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CmPPR4 gene controls drought resilience in melon ecotypes

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Summary

Global climate change has rendered drought stress an increasing threat to sustainable crop production. Melon (*Cucumis melo*) crop is widely cultivated worldwide, and has been classified into two subspecies *C. melo* ssp. *melo* and *C. melo* ssp. *agrestis* with greater drought tolerance variation. However, the genetic basis for the difference in drought resilience between two subspecies ecotypes remains unclear. In this study, we constructed an F_8 recombinant inbred lines (RILs) population generated by crossing drought-tolerant *C. melo* ssp. *melo* with drought-sensitive *C. melo* ssp. *agrestis* and identified a *CmPPR4* gene that encoded a pentatricopeptide repeat (PPR) protein highly associated with drought tolerance. A single nucleotide polymorphism (SNP) variation in *CmPPR4* resulted in a nonsynonymous mutation, leading to reduced drought resilience in *C. melo* ssp. *agrestis*. The geographical distribution of *CmPPR4* genotypes among 297 melon accessions closely parallels global annual precipitation patterns. Furthermore, the diminished drought tolerant capacity in RNA silencing seedlings and enhanced drought tolerance in overexpression lines further confirmed *CmPPR4* as a crucial regulator of drought tolerance in melon. Collectively, our findings provide new insights into the crucial role of *CmPPR4* in regulating drought tolerance of melon ecotypes, promoting molecular breeding of water-saving and drought-resilient melon cultivars.

Keywords: *CmPPR4*, drought tolerance, melon, QTL mapping.

Introduction

Anthropogenic activities are exacerbating environmental issues pertaining to global climate change. According to the World Economic Forum Global Risks Perception Survey 2021–2022, 'Climate action failure' and 'Extreme weather' are the top two global risks over the next decades (McLennan, 2022). Given that agricultural productivity hinges on weather and climate stability, climate change poses multifaceted challenges to crop production, notably concomitant with increased drought stress. Global climate change has rendered drought stress an increasing threat to sustainable crop production by negatively affecting plant growth, physiology and reproduction (Gupta *et al*., 2020; Mancosu *et al*., 2015; Zhu, 2016). Severe water deficiency leads to more than an 80% decrease in rice grain productivity annually (Li *et al*., 2015; Yadav *et al*., 2023), and maize yields can suffer losses ranging from 10% to 50% (Daryanto *et al*., 2016; Lobell and Burke, 2010; Olesen and Bindi, 2002). Soybean, as a drought-sensitive crop, is particularly vulnerable to water scarcity, impacting critical growth stages such as germination, floral initiation, pollination and fertilization, potentially reducing annual production by up to 40% (Shaffique *et al*., 2024; Shaheen *et al*., 2016). A comprehensive analysis across various vegetables, including bush beans, green beans, cabbage, peppermint, spearmint, yellow straight neck squash, zucchini and bell peppers, demonstrated that biomass under drought stress can decrease by nearly 15%–49% compared to well-watered conditions (Dong *et al*., 2020; Kim *et al*., 2020).

Accumulating knowledge has been gained about how drought stress impacts fundamental physiological processes, cellular functions and biochemical targets (Bohnert *et al*., 1995; Krasensky and Jonak, 2012; Shinozaki and Yamaguchi-Shinozaki, 2007; Xiong and Zhu, 2002). Plants can sense drought signals through their roots, closing the stomata and shaping the under- and above-ground architecture to withstand water loss (Gupta *et al*., 2020). As a complex regulatory network with numerous sensing and signalling genes (Zhu, 2016), many drought-related genes participating in hormone biosynthesis/response pathways were identified such as *ERA1* (enhanced response to ABA1) (Pei *et al*., 1998), *SnRK2* (SNF1-related protein kinase 2) (Okamoto *et al*., 2013; Park *et al*., 2015; Wang *et al*., 2018), *NAC* (Hu *et al*., 2006; Mao *et al*., 2015) and *WRKY* transcription factors (Chen *et al*., 2017a; Chen and Yin, 2017b). Besides, active *NF-YB1* (nuclear factor Y B subunits1) genes as the hormone biosynthesis/response genes can confer plants with higher drought tolerance in *Arabidopsis* and maize (Nelson *et al*., 2007), *AtCSLD5* (cellulose synthase-like

protein) gene can affect drought tolerance by adjusting cell wall chemical composition and ROS accumulation (Zhu *et al*., 2010), *DROT1* gene can render drought adaptation by modulating cell wall structure in upland rice (Sun *et al*., 2022), *ZmVPP1* (vacuolar-type H+ pyrophosphatase) and *ZmRtn16* (reticulon-like protein) gene can contribute to endure water deficiency by facilitating the vacuole H^+ in maize (Tian *et al*., 2023; Wang *et al*., 2016) and some *PPR* (pentatricopeptide repeat protein) genes can respond to drought stress signals and regulate drought tolerance in Arabidopsis, maize and soybean (Su *et al*., 2019; Wei and Han, 2016; Zsigmond *et al*., 2008). However, few causal genes have been mapped and validated to be effectively utilized in crop breeding programmes. As a result, this knowledge-to-application gap causes the limited success in developing novel drought-resilient crop varieties, which is also reflected in the market release of only three GMO cultivars with improvement of drought tolerance traits: DroughtGard™ maize by BASF, HB4 soybean and wheat by Bioceres (Gupta, 2024; Ribichich *et al*., 2020; Wang *et al*., 2015a). DroughtGard™ maize was developed through constitutive expression of cold shock protein B (CSPB) from *Bacillus subtilis* to improve performance of maize (Wang *et al*., 2015a). HB4 soybean and wheat were developed by expressing the sunflower transcription factor HaHB4 (Gupta, 2024, Ribichich *et al*., 2020).

Hence, more efforts are needed to explore the causative genes for drought tolerance from diverse crops and develop functional markers for enhancing drought resilience by design breeding. In exemplar, melon (*Cucumis melo* L.) is a major cash crop with great economic values which is cultivated worldwide. One of the primary challenges in melon farming is its higher water demand (Cabello *et al*., 2009; Chevilly *et al*., 2021). As an ancient species with a long domestication history, the earliest recorded melon can be traced back to 3700 BC in Lower Egypt (van Zeist and de Roller, 1993). Based on ovarian pubescence, melons can be classified into two subspecies, the *C. melo* ssp. *melo*, and *C. melo* ssp. *agrestis* (Jeffrey, 1980; Kerje and Grum, 2000; Kirkbride, 1993). Due to their original climate zone of domestication and cultivation, these two subspecies have evolved into distinct ecotypes. *C. melo* ssp. *agrestis* thrives in regions of Asia and Australia regions with ample water resources and are sensitive to drought stress. In contrast, *C. melo* ssp. *melo* is found predominantly in India, central and western Asia, Africa, Europe and the United States, adapting to arid environments and displaying drought tolerance (Fabeiro *et al*., 2002; Kerje and Grum, 2000; Sensoy *et al*., 2007). The hidden drought-tolerant gene resources of ecotype *C. melo* ssp. *melo* can be further exploited to improve drought adaptation for ecotype *C. melo* ssp. *agrestis* and interspecific hybrids between these two ecotypes, broadening their adaptabilities to arid or semi-arid climate zones. However, the genetic basis for the differentiation in drought tolerance between the two subspecies remains largely unclear.

