Breeding Science 71: 417–425 (2021)
doi: 10.1270/jsbbs.20149

Research Paper

CsKDO is a candidate gene regulating seed germination lethality in cucumber

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Seed germination plays an important role in the initial stage of plant growth. However, few related studies focused on lethality after seed germination in plants. In this study, we identified an Ethyl methanesulfonate (EMS) mutagenesis mutant *Csleth* with abnormal seed germination in cucumber (*Cucumis sativus* L.). The radicle of the *Csleth* mutant grew slowly and detached from the cotyledon until 14 d after sowing. Genetic analysis showed that the mutant phenotype of *Csleth* was controlled by a single recessive gene. MutMap$^+$ and Kompetitive Allele Specific PCR (KASP) genotyping results demonstrated that *Csa3G104930* encoding 3-deoxy-manno-octulosonate cytidylyltransferase (*CsKDO*) was the candidate gene of the *Csleth* mutant. The transition mutation of aspartate occurred in *Csa3G104930* co-segregated with the phenotyping data. *CsKDO* was highly expressed in male flowers in wild type cucumbers. Subcellular localization results showed that CsKDO was located in the nucleus. Overall, these results suggest *CsKDO* regulates lethality during seed germination in cucumber.

Key Words: cucumber, EMS mutation, genotyping, MutMap$^+$, seed germination.

Introduction

The growth process after seed germination is critical to crops. After absorbing water, dried seeds swell with water, marking the first stage of seed germination (Bewley 1997). In the second stage, the seed size increases and metabolism begins, and protein storage mobilization is essential in the germination of dicotyledons (Tiedemann *et al.* 2000). In the last stage, the endosperm is ruptured and the radicle becomes prominent (Manz *et al.* 2005, Müller *et al.* 2006).

At present, great progress has been achieved in the research of plant seed germination. In specific, previous studies identified that genes *GID1*, *MAIN*, *MAIL1*, *ga1-1 OsTPP7*, *OsIPMS1*, *Zm00001d026317*, and *Zm00001d014814* regulate seed germination in Arabidopsis, rice, and maize (De Luxán-Hernández *et al.* 2020, Gallardo *et al.* 2002, Han *et al.* 2020, Hauvermale and Steber 2020, Kretzschmar *et al.* 2015). In Arabidopsis, mutations of the *MAIN* or *MAIL1* gene plays a role in the protein complex. PROTEIN PHOSPHATASE 7-LIKE in Arabidopsis can cause meristematic cell death and prevent primary root growth after germination (De Luxán-Hernández *et al.* 2020). Hauvermale and Steber (2020) found that the transition from embryo to seedling in Arabidopsis requires GA and GA receptor *GA-INSENSITIVE DWARF1* (*GID1*). In *ga1-3* GA biosynthesis mutant, *GID1* overexpression causes abnormal seed germination, such that the cotyledons appear before the radicle and primary roots fail to develop, leading to plant death. He *et al.* (2019) showed that disruption of the isopropylmalate synthase gene *OsIPMS1* in rice can reduce seed vigor and that *OsIPMS1* can affect the vigor, starch hydrolysis, glycolytic activity, and energy amount of germinated seeds. Han *et al.* (2020) showed that the expression levels of two *bHLH* TFs in maize increase during seed germination and identified other TF family proteins related to seed germination. The above studies provide a reference to understand the general mechanism underlying plant seed germination, but the molecular mechanism behind cucumber seed germination remains unknown.

Dumont *et al.* (2016) found that 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) is a monosaccharide found only in the pectin rhamnogalacturan-II (RG-II) of the plant cell wall. CMP-KDO is a nucleotide sugar that can integrate KDO into lipopolysaccharide and capsular polysaccharide transferase, which acts as a substrate in this series of reactions (Jelakovic and Schulz 2002). KDO is found in the core of lipopolysaccharide of Gram-negative bacteria. After being dephosphorylated, KDO-8-P is coupled with CMP by

Communicated by Baorong Lu
Received November 12, 2020. Accepted April 18, 2021.
First Published Online in J-STAGE on August 18, 2021.
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CMP-KDO synthase to form an activated CMP-KDO sugar nucleotide (Royo *et al.* 2000). Then, it transported to the Golgi apparatus and participates in the RG-II backbone (Deng *et al.* 2010, Dumont *et al.* 2014). Dumont *et al.* (2016) showed that KDO is associated with pectin synthesis in the cell wall. Kobayashi *et al.* (2011) characterized CTP:KDO cytidylyltransferase (CMP-KDO synthetase; CKS) in Arabidopsis and found that the *cks* mutation causes pollen infertility by inhibiting pollen tube elongation. Despite the progress in KDO-related research, the mechanism by which the *KDO* gene regulates plant seed germination and development remains unclear.

The classic technique for cloning candidate genes in plants is map-based cloning (Wing *et al.* 1994). With the development of genome sequencing, other techniques, such as MutMap, have been developed for the rapid cloning of mutant genes (Abe *et al.* 2012). However, regardless of whether map-based cloning or MutMap is used, cross between mutants and wild type (or different ecological types) is necessary to build genetic groups, making causal genes hard to identify from mutants with early lethality or cross-pollination difficulties. With MutMap$^+$ technology, cross between wild type and mutant is unnecessary; thus, this method is suitable for identifying infertility lethal traits or hampering crossing mutants (Fekih *et al.* 2013). Using MutMap$^+$, Fekih *et al.* (2013) identified the causal genes of two prematurely dead rice mutants. Liu *et al.* (2019) also identified *CsPID*, a gene that can regulate the morphogenesis of cucumber lateral organs and the development of the ovule.

In the present study, we identified a lethal mutant (*Csleth*) from our previous ethylmethylsulfone (EMS) mutagenized library (Xue *et al.* 2016). The *Csleth* mutant grew slowly and detached from the cotyledon until 14 d after sowing. *Csa3G104930* (*CsKDO*) was identified as the causal gene regulating lethality during seed germination in cucumber. *CsKDO* was highly expressed in male flowers, and CsKDO protein was localized in the nucleus. This study provided insights into the function of *CsKDO* in regulating the seed germination of cucumber.

