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Review

MdCER2 conferred to wax accumulation and increased drought tolerance in plants

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ABSTRACT

Drought can activate many stress responses in plant growth and development, including the synthesis of epidermal wax and the induction of abscisic acid (ABA), and increased wax accumulation will improve plant drought resistance. Therefore, an examination of wax biosynthesis genes could help to better understand the molecular mechanism of environmental factors regulating wax biosynthesis and the wax associated stress response. Here, we identified the *MdCER2* gene from the 'Gala' (*Malus* × *domestica Borkh.*) variety of domestic apple, which is a homolog of *Arabidopsis AtCER2*. It possesses a transferase domain and the protein localizes on the cell membrane. The *MdCER2* gene was constitutively expressed in apple tissues and was induced by drought treatment. Finally, we transformed the *MdCER2* gene into *Arabidopsis* to identify its function, and found ectopic expression of *MdCER2* promoted accumulation of cuticular wax in both leaves and stems, decreased water loss and permeability in leaves, increased lateral root number, changed plant ABA sensitivity, and increased drought resistance.

1. Introduction

Plants are subjected to various stresses during growth and development. Among them, drought is a form of abiotic stress that can disrupt the normal growth and development patterns of plants, especially impacting the physiology and morphology of terrestrial plants (Kazuko and Kazuo, 2006). Plants have evolved many unique adaptive mechanisms in response to drought, including induction of the accumulation of a variety of stress response hormones, whether involved in short-term or long-term growth and development (Fang and Xiong, 2015). The hormone response induced by abscisic acid (ABA) is unique in plant drought resistance. ABA is a key hormone that regulates water status and stomatal movement (Lim et al., 2015). In addition, the existence of epidermal wax is a more prominent mechanism for dealing with drought. It has also become clear that the physiological role of cuticle wax, beyond its primary function as a transpiration barrier, plays important roles in processes ranging from development to microbial interactions (Yeat and Rose, 2013; Zhu et al., 2014).

The unique mechanism of cutin defense is closely tied to cuticular wax. Many studies have reported that cuticular wax undergoes synthesis in four stages, in cellular compartments spanning from plastids to the endoplasmic reticulum to the extracellular space (Bernard et al., 2012). The first stage is composed of the synthesis of fusions of C16 and C18 fatty acid monomers to aceyl carrier protein (ACP) to form C16-ACP and C18-ACP, which mainly involves the plasticidal fatty acid synthase (FAS) complex, ACP, and a long-chain acyl-CoA synthetase (LACS). The second stage, the rate limiting step, involves the elongation of C16 and C18 fatty acids into very long chain fatty acids (VLCFAs) composed of 20–36 carbons. It mainly consists of the fatty acid elongase (FAE) complex, 3-ketoacyl CoA synthase (KCS), 3-ketoacyl CoA reductase (KCR), 3-hydroxyacyl CoA dehydratase (PAS2), trans-2-enoyl-CoA reductase (ECR), and andglycerol 3-phosphate acyltransferase (GPAT). The third stage is the synthesis of different waxy products by VLCFA distribution. Finally, a variety of keratinous wax components are transported out of the cell to perform their function.

The internal regulatory mechanism controlling wax synthesis is

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complex. More than 190 genes are reported to be involved in waxy synthesis in *Arabidopsis*, and 89 *Arabidopsis* mutants affect wax accumulation (Borisjuk et al., 2014). Transcriptional regulation is one of the most important regulatory mechanisms involved in this process. Five main transcription factors have been identified in this process to date: Ethylene responsive factors, WRINKLED transcription factors, CBF/DREB transcription factors, MYB transcription factors, and HD-Zip class IV transcription factors. *AtWIN1/SHN1* affects cuticular components including C30/34 FA, C28/30 aldehyde, C27/C29/C33 alkane, C16:0 and C18:1 ω-HFAs, and C31 and C29 alkanes (Broun, 2004; Aharoni et al., 2004). *OsWR1* participates in the synthesis of wax components by regulating multiple downstream target genes (Wang et al., 2012). The transcription factor *AtMYB96* is the most representative waxy drought resistance gene. It induces both stomatal closure via *RD22* and cuticular wax biosynthesis by upregulating directly cuticular wax biosynthetic enzyme genes (Seo et al., 2011). In addition, more and more studies have shown that wax synthesis also involves indirect transcriptional regulation. It has been reported that *AtCER9* can change the synthesis of wax in stems and leaves. It encodes a protein with sequence likeness to yeast Doa10, an E3 ubiquitin ligase involved in ER-associated degradation of misfolded proteins (Lü et al., 2009). A recent study showed that the *Arabidopsis* F-box protein SAGL1 mediates proteasome-dependent degradation of ECERIFERUM3, thereby negatively regulating cuticular wax biosynthesis in response to changes in humidity (Kim et al., 2019). This demonstrates a mechanism for how *DHS* negatively regulates wax biosynthesis through promoting the degradation of ROC4 for the condition of drought-tolerant rice (Wang et al., 2018a,b).

Besides transcription factors, structural genes function in a more direct way to regulate wax accumulation. The *CERs* gene family, for example, regulates wax biosynthesis and drought response. *CER1*-overexpressing plants show reduced soil water deficit susceptibility and decreased cuticle permeability (Bourdenx et al., 2011). A recent report demonstrated that altering the activity of *CER1-LIKE1* influences VLC-n-alkane biosynthesis and wax crystallization (Pascala et al., 2019). *AtCER3* can affect the synthesis of stem wax (Wang et al., 2018a,b). Moreover, *CER1* interacts with *CER3* to catalyze the redox-dependent synthesis of VLC-alkanes from VLC acyl-CoAs (Bernard et al., 2012). *AtCER4* is involved in the synthesis of very-long-chain primary alcohols with C24–C28 (Rowl et al., 2006). *AtCER11* is involved a dephosphorylation step in secretory trafficking in plant cells (Shi et al., 2019). Many articles have reported roles for *AtCER2* and its different congeners in wax accumulation. Wang et al. demonstrated that *WSL4* participates in VLCFA elongation beyond C22, and elongation beyond C24 requires the participation of *OsCER2* (2017). Another group provides evidence for the function of *CER2* in C28 elongation based on experiments in yeast (Haslam et al., 2012). The results of other assays show that the changes in acyl chain length caused by each CER2-LIKE protein are of substantial importance for cuticle formation and pollen coat function. *CER2* and *CER2-LIKE2* are both involved in VLCFA elongation to C30 (Haslam et al., 2015). These findings proved that *CER2* is deeply integral to the process of long chain fatty acid extension.

