Genes & Genomics (2019) 41:1241–1251
https://doi.org/10.1007/s13258-019-00852-4

Online ISSN 2092-9293
Print ISSN 1976-9571

RESEARCH ARTICLE

Validation of MADS-box genes from apple fruit pedicels during early fruit abscission by transcriptome analysis and real-time PCR

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Received: 21 April 2019 / Accepted: 12 July 2019 / Published online: 26 July 2019

Abstract

Background Fruit abscission in an isolated region called abscission zone (AZ) is regulated by several genes including *JOINTLESS*, *MACROCALYX* and *SEPALLATA*, MADS-box genes, in tomato.

Objective The surviving central pedicels and the abscised lateral pedicels were examined in fruit clusters in order to investigate apple MADS-box genes from fruit pedicels of self-abscising apple 'Saika' during early fruit abscission.

Methods After performing RNA-Seq, transcription profiling was conducted on the MADS-box genes from apple central and lateral pedicels. The *JOINTLESS* homolog of apple (*MdJOINTLESS*) was amplified using degenerate primers annealing to a highly conserved domain based on the orthologous genes of various crops, including *JOINTLESS* gene of tomato. The expression pattern of *MdJOINTLESS* was investigated in central and lateral pedicles by real-time PCR.

Results Some homologs were found which similar to *JOINTLESS*, *MACROCALYX* and *SEPALLATA* of tomato MADS-box genes from transcriptome analysis and RACE. Using phylogenetic analyses with the MADS-box gene family, *MdJOINTLESS* was classified into the *SHORT VEGETATIVE PHASE* (*SVP*) clade that included Arabidopsis and other crops. The expression level of *MdJOINTLESS* in central pedicel was more than twice as high as that of lateral pedicel.

Conclusion In the current study, we could find apple homologs of *JOINTLESS*, *MACROCALYX*, *SEPALLATA*, which were known to regulate pedicel AZ development in tomato. Furthermore, *MdJOINTLESS* might contribute to auxin gradation, influencing hierarchical ranking of auxin transport between fruit pedicels of self-abscising apple.

Keywords *Malus*×*domestica* · Abscission zone · Self-abscission · MADS-box · Transcriptome analysis

Introduction

Fruit abscission typically occurs in a developmentally separated region called the abscission zone (AZ). The AZ, formed at the junction between the fruit and the branch of the plant, is composed of six to eight layers of small, square, and densely packed cells (Sexton and Roberts 1982; Sun et al. 2009). The cells in the AZ are arrested during growth and development and arranged in transverse sections relative to the adjacent vascular cells (Sun et al. 2009). Moreover, the treachery elements in the AZ are less developed and their lignins are less abundant (Patterson 2001). Below the AZ, the protective layers are formed in the proximal region of the peduncle. Abscission is processed by the expanded cells of the AZ and the immature vascular system that does not support the pedicels and fruits.

AZ differentiation is known to be regulated by several genes and transcription factors. In tomato, the *LATERAL SUPRRESSOR* gene, which encodes a member of VHIID protein family, is required for AZ development in the pedicel (Schumacher et al. 1999). The MADS-box protein, JOINTLESS (J) also plays a crucial role in the development of the AZ in the tomato (Mao et al. 2000) and interacts with the other MADS-box protein, such as MACROCALYX (MC) and SlMBP21, forming protein complexes that regulate AZ formation (Liu et al. 2014; Nakano et al. 2012). SlMBP21 is thought to attach J to MC (AP1 family), as a glue, and was reported that SlMBP21 belongs to SEPALLATA (SEP)

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family in the quartet model describing floral organ formation (Liu et al. 2014).

In *Arabidopsis*, the double mutant lacking the transcription factors *BLADE-ON-PETIOLE 1* (*BOP1*) and *BOP2* failed to abscise the floral organs, even after senescence and wilting (McKim et al. 2008). *BOP1* and *BOP2* encode BTB/POZ domain and ankyrin repeat-containing proteins, which redundantly regulate AZ differentiation in floral organs (McKim et al. 2008). The INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptide ligand is known to trigger abscission mechanism by the receptor-like kinases, HAESA (HAE) and HAESA-LIKE2 (HSL2) (Butenko et al. 2003; Jinn et al. 2000; Stenvik et al. 2008), and the signal is transduced into the nucleus by mitogen activating protein kinase cascade (Cho et al. 2008). Shi et al. (2011) reported that *KNOTTED-like homeobox* (*KNOX*) genes may regulate transcription of genes related to cell separation. *AUXIN RESISTANT 1* (*AUX1*), *LIKE AUX1* (*LAX1*), and *LAX3* were strongly expressed at the site for floral organ abscission and the *AXR3* gene, which induced in *Aux/IAA* transcription factor family, was interfered with auxin signaling at the AZ (Basu et al. 2013), suggesting the involvement of auxin signaling in abscission (Heo et al. 2016).

In rice, differentiation of the pedicel AZ is regulated by *SHATTERING 4* (*SH4*) (Li et al. 2006), *qSH1* (Konishi et al. 2006), and *SHATTERING ABORTION 1* (*SHAT1*) (Zhou et al. 2012). *SH4* and *qSH1* both activate AZ formation at the pedicel. *SH4* encodes a Myb3 DNA-binding domain-containing protein (Li et al. 2006), whereas *qSH1* is a BELL homeobox gene such as *REPLUMLESS* (or *VAAMANA*) (Konishi et al. 2006). *SHAT1* belongs to the *AP2* transcription factor family and is involved in rice grain shattering (Zhou et al. 2012).

In apple, the genes related to polar auxin transport might be involved in abscission induction during the shedding of immature fruit (Dal Cin et al. 2009a, b). Dal Cin et al. (2009a) reported that *MdLAX1*, *MdLAX2*, *MdPIN1*, and *MdPIN10* genes may be responsible for the abscission induction and the expression level of *auxin hydrogen symporter* (*AHS*) gene was lower in the cortex of abscising fruit (Dal Cin et al. 2009b). Our previous study revealed that *IAA3/SHY2* or *IAA14/SLR* were differentially expressed in the surviving central fruits or the abscised lateral fruits (Heo et al. 2016). The *Aux/IAA* genes which were related to auxin signal transduction might participate in fruit pedicel abscission by performing comparative analysis using RNA-Seq and validating the expression profiles by qPCR (Heo et al. 2016).

