, rte1-3, and rth-1, respectively. qRT-PCR analysis showed that accumulation of the ETR1 receptor mRNA in the nucleus of cpr5-T3 reached over 60% of its total level, while those in the nucleus of WT, rte1-3, or rth-1 were about 30%. As a control, the Actin1 mRNA accumulation in the nucleus of cpr5-T3 was almost the same as in WT, rte1-3, or rth-1 (Fig. 5).

**CPR5 may regulate the nucleocytoplasmic transport of the mRNAs in ethylene signaling pathway**

We further examined nucleocytoplasmic transport of the other ethylene-related mRNAs in cpr5-T3, including the ethylene receptors genes (ETR2, ERS1, ERS2, and EIN4), the downstream components (CTR1, EIN2, EIN3), and some of the ETHYLENE RESPONSIVE FACTORS (ERFs) in the pathway (Fig. 5). To our surprise, it was observed that the mRNAs of the ETR2, ERS1, ERS2, EIN4, and CTR1 were significantly aggregated in the nucleus of cpr5-T3, and their accumulation levels were more than 60% of totals, being similar to that of the ETR1. In contrast, the mRNAs of the EIN2, EIN3, and ERFs (ERF1, ERF2, ERF4, ERF11, ERF105) in ethylene signaling pathway did not dramatically accumulate in the nucleus of cpr5-T3 compared to that of WT. As controls, the abnormal mRNAs accumulations were not detected in the nucleus of rte1-3 or rth-1 (Fig. 5).

In addition, we examined the mRNAs accumulations of the genes which were not the components of ethylene signaling pathway such as ADF1, an regulator of actin cytoskeleton (Dong et al. 2001). Experiments revealed a significant increase of the ADF1 mRNA accumulation in the cpr5-T3 mutant. Moreover, the nuclear mRNA accumulation assay was carried out for the genes mainly involved in flowering pathway such as LEY, REM16, GI,
and AP1 (Yu et al. 2020), and the observations showed that increased nuclear aggregations of the mRNAs were detected for AP1 and GI, but not LFY and REM16 in cpr5-T3 (Fig. 5). Taken together, these studies suggested that CPR5 regulated mRNA nucleocytoplasmic transport may exist in different pathways.

Discussion

Previous studies showed that the rte1 mutations can restore the ethylene sensitivity of etr1-2 but not etr1-1 (Resnick et al. 2006), while the cpr5 mutations can restore the ethylene sensitivity of both etr1-2 and etr1-1 (Wang et al. 2017), indicating that CPR5 may function differently from RTE1 in regulating the ETR1 receptor signaling. The etr1-2 encodes an Ala102-to-Thr substitution in the putative ethylene binding domain, while the etr1-1 mutation (Cys65-to-Tyr) fully blocks ethylene binding (Schaller and Bleecker 1995; Hall et al. 1999). These studies suggested that CPR5 differed from RTE1 in regulating the ETR1 receptor signaling. As supported, the present study provided evidence indicating that CPR5 may act as a nucleoporin in regulating the nucleocytoplasmic transport of the mRNAs in ethylene signaling pathway, whereas RTE1 or its homolog (RTH) had no effect on the mRNAs nucleocytoplasmic transport (Figs. 4, 5).

RTE1 is mainly localized in the ER and Golgi and required for the function of ethylene receptor ETR1 (Resnick et al. 2006; Zhou et al. 2007; Dong et al. 2008). Genetic analyses revealed that Arabidopsis RTE1 is required for the ETR1 receptor signaling, but not for the other receptors, suggesting that RTE1 may play a specific role in regulating the ETR1 signaling (Resnick et al. 2006). The genetic analyses using the double mutants of cpr5-T3 with each of the ethylene insensitive mutants (etr1-2, etr1-1, ers1-10, etr2-1, ein2-1) showed that the ethylene insensitivity of the mutant etr1-2 and etr1-1 was suppressed by cpr5-T3, while those of the others (ers1-10, etr2-1, ein2-1) were not suppressed by cpr5-T3 (Wang et al. 2017), suggesting that the ETR1 receptor may also be the main target of CPR5 in ethylene signaling pathway. As an ETR1 associated protein, CPR5 may regulate the ETR1 receptor signaling via protein–protein interaction with the receptor. As supported, both CPR5 and RTE1 proteins are localized in the ER and Golgi (Gu et al. 2016; Wang et al. 2017), and both of them can interact directly with the ETR1 receptor (Wang et al. 2017). In this study, we further analyzed the binding domains of ETR1 by CPR5 or RTE1, and the results revealed that RTE1 could bind to a smaller region which contains two transmembrane domains, and the CPR5 binding required at least three transmembrane domains and the GAF domain of ETR1 (Figs. 1, 2).

