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ORIGINAL ARTICLE

The *qLTG1.1* candidate gene *CsGAI* regulates low temperature seed germination in cucumber

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Abstract

Key message **The *CsGAI* gene, identified by map-based, was involved in regulating seed germination in low temperature via the GA and ABA signaling pathways.**

Abstract Low temperature reduces the percentage of seeds germinating and delays seed germinating time, thus posing a threat to cucumber production. However, the molecular mechanism regulating low temperature germination in cucumber is unknown. We here dissected a major quantitative trait locus *qLTG1.1* that controls seed germination at low temperature in cucumber. First, we fine-mapped *qLTG1.1* to a 46.3-kb interval, containing three candidate genes. Sequence alignment and gene expression analysis identified *Csa1G408720* as the gene of interest that was highly expressed in seeds, and encoded a highly conserved, low temperature-regulated DELLA family protein *CsGAI*. GUS expression analysis indicated that higher promoter activity underscored higher transcriptional expression of the *CsGAI* gene. Consistent with the known roles of *GAI* in ABA and GA signaling during germination, genes involved in the GA (*CsGA2ox*, *CsGA3ox*) and ABA biosynthetic pathways (*CsABA1*, *CsABA2*, *CsAAO3* and *CsNCED*) were found to be differently regulated in the tolerant and sensitive genotypes under low temperatures, and this was reflected in differences in their ratio of GA-to-ABA. Based on these data, we proposed a working model explaining how *CsGAI* integrates the GA and ABA signaling pathways, to regulate cucumber seed germination at low temperature, thus providing new insights into this mechanism.

Introduction

Cucumber (*Cucumis sativus* L.) is one of the major economically important vegetable crops around the world. As a typical chilling-sensitive crop originated from tropical area (Bulder et al. 1991; Cabrera et al. 1992; Kłosińska et al. 2013), cucumber normally germinates at a temperature of 24–28 °C (Wehner 1982, 1984a). Previous studies indicated that the low temperature (LT) range of cucumber seed germination is from 13 °C to 17 °C (Nienhuis et al. 1983; Wehner 1984a, b). However, straight seeded is a major planting pattern at early season and exposing to sub-optimal temperature may delay germination and emergence, so low temperature germination (LTG) is vital for cucumber growth well. LT stress is shown to delay seed germinating time, reduce the percentage of seeds germinating, decrease yield, and is a serious obstacle for cucumber production (Nienhuis et al. 1983; Røeggen 1987). Thus, it is critical to identify genes responsible for LTG to develop novel germplasm with high LTG ability.

Previous studies showed that LTG ability in cucumber was controlled by multiple genes (Gu et al. 2002; Kłosińska et al. 2013); however, very few quantitative trait locus (QTL) analyses of LTG in cucumber have been reported so far. Many indices were used to evaluate seed germination ability at LT, such as germination rate (GR), germination energy (GE), germination index (GI), days to germination (DIG),

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and radicle length (RL) (Kłosińska et al. 2013; Song et al. 2018; Wehner 1984b; Yagcioglu et al. 2019). Dong (2017) identified eight loci associated with LT tolerance on chromosome 4, chromosome 6 and chromosome 7 for LTG using a $F_{2:3,}$ population. Three QTLs related to LTG ability (major-positive effect *qLTG1.1*, minor-positive effect *qLTG2.1* and *qLTG4.1*) were detected using RILs in our lab(Song et al. 2018). Furthermore, a recent study identified a major-positive effect locus *qLTG1.2*, and two minor-positive effect loci (*qLTG2.1* and *qLTG4.1*) using RILs population and $F_{2:3}$ families (Yagcioglu et al. 2019). However, LTG related-genes and their molecular mechanism still need further investigation in cucumber.

The plant hormones gibberellic acid (GA) and abscisic acid (ABA) play crucial roles in seed dormancy and germination (Graeber et al. 2012; Shu et al. 2016), with GA promoting germination (Richards et al. 2001) and ABA promoting seeds dormancy (Liu et al. 2016; Tyler et al. 2004). DELLA transcription factors are key repressors of the GA signaling pathway, and are negatively regulated by GA (Feng et al. 2008). DELLAs have also been shown to interact with the transcription factor ABA INSENSITIVE5 (ABI5), which controls germination by positively regulating a set of ABA-responsive genes (Liu et al. 2016; Xi et al. 2010; Yang et al. 2020). Additionally, ABA synthesis and metabolism related genes, such as zeaxanthin epoxidase (*ABA1/ZEP*) (Agrawal et al. 2001), short-chain dehydrogenase/reductase family member (*ABA2/SDR*) (González-Guzmán et al. 2002), abscisic aldehyde oxidase (*AAO3*), ABA 8'-hydroxylase (*CYP707*) (Kushiro et al. 2014), and 9-cis-Epoxycarotenoid dioxygenase (*NCED*) (Tan et al. 1997), also play crucial role in seeds germination. In *Arabidopsis*, there are five members of the DELLA family: GA-INSENSITIVE (GAI), REPRESSOR OF GA1-3 (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3 (Daviere and Achard 2016; Lee et al. 2002; Peng et al. 1997; Silverstone et al. 1998; Tyler et al. 2004). *GAI, RGA*, and *RGL2* are the major suppressors of seed germination in *Arabidopsis* (Chahtane et al. 2018; Piskurewicz et al. 2008, 2009). However, it is unclear how the DELLAs regulate seed germination and response to LT-stress in cucumber.

Many transcription factors in regulatory pathways are activated to perceive and respond to LT-stress (Ding et al. 2020). These transcription factors include the U-box type E3 ubiquitin ligases (PUB25/26)(Wang et al. 2019), BRASSINOSTEROID-INSENSITIVE2 (BIN2) (Ye et al. 2019), high expression of osmotically responsive gene 1 (HOS1) (Dong et al. 2006), cold-responsive protein kinase 1 (CRPK1) (Liu et al. 2017), mitogen-activated protein kinase 3 (MPK3) and MPK6 (Li et al. 2017), ETHYLENE INSENSITIVE4 (EIN4) (Shi et al. 2012) and PHYTOCHROME-INTERACTING FACTOR 3 (PIF3) (Jiang et al. 2017). They directly or indirectly regulate downstream genes, such as INDUCER OF CBF EXPRESSION 1 (ICE1) (Chinnusamy et al. 2007; Shi et al. 2018b; Zhu 2016). ICE1, in turn, is able to activate C-REPEAT BINDING FACTOR (CBF) expression (Chinnusamy et al. 2003; Fursova et al. 2009), thereby precisely modulating the expression of a set of downstream COR (Cold-regulated) genes involved in LT tolerance (Shi et al. 2018b). Additionally, with cold exposure, *CBF3* promotes the accumulation of DELLA proteins, which in turn positively regulate *CBF3* in *Arabidopsis* creating a positive feedback loop (Zhou et al. 2017). Achard et al. (2008) suggested that cold-induced *CBF1* reduced bioactive GA by up-regulating GA 2-oxidase genes (*GA2ox*) in the GA signaling pathway, and that the enhanced accumulation of DELLAs ultimately inhibits seed germination. However, the mechanisms underscoring the integration of environmental factors and hormone signals to regulate seed germination have not been well studied in cucumber.

