Genetic Analysis and Related Gene Primary Mapping of Heat Stress Tolerance in Cucumber Using Bulk Segregant Analysis

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Abstract. Heat stress (HS) negatively influences plant development and growth, especially production and quality. Cucumber is a widely cultivated plant in the gourd family Cucurbitaceae that is often exposed to high temperatures during summer and protected cultivation. In this study, we performed whole-genome re-sequencing of two pools, one heat-tolerant and one heat-sensitive, of the F₂ population derived from L-9 (heat-resistant) and A-16 (heat-sensitive). The genetic analysis showed that the heat resistance of L-9 cucumber seedlings was controlled by a single recessive gene. By combining bulked segregant analysis (BSA) technology, the crucial gene related to HS was preliminarily mapped to a 1.08-Mb region on chromosome 1. To fine-map the locus, Indel markers were designed according to the genomic sequence. Finally, the gene was narrowed to a 550-kb region flanked by two Indel markers, namely Indel-H90 and Indel-H224, that contained 56 candidate genes. Re-sequencing results indicated that 10 candidate genes among the 56 in the candidate region showed single base pair differences in the exons. Quantitative reverse-transcription polymerase chain reaction showed that 6 genes among the 10 candidate genes were significantly decreased when exposed to high temperatures. These results not only were useful for the isolation and characterization of the key genes involved in HS but also provided a basis for understanding the mechanism of heat tolerance regulation.

In the recent years, high temperatures due to global warming have led to catastrophic crop losses and widespread famine world-

wide (Bita and Gerats, 2013). HS could cause changes in plant respiration and photosynthesis, resulting in shortened life cycles and diminished plant productivity (Hall et al., 2001; Ray et al., 2015). HS causes considerable morphological damage, including scorching and sunburn of leaves, leaf senescence, shoot and root growth inhibition, fruit discoloration, and fruit damage (Ismail and Hall, 1999; Vollenweider and Gunthardt-Goerg, 2005). It also causes cellular damage such as the accumulation of reactive oxygen species (ROS) and antioxidants, content changes of secondary metabolites, and production of related HS proteins (Wahid et al., 2007; Xu et al., 2006). To survive HS, plants have to regulate the transcription of stress-related genes, signal transduction pathways (Wahid et al., 2007) such as heat shock proteins (HSPs) (Bowen et al., 2002; Xu et al., 2006) and heat shock transcription factors (HSFs) (Panchuk et al., 2002), and the HS signal transduction pathway (Joyce et al., 2003; Suzuki and Mittler, 2006).

Cucumber (*Cucumis sativus* L., $2n = 2x = 14$) is a widely cultivated plant in the gourd

family Cucurbitaceae. It originated from the southern Himalayas and prefers to grow in warm conditions (Plader et al., 1998). High temperatures lead to negative impacts on cucumber development, especially production and quality, during summer and protected cultivation (Wahid et al., 2007). Therefore, breeding cucumber cultivars with thermostability is a useful strategy for improving the heat tolerance of plants (Wahid et al., 2007). Furthermore, the identification and characterization of genes involved in HS responses could greatly facilitate the development of improved cucumber cultivars with enhanced heat tolerance.

Multiple studies of the stress response of vegetables under various abiotic stress conditions have been performed (Gous et al., 2016; Wang et al., 2018; Zhou et al., 2013). Studies of HS on vegetables have mainly focused on HSPs and relative enzymes. For instance, the accumulation of small HSPs in peanuts could improve the resistance to HS (Chakraborty et al., 2018). *CaHSP24* in pepper is significantly induced by high temperatures, thus leading to its thermotolerance (Zhu et al., 2011). In cucumber, the HSF family was identified and analyzed by a genome-wide assay (Zhou et al., 2016). Under high-temperature stress, ABA could induce the expression of *HSP70*, leading to its tolerance to heat in cucumber (Li et al., 2015). MicroRNAs were related to improvements in the high-temperature tolerance of cucumber seedlings (Wang et al., 2018). Ten quantitative trait loci (QTLs) (rate of contribution, 6% to 17%) influencing HS in cucumber have been detected (Chen et al., 2008). Yang (2006) obtained three QTLs that impact heat tolerance in cucumber with 6.18% to 10.56% genetic variability. However, no related gene has been fine-mapped or isolated in cucumber, and little is known about the molecular mechanism involved in HS.