In this study, we employed an advanced F_8 recombinant inbred lines (RILs) population derived from a cross between drought-tolerant accession 'XLH' (*C. melo* ssp. *melo*) and drought-sensitive accession 'HPSG' (*C. melo* ssp. *agrestis*). Through gene mapping, virus-induced gene silencing (VIGS), and overexpression transgenic approaches, we identified the *CmPPR4* gene, which encodes a pentatricopeptide repeat (PPR) protein. This gene is strongly associated with drought tolerance in melon, shedding light on the pivotal role of *CmPPR4* in the variation of drought tolerance across melon ecotypes.

Results

Screening and identification of phenotypic variations in drought tolerance

To investigate differences in drought tolerance between two melon subspecies, we first systematically evaluated the ability of drought tolerance of the parents of the mapping population, that is, *C. melo* ssp. *melo* accession 'XLH' and the *C. melo* ssp. *agrestis* accession 'HPSG'. After the 7-day-long drought treatment with 20% PEG, seven physiological parameters of the leaves and roots were measured. For the green part of the seedlings, the content of the chlorophyll, the *Fv/Fm* and the chlorophyll fluorescence curve were measured. The content of chlorophyll in 'HPSG' significantly upregulated under drought, while there was no difference in 'XLH' seedlings (Figure 1a). *Fv/Fm* can reflect the maximum quantum efficiency of PSII photochemistry, but in our assay, the value of the *Fv/Fm* between the two species was approximate 0.8 with no significant variation (Figure 1b). The chlorophyll fluorescence kinetics analysis contains large messages about the photosynthesis biophysics under stress. In the analysis of the OJIP curve, we found that the variation tendency from O (origin, minimal fluorescence) to J (intermediate inflection points at about 2 ms) to I (intermediate inflection points at about 20 ms) was the same in general, while from I to P (peak, maximum fluorescence), the fluorescence intensity of 'XLH' under drought rose slowly than 'HPSG' (Figure 1c). For the root architecture traits treated under different concentrations of PEG (10%, 20% and 30%), the main root length (MRL), the number of lateral roots (NLR), total root system (TRS) and lateral root system (LRS) were measured. To directly compare the results, the parameters of the root traits were converted into the stress coefficients, and no significant difference was detected between the two subspecies under different concentrations of PEG treatment (Figure 1d). Overall, the difference in the ability of drought tolerance between two accessions are mainly in leaves. Then we measured the survival rate according to the phenotypic differences in leaves. After a 14-day-long drought treatment and 7-day-long rewatering, leaves of 'XLH' were yellowish but still vigorous, while almost all leaves of 'HPSG' were wilted, and the survival rate of 'XLH' seedlings was 96.67% while the survival rate of 'HPSG' seedlings was 6.67% (Figure 1e, f), indicating the greater drought tolerance of 'XLH' seedlings than 'HPSG' seedlings.

Thereout, we employed a resequencing F_8 RIL population derived from crossing drought-tolerant 'XLH' and drought-sensitive 'HPSG' using the single seed descent method (Yang *et al*., 2020), and evaluated the survival rate of 104 RILs under 14-day-long drought and 7-day-long rewatering treatment. As a quantitative trait, the frequency distribution of the RIL population tends to be complex (Figure 1g). RILs were divided into three groups, the drought-sensitive group with a survival rate between 0% and 33%, the intermediate group with a survival rate between 33% and 67% and the drought-tolerance group with a survival rate between 67% and 100%. There are 35 lines belonging to the drought-sensitive group, 38 lines belonging to the intermediate group and 25 lines belonging to the drought-tolerance group (Table S1).

QTL mapping of candidate genes associated with drought tolerance

To identify the candidate genes for drought tolerance in melon seedlings, QTL mapping based on survival rate after drought