Given this gap in knowledge, the objectives of this study were to identify candidate genes responsible for LTG and to clarify how the candidate gene contributes to the underlying regulatory mechanisms for LTG. Thus far, key components of the molecular mechanism determining seed germination at LT in cucumber remains largely elusive. To meet our objectives, we first fine-mapped a major QTL controlled LTG using a backcross population. Second, we identified and then validated a candidate gene, i.e., *Csa1G408720*, which encodes a DELLA protein CsGAI as a key regulator of LTG. This was accomplished by comparative sequence analysis, expression analysis, hormones measurements, and by assessing *CsGAI* promoter activity. Third, we speculated the cognate regulatory pathway of the candidate gene and elucidate how the candidate gene might control seed germination under LT-stress. This study will aid molecular marker-assisted breeding and molecular mechanism of germination ability in response to LT in cucumber.

Materials and methods

Plant materials

Parental lines '65G' and '02245' that previously used for QTL identification (Song et al. 2018) were used to develop the F_1, F_1 was consistently cross with recurrent parent to construct backcross (BC) population, $BC_4F_{2:3}$ were used for fine-mapping (Fig. S1). Two lines 'HR78' (with low germination ability at 13 °C) and 'HR170' (with high germination ability at 13 °C) are near isogenic lines derived from parents, they have different genotype of major locus *qLTG1.1* but identical genotypes of two minor loci *qLTG2.1* and *qLTG4.1*. All materials were provided by the Cucumber Research Group, Institute of Vegetable and Flowers, Chinese Academy of Agricultural Science, Beijing. The materials

were grown in greenhouse in Nankou, Beijing, China in Fall 2017, 2018 and 2019.

Low temperature germination assay

Fifteen seeds per triplicate were placed in the same incubator at 13 °C for 14 days in the dark and while using 28 °C for 3 days as the control (Kłosińska et al. 2013). 'HR78', 'HR170' and recombinant individuals were divided into three replicates by a randomized complete block. The number of germinated seeds was recorded on daily basis during LT treatment. The percentage of seeds germinating was calculated to indicate LTG ability as shown in our previous study (Song et al. 2018), that is the percentage of seeds germinating (%) = (*n*/15)*100%, where "*n*" is the number of germinated seeds at 13 °C. The relative the percentage of seeds germinating values calculated by (the percentage of seeds germinating at 13 °C)/(the percentage of seeds germinating at 28 °C) were used for subsequent fine mapping and statistical analysis.

Whole genome background screening

A total of 127 insertion–deletion (InDel) and simple sequence repeat (SSR) markers were used for genetic mapping in our earlier study (Shi et al. 2018a; Song et al. 2018). A total of 50 polymorphic markers distributed uniformly on seven chromosomes were selected for genomic background analyses in each BC population (Fig. S1b). The individual with the highest background was used to construct the BC_2F_1 and all subsequent populations until BC_4F_1 (Fig. S1a), and their background homozygosity were from 50% to 93.5%. A total of six flanking markers, including InDelLTG33 and InDelLTG31 for *qLTG1.1*, 2SSR00204 and 2SSR22338 for *qLTG2.1*, 4SSR23549 and 4SSR07431 for *qLTG4.1*, were used to screen recombinant individuals in the BC_1 and BC_2 populations. Two flanking markers of *qLTG1.1*, InDelLTG33 and InDelLTG31 were used in the BC_3 and BC_4 populations (Fig. S1a). Finally, the heterozygous loci (highlighted in red in Fig. S1b) only existed on Chr. 1. Therefore, it was self-pollinated to produce F_2 progeny for map construction and $F_{2:3}$ progeny for genotyping.

Molecular marker and map construction

In our previous study, three QTLs including the major locus *qLTG1.1* for LTG was identified (Song et al. 2018). Six flanking markers were used to screen recombinant individuals of the BC population and to construct a linkage map. Genomic DNA of each individual was extracted from the second true leaf of seedlings at the two-leaf stage using the CTAB method (Wei et al. 2019). The polymerase chain reaction (PCR) amplification and agarose gel electrophoresis were performed as described by Song et al (2018). According to the 9930 V2 Cucumber Genome (http://cucurbitgenomics.org) and the whole genome resequencing information of parents, all new InDel and SNP primers were designed and first verified to be polymorphic between two parental lines. Finally, eight SNPs and InDels molecular markers were used to phenotype the recombinant individuals. All the PCR primers (Table S1) were designed with DNAMAN7.0 (Woffelman 2004).

Gene prediction, cloning and sequence analyses of candidate genes

Using the cucumber genomic information website, three annotated genes within *qLTG1.1* interval were identified and were then cloned. PCR amplification was performed with a total volume of 20 µL reaction: 2 µL of genomic DNA, 1 µL of both forward and reverse primers, 10 µL high fidelity enzyme Mix (Vazyme, Nanjing, China) and 6 µL ddH_2O. The PCR conditions were as follows: 94 °C for 5 min, 35 cycles of (94 °C for 15 s, 55 °C For 15 s, 72 °C for 90 s), 72 °C for 5 min, and a final incubation at 4 °C. The PCR products were sequenced by Sangon Biotech (Beijing, China). The gene sequence of 'HR78' and 'HR170' were aligned using DNAstar (Swindell and Plasterer 1997). The regulatory elements in the promoter region were predicted using the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002).

Real-time quantitative reverse transcription polymerase chain reaction analysis

For cucumber, the time-course expression of the three candidate genes, together with the genes in the GA and ABA signaling pathways were examined during seed germination. Cucumber seeds exposed to 13 °C in an incubator for 0, 10 and 24 h, and for 3, 7 and 14 d. Seeds exposed to 28 °C as control were collected at 0, 10, and 24 h, and 3 d. Additionally, various young tissue samples (root, stem, leaves, young stems, female flower, male flower, tendrils, roots, ovary) and various stage of seed (30 DAF, 45 DAF, 60 DAF and imbibed seeds) of 'HR78' and 'HR170' were collected. For Arabidopsis, T_2 transgenic lines (*CsGAI* $Pro^{2000-HR170}$ and *CsGAI* $Pro^{2000-HR78}$) were grown on 1/2 MS agar media. Two-week-old seedlings were transferred to soil medium in a growth chamber at 24 °C with 16 h/8 h day/night. The rosettes of each individual were collected for RNA extraction. Three biological replicates were set for each treatment.

Total RNA was extracted, and the first-strand complementary DNA (cDNA) was synthesized using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan), respectively, according to the manufacturer's instructions.

Quantitative reverse transcription PCR analysis (qRT-PCR) was performed using SYBR Premix Ex TaqTM II (TaKaRa, Kyoto, Japan). The PCR primers (Table S1) were designed with DNAMAN7.0 (Woffelman 2004). *Actin1* (*Csa3G806800*) was employed as the reference gene to normalize gene expression values (Xie et al. 2018). Each qRT-PCR experiment was performed with three biological replicates. The analysis of gene relative expression data was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Extraction and Quantification of exogenous GA and ABA

For endogenous hormone quantification, the seeds of 'HR78' and 'HR170' in control (0, 10, and 24 h, and 3 d at 28 °C) and LT (0 h, 24 h, 3 d and 7 d at 13 °C) environments were collected. Endogenous hormones in 0.2 g seeds was quantified by ELISA (euzymelinked immunosorbent assay) method. Three independent biological replicates were performed.

Application of exogenous GA, PAC and ABA

Fifteen seeds per replicate of 'HR78' and 'HR170' were treated with different hormone concentration: GA3 (Coolaber, Beijing, China) (100, 400, 1000, 2000, and 4000 mg/L), ABA (Sigma, USA) (5, 50, and 100 µmol/L), PAC (Sigma, USA) (10, 50, and 100 µmol/L). All seeds were treated in the same incubator at 13 °C in dark for 14 days. The optimal concentration of hormones was then used to investigate its effect on germination ability under LT in 'HR78' and 'HR170'. Three biological replicates were used for each treatment.

