

EXPRESSION AND CHARACTERISATION OF CUCUMBER FRUIT FLESH THICKNESS-RELATED GENE *CSA2M058670.1*

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ABSTRACT

Bulked segregant analysis combined with specific length amplified fragment sequencing techniques have been applied to determine the fine genetic mapping of fruit flesh thickness-related genes in cucumber. Herein, the *Csa2M058670.1* gene was subjected to real time fluorescence quantitative PCR (qRT-PCR) and sequence analysis, indicating a strong correlation with cucumber fruit flesh thickness. Expression and characterisation of the *Csa2M058670.1* gene were performed based on previous studies. The results of the fluorescence-based quantitative PCR showed that *Csa2M058670.1* was expressed in all organs, but levels were highest in fruit peel, fruit flesh, and female flowers. Furthermore, *Csa2M058670.1* expression was induced by abiotic stresses including drought, low temperature, and high salt. Domain analysis revealed that the protein encoded by *Csa2M058670.1* possesses an SET (Su(var), Enhancer of zeste CE(z), and Trithorax) domain that may control cell division and differentiation. Therefore, we speculated that *Csa2M058670.1* might affect fruit flesh thickness in cucumbers by influencing cell division.

Keywords: cucumber, gene, fruit flesh thickness

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an annual creeping plant (i.e. a “climber”), and is an important vegetable consumed worldwide. It is one of the most extensively cultivated vegetable species in China [Yang et al. 2014]. With economic development and continuous improvements in living standards, the demand for high-quality cucumber fruits is increasing [Zhong et al. 2017]. Therefore, improving fruit characteristics is a critical objective for current cucumber breeding research.

Fruit shape is generally determined by fruit length, fruit diameter, fruit weight, fruit stem length, fruit shape index, and other characteristics. It is an essential parameter of fruit appearance quality [Song et

al. 2016]. Meanwhile, fruit weight is one of the most critical factors determining yield, and flesh thickness makes a large contribution to fruit weight. In a preliminary study on heritability and genetic correlation of quality traits in processed cucumbers carried out by Xu et al. [2001], a positive correlation was observed between the genetic determinants of fresh fruit weight and flesh thickness [Xu et al. 2001]. The correlation coefficient between fruit flesh thickness and fruit quality was 0.67, 0.46, and 0.42 according to Sun et al. [2010], Wang et al. [1998] and Li et al. [2008], respectively. Meanwhile, Le et al. [2011] revealed that the correlation coefficient of the two aforementioned indices was up to 0.741 1, and the coefficient of

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determination was 0.5492; in other words, 54.92% of fruit quality is determined by flesh thickness. Flesh thickness is one of the vital structural characteristics of cucumber, and is positively correlated with its utilisation, regardless of its use in processed or fresh foods [Zhu et al. 2016]. Previous research on cucumber fruit fresh mainly focusses on fruit diameter [Bo et al. 2015], flesh colour [Li et al. 2013] and flesh crispness. However, there are few studies related to mechanism underpinning cucumber fruit flesh thickness.

BSA and SLAF-seq techniques have been used previously for fine genetic mapping of cucumber fruit flesh thickness on a quantitative trait locus (QTL) of 0.19 Mb in length on chromosome 2. Further verification of the QTL was provided by QTL mapping based on a traditional simple sequence repeat (SSR) marker in 138 cases of the F2 generation. Gene prediction results indicated that the 0.19 Mb region contained 20 genes, and fluorescence-based quantitative PCR revealed that among these 20 genes, a variety with thicker fruit flesh was the only one able to maintain a higher expression level of the *Csa2M058670.1* gene. Subsequent analysis of the *Csa2M058670.1* gene from varieties with thicker and thinner fruit revealed a 4 bp deletion in the promoter region in varieties with thinner flesh. This deletion affects expression of the *Csa2M058670.1* gene, which may account for the thinner flesh [Xu et al. 2015].

The present work focuses on the expression and characterisation of the *Csa2M058670.1* gene in cucumber, and the findings provide a theoretical basis for revealing the molecular mechanism underpinning flesh thickness.