In terms of MADS-box genes, Nakano et al. (2015) reported three *J* homologs exist in apple 'Fuji' genome. *MdJa1, 2* and *MdJb*, respectively, interacted with tomato *MC* and *SlMBP21* (*SEP*), forming a tetramer that act as transcription factor for regulating pedicel AZ specification or development. *MdJb* complemented the *j* mutant phenotype in tomato, inducing AZ development. However, *MdJa* did not restore the *J* deficient phenotype, showing incomplete AZ structure in pedicel and the genes that *J* induced in wild type tomato were not expressed, such as polygalacturonase, cellulase and AZ-specific transcription factors (Nakano et al. 2015).

Fruit trees generally produce excessive fruitlets to the extent that trees can not support the fruit load (Heo et al. 2015). Moreover, most apple cultivars bear five flowers in a flower cluster and remain to grow not having any abscission in their pedicels. Therefore, fruit thinning is an essential practice for producing apples that have commercial quality. Chemical thinning has been widely used with similar substance to plant hormones, such as auxin or ethylene. By spraying chemicals, AZ cells perceive an abscission-stimulating signal and cell adhesion at AZ finally become to be loosened through cell wall remodeling mechanism, activating ethylene-induced enzymes (Nakano et al. 2015; Roberts et al. 2002). However, thinning chemicals have harmful side effects on trees, environment and even fruits, causing resetting, and malformation. In Korea, hand thinning is generally practiced to obtain bigger and sweeter fruits, but it is still labor- and time-intensive work. Therefore, the breeding of self-thinning cultivars or development of new thinning chemical based on abscission mechanism can be an effective alternatives and suit modern apple production for low labor input.

Apple cultivars were classified into three groups as a non-abscising, a June drop, and a self-abscising, according to the fruit abscission patterns from full bloom (FB) to 30 days after full bloom (DAFB) (Heo et al. 2015). Unlike June drop and pre-harvest drop, the self-abscission characteristics shows the only one central fruit remains to grow and the other lateral fruits are abscised in a fruit cluster. Here, we validated the MADS-box genes reported from Nakano et al. (2015) in different apple cultivar, which were expressed in fruit pedicels of self-abscising apple during early fruit abscission. The comparative and phylogenetic analyses were also performed to identify the apple MADS-box (*MdMADS*) genes which might be involved in the AZ development.

Materials and methods

Plant materials

Ten-year-old, self-abscising 'Saika' apple (*Malus* × *domestica* Borkh.) trees, grafted on M.9 rootstocks were grown at the National Institute of Horticultural and Herbal Science, Korea. All flowers were artificially pollinated with mixed pollens collected from various cultivars to avoid the self-incompatibility resulting from the same S-genotypes. The

central pedicels (CP) and lateral pedicels (LP) in clusters were collected from FB to 20 DAFB. They were immediately frozen in liquid nitrogen and stored at − 80 °C until use.

Microscopic observation

The pedicel tissues including AZ were prepared under a light microscope (Axioskop 2, Carl Zeiss Inc., Oberkochen, Germany) and the specimen fixation, post-fixation treatment, and observation were performed as described by Heo et al. (2016).

Genomic DNA and RNA extraction

Frozen pedicel tissue samples were pulverized with a mortar and pestle using liquid nitrogen. One gram of frozen sample was suspended in 2.5 mL of extraction buffer consisting of 2% (w/v) cetyltrimethyl ammonium bromide (CTAB), 100 mM Tris–Cl (pH 8.0), 20 mM EDTA, 1.42 M NaCl, 5 mM ascorbic acid, and 2% (w/v) polyvinyl pyrrolidone. Genomic DNA was isolated according to the method of Heo et al. (2016), with minor modifications. Approximately 100 mg of powdered tissue was used to extract total RNA using to the CTAB method (Chang et al. 1993). The extracted RNA was precipitated, and the resulting pellet was resuspended in diethylpyrocarbonate-treated water. RNA quality was assessed by electrophoresis on an 1% agarose gel and quantified using a NanoDrop ND-1000 spectrophotometer (ThermoScientific, Waltham, MA, USA).

Pyrosequencing, trimming, assembly, and annotation of the pedicel transcriptome

As described in our previous research (Heo et al. 2016), the pedicel transcriptome was generated by pyrosequencing on Roche 454 GS-FLX sequencer (Basel, Switzerland). The reads were mapped against *Malus* × *domestica* reference sequences from Phytozome (http://www.phytozome.net). The expression values were normalized to reads per kilobase of exon model per million (RPKM) mapped read values and the contigs related to MADS-box genes were screened by annotation informations.

Identification of *JOINTLESS* homologue, *MdJOINTLESS*

For isolation of the *JOINTLESS* homologue, *MdJOINTLESS*, 1 µg of total RNA was subjected to first-strand cDNA synthesis using T (18) primers and PowerScript reverse transcriptase (Clontech, Mountain View, CA, USA). For second-strand cDNA synthesis, two degenerate primers were designed using the J-CODEHOP program targeting the conserved amino acid sequences MAREKIQIK from the MADS-box domain and RQMRGEDLQG from the K-box region. The sequences of the primers were 5′-ATGGCKAGAGARAARATTMAGATMAAGAA-3′ and 5′-CCTTGRAGHTCYTCHCCYCTCATYTGCCT-3′. Second-strand cDNA synthesis was performed according to the following procedure: initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 3 min, and a final extension for 5 min at 72 °C. A single DNA band was detected by agarose gel electrophoresis and was extracted using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted DNA was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions, and sequenced. Rapid amplification of cDNA ends (RACE) was performed using the SMARTer RACE cDNA amplification kit (Clontech) with adaptors and specific primers that were based on a partial cDNA sequence. The polymerase chain reaction (PCR) products were cloned and sequenced as described above.

Quantitative real-time reverse-transcription PCR

The cDNA was synthesized from 1 µg of total RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR reactions were performed with Applied Biosystems 7500 Fast Real-Time PCR System and TaqMan Universal PCR Master Mix (Applied Biosystems). The mixture is composed of a 20 µl reaction solution containing 10 µl 2× master mix, 7 µl ddH_2O, 2 µl of template, and 1 µl 20× TaqMan assay mix. The primer and probe sequence for *MdJOINTLESS* gene are as follows: F: GGGATGCAATTGATGGAAGAGAATG, R: GCCTCC GGCCATCAGA TTT and TaqMan probe: FAM-TCCGCC ACTTGCTGTC-TAMRA. Transcript values normalized and calibrated relative to *Malus* × *domestica* actin (CN935584) protein as a housekeeping internal standard gene according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The sequences are: F: GCCTCCAGAGTATATGCCAGA GAAT, R: TCTGCTTTTGTTATA TGTTGGTTTTGTGG and TaqMan probe: FAM-CAAGTATGGCACCT CCC-TAMRA. Three biological and technical replicates were used in this experiment.