Although *CER2* has been examined in detail in several species, its function in apples has not been reported. Cuticular wax has significant role in apple resistance to stresses and fruit quality. Therefore, we isolated the *MdCER2* gene from apple, and analyzed its function under drought conditions and varying exposure to ABA, which provides new insights for further studying the molecular mechanisms of cuticular wax biosynthesis in plants.

2. Material and methods

2.1. Plant materials and growth conditions

The materials used in this experiment were 'Gala' apple seedlings and fruits, apple tissue culture seedlings, *Arabidopsis thaliana* (*Columbia*) and tobacco (*Nicotiana benthamiana*). The growing roots, young stems, new leaves, flowers, and fruit of the apple seedlings were collected from the fruit tree experimental station of Shandong Agricultural University from June to September 2018.

Apple tissue culture seedlings were grown on MS medium containing 0.5 mg l^{-1} 6-BA and 0.5 mg ml^{-1} NAA for 25 days (room temperature 24 °C), then treated with 10% polyethylene glycol (PEG 6000) and 50 μmol L^{-1}ABA for 0, 1, 3, 6, 12, and 24 h, respectively. Then cryopreserved with liquid nitrogen immediately after treatment.

Wild type (WT) *Arabidopsis* grew at 22 °C under a 16-h light/8-h dark photoperiod. After 20 days of growth, transgenic *Arabidopsis* was obtained as described (Zhang et al., 2019a). After screening, the T3 generation plants were obtained and used in the experiment.

Tobacco grows at room temperature until at least five leaves can be used for injection to perform the *MdCER2* localization experiment (Wang et al., 2014).

2.2. Subcellular localization

The primers *MdCER2-TOPO-F* (AAGGAGCCCTTCACCATGGTTTCA TCAACCAGCTTGAGG) and *MdCER2-TOPO-R* (GGCGCGCCCACCCTTA GCAATACTCCAATTCCTTTGG) were designed to construct TOPO vector (The Gateway® System). Then, the MdCER2-GFP was obtained by connecting to the GFP carrier by LR substitution reaction to exchange the *MdCER2* gene to the PAL1107 vector. Subcellular localization of *MdCER2* was identified by transient expression in *Nicotiana benthamiana* as described (Qi et al., 2019a,b).

2.3. RNA extraction and qRT-PCR analysis of gene expression

Total RNA of different apple tissues and *Arabidopsis* seedlings were obtained using the RNA Plant Plus Reagent kit (Tiangen Biotech, Beijing, China). cDNA was obtained using the PrimeScript™ RT reagent Kit (TaKaRa, Shiga, Japan) and stored at −80 °C (An et al., 2018).

The upstream primer 5′-ATCCGGATAACCGAAACGGG-3′ and downstream primer 5′-AGGCAGAAAACGCATCTCCA-3′ of *MdCER2* were designed for qRT-PCR analysis. qRT-PCR was performed using the UltraSYBR Mixture (with ROX) kit (CoWin Biosciences, Taizhou, Jiangsu, China) under the following conditions: pre-denaturation at 94 °C for 10 min, denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s, and elongation at 65 °C for 15 s for 40 cycles. Samples were collected at step 3 of each cycle for fluorescence analysis. The $2^{-\Delta\Delta Ct}$ method was applied for data analysis.

2.4. Scanning electron microscopy (SEM)

After 30 days of growth, we cut off the leaves and stems of *Arabidopsis* to observe surface wax morphology. The leaves and stems were vacuum-dried at −80 °C in a freeze drier for 12 h. Then, the sample was placed on a round table and treated with gold spray for observation with a scanning electron microscope as described (Lü et al., 2009).

2.5. Wax extracting method

Cuticular wax was extracted exhaustively using the following steps: chloroform extraction, nitrogen blow drying, derivatization reaction, and sample analysis following that described previously (Zhang et al., 2019b).

2.6. Water loss experiment

Water loss in 25-day old *Arabidopsis* leaves was carried out as follows: Rosette leaves were cut and immediately incubated in deionized water for 3 h under dark conditions. Excess water was removed with filter paper. The leaves were then placed in the dark, and their weight was measured every 30 min for 180 min. The rate of water loss of the

leaves was then calculated. Each experiment was carried out five times independently.

2.7. Chlorophyll extraction

After 25 days of growth, *Arabidopsis* rosette leaves were removed for a chlorophyll extraction assay. The leaves were soaked in the dark with 80% anhydrous ethanol and their absorbance at 665 and 649 was measured every 10 min until 80 min (Yu et al., 2018). The total micromoles of chlorophyll were calculated by: 6.63*(A665) + 18.08*(A649). This experiment was repeated five times.

2.8. PEG and ABA treatment

Arabidopsis seeds were seeded on normal MS medium. After germination and growth for 5 days, *Arabidopsis* seedlings were transferred to MS, MS + 10 μmol L^{-1} ABA, MS + 30 μmol L^{-1} ABA, MS + 4% PEG 4000, and MS + 6% PEG 4000 medium. Seedlings were then grown in growth chambers at 24 °C under 16 h of light per day (An et al., 2016). After seven days of treatment, the root length and number of lateral roots of 30 seedlings of each experimental condition were measured at a time, and the experiment was repeated five times.

2.9. Drought treatment

The drought treatment assay was carried out with single pot seedling and four pot seedlings, respectively. The seedlings were grown in 50% vermiculite and 50% coarse vermiculite. After 20 days of normal plant growth, the seedlings were no longer watered until the leaves wilted and turned yellow.