Materials and Methods

Plant materials

The lethality mutant *Csleth* and wild type of M_3 generation were isolated from the selfing of heterozygous individuals of the M_2 generation in our previous EMS mutagenized library. All plants were grown under 12 h light/12 h dark at 25°C.

Mutant selection and phenotypic characterization

EMS-mutagenized M_2 seeds (10 seeds/M_2 line × 295 M_2 lines; a total of 2950 seeds) were screened and grown on an acrylic plate culture system with vermiculite as a substrate for cultivation, and wild type seeds were sown into the same acrylic plate as a control. The acrylic plate was placed at an angle of 30° for 15 d. The radicle length was measured with a ruler. The *Csleth* mutant was identified as the radicle grew slowly and then detached from the cotyledon after growth. In consideration of the early development lethality of the *Csleth* mutant, M_2 generation individuals were selfed with the wild type phenotype and produced the seeds with segregated traits of the wild type and mutant phenotypes (*Csleth*) in the M_3 line. Then, the seeds were sown on an acrylic plate culture system for phenotype identification. The *Csleth* mutant was obtained after the second screening.

Whole genome re-sequencing

The DNA of M_3 plants was extracted using the CTAB method (Clark and Edwards 1997). In the M_3 population, the DNA of 11 plants with the mutant phenotype and 34 plants with the wild type phenotype were mixed equally to construct a mutant bulk and a wild type bulk, respectively. Genome re-sequencing for the bulks was performed at Annoroad Genomics (Beijing) by using an Illumina Hi-Seq 2000 sequencer (100 bp).

SNP calling and filtering

Short reads of large amounts of DNA were aligned with the reference genome of cucumber (http://cucurbitgenomics.org/organism/2, version 2i) (Huang *et al.* 2009) by using Burrows-Wheeler Aligner software (Li and Durbin 2009). As described by Abe *et al.* (2012), the SNP call filter "Coval" was applied to improve the accuracy of SNP. SNPs with SNP index >0.3 detected in only two large pieces of DNA were selected to calculate Δ(SNP-index). R scripts were used to apply sliding window analysis in 1 Mb window size and 100 kb increments (Takagi *et al.* 2013). Finally, causal mutations were mapped in the window, with an average p value < 0.1.

SNP genotyping with KASP

M_3 individual plants were used for KASP genotyping. Primers for all candidate SNPs were designed on the basis of the SNP mutation information (**Supplemental Table 1**). Specific genotyping was conducted in accordance with the method described by Hao *et al.* (2018). After completing the reaction, the fluorescence data were read using the LGC Genomics genotyping platform (Zhao *et al.* 2017).

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

The total RNA of male flowers, roots, shoot apices, and stems of wild type plants was isolated by using TRIZOL reagent (Invitrogen, USA). First-strand cDNA was synthesized using a cDNA synthesis kit (Toyobo, Japan). Expression data were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). *CsACTIN* (*Csa2G139820*) was selected as an internal reference to standardize the expression data (Wan *et al.* 2010). The primers for qRT-PCR analysis are listed in **Supplemental Table 2**.

Bioinformatics analysis of candidate genes

The gene structure and the full length of DNA were searched in the Cucurbit genomic database (http://cucurbitgenomics.org/organism/20, version 3i) (Li *et al.* 2019). Protein sequences and homologs were obtained from GenBank (https://www.ncbi.nlm.nih.gov) to study the phylogenetic relationship between CsKDO in cucumber and other species, and then CsKDO was compared with homologous protein sequences in different species by using DNAMAN 8.0 software. To build a phylogenetic tree, we used Clustal W in MEGA 6.0 for multi-sequence alignment and built an adjacent tree based on the bootstrap test of MEGA 6.0 through 1000 repeated tests.

Subcellular localization

To study the subcellular localization of CsKDO protein, we cloned the coding region of the *CsKDO* gene without a stop codon and then fused it with eGFP in pSUPER-1300 to form the pSuper1300::CsKDO-eGFP vector. pSuper1300::eGFP served as the blank vector control. Bacterial solution was injected on the back of tobacco (*Nicotiana benthamiana*) leaves (5–6 weeks old). After 48–72 h of injection, the subcellular localization was measured under a laser confocal microscope. The fluorescence signal was detected using a Zeiss LSM 7100 laser scanning confocal microscope.

Results

Isolation and phenotypic characterization of the lethality mutant Csleth

A total of 2950 seeds from 295 EMS-mutagenized M_2 lines were sown on an acrylic plate culture system with vermiculite as a substrate for cultivation to identify novel mutants related to seed germination in cucumber. M_2 lines were selected as the radicle grew slowly and then detached from the cotyledon after growth. In consideration of the early development lethality of the *Csleth* mutant, individual heterozygous M_2 plants were self-pollinated, and seeds of an M_3 line with segregation were obtained and then confirmed another time. After this screening, the *Csleth* mutant was obtained (**Fig. 1A**). The radicle growth rate of the mutant was significantly lower than that of the wild type (**Fig. 1B**). The radicle of the *Csleth* mutant developed slowly in the early stage. Compared with that of the wild type, the radical length of the mutant did not show obvious change about 5 d after sowing. The radicle of the wild type developed normally, and the length was 14.8 ± 0.16 cm at 14 d after sowing. However, the *Csleth* mutant remained at 0.83 ± 0.11 cm, and the radicle grew slowly throughout the growth period. True leaves of wild type plants emerged at 14 d after sowing, whereas the cotyledon of the *Csleth* mutant stopped growing and the radicle separated from the cotyledon, which was a lethal phenotype (**Fig. 1**).