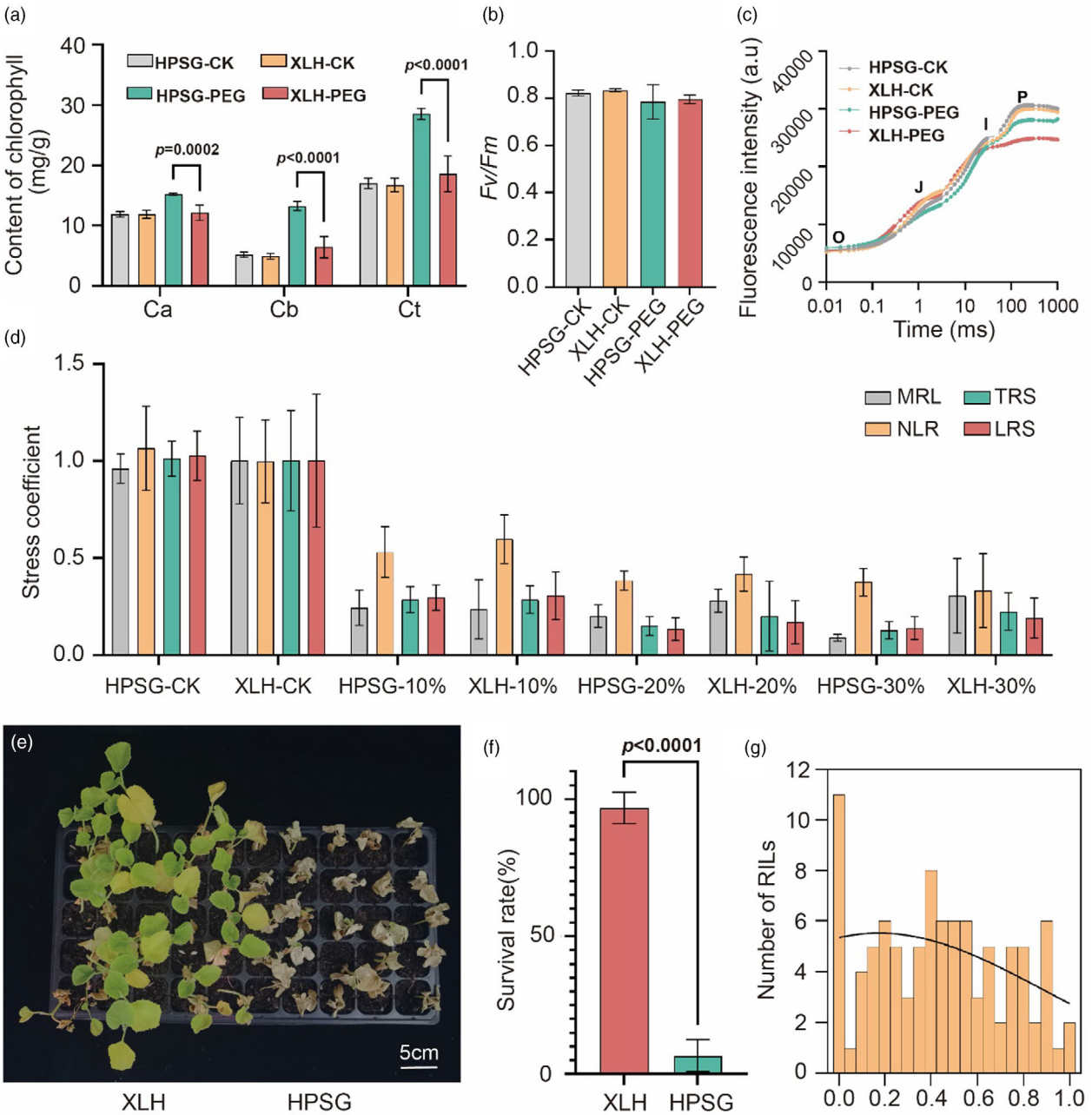

Figure 1 Phenotypic difference of drought tolerance in two melon subspecies and RILs. (a) The difference of chlorophylls contents in drought-tolerant parental line 'XLH', (*C. melo* ssp. *melo*) and drought-sensitive parental line 'HPSG' (*C. melo* ssp. *agrestis*) under control and drought treatment. Ca, chlorophyll a; Cb, chlorophyll b, Ct, total chlorophyll. (b) The maximum quantum efficiency of PSII photochemistry shown as *Fv/Fm* of the parent lines under control and drought treatment. (c) The OJIP chlorophyll fluorescence curve of the parent lines under control and drought treatment. O stands for origin or minimal fluorescence, J stands for intermediate inflection points at about 2 ms, I stands for intermediate inflection points at about 20 ms and P stands for peak or maximum fluorescence. (d) The root architecture traits of the parent lines under control and drought treatment. 10%, 20% and 30% were the concentrations of PEG treated with melon seedlings; MRL, the main root length; NLR, the number of lateral roots; TRS, total root system; LRS, lateral root system. (e) Photograph of the drought-tolerant 'XLH' and drought-sensitive 'HPSG' seedlings under drought treatment. (f) The survival rate of the drought-tolerant 'XLH' and drought-sensitive 'HPSG' seedlings under drought treatment. (g) Frequency distribution of survival rate of RILs after drought treatment. Statistical significance is analysed using the ordinary one-way ANOVA test and *t*-test.

treatment was conducted. Using the resequencing data of the F_8 RIL population and genome of one of the parent lines 'HPSG' assembled in our previous work (Yang *et al*., 2020), a major QTL was identified on chromosome 5 named as qDT_Cm05 (Figure 2a, Table S2). The length of the major QTL qDT_Cm05 was 200 kb (27, 150, 000–27, 350, 000) long with eight genes harbouring in the candidate region under the default threshold (Figure 2b). The eight genes are *MELO11404* encoding a