2.10. Data analysis

Each experiment was carried out five times. DPS software was used to analyze the data, and the single factor Tukey method was used to analyze the relative expression levels. Different letters represent different significant differences at $P < 0.05$.

2.11. Bioinformatics analysis of MdCER2

Gene sequences of 10 different species were obtained from BLAST, DNAMAN was used to create a multiple sequence alignment, and MEGA. 7 was used to generate a phylogenetic tree. The online software SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) and PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) were used to predict the second-level structure and the three-dimensional structure, respectively. The gene structure and domain were found by GSDS 2.0 (http://gsds.cbi.pku.edu.cn) and SMART (smart.embl-heidelberg.de). ProtParam (http://web.expasy.org/protparam) was conducted to analyze the molecular weight and isoelectric point of proteins. Protein interaction was predicted by the online software STRING (https://string-db.org).

3. Results

3.1. Gene cloning and sequence analysis of MdCER2

The *MdCER2* gene of apple was identified from NCBI sequence data using *AtCER2* as bait. *MdCER2* is 1299 bp in length, encodes 432 amino acids, and has two exons and a single intron (Fig. 1a). In addition, the sequence similarity of *CER2* between apple and other plant species was analyzed, including peach, plum, waxberry, white pear, rose, durian, cocoa, poplar, rubber tree, and castor oil, and an evolutionary tree was generated based a multisequence alignment (Fig. 1b). The results show that the *MdCER2* sequence is highest in similarity to white pear (both belong to the *Rosaceae* family). Based on the second-level structure and the three-dimensional structure of *MdCER2*, the molecular weight of MdCER2 is predicted to be 48 kD and the theoretical PI is 5.84. We compared the protein sequences of *AtCER2* and *MdCER2*, and found that the two sequences were highly similar (Fig. 1c). Then, we analyzed the domains of the two proteins, and found that they both have only one transferase domain with predicted transferase activity (Supplemental Fig. 1).

3.2. Cis-regulatory analysis

Using PLANTCARE software, we studied the 1400 bp upstream of the *MdCER2* transcription initiation site (TSS) to explore the potential response of *MdCER2* to various regulatory inputs. As shown in Table 1, several *cis*-regulatory elements were identified in the promoter sequence of *MdCER2*, including ABRE and SARE sites, which have been shown to respond to abscissic and salicylic acid, respectively. *MdCER2* also contains some plant hormone related sequences, such as MBSI, involved in flavonoid biosynthetic gene regulation. In addition, it also includes five sites related to optical signal components, including BOX4, G-BOX, and others. These results suggest that *MdCER2* may be affected by external environmental factors such as light to regulate growth and development.

3.3. MdCER2 localization and expression pattern in different apple tissues

To study the localization of MdCER2 protein, two expression vectors of MdCER2-GFP and AtCBL1-RFP were constructed using the full sequence of *MdCER2*, and transiently injected into the epidermal cells of tobacco leaves. Two-photon laser confocal microscopy revealed that *MdCER2* is located on the cell membrane (Fig. 2a).

The specific expression pattern of *MdCER2* in different apple tissues, including root, stem, leaf, and fruit was then analyzed by qRT-PCR (Fig. 2b). *MdCER2* constitutively expressed in all tissues tested. The highest expression was observed in fruit, and then stem and leaf, and the lowest expression level was observed in the root, suggesting its potential function in plant aerial organs.

3.4. Change of wax load in stems and leaves

To identify the function of *MdCER2*, an *MdCER2* overexpression vector was constructed and transformed into wild type *Arabidopsis*, and three ectopically expressing *MdCER2* strains with different *MdCER2* transcription levels of *Arabidopsis* were generated (Fig. 3a). Based on the tissue-specific expression levels of *MdCER2*, we next analyzed the wax load in stem and leaf in WT and *MdCER2* ectopically-expressed lines of *Arabidopsis* to detect whether MdCER2 protein expression is related to wax accumulation. Fig. 3b and c shows that the wax content is higher in leaves of *Arabidopsis* expressing *MdCER2* than those of the wild type, and was significantly higher in transgenic stems compared to the WT.

The morphology of wax crystals was observed by SEM (Fig. 3d and e). In stem tissue, there was more wax observed in transgenic lines than in wild type, but there was no significant difference in the wax crystals' morphology. In leaves, the wax was observed as lamellar, and the quantity is slightly higher in transgenic *Arabidopsis* than in WT.

3.5. MdCER2 affects leaf permeability in plants

A previous study has shown that a change in wax load in leaves affects the permeability of leaf epidermis (Schönherr, 1976). Therefore, we performed a leaf water loss experiment and a chlorophyll extraction experiment to observe the permeability of *MdCER2* transgenic and WT plant leaf epidermis. The rate of water loss was measured for six time periods. As shown in Fig. 4a, the water loss rate of wild type plants was higher than that of *MdCER2* transgenic plants at each time point