In the present study, we combined the BSA (Michelmore et al., 1991) method and whole-genome re-sequencing to analyze heat-tolerant and heat-sensitive pools in cucumber. The genome-wide SNP analysis allowed us to detect a genomic region that harbored the target gene involved in HS. Genetic analysis and fine-mapping of the HS gene were performed to provide a basis for molecular cloning, the mechanism of heat tolerance, and future marker-assisted selection breeding to develop new heat-resistant cucumber cultivars.

Materials and Methods

Plant materials. Two cucumber cultivars, L-9 (South China cucumber variety, heat-resistant) and A-16 (North China cucumber variety, heat-sensitive), were used as parents for the F₂ population in this study. One-hundred sixty seeds of parents F₁ and F₂ were germinated overnight on a wet filter in a culture dish at 28 °C in a dark environment. Then, seedlings were grown in a feeding block for 14 h/10 h at 28 °C/18 °C during

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Min Wang and Yu'e Lin designed the experiment. Min Wang performed most of the experiments. Wenrui Liu, Biao Jiang, Qingwu Peng, Xiaoming He, and Zhaojun Liang participated in revising the paper and performed some experiments. Min Wang wrote the paper. Yu'e Lin edited the paper.

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day/night, respectively, in a culture room (5, 500 lux). When plants were grown to the one true leaf stage, they were transferred to HS conditions (14 h/10 h at 42 °C/36 °C during day/night) for 7 d. Then, seedlings were recovered for 3 d under normal conditions. Before HS treatment, normal leaves of parents, F₁ and 160 F₂ were pooled and sampled, respectively. Then, these samples were immediately frozen in liquid nitrogen and consistently stored at -80 °C for further analyses.

Generation of BSA data. Genome DNA of parents F₁ and F₂ were isolated using the CTAB method (Murray and Thompson, 1980) and used for the BSA. One heat-tolerant DNA pool (T pool) and one heat-sensitive DNA pool (S pool) were constructed by mixing an equal amount of DNA from 30 heat-tolerant and 30 heat-sensitive F₂ plants. Then, these raw reads were carefully filtered to produce clean reads according to three stringent filtering standards: 1) removing reads with $\geq 10\%$ unidentified nucleotides (N); 2) removing reads with $>50\%$ bases with Phred quality scores ≤ 20 ; and 3) removing reads aligned to the barcode adapter.

Variants identification and annotation. To identify SNPs and Indels that differed between parents and pools, Burrows-Wheeler Aligner (BWA) was used to align the clean reads from each sample against the public reference genome (Li and Durbin, 2009). Variant calling was performed for all samples using the GATK Unified Genotyper. SNPs and Indels were filtered using GATK Variant Filtration with proper standards (Window 4, filter QD < 4.0 || FS > 60.0 || MQ < 40.0 , G_filter GQ < 20); those exhibiting segregation distortion or sequencing errors were discarded. To determine the physical positions of each SNP, the software tool ANNOVAR (Wang et al., 2010) was used to align and annotate SNPs or Indels.

