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Authors

Zhang, Lili

Xu, Yanjie

Liu, Xuening

et al.

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RESEARCH PAPER

The chrysanthemum DEAD-box RNA helicase CmRH56 regulates rhizome outgrowth in response to drought stress

Lili Zhang[1,†,‡], Yanjie Xu[1,‡], Xuening Liu[1], Meizhu Qin[1], Shenglan Li[1], Tianhua Jiang[1], Yingjie Yang[1], Cai-Zhong Jiang[2,3], Junping Gao[1], Bo Hong[1,*] and Chao Ma[1,*]

[1] State Key Laboratory of Agrobiotechnology, Beijing Key Laboratory of Development and Quality Control of Ornamental Crops, Department of Ornamental Horticulture, College of Horticulture, China Agricultural University, Beijing 100193, China.
[2] Crops Pathology and Genetic Research Unit, United States Department of Agriculture, Agricultural Research Service, Davis, CA, 95616, USA.
[3] Department of Plant Sciences, University of California Davis, Davis, CA, 95616, USA.

[†]Present address: Beijing Key Laboratory of Agricultural Genetic Resources and Biotechnology, Institute of Biotechnology, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China.

‡These authors contributed equally to this work.
* Correspondence: mac@cau.edu.cn or hongbo1203@cau.edu.cn

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Abstract

Plants have evolved complex mechanisms to reprogram growth in response to drought stress. In herbaceous perennial plant species, the rhizome, which is normally an organ for propagation and food storage, can also support plant growth in stressful environments, and allows the plant to perennate and survive stress damage. However, the mechanisms that regulate rhizome growth in perennial herbs during abiotic stresses are unknown. Here, we identified a chrysanthemum (*Chrysanthemum morifolium*) DEAD-box RNA helicase gene, *CmRH56*, that is specifically expressed in the rhizome shoot apex. Knock down of *CmRH56* transcript levels decreased the number of rhizomes and enhanced drought stress tolerance. We determined that CmRH56 represses the expression of a putative gibberellin (GA) catabolic gene, *GA2 oxidase6* (*CmGA2ox6*). Exogenous GA treatment and silencing of *CmGA2ox6* resulted in more rhizomes. These results demonstrate that CmRH56 suppresses rhizome outgrowth under drought stress conditions by blocking GA biosynthesis.

Keywords: *Chrysanthemum morifolium*, *CmGA2ox6*, *CmRH56*, DEAD-box RNA helicase, drought stress, rhizome.

Introduction

Rhizomes are modified stems and consist of leaves, stems, or internodes, and contain meristematic tissue that can produce a daughter plant (Jernstedt and Bouton, 1985; Li and Beuselinck, 1996). As a propagation strategy and as storage organs, rhizomes support plant growth under abiotic stresses and allow plants to perennate and thus survive stress-induced damage (Ma *et al.*, 2020).

Rhizome growth is an induced response that adjusts to conditions in the surrounding environment (Marshall *et al.*, 2001; Zhou *et al.*, 2014). Indeed, rhizome growth can slow substantially, and even stop completely, until more favourable conditions are restored (Del Tredici, 2001). Rhizomes offer a major advantage in permitting plants to survive harsh environments. Under stressful environmental conditions, rhizomes can survive underground, where they store and allocate nutrients to support plant growth, while they can develop into aerial shoots when conditions become suitable for growth (C. Ma *et al.*, 2010; X.Q. Ma *et al.*, 2020). In addition, the rhizome meristem can produce nodes and adventitious roots to extend the root system of the mother plant (Guo *et al.*, 2021). However, the molecular mechanisms involved in rhizome initiation, as well as the growth and development in response to harsh environmental conditions are largely unknown.

Phytohormones such as auxin, abscisic acid (ABA), and gibberellin (GA) participate in rhizome development and growth (Guo *et al.*, 2021). Among them, GA plays a critical role in rhizome development. For example, several genes related to the GA biosynthesis and signalling pathway are expressed to high levels in rhizomes compared with regular stems in species such as lotus (*Nelumbo nucifera*), bamboo (*Phyllostachys praecox*), and *Oryza longistaminata* (Wang *et al.*, 2010; Hu *et al.*, 2011;Cheng *et al.*, 2013; He *et al.*, 2014). In tall fescue (*Festuca arundinacea*), the expression levels of several genes related to the GA pathway decreased in rhizomes experiencing drought stress, suggesting that drought-inhibited rhizome growth may also be modulated by the GA pathway (Ma *et al.*, 2020). However, the mechanisms that regulate the GA pathway to modulate rhizome growth have not been fully characterized.

RNA helicases are ubiquitous enzymes in eukaryotes that function in RNA metabolism (Rocak and Linder, 2004) and are divided into five superfamilies (SFs), SF1 to SF5. DEAD-box RNA helicases belong to RNA helicase SF2, which also comprises the most members. Increasing evidence has demonstrated that plant DEAD-box RNA helicases carry out various functions, such as developmental regulation (Stonebloom *et al.*, 2009; Burch-Smith *et al.*, 2011; Asakura *et al.*, 2012; Hsu *et al.*, 2014) and responding to abiotic stress (Gong *et al.*, 2005; Kant *et al.*, 2007; Huang *et al.*, 2010; Khan *et al.*, 2014). Moreover, DEAD-box RNA helicases have been reported to participate in plant development and responses to abiotic stresses by modulating phytohormone pathways such as that of auxin (Huang *et al.*, 2016a), ABA (Baek *et al.*, 2018) and GA (Huang *et al.*, 2016b). However, whether RNA helicases are involved in rhizome formation and growth in response to environmental cues is unknown.

Chrysanthemum (*Chrysanthemum morifolium*) is a herbaceous perennial plant that is commercially important worldwide as an ornamental crop. Rhizomes are the main organ responsible for its propagation and perennation. Both rhizomes and aerial shoots are derived from axillary buds on the mother plant, despite their distinct developmental patterns (Yoshida *et al.*, 2016). In chrysanthemum, the formation of axillary meristems can be recognized in early developmental stages of leaf primordia. Thus, changes in rhizome morphology in response to environmental cues are associated with growth and development of the rhizomes. Abiotic stresses, such as cold and drought, can inhibit chrysanthemum rhizome growth. However, the molecular mechanisms underlying rhizome growth in response to adverse growth conditions such as drought stress are not well understood.

During our studies of rhizome drought-responsive gene expression in chrysanthemum, we identified a gene annotated as an RNA helicase that was specifically expressed in the rhizome shoot apex, and whose transcript abundance dramatically decreased in response to drought. We hypothesized that this RNA helicase may play an important role in the regulation of rhizome development and growth under adverse environmental conditions. In this study, we tested this hypothesis and report that *CmRH56*, a chrysanthemum DEAD-box RNA helicase, influences GA metabolism to modulate rhizome outgrowth in response to drought stress.