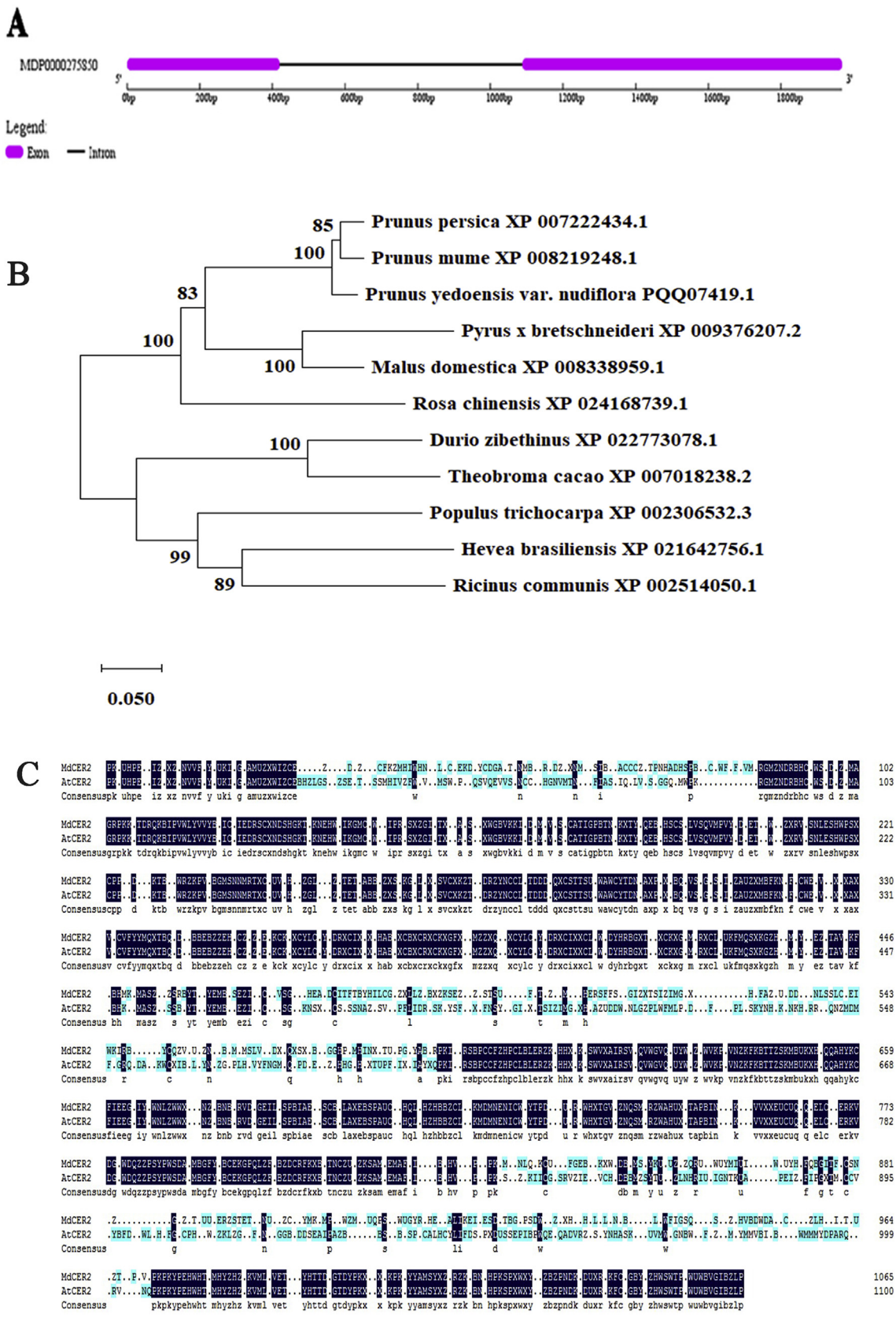

Fig. 1. Schematic diagram of the *MdCER2* sequence and phylogenetic analysis of the *CER2* gene family in different species. (a) Schematic diagram of *MdCER2* genomic and cDNA sequences. (b) Phylogenetic tree of the *CER2* gene family in different species, including peach (*Prunus persica*), plum (*Prunus domestica*), waxberry (*Prunus yedoensis*), white pear (*Pyrus bretschneideri*), apple (*Malus domestica*), rose (*Rosa chinensis*), durian (*Durio zibethinus*), cocoa (*Theobroma cacao*), poplar (*Populus trichocarpa*), rubber tree (*Hevea brasiliensis*), and castor oil (*Ricinus communis*). (c) Alignment of amino acid sequences of *MdCER2* and *AtCER2* proteins.

Table 1
MdCER2 promoter *cis*-regulatory element analysis.

Cis-element name	Cis-element sequence	Function	Number
ABRE	ACGTG	cis-acting element involved in the abscisic acid responsiveness	2
MBSI	AAAAAAC(G/C)GTTA	MYB binding site involved in flavonoid biosynthetic genes regulation	1
Box 4	ATTAAT	Part of a conserved DNA module involved in light responsiveness	4
G-box	CACGTC	cis-acting regulatory element involved in light responsiveness	1
	TACGTG		
MYB	TAACTG		1
MYC	CATGTG	cis-acting element involved in salicylic acid responsiveness	6
SARE	CAATTG		1
	TTCGAC		
	CATCTT		

examined. Similarly, after soaking leaves with 80% anhydrous ethanol, the chlorophyll leached out of the leaves of wild type plants faster than that of *MdCER2* transgenic plants (Fig. 4b). These results indicate that *MdCER2* affects the permeability of plant leaves, which suggest its potential role in drought tolerance.

3.6. MdCER2 transgenic Arabidopsis is sensitive to ABA

In the *MdCER2* promoter sequence, we found an ABRE *cis*-regulatory element that is involved in the ABA response. Here, we examined whether transcription of *MdCER2* is regulated by ABA. The qRT-PCR results show that the presence of ABA significantly decreases the expression of *MdCER2*, and *MdCER2* is highly sensitive to ABA treatment (Fig. 5a). Subsequently, we used WT and *MdCER2* transgenic *Arabidopsis* to analyze their ABA response. *Arabidopsis* seeds were germinated and grew on normal MS medium for 5 days, then transferred to MS medium containing 10 μmol L^{-1} ABA or 30 μmol L^{-1} ABA for 7 days, respectively. As is shown in Fig. 5b and c, the root length of *MdCER2* transgenic plant was significantly shorter than that of wild type under ABA treatment, but there was no obvious difference in the number of lateral roots among different lines (Fig. 5b, d). This confirms that *MdCER2* is sensitive to ABA signaling.

3.7. MdCER2 enhances plant drought resistance

ABA sensitivity is often closely related to plant drought tolerance, so we examined the expression levels of *MdCER2* under different drought treatment times by qRT-PCR. The results show that the expression level of *MdCER2* increases gradually within 12 h of drought treatment, and then decreases (Fig. 6a). We then analyzed the phenotype of WT and *MdCER2* transgenic *Arabidopsis* under 7 days of drought treatment and found that, in WT *Arabidopsis*, more leaves turn yellow and wilt than in *MdCER2* transgenic plants under drought treatment, while there is no difference among the different *Arabidopsis* lines under normal watering condition (Fig. 6b). We similarly detected the leaf chlorophyll content (Fig. 6c). Under normal watering condition, there is no obvious difference in chlorophyll content of leaves of the four lines. However, after drought treatment, the chlorophyll content of WT *Arabidopsis* leaves is significantly lower than that of transgenic plants.