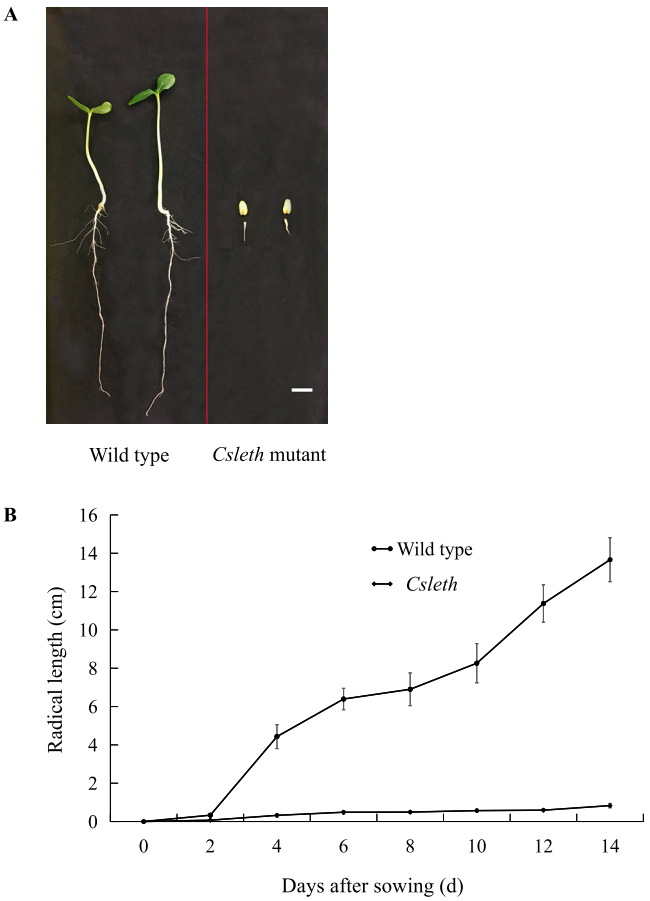


Fig. 1. Phenotype identification of the cucumber *Csleth* mutant. A: Phenotypic characterization of wild type and mutant *Csleth* in cucumber. The plants are at the 14 d after sowing stage. Scale bar = 1 cm. B: The growth rate of wild type and mutant *Csleth* from 0 to 14 d after sowing.

Identification of the candidate genes for Csleth in cucumber

Given the seed early development lethality of the *Csleth* mutant, an M_3 line with segregation was obtained from selfing an individual heterozygous M_2 plant. Among the M_3 progeny, eight plants showed lethality phenotype, and 42 plants showed wild type phenotype, which fitted Mendel's segregation ratio of 1:3 ($\chi^2 = 1.707$, $p > 0.05$). These data revealed that the phenotype of *Csleth* mutant was regulated by a single recessive gene.

In order to identify the candidate gene of *Csleth* mutants with more mutant plants, another large number M_3 generation was used. Two bulks, one wild type bulk with 34 progeny and one mutant bulk of 11 progeny, were established to identify the candidate gene by MutMap$^+$ (Fekih *et al.* 2013). The DNA copies of two bulks were re-sequenced. A total of 84,241,922 (94.77%) and 86,131,106 (94.29%) clean reads were obtained for the wild type and mutant bulks, respectively. In addition, 76,893,170 (91.28%) and 74,568,719 (86.58%) reads were mapped to the cucumber reference genome (http://cucurbitgenomics.org/organism/2,

version 2i) in the two bulks (**Supplemental Table 3**). The difference in th SNP-index values of the mutant and wild type bulks was the ΔSNP-index. From this, scatter plots and graphs of the mutant SNP-index, the wild type SNP-index, and the ΔSNP-index as a function of chromosome position were obtained (**Fig. 2**). The ΔSNP-index should be 0 in most regions of the genome, but its value should be significantly positive in regions containing mutation sites that cause mutation phenotypes.

According to the filter conditions: (1) The SNP-index in the mutant is close to 1; (2) G to A or C to T mutations; (3) Whether it is a non-synonymous mutation or a stop codon mutation. Five candidate SNP mutation sites located in chromosome 3 of cucumber were screened out, including four non-synonymous mutation sites in the exon region of the gene and one mutation site in the stop codon region of the gene (**Table 1**). Gene annotation indicated that the candidate genes containing these five SNPs were involved in 3-deoxy-manno-octulosonate cytidylyltransferase (SNP 5265242), receptor-like kinase (SNP 5985717), tetratricopeptide-like helical (SNP 7327506), Arabidopsis *At2G44640* genes related to lipid transporter from the ER to the chloroplast (SNP 8204046), and embryo sac development arrest 6 protein (SNP 9037163).

To identify the candidate SNP, we performed the KASP technique to genotype the five SNPs in a M_3 population accompanied with phenotypic data. KASP genotyping was conducted twice. A small number of M_3 plants containing five mutant plants and 34 wild type plants were used in the first KASP genotyping analysis. Results showed that two (SNP 5265242 and SNP 7327506) of the five SNPs showed co-segregation of genotyping and phenotyping data (**Supplemental Table 4**). To distinguish the two SNPs, we performed another genotyping test using a large M_3 population with 68 individuals (57 wild type, 11 mutants). Results showed that SNP 5265242 of *Csa3G104930* co-segregated with the phenotyping data in the M_3 population. Plants with mutant phenotypes had A:A genotypes, whereas those with wild type phenotypes had A:G or G:G genotypes. However, later results (**Supplemental Table 5**) showed that SNP

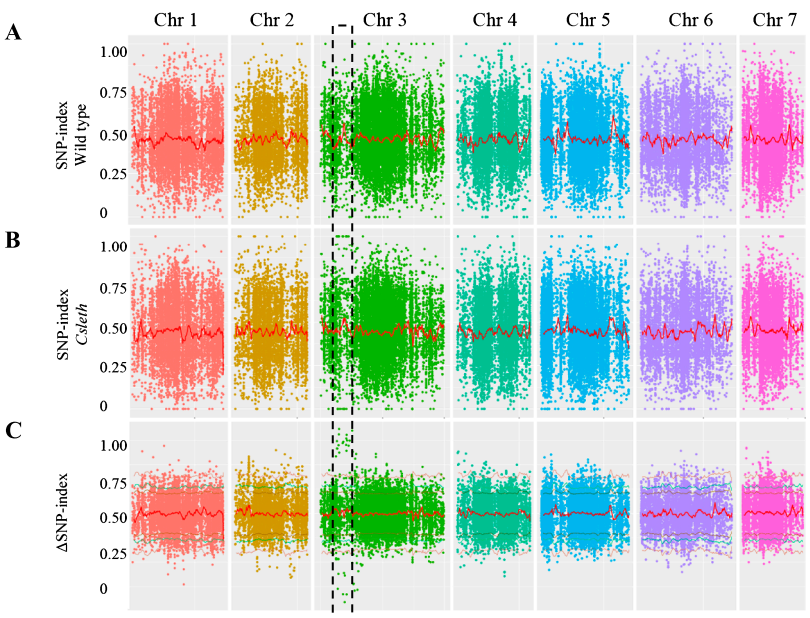


Fig. 2. Scatter plots of all candidate sites generated by the MutMap$^+$ method. A: SNP-index plots for wild type plants. B: SNP-index plots for *Csleth* mutant (Five candidate SNP mutation sites with SNP-index equal to 1 were represented in the black dashed box). C: ΔSNP-index obtained by subtracting mutant SNP-index bulk from wild type bulk (The point represents the SNP-index, the red line represents the average SNP-index in the sliding window. The black dashed box indicates the five candidate SNP mutation sites).