Subcellular localization of the CsGAI protein

The full-length coding sequence of *CsGAI* without the stop codon was cloned into the pSUPER1300 vector between the *Xba*I and *Kpn*I-HF sites. The *CaMV35S* promoter was used to drive the expression of *GFP-CsGAI*. The control vector *pSUPER1300-GFP* and *CaMV35S::GFP-CsGAI* fusion were infiltrated into epidermal cells of tobacco leaves (4- to 6-weeks old) using the *Agrobacterium* mediated method(Sparkes et al. 2006). Fluorescence images were taken with a confocal laser-scanning microscope (Zeiss LSM510) excited at a 488-nm wavelength. The primers for vector construction were listed in Table S1.

Phylogenetic analysis of CsGAI protein homologs

To further understand the function of *CsGAI,* phylogenetic trees were generated. DELLA proteins in cucumber were obtained from the Cucurbit Genomics Database, and the deduced amino acid sequences of the *CsGAI* were employed to search homologous proteins in seven other species including *Cucumis melo, Cucumis melo var.makuwa, Cucurbita moschata, Cucurbita pepo subsp.pepo, Cucurbita maxima, Arabidopsis thaliana, Oryza sativa, Triticum aestivum, Zea mays* using the protein BLASTP tool in the NCBI database (Altschul et al. 1997) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Homologous amino acid sequences were downloaded in FASTA format for alignment in ClustalW (Thompson et al. 1994), and a phylogenetic tree was constructed using a maximum parsimony method with MEGA7.0 software (Kumar et al. 2016).

GUS assay constructs, histochemical staining, and GUS transcript assessment

The 2.0-kb promoter fragments upstream of the ATG start codons were independently amplified by PCR using 'HR78' and 'HR170' genomic DNA as the templates. The fragments were then inserted upstream of the *GUS* gene in the binary vector. Fully expanded 5-week-old tobacco leaves were infiltrated with *Agrobacterium tumefaciens* (OD600 = 1.0) harboring the recombinant plasmids. The GUS activities were measured using a GUS staining kit (Solarbio science & technology Co., Ltd. Beijing, China). Meanwhile, recombinant plasmids $CsGAIPro^{2000-HR78}$*::GUS* and $CsGAIPro^{2000-HR170}$*::GU*S was used to transform *Arabidopsis* Col-0 plants using the floral dip method(Dong et al. 2020). Transgenic seedlings were selected for their resistance to the 30 mg/mL hygromycin and further confirmed by PCR using *hyg* specific primer PCR (Table S1). The resistant T_1 transgenic seedlings were selected and propagated until T_2 homozygotes could be selected for further analysis. The GUS transcript level of $CsGAIPro^{2000-HR78}$*::GUS* and $CsGAIPro^{2000-HR78}$*::GU*S were measured(Park et al. 2011).

Statistical analysis

All tests for significant differences were conducted in Microsoft Excel, and the mean values for each measured parameter were compared using two-way analysis of variance or two-tailed, two-sample Student's *t*-test in Excel (2016).

Results

Narrowing down *qLTG1.1* into a 46.3-kb region via map-based cloning

We previously generated a RIL population to map the loci controlling seed germination in response to LT in cucumber,

and identified three loci named *qLTG1.1*, *qLTG2.1* and *qLTG4.1* on chromosomes 1, 2 and 4, respectively (Song et al. 2018). To further reduce the 569-kb interval of *qLTG1.1* (Fig. 1a), we first generated near-isogenic lines (NILs) (Fig. S1a). A segregating population of $F_{2:3}$ plants were derived from the NILs, and 11 recombinants were identified using two flanking markers (IndelLTG31 and IndelLTG33). Additional markers were designed based on parental resequencing information, of which 6 polymorphic InDel markers and 2 polymorphic SNP markers were employed to construct the genetic map (Table S1). Finally, based on the genotypic data of each recombinant plant and the relative position of the 8 markers (Fig. 1b, c), *qLTG1.1* was mapped into the 46.3-kb region flanked by SNP14.95 and IndelLTG1. To exclude potential disturbance due to minor-effect loci co-localizing with *qLTG1.1*, each individual offspring polymorphic at the major locus *qLTG1.1* but with identical alleles at the minor loci *qLTG2.1* and *qLTG4.1* were selected. Two near isogenic lines, 'HR78' and 'HR170', which had the sensitive and tolerant allele respectively, met this criteria, and were chosen for subsequent study.

CsGAI was identified as the candidate gene controlling LTG

Three genes, i.e., *Csa1G408700, Csa1G408710,* and *Csa1G408720* were found within the 46.3-kb region (Fig. 1d; Table S2). To further analyze the sequence of these genes, the promoter (a 2.0-kb upstream sequence from the translation start codon ATG), full-length DNA and promoter of each gene from 'HR78' and 'HR170' were cloned and sequenced (Table S2). *Csa1G408700* showed 100% nucleotide sequence identity (Fig. S2a), while *Csa1G408710* had one SNP within the second intron (Fig. S2b). Interestingly, there were three SNPs and nine InDels in the promoter region of *Csa1G408720*, and the -1876th Indel, -1870th Indel and -923th SNP were located in *cis*-acting element CAAT-box, TATA-box and MYC elements, respectively (Fig. 2a). These *cis*-elements in promoter regions are closely related with gene transcription expression, and with response to stress. Several low temperature-responsive *cis*-acting elements were identified in the promoter region of *Csa1G408720,* for example, an ABA-responsive element

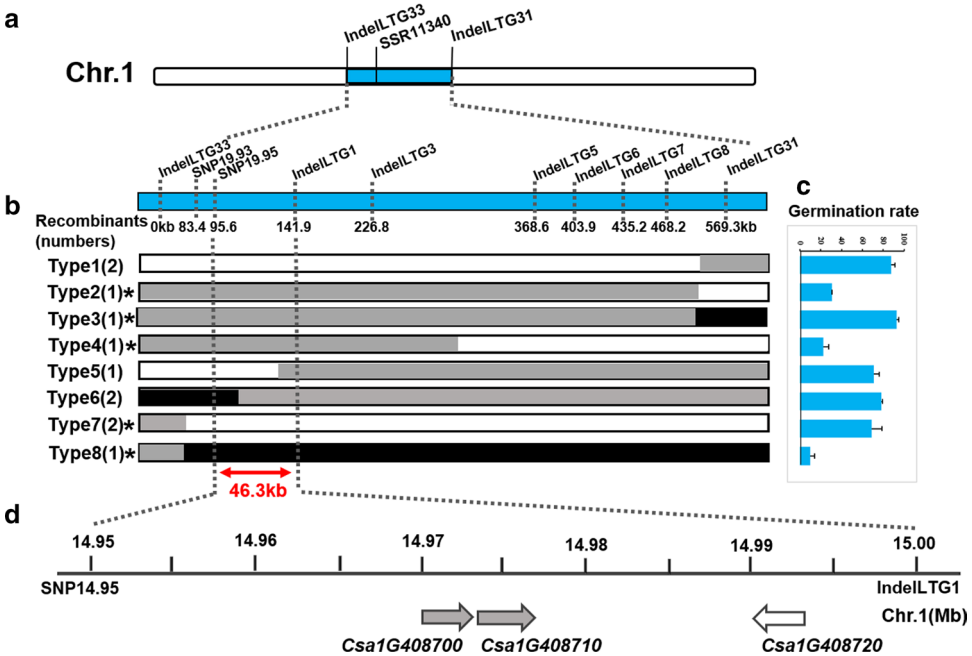


Fig. 1 Map-based cloning of *qLTG1.1*. **a** Analysis of major-effect QTL for low temperature germination using RILs population generated from parents. **b** Key recombinants used for the genetic and physical mapping of *qLTG1.1*. The relative physical distances of eight InDels and SNPs markers are indicated on the map based on their physical position in '9930' cucumber V2 genome; white and black columns represent homozygous fragments from 'HR170' to 'HR78', respectively; gray columns represent heterozygosity. "*"represents heterozygosis. **c** Phenotypic data of recombinants were shown on the right. The percentage of seeds germinating of 'HR170' is high and 'HR78' was low. Bar graphs representing the percentage of seeds germinating of each genotype. 0 < the percentage of seeds germinating < 40 amounts to a low the percentage of seeds germinating, 60 < the percentage of seeds germinating < 100 amounts to a high the percentage of seeds germinating. **d** The physical location of the three candidate genes between SNP14.95 and IndelLTG1 in the '9930' reference genome. Arrow indicated direction of the three genes. The final mapping region contains following three genes*: Csa1G408700*, *Csa1G408710*, and *Csa1G408720*