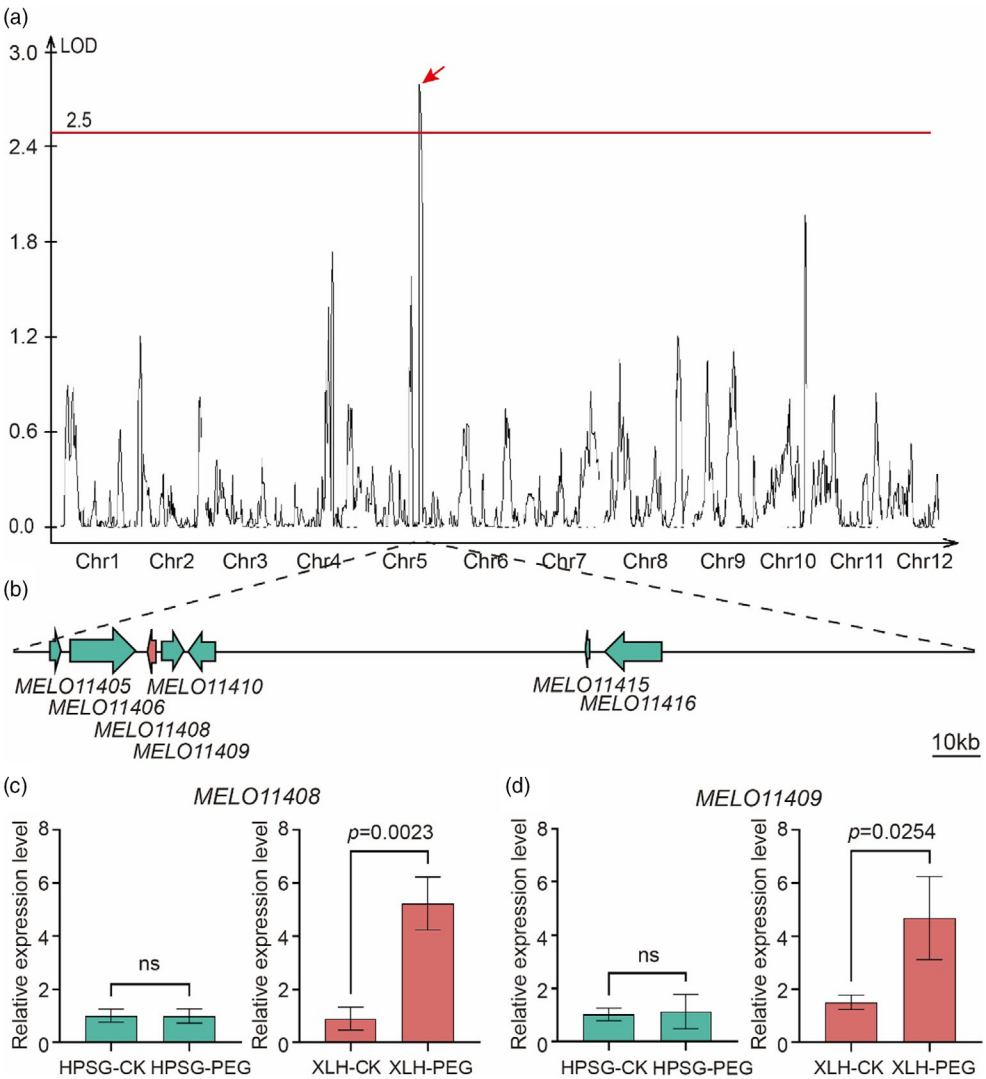

Figure 2 QTL mapping and identification of candidate genes for drought tolerance. (a) Results of QTL mapping of drought tolerance in RILs. Graph of the LOD values used for trait association analysis. The x-axis indicates the 12 chromosomal positions and the y-axis indicates the LOD (Logarithm of Odds Ratio) values. (b) Graph of the gene distribution pattern of the candidate interval harbouring eight genes. The red arrow indicates the *MELO11408* (*CmPPR4*) locus. Scale bar, 10 kb. (c and d) Relative expression levels of *MELO11408* (c) and *MELO11409* (d) under different treatments with water and PEG. Tissues from the third leaves of 1-month-old seedlings after 7-day-long control and PEG treatment were sampled for analysis. Statistical significance is analysed using the *t*-test.

vesicle-associated protein 2–2 isoform X1, *MELO11405* encoding an acyl carrier protein, *MELO11406* encoding a coiled -coil domain-containing protein SCD2, *MELO11408* encoding a pentatricopeptide repeat-containing family protein, *MELO11409* encoding a superoxide dismutase, *MELO11410* encoding an ATP-dependent RNA helicase, *MELO11415* encoding an unknown protein and *MELO11416* encoding an acyltransferase-like protein, respectively (Table S3). To further validate the function of the eight candidate genes in response to drought stress, the gene expression level was analysed after drought treatment for 7 days in both 'XLH' and 'HPSG'. Only *MELO11408* and *MELO11409* were upregulated by drought stress treatment in drought-tolerant 'XLH' (Figure 2c, d), suggesting that these two genes might be the key genes in regulating drought tolerance in melon.

A nonsynonymous mutation in *CmPPR4* is relevant to drought tolerance variants

To further confirm the key gene in regulating drought tolerance in melon, we analysed the sequence variations of the two candidate genes, *MELO11408* and *MELO11409*. Based on the resequencing data of the RILs and the cloning results of the DNA and cDNA in the parent lines, drought-sensitive 'HPSG' and drought-tolerant 'XLH', we identified 11 SNPs associated with *MELO11408* and *MELO11409*. Among these, only one nonsynonymous mutation was found in the exon of *MELO11408* (Table S3). *MELO11408* encoded a pentatricopeptide repeat-containing family protein (CmPPR4). CmPPR4 belongs to the P subfamily with 4 typic P-type motifs, and the abbreviated protein form of CmPPR4 is 135-P–P–P-36-P-32

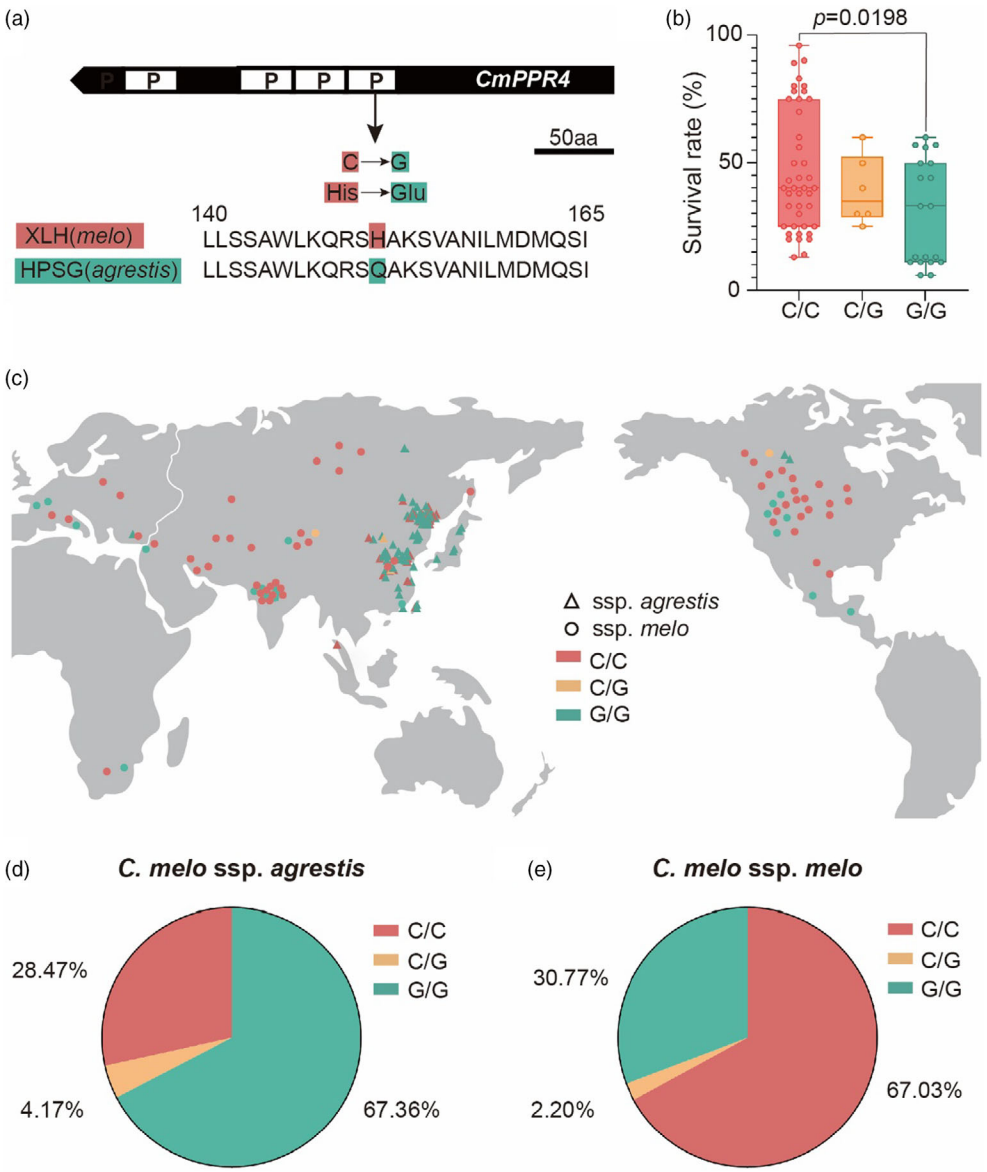

Figure 3 A single SNP variation in *CmPPR4* differentiates drought tolerance from melons. (a) Protein sequence variations in *CmPPR4* between two melon subspecies. A nonsynonymous mutation on the first PPR motif of CmPPR4 from cytosine (C) to guanine (G) results in the amino acid variation from histidine (H) to glutamine (Q). (b) Box graph integrating the genotype and phenotype of the RILs. Red parts represented the C/C genotype same as the drought-tolerant 'XLH' melon, green parts represented the G/G genotype same as the drought-sensitive 'HPSG' melon and orange parts represented the C/G genotype same as the F_1. (c) Geographical distribution of genomic variations of *CmPPR4* in 297 melon accessions. Ninety one *C. melo* ssp. *melo* accessions are represented by dots and 144 *C. melo* ssp. *agrestis* accessions represented by triangles. Accessions with drought-tolerant C/C genotype are filled with red colour, accessions with drought-sensitive G/G genotype are filled with green colour and accessions with intermediate C/G genotype are filled with orange colour. (d and e) Pie graph of the genotyping in 297 melon accessions. Statistical significance is analysed using the Mann-Whitney test.