Analysis of BSA data. The SNP index and Δ (SNP index) were calculated to identify candidate regions for the HS gene (Lu et al., 2014; Takagi et al., 2013). An SNP index is a proportion of reads harboring the SNP that differs from the reference sequence and Δ (SNP index) when obtained by subtraction of the SNP index of the T pool from that of the S pool. The SNP index represents frequencies of parental alleles in the population of bulked individuals. The SNP index was calculated at all SNP positions by in-house Perl scripts with the following values: SNP index > 3 and SNP depth > 6 . A slide window analysis was applied to SNP index plots with a 2-Mb window size and 100-kb increments. The Δ (SNP index) was calculated based on subtraction of the SNP index from two bulk pools. The Δ (SNP index) was zero in most of the genomic regions, but a few genomic regions exhibited positive or negative Δ (SNP index) values. The related locus was identified in these positive or negative peak regions (with a 95% confidence interval) for 10,000 boot strap replicates.

Fine-mapping of the candidate gene. A total of 302 Indel markers that were well-distributed in the candidate region on chromosome 1 were used for the gene linkage analysis. New Indel markers were developed based on the DNA sequence polymorphisms between L-9 and A-16 according to the re-sequencing genome results.

Quantitative real-time polymerase chain reaction identification. A quantitative real-time polymerase chain reaction (PCR) analysis was performed using the total RNA from seedling leaves of parents and the normal and HS treatments. Additionally, 20 μ L cDNA was obtained using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative reverse-transcriptase PCR (qRT-PCR) (20 μ L reaction volume) was performed with 0.5 μ L of cDNA, 0.2 μ M of primer mix, SYBR Premix Ex Taq Kit (TaKaRa, Tokyo, Japan), and the ABI PRISM 7900HT system (Applied Biosystems, Foster City, CA). Cucumber α -TUBULIN (*TUA*) gene was used normally. All qRT-PCR primers are listed in Table 3.

Statistical analysis. Significant differences were detected by IBM SPSS Statistics 20 (using Student's *t* test). Relative gene expressions were calculated using the $2^{-\Delta\Delta C_t}$ method (Jarošová and Kundu, 2010). In addition, GraphPad Prism 5 was used for chart preparation.

Results

Phenotypes analysis of parents and F₂ plants. Ten-day seedlings of parents F₁ and F₂ plants grown under normal conditions (Fig. 1A) were treated with HS for 7 d and recovered for 3 d (Fig. 1B). L-9, A-16, and F₁ showed vigorous development before treatment; however, A-16 and F₁ exerted dry wilting after HS, and their leaves turned chlorotic and yellow (Fig. 1A and B). Most F₂ plants were sensitive to heat, and leaves died after wilting (Fig. 1C and D).

To understand the genetic analysis of cucumber heat tolerance, we analyzed the number of heat-tolerant and heat-sensitive plants. Results showed that all F₁ plants were sensitive to heat. Among the F₂ population, 112 plants showed sensitivity to high temperatures and 48 plants demonstrated resistance to HS (Table 1), indicating that cucumber thermostability was controlled by a single dominant gene.

Identification of the candidate region by genome re-sequencing. Using Illumina high-throughput sequencing, we finally obtained 65,406,980 and 61,728,581 short reads (100 bp in length) from the T pool and S pool, respectively. These short reads were aligned to the 9930 reference genome (Huang et al., 2009), and the average paired mapped reads were 27,516,956, with an average align ratio of 88.3% (Table 2). Next, the SNP index was calculated and computed to identify the SNP. SNP index graphs of the T pool (Fig. 2A) and S pool (Fig. 2B) were generated by plotting the average SNP index against the position of each sliding window in the reference genome assembly. The δ (SNP index) was calculated and plotted against the genome positions (Fig. 2C) by combining the information of the SNP index of these two extreme pools.

Fine-mapping of the candidate gene locus. A previous study reported that SNPs with a SNP index < 0.3 for two bulked sequences were filtered out during SNP calling to avoid sequencing or alignment errors (Takagi et al., 2013). These band regions of the SNP index for the T pool and S pool ranged between 0.3 and 0.7. The region on chromosome 1 from 19.51 to 20.59 Mb (Fig. 3A) was the most probable interval. To fine-map the candidate gene locus, we used the F₂ genetic population and designed multiple Indel markers in this region. Subsequently, using this mapping strategy, the candidate gene was primarily

Fig. 1. Phenotype analysis of before and after heat stress (HS). (A and B) L-9, A-16, and F₁ plants under normal condition (A) and after recovery of HS (B). (C and D) F₂ phenotype after HS treatment. Bar in (A–D): cm.