Materials and methods

Plant materials and treatments

A chrysanthemum cultivar (*Chrysanthemum morifolium* 'Fall Color') used as plant material in this study was propagated by tissue culture as reported previously (Wei *et al.*, 2017). Forty-day-old plants were transplanted into 14 cm diameter pots containing peat and vermiculite (1:1, v/v; 144 g per pot), and were grown in a growth chamber at 23 ± 1 °C, under 100 μmol m^{-2} s^{-1} photosynthetically active radiation (fluorescent lamps, SINOL, China, SN-T5, 16W), and 16 h light/8 h dark photoperiod. Soil weight was determined as W_t, and another 144 g of soil was oven-dried at 65 °C for 72 h (W_d). The relative water content (RWC) was calculated as follows RWC (%) = $(W_t\text{-}W_d)/W_t \times 100$.

For different degrees of drought stress treatments, plants grown for 2 weeks after transplanting were watered to saturation, and then controlled water treatments were applied to gradually reduce the RWC of the soil to 50%, 20%, and 10%, which took approximately 17, 25, and 30 d, respectively. To maintain RWC at 50%, 20%, and 10% for ~30 d, daily water replenishment was carried out by adding the appropriate volume of water to the soil around the plant roots. Plants were randomly placed on the same culture shelf. Three independent experiments were performed with 10 plants in each experiment.

To determine plant survival rate under drought stress, wild-type (WT) and transgenic plants were planted in one pot. Plants grown for 2 weeks after transplanting were watered to saturation, and then water was withheld for ~35 d. Plant survival rate and rhizome regeneration rate were determined after 30 d of re-watering. Three independent experiments were performed with 10 plants of each line in each experiment.

To observe the occurrence time of rhizomes in *CmRH56*-OX and *CmRH56*-RNAi lines, 12 plants were used for each line. Under long-day conditions, the occurrence of rhizomes was observed 7 d after transplanting, and the days from transplanting to first occurrence of rhizome were recorded for *CmRH56*-OX and *CmRH56*-RNAi lines.

For gibberellin A_3 (GA_3) and paclobutrazol (PAC) treatments, plants were irrigated with 100 μM GA_3 or 100 μM PAC solutions. GA_3 and PAC solutions were applied every 10 d, and the number of rhizomes was determined 50 d after treatments. Three independent experiments were performed, and for each experiment 10 plants of each line were used.

Sub-cellular localization of CmRH56

First-strand cDNAs were synthesized from 1 μg total RNA extracted from chrysanthemum rhizome. The *CmRH56* open reading frame without the stop codon was amplified by PCR containing 1 μl cDNA as template with primers to incorporate *Bam*HI and *Sal*I sites in the amplified fragment, which was then cloned into the pEZS-NL vector (D. Ehrhardt, Carnegie Institution, Stanford, CA, USA). Primer information is given in Supplementary Table S1. The resulting construct was transformed into Arabidopsis protoplasts using polyethylene glycol (PEG)–mediated transformation. After culturing the transfected cells for 16~20 h at 22 °C, images were taken with an Olympus FV1000 confocal laser scanning microscope (Olympus, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Reverse transcription quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from chrysanthemum rhizomes using TRIzol reagent (Invitrogen, USA), and RNA quality was assessed by gel electrophoresis on a NanoDrop One microvolume UV-Vis spectrophotometer (ThermoFisher, USA). The removal of trace DNA from RNA samples and first-strand cDNA synthesis were performed using the FastQuant RT Kit with gDNase (Tiangen Biotech, China). qPCR was conducted in standard mode using an ABI StepOne Real-Time PCR System (Applied Biosystems, USA). The transcript levels of each gene were analysed from at least three biological replicates from three individual plants and three technical replicates. The chrysanthemum *Ubiquitin* gene (Wei *et al.*, 2017) was used as an internal control. The stable expression of *Ubiquitin* under all the experimental conditions in this study was tested relative to the other reference gene *EF1a* (Wang *et al.*, 2021; Supplementary Fig. S1). Relative transcript levels were calculated by the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). The primers and annealing temperature of each primer pair used for qPCR are listed in Supplementary Table S1. The accession numbers for the genes in this study are provided in Supplementary Table S2.

Chrysanthemum transformation

To construct the *35S:CmRH56* overexpressing vector, the *CmRH56* coding sequence was amplified containing 1 μl cDNA as template, and cloned into the pBIG vector (Becker, 1990).

To construct the *CmRH56*-RNAi and *CmGA2ox6*-RNAi vectors, 261 bp *CmRH56* and 238 bp *GA2 oxidase6* (*CmGA2ox6*) sense and antisense fragments were amplified and directionally inserted on either side of the *PDK* (*Pyruvate orthophosphate dikinase* from *Flaveria trinervia*) intron in the pHANNIBAL vector. The resulting ihpRNA constructs were then cloned into the pART27 binary vector (Gleave, 1992).

The resulting plasmids were transformed into chrysanthemum using the Agrobacterium (*Agrobacterium tumefaciens*)-mediated leaf disc transformation method (Wei *et al.*, 2017). PCR primers used for vector construction are listed in Supplementary Table S1. The accession numbers for the vectors used in the study are listed in Supplementary Table S2.

In situ hybridization

Chrysanthemum rhizomes and axillary shoot apices were fixed in 3.7% (v/v) formaldehyde-acetic-alcohol. Sample fixation, sectioning, and hybridization were performed as described previously (Zhang *et al.*, 2013). The *CmRH56* probe (236 bp) and the *CmGA2ox6* probe (299 bp) were linearized fragments designed based on the unique region of the corresponding coding sequences. The primers are listed in Supplementary Table S1.

Detection of poly(A) RNA by in situ hybridization

Total mRNA *in situ* localization was performed as described previously (Germain *et al.*, 2010). Briefly, small pieces of leaves from 40-day-old WT and *CmRH56*-RNAi chrysanthemum plants were immersed in 1 ml of fixation solution (120 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 2.7 mM KCl, 0.1% [v/v] Tween 20, 80 mM EGTA, 5% [w/v] formaldehyde, 10% [v/v] DMSO, and 50% [v/v] heptane). The samples were incubated in 1 ml methanol twice and in 1 ml ethanol three times, and then immersed in 1 ml ethanol/xylene (1:1, v/v) for 30 min at 24 °C. Methanol/fixation solution without formaldehyde (1:1, v/v) was added for 5 min twice. Samples were incubated in 1 ml PerfectHyb Plus (Sigma) at 50 °C for 1 h. The samples were incubated in 1 μl of 10 μM Alexa Fluor-488-labeled 48-mer oligo(dT) (Invitrogen) at 50 °C overnight. Images were taken with an Olympus FV1000 confocal laser scanning microscope (Olympus) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Results

Rhizome outgrowth is decreased in response to drought stress

To investigate the effects of drought stress on rhizome growth in chrysanthemum, we examined changes in rhizomes experiencing different degrees of drought stress in a controlled environment growth room. To this end, we withheld water to reach 50% RWC (slight drought), 20% RWC (moderate drought), or 10% RWC (severe drought) for 30 d. We observed that growth, measured by plant height, decreased under all drought conditions tested, with fewer rhizomes per plant compared with well-watered control plants. In addition, the number of rhizomes gradually decreased with lower RWC (Fig. 1A, B).