MATERIALS AND METHODS

Materials and growth conditions. *Cucumis sativus* L. line 3407 was obtained from the China Agriculture University and used in this study. Plants were grown at a phytotron under a 16 h light : 8 h dark 25°C : 18°C day/night photoperiod. Cucumber plants at the 3–4 true leaf stage were used for different stress treatments. For salt treatments, various concentrations (50, 100 and 150 mM) sodium chloride (dissolved in deionised water) was used to irrigate soil, and control plants received the same volume of deionised water. For low temperature treatment, plants were exposed

to 12°C for 24 or 48 h. For drought treatment, plants were treated with 10% (w/v) polyethylene glycol-6000 (PEG-6000) and harvested at different time points. After treatment, leaves were used for RNA extraction.

Gene expression analysis by quantitative RT-PCR.

Total RNA was extracted using a Column Plant RNA out kit, and cDNA was synthesised using Power Script Reverse Transcriptase. Quantitative real-time RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China) on an Applied Biosystems 7500 RT-PCR system. The cucumber gene encoding α -Tubulin (TUA) was used as an internal control, and three biological replicates were performed for each sample. Relative transcript abundance was calculated by the method of $2^{-\Delta\Delta Ct}$.

Sequence alignment and phylogenetic analysis.

Amino acid sequences of proteins related to *Csa2M058670.1* in various species were obtained from the National Center for Biotechnology Information [<http://www.ncbi.nlm.nih.gov>] via BLAST analysis with cucumber *Csa2M058670.1* as the query sequence. Multiple sequence alignment of related *Csa2M058670.1* proteins was performed by DNAMAN, and a phylogenetic tree was constructed using the neighbour-joining method in MEGA 5 software.

Analysis of physical and chemical properties.

In this study, the properties of the *Csa2M058670.1* protein were analysed using the ExPASy ProtParam tool [<https://web.expasy.org/cgi-bin/protparam/protparam>].

Protein three-dimensional structure prediction.

The structure of *Csa2M058670.1* was predicted using SWISS-MODEL [<https://swissmodel.expasy.org/interactive>], downloaded in PDB format, and imported into SPDBV for visualisation and analysis.

Transmembrane region prediction. Prediction of *Csa2M058670.1* transmembrane regions was performed using the TMHMM server [<http://www.cbs.dtu.dk/services/TMHMM-2.0/>].

RESULTS

Expression of the *Csa2M058670.1* gene in cucumber.

The expression pattern of *Csa2M058670.1* in different cucumber tissues was analyzed by qRT-PCR. The results showed that *Csa2M058670.1* was expressed in all organs, with highest expression in fruit epidermis, fruit flesh, and female flowers

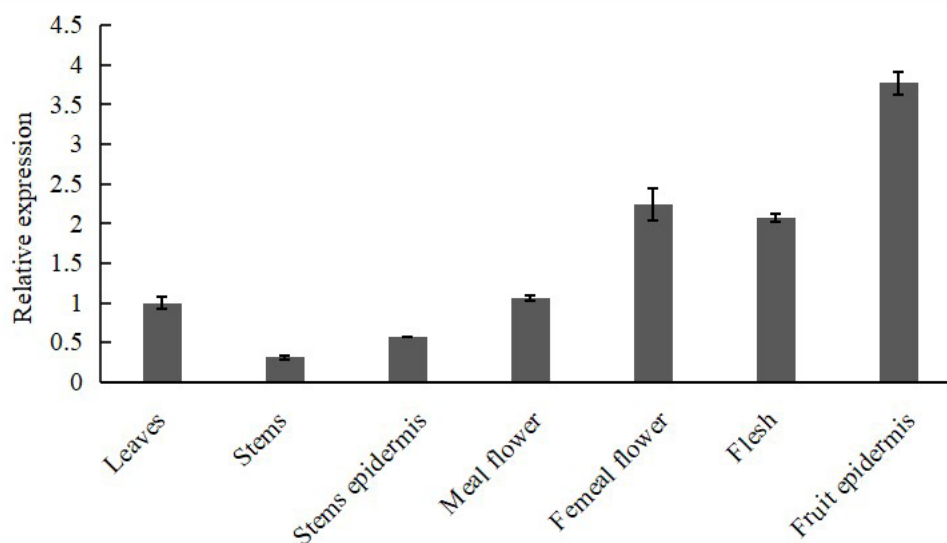


Fig. 1. Expression of *Csa2M058670.1* in different organs of cucumber

(Fig. 1), indicating that *Csa2M058670.1* plays a significant role in fruit development in cucumber.