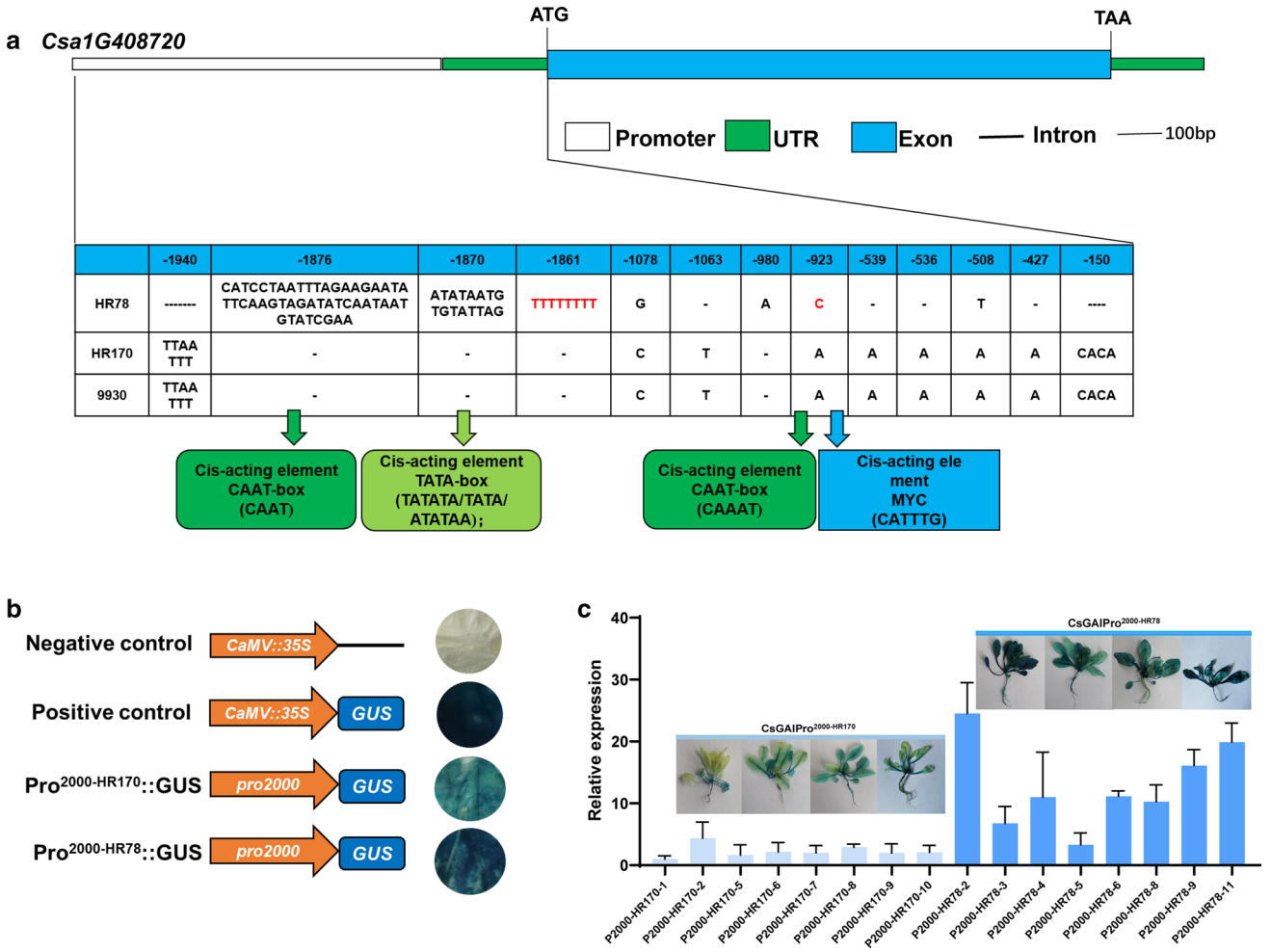


Fig. 2 Qualitative analyses of the activity of the *CsGAI* promoter. **a** Gene structure and nucleic acid variation in the *Csa1G408720* promoter between 'HR78' and 'HR170' lines. Functional element prediction of promoter in mutation locus. **b** *GUS* activity of the *CsGAI* promoter in tobacco leaves based on GUS histochemical staining. The *Pro*$^{2000-HR78}$*::GUS* construct consists of GUS fused to the promoter cloned from the LT-sensitive 'HR78', while the *Pro*$^{2000-HR170}$*::GUS* construct contained the promoter cloned from the LT-tolerant 'HR170'. GUS activity in both lines was significantly higher and lower than the negative and positive controls respectively. **c** Relative *GUS* transcript expression level in CsGAI *Pro*$^{2000-HR78}$*-GUS* and CsGAI *Pro*$^{2000-HR170}$*-GUS* plants

(ABRE), a MYB-binding site (MBS), and Methyl jasmonate-responsive element (TGACG motif) (Fig. S3).

Based on the above results, the promoter region is important for regulating the expression of the gene (Riewe et al. 2016). Sequence alignment of the *CsGAI* sequence from the LT-tolerant 'HR170' and LT-sensitive 'HR78' indicated that there were three SNPs and nine InDels in the promoter region (Fig. 2a). To determine if the polymorphisms in *CsGAI* gene's promoter region, could influence expression, we cloned the promoter sequence of the 'HR78' (*CsGAI Pro*$^{2000-HR78}$*::GUS*) and 'HR170' (*CsGAI Pro*$^{2000-HR170}$*::GUS*) genotypes. Their activities were determined using an *Agrobacterium*-mediated GUS transient assay in tobacco leaves followed by the detection of GUS using histochemical staining, along with the quantification of GUS transcripts in the *Arabidopsis* leaves. The *CsGAI* promoter activity in the *CsGAI Pro*$^{2000-HR78}$*::GUS* plants led to higher GUS expression than the promoter activity in the *CsGAI Pro*$^{2000-HR170}$*::GUS* lines (Fig. 2c). These results are in good agreement with tobacco and transgenic *Arabidopsis* plants. These results indicated that the promoter variation of *CsGAI* gene effected seed germination in low temperature.