CmRH56 *expression is lower in rhizomes exposed to drought stress*

We examined the expression levels of drought-responsive genes in chrysanthemum in a dataset obtained from our previous studies (Ma *et al.*, 2010; Xu *et al.*, 2013). We identified a gene encoding a DEAD-box RNA helicase that is differentially expressed in rhizomes exposed to drought stress. Sequence comparisons of DEAD-box proteins from multiple species revealed that the encoded protein contains nine conserved motifs and belongs to the DEAD-box helicase sub-family (Supplementary Fig. S2A). Phylogenetic analysis showed that the chrysanthemum protein is most closely related to Arabidopsis DEAD-box RNA helicases RH56 and RH15, with the highest identity to RH56, prompting us to name it CmRH56 (Supplementary Fig. S2B). RT–qPCR analysis showed that *CmRH56* expression is down-regulated in rhizomes exposed to different degrees of drought stress (Fig. 2B). In addition, *in situ* hybridization revealed that *CmRH56* is highly expressed in rhizome shoot apices, but was expressed below detection levels in axillary shoot apices (Fig. 2A).

We also determined the sub-cellular localization of CmRH56 as a fusion protein between CmRH56 and green fluorescent protein (CmRH56-GFP) by transiently transfecting Arabidopsis protoplasts with the encoding construct. We observed that CmRH56-GFP localizes to the nucleus, while we detected the GFP control fluorescence in both the

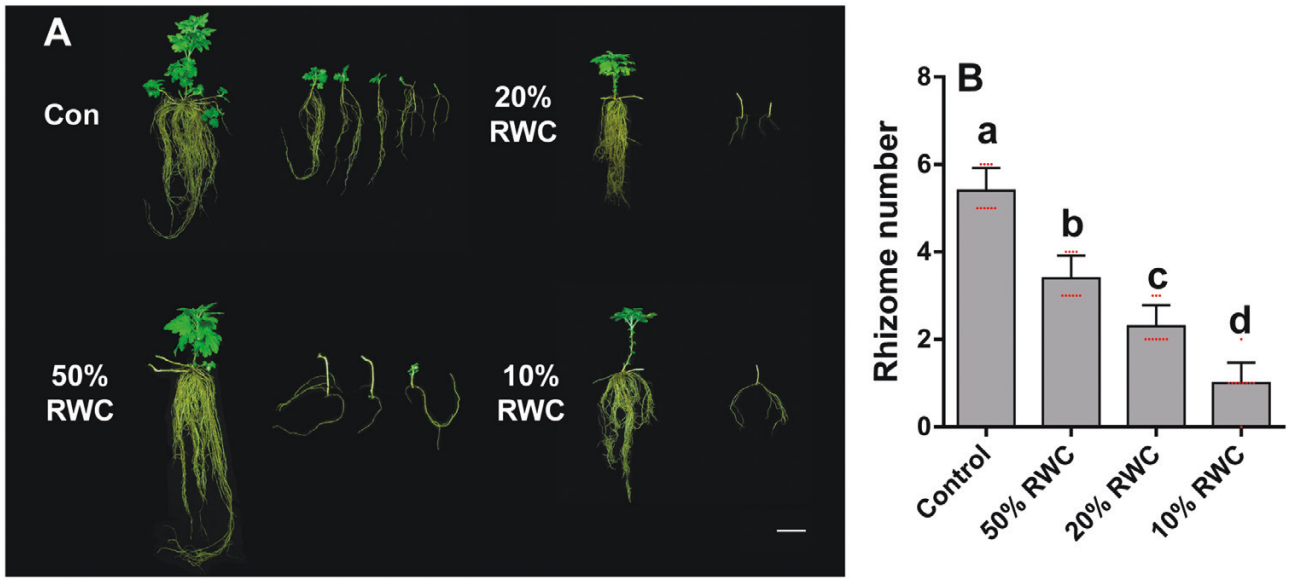

Fig. 1. Chrysanthemum plants produce fewer rhizomes in response to drought stress. (A) Rhizome phenotypes under different degrees of drought stress. Plants were subjected to different degrees of drought stress by mitigating irrigation to reach 50%, 20%, or 10% relative water content (RWC) for 30 d. All rhizomes from each plant were separated and are shown at the right. (B) Rhizome number under different degrees of drought stress. Results are the means of 10 biological replicates with standard deviation. Different letters indicate significant differences according to a Tukey-Kramer test (P<0.05). Con, control. Scale bar, 5 cm.

cytoplasm and the nucleus, suggesting that CmRH56 is a nuclear protein (Fig. 2C).

The DEAD-box RNA helicase CmRH56 participates in rhizome outgrowth in response to drought stress

To elucidate the roles of CmRH56 in drought tolerance, we generated transgenic chrysanthemum lines in which *CmRH56* transcript levels were lowered by RNA interference (RNAi) or ectopically overexpressed (OX) constructs. A reduction or increase in *CmRH56* expression in the transgenic lines compared with WT plants was confirmed by RT-qPCR analysis (Fig. 3B). We established that the aerial parts of *CmRH56*-RNAi and *CmRH56*-OX plants exhibit normal phenotypes, based on comparison with WT plants, when grown under non-stress conditions (Fig. 3A; Supplementary Fig. S3). However, under drought stress conditions, *CmRH56*-RNAi plants showed a higher survival rate, while *CmRH56*-OX plants were characterized by a lower survival rate than WT plants (Fig. 3A, C). We noticed that most surviving RNAi plants regenerated entire plants from axillary buds of the main plants. In contrast, *CmRH56*-OX plants produced new growth through rhizome survival (Fig. 3A, C).

We further tested the effects of ectopic *CmRH56* expression on drought stress tolerance associated with rhizomes. We determined that rhizome number in *CmRH56*-RNAi plants was significantly lower than in WT plants (P<0.05), while rhizome number in *CmRH56*-OX plants was significantly higher than in WT under both normal and drought stress conditions (P<0.05, Fig. 4A, C). In addition, we observed that *CmRH56*-OX plants develop rhizomes earlier relative to the WT, while *CmRH56*-RNAi plants had delayed rhizome development (Fig. 4B, D). Specifically, the rhizomes in the *CmRH56*-OX lines appeared about 3 d earlier than in the WT, while the rhizomes of *CmRH56*-RNAi lines appeared about 7 d later than in the WT (Fig. 4D).