Effects of abiotic stress on *Csa2M058670.1* expression. Different abiotic stress treatments were conducted on wild-type cucumber seedlings at the three leaf and one leaflet stage, followed by analysis of *Csa2M058670.1* transcription. The results indicated that *Csa2M058670.1* expression was increased during drought, salt stress, and especially under low temperature treatment. Following treatment with NaCl at different concentrations, the results revealed that *Csa2M058670.1* expression was elevated with increasing salt concentration; at 100 and 150 mM, expression was nearly four-fold higher than at 0 mM NaCl. The results of drought treatment revealed that *Csa2M058670.1* expression was increased gradually with prolonged drought stress; after a 3 h treatment, *Csa2M058670.1* expression was 3 times higher than untreated controls. Following low temperature treatment, *Csa2M058670.1* expression was increased with increasing treatment duration; *Csa2M058670.1* expression levels after 48 h were 76 times higher than untreated controls (Fig. 2).

Protein sequence and phylogenetic tree analysis. To further analyse the relatedness between *Csa2M058670.1* and homologs in other species, the sequence of *Csa2M058670.1* was compared with that

Cucurbita moschata XP_022937782.1, *Citrus sinensis* KDO46508.1, *Arachis duranensis* XP_020999588.1 and *Glycine soja* KHN17593.1. Sequence identity ranged between 49.81–74.15%, with highest similarity shared with *C. moschata* XP_022937782.1 (74.15%). Protein domain prediction by SMART showed *Csa2M058670.1*, *C. moschata* XP_022937782.1, *A. duranensis* XP_020999588.1 and *G. soja* KHN17593.1 all possess SET structural domains [Zhang et al. 2009] spanning amino acid residues 173–397 (Fig. 3).

A phylogenetic tree was constructed to further analyse the evolutionary relationships between *Csa2M058670.1* and its homologs. The tree as constructed using sequences from 15 species including pumpkin, sweet orange and soybean. *Csa2M058670.1* and *C. moschata* XP_022937782.1 are clustered in the same evolutionary branch (Fig. 4).

Analysis of protein physicochemical properties. The 1566 bp *Csa2M058670.1* cDNA contains an open reading frame encoding a 521 amino acid polypeptide with a molecular weight of 128637.94, a predicted isoelectric point (pI) of 5.03, and the molecular formula $C_{4788}H_{3021}N_{1566}O_{2023}S_{273}$. The protein was predicted to be unstable, with an instability index of 40.66, and a fat coefficient of 30.08. The protein is predicted to be water-soluble, with a total hydrophilicity of 0.680.