Table 1. Segregation ratios of heat-resistant and heat-sensitive plants in the L-9 × A-16 genetic population.

Population	Total plants	Sensitivity	Resistance	Expected ratio	χ^2	P
L-9	16	0	16	—	—	
A-16	16	16	0	—	—	
F ₁	16	16	0	—	—	
F ₂	160	112	48	3:1	2.13	0.15

Table 2. Comparative statistical analysis between clean reads and paired mapped reads.

Sample	All reads	Single mapped reads	Paired mapped reads	Unmapped reads	Align ratio (%)
L-9	65670428	1867834	27828643	8145308	87.6
A-16	64058461	1857682	27220014	7760751	87.88
Thero	65406980	1527001	28306712	7266555	88.89
Sensitive	61728581	1448441	26712456	6855228	88.89

Table 3. Primers used in the gene mapping of the study.

Primers	Forward	Reverse
Indel-H24	CTTCATTGGCCTAACCTTG	TGGTTTCTTCACGAGGAGTG
Indel-H2	TCAAATTCATATGCAACATAAATACA	GGATGAAGGAGACATTATTTC
Indel-H37	TGGTTGTTTGTCTTTACTTTTTC	TTTTTGAAACGGAGACAAACTTC
Indel-H90	GGTGAGGTGAGGTAAATTGTGTC	TGAAAATTGGGGTTTTC
Indel-H224	CAAGATTCAAGAGAGAACTTTTCG	TTTGACATAAGAATTAGGAAGATTA

narrow the candidate region, we designed many more SSR or Indel markers; however, we were not successful because no polymorphism of these markers was detected in the region.

Analysis of candidate genes related to cucumber HS. After analyzing the bulked segregant sequencing results, we finally mapped the candidate gene in the region of 550 kb on chromosome 1. According to the re-sequencing genome data, we found that 10 of these candidate genes showed single differences in the CDS region. Among these 10 candidate genes, only the function of *Csa1M560770* was unknown. The others functioned in DNA primase/helicase (*Csa1M542430*), receptor-like protein kinase 4 (*Csa1M542460*), short-chain dehydrogenase/reductase family protein (*Csa1M542480*), calmodulin-binding protein (*Csa1M561930*), and other biological pathways (Table 4).

Next, to determine the expression changes under normal and HS conditions, we performed a qRT-PCR assay for these candidate genes. Results showed that 7 of the 10 gene expressions were significantly decreased in A-16 compared with L-9 under HS, whereas two genes showed no difference (Fig. 4). Among them, four gene expressions in L-9 were much higher than that in A-16: *Csa1M542460* (19.12-times higher), *Csa1M561420* (24.93-times higher), *Csa1M561930* (16.78-times higher), and *Csa1M561940* (15.13-times higher) (Fig. 3). According to previous reports (Kotak et al., 2007; Li et al., 2004; Liu et al., 2005), the gene *Csa1M561930*, which encodes a calmodulin-binding protein, was the locus most likely related to HS.

Characterization of Csa-HR1.1. Previous studies addressed the possible roles of Ca^{2+} -dependent signaling during the heat response (review by Kotak et al., 2007) because heat could induce cytosolic Ca^{2+} transients in *Arabidopsis* cell cultures with the help of calmodulin (Liu et al., 2005). According to the gene function in the candidate region, only *Csa1M561930* encoding a calmodulin-binding protein was related to plant heat resistance; it showed significantly decreased expression in A-16 under HS, possibly as the candidate gene. Therefore, we performed a re-sequenced analysis using the next assay. Results showed that there was a real single base difference between L-9 and A-16, resulting in the change in amino acids