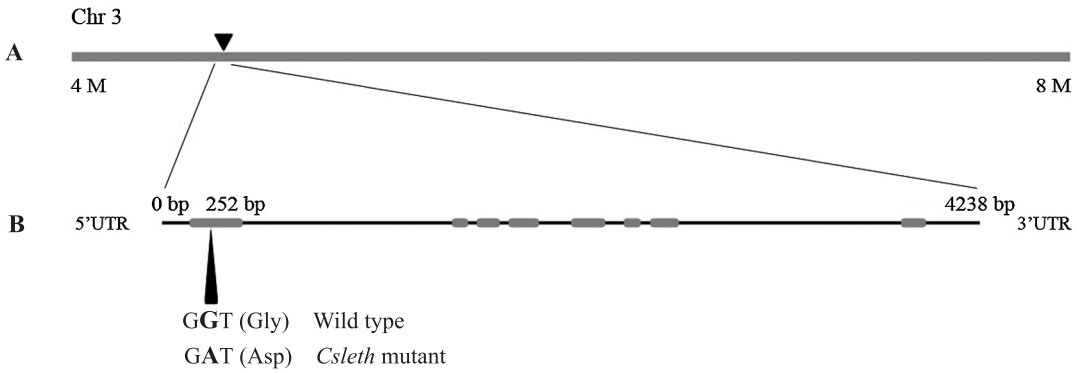
Table 1. List of candidate SNPs

Chromosome	Position	Gene	Reference	Alteration	SNP_index *Csleth*	Type	Amino Acid change	Annotation
3	5265242	*Csa3G104930*	C	T	1	Exonic	G to D	the Glyco-trans-GTA-type superfamily protein
3	5985717	*Csa3G114500*	C	T	1	Exonic		
3	7327506	*Csa3G122560*	C	T	1	Exonic		
3	8204046	*Csa3G128880*	C	T	1	Exonic		
3	9037163	*Csa3G134830*	G	A	1	Exonic		

7327506 did not co-segregate with the phenotype of M_3 individuals. These results indicated that *Csa3G104930*, a gene encoding 3-deoxy-manno-octulosonate cytidylyltransferase, which harbors SNP 5265242, is the causal gene of the *Csleth* mutant. In this study, the causal gene of the *Csleth* mutant is hereafter referred to as *CsKDO*.

Sequence analysis of CsKDO

Gene sequence analysis revealed that *CsKDO* is 4238 bp in length, containing 8 exons and 9 introns by consulting Cucurbit Genomics Database (http://cucurbitgenomics.org/organism/20, version 3i). A transition from glycine (Gly) to aspartate (Asp) was occurred in the first exon of *CsKDO* gene due to a G to A substitution at 252 bp (**Fig. 3B**). *CsKDO* encodes the 3-deoxymanmanate cytidyl acyltransferase, which catalyzes the production of sugar nucleotide CMP-3-deoxy-D-manmanate (CMP-KDO). KDO is activated during the biosynthesis of Rhamnogalacturonan II (RG-II), a pectin polysaccharide with a complex primary cell wall. RG-II is essential for cell wall integrity of fast-



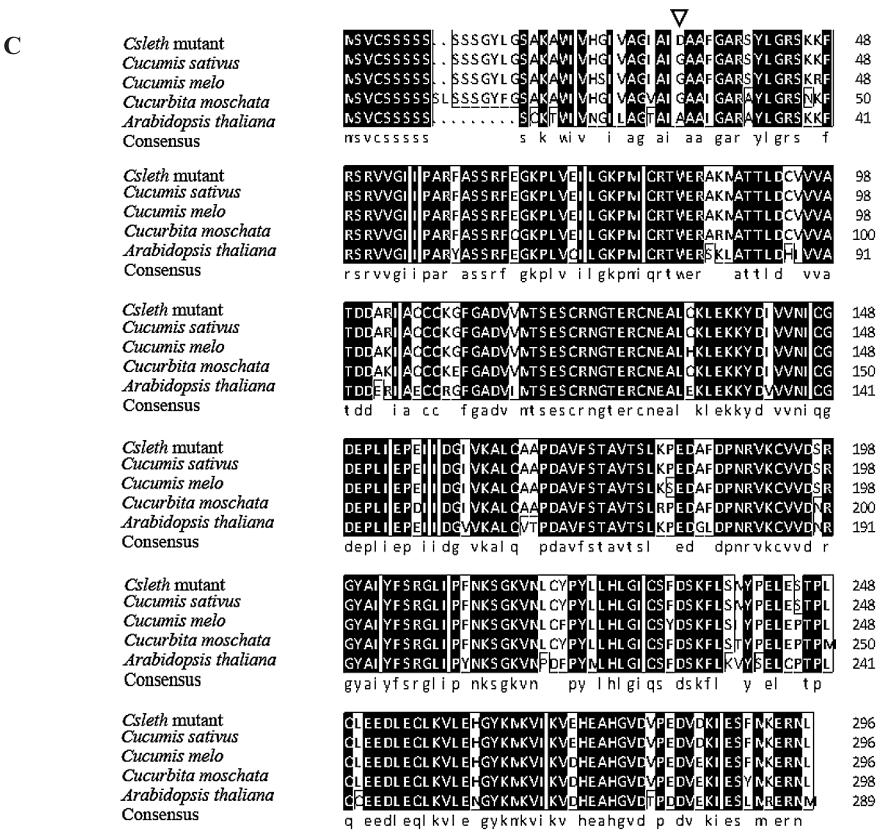


Fig. 3. Analysis of the candidate gene *CsKDO*. A: The relative position of mutations on the chromosome 3. B: Gene structure of *CsKDO*. Gray rectangles represent exons; solid lines represent intron. C: Aligned with the amino acid sequence of *CsKDO*, other cucurbitaceae and *Arabidopsis thaliana*. The black arrow indicates the *Csleth*-SNP (Chr3, 5265242) of *CsKDO* and the corresponding amino acid sequence.

growing tissues and for pollen tube growth and elongation (O'Neill *et al.* 1996).