(https://ppr.plantenergy.uwa.edu.au/) (Figure 3a). Since the characteristic of the PPR family is the architecture of multiple tandem repeats of helix-turn-helix motifs, it is noteworthy that there may be a huge impact of the nonsynonymous mutation from cytosine (C) in 'XLH' to guanine (G) in 'HPSG' on the first P-type motif of *CmPPR4* for the gene expression, protein structure and function. The following genotyping results of the RIL population using the KASP SNP marker at the nonsynonymous mutation site confirmed the function of this SNP variation in governing melon drought tolerance (Table S4). Integrating the genotyping and phenotyping results, RILs with the C/C genotype generally represented higher survival rates than the G/G genotype and the survival rates of RILs with the C/G genotype were in the middle of the C/C and G/G genotype (Figure 3b).

As 'XLH' belongs to *C. melo* ssp. *melo* while 'HPSG' belongs to *C. melo* ssp. *agrestis*, we performed an analysis of the nonsynonymous mutation in 297 melon varieties using the previously reported resequencing data by Liu *et al*. (2020a). The accessions of the drought-tolerant C/C genotype were

distributed mainly in Western Asia, Europe and the North American regions with relatively arid environments, while accessions of the drought-sensitive G/G genotype were distributed mainly in East Asia with abundant water sources (Figure 3c). In a total of 91 *C. melo* ssp. *melo* accessions, 67.03% of the accessions were the C/C genotype same as 'XLH', 2.20% were the C/G genotype and 30.77% were the G/G genotype (Figure 3d). In a total of 144 *C. melo* ssp. *agrestis* accessions, 67.36% of the accessions were the G/G genotype same as 'HPSG', 4.17% were the C/G genotype and 28.47% were the C/C genotype (Figure 3e, Table S4). The high agreement between the genotype and the melon ecotypes suggests that *CmPPR4* might be the key gene determining the differentiation in drought tolerance between two melon subspecies.

TRSV-based RNA silencing of *CmPPR4*^{C} reduces drought tolerance

To further validate the function of CmPPR4 in regulating drought tolerance, *CmPPR4*^{C} was knocked down in drought-tolerant *C. melo* ssp. *melo* 'XLH' plants using a *CmPPR4*-targeted TRSV (tobacco ringspot virus-based) vector referring to Fang *et al*. (2021). Setting plants infected with TRSV-*CuPDS* (*CUCURBIT PHYTOENE DESATURASE*) as a biological indicator, the moment TRSV-*CuPDS* filtrated plants appeared photo-bleaching phenotype (Figure S1a), drought-tolerant 'XLH' plants inoculated with empty vector TRSV-control or TRSV-*CmPPR4*^{C} were treated with 20% PEG for 14 days and rewatered for 7 days. Then, the phenotype of plants infected by TRSV-control and TRSV-*CmPPR4*^{C} were investigated and the leaves of treated plants were sampled to analyse the silencing efficiency of TRSV and the expression level of *CmPPR4*. After the 14-day-long drought and 7-day-long rewatering treatment, there were significant differences between plants infected by TRSV-control and TRSV-*CmPPR4*^{C}. The height of plants incubated with TRSV-*CmPPR4*^{C} was significantly shorter than TRSV-control and the leaves of plants incubated with TRSV-*CmPPR4*^{C} were withered and yellow to a greater extent (Figure 4a). The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) showed that the relative expression level of *CmPPR4* in all plants infected by TRSV-*CmPPR4*^{C} was remarkably decreased than plants infected by TRSV-control, which indicated the high RNA silencing efficiency of TRSV (Figure 4b, Figure S2b). As the negative control, almost all seedlings incubated with TRSV-control were alive, while only 36.93% of seedlings incubated with TRSV-*CmPPR4*^{C} survived on average (Figure 4c). These data suggested RNA silencing *CmPPR4*^{C} in drought-tolerant melon can weaken the ability to withstand drought stress.

Overexpression of *CmPPR4*^{C} enhances drought tolerance in *C. melo* ssp. *agrestis*

To further confirm the function of *CmPPR4*, we generated two overexpression lines of *CmPPR4*^{C} in drought-sensitive *C. melo* ssp. *agrestis* 'HPSG' seedlings, named as HPSG-OE-*CmPPR4*^{C}-1 and HPSG-OE-*CmPPR4*^{C}-2, respectively. The expression level of the 2 overexpression lines was detected, in HPSG-OE-*CmPPR4*^{C}-1 *CmPPR4* slightly increased 2.279 folds compared to 'HPSG', while in HPSG-OE-*CmPPR4*^{C}-2 *CmPPR4* increased 3.232 folds compared to 'HPSG' (Figure 4e). Although there was no remarkable difference in overexpression lines at the expression level of *CmPPR4*, the phenotype of overexpression lines was evident. After drought treatment, seedlings of overexpression lines were more vigorous than 'HPSG', all HPSG-OE-*CmPPR4*^{C}-2 seedlings survived with an average survival rate of 100% and HPSG-OE-*CmPPR4*^{C}-1 seedlings with an average survival rate of 96.67% (Figure 4d, f). These results suggested that overexpression of *CmPPR4* in the background of 'HPSG' can enhance drought tolerance, and further verified that *CmPPR4* can positively regulate drought tolerance in melon seedlings.