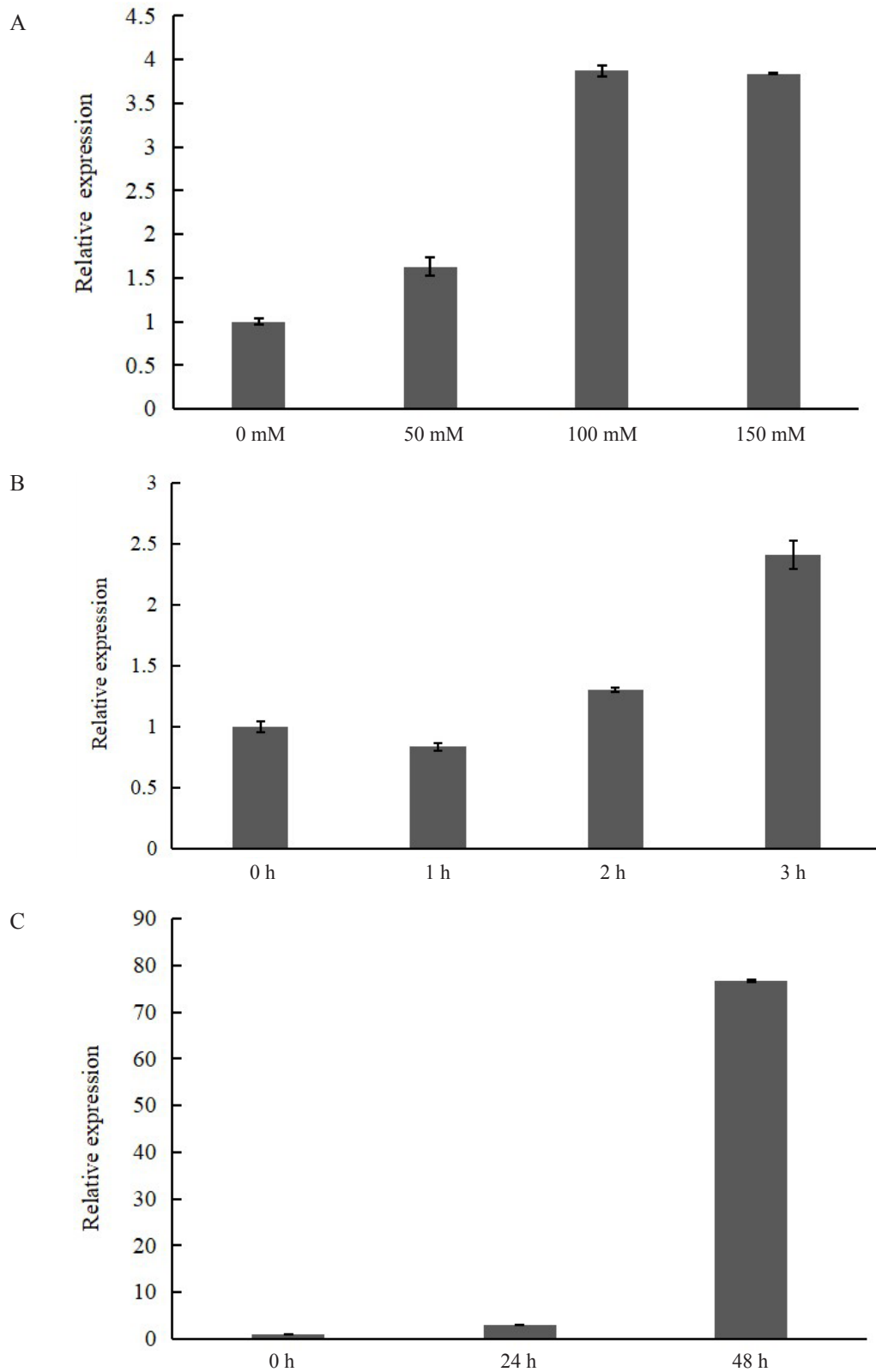


Fig. 2. (A) qRT-PCR analysis of *Csa2M058670.1* in cucumber plants treated with various concentrations of NaCl (0, 50 mM, 100 mM, and 150 mM) for 7 days, respectively. (B) qRT-PCR analysis of *Csa2M058670.1* in cucumber plants treated with drought for 0, 1, 2 and 3 hours, respectively. (C) qRT-PCR analysis of *Csa2M058670.1* in cucumber plants treated with low-temperature (12°C) for 0, 24 and 48 hours, respectively