To confirm these results, we tested *Arabidopsis* seedlings under PEG treatment (Fig. 5b). After 5 days of growth, we transferred *MdCER2* transgenic and WT *Arabidopsis* from normal MS medium to MS medium containing 4% or 6%PEG, and treated for 7 days. Then, we measured the root length and number of lateral roots. Fig. 6d shows that the roots of PEG-treated *MdCER2* transgenic plant are longer than those of WT, and the number of lateral roots is significantly increased (Fig. 6e). These results indicate that *MdCER2* can improve the plant drought tolerance.

Finally, we used the online software STRING to predict protein-protein interactions and found that MdCER2 protein might interact with MdMYB96 (Supplemental Fig. 2), which suggests a possible pathway for regulating plant drought tolerance. However, this interaction needs to be further verified by experiments.

4. Discussion

The *CER* gene family is involved in the response to biological and abiotic stresses in many plant species, which suggest areas for the subsequent study of *CER* genes in apples. Qi et al. showed that, when compared to *AtCERs*, *MdCERs* had high similarity in 3D structures, indicating MdCER proteins might have similar functions as AtCERs (Qi et al., 2019a). Qi also identified the first *CER* gene from apple, *MdCER1*. *MdCER1* is associated with the accumulation of cuticular wax and drought resistance (Qi et al., 2019b). In the present study, we identify

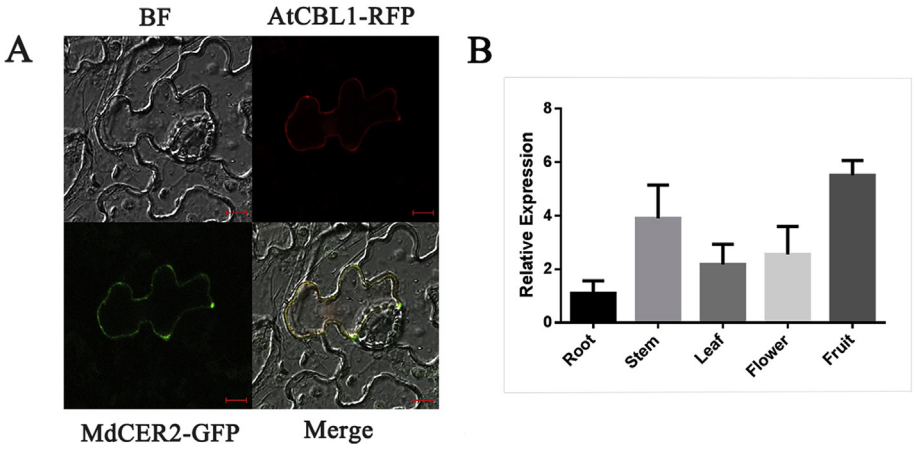

Fig. 2. *MdCER2 localization and* expression analysis. (a) Subcellular localization of *MdCER2*. (b) *MdCER2* expression in apple root, stem, leaf, flower, and fruit as detected by qRT-PCR.