Discussion

The environments of the crop origin and distribution play a crucial role in shaping the adaptation of plants by ecological factors such as climate, soil conditions and microbial communities (Desaint *et al*., 2021; Milanovic *et al*., 2020). Plants evolving in different geological habitats may develop different tolerance mechanisms to cope with the biotic and abiotic stresses. Plants originating from arid climates may have vigorous root systems and compact plant architecture for reducing water loss, while plants grown in areas with high humidity may have shallow root systems and higher transpiration capacity (Gupta *et al*., 2020; Hund *et al*., 2009; Zhu, 2016). As an economically important cucurbit crop, melon is cultivated around the world, spanning different ecological regions with varying precipitation levels at different latitudes. In fact, owing to the different areas of origin and domestication, the two subspecies are clearly differentiated into two distinct ecotypes. The *C. melo* ssp. *melo* is drought-tolerant ecotype, while the *C. melo* ssp. *agrestis* is drought-sensitive ecotype (Kerje and Grum, 2000; Luan *et al*., 2008; van Zeist and de Roller, 1993). Many physiological parameters varied in drought-tolerant *C. melo* ssp. *melo* and drought-sensitive *C. melo* ssp. *agrestis* at different growth stages (Dasgan *et al*., 2015; Fila *et al*., 2019; Sharma *et al*., 2019). In this study, we assessed the response of both above- and below-ground biomass of melon plants to drought stress conditions. Our findings indicate that the difference in drought tolerance between two accessions primarily manifests in the above-ground biomass, contrasting to our previous observations in watermelon (Mahmoud *et al*., 2022, 2023). Accordingly, we used the survival rate of the above-ground portions to evaluate the RIL population derived from crossing a drought-tolerant *C. melo* ssp. *melo* with a drought-sensitive *C. melo* ssp. *agrestis*. By gene mapping approaches, we successfully identified a single SNP variation on the *CmPPR4* gene highly associated with drought tolerance (Figures 2–4). Interestingly, the geographical distribution of two *CmPPR4* haplotypes among 297 melon accessions closely fits the global annual precipitation patterns (Asadieh and Krakauer, 2016). Melons carrying the *CmPPR4*^{G} haplotype (most are *C. melo* ssp. *agrestis*) are predominantly found in regions with abundant annual precipitation, whereas those with the *CmPPR4*^{C} haplotype (most are *C. melo* ssp. *melo*) are mainly found in areas with less annual precipitation. These findings imply that *CmPPR4* may play a crucial role in determining the drought tolerance disparity between two melon subspecies. As a superfamily with the symbolic PPR motif, numerous PPR proteins have been studied in food crops (Small and Peeters, 2000). According to the length, the PPR motif can be divided into three types. The classic P-type motif has 35 amino acids, the L(long)-type motif has 35–36 amino acids and the S(short)-type motif has 31 amino acids. Based on the motif constitution, the PPR proteins are classified into the P subfamily and the PLS subfamily. The P subfamily proteins only contain the typical P-type motif, while the PLS subfamily proteins contain characteristic triplets of the P-, L- and S-type motifs (Barkan and Small, 2014; Chateigner-Boutin and Small, 2010;

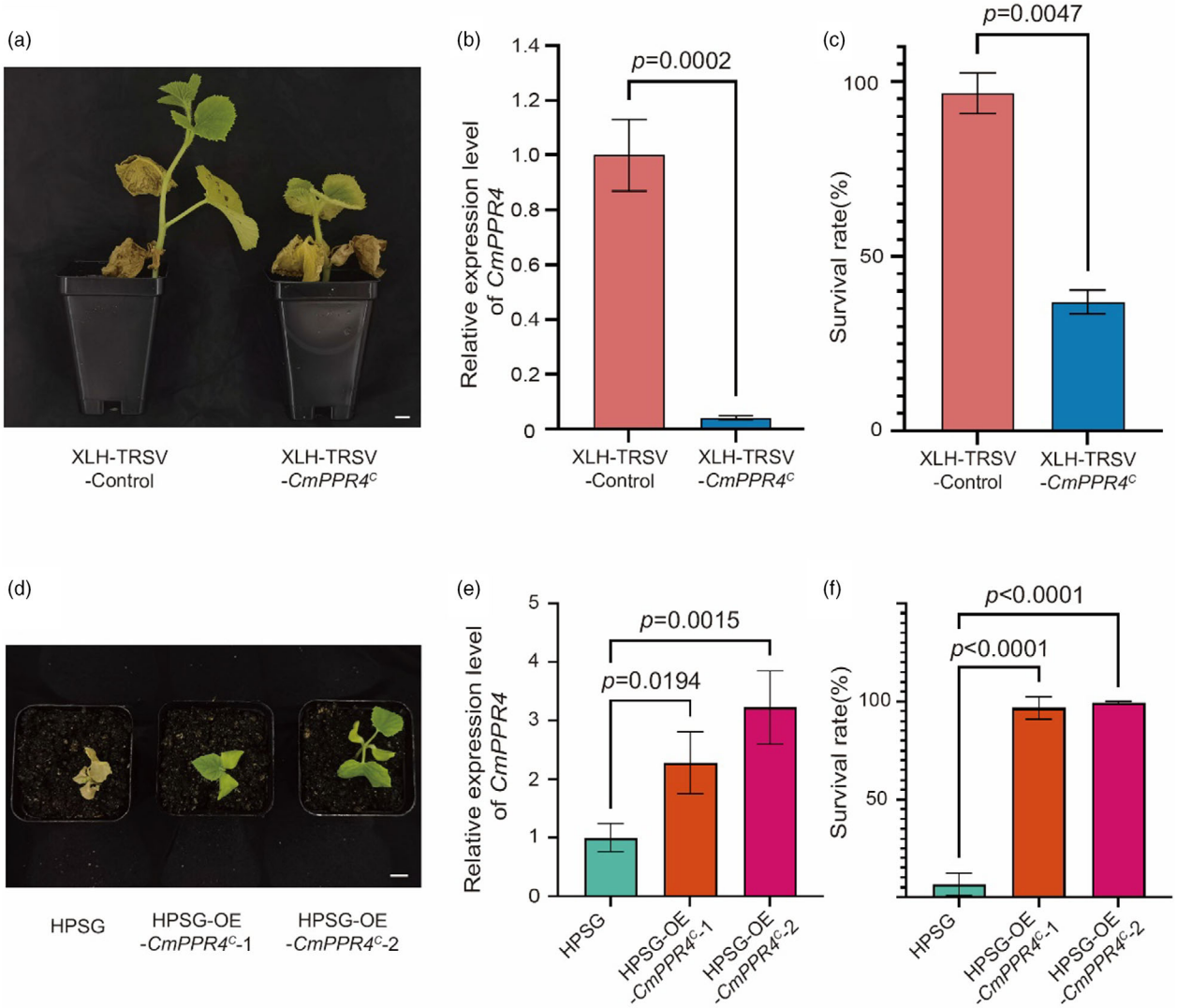

Figure 4 Functional validation of *CmPPR4* in regulating drought tolerance in melons. (a) Photograph of melon seedlings filtrated with XLH-TRSV-Control and XLH-TRSV-*CmPPR4*^{C} under drought treatment. (b) The relative expression levels of *CmPPR4* in melon seedlings filtrated with XLH-TRSV-Control and XLH-TRSV-*CmPPR4*^{C} under drought treatment. (c) The survival rates of melon seedlings filtrated with XLH-TRSV-Control and XLH-TRSV-*CmPPR4*^{C} under drought treatment. (d) Photograph of the *CmPPR4*^{C} overexpression lines under HPSG background after drought treatment. (e) The relative expression levels of *CmPPR4* for the *CmPPR4*^{C} overexpression lines under HPSG background after drought treatment. (f) The survival rates of the *CmPPR4*^{C} overexpression lines under HPSG background after drought treatment. Statistical significance is analysed using the *t*-test and ordinary one-way ANOVA.