Phylogenetic analysis

To survey the phylogenetic relationship of MdMADS proteins with those of other plants, the phylogenetic tree was constructed using MEGA X. Dendrograms were generated using neighbor-joining method with default parameters. The MADS-box genes of other crops were assorted with *MdMADS* genes reported by Velasco et al. (2010).

Results

AZ of self-abscising apple 'Saika'

As described in Fig. 1, apple cultivars which have the self-abscission characteristics retain their central fruits and abscise lateral fruits within 30 DAFB (Heo et al. 2015). During early fruit abscission, the AZ was developed, and the abscission process occurred in only lateral pedicel of the self-abscising apple cultivar 'Saika' (Fig. 1). At 20 DAFB in the LP, the AZ was developed at the junction between the pedicel and the peduncle, with the protective layers forming immediately below the AZ (Fig. 2a). The AZ was composed of six to eight layers of small, square, and densely packed cells (Fig. 2a). The cells of the AZ were transversely arranged relative to vascular system and adjacent cells (Fig. 2b).

Hierarchical clustering analysis

In the previous research (Heo et al. 2016), the self-abscising cultivar 'Saika' was used for RNA-Seq and differentially expressed genes analysis between the surviving central

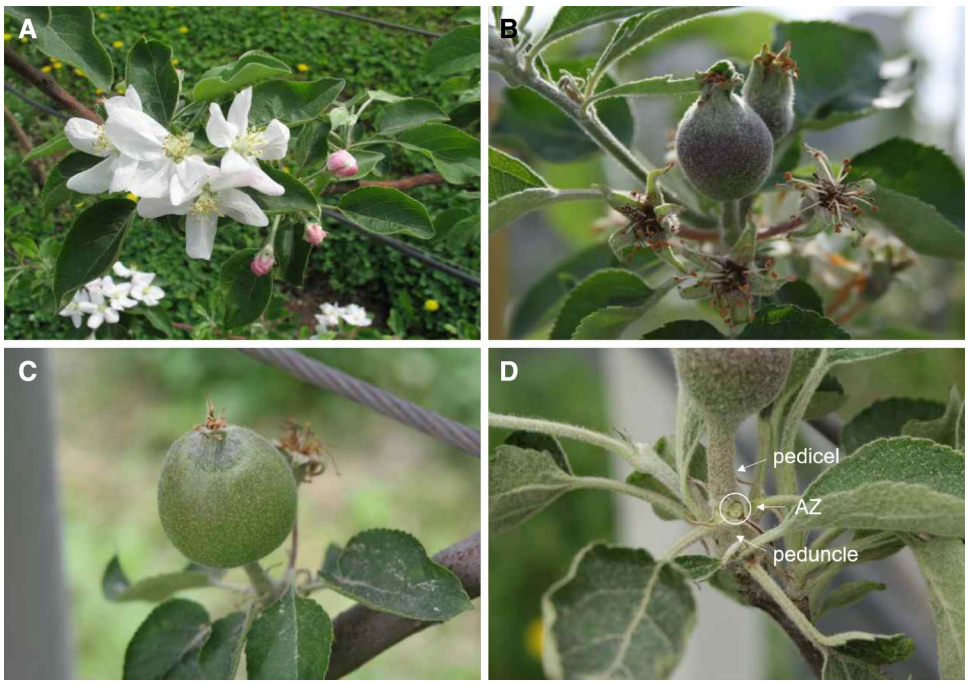

Fig. 1 Early fruit abscission of the self-abscising apple 'Saika' from full bloom (FB) to 30 days after full bloom (DAFB). All lateral fruit were abscised within 30 DAFB. **a** FB, **b** 15 DAFB, **c** 25 DAFB, and **d** 30 DAFB. Each parts of the fruit cluster is designated by arrowheads

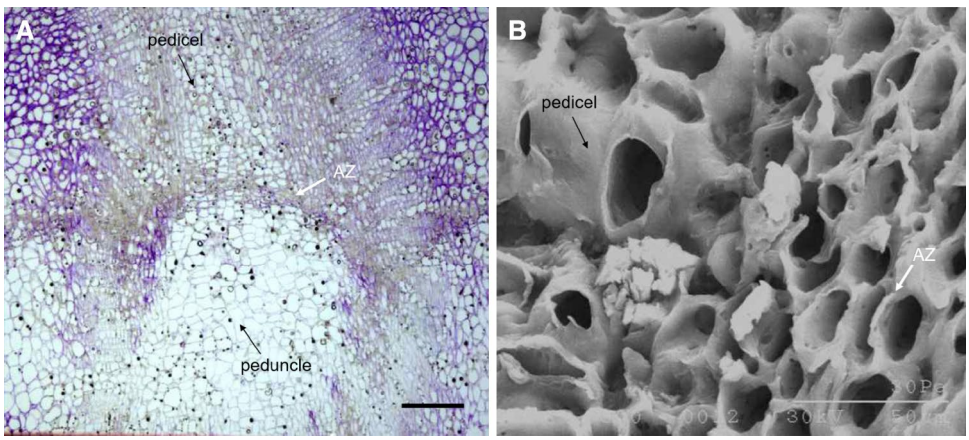

Fig. 2 The abscission zone (AZ) developed at the pedicel of the lateral fruits in the self-abscising apple 'Saika'. The AZs located between the pedicel and peduncle were manually dissected from longitudinal sections (**a**). Scanning electron micrograph of the pedicel AZ (**b**). The square cell layers are seen at the lower right corner. These cells were transversely arranged relative to the adjacent cells. Scale bar: 100 μm in **a**; 50 μm in **b**. Each parts of the fruit cluster is designated by arrowheads

pedicels and the abscised lateral pedicels. Total 65,876 contigs were assembled and mapped to apple reference transcriptome. Among the assembled transcripts, MADS-box genes were screened and assigned according to hierarchical clustering (Fig. 3). Twenty-one contigs containing *MdMADS* genes were found to be expressed in fruit pedicels, while other MADS-box genes exhibit no expression by RNA-Seq. *MdMADS* genes were divided into three groups, with six genes each appearing specifically in CP and LP. The gene that expressed in both CP and LP generally belonged to *AG* family. In this study, apple *MC* homolog (*MdMC*) was MDP0000269921 and *SlMBP21* (*MdSEP*) homolog was MDP0000326390 (Fig. 3). *MdMC* gene was differentially expressed in LP, but *MdSEP* gene was similarly expressed in both pedicels.