Previous studies demonstrated that DEAD-box RNA helicases participate in RNA metabolism (Rocak and Linder, 2004). We therefore tested the function of CmRH56 in mRNA export by localizing poly(A) mRNA in chrysanthemum cells. Compared with WT plants, we observed a block in total mRNA export in *CmRH56*-RNAi chrysanthemum, as evidenced by the accumulation of a fluorescent probe inside the nucleus (Fig. 5). This result thus suggested that CmRH56 may participate in mRNA export.

GA biosynthesis is involved in CmRH56-regulated rhizome growth in response to drought stress

Given the known role for GA in rhizome growth, we investigated whether CmRH56 might participate in rhizome growth by affecting the GA pathway. We observed that exogenous GA treatment of WT plants caused an increase in rhizome number, whereas treatment with the GA biosynthesis inhibitor paclobutrazol (PAC) caused the opposite effect with a decrease in the number of rhizomes, indicating that GA plays a role in controlling rhizome outgrowth in chrysanthemum (Fig. 6). The exogenous application of GA to *CmRH56*-RNAi plants rescued their lower rhizome number phenotype, while

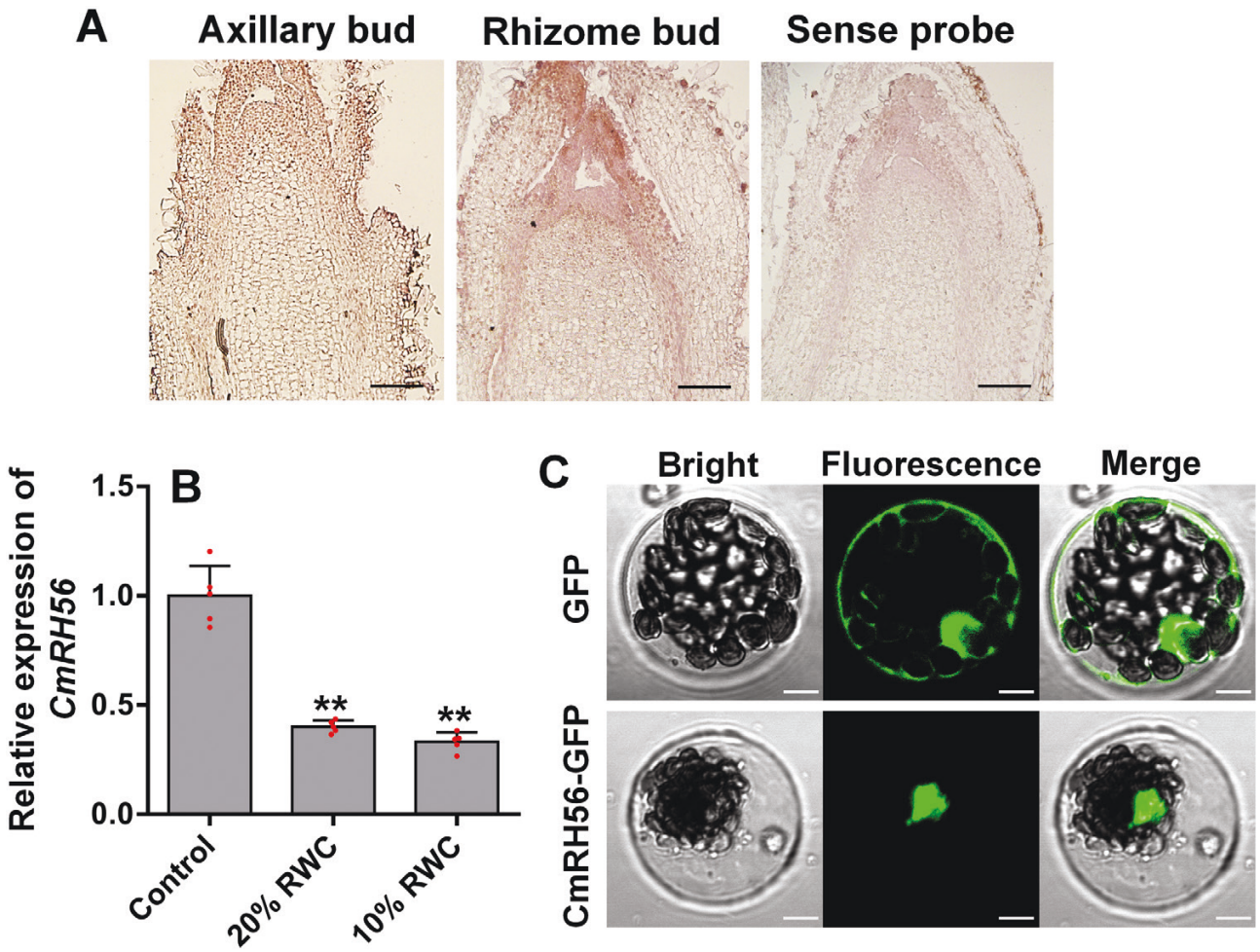

Fig. 2. *CmRH56* expression and sub-cellular localization of CmRH56-GFP fusion protein. (A) *CmRH56* mRNA accumulation pattern in rhizomes and axillary meristems, as shown by *in situ* hybridization. The sense *CmRH56* probe was used as a negative control. Scale bars, 200 μm. (B) Relative *CmRH56* transcript levels in chrysanthemum rhizomes grown under different degrees of drought stress, as determined by RT–qPCR. Plants were grown under different degrees of drought stress by withholding irrigation to reach 20% or 10% RWC. Chrysanthemum *Ubiquitin* gene was used as an internal control. Results are the means of five biological replicates with standard deviation. Statistical significance between different drought treatments and the control was determined using a Dunnett test (**P<0.01). (C) Sub-cellular localization of CmRH56-GFP fusion protein in Arabidopsis protoplasts. GFP, green fluorescent protein; CmRH56-GFP, CmRH56-GFP fusion protein. Images shown are brightfield (left), GFP fluorescence (middle), and merged (right). Scale bars, 10 μm.

treatment with PAC in *CmRH56*-RNAi plants caused a decrease in rhizome number (Fig. 6), suggesting that the GA treatment alleviates the suppressive effect of *CmRH56* RNAi-mediated silencing.

We then analysed the expression of GA-related genes in *CmRH56*-RNAi and WT plants by RT–qPCR (Supplementary Fig. S4). We observed that the expression of a gene encoding a putative GA catabolism enzyme, GA2 oxidase (GA2ox), is up-regulated in *CmRH56*-RNAi plants compared with WT plants (Fig. 7A). Multiple sequence alignment confirmed that the encoded protein belongs to the GA2ox family (Supplementary Fig. S5A). Phylogenetic analysis revealed Arabidopsis GA2ox6 as the closest protein to the chrysanthemum GA2ox, which we thus named CmGA2ox6 (Supplementary Fig. S5B). The expression of *CmGA2ox6* increased significantly in rhizomes under drought stress in WT plants (P<0.01; Fig. 7C), and *in situ* hybridization established that *CmGA2ox6* is highly expressed in rhizome shoot apices, but was not detectable in axillary shoot apices (Fig. 7B).