It is worth to note that all the etiolated seedlings of cpr5-T3, rte1-3, and cpr5-T3 rte1-3 were ethylene hypersensitive, and the hypocotyl lengths of the double mutant cpr5-T3 rte1-3 appeared similar to those of the mutant cpr5-T3 or rte1-3 (Fig. 3), suggesting that synergistic effect may not exist in their regulation of the ETR1 receptor signaling. Nevertheless, it is suggested that the
enhanced ethylene sensitivity of cpr5-T3 may be largely attributed to the defected nucleocytoplasmic transport of the ETR1 mRNA and the other ethylene-related mRNAs (ETR2, ERS1, ERS2, EIN4, CTR1) in the mutant, while the phenotype of rte1-3 may not be caused by alteration of the nucleocytoplasmic transport of mRNAs (Figs. 4, 5).

CPR5 is originally isolated from the research on plant pathogenesis (Bowling et al. 1997; Boch et al. 1998). Arabidopsis CPR5 has five transmembrane domains at its C-terminus and nuclear localization signal at the N-terminus. Subcellular localization analysis indicated that CPR5 is localized in the ER and Golgi membrane, and the nuclear membrane (Gu et al. 2016). CPR5 was reported to be able to act as a nucleoporin in controlling of triggering immunity (Gu et al. 2016). For example, overexpression of CPR5 resulted in the retention of stress and hormone related NPR1, JAZ1 and ABI5 in the cytoplasm (Gu et al. 2016). However, whether CPR5 regulates ethylene signaling via controlling of nucleocytoplasmic transport of mRNAs has not been reported. The present study provided evidence showing that CPR5 may play a crucial role in regulation of the nucleocytoplasmic transport of the mRNAs in ethylene signaling pathway. When the mutant cpr5-T3 was examined for poly(A)—mRNAs by in situ hybridization, obvious mRNAs aggregation was detected in the nucleus of cpr5-T3 (Fig. 4). Further qRT-PCR analysis showed that the ethylene signaling-related mRNAs including those of ETR1, ETR2, ERS1, ERS2, EIN4, and CTR1 were dramatically accumulated in the nucleus of cpr5-T3, but not in the controls (WT, rte1-3, rth-1) (Fig. 5). Interestingly, there were no significant accumulations of the mRNAs of EIN2, EIN3, and some ethylene-induced ERFs in the nucleus of cpr5-T3, suggesting that CPR5 may regulate the nucleocytoplasmic transport of the mRNAs in ethylene signaling pathway. It is worth to note that the negative regulators of ethylene signaling (ETR1, ETR2, ERS1, ERS2, EIN4, and CTR1) were obviously affected, whereas the positive factors (EIN2, EIN3) were barely affected in the mutant cpr5-T3. This observation may give a reason why the cpr5-T3 mutant exhibited enhanced ethylene sensitivity phenotype. On the other hand, some downstream ERFs may function as both positive and negative regulators in ethylene signaling, none of them seem to be compromised in their export out of the nucleus. For example, it was reported that some EAR-domain containing ERFs can function as both positive or negative regulators of transcription and ethylene responses (Lyons et al 2013).
Fig. 5 Analysis of the relative accumulation of mRNAs in the nucleus of WT (Col-0), cpr5-T3, rte1-3 or rth-1 by qPCR. Plants grown on 1/2 MS medium for 10 days were used for RNA extract.

Interestingly, the increased aggregations of the nuclear mRNAs were observed for AP1 and GI but not for LFY and REM16 in the mutant cpr5-T3 in flowering pathway (Fig. 5), suggesting that the CPR5-mediated regulation of mRNAs nucleocytoplasmic transport also existed in the other pathways. Further research about the regulation mechanism and target selectivity is needed in the future study.

Nucleoporin is a basic unit of the nuclear pore complex (NPC) which controls the two-way transport of RNA and protein between nucleus and cytoplasm. Yeast NPC contains 35 to 50 unique proteins (Yang et al. 1998; Rout et al. 2000), while mammalian NPC is a larger complex consisting of 80 to 100 unique proteins (Görlich and Kutay 1999). In plants, at least 30 nucleoporins were identified, involved in different events including flowering, pathogen interaction, nodulation, cold response, and hormone signaling (Zhang and Li 2005; Dong et al. 2006; Parry et al. 2006; Meier and Brkljacic 2009; Tamura et al. 2010). CPR5 was thought to be a plantspecific transmembrane nucleoporin that may contribute to the stability of the NPC core scaffold (Gu et al. 2016). Nucleoporins play multiple roles in various biological processes. In this study, we provided evidence supporting that CPR5 may function as a nucleoporin in regulating ethylene response via affecting the nucleocytoplasmic transport of the mRNAs in ethylene signaling pathway (Fig. 5).