CsKDO encoding a protein with 894 amino acids, with a G to A mutation at 252 bp (**Fig. 3B**). According to the structural properties indicated by the InterPro program (http://www.ebi.ac.uk/interpro/), the protein contains seven ligand binding sites, located at the 57th, 58th, 59th, 98th, 125th, 147th, and 149th amino acids. Conserved domain analysis of the amino acid encoded by CsKDO was performed using the NCBI database. Protein sequences of *Cucurbita moschata*, *Cucumis melo*, and *Arabidopsis thaliana* in the NCBI database were compared to obtain alignment scores in different species. The homology of the proteins was as high as 93.91%, with no highly conserved domains (**Fig. 3C**).

Using the protein sequence of CsKDO as the search sequence, we searched for homologous proteins by BLASTP and constructed a phylogenetic tree for analysis to explore further the genetic relationship of the protein in different species. Results showed that the CsKDO protein had high homology with the KDO proteins in *Manihot esculenta*, *Cucumis melo*, *Nicotiana attenuata*, *Arachis ipaensis*, and other plants. The highest and lowest homologies were observed with *Cucumis melo* (89.23%) and *Punica granatum* (78.55%), respectively (**Fig. 4**).

Expression and subcellular localization analysis

The expression of *CsKDO* was also investigated in different organs of wild type plants. The expression level of *CsKDO* was higher in male flowers than in other organs, such as shoot apices, root, and stems (**Fig. 5**). Subcellular localization results showed that CsKDO was located in the nucleus (**Fig. 6**).

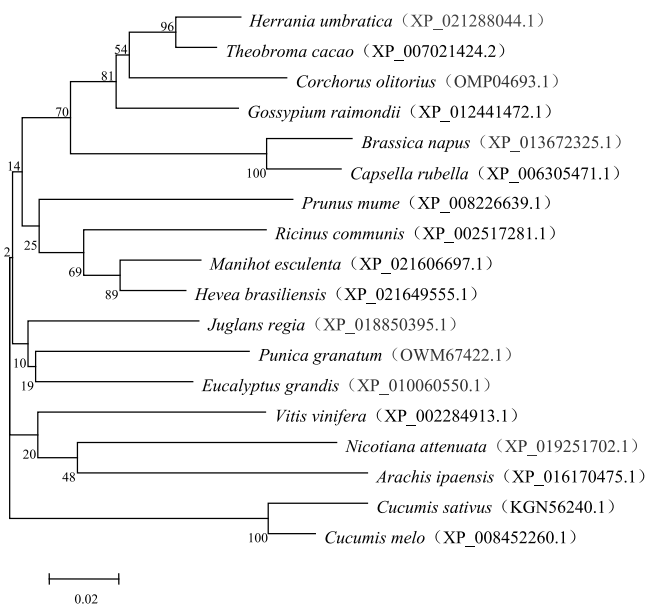


Fig. 4. Phylogenetic analysis of *CsKDO* in cucumber and its homologs.

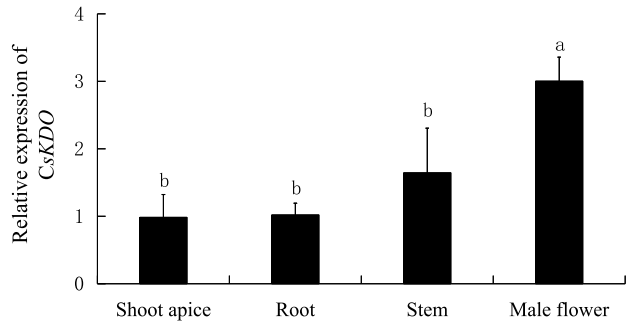


Fig. 5. Relative expression level of *CsKDO* in different tissues of the wild type. Letters indicate the significant difference of traits between wild type and *Csleth* mutant with p value < 0.05. Data were presented as means ± SD of three replicates. Tukey's test was conducted for statistical analysis.

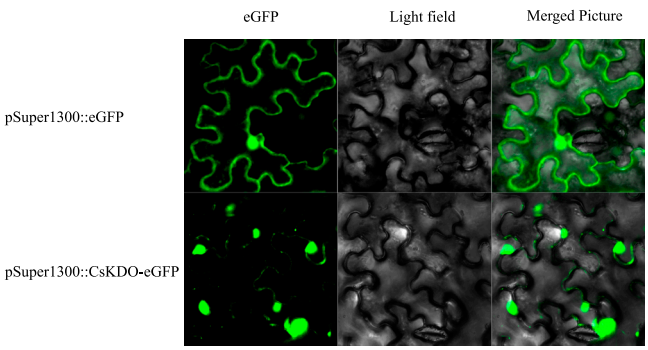


Fig. 6. Subcellular localization of CsKDO in cucumber. Subcellular localization showing that CsKDO is localized in the nucleus. eGFP driven by the pSUPER promoter was used as a control.

Discussion

Lethal phenotypes are ubiquitous in nature. They are closely related to the growth and development of plants and include the initial growth processes, such as seed germination and nutrient absorption. In this study, we obtained a cucumber lethality mutant and identified a gene (*CsKDO*) encoding 3-deoxy-manno-octulosonate cytidylyltransferase. This gene is homologous to Arabidopsis *At1g53000* and is a candidate to the *Csleth* mutant.