Fig. 2. SNP-index graphs of T-pool (A), S-pool (B), and delta (SNP-index) (C) from sequencing analysis. X-axis represents the position of seven cucumber chromosomes and Y-axis represents the SNP-index.

mapped between two Indel markers, Indel H2 and Indel H224 (Fig. 3B). Next, we used the polymorphism primers to narrow the primary region. Finally, the gene narrowed the locus

to a 550-kb region flanked by two Indel markers, Indel H90 and Indel H224 (Fig. 3B; Table 2). The final mapping region contained 56 candidate genes (Fig. 5C). To further

Fig. 3. Delta SNP-index graphs from sequencing analysis. X-axis represents the position of chromosome 1 of cucumber and Y-axis represents the SNP-index. Red box represents the candidate region related to heat stress in cucumber.

Table 4. Analysis of candidate genes related to cucumber heat stress.

Chromosome	Function gene	Description
Chr.1	<i>Csa1M542430</i>	DNA primase/helicase
Chr.1	<i>Csa1M542460</i>	Putative receptor-like protein kinase 4
Chr.1	<i>Csa1M542480</i>	Short-chain dehydrogenase/reductase
Chr.1	<i>Csa1M554570</i>	Gamma-soluble NSF attachment protein
Chr.1	<i>Csa1M554580</i>	Lysine-specific demethylase 5A
Chr.1	<i>Csa1M560770</i>	Unknown protein
Chr.1	<i>Csa1M561360</i>	Pectin acetyltransferase, putative
Chr.1	<i>Csa1M561420</i>	Transcription regulatory protein SNF5
Chr.1	<i>Csa1M561930</i>	Calmodulin-binding protein
Chr.1	<i>Csa1M561940</i>	Glycine-rich protein

(Supplemental Fig. 1A and B). The protein structure included a DUF1645 domain (Supplemental Fig. 1C). To detect the expression of the tissue pattern, we performed qRT-PCR. We found that *Csa1M561930* was highly expressed in the fruit and flower bud, with lower expression in the stem tendril and cotyledon (Supplemental Fig. 1D).

Discussion

HS was considered a severely negative factor that led to decreased yield and quality in cereal crops (Bita and Gerats, 2013; Ray et al., 2015). At present, little is known about the molecular mechanism of heat resistance in cucumber, especially because no related genes have been isolated and characterized. In this study, heat tolerance of the cucumber seedling was controlled by a single recessive gene based on the genetic analysis. By combining BSA sequencing technology, we finally mapped the gene related to heat

resistance in a 550-kb region on chromosome 1, with 56 candidate genes in that region. Previous studies reported that genetic regulation of cucumber heat resistance was complex and easily influenced by the external environment (Yu and Wang, 2003; Zhang and Qian, 2007). One study reported that the trait was quantitative and controlled by several genes with a major locus (Zhang and Qian, 2007). Yang (2006) detected three QTLs with 6.18%, 10.36%, and 10.56% contribution rates. One study obtained one SSR marker and nine SRAP markers, which explained the 6% to 17% variation rates (Yu and Wang, 2003). However, in our study, we identified that cucumber heat resistance was a quality trait controlled by a single gene, which was in contrast to previous results. This difference might be attributed to the identification method and various cucumber cultivars. Isolation and characterization of the target gene involved in heat tolerance