Csa2M058670.1	N A T E N Q F S S S D E V R D D D G R F L F L A L D R N D R L F F K K K K L L E R Q G F K S E N L I Y L R Q S N C P E E V D T V L K E L V	69
Cucurbita_moschata_XP_022937782.1	N E I E N L F S A S D E V R D V N D C S I Q L A L N C N D E L F S K K K K L L E R Q G F K S E N C I Y L R Q S L C S E E V D T V L K E L V	69
Glycine_soja_KHN17593.1 M D T D E E C S I V L E L S E R D F F D K K K L L Q S K G F S P K E R I Y L R S S R P G W N A T V E L L	57
Arachis_duranensis_XP_020999588.1 M D T G E E Y S L V I E L S E N D L F F E C K K K L L E N K G F R P K E R I Y L R G T S R P G W N A T V K L L	57
Citrus_sinensis_KDO46508.1	N A D V E A C S S R F S L E N G D C S L V L E L T E D D L F F K K K K L L N D K G F G V K E C H L Q S N C P E D S I S N S L E T N L	69
Csa2M058670.1	Q I S R I I H L N E P E N Y F G E N D E G T P V F Y S P R N E V E T F D S I I S L L D L S L S S C T P A F S V L Q E L R R A V I H N	138
Cucurbita_moschata_XP_022937782.1	Q I A R I I H L N E P E N Y F G E D D A C T P A D S Y S P R N E M E A L N T I I S L V D I C L S S C K P V Q L N V L Q E L R R A A I R M	138
Glycine_soja_KHN17593.1 V L L D I S L S I C T H L H T N I L Q E L R Q T L D L I	126
Arachis_duranensis_XP_020999588.1	Q I A R I I Q L N E L E L Y F A E D D G C P L V E F Y S P R N E L E A F D S I L S L V D I S L S C A N L H T N I L E L L R Q T I L D L I	126
Citrus_sinensis_KDO46508.1	R I G R I I H V D E L E L Y F G E F L T Q M C F Y S P R N E L E L N S I L A R V N T L S R Q M H K K N V W Q C L Q A A I I L K I	136
Csa2M058670.1	H E Y G N V F S N V A K T L E N S C E K C N L L E V G E S N G V R T S L K I A Y V E G A G R G A I A R E D L D V G D I V L E I P L A I	206
Cucurbita_moschata_XP_022937782.1	H K Y G D V Y S M D A K T L D S..... C V K E N C L L C W G E S N G V R T S L K I A Y V E G A G R G A I A R E D L D V G D I V L E I P L D I	206
Glycine_soja_KHN17593.1	S D F G D K N S V K G V V E N N S C D P E R L L E W G E S N G L N T Q L K I A Y I E G A S R G A I A R K D L V G D I A L E I P V S I	195
Arachis_duranensis_XP_020999588.1	S D F G D K N S V K G I V E R D H G I D C P E R L I K W A E S N G V R T Q L K I A Y V O G A G R G A I A R E D L D V G D I A L E I P S I	195
Citrus_sinensis_KDO46508.1	Q E F G N Q I K E V A R I D R N Y T C E K E K C L V W G E S N G V K T K L E I A Y V G A G R G A V A R E D L D V G D I A L E I P V S I	205
Csa2M058670.1	I I S E E L V C K S I N Y E V L S K V E G L P E T M I L L V S N K E K H I V D S E F R V Y F D L P E A F N	261
Cucurbita_moschata_XP_022937782.1	V I S E E L V C K T I N Y E L S K I E G M S E T M L L V S N K E K H I A D S K F K V Y F D L P E A F N	261
Glycine_soja_KHN17593.1	I I S E E L V E H E T D M Y V L K E H I D G I S S E T I L L L V S N K E K H I N D S K F K I Y F D L P E R F T A A Y L F N L F K T P S E	264
Arachis_duranensis_XP_020999588.1	I I S E E L V C E T D M Y V L K E V D G I S S E T I L L L V S N K E K H I N S K F K I Y F D L P E K F N	250
Citrus_sinensis_KDO46508.1	I L S R D L V E K S D N Y V L G K I E G A S S E T M L L V S N K E K E N G S K F K N Y F D S L P K E F	260
Csa2M058670.1 T G L S F G V C A M T L V G T L L F D E L M Q A K E H L R K Q Y N E L F P A L C N N F P D I F P P E F Y S W E F L W A C E L Y	327