Isolation and sequence analysis of *J* homolog from apple fruit pedicels

The full-length protein sequences of MADS-box proteins of Arabidopsis, field mustard, sweet potato, bean, and tomato were aligned using ClustalW2 for designing degenerate primers to discover J-like proteins in apple (Fig. 4a). The reverse transcription (RT)-PCR produced a single cDNA band at the expected size of 350 bp, from which full-length cDNA was reconstructed using 3′-RACE. The final product, named *MdJOINTLESS* (*MdJ*, GenBank accession No. ABD66219.2), is a MADS-box gene isolated from the pedicel tissue of young fruit. *MdJ* showed the highest sequence similarity to members of the *SHORT VEGETATIVE PHASE* (*SVP*) family of MADS-box genes (Fig. 4b). The *MdJ* gene encodes a putative 224 amino acid protein that is 77% and 78% identical to *J* (tomato) and *IbMADS3* (sweet potato), respectively (Fig. 3b). The tree was divided into some distinct clades according to the representing *MdMADS* genes with those of other crops, such as *Arabidopsis* and tomato. The orthologs of *SVP* gene are known to be repressors of floral transition during the vegetative phase (Hartmann et al. 2000), and after the floral transition, they are expressed in the floral meristem (Gregis et al. 2013).

Analysis of expression pattern of *MdJ* genes in pedicels during early fruit abscission

The DNA gel blot revealed a single hybridization band with *Eco*RV and two bands with *Hin*dIII (data not shown). These

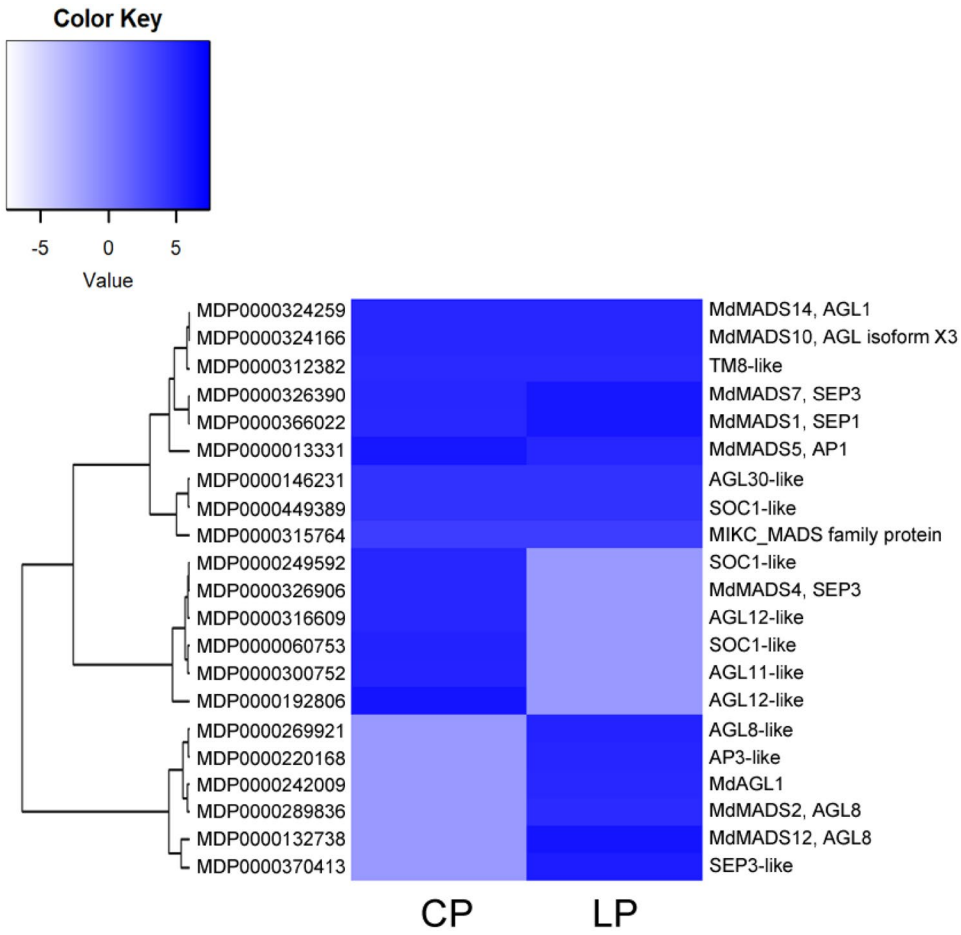

Fig. 3 The heatmap representing MADS-box genes from the surviving central pedicels (CP) and the abscised lateral pedicels (LP) of self-abscising apple 'Saika'. Apple MADS-box genes with their contig numbers are presented with clades