To further analyze the three candidate genes, their expression pattern under LT-germination was studied. RNA from seeds of 'HR78' and 'HR170' exposed to LT for 0, 10, and 24 h, 3 d and 7 d were extracted, respectively, and then, qRT-PCR was performed to quantify the expression level of each gene. No expression changes were found in either *Csa1G408700* (Fig. S4a) or *Csa1G408710* (Fig. S4b) during LT-germination. However, the expression of *Csa1G408720* was significantly lower in 'HR170' after 24 h in the cold, compared with 'HR78' (Fig. S4c). The results is consistent

with the differential promoter activity of *CsGAI* gene. This difference was dependent on LT, as there was no disparity detected during seed germination at 28 °C (Fig. 3a). In addition, the expression pattern of *Csa1G408720* was consistent with the germination pattern of 'HR78' and 'HR170' (Fig. 3b). The temporal expression pattern of *Csa1G408720* was examined in several tissues vegetative and reproductive tissues, and at various stage of seeds development in both 'HR78' and 'HR170'. The highest expression of *Csa1G408720* was observed in seeds, both during dormancy and after imbibition (Fig. 3c), which was consistent with the expression of its orthologue in *Arabidopsis* (http://bar.utoronto.ca/eplant/). Therefore, we proposed that *Csa1G408720* is the most likely candidate gene for LTG.

Subcellular localization and phylogenetic analysis of *CsGAI*

Gene annotation suggests that *Csa1G408720* encodes the DELLA transcription factor *GAI*, therefore we changed its nomenclature to *CsGAI*. *CsGAI* is a member of the GRAS gene family DELLA subfamily, and consists of both a DELLA and GRAS domain (Fig. S5). Its ortholog in *Arabidopsis*, *AT1G14920,* encodes DELLA family member GAI, which is a key transcription factor that regulates seed germination by modulating the expression of GA responsive genes (Piskurewicz et al. 2008; Wang 2014). We performed a transient expression assay to determine the subcellular location of *CsGAI*, and showed that the *GFP-CsGAI* fusion protein was localized to the nucleus (Fig. 4a). This is consistent with *CsGAI* putative role as a transcriptional regulator. To test if the function of *CsGAI* is conserved in cucumber, we performed phylogenetic analysis with 15 DELLAs orthologues from nine species, including fruits, vegetables and cereals (Fig. 4b). The results showed that the DELLA protein encoded by *CsGAI* is highly conserved in *Arabidopsis* and many other plant species, which suggests that they may share similar functions. Interestingly, dicotyledonous and monocotyledonous plants clustered into two large clades. Within the dicotyledonous DELLA subclade, other cucurbitaceous DELLA proteins were close to cucumber DELLAs, which suggested that the clustering was largely consistent with the taxonomic position in the tree.

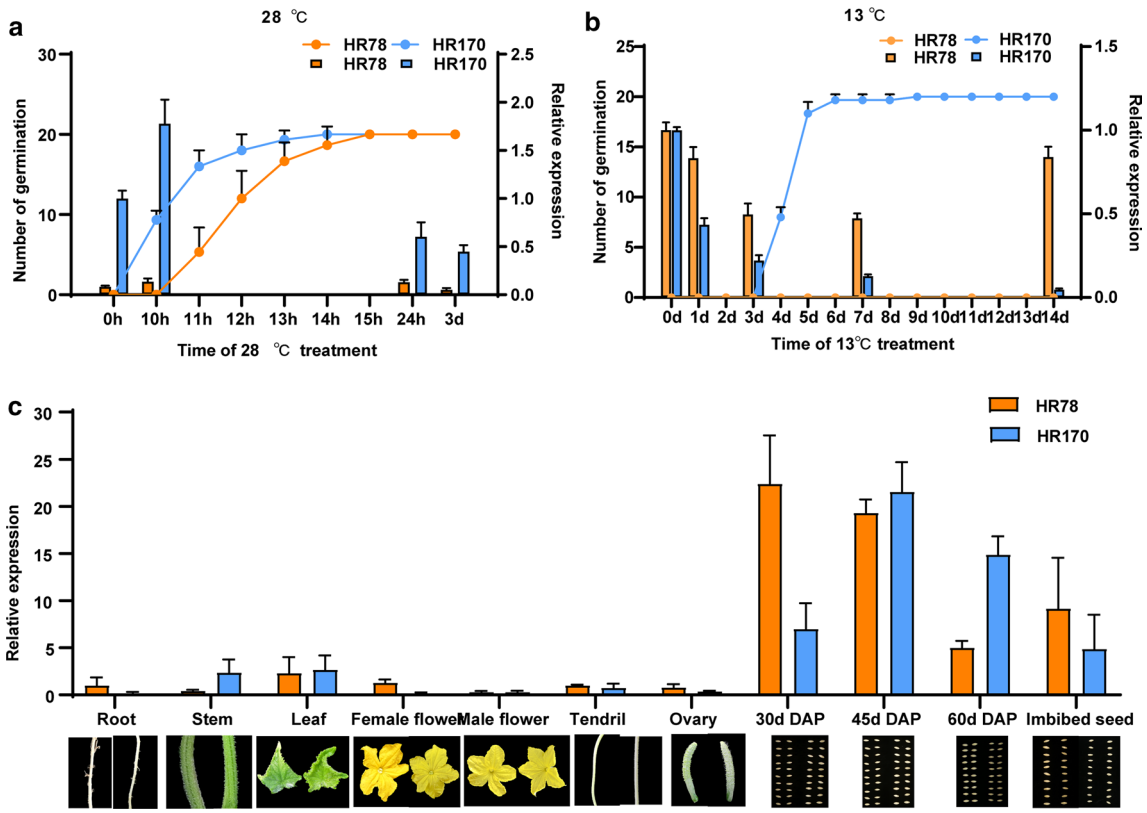


Fig. 3 Spatio-temporal expression of *CsGAI* in cucumber. Relationship between number of germination and relative expression in 28 °C **a** and 13 °C **b** treatment was shown between 'HR78' and 'HR170'. The line chart represents 'Number of germination' and the column diagram represents 'Relative expression'. **c** Data represent the expression of *CsGAI* relative to that of actin, measured by qRT-PCR in different organs and stages of seeds in 'HR78' and 'HR170'. DAF: days after anthesis

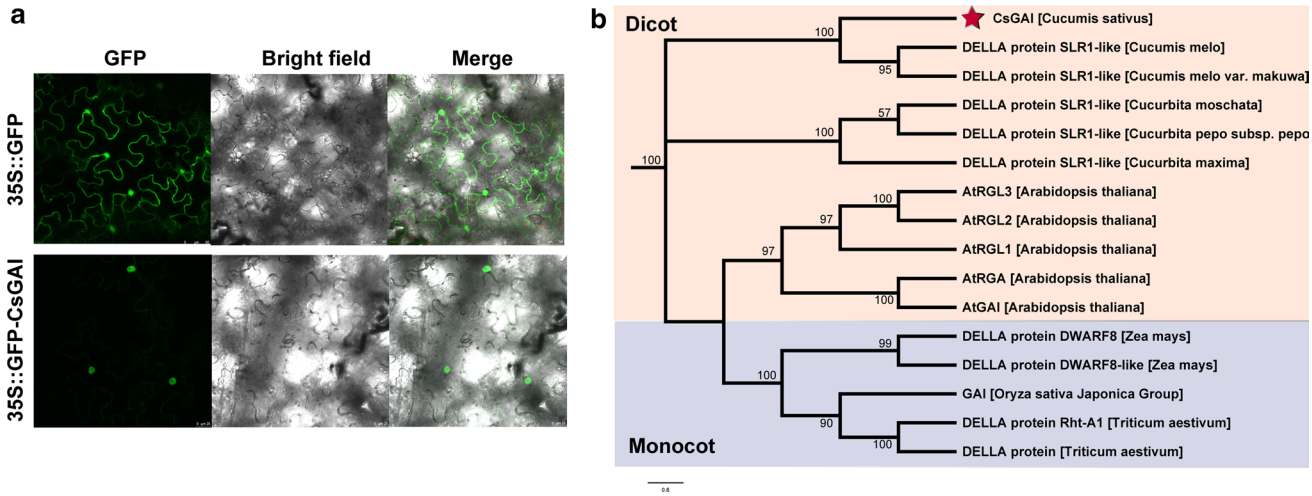


Fig. 4 Subcellular localization and phylogenetic analysis of protein encoded by *CsGAI*. **a** *35S::GFP* and *35S::CsGAI* constructs were transiently expressed in tobacco leaf epidermal cells. The fusion GFP-CsGAI protein was localized to the nucleus. Scale bars were located in bottom right corner of picture. **b** Phylogenetic analysis of the protein encoded by *CsGAI* (indicated with a red pentacle), and its 15 related-DELLA proteins family from nine species