We next silenced *CmGA2ox6* in chrysanthemum using RNAi (Fig. 7E) and counted more rhizomes in *CmGA2ox6*-RNAi plants than in WT plants under both control and drought conditions (Fig. 7D, F).

Discussion

In herbaceous perennials, the modulation of rhizome growth under abiotic stresses is an adaptive strategy that enables plants to survive in harsh environments (C. Ma *et al.*, 2010;

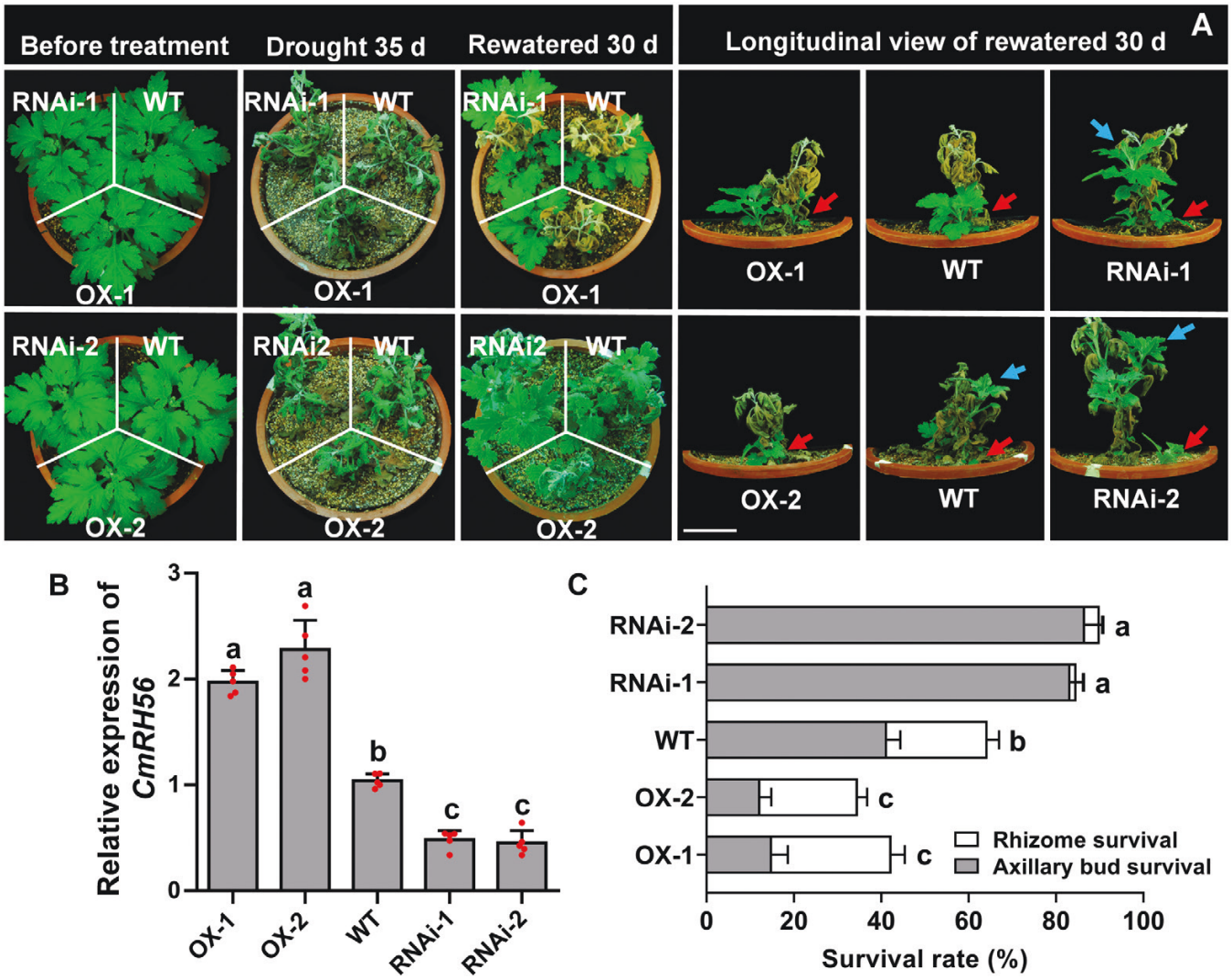

Fig. 3. Drought stress tolerance of *CmRH56*-OX and *CmRH56*-RNAi chrysanthemum plants. (A) Phenotypes of *CmRH56*-OX and *CmRH56*-RNAi plants under drought stress conditions. OX-1 and OX-2 correspond to two independent *CmRH56*-OX lines. RNAi-1 and RNAi-2 correspond to two independent *CmRH56*-RNAi lines. OX, RNAi, and wild-type (WT) plants were planted in one pot, and water was withheld for 35 d before a 30 d recovery period with regular watering. Blue arrows indicate resurrected axillary buds, and red arrows indicate resurrected rhizome buds. Scale bar, 5 cm. (B) Relative *CmRH56* transcript levels in *CmRH56*-OX, *CmRH56*-RNAi, and WT plants, as determined by RT–qPCR. Chrysanthemum *Ubiquitin* gene was used as an internal control. Results are the means of five biological replicates with standard deviation. (C) Rhizome and axillary bud survival rates in *CmRH56*-OX, *CmRH56*-RNAi, and WT plants grown under drought stress conditions. Three independent experiments were performed, and for each experiment, 10 plants were used for each line. Different letters indicate significant differences between survival rates according to a Tukey-Kramer test ($P<0.05$).

X.Q. Ma *et al.*, 2020). However, the mechanisms that regulate rhizome growth in response to abiotic stresses are largely unknown. Here, we identified a DEAD-box RNA helicase in chrysanthemum, CmRH56, that plays an important role in the regulation of rhizome outgrowth under drought stress. The regulatory roles of RNA helicases in response to biotic and abiotic stress have been demonstrated in numerous studies (Gong *et al.*, 2005; Kant *et al.*, 2007; Asakura *et al.*, 2012; Hsu *et al.*, 2014; Khan *et al.*, 2014). Accumulating evidence showed that the expression of genes encoding DEAD-box RNA helicases is transcriptionally regulated in response to adverse environmental conditions (Vashisht and Tuteja, 2006; Guan *et al.*, 2013). For example, in Arabidopsis, the two DEAD-box RNA helicase genes *STRESS RESPONSE SUPPRESSOR1* (*STRS1*) and *STRS2* were identified in a functional genomics screen (Kant *et al.*, 2007), and their expression is down-regulated by multiple abiotic stresses, such as salt, drought, and heat stress. Mutations in *STRS1* and *STRS2* enhanced plant tolerance against these stresses (Kant *et al.*, 2007). Our results also showed that the expression of *CmRH56* decreases in response to drought stress and that *CmRH56* silencing enhances drought stress tolerance (Figs 2, 3). Our study elucidates the