Compared to the studies in yeast and metazoa, the nucleocytoplasmic transport of mRNAs remains less understood in plants (Ehrnsberger et al. 2019a). An early study showed defect of nucleocytoplasmic transport of mRNAs in the Arabidopsis nap160 mutant (Dong et al. 2006), but the ethylene response of the mutant was not clear. It was reported that the quadruple mutant (aly1aly2aly3aly4)
showed defects in plant growth, flowering and seed production, and a nuclear accumulation of mRNAs (Pfaff et al. 2018). The UIF-like proteins bind to RNA and UAP56, and cooperate with ALY1-4 to regulate nucleocytoplasmic mRNA transport (Ehrnsberger et al. 2019b). UAP56 encodes a DEAD-box RNA helicase, and acts together with ALY and MOS11 in regulating nuclear accumulation of mRNAs. The mos11 knockout plants show nuclear accumulation of mRNAs (Germain et al. 2010). Recently, it was reported that two CaMV proteins, the coat protein P4 and reverse transcriptase P5, play important roles in nuclear export of mRNAs (Kubina et al. 2021). In the present study, we provided evidence demonstrating that Arabidopsis CPR5 play a role in nuclear accumulation of mRNAs in ethylene signaling pathway. Although it was thought that pre-mRNA splicing may play an essential role for the nuclear export of mature mRNAs, the regulation mechanism needs further investigation.

**Materials and methods**

**Plant materials and ethylene response assays**

Seeds of the wild type (WT, Col-0) or mutants were surface sterilized and then sowed on 1/2 MS (Murashige and Skoog) medium or in soil in a controlled environment growth chamber set at 21 °C with 16 h light/8 h in darkness. The Arabidopsis ethylene response mutants were either obtained from the previous studies (Dong et al. 2008; Wang et al. 2017; Zheng et al. 2017), or generated by genetic crossing. The F2 progeny from the crosses were screened by specific PCR markers, as previously described (Resnick et al. 2006; Wang et al. 2017).

The ethylene response of Arabidopsis seedling was examined in the presence of ACC at different concentrations (0, 0.5, 5, 10, 20, 100 μM) as previously described (Wang et al. 2017). After sterilization, seeds were sowed on 1/2 MS medium containing ACC, and treated at 4 °C for 3 days. Thereafter, the plates were moved to a growth chamber for 8 h under white light, and then wrapped with aluminum foil and placed in a growth chamber for indicated periods. Measurement of hypocotyl length and statistical data were evaluated by Student’s t test.

**Yeast split-ubiquitin assay**

For yeast split-ubiquitin assay, the portions of Arabidopsis ETR1 (1–368 aa; 369–596 aa; 597–739 aa; 1–78 aa; 79–139 aa; and 140–368 aa) were each PCR-amplified from an existing template, cloned into the pMD18-T vector and then transferred into a bait vector pPR3-N or a prey vector pBT3-STE through the restriction sites shown in the supplementary table (Table S1). The constructions were verified by sequencing of the inserts. The bait or prey vector harboring a full-length of ETR1, CPR5, or RTE1 was obtained from the previous study (Wang et al. 2017).

**Bimolecular fluorescence complementation (BiFC) assay**

To generate the constructs harboring the fusions of cYFP-ETR1 (1–368 aa) and cYFP-ETR1 (1–78 aa), the coding sequences of cYFP (466–717 aa), ETR1 (1–368 aa), and ETR1 (1–78 aa) were each PCR-amplified from existing templates as previously described (Wang et al. 2016), and cloned into the pMD18-T vector. After sequencing verification of the inserts, the fusions were cloned into a binary vector, pCambia1300-3HA, through the restriction sites (Xba I and Kpn I for cYFP; Kpn I and BamH I for ETR1 and ETR1 (1–78 aa). The constructs containing the fusions of nYFP-CPR5 and nYFP-RTE1 were obtained from the previous study (Wang et al. 2017). Transformation mediated by Agrobacterium (GV3101) using onion epidermal cells was according to the previous study (Xu et al. 2014). Fluorescent signal from transfected cells was examined under a fluorescence microscope (Nikon ECLIPSE Ti-S, Japan) for a preview and a laser scanning confocal microscope (Leica TCS SP5, Germany) for phototopictures. The primers used for the constructs of BiFC assay are listed in supplementary table (Table S2).