Cucurbita_moschata_XP_022937782.1 T G L S F G V C A M T D G T L L F G E I N Q A K E H L R E Q Y N E L F P I L C N N F P D V F P E P Y S W E K F L	327
Glycine_soja_KHN17593.1	S S S T G L S F G V F S H C A I T M D G T L L L E E I N Q A R Q H L A Q Y D E L F P A L C N N F P D I F P P E L Y T W E K F L W A C E L Y	333
Arachis_duranensis_XP_020999588.1 T G L S F G V F S H C A I T M D G T L L L E E I N Q A R Q H L A Q Y D E L F P A L C N N F P E I F P V E L Y T W E K F L	316
Citrus_sinensis_KDO46508.1 T G L S F G V D A I N A L E G T L L L E E I N Q A K E H L R T Q Y D E L F P R L C N D P D I F P P K F Y T W E F L W A C E L Y	326
Csa2M058670.1	S N S L K I M F P D C N V R T C L V P I A G F L N H S L P H I L H Y G K V D S D T D S L K F R L S R P C R A G E E C L S Y G N S G S	396
Cucurbita_moschata_XP_022937782.1	S N S N K I M S D G S L I T S C L V P I A G F L N H S L P H I L H Y S K A D S D T N S L K F R L S R P C R A G E E C L S Y G N S A S	396
Glycine_soja_KHN17593.1	S N S N K I M S D G K L R T C L I P I A G F L N H S L D Y N C K L M P M I G E L Y N T I S	395
Arachis_duranensis_XP_020999588.1	S N S N K I M F P D G K L I T C L I P I A G F L N H S L C P H I L HY G K V L A T T N S L K F R L S R P C S G E E C C L S Y G N F S S	385
Citrus_sinensis_KDO46508.1	S N S N K I I F A D G K L R T C L I P I A G F L N H S L N P H IV H Y G R V D S A T N S L K F P L S R P C N IG E Q C C L S Y G N F S T	395
Csa2M058670.1	H L V T F Y G F L P E G E N V D V I P L D I D F G D D N N N I T S D W S T H M L C G	440
Cucurbita_moschata_XP_022937782.1	H L V D F Y G F L P E G E N V D V I P L D I D F G D D A..... S D I I T S D W S T H N V R G I T W L S K N Q S I F H Y G L P S P L L E C L C	463
Glycine_soja_KHN17593.1	H L I T F Y G F L P E G E N S Y D V I P L D I D G S D V S N E D I P V S N W T H M L C G S L E N E	446
Arachis_duranensis_XP_020999588.1	H L I T F Y G F L P E G E N Y D I P L D I D G S D V Y V E D K C P N W T H V R G	431
Citrus_sinensis_KDO46508.1	H L I T F Y G F S P E G E N Y D V I P L D I D V G Q A D C F E D C P S N W T H V R G I T W L S K N E N I F N Y G L P S P L L D Y L R	464
Csa2M058670.1 S N E C E I F V L N E L L S I F S E N E K L E D E D E S R I S T E W D I K L A L E Y K D L Q R K I V S	494
Cucurbita_moschata_XP_022937782.1	K A R C P E L R I K L K S L E N E V L N D L L S I F D G M E N L E D V N E D S R S T E W D I K L A L N Y K D L Q R R I V S	528
Glycine_soja_KHN17593.1 L E V L E N L K D I F D D M E N N G K I D L D D R E N C S W E K L A M D F K D L Q R R I A C	494
Arachis_duranensis_XP_020999588.1 T W S N N E S L F Y Y G L P S P L D H L R R S R S P M L N K T I F	467
Citrus_sinensis_KDO46508.1	R V R N E N Q Y E K I L Q P D L E I E L V L E D I L S T F S N N A M E I L G I T D F G D G E N S W M K L A L E F K D L Q R R I I S	532
Csa2M058670.1	S C L T S C F S G L K N V E I A L C D C N K E D T R	520
Cucurbita_moschata_XP_022937782.1	S C L I S C H A G L K T V E L A L Y E C N E D T	554
Glycine_soja_KHN17593.1	S V S T S C H T G M D N L K N E L C K C N A E D I L	520
Arachis_duranensis_XP_020999588.1	467
Citrus_sinensis_KDO46508.1	S I L T S C S A G R S L V E S E L S R V N S K D	556