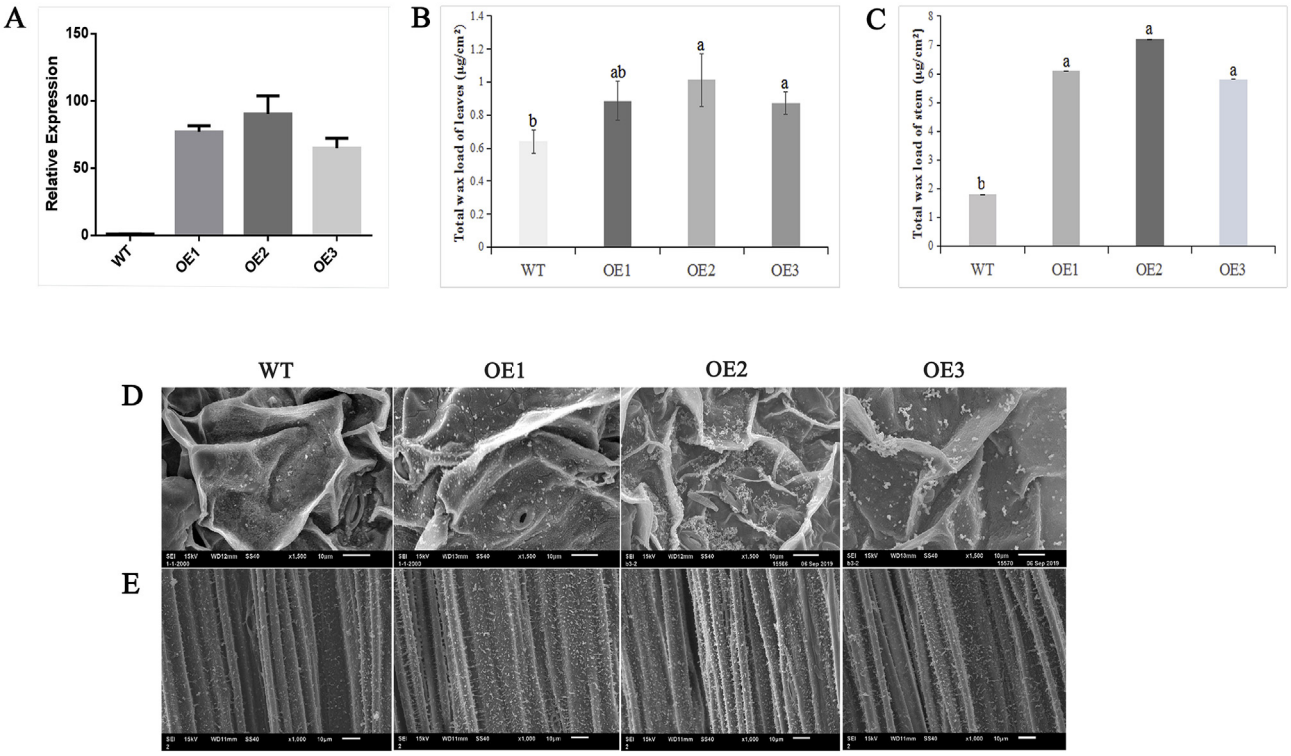

Fig. 3. Change of wax load. (a) qRT-PCR analysis of ectopic expression of *MdCER2* in *Arabidopsis*. (b) Total cuticular wax content of leaves from WT and *MdCER2* transgenic *Arabidopsis* lines. (c) Total cuticular wax content of stems from WT and *MdCER2* transgenic *Arabidopsis* lines. (d) Scanning electron microscopy showing the relative of wax structures of leaves in WT and *MdCER2* transgenic plants. The magnification times of the SEM was 1500x. (e) Scanning electron microscopy showing the relative of wax structures of stems in WT and *MdCER2* transgenic plants. The magnification times of the SEM was 1000x.

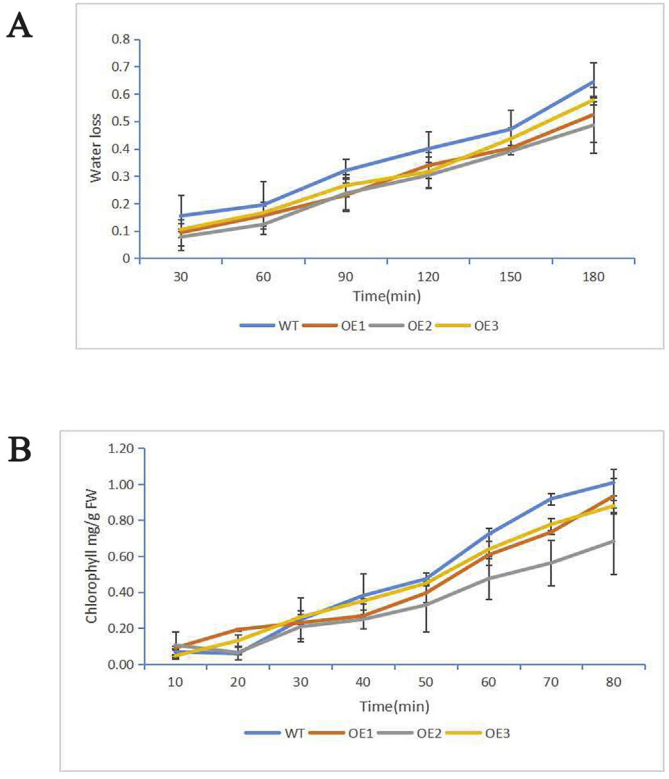

Fig. 4. Examination of Water loss and permeability of Arabidopsis leaves. (a) The rate of water loss from leaves in WT and *MdCER2* transgenic plants. (b) Chlorophyll leaching assay in rosette leaves of WT and *MdCER2* transgenic plants. FW indicates fresh weight.

another *CER* gene from apple, a homolog of *AtCER2*, to further investigate the apple *CER* family. Phylogenetic analysis indicates that *CER2* genes from different species of dicotyledonous plants have a high degree of similarity, demonstrating that the gene has been conserved in the course of evolution. We also show that there is high sequence similarity between *MdCER2* and *AtCER2*, and it is presumed that they have similarly conserved functions. In a study of *AtCER2*, *CER2* was classified, based on sequence homology, as a BAHD acyltransferase (Haslam et al., 2012). Similarly, domain analysis showed that *MdCER2* and *AtCER2* each have only one transferase domain. This indicates that *MdCER2* may also produce C28 and C30 long-chain fatty acids during long-chain fatty acid synthesis. The protein interaction prediction suggests that *MdCER2* might interact with multiple transcription factors, indicating that *MdCER2,* as a target gene, might be involved in multiple waxy biosynthetic pathways.

Previous studies have shown that most wax synthase proteins in *Arabidopsis* are located in the endoplasmic reticulum (ER), such as AtCER17 (Yang et al., 2017). In Apple, it was found that MdCER1 was also located in ER (Qi et al., 2019b). However, we found that MdCER2 localizes to the cell membrane, which is different than AtCER2, whose localization has been placed in either the ER or the nucleus in different studies (Xia et al., 1997; Haslam et al., 2012). *AtCER2* is a acyl-transferase, but does not have the activity of a acyl-transferase (Aarts et al., 1995). Therefore, we propose that the localization to the cell membrane may relate to *MdCER2*'s transferase activity, or play a role in the wax synthetic pathway. Next, we analyzed the expression pattern of *MdCER2*. The apple *CER* family is mostly expressed in apple leaves and stems, with low distribution levels in the roots, indicating that the *MdCER* genes are highly distributed in the organs exposed to the air above the ground, which are with covered cuticles. Our results for *MdCER2* are in concert with the broader family expression. To efficiently adapt to the complex and changeable environment, terrestrial plants have evolved many different adaptive mechanisms, including