Cheng *et al*., 2016; Small and Peeters, 2000). Most PPR proteins are mitochondria- or chloroplast-targeted; some of them can function in post-transcriptional regulation (Barkan and Small, 2014), fertility restoration in cytoplasmic male sterility plants (Bentolila *et al*., 2002), embryogenesis (Cushing *et al*., 2005), photosynthesis (Hashimoto *et al*., 2003; Kotera *et al*., 2005; Meierhoff *et al*., 2003), plant development (Hammani *et al*., 2011; Yuan and Liu, 2012; Zhao *et al*., 2020) and responses to stress (Su *et al*., 2019; Wei and Han, 2016; Yuan and Liu, 2012). Studies on the model plants have proved that many PPR proteins are linked to abiotic stress. For instance, a mitochondrial PPR protein PPR40 was reported to be involved in the generation of reactive oxygen species (ROS) under salt, abscisic acid (ABA) and oxidative stress in *Arabidopsis* (Zsigmond *et al*., 2008). Besides, ABO5, PGN, SLG1, SLO2 and PPR96 were also verified to participate in responses to different abiotic stresses in *Arabidopsis* (Laluk *et al*., 2011; Liu *et al*., 2010, 2016; Yuan and Liu, 2012; Zhu *et al*., 2014). In rice, two chloroplast-targeted PPR proteins OsV4 and tcd10 were reported to be involved in chloroplast development under cold stress (Gong *et al*., 2014; Wu *et al*., 2016). In maize, the expression level of 7 *ZmPPRs* was detected to be upregulated under drought stress (Wei and Han, 2016). In soybean, GmPPR4 was identified to be involved in drought tolerance (Su *et al*., 2019).

But whether it plays a key role in abiotic stress is still unknown. In this study, we first mapped the *CmPPR4* gene from melon and demonstrated that the haplotype *CmPPR4*^{C} acts as a positive regulator of drought tolerance through VIGS in drought-tolerant *C. melo* ssp. *melo* accession 'XLH' and overexpression in drought-sensitive *C. melo* ssp. *agrestis* 'HPSG'. Collectively, our current study first demonstrates that *CmPPR4* encoding a pentatricopeptide repeat protein is highly responsible for drought tolerance

variation in melon. A single nucleotide polymorphism (SNP) alteration gives rise to a nonsynonymous mutation in *CmPPR4*, leading to the difference in drought tolerance between two melon subspecies. The haplotype-resolved marker from the SNP variant on *CmPPR4* is promising in molecular breeding of climate-resilient melon varieties, expanding their adaptations to increasing drought stress.

Experimental procedures

Plant materials and evaluation of drought tolerance phenotypes

The population consists of 104 F_8 RIL lines derived from a cross between the drought-tolerant accession 'XLH' (*C. melo* ssp. *melo* var. *inodorus*) and the drought-sensitive accession 'HPSG' (*C. melo* ssp. *agrestis* var. *conomon*), using single seed descent method. This population was resequenced as described in our previous work (Yang *et al*., 2020). The parental lines and the RIL population were cultivated in the phytotron in Hangzhou, Zhejiang, China, with 16 h light at 28°C and 8 h dark at 22°C. After 1-month cultivation, seedlings with 3–4 leaves including cotyledons were treated with adequate 20% polyethylene glycol (PEG) for 14 days and then rewatered for 7 days referring to a modified method on cucumber (Wang *et al*., 2015b). Drought tolerance was evaluated by the average survival rate of treated seedlings (number of seedlings survival/total number of seedlings). Ten seedlings for each line were guaranteed to be under drought treatment. The content of chlorophyll was measured 7 days after drought treatment following the spectrophotometric methods with at least three biology replications (Walkerce, 1985). The chlorophyll fluorescence parameters containing *Fv/Fm* and OJIP curve were measured 7 days after drought treatment using the POCKET-PEA with at least three biology replications (Hansatech Instruments Ltd). The root architecture traits were investigated using the germination pouches and analysed by the EZ-Root-VIS with six biology replications under treatment of different concentrations of PEG (Mahmoud *et al*., 2022). The statistical data were converted into stress coefficients by calculating the proportion of the difference between values under stress and values under control for subsequent comparison.

Statistical analysis and QTL mapping approaches

All data were first managed by Microsoft Excel 2016 and tested by IBM SPSS Statistics 25, pictures were drawn by GraphPad Prism 9.5.1 and Adobe Illustrator 2024. QTL mapping was performed based on the chromosome-scale genetic map of one of the parent lines 'HPSG' and resequencing data of 104 F_8 RILs as described in our previous work (Yang *et al*., 2020). After calculating the genetic distance between adjacent markers according to the Kosambi mapping function, WinQTLcart software (parameters: CIM, permutation time—500, *p*-value—0.05) was used for generating QTL mapping results of the survival rate under drought.

Sequence and expression analysis of candidate genes

Total RNA was isolated from the third leaves of 1-month-old seedlings of parent lines after 7 days under drought and control treatment using the Trizol Reagent (ThermoFisher Scientific, Waltham, USA). Qualified RNA was reverse transcripted into cDNA following the protocol of ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Based on the sequencing data of the reference genome and data on CuGenDB (http://www.cucurbitgenomics.org/), cloning primers were designed for candidate genes (Table S5). Then, PCR was conducted to amplify candidate genes using the KOD One PCR Master Mix (TOYOBO, Osaka, Japan). The product was constructed to Blunt Zero cloning vector (Transgen Biotech, Beijing, China) for the following sequencing. Sequencing results were aligned by SnapGene 4.1.8 and blasted on NCBI (https://www.ncbi.nlm.nih.gov/).