Strohmaier *et al.* (1995) found two *Escherichia coli* regulatory genes, *kdsA* and *kdsB*, which are involved in 3-deoxy-D-manno-octulosonic acid metabolism and biosynthesis of enterobacterial lipopolysaccharide and expressed in the growth phase regulated primarily at the transcriptional level in Escherichia coli K-12. In other crop, Royo *et al.* (2000) first discovered the maize root development-related gene *ZmCKS*, which is involved in the synthesis of corn CMP-KDO enzymes. In Arabidopsis, CMP-KDO synthesis gene has also been reported, the *CKS* gene is expressed in mature pollen and pollen tube, and its expression is also high in the shoot tip (Winter *et al.* 2007). Analysis of the *cks* mutant showed that the gene is

expressed in the leaves, stems, roots, and cilia of *Arabidopsis thaliana*, but the highest expression level can be found in the roots. In 2009, a report pointed that KDO is an essential component of the plant cell wall, and this gene may affect the catalytic performance of KDO synthetase (Misaki *et al.* 2009). Séveno *et al.* (2010) pointed that Kdo transferase (KDTA) deletion mutants do not affect the phenotypic changes and structural changes of RG-II in Arabidopsis and that *AtKDTA* is involved in mitochondrial molecular synthesis rather than cell wall RG-II. Smyth *et al.* (2013) reported that the 2β-deoxy-Kdo as an in vivo inhibitor of AtkdsB can affect the root cell elongation even lead to the growth and development defect in Arabidopsis. The study showed that this phenomenon may be attributed to the specific inhibition of AtkdsB enzyme by 2β-deoxidized KDO in Arabidopsis seedlings, resulting in structural changes or decreased RG-II abundance, which can also give us a new method for identification of RG-II/KDO related genes. In the meantime, a previous study reported that CMP-KDO is related to the synthesis of pectin in the cell wall (Dumont *et al.* 2016). However Kobayashi *et al.* (2011) found that the Arabidopsis *At1g53000* gene encodes a mitochondrial-associated CMP-KDO (3-deoxy-D-mannose-octasulfonate) synthetase and incorporated RG II (type II rhamnogalacturon) in KDO previously responsible for activating KDO; the heterozygous mutant of the gene is defective in pollen development and pollen tube elongation (Kobayashi *et al.* 2011). They also pointed out that the *At1g53000* gene encodes a *cks* mutant of *Arabidopsis thaliana*, subcellular localization of the gene indicates that it is expressed in the mitochondria of plant cells and is related to mitochondrial inner membrane synthesis (Kobayashi *et al.* 2011). The results obtained in the present study showed that cucumber CsKDO was a nuclear localization, which is different from its homologous from other crops including Arabidopsis *AT1G53000* gene, indicating that functions between *CsKDO* and its homologous might be different, which might be caused by their localization differences.

RG-II is a pectin molecule that is present in the primary cell wall of plants, it exist in the form of dimer, which is cross-linked by the borate diester bond between two arachidonic residues, containing 12 different glycosyl residues including aceric acid, apiose, 3-deoxy-D-lyxo-hept-2-ulosaric acid 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) and so on (Bar-Peled *et al.* 2012, Kobayashi *et al.* 1996, O'Neill *et al.* 1996, 2004, Zhao *et al.* 2020). The synthesis of dimer promotes the formation of pectin network, which promotes the mechanical properties of the primary wall and is necessary for the normal growth and development of plants, the absence of this dimer will lead to abnormal biochemical and biomechanical properties of the cell wall, even affect the productivity of plants (Julien *et al.* 2018). Many evidence shows that the alteration and cross-linking of RG-II structure have serious effects on the growth, development and viability of plants (Sechet *et al.* 2018). Defects in RG-II biosynthesis lead to changes in plant growth and cell wall structure (Dumont *et al.* 2014, Fleischer *et al.* 1999, O'Neill *et al.* 2001, Voxeur *et al.* 2011). However, the exact function of RG-II is unknown. In plants, 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) is a monosaccharide that is only found in the cell wall pectin, rhamnogalacturonan-II (RG-II) (Dumont *et al.* 2016). The inhibited mutant of CMP-KDO synthesis is lethal, which further indicates the important role of RG-II in plant growth (Ahn *et al.* 2006, Delmas *et al.* 2003, 2008, Mølhøj *et al.* 2003). Therefore, we speculate that the mutations of *Csa3G104930* and its homologous genes are likely to inhibit the synthesis of CMP-KDO during the germination of cucumber seeds. CMP-KDO is related to RG-II synthesis, and the inhibition of its synthesis may affect plant primary cell wall growth, as a result, the plant is stunted early in the stage, leading to plant death. This hypothesis further confirms that the cucumber lethality mutation phenotype is caused by the *CsKDO* gene mutation which identified in our study.

In conclusion, we obtained the stable dysplasia mutant *Csleth* by screening the EMS-mutated M_3 generation pedigree and found the *CsKDO* gene. The mutant significantly differed from the wild type in terms of lethality during seed germination. Predecessor analysis results suggest that mutations of the *CsKDO* gene block the synthesis of CMP-KDO in plant cells, affecting plant development and leading to cucumber lethal during seed germination. This study may serve as a theoretical reference for cucumber development during seed germination and promote the research of crop growth in the future.

Author Contribution Statement

TW and NH conceived the experiment. CW performed the research. CW and NH constructed the mutant and M_3 populations for experiments. YX, YD, and KH collected data for experiments. CW and TW wrote the manuscript. All authors reviewed and approved this submission.

Acknowledgments

We are thankful to the National Key Research and Development Program of China (2018YFD1000800), National Natural Science Foundation of China (31972429, 31972407, 32011540003), and Hunan Provincial Natural Science Foundation of China (2020JJ4363).

Literature Cited

Abe, A., S. Kosugi, K. Yoshida, S. Natsume, H. Takagi, H. Kanzaki, H. Matsumura, K. Yoshida, C. Mitsuoka, M. Tamiru *et al.* (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. Nat. Biotechnol. 30: 174–178.

Ahn, J.W., R. Verma, M. Kim, J.Y. Lee, Y.K. Kim, J.W. Bang, W.D. Reiter and H.S. Pai (2006) Depletion of udp-D-apiose/udp-D-

xylose synthases results in rhamnogalacturonan-II deficiency, cell wall thickening, and cell death in higher plants. J. Biol. Chem. 281: 13708–13716.