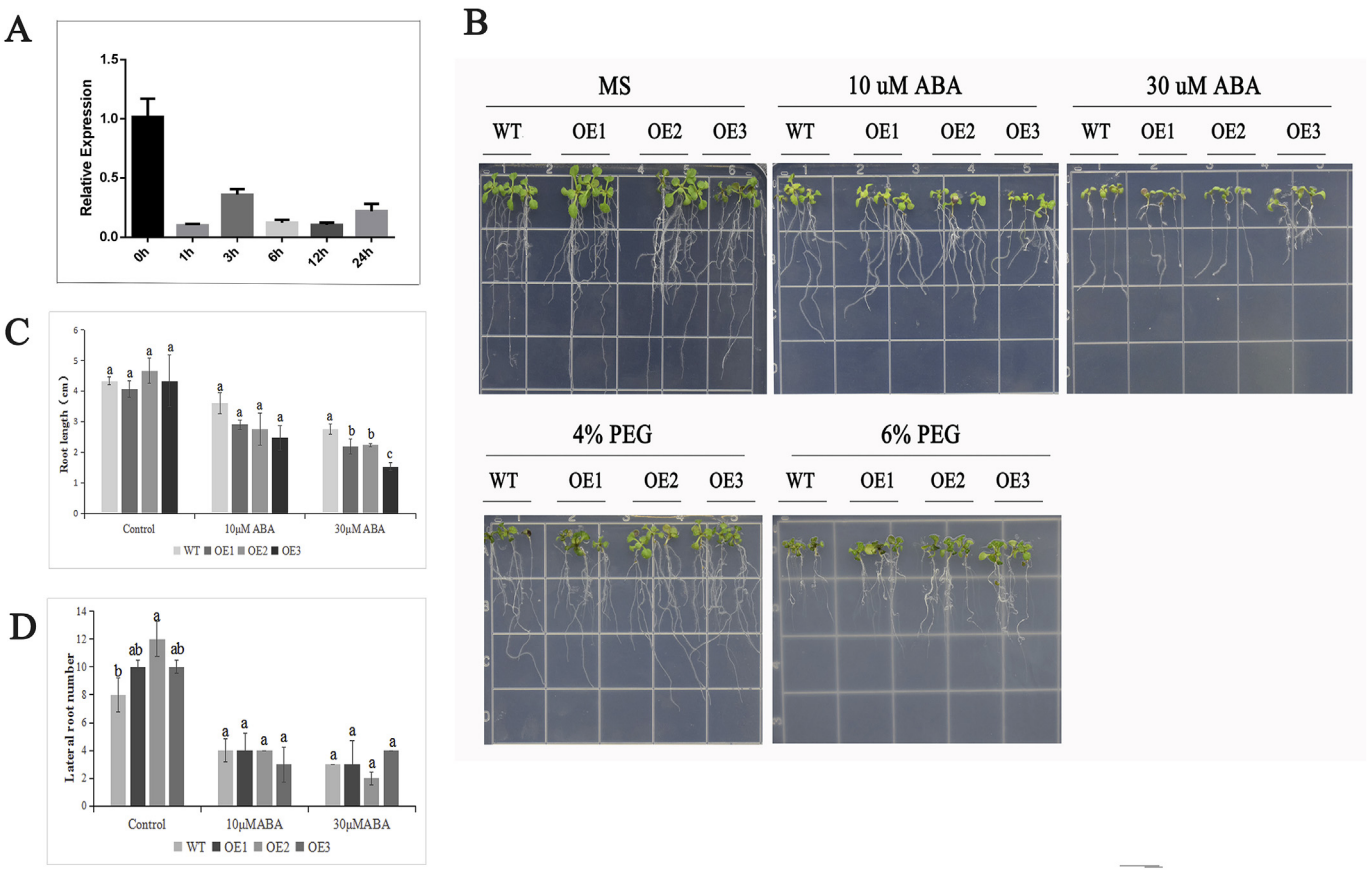

Fig. 5. Transgenic expression of *MdCER2* in *Arabidopsis* enhances ABA sensitivity. (a) Expression analysis of *MdCER2* in response to ABA. (b) Phenotype of *MdCER2* transgenic and WT *Arabidopsis* in MS, MS + 10 μmol L^{-1} ABA, and MS + 30 μmol L^{-1} ABA media. Growth of WT and *MdCER2* Arabidopsis in MS, MS + 4% PEG, and MS + 6% PEG media, scale bar = 1 cm. (c) The primary root length of WT versus transgenic *Arabidopsis*. (d) The number of lateral roots. The means and standard deviations were calculated from the results of five independent experiments.

changing the properties of epidermal wax in some organs above the ground.

In this study, *MdCER2* transgenic plants have higher wax accumulation than WT in both leaves and stem. This result was confirmed by SEM. It was also found that the wax crystal structure of the transgenic plant exhibited snowflake-like wax accumulation in leaves, which is closely related to the drought tolerance of transgenic plants. It has been demonstrated in a series of species in addition to *Arabidopsis* that there is a relationship between wax and epidermis permeability (Shepherd and Griffiths, 2006). Epidermis permeability can be measured by the rates of water loss and chlorophyll-leaching (Wang et al., 2014). Therefore, our observation that reduced water loss and chlorophyll leaching is positively correlated with the content of more wax in the epidermis of *MdCER2* transgenic plant is further confirmation that *MdCER2* has a unique effect on epidermis permeability. *MdCER2* may affect the regulation of wax biosynthesis and play an active role in the drought resistance.

From upstream sequence analysis of *MdCER2*, a MBSI acting element, a MYB binding site involved in flavonoid biosynthetic gene regulation, was found, indicating that the gene might play an important role in the regulation of fruit quality. In addition, the gene contains many elements related to light. Light is able to induce wax accumulation (Hooker et al., 2002), and it has been pointed out that the protective effect of ultraviolet radiation is due to the light-scattering characteristics of the cuticular layer (Seo and Park, 2011; Li et al., 2013). Therefore, we speculate that light is highly likely to affect wax accumulation and fruit quality by regulating the expression of *MdCER2*. We also found an ABA response element. ABA plays a central role in modulating the effect of stress via maintaining plant homeostasis. (Liu et al., 2017). ABA deficiency consistently results in differences in the composition of epidermis waxes and leaf cutin (Martin et al., 2017). Furthermore, decreased expression of genes involved in wax or cutin formation has been shown consistently among ABA mutants (Martin et al., 2017). ABA can induce the transcription of *MYB96* to regulate drought resistance (Borisjuk et al., 2014), and guide the expression of *AtCER6* (Hooker et al., 2002). We demonstrate that ABA could not induce high *MdCER2* expression, but *MdCER2* transgenic plants show hypersensitivity to ABA. Therefore, we speculate that *MdCER2*, as a target gene, may be regulated by some upstream transcription factors, resulting in its susceptibility to ABA. The accumulation of the phytohormone ABA is a major response to water deficiency in plants (Osakabe et al., 2013). After drought treatment, *MdCER2* transgenic plants have increased drought resistance compared to WT. Therefore, it is reasonable to believe that high waxy content in plants is a key factor in regulating plant drought resistance, and drought stress could be effective to induce the expression of *MdCER2* to increase wax accumulation and enhance tolerance, which forms a regulatory pathway among drought, *MdCER2*, wax accumulation, and drought resistance. It is known to all that ABA content will increase after drought treatment. However, ABA treatment decreased, while drought stress induced the expression of *MdCER2* in our result. Zong et al. reported that the feedback up-regulation of ABA synthesis may contribute to the enhancement of ABA signaling, especially under persistent drought stress, plants have evolved other mechanisms to avoid over-amplification. (2016). Therefore, we speculate that when drought induces high expression of *MdCER2*, excessive expression of the gene may adversely affect plant growth and development. In this case, the accumulation of drought-induced ABA causes a protection mechanism that prevents the