For detecting the expression level of candidate genes, specific primers (Table S5) were designed on the genscript website (http://www.genscript.com). cDNA of different treatments was used as the template. The reaction was set following the standard steps of SYBR Green supermix (TOYOBO, Osaka, Japan) using the StepOnePlus Real-Time PCR System (Applied Biosystems, ThermoFisher, USA) with three biology repetitions and three technical repetitions for each treatment. The relative expression value was further analysed by applying the $2^{-\Delta\Delta CT}$ algorithm using the actin gene as the internal reference.

Genotypings of the RILs and 297 resequencing melon accessions

The targeted single nucleotide polymorphism (SNP) in the RIL population was genotyped by the kompetitive allele-specific PCR (KASP) platform. The KASP TF V4.0 2x Mastermix (LGC, Hoddesdon, United Kingdom) was blended with a mixture of the primer combination (Fam, Hex, Common) with a volume ratio of 2:2:5 (Table S5). The KASP reaction was performed following the protocol and the amplification results were detected using an LGC Genomics system (Hoddesdon, United Kingdom) (Liao *et al*., 2020). The published resequencing data of 297 melon accessions (Liu *et al*., 2020a) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/) under BioProject ID PRJNA529037 and used for further analysis. After aligning the homology gene of *CmPPR4* to the reference genome (DHL92 V3.5.1; Garcia-Mas *et al*., 2012) of the 297 melon accessions, we extracted genotyping data for the targeted SNP. The genotyping results of the nonsynonymous mutation, taxonomic information and the global geographical distribution of the 297 melon accessions were integrated. The distribution graphs about the distribution of genomic variations of *CmPPR4* across the 297 melon accessions were created using Adobe Illustrator 2024 and GraphPad Prism 9.5.1.

VIGS assay

The miRNA silencing vectors were offered by Li's lab from Shandong Agricultural University. A 137 bp short fragment from the coding region of *CmPPR4* was inserted into the TRSV2 vector using TRSV-*CmPPR4*-F/R primers (Table S5). Then the constructed plasmid was transformed into *A. tumefaciens* GV3101 cells as the standard protocol (Hofgen and Willmitzer, 1988). The resuspended mixture of these miRNA silencing vectors was first inoculated into *N. benthamiana* plants, and collected after 6 days for infiltrating melon seedlings. The above infiltrating procedures were referred to a tobacco ringspot virus-based vector system (Fang *et al*., 2021). The moment seedlings infiltrated with TRSV-*CuPDS* (*CUCURBIT PHYTOENE DESATURASE*) plasmid showed photo-bleaching phenotype, seedlings infiltrated with TRSV-*CmPPR4*^{C} were treated under drought with at least five biology repetitions. After a 14-day drought and 7-day rewatering routine, the drought tolerance ability of treated seedlings was evaluated. Samples for further analysis were harvested 7 days after drought treatment.

Generation of overexpression lines

The coding sequence of *CmPPR4* without stop codon was cloned by the OE-*CmPPR4*-F/R primers (Table S5) and inserted into the pMDC83 binary expression vector under the control of the CaMV 35S promoter. Then the constructed plasmid was transformed into *A. tumefaciens* GV3101 cells as the standard protocol (Hofgen and Willmitzer, 1988). Explants grown from wounded cotyledons were generated following a modified tissue culture procedure in watermelon (Chen *et al.*, 2023). Briefly, sterilized seeds without hull were sowed on the germination medium (MS 2.22 g/L with sucrose 25 g/L and phytagel 4 g/L) and cultivated in the dark at 26–28°Cfor 4 days. Then, the central section of every cotyledon was cut into four parts as explants, placed on the pre-culture medium (MS 4.43 g/L with sucrose 25 g/L, 6-BA 1.5 mg/L, and phytagel 4 g/L), and cultivated with 16 h light and 8 h dark at 26°C for 2–3 days. After that, explants were infiltrated with GV3101 cells carrying the constructed vectors and co-cultivated on the co-culture medium (MS 4.43 g/L with sucrose 25 g/L, 6-BA 1.5 mg/L, and phytagel 4 g/L) in dark at 26–28°Cfor 3–4 days. Afterwards, explants were rinsed and transferred onto the screening medium (MS 4.43 g/L with sucrose 25 g/L, 6-BA 1.5 mg/L, phytagel 4 g/L, kanamycin 150 mg/L and Timentin 100 mg/L) and cultivated with 16 h light and 8 h dark at 26°C till generating new shoots. New shoots were subsequently transferred onto the elongation medium (MS 4.43 g/L with sucrose 25 g/L, 6-BA 0.05 mg/L, phytagel 4 g/L, kanamycin 120 mg/L and Timentin 100 mg/L) for 2 weeks. Then, vigorous shoots were transferred onto the rooting medium (MS 4.43 g/L with sucrose 25 g/L, 6-BA 0.05 mg/L, phytagel 4 g/L, kanamycin 60 mg/L and Timentin 40 mg/L). When five roots were formed, the aseptic seedlings were transferred into the soil. Afterwards, DNA was extracted and the existence of the plasmid was first verified by PCR using primers on the vector. Positive overexpression lines were further cultivated and pollinated. The expression level of *CmPPR4* in seedlings from the T_2 generation was measured by qRT-PCR and these T_2 seedlings were used for further analysis.

Author contribution

XL and MZ designed the project. YX conducted most of the experiments; MX, QR, YZ, HS, MY, HZ, AB, HC, RJ and KZ performed some of the fieldwork and measured the phenotypes. YB, YM, ZH and JY performed some data analysis; YX wrote the manuscript, and MZ and XL revised it.

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Conflict of interest

The authors declare that there is no conflict of interest.

Significance statement

The *CmPPR4* gene encoding a pentatricopeptide repeat protein was mapped and verified to be associated with drought tolerance of melon ecotypes.

Data availability statement

All data generated in this study are included in this article and its supplementary information files.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Relative expression levels of genes in the candidate region with no significant differences. Relative expression levels of *MELO11404* (a), *MELO11405* (b), *MELO11406* (c), *MELO11410* (d), *MELO11415* (e) and *MELO11416* (f) under different treatments with water and PEG. Tissues from the third leaves of 1-month-old seedlings after 7-day-long control and PEG treatment were sampled for analysis. Statistical significance is analysed using the *t*-test.

Figure S2. Detection of RNA silencing efficiency using TRSV system. (a) The photo-bleaching phenotype of the plant infected with TRSV-*CuPDS*. (b) The relative expression level of *CmPPR4* in plants infected by TRSV-*CmPPR4*^{C}.

Table S1. Survival rates of the RILs population under drought treatment.

Table S2. Variations in the QTL qDT_Cm05.

Table S3. Information of eight candidate genes.

Table S4. Genotyping of the 297 resequencing melon accessions and the 104 RILs.

Table S5. Primers for gene cloning, qPCR, KASP and vector construction.