Bar-Peled, M., B.R. Urbanowicz and M.A. O'Neill (2012) The synthesis and origin of the pectic polysaccharide rhamnogalacturonan II—insights from nucleotide sugar formation and diversity. Front. Plant Sci. 3: 92.

Bewley, J.D. (1997) Seed germination and dormancy. Plant Cell 9: 1055–1066.

Clark, M.S. and K.J. Edwards (1997) Plant molecular biology. A laboratory manual. Plant Growth Regul. 23: 210.

De Luxán-Hernández, C., J. Lohmann, W. Hellmeyer, S. Seanpong, K. Wöltje, Z. Magyar, A. Pettkó-Szandtner, T. Pélissier, G. De Jaeger, S. Hoth *et al.* (2020) PP7L is essential for MAIL1-mediated transposable element silencing and primary root growth. Plant J. 102: 703–717.

Delmas, F., J. Petit, J. Joubès, M. Séveno, T. Paccalet, M. Hernould, P. Lerouge, A. Mouras and C. Chevalier (2003) The gene expression and enzyme activity of plant 3-deoxy-D-manno-2-octulosonic acid-8-phosphate synthase are preferentially associated with cell division in a cell cycle-dependent manner. Plant Physiol. 133: 348–360.

Delmas, F., M. Seveno, J.G.B. Northey, M. Hernould, P. Lerouge, P. McCourt and C. Chevalier (2008) The synthesis of the rhamnogalacturonan II component 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) is required for pollen tube growth and elongation. J. Exp. Bot. 59: 2639–2647.

Deng, Y., W. Wang, W.Q. Li, C. Xia, H.Z. Liao, X.Q. Zhang and D. Ye (2010) *MALE GAMETOPHYTE DEFECTIVE 2*, encoding a sialyltransferase-like protein, is required for normal pollen germination and pollen tube growth in *Arabidopsis*. J. Integr. Plant Biol. 52: 829–843.

Dumont, M., A. Lehner, S. Bouton, M.C. Kiefer-Meyer, A. Voxeur, J. Pelloux, P. Lerouge and J.C. Mollet (2014) The cell wall pectic polymer rhamnogalacturonan-II is required for proper pollen tube elongation: implications of a putative sialyltransferase-like protein. Ann. Bot. 114: 1177–1188.

Dumont, M., A. Lehner, B. Vauzeilles, J. Malassis, A. Marchant, K. Smyth, B. Linclau, A. Baron, J.M. Pons, C.T. Anderson *et al.* (2016) Plant cell wall imaging by metabolic click-mediated labelling of rhamnogalacturonan II using azido 3-deoxy-D-*manno*-oct-2-ulosonic acid. Plant J. 85: 437–447.

Fekih, R., H. Takagi, M. Tamiru, A. Abe, S. Natsume, H. Yaegashi, S. Sharma, S. Sharma, H. Kanzaki, H. Matsumura *et al.* (2013) MutMap^{+}: Genetic mapping and mutant identification without crossing in rice. PLoS ONE 8: e68529.

Fleischer, A., M.A. O'Neill and R. Ehwald (1999) The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan II. Plant Physiol. 121: 829–838.

Gallardo, K., C. Job, S.P.C. Groot, M. Puype, H. Demol, J. Vandekerckhove and D. Job (2002) Proteomics of Arabidopsis seed germination. A comparative study of wild-type and gibberellin-deficient seeds. Plant Physiol. 129: 823–837.

Han, Z., B. Wang, L. Tian, S. Wang, J. Zhang, S. Guo, H. Zhang, L. Xu and Y. Chen (2020) Comprehensive dynamic transcriptome analysis at two seed germination stages in maize (*Zea mays* L.). Physiol. Plant. 168: 205–217.

Hao, N., Y. Du, H. Li, C. Wang, C. Wang, S. Gong, S. Zhou and T. Wu (2018) *CsMYB36* is involved in the formation of yellow green peel in cucumber (*Cucumis sativus* L.). Theor. Appl. Genet. 131: 1659–1669.

Hauvermale, A.L. and C.M. Steber (2020) GA signaling is essential for the embryo-to-seedling transition during Arabidopsis seed germination, a ghost story. Plant Signal. Behav. 15: 1705028.

He, Y., J. Cheng, Y. He, B. Yang, Y. Cheng, C. Yang, H. Zhang and Z. Wang (2019) Influence of isopropylmalate synthase *OsIPMS1* on seed vigour associated with amino acid and energy metabolism in rice. Plant Biotechnol. J. 17: 322–337.

Huang, S., R. Li, Z. Zhang, L. Li, X. Gu, W. Fan, W.J. Lucas, X. Wang, B. Xie, P. Ni *et al.* (2009) The genome of the cucumber, *Cucumis sativus* L. Nat. Genet. 41: 1275–1281.

Jelakovic, S. and G.E. Schulz (2002) Catalytic mechanism of CMP: 2-keto-3-deoxy-*manno*-octonic acid synthetase as derived from complexes with reaction educt and product. Biochemistry 41: 1174–1181.

Julien, S., H. Soe, U. Breeanna, A. Abigail, F. Wei and I. Toshiki (2018) Suppressing arabidopsis GGLT1 affects growth by reducing the L-galactose content and borate cross-linking of rhamnogalacturonan II. Plant J. 96: 1036–1050.

Kobayashi, M., T. Matoh and J. Azuma (1996) Two chains of Rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. Plant Physiol. 110: 1017–1020.

Kobayashi, M., N. Kouzu, A. Inami, K. Toyooka, Y. Konishi, K. Matsuoka and T. Matoh (2011) Characterization of Arabidopsis CTP: 3-deoxy-D-*manno*-2-octulosonate cytidylyltransferase (CMP-KDO synthetase), the enzyme that activates KDO during rhamnogalacturonan II biosynthesis. Plant Cell Physiol. 52: 1832–1843.

Kretzschmar, T., M.A.F. Pelayo, K.R. Trijatmiko, L.F.M. Gabunada, R. Alam, R. Jimenez, M.S. Mendioro, I.H. Slamet-Loedin, N. Sreenivasulu, J. Bailey-Serres *et al.* (2015) A trehalose-6-phosphate phosphatase enhances anaerobic germination tolerance in rice. Nat. Plants 1: 15124.