should be performed in future studies to verify our results. BSA is considered a simple and efficient alternative method for exploring molecular markers linked to target genes or QTLs related to important traits because it genotypes a pair of bulked DNA samples from two sets of individuals with distinct or opposite extreme phenotypes (Giovannoni et al., 1991; Lu et al., 2014; Michelmore et al., 1991; Takagi et al., 2013). For example, QTLs were detected by whole-genome re-sequencing of DNA from two populations showing extremely opposite traits for a given phenotype in a segregating progeny (Takagi et al., 2013). Candidate genes in cucumber associated with resistance to powdery mildew were identified by combining BSA and SLAF-Seq (Zhang et al., 2015). Furthermore, a major QTL controlling early flowering in cucumber was discovered using next-generation sequencing (Lu et al., 2014). Therefore, in our study, we constructed two pools with opposite heat resistance (heat-tolerant pool and heat-sensitive pool) to perform whole-genome re-sequencing, which offered an efficient way to explore the possible genes related to heat tolerance in cucumber. To deeply explore the most likely candidate gene related to HS, several molecular SSR or Indel markers were designed in the primary region. Results showed that only a few Indel markers had a polymorphism between L-9 and A-16. Finally, because no Indel polymorphism primers were detected in the 550-kb region, the candidate region could not be further narrowed. After combining sequencing data, we observed 10 out of the 56 candidate genes. Among these 10 genes, *Csa1M561930*, which encoded a calmodulin-binding protein, was the locus possibly related to HS. Previous studies have demonstrated that CaMK plays a crucial role in plant heat tolerance (Kotak et al., 2007; Liu et al., 2005). Ca^{2+} transients were only detected after recovery of HS in Arabidopsis (Larkindale et al., 2005). In addition, Ca^{2+} could increase in vitro DNA binding with HSFs (Li et al., 2004). Here, *Csa1M561930* was detected based on the difference between L-9 and A-16, which indicated that the gene might be the locus for cucumber HS. Although *Csa1M561930* in this study was considered the possible gene for cucumber HS, we could not rule out the possibility of other candidate genes that also showed single base pair differences between parents. For example, *Csa1M542460*, which encodes putative receptor-like protein kinase 4, was reported to be involved in abiotic and biotic stresses such as bacterial pathogen *Pseudomonas syringae* (Chen et al., 2003), cold, and salt (Hong, 1997). *Csa1M542480*, which encodes a short-chain dehydrogenase/reductase, was reported to be related to plant defense responses (Hwang et al., 2012). However, these candidate genes, except for *Csa1M561930*, were not directly involved in HS and had certain roles in other abiotic environmental stresses. We first performed BSA sequencing to fine-map the crucial gene related to HS. After

Fig. 4. Expression of candidate genes under normal and heat stress conditions. Data is presented as the mean $\pm$ standard deviation ($n = 9$). $0.01 \leq P \leq 0.05$, $**P \leq 0.01$, Student's t test.

combining the genetic analysis and whole-genome re-sequencing, the key gene was located in the 550-kb region flanked with Indel H90 and Indel H224 on chromosome 1. Our study provides a foundation for the further isolation and characterization of key genes involved in HS and provides useful information for understanding molecular regulation of heat tolerance.