Alleviation of germination inhibition by increased endogenous GA and decreased endogenous ABA at LT in 'HR170'

Members of the DELLA family have roles regulating seed germination via the GA and ABA signaling pathways in *Arabidopsis* (Boccaccini et al. 2014; Hu et al. 2019; Piskurewicz et al. 2008). To test if *CsGAI* regulates cucumber seed germination in LT via these hormonal signaling pathway, the endogenous GA and ABA content of 'HR78' and 'HR170' in seeds germinated at 28 °C were measured at different time points over 3 days, and at 13 °C over 7 days. As Fig. 3 shows, both 'HR78' and 'HR170' seeds germinated within 15 h at 28 °C, however, while 'HR170' germinated within 6 d at 13 °C, 'HR78' did not. Accordingly, an increase in GA and a decrease in ABA was detected in both 'HR78' and 'HR170' at 28 °C (Fig. 5a, c). Interestingly, at 13 °C, GA content was significantly reduced while ABA content increased, leading to a lower ratio of GA-to-ABA from 3 days before seed germination to 7 d after seed germination in 'HR78', while the opposite was true in 'HR170,' i.e., the ratio of GA-to-ABA was higher (Fig. 5b, d). Therefore, we propose that the differing LTG ability of 'HR78' and 'HR170' is due to differences in the regulation of GA and ABA in response to LT-stress, which operated in diametrically opposing ways.

Inhibition of germination by exogenous GA inhibitor and ABA in 'HR170' at LT

To test our hypothesis, exogenous GA3, a GA inhibitor (PAC) and ABA were applied to isogenic lines 'HR78' and 'HR170'. Four GA3 concentrations (100, 400, 1000, 2000, 4000 mg/L), three PAC concentrations (5, 50, 100 µmol/L) and ABA concentrations (5, 50, 100 µmol/L) were first tested and the optimal concentration of GA3 (100 mg/L), PAC (50 µmol/L), and ABA (50 µmol/L) were used. Results showed that some seeds of 'HR78' could germinate when GA3 was applied (Fig. 5e), and that the germination of 'HR170' at 13 °C was suppressed by the GA inhibitor (PAC) and by ABA (Fig. 5f, g). These results support the view that different GA and ABA content in 'HR78' and 'HR170' resulted in differences in their seed germination ability at LT.

Higher expression level of GA biosynthetic genes and lower expression level of ABA biosynthetic genes in 'HR170' at LT

To investigate how GA and ABA levels respond differently to LT in the LT-tolerant 'HR170' and LT-sensitive 'HR78' at the transcriptional level, we compared the expression of genes involved in ABA and GA metabolism in the two genotypes by qRT-PCR (Fig. S6). The expression levels of ABA biosynthetic genes (*CsABA1*, *CsABA2, CsAAO3* and *CsNCED*) were significantly lower in 'HR170' after 10 h incubation at 13 °C compared to 'HR78' (Fig. 5h–k), however, the ABA catabolic gene *CsCYP707A* was higher in 'HR170' (Fig. 5l). The *GA20ox3* and *GA3ox1* genes encode enzymes that catalyze successive steps in the synthesis of bioactive GAs (Chiang et al. 1995; Lantzouni et al. 2020; Phillips et al. 1995). Their expression levels were significantly higher in 'HR170' compared to 'HR78' at 13 °C (Fig. 5m, n). In contrast, the expression levels of *CsGA2ox3,* which functions in deactivating bioactive GAs (Thomas

et al. 1999), was lower in 'HR170' (Fig. 5o). These results suggested that at LT, the GA and ABA metabolic genes were differently regulated in the LT-tolerant 'HR170' and LT-sensitive 'HR78'.

CsCBF1 might repress seed germination via reducing GA biosynthesis genes at LT

We next asked how *CsGAI* responds to low temperature during seed germination. In *Arabidopsis*, DELLA accumulation inhibits seed germination by reducing the expression of GA biosynthetic genes and increasing the expression of GA degradative genes (Achard et al. 2008; Sun 2011). In this process, the GA receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1), connects GA and the DELLA protein (Griffiths et al. 2006). Since the expression levels of *CsGA20ox3* and *CsGA3ox1* were significantly higher in 'HR170', the expression of *CsCBF1* and *CsGID1* were further examined in 'HR78' and 'HR170' using a time-course qRT-PCR. Interestingly, *CsCBF1* in 'HR170' was transiently up-regulated after 10 h incubation at 13 °C, but expression was the same in both genotypes thereafter (Fig. 6a). *CsGID1* expression in 'HR170' was also transiently up-regulated at 13 °C, but after 24 h incubation (Fig. 6b).