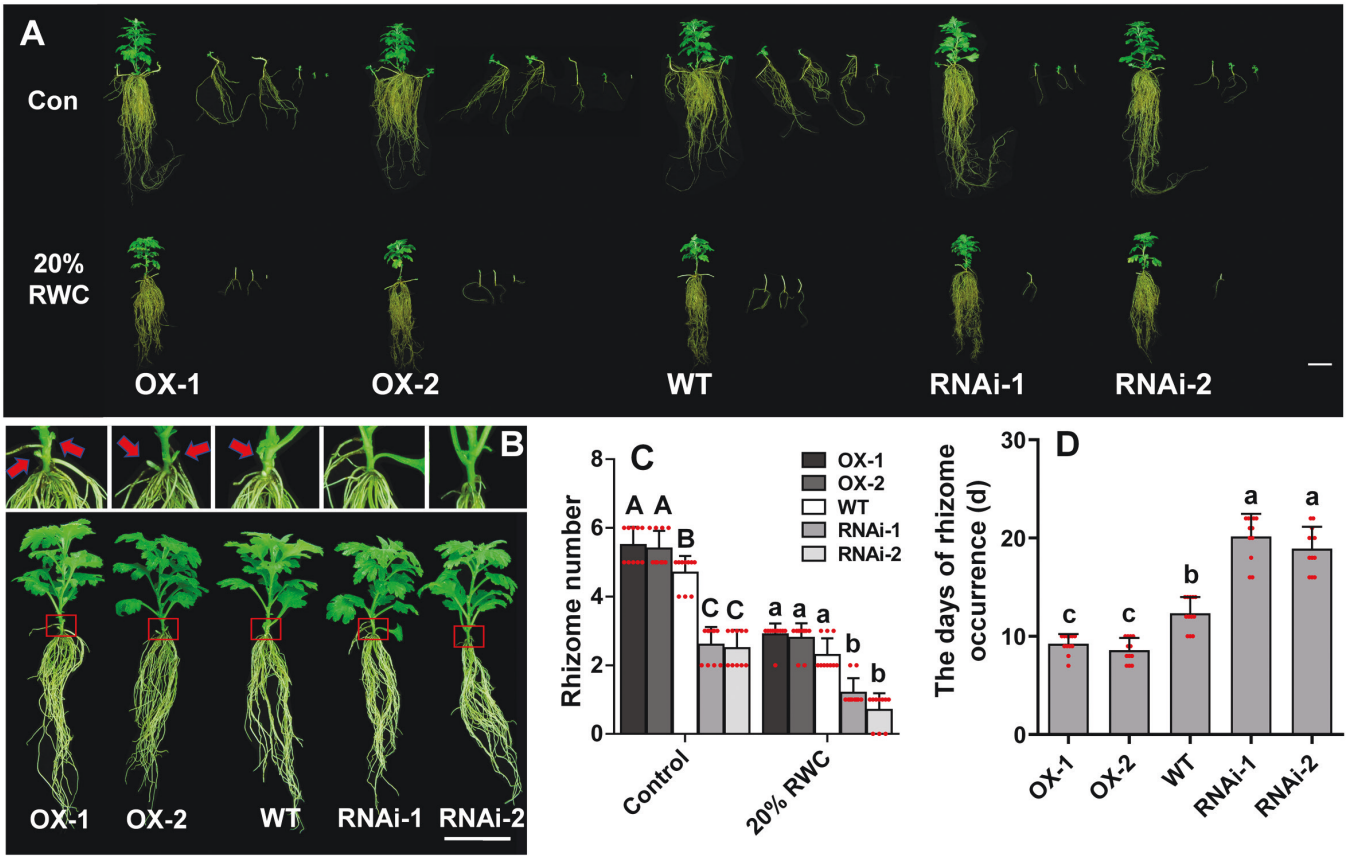

Fig. 4. Rhizome growth in *CmRH56*-OX and RNAi chrysanthemum plants under normal growth and drought stress conditions. (A) Phenotypes of *CmRH56*-OX and *CmRH56*-RNAi chrysanthemum plants grown under normal and drought stress conditions. Drought stress conditions were applied by withholding irrigation to reach 20% relative water content (RWC) for 30 d. All rhizomes from each plant were separated and are shown at the right. Scale bar, 5 cm. (B) Phenotype of rhizome occurrence in WT, *CmRH56*-OX and *CmRH56*-RNAi chrysanthemum plants 14 d after transplanting. Red arrows indicate rhizomes. The images in the upper row are enlarged sections (red square boxes) of the lower image. Scale bar, 5 cm. (C) Rhizome number in WT, *CmRH56*-OX and *CmRH56*-RNAi chrysanthemum plants under normal and drought stress (20% RWC) conditions. Results are the means of 10 biological replicates with standard deviation. Different letters indicate significant differences according to Tukey-Kramer test ($P<0.05$), with uppercase and lowercase letters indicating significant differences between transgenic plants and WT under normal conditions (control) or under drought stress (20% RWC), respectively. (D) Number of days until rhizomes were first observed in WT, *CmRH56*-OX and *CmRH56*-RNAi chrysanthemum plants grown under normal conditions. Results are the means of 12 biological replicates with standard deviation. Different letters indicate significant differences according to a Tukey-Kramer test ($P<0.05$).

biological functions of DEAD-box RNA helicases during abiotic stress and opens new avenues for studying the regulatory roles of RNA helicase–mediated rhizome growth in plant stress responses, which have not been reported previously.

GA is an important plant hormone that plays critical roles in the regulation of plant growth and development (Hedden and Phillips, 2000). Multiple GA-deficient mutants in various plants have demonstrated that the absence of GA accumulation or a lack of GA sensitivity results in typical GA-deficient dwarf phenotypes and the enhancement of drought stress tolerance (Colebrook *et al.*, 2014; Plaza-Wüthrich *et al.*, 2016). Recently, enhanced drought resistance was reported in transgenic plants expressing the mutant allele *gibberellin acid insensitive-1* (*gai-1*) encoding a DELLA protein under the control of a drought stress–inducible promoter when experiencing severe drought stress, although this was also accompanied by a temporary inhibition of cell growth (Zhang *et al.*, 2021). In *F. arundinacea*, the expression of a GA biosynthetic gene (*GA3ox*) and that of GA signalling pathway genes [*GA INSENSITIVE DWARF1* (*GID1*), *GID2*, and *DELLA*] was lower in rhizomes exposed to drought stress relative to control conditions, suggesting that drought-inhibited rhizome growth may be modulated by the GA pathway (Ma *et al.*, 2020). Here, we provide empirical evidence (Figs 6, 7) for the involvement of the GA pathway in rhizome outgrowth in response to drought stress via CmRH56 by influencing *GA2ox6* expression in a tissue-specific manner. CmRH56 may therefore regulate GA content by modulating phytohormone catabolism. Active GA accumulation would decrease in the rhizome shoot apex, resulting in an inhibition