A

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                  *        20        *        40        *        60        *        80        *       100       *
MdJOINTLES : MAREKIQIKKIDNATARQVTFSKRRRGLLKKAEELSVLCDADIALIIFSSTGKLFEYASSSMKEILERHNLHSKNLEKLEQPSLQLQLVENSNYTRLSKEIAAKSHQLRQMRGEE : 115
MdJa1      : MAREKIQIKKIDNATARQVTFSKRRRGLLKKAEELSVLCDADIALIIFSSTGKLFEYASSSMKEILERHNLHSKNLEKLEQPSLQLQLVENSNYTRLSKEIAAKSHQLRQMRGEE : 115
MdJa2      : MAREKIQIKKIDNATARQVTFSKRRRGLLKKAEELSVLCDADIALIIFSSTGKLFEYASSSMKEILERHNLHSKNLEKLEQPSLQLQLVENSNYTRLSKEIAAKSHQLRQMRGEE : 115
MdJb       : MAREKIQIKKIDNATARQVTFSKRRRGLEKKAEELSVLCDADIALIIFSSTGKLFEYASSSMKEILERHNLHSKNLTKLEQPSLELQLVENSNYSRLSKEIAAKSHQLRQMRGEE : 115
AtSVP      : MAREKIQIRKIDNATARQVTFSKRRRGLEKKAEELSVLCDADVALIIFSSTGKLFEFCSSSMKEVLERHNLQSKNLEKLDQPSLELQLVENSDHAIRMSKEIADKSHRLRQMRGEE : 115
BcSVP      : MAREKIQIRKIDNATARQVTFSKRRRGLEKKAEELSVLCDADVALIIFSSTGKLFEFCSSSMREVLERHNLQSKNLEKLDQPSLELQLVENSDHAILSKEIAEKSHRLRQMRGEE : 115
IbMADS3    : MAREKIKIKKIDNATARQVTFSKRRRGLEKKAEELSVLCDADVALIIFSSTGKLFEYASSSMKGILERRNLHSKNLEKMLQPSLELQLVENANHSRLSKEIADMTHRLRQMRGED : 115
PsSVP      : MAREKIQIKKIENATARQVTFSKRRRGLEKKAEELSVLCDADVALIIFSSTGKLFEYSNLSMREILERRHLHSKNLAKMEPPSLELQLVENSNCTRLNKEIAEKSHQLRQMRGED : 115
JOINTLESS  : MAREKIQIKKIDNSTARQVTFSKRRRGLEKKAEELSVLCDADVALIIFSSTGKLEDYSSSSMKQILERRDLHSKNLEKLDQPSLELQLVENSNYSRLSKEISEKSHRLRQMRGEE : 115
             MAREKIqI4KIdNaTARQVTFSKRRRGL KKAEELSVLCDAD6ALIIFSSTGKLFe5 ssSM4 6LERh LhSKNLeK6 2PSL2LQLVENs1  r6sKEIa k3H LRQMRGEe

                 120       *       140       *       160       *       180       *       200       *       220       *
MdJOINTLES : IQGLNLEELQQLEKSLETGLGRVIEKKSEKIMKEIGDLQRNGMQLMEENERLRQQVAEKSDGRRLVQVDSENMFTEEGQSSESVTNPCNSNNGPQDYDSSDTSLKLGCV------ : 224
MdJa1      : IQGLNLEELQQLEKSLETGLGRVIEKKSEKIMKEIGDLQRNGMQLMEENERLRQQVAEKSDGRRLVQVDSENMFTEEGQSSESVTNPCNSNNGPQDYDSSDTSLKLGLPYSG--- : 227
MdJa2      : IQGLNLEELQQLEKSLETGLGRVIEKKSEKIMKEIGDLQRNGMQLMEENERLRQQVAEKSDGRRLVQVDSENMFTEEGQSSESVTNPCNSNNGPQDYDSSDTSLKLGCV------ : 224
MdJb       : IQGLSLEELQQLEKSLEAGIGGVVEKKSEKIMKEINDLQRNAMQLTEENERLRQQVVEKSNGRRLVHVDSELLITEEGQSSESVTNLCKSNSGPQDYDNSVTSLKLGCA------ : 224
AtSVP      : LQGLDIEELQQLEKALETGLTRVIETKSEKIMSEISDLQRKGMQLVDENKRLRQQGTQLEEENERLGMQICQNVHAHGGAESENAAVYEEGQSSESITNAGNSTGAPVDSESSDT : 230
BcSVP      : LQGLNIEELQQLEKALESGLTRVIETKSEKIMNEISVLQRKGMQLVDENKRLRQQGTQLEEENERLGQQIYQNVHERYGGGESENIAVYEEGHSSESITNAGNSTGAPVDSESSD : 230
IbMADS3    : LQGMSIEELQQLERSLETGLSRVIEKKGEKLMKEINELQCKGNNLMEEKERLTQQVMAISNGQRVTAVINSLNMLNEEGLSSESITNVCNSTSPPQDYDDSSDTSLKLGLPY--- : 227
PsSVP      : LQGMNVEQLQELERSLETGIGRVIENKGEKLMMEIQELQFKGRQLMEENLRLRRHVTGMMNNGKIMGGVECENVVIEEGQSSESITNVYNSTGPPQDYES---------------- : 215
JOINTLESS  : LQGLNIEELQQLERSLETGISRVIEPKGDKLMREINQLQCKGNHLMEENEKLRQQVMEISNNNNNNNGYREAGVVIFEPENGFNNNNNEDGQSSESVTNPCNSIDPPPQDDDSS : 230
             6QG6 6E2LQqLE4sLE GL rV6E K eKiM EI  LQ  gmqImeEn 4L qqv

                  240       *       260
MdJOINTLES : ------------------------------------ :   -
MdJa1      : ------------------------------------ :   -
MdJa2      : ------------------------------------ :   -
MdJb       : ------------------------------------ :   -
AtSVP      : SLRLGLPYGG------------------------- : 240
BcSVP      : ISLRLGLPYGG------------------------- : 241
IbMADS3    : ------------------------------------ :   -
PsSVP      : ------------------------------------ :   -
JOINTLESS  : DTSLKLGLATLLRLKRSKARCGYFCMLLEEGEKKK : 265
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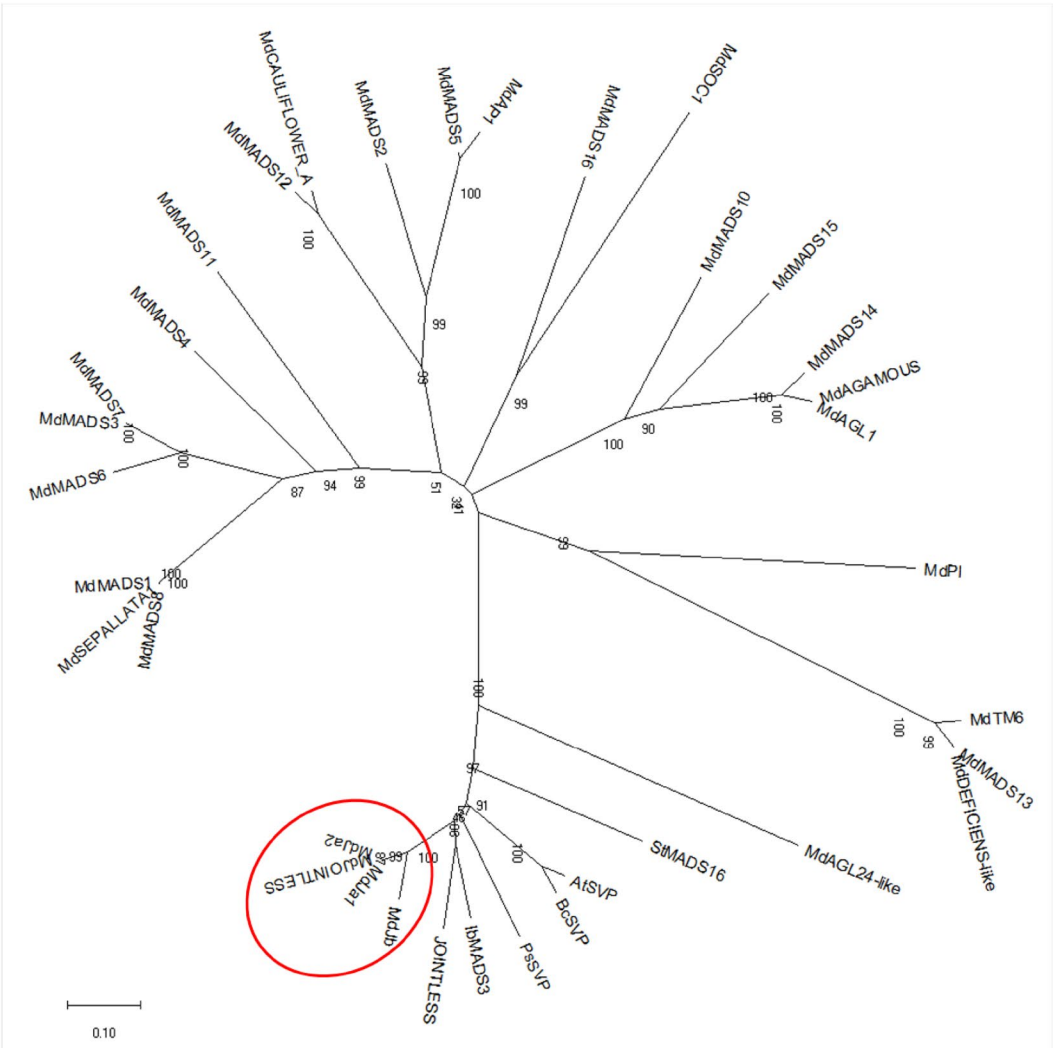
B