Li, H. and R. Durbin (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25: 1754–1760.

Li, Q., H. Li, W. Huang, Y. Xu, Q. Zhou, S. Wang, J. Ruan, S. Huang and Z. Zhang (2019) A chromosome-scale genome assembly of cucumber (*Cucumis sativus* L.). GigaScience 8: giz072.

Liu, X., N. Hao, H. Li, D. Ge, Y. Du, R. Liu, C. Wen, Y. Li, X. Zhang and T. Wu (2019) PINOID is required for lateral organ morphogenesis and ovule development in cucumber. J. Exp. Bot. 70: 5715–5730.

Livak, K.J. and T.D. Schmittgen (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402–408.

Manz, B., K. Muller, B. Kucera, F. Volke and G. Leubner-Metzger (2005) Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. Plant Physiol. 138: 1538–1551.

Misaki, R., H. Kajiura, K. Fujii, K. Fujiyama and T. Seki (2009) Cloning and characterization of cytidine monophosphate-3-deoxy-D-*manno*-octulosonate synthetase from *Arabidopsis thaliana*. J. Biosci. Bioeng. 108: 527–529.

Mølhøj, M., R. Verma and W.D. Reiter (2003) The biosynthesis of the branched-chain sugar D-apiose in plants: functional cloning and characterization of a UDP-D-apiose/UDP-D-xylose synthase from *Arabidopsis*. Plant J. 35: 693–703.

Müller, K., S. Tintelnot and G. Leubner-Metzger (2006) Endosperm-limited brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*.

Plant Cell Physiol. 47: 864–877.

O'Neill, M.A., D. Warrenfeltz, K. Kates, P. Pellerin, T. Doco, A.G. Darvill and P. Albersheim (1996) Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester: *in vitro* conditions for the formation and hydrolysis of the dimer. J. Biol. Chem. 271: 22923–22930.

O'Neill, M.A., S. Eberhard, P. Albersheim and A.G. Darvill (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. Science 294: 846–849.

O'Neill, M.A., T. Ishii, P. Albersheim and A.G. Darvill (2004) Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu. Rev. Plant Biol. 55: 109–139.

Royo, J., E. Gómez and G. Hueros (2000) A maize homologue of the bacterial CMP-3-deoxy-D-manno-2-octulosonate (KDO) synthetases. Similar pathways operate in plants and bacteria for the activation of KDO prior to its incorporation into outer cellular envelopes. J. Biol. Chem. 275: 24993–24999.

Sechet, J., S. Htwe, B. Urbanowicz, A. Agyeman, W. Feng, T. Ishikawa, M. Colomes, K.S. Kumar, M. Kawai-Yamada, J.R. Dinneny *et al.* (2018) Suppression of Arabidopsis GGLT1 affects growth by reducing the L-galactose content and borate cross-linking of rhamnogalacturonan-II. Plant J. 96: 1036–1050.

Séveno, M., E. Séveno-Carpentier, A. Voxeur, L. Menu-Bouaouiche, C. Rihouey, F. Delmas, C. Chevalier, A. Driouich and P. Lerouge (2010) Characterization of a putative 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) transferase gene from *Arabidopsis thaliana*. Glycobiology 20: 617–628.

Smyth, K.M., H. Mikolajek, J.M. Werner and A. Marchant (2013) 2β-deoxy-Kdo is an inhibitor of the *Arabidopsis thaliana* CMP-2-Keto-3-deoxymanno-octulosonic acid synthetase, the enzyme required for activation of Kdo prior to incorporation into rhamnogalacturonan II. Mol. Plant 6: 981–984.

Strohmaier, H., P. Remler, W. Renner and G. Högenauer (1995) Expression of genes *kdsA* and *kdsB* involved in 3-deoxy-D-*manno*-octulosonic acid metabolism and biosynthesis of enterobacterial lipopolysaccharide is growth phase regulated primarily at the transcriptional level in *Escherichia coli* K-12. J. Bacteriol. 177: 4488–4500.

Takagi, H., A. Abe, K. Yoshida, S. Kosugi, S. Natsume, C. Mitsuoka, A. Uemura, H. Utsushi, M. Tamiru, S. Takuno *et al.* (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.

Tiedemann, J., B. Neubohn and K. Müntz (2000) Different functions of vicilin and legumin are reflected in the histopattern of globulin mobilization during germination of vetch (*Vicia sativa* L.). Planta 211: 1–12.

Voxeur, A., L. Gilbert, C. Rihouey, A. Driouich, C. Rothan, P. Baldet and P. Lerouge (2011) Silencing of the GDP-D-mannose 3,5-epimerase affects the structure and cross-linking of the pectic polysaccharide rhamnogalacturonan II and plant growth in tomato. J. Biol. Chem. 286: 8014–8020.

Wan, H., Z. Zhao, C. Qian, Y. Sui, A.A. Malik and J. Chen (2010) Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. Anal. Biochem. 399: 257–261.

Wing, R.A., H.B. Zhang and S.D. Tanksley (1994) Map-based cloning in crop plants. Tomato as a model system: I. Genetic and physical mapping of *jointless*. Mol. Gen. Genet. 242: 681–688.

Winter, D., B. Vinegar, H. Nahal, R. Ammar, G.V. Wilson and N.J. Provart (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE 2: e718.

Xue, C.B., C. Wang, L.X. Fan, N. Hao, D.D. Zou, Q. Zhang, H.X. Chen and T. Wu (2016) Ethylmethanesulfonate mutagenesis of cucumber for large-scale mutant screens. Pak. J. Bot. 48: 2261–2266.

Zhao, X., B. Ebert, B. Zhang, H. Liu, Y. Zhang, W. Zeng, C. Rautengarten, H. Li, X. Chen, A. Bacic *et al.* (2020) UDP-Api/UDP-Xyl synthases affect plant development by controlling the content of UDP-Api to regulate the RG-II-borate complex. Plant J. 104: 252–267.

Zhao, Y., X. Liu, H. Zhao, C. Yuan, G. Qi, Y. Wang and Y. Dong (2017) Comparison of methods for SNP genotyping in soybean. Mol. Plant Breed. 15: 3540–3546.