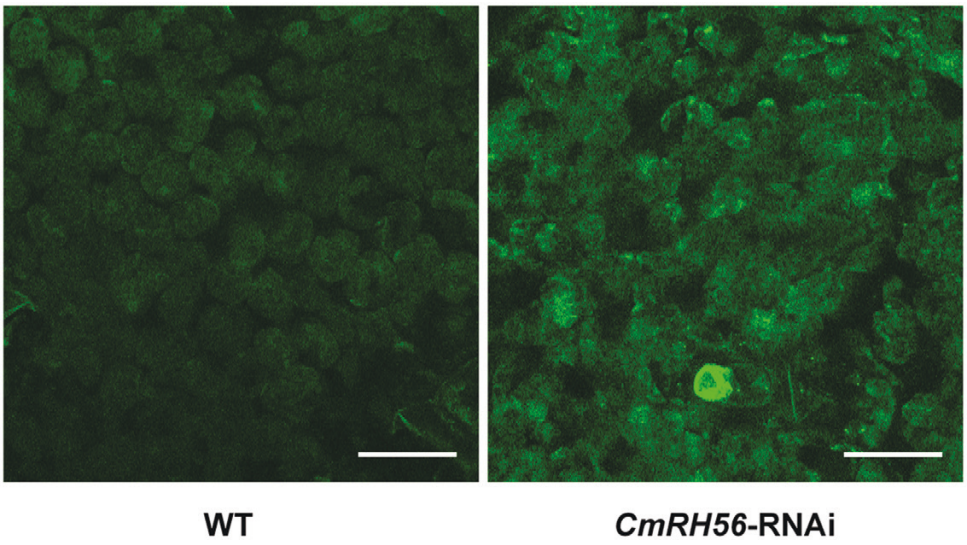

Fig. 5. *CmRH56*-RNAi chrysanthemum plants are defective in mRNA export from the nucleus. *In situ* hybridization with Alexa 488–labelled oligo(dT) probe, performed with WT and *CmRH56*-RNAi chrysanthemum leaves. Scale bar, 60 μm.

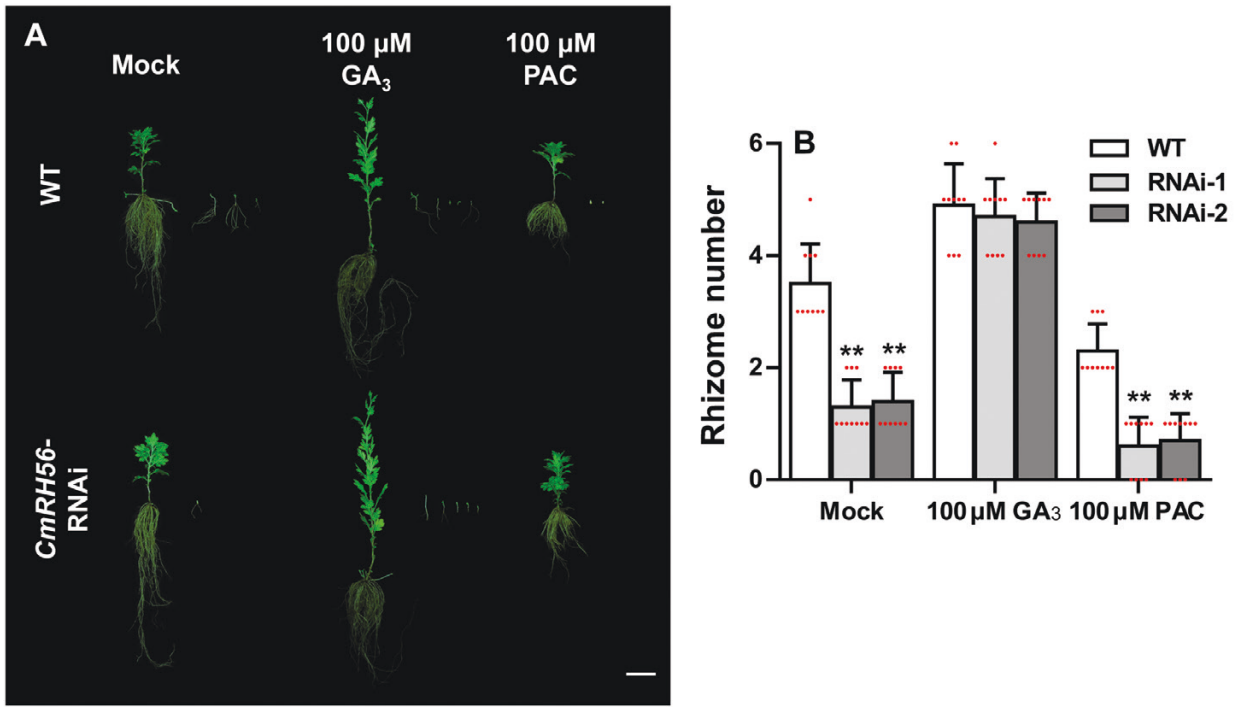

Fig. 6. Rhizome number of *CmRH56*-RNAi chrysanthemum plants under GA and PAC treatments. (A) Rhizome phenotype of *CmRH56*-RNAi and WT chrysanthemum plants treated with 100 μM GA or 100 μM PAC. Mock samples were treated with 0.1% dimethyl sulfoxide. All rhizomes from each plant were separated and are shown at the right. Scale bar, 5 cm. (B) Rhizome number in *CmRH56*-RNAi and WT chrysanthemum plants grown under GA and PAC treatments. Results are the means of 10 biological replicates with standard deviation. Statistical significance between different drought treatments and the control was determined using a Dunnett test ($^{**}P<0.01$).

of rhizome initiation, growth, and development under adverse environmental conditions. Our results also showed that total mRNA export is blocked in *CmRH56*-RNAi chrysanthemum lines (Fig. 5). The data suggest that CmRH56 may control *GA2ox6* mRNA export. However, the specific regulatory roles of CmRH56 in *GA2ox6* mRNA metabolism remain to be determined. In Arabidopsis, the DEAD-box RNA helicase UAP56 (U2AF65-associated protein56) plays a critical role in initiating mRNA export through loading mRNAs into different types of mRNA export adaptors, including four Ally