Literature Cited

- Bitá, C.E. and T. Gerats. 2013. Plant tolerance to high temperature in a changing environment, scientific fundamentals and production of HS-tolerant crops. *Front. Plant Sci.* 4:1.
- Bowen, J., L.Y. Michael, K.I.M. Plummer, and I.A.N. Ferguson. 2002. The heatshock response is involved in thermotolerance in suspension-cultured apple fruit cells. *J. Plant Physiol.* 159:599–606.
- Chakraborty, K., S.K. Bishi, A.L. Singh, P.V. Zala, M.K. Mahatma, K.A. Kalariya, and R.A. Jat. 2018. Rapid induction of small heat shock proteins improves physiological adaptation to high temperature stress in peanut. *J. Agron. Crop Sci.* 204:285–297.
- Chen, F.X., G.H. Zhang, W.C. Qian, Y.K. Han, D.F. Chen, S.L. Du, and X.W. Chen. 2008. Molecular markers linked to high temperature resistance QTLs in cucumber. *Acta Sci. Natur. Univ. Nankaiensis* 41:49–54. (In Chinese).
- Chen, K., L. Du, and Z. Chen. 2003. Sensitization of defense responses and activation of programmed cell death by a pathogen-induced receptor-like protein kinase in arabidopsis. *Plant Mol. Biol.* 53:61–74.
- Giovannoni, J.J., R.A. Wing, M.W. Ganai, and S.D. Tanksley. 1991. Isolation of molecular markers from specific chromosome intervals using DNA pools from existing mapping populations. *Nucleic Acids Res.* 19:6553–6558.
- Gous, P.W., L. Hickey, J.T. Christopher, J. Franckowiak, and G.P. Fox. 2016. Discovery of QTL for stay-green and heat-stress in barley (*Hordeum vulgare*) grown under simulated abiotic stress conditions. *Euphytica* 207:305–317.
- Hall, A.E. 2001. Crop responses to environment. CRC Press LLC, Boca Raton, FL.
- Hong, S. 1997. Identification of a receptor-like protein kinase gene rapidly induced by abscisic acid, dehydration, high salt, and cold treatments in arabidopsis thaliana. *Plant Physiol.* 113(4):1203–1212.
- Huang, S.W., R.Q. Li, Z.H. Zhang, L. Li, X.F. Gu, and W. Fan, et al. 2009. The genome of the cucumber, *Cucumis sativus* L. *Nat. Genet.* 41:1275–1281.
- Hwang, S.G., N.C. Lin, Y.Y. Hsiao, C.H. Kuo, P.F. Chang, W.L. Deng, M.H. Chiang, H.L. Shen, C.Y. Chen, and W.H. Cheng. 2012. The Arabidopsis short-chain dehydrogenase/reductase 3, an abscisic acid deficient 2 homolog, is involved in plant defense responses but not in abscisic acid biosynthesis. *Plant Physiol. Biochem.* 51:63–73.
- Ismail, A.M. and A.E. Hall. 1999. Reproductive-stage heat tolerance, leaf membrane thermostability and plant morphology in cowpea. *Crop Sci.* 39:1762–1768.
- Jarošová, J. and J.K. Kundu. 2010. Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR. *BMC Plant Biol.* 10:146.
- Joyce, S.M., A.C. Cassells, and J.S. Mohan. 2003. Stress and aberrant phenotypes in vitro culture. *Plant Cell Tissue Organ Cult.* 74:103–121.
- Kotak, S., J. Larkindale, U. Lee, P. von Koskull-Döring, E. Vierling, and K.D. Scharf. 2007. Complexity of the HS response in plants. *Curr. Opin. Plant Biol.* 10:310–316.
- Larkindale, J., M. Mishkind, and E. Vierling. 2005. Plant responses to high temperature, p. 100–144. In: *Plant abiotic stress*. Blackwell Publishing.
- Li, B., H.T. Liu, D.Y. Sun, and R.G. Zhou. 2004. Ca^{2+} and calmodulin modulate DNA-binding