Fig. 3. Sequence alignment of amino acid residues of *Csa2M058670.1* with other related proteins (the identical and similar residues are shown in black and gray, respectively. Double-line represents the SET domain)

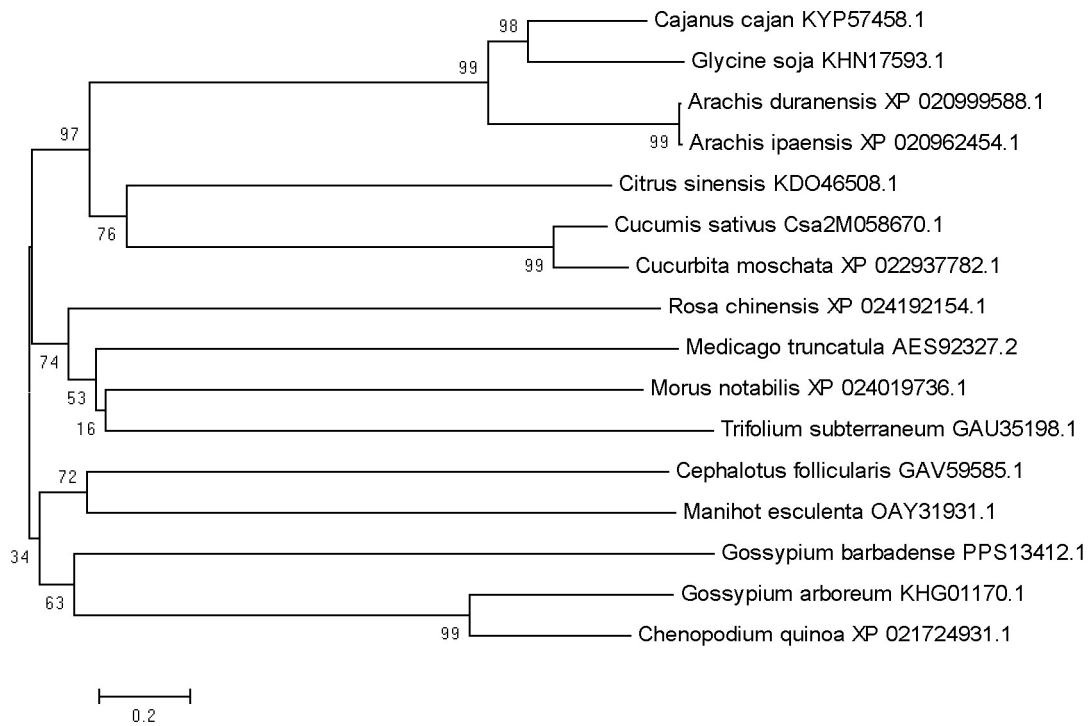


Fig. 4. Phylogenetic tree analysis of *Csa2M058670.1* with different species



Fig. 5. Three-dimensional structure analysis of protein

Table 1. The amino acid composition of the *Csa2M058670.1* protein sequence

amino acid	content
Ala	30.1%
Cys	17.4%
Gly	23.4%
Thr	29.1%

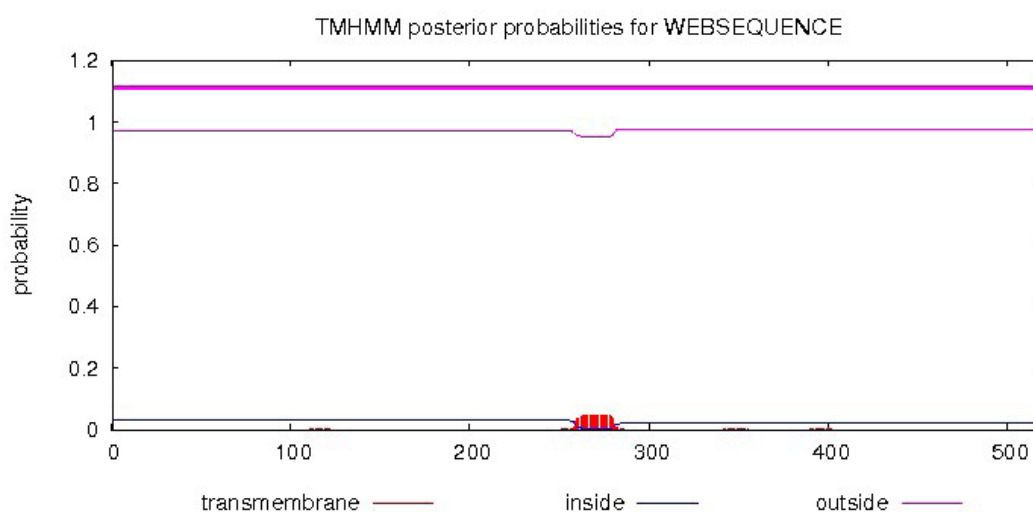


Fig. 6. Prediction on the protein transmembrane region by TMHMM 2.0

The amino acid composition is shown in Table 1. Alanine and threonine are the most abundant amino acids.