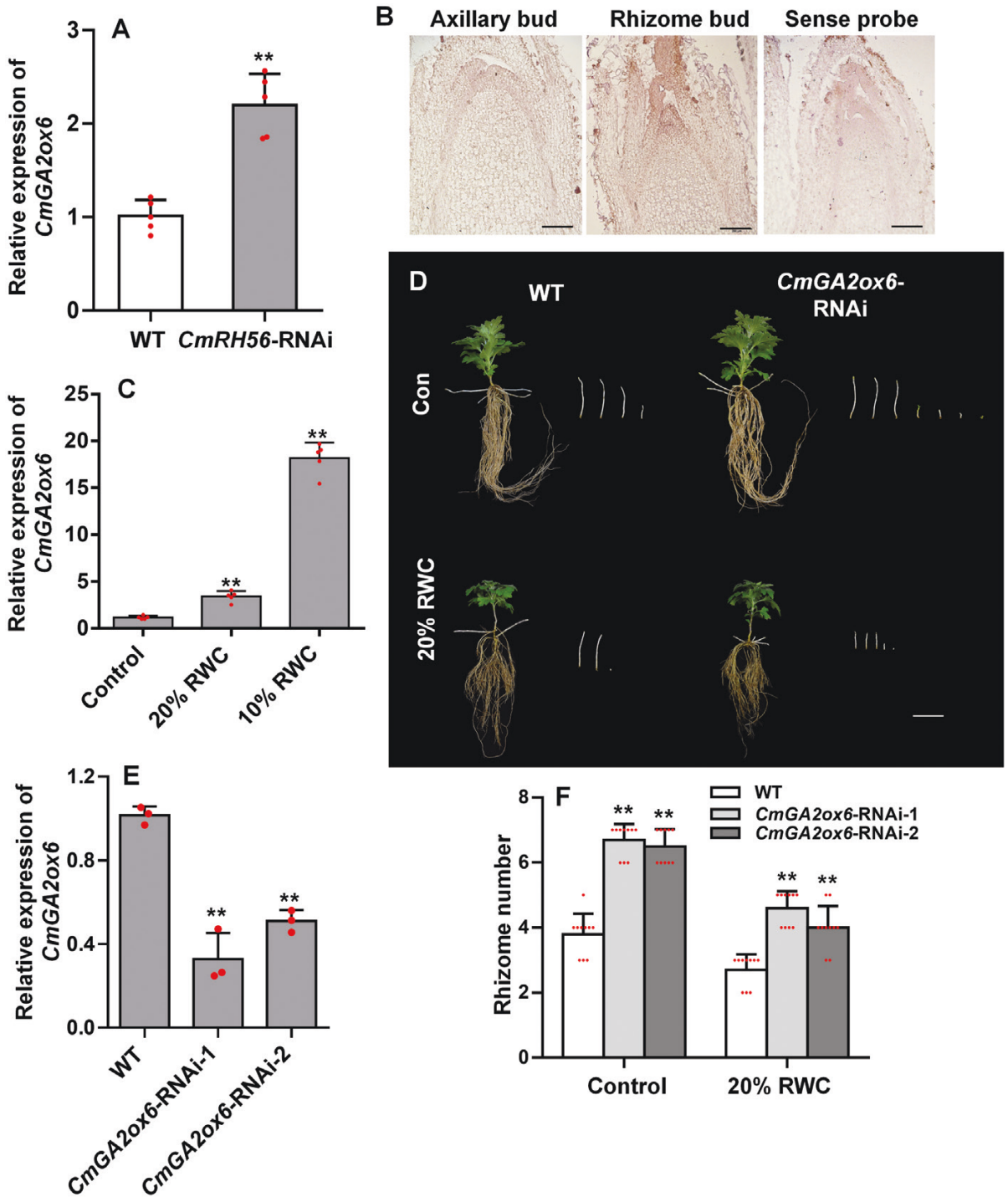

Fig. 7. CmRH56 influences rhizome outgrowth by modulating GA biosynthesis. (A) Relative *CmGA2ox6* transcript levels in *CmRH56*-RNAi and WT plants, as determined by RT–qPCR. Chrysanthemum *Ubiquitin* gene was used as an internal control. (B) *CmGA2ox6* mRNA accumulation pattern in rhizomes and axillary meristems, as shown by *in situ* hybridization. The sense *CmGA2ox6* probe was used as negative control. Scale bars, 200 μm. (C) Relative *CmGA2ox6* transcript levels in rhizomes grown under different degrees of drought stress (20% RWC and 10% RWC), as determined by RT–qPCR. Chrysanthemum *Ubiquitin* gene was used as an internal control. (D) Rhizome phenotype in *CmGA2ox6*-RNAi and WT chrysanthemum plants in response to drought stress. Chrysanthemum plants were grown under drought stress conditions by withholding irrigation to reach 20% RWC. Control treatment plants were well-watered. All rhizomes from each plant were separated and are shown at the right. Scale bar, 5 cm. (E) Relative *CmGA2ox6* transcript levels in WT and *CmGA2ox6*-RNAi plants, as determined by RT–qPCR. (F) Rhizome number in *CmGA2ox6*-RNAi and WT chrysanthemum plants in response to drought stress (20% RWC). Results are the means of 10 biological replicates with standard deviation. Statistical significance between WT plants and the different transgenic lines under control or under drought stress (20% RWC) was determined using a Dunnett test (**P<0.01).

of AML-1 and LEF-1 (ALY) proteins (Taniguchi and Ohno, 2008; Pfaff *et al.*, 2018) and UAP56-INTERACTING EXPORT FACTOR1 (UIEF1) and UIEF2 (Ehrnsberger *et al.*, 2019). Experimental analyses suggested that certain export adaptors are responsible for the export of specific subsets of mRNAs. Therefore, the identification of CmRH56-interacting partners participating in mRNA export may reveal the detailed mechanism of *GA2ox6* mRNA export.

In conclusion, our results demonstrate that under drought stress, the chrysanthemum DEAD-box RNA helicase CmRH56 participates in rhizome outgrowth by influencing GA metabolism.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. *Ubiquitin* expression levels under all experimental conditions used in this study.

Fig. S2. Multiple sequence alignment and phylogenetic analysis of CmRH56 with DEAD-box proteins from other plant species.

Fig. S3. Phenotypic characterization of *CmRH56* transgenic chrysanthemum plants.

Fig. S4. Expression of GA pathway genes in *CmRH56*-RNAi and WT plants by RT–qPCR.

Fig. S5. Multiple sequence alignment and phylogenetic analysis of CmGA2ox6 with GA2ox proteins from other plant species.

Table S1. Primers used in this study.

Table S2. Accession numbers for the genes and vectors mentioned in this study.

Author contributions

CM and BH conceived and designed the experiments; LZ and YX performed most of the experiments; SL, XL, and MQ contributed to the chrysanthemum transformation and drought stress treatments; TJ contributed to *in situ* hybridization; C-ZJ, and JG provided technical support and conceptual advice; CM, LZ, and BH analysed the data and wrote the article.

Conflict of interest

The authors declare no competing interests.

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Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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