CsGAI regulates seed germination via ABA responsive genes

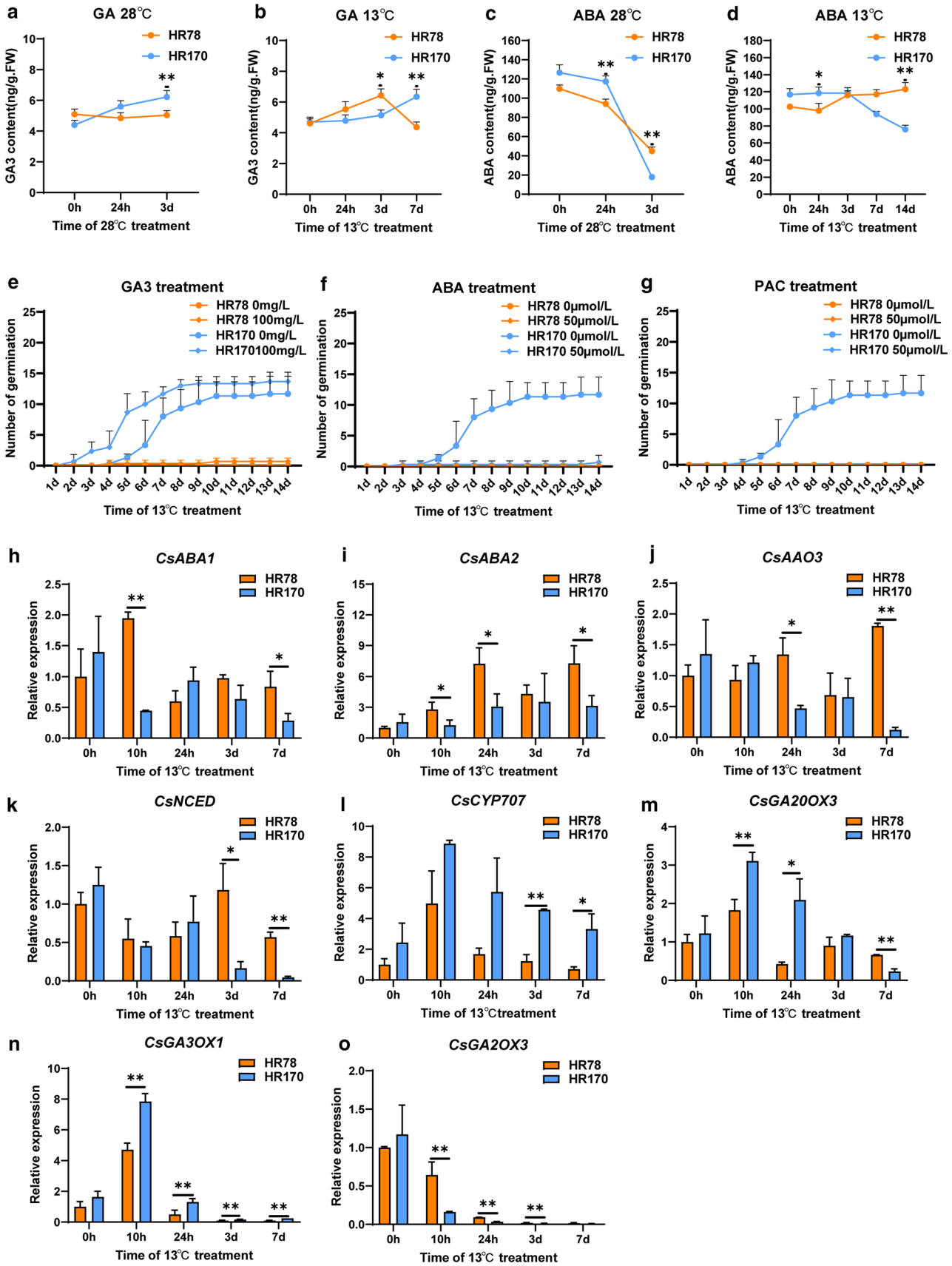
We further asked how *CsGAI* regulates seed germination via the GA and ABA signaling pathway. In *Arabidopsis*, *ABI5* is a critical modulator of ABA signaling pathways functions in repressing seed germination (Chen et al. 2008; Guan et al. 2014; Hu and Yu 2014; Piskurewicz et al. 2008). DELLAs including *GAI* could interact with *ABI5* to regulate downstream ABA responsive genes, such as *EMBRYOGENESIS ABUNDANT1* (*EM1*) and *EM6* (Carles et al. 2002), which directly regulate seed germination (Gaubier et al. 1993; Manfre et al. 2006). Therefore, the expression of the cucumber ortholog of *ABI5*, and, ABA responsive genes including *CsABI5*, *CsEM1*, and *CsEM6* were investigated in 'HR78' and 'HR170'. The time-course qRT-PCR showed that the expression level of *CsABI5* was significantly lower in 'HR170' compared to 'HR78' starting from 3 d at 13 °C (Fig. 6d). *CsEM1* (Fig. 6e) and *CsEM6* (Fig. 6f) showed similar lower expression pattern with *CsGAI* (Fig. 6c) and *CsABI5*.

Discussion

In this work, we identified a candidate gene that underscores LTG in cucumber and provided evidence that it could function in the ABA and GA pathway. Several studies on QTL mapping for LTG in cucumber have been reported thus far, but, the results differed due to varied plant materials and different mapping population (RILs or $F_{2:3}$ lines). Yagcioglu et al (2019) identified one QTL *qLTG1.2* on chromosome 1, one on chromosome 2 and one on chromosome 4 using RILs and $F_{2:3}$, and obtained two markers CG104 (17,549,216 bp) and CG137 (17,901,284 bp) that were closely linked to *qLTG1.2* on chromosome one (Fig. S7). Dong et al. (2017) identified eight QTLs associated with LTG, of which five QTLs on chromosome 4, one on chromosome 6 and two on chromosome 7 using RILs (Fig. S7). However, in these studies, the flanking markers were too far apart to candidate gene in the above two studies. In our study, a 569-kb major effect QTL *qLTG1.1* for LTG was successfully narrowed to a 46.3-kb region between SNP19.95 (Chromosome 1: 14,950,833) and IndelLTG1 (Chromosome 1:14,997,165), containing three genes. *CsGAI*, a key regulator in GA signaling pathway, was identified as the candidate gene responsible for LTG in this work (Figure 1). The fine-mapped 46.3-kb region was only ~ 2.5 Mb away from marker CG104 of *qLTG1.2* identified by Yagcioglu et al. (2019). The relationship between *CsGAI* and *qLTG1.2* loci needs to be further investigated.

The role of DELLAs in modulating seed germination has been widely reported in model plants (Dill and Sun 2001; Achard et al. 2008; Piskurewicz et al. 2008; Willige et al. 2007). DELLAs are able to activate or repress the expression of several genes (Lim et al. 2013; Liu et al. 2016; Sun 2011; Ueguchi-Tanaka et al. 2005; Willige et al. 2007), including *GRF4* (Li et al. 2018), *MFT* (Xi et al. 2010), and *ABI5* (Nakashima et al. 2009). *ABI5* is a key member of the ABA signaling pathway that directly binds the promoters and regulates the late embryonic and abundant (LEA) genes including *EM1* and *EM6* (Carles et al. 2002; Finkelstein and Lynch 2000), thus serving as the final downstream repressor of seed germination in ABA signaling pathway (Hu et al. 2019; Piskurewicz et al. 2008; Reeves et al. 2011). However, little is known of how DELLAs regulate seed germination in cucumber. In our study, at 13 °C LT, endogenous ABA levels were lower in 'HR170' and this was associated with the repression of ABA synthesis genes (*CsABA1*, *CsABA2*, *CsNCED,* and *CsAAO3*) and the activation of the ABA degradation gene *CsCYP707* (Fig. 5h–l). Reduced *CsABI5* expression level leads to decreased expression of ABA-responsive genes (*CsEM1* and *CsEM6*) (Fig. 6d–f), therefore releasing ABA inhibition, and promoting seed germination.

DELLAs play important role in plant response to LT-stress (Colebrook et al. 2014; Lantzouni et al. 2020; Sakata et al. 2014; Sun 2011). *GA20ox, GA3ox* (*GA2ox*) in GA metabolism were up-regulated (down-regulated) by LT-stress in *Arabidopsis* seed germination (Ogawa et al. 2003; Yamauchi et al. 2004). Higher GA level would induce GID1 expression, thus triggering DELLAs degradation by


a
GA 28℃
HR78
HR170
GA3 content(ng/g.FW)
Time of 28℃ treatment
b
GA 13℃
Time of 13℃ treatment
c
ABA 28℃
ABA content(ng/g.FW)
d
ABA 13℃
e
GA3 treatment
HR78 0mg/L
HR78 100mg/L
HR170 0mg/L
HR170100mg/L
Number of germination
f
ABA treatment
HR78 0μmol/L
HR78 50μmol/L
HR170 0μmol/L
HR170 50μmol/L
g
PAC treatment
h
CsABA1
Relative expression
i
CsABA2
j
CsAAO3
k
CsNCED
l
CsCYP707
m
CsGA20OX3
n
CsGA3OX1
o
CsGA2OX3