◂**Fig. 4** Multiple alignment (**a**) and phylogenetic analysis (**b**) of the deduced amino acid sequence of *MdJOINTLESS* with other MADS-box protein homologs. **a** Comparison of *MdJOINTLESS* with *SVP* from *Brassica campestris* (DQ922944), *IbMADS3* from *Ipomoea batatas* (AB054255), *SVP* from *Arabidopsis thaliana* (NP_179840), *SVP* from *Pisum sativum* (AY830919), and *JOINTLESS* from *Solanum lycopersicum* (AF275345). Dark boxes indicate amino acid identity or similarity between all six sequences, whereas grey boxes indicate amino acid identity or similarity among any four sequences. **b** An unrooted tree showing the phylogenetic relationships between *MdJOINTLESS* and MADS-box proteins from apple (*Malus*×*domestica* Borkh.) and other plants, including: *StMADS16* (AF008651) from *Solanum tuberosum*; *MdMADS1* (AAC25922), *MdMADS2* (AAC83170), *MdMADS3* (AAD51422), *MdMADS4* (AAD51423), *MdMADS5* (CAA04321), *MdMADS6* (CAA04322), *MdMADS7* (CAA04323), *MdMADS8* (CAA04919), *MdMADS10* (CAA04324), *MdMADS11* (CAA04325), *MdMADS12* (CAC86183), *MdMADS13* (CAC80856), *MdMADS14* (CAC80857), *MdMADS15* (CAC80858), *MdMADS16* (AB370212), *MdTM6* (AB081093), *MdPI* (CAC28021), *MdAGAMOUS* (AAQ03090), *MdAP1* (AAL61543), and *MdSOC1* (DQ887181). The phylogenetic tree was generated in MEGA X using the neighbour-joining method with the *P*-distance amino acid substitution model and 1000 bootstrap replicates

data suggest that apple genome has *J* homologs. Although 'Saika' is a triploid apple, it seems to be common that low-copy-number of *MdJ* is in diploid apple cultivars. Quantification of expression levels of *MdJ* gene was performed through quantitative real-time PCR analysis in CP and LP of self-abscising apple (Fig. 5). Based on normalization of gene expression to that of *MdActin*, *MdJ* was expressed higher in CP than in lateral fruit at FB. *MdJ* was detectable in both pedicels at 10 DAFB and its expression levels were lower than those at FB. However, the relative expression of *MdJ* was higher in CP than in LP (Fig. 5). In LP, the expression of *MdJ* showed similar level from FB to 10 DAFB, while, in CP, the expression level at 10 DAFB was the half of that at FB. According to the quantitative real-time polymerase chain reaction (RT-qPCR) result, *MdJ* might not be involved in formation of AZ in apple. The highest expression level of *MdJ* was found actually in CPs, although its expression should have up-regulated in LPs.

Phylogenetic analysis with *MdMADS* and MADS-box genes from other crops

MADS-box genes contribute to reproductive development by regulating flowering timing and floral organogenesis (Theissen and Saedler 2001; Weigel 1995). However, several genes are also involved in vegetative development, including the regulation of the AZ (Liu et al. 2014; Mao et al. 2000; Nakano et al. 2012). The MADS-box genes in pedicel transcriptome from 'Saika' were compared to the *MdMADS* genes reported from apple genome sequencing project (Velasco et al. 2010) (Fig. 6).

The *SlMBP21, MC*-like genes that are involved in pedicel AZ formation in tomato were discovered in pedicel transcriptome. Considering only MADS-box genes that were specific to the central or lateral fruitlets, except those that were expressed at the same time, the central fruitlets had a high expression of genes belonging to *AG*, *AP2* groups and the *MdMADS* genes which belong to *AP1*, *SEP*, *AP3*, and *AG* groups were highly expressed in lateral fruitlets. *AP1* family contained *MC* gene of tomato and the similar contigs to *SlMBP21* gene were included in *SEPALLATA* family. The *SEPALLATA*-like genes were significantly upregulated in pedicel as follows: MDP0000326390, MDP0000366022, MDP0000370413, MDP0000300752, and MDP0000326906. The following *MC* homologs were also discovered: MDP0000269921, MDP0000013331, MDP0000289836 and MDP0000132738.