**RNA extraction and qRT-PCR analysis**

The total RNA was extracted from the 21-day-old Arabidopsis seedlings according to TRNzol (TIANGEN, China). Half of the same sample was used for the nuclear RNA extraction essentially as described by Wang et al. (2011) with minor modifications. 3 g of the fresh seedlings were ground into powder in liquid nitrogen, and 6 mL extract A (0.3 M sucrose, 50 mM Tris–HCl, 40 U/mL RNase inhibitor, pH 8.5) was added to the ground material. After further grinding, the residue was filtered by microcloth, and the filtrate was collected in the RNase free tube. The residue was added to extract A to continue grinding and filtering. The two filtrates were mixed and centrifuged at 500 g under 4 °C for 5 min. The supernatant was discarded, and 1 mL of extract B (20 mM Tris–HCl, pH 8.5, 25% glycerol, 2.5 mM MgCl2, and 0.2% Triton X-100) was added to the sediment and suspended. The samples were centrifuged at 1300 g under 4 °C for 2 min. After repeating two times, 300 μL of extract C (20 mM Tris–HCl, pH 8.5, 0.25 M sucrose, 10 mM MgCl2, 0.5% Triton X-100, and 350 U/mL RNase inhibitor) was added to the sediment and suspended. The samples were centrifuged at 13000 g under 4 °C for 2 min. After repeating two times, 300 μL of extract D (20 mM Tris–HCl, pH 8.5, 1.7 M sucrose, 10 mM MgCl2, 0.5% Triton X-100, and 350 U/mL RNase...
inhibitor) was added to a new RNase-free tube. Then, the heavy suspension from the extract C was slowly added to the upper layer of extract D, and centrifuged at 1300g under 4 °C for 10 min. The supernatant was discarded, and 1 mL TRNzol reagent was added to the sediment, and the nuclear RNA was extracted accordingly.

cDNA was synthesized with oligo(dT) primers using PrimeScript™ 1st strand cDNA synthesis kit (Takara, Japan). Quantitative RT-PCR analysis was performed on an Agilent Real-Time qPCR apparatus (Mx3000P system) using SYBR Premix ExTaq TM II (Takara, Japan). Biological replicates for each set of experiments were carried out three times, and the mean value of three replicates was normalized using Tubulin8 as an internal control. Quantitative RT-PCR was conducted at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s. The primers used for quantitative RT-PCR are listed in supplementary table (Table S3).

Poly(A)-mRNA in situ hybridization

Examination of the Poly(A)-mRNA in situ localization was performed as previously described (Engler et al. 1994; Gong et al. 2005). Fresh leaves taken from the 21-day-old seedlings were put into a fixation cocktail containing 10 ml of 50% fixation buffer (120 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, 2.7 mM KCl, 0.1% Tween 20, 80 mM EGTA, 1% DMSO, 5% formaldehyde) and 50% heptane. After shaking gently at room temperature for 30 min, the samples were transferred into anhydrous methanol for dehydration for 5 min, and repeated once. Then the samples were transferred into anhydrous ethanol for further dehydration for 5 min, and repeated twice. The samples were incubated in a mixture with 50% ethanol and 50% xylene for 30 min. After washing twice with anhydrous ethanol for 5 min at each time, the samples were washed twice with anhydrous methanol for 5 min each. The samples were transferred into a mixture with 50% methanol and 50% fixation buffer (excluding 5% formaldehyde) for 5 min, and then transferred into a fixation buffer containing 5% formaldehyde for 30 min at room temperature. After fixation, the samples were washed twice with the fixation buffer without 5% formaldehyde, and washed once with 1 ml of perfect Hyb Plus hybridization buffer (h-7033, Sigma Aldrich). Then 1 ml of hybridization buffer was added to each sample for prehybridization at 50 °C for 1 h. After prehybridization, 5 pmol of 45-mer oligo(dT) labeled with one molecule of 6-FAM fluorescein at N-terminus (synthesized by Sangon Biotech, China) at 50 °C in darkness for more than 8 h. After hybridization, the samples were washed with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) and 0.1% SDS for 60 min, and then washed with 0.2× SSC and 0.1% SDS at 50 °C for 20 min in darkness. The samples were observed immediately using a laser scanning confocal microscopy (Leica TCS SP5, Germany) with a 488 nm excitation laser, and photopictures were taken from the leaf mesophyll cells. Each experiment was repeated at least three times, and similar results were obtained.

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Author contributions JC, XS, and BM performed most of the research and wrote the first draft of the manuscript. YL, NL, LQ, and YY performed mutant screening and some qRT-PCR analysis. CHD designed the experiments and revised the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest for publication.

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