- activity of maize heat shock transcription factor in vitro. *Plant Cell Physiol.* 45:627–634.
- Li, H., S.S. Liu, C.Y. Yi, F. Wang, J. Zhou, X.J. Xia, K. Shi, Y.H. Zhou, and J.Q. Yu. 2015. Hydrogen peroxide mediates abscisic acid-induced HSP70 accumulation and heat tolerance in grafted cucumber plants. *Plant Cell Environ.* 37:2768–2780.
- Li, H. and R. Durbin. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 14:1754–1760.
- Liu, H.T., D.Y. Sun, and R.G. Zhou. 2005. Ca²⁺ and AtCaM3 are involved in the expression of heat shock protein gene in *Arabidopsis*. *Plant Cell Environ.* 28:1276–1284.
- Lu, H., T. Lin, J. Klein, S. Wang, J. Qi, Q. Zhou, J. Sun, Z. Zhang, Y. Weng, and S. Huang. 2014. QTL-seq identifies an early flowering QTL located near flowering locus T in cucumber. *Theor. Appl. Genet* 127:1491–1499.
- Michelmore, R.W., I. Paran, and R.V. Kesseli. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis, a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA* 88:9828–9832.
- Murray, M. and W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8:4321–4326.
- Panchuk, I.I., R.A. Volkov, and F. Schoffl. 2002. HS- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in *Arabidopsis*. *Plant Physiol.* 129: 838–853.
- Plader, W., S. Malepszy, W. Burza, and Z. Rusinowski. 1998. The relationship between the regeneration system and genetic variability in the cucumber (*Cucumis sativus* L.). *Euphytica* 103:9–15.
- Ray, D.K., J.S. Gerber, G.K. MacDonald, and P.C. West. 2015. Climate variation explains a third of global crop yield variability. *Nat. Commun.* 6:5989.
- Takagi, H., A. Abe, K. Yoshida, S. Kosugi, S. Natsume, C. Mitsuoka, A. Uemura, H. Utsushi, M. Tamiru, S. Takuno, H. Innan, L.M. Cano, S. Kamoun, and R. Terauchi. 2013. QTL-seq, rapid mapping of quantitative trait loci in rice by whole genome resequencing of DNA from two bulked populations. *Plant J.* 74:174–183.
- Suzuki, N. and R. Mittler. 2006. Reactive oxygen species and temperature stresses, a delicate balance between signaling and destruction. *Physiol. Plant.* 126:45–51.
- Vollenweider, P. and M.S. Gunthardt-Goerg. 2005. Diagnosis of abiotic and biotic stress factors using the visible symptoms in foliage. *Environ. Pollut.* 137:455–465.
- Wahid, A., S. Gelani, M. Ashraf, and M.R. Foolad. 2007. Heat tolerance in plants: An overview. *Environ. Expt. Bot.* 61:199–223.
- Wang, K., L. Mingyao, and H. Hakon. 2010. ANNOVAR, functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* 38:e164.
- Wang, Y., S. Guo, L. Wang, X. He, S. Shu, J. Sun, and N. Lu. 2018. Identification of microRNAs associated with the exogenous spermidine-mediated improvement of high-temperature tolerance in cucumber seedlings (*Cucumis sativus* L.). *BMC Genomics* 19:285.
- Xu, S., J. Li, X. Zhang, H. Wei, and L. Cui. 2006. Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under HS. *Environ. Expt. Bot.* 56:274–285.
- Yang, D.F. 2006. Mapping quantitative traits for the heat tolerance in cucumber. Master Thesis (In Chinese).
- Yu, S.C. and Y.J. Wang. 2003. Inherence of heat tolerance in cucumber. *Huabei Nongxuebao* 18:87–89. (In Chinese).
- Zhang, P., Y. Zhu, L. Wang, L. Chen, and S. Zhou. 2015. Mining candidate genes associated with powdery mildew resistance in cucumber via super-BSA by specific length amplified fragment (SLAF) sequencing. *BMC Genomics* 16:1058.
- Zhang, P. and Z.W. Qian. 2007. Genetic analysis of heat tolerance in cucumber. *J. Northeast Agr. Univ.* 38:486–490. (In Chinese).
- Zhou, S., P. Zhang, Z. Jing, and J. Shi. 2013. Genome-wide identification and analysis of heat shock transcription factor family in cucumber (*Cucumis sativus* L.). *Plant Omics Journal of Plant Molecular Biology & Omics* 6:449–455.
- Zhu, W., M. Lu, Z. Gong, and R. Chen. 2011. Cloning and expression of a small heat shock protein gene CaHSP24 from pepper under abiotic stress. *Afr. J. Biotechnol.* 1025:4968–4976.

Supplemental Fig. 1. Characterization and tissue expression of *Csa1M561930*. (**A** and **B**) *Csa1M561930* showed single base difference between L-9 and A-16. (**C**) Analysis of *Csa1M561930* protein domain. (**D**) Relative expression of *Csa1M561930* in different tissues. Red box represents the mutated base pair.