Discussion

Abscission is progressed by four steps: (1) differentiation of abscission zone, (2) excess of the threshold to abscission-promoting signals during the pre-abscission stage, (3) activation of abscission, and (4) suturing abscission zone in the proximal region (Nakano et al. 2013; Patterson 2001). In step 1, the differentiation of AZ is initiated by transcription factors and other regulators. In tomato, *J* (Mao et al. 2000) forms protein complexes to develop the AZ by interacting with other MADS-box genes, *MC* (Nakano et al. 2012) and *SEP* (Liu et al. 2014). The important point is that the AZ was formed in step 1, but abscission process was not advanced yet.

In step 2, The *AUX/IAA* genes which related to auxin signal transduction may act as the trigger to initiate abscission process. The *AXR3* gene, a member of *AUX/IAA* transcription factor family, delayed shedding of floral organs by interfering with auxin signaling at the AZ (Basu et al. 2013). Meir et al. (2006) showed that expression levels of *Mj-Aux/IAA1* and *Mj-Aux/IAA2* were repressed by removal of IAA sources in the AZ of *Mirabilis jalapa*. Microarray analysis of the transcriptome in the AZ of tomato flowers showed that *AUX/IAA* genes were down-regulated early after the depletion of auxin and that early modified expression of these genes might mediate auxin regulation of ethylene sensitivity in the AZ (Meir et al. 2010).

In step 3, cell wall degrading and modifying enzymes that expressed by ethylene were activated in the separation layers of AZ (Belfield et al. 2005; Cho and Cosgrove 2000). The combined function of enzymes, such as cellulase, polygalacturonase, expansin, xyloglucan endohydrolase and endotransglycosylase, could modify cell wall and finally detach organ.

In step 4, late phase of abscission, the genes belong to lignin biosynthesis pathway were significantly up-regulated by ethylene exclusively in the AZ. The role of lignin

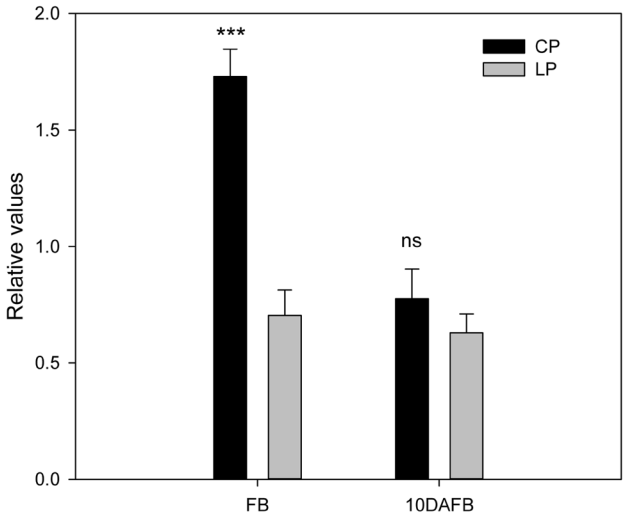


Fig. 5 Relative expression levels of *MdJOINTLESS* gene between central pedicel (CP) and lateral pedicel (LP) of self-abscising apple 'Saika' from full bloom (FB) to 10 days after full bloom (DAFB). Data were normalized using housekeeping *MdActin* gene. Vertical bars are the standard errors of the means. Statistical analysis was performed using Student's *T* test (***$p < 0.001$, NS, not significant). The primers and probe sequences are listed in "Materials and methods"

deposition may be associated with the generation of protective layers at the tissues remaining in the plant, such as receptacle or peduncle, during the last step of abscission process (Van Nocker 2009) and lignification could facilitate cell wall separation mechanically (Merelo et al. 2017). On the contrary, repression of lignin biosynthesis may result in grain shattering in rice (Yoon et al. 2017).

In tomato, AZ exists in fruit pedicel and *J* has an important role for AZ development. This gene belongs to the *SVP* clade in MADS-box gene family which are generally expressed in vegetative organs and are involved in floral transition and bud dormancy. The *SVP* genes differ in function compared with other MADS-box genes that are involved in floral organ determination. Based on phylogenetic analyses with MADS-box genes derived from pedicel transcriptome of self-abscising apple, many MADS-box genes were also expressed in both central and lateral pedicels which did not belong to the *SVP* clade. Some genes were estimated to be similar with *SEP* (*SlMBP21*) and *MC* gene in tomato and most MADS-box genes belonged to AGAMOUS-LIKE (AGL) gene clade. To identify the *J* homolog that played a similar role in apple, fruit pedicels were used from self-abscising apples during early abscission. Based on multiple sequence alignment and phylogenetic analysis, *MdJ* was identified as *J* homolog in apple, while we could find only