Protein three-dimensional structure prediction. The predicted 3D structure of the *Csa2M058670.1* protein has 13 α -helices and 8 β -strands (Fig. 5).

Prediction and analysis of transmembrane domains. As shown in Figure 6, three transmembrane helices were predicted for the *Csa2M058670.1* protein, and the number of intramembranous helical amino groups was 68.6888. When the value of the latter exceeded 18, a transmembrane region was predicted, suggesting the existence of a transmembrane region. There were 39.8875 intramembranous helical amino groups in the first 60 amino acids of the protein, indicating that *Csa2M058670.1* may include a signal peptide.

DISCUSSION

***Csa2M058670.1* plays an important role in cucumber fruit development.** In this study, the results of qRT-PCR in different tissues revealed that *Csa2M058670.1* has highest expression levels in female flowers, fruit flesh, and fruit peel. Previous studies showed that the expansion CsEXPB1 in cucumber is related to fruit setting, cell division and fruit swelling. The qRT-PCR results for the CsEXPB1 gene in different tissues of cucumber indicate that CsEXPB1 was highly expressed in female flowers. Additionally, expression in fruit is consistently increased after pollination [Meng et al. 2015]. Similarly, *Csa2M058670.1* expression was significantly higher in female flowers, fruit flesh, and fruit peel than in other tissues. There-

fore, it we propose that *Csa2M057670.1* may be essential throughout the development of cucumber fruits.

***Csa2M058670.1* is induced by abiotic stress.**

Real-time fluorescence quantitative PCR revealed that *Csa2M057670.1* gene expression was increased following drought, low temperature, and salt abiotic stresses. A previous study conducted by Liu et al. [2015] showed that expression of the *SINAC80* gene related to fruit development in tomato was higher under low temperature and salt stress relative to control groups. A study on strawberry by Wang et al. [2015] found that fruit development-related *FaChi4* expression was increased under drought stress. Similarly, the *Csa2M058670.1* gene was induced by abiotic stress.

***Csa2M058670.1* may be related to fresh thickness.** Domain analysis of the *Csa2M057670.1* protein sequence revealed the existence of a SET domain. SET domains are important in the growth and development of plants because they control cell division and differentiation [Xue and Tian 2005]. For example, the *PR-SET7* gene is closely related to cell proliferation, and deletion of *PR-SET7* induces DNA damage, cell cycle arrest, and even apoptosis [Liang et al. 2013]. Moreover, the *NTSET1* gene in tobacco can prevent cell elongation via its influence on cell division and differentiation, and mutations can lead to dwarfing [Song and Cao 2007]. Prior studies in sweet cherry, banana, melon, peach, and avocado, among many other crops showed that differences in fruit size among various cultivars is predominantly determined by the number of cells in fruits [Scorzal et al. 1991, Cowan et al. 1997, Higashi et al. 1999, Jullien et al. 2001, Olmstead et al. 2007]. In addition, several studies suggest that the number of fruit cells and cell size can jointly affect the final size of fruits [Li et al. 2001, Harada et al. 2005]. A change in organ size in plants can be achieved by changing the size and number of cells by manipulating cell division and cell elongation. Thus, based on these findings, we deduced that *Csa2M058670.1* might affect the formation of flesh thickness in cucumbers by acting on cell division.

In conclusion, we investigated the expression pattern of *Csa2M058670.1* in different tissues of cucumber, and analyses sequence and structural features. *Csa2M058670.1* was induced by abiotic stress, and may affect the formation of fruit thickness in cucumber by influencing cell division. However, further

exploration and verification are needed to determine specific molecular mechanisms.

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