◂**Fig. 5** Endogenous and exogenous hormone analysis, and expression analysis of ABA and GA metabolic pathways genes in low temperature germination. Line charts represent endogenous hormone (GA, ABA) content at 13 °C (**a**, **c**) and 28 °C (**b**, **d**). Number of 'HR78' and 'HR170' seeds which germinated after exogenous application of hormone GA (**e**), PAC (**f**) and ABA (**g**) treatment under low temperature treatment. The blue line represents 'HR170' and the orange line represents 'HR78'. (**h**–**o**) Relative expression levels of ABA metabolism related-genes (*CsABA1*, *CsABA2*, *CsAAO3*, *CsNCED*, *CsCY707*) and GA metabolism related-genes (*CsGA2ox3*, *CsGA3ox1*, *CsGA2ox3*) at 0 h, 10 h, 24 h, 3 d and 7 d under LT. The values are given as means from three biological replicates. *Significant difference ($P < 0.05$). **Significant difference ($P < 0.01$)

ubiquitin–proteasome pathway (Ariizumi and Steber 2007; Griffiths et al. 2006; Mace et al. 2013) and nonproteolytic gibberellin signaling also repressed DELLAs (Ariizumi et al. 2013), which could further induce DELLA degradation during LT-stress(Lantzouni et al. 2020) . Important regulators of cold responsive CBFs, whose overexpressed phenotypes could be suppressed by GA application or in *della* mutant (Achard et al. 2008; Jia et al. 2016), might be rapidly and timely induced by LT-stress (Barrero-Gil and Salinas 2017; Shi et al. 2018b). CBFs also promoted DELLAs stabilization by the activation of the GA catabolic gene instead of interacting with DELLAs (Achard et al. 2008; Zhou et al. 2017). In our study, *CsCBF1, CsG1D1 and CsGAI* were expressed differently in the LTG tolerant 'HR170' at distinct timepoints during germination (Fig. 6a–c). The expression of these genes occurred in waves, *CsCBF1* was higher after 10 h, *CsG1D1* at 24h and *CsGAI* was repressed relative to the LTG sensitive line from 24 until 7 days. Then, the increased expression of *CsGA3ox1* and *CsGA20ox3*, combined with the down-regulation of *CsGA2ox3* that accelerated GA accumulation were observed in 'HR170' (Fig. 5m–o). Additionally, endogenous GA levels were up-regulated at 28 °C and 13 °C in 'HR170' (Fig. 5a,b). Furthermore, the application of the GA inhibitor PAC was shown to inhibit 'HR170' germination at 13 °C (Fig. 5f, g), which illustrated the crucial role of GA in cucumber seed germination under LT-stress. Thus, *CsGAI* might respond to LT through the regulation of GA biosynthetic and signaling transduction genes.

Emerging evidence in this study indicates that *CsGAI* integrates hormone signals to regulate seed germination in low temperature. Thus, we proposed a working model to explain the possible mechanism of *CsGAI* mediated LTG in LT-tolerant cucumber 'HR170' (Figure 7). In 'HR170', we speculate that LT-induced *CsCBF1* stimulates the expression of GA synthetic genes (*CsGA3ox1* and *CsGA20ox3*) and represses the expression of GA catabolic gene (*CsGA2ox3*), results in increased GA content and reduced *CsGAI* accumulation. Subsequently, the reduced *CsGAI* that regulated *CsABI5* lead to the down-regulation of downstream ABA responsive genes (*CsEM1* and *CsEM6*), thus releasing the inhibition on seed germination and finally enhance seed germination. However, the precise mechanism by which *CsGAI* between LT and seed germination in cucumber needs to be verified.

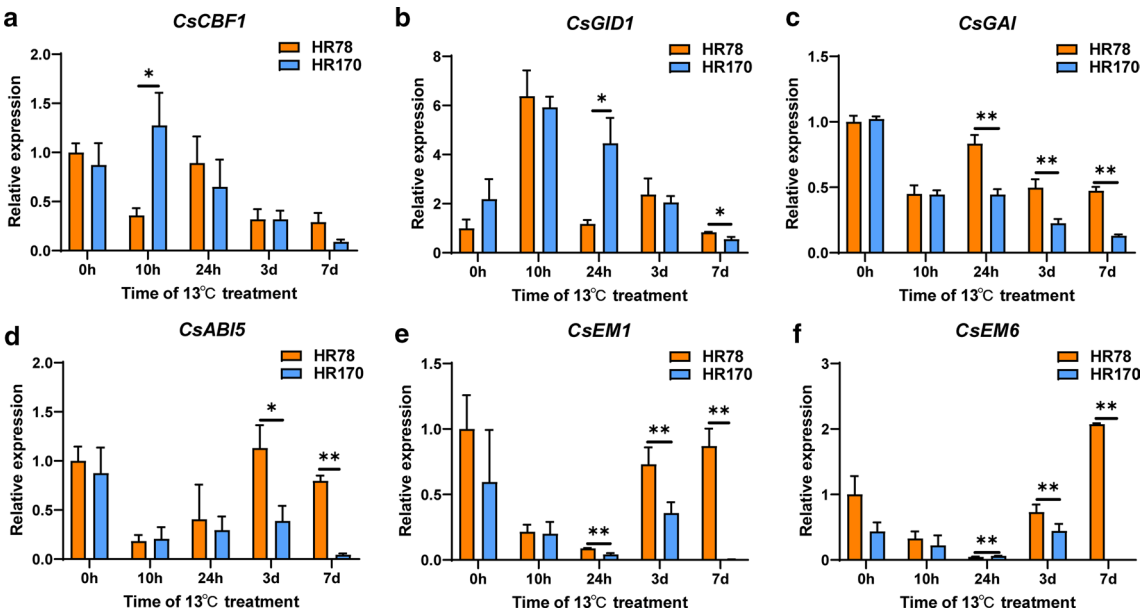


Fig. 6 Expression analysis of low temperature responsive genes. **a**–**c** Relative expression levels of LT responsive gene (*CsCBF1*) and GA receptor (*CsGID1*) at 0 h, 10 h, 24 h 3 d and 7 d under LT. **d**–**f** Relative expression levels of *CsABI5*, *CsEM1* and *CsEM6* to regulate seed germination at 0 h, 10 h, 24 h, 3 d and 7 d under LT. The values are given as means ± SD from three biological replicates. *Significant difference ($P < 0.05$). **Significant difference ($P < 0.01$)

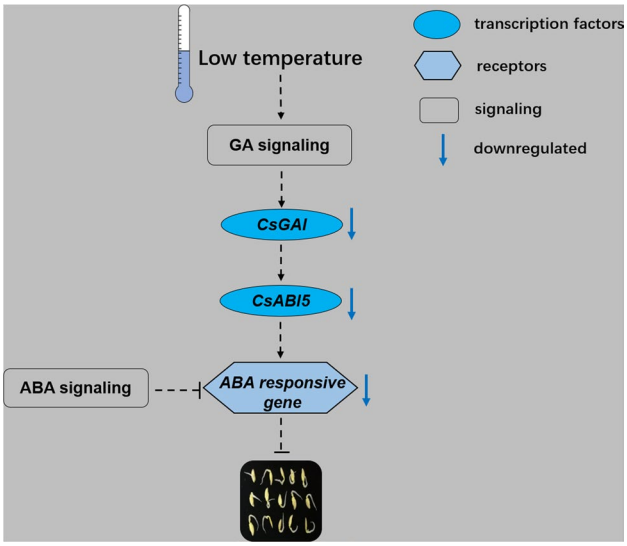


Fig. 7 A working model explaining the possible mechanisms of *CsGAI*-mediated low temperature germination in cucumber. In LT-tolerant 'HR170', LT-induced GA signaling response results in reduced *CsGAI* accumulation. CsGAI might involve in ABA signaling via interacting with CsABI5 to regulate downstream ABA responsive genes, and finally enhance seed germination. Dotted arrow represents unproved by experiment

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Author contributions statement CXL and SYD analyzed the data and drafted the manuscript, CXL performed the experiments, DMB helped to analyze the data and drafted the manuscript, HM helped to analyze the data, JQS, XPL and WPW helped to collect the data, XFG and SPZ designed the experiments. All authors read and approved the final draft of the manuscript.

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Data availability The data that support the findings of this study are available in the article and supplementary materials.

Declarations

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

Ethical approval The authors declare that this study complies with the current laws of the countries in which the experiments were performed.

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