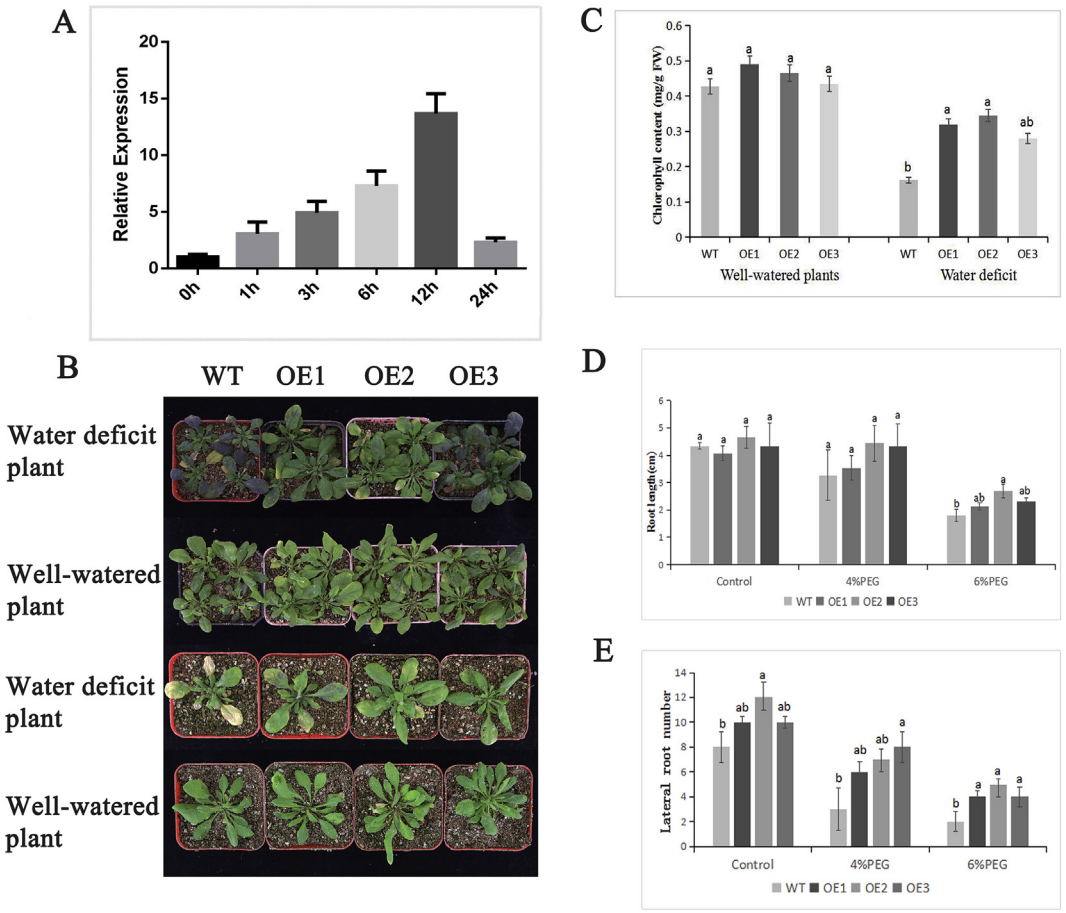

Fig. 6. *MdCER2* improves drought resistance. (a) The expression of *MdCER2* in response to drought, as determined by qPCR. (b) Watered and unwatered WT and *MdCER2* transgenic *Arabidopsis* growing in soil. (c) Chlorophyll leaching assay in rosette leaves of WT and *MdCER2* transgenic *Arabidopsis*, expressed in μmol/mg. FW = fresh weight. (d) The root length of WT versus transgenic *Arabidopsis*. (e) The number of lateral roots in WT versus transgenic *Arabidopsis*. The means and standard deviations were calculated from the results of five independent experiments.

increase of *MdCER2* expression.

MYB96 is an important regulator of drought stress. There is a molecular link that mediates ABA-auxin cross-talk in drought stress response and lateral root growth, providing an adaptive mechanism under drought stress conditions (Seo et al., 2009). And *MYB96* is a positive regulator of *ABI4* in the control of seed germination (Lee et al., 2015). According to the protein-protein interaction prediction, we found that there may be an interaction between *CER2* and *MYB96*. We speculate two regulatory pathways between *CER2*, *MYB96,* and drought. First, *MYB96*, as a transcription factor, may act on the upstream sequence of *CER2* to activate or suppress its transcription and thereby regulate the accumulation of wax in drought. Second, CER2 and MYB96 protein directly interact with each other to positively or negatively regulate plant drought resistance.

In conclusion, our study identified a wax-related gene, *MdCER2*, in apple. MdCER2 functions to enhance plant drought resistance by increasing the accumulation of wax or by interacting with MdMYB96, which needs to be further investigated.

Author contributions

YYL and YJH initiated and designed the research. MSZ, HJ, YC, YXW, and CXY performed the experiments. MSZ analyzed the data. YYL contributed reagents/materials/analysis tools. MSZ and YYL wrote the manuscript. All authors have read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.02.013.

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