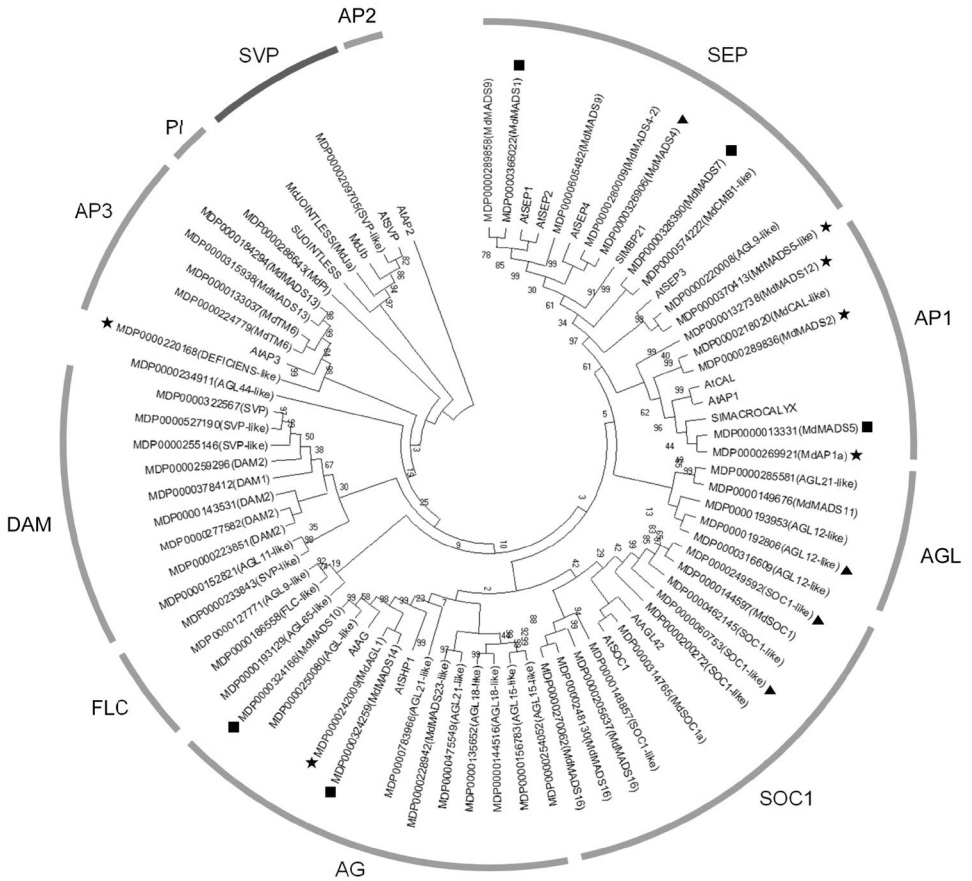


Fig. 6 Phylogenetic tree of apple MADS-box genes, using the neighbour-joining method. The MADS-box gene or transcripts which expressed in central pedicel, lateral pedicel, and both pedicels marked with triangle, star, and square shape, respectively. The MADS-box genes, except apple transcripts, have the abbreviation of scientific name in front of their gene name: At, *Arabidopsis thaliana*; Sl, *Solanum lycopersicum*; Md, *Malus*×*domestica*. The abbreviation for MADS-box clades are as follows: *SEP* SEPALLATA, *AP1* APETALA1, *AGL* AGAMOUS-LIKE GENE, *SOC1* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1, *AG* AGAMOUS, *FLC* FLOWERING LOCUS C, *DAM* DORMANCY ASSOCIATED MADS-BOX, *AP3* APETALA3, *PI* PISTILLATA, *SVP* SHORT VEGETATIVE PHASE, *AP2* APETALA2

one *J* homolog using RACE method. According to our DNA blot analyses (data not shown), 'Saika' has three *J* homologs in its genome. In addition, three *SVP* homologs were reported to exist in 'Golden Delicious' genome (Velasco et al. 2010), and three homologs, *MdJa1*, *MdJa2* and *MdJb*, were also found in 'Fuji' pedicels (Nakano et al. 2015). These *J* homologs and our *MdJ* sequence had the same length and were highly homologous to each other. Even our *MdJ* sequence was 100% identical to *MdJa2*. Ectopic expression of the *MdJa* restored *J*-deficient tomato mutant and *MdJa* physically interacted with *MC* and *SlMBP21* of tomato (Nakano et al. 2015). The amino acid sequences of tomato *J*, apple *MdJ* and *Arabidopsis SVP* showed significant similarity each other. The *SVP* orthologs of other crops repressed the development of floral organs in the meristem and the floral differentiation during bud dormancy (Li et al. 2009; Yamane et al. 2011; Wu et al. 2012). The *SVP* gene might suppress cell differentiation in pedicels AZs. Apple pedicel AZs showed a group of small cells that were arranged transversely to adjacent cells and arrested in an undifferentiated state (Fig. 2). In tomato, these undifferentiated cells were not discovered in *j* mutant pedicels (Mao et al. 2000; Nakano et al. 2015). These results suggest that *MdJ* may play a fundamental role in repressing cell differentiation in pedicel AZ and prohibiting transition to reproductive phase.

The expression pattern of *MdJ* was investigated in central and lateral pedicles by real-time PCR. At FB, the expression of *MdJ* in central pedicel was more than twice as high as that of lateral pedicels. After 10 days from FB, the expression was still higher in CP than LP, but it did not show much difference between central and lateral pedicels. The expression of *MdJ* was higher in CF from FB than that from 10 DAFB and this reason is seemed that *MdJ* may be involved not only in the formation of AZ but also in other function. *MdJ* has three homologs in apple genome, which are likely to be functionally diversified. Ectopic overexpression of *MdJa* showed a strong apical dominance phenotype in transgenic 'Royal Gala' apple, constraining lateral shoot outgrowth during the first year (Wu et al. 2017). At the second year, the bud break was significantly delayed and leaf senescence was not generated in transgenic lines (Wu et al. 2017). According to these results, *MdJ* affects apical dominance of shoot in meristem being expressed within pedicels of fruit clusters in self-abscising apple.

Bangerth (2000) explained that early abscission of young fruits results from correlative dominance between fruit pedicels by competing polar basipetal IAA transport (Fig. 7). This dominance may be due not only to earlier pollination and fertilization but also to the position of pedicels in one cluster (Celton et al. 2014). In fruit cluster, central fruit (central position) exerts a dominance over lateral fruits enclosed with central pedicel. The auxin gradient may be formed between central pedicel and lateral pedicels, and affects gene

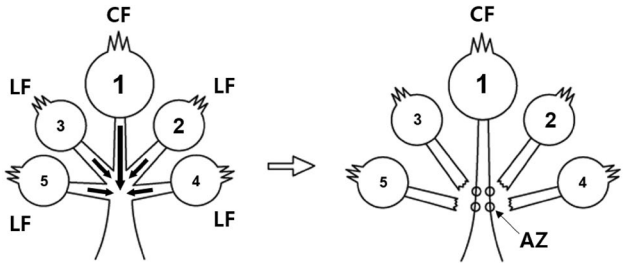


Fig. 7 Correlative dominance in apple fruit cluster. Numbers inside the fruit represent the hierarchical ranking according to the order of flowering, pollination and fertilization. The arrowheads indicate the competition of auxin transport between central fruit (CF) and lateral fruits (LF)

expression related to auxin signal transduction. According to our previous research, the competition of auxin transport between CP and LP has resulted in determining the survival of pedicels elevating expression of *IAA3/SHY2* gene in the dominant CP and *IAA14/SLR* in the inferior LPs according to hierarchical ranking (Heo et al. 2016). *IAA3/SHY2* homolog was differentially expressed in CP and *IAA14/SLR* homolog was strongly up-regulated in LP from self-abscising apple (Heo et al. 2016). The function of *Aux/IAA* transcription factor genes in pedicel AZ of apple is not yet clear, while these genes have been well investigated to play a key role in development of lateral root. *IAA3/SHY2* was reported to participate in auxin-mediated meristem differentiation (Koren et al. 2013) or lateral root emergence (Lavenus et al. 2013), which mediate auxin signal transduction. The mechanism of lateral root emergence allows *IAA14/SLR* signaling cascade to trigger *IDA-HAE-HSL2* signaling cascade in *Arabidopsis* (Okushima et al. 2007). Chrysanthemum gene *CmANR1*, homologous to MADS-box gene *AtANR1*, plays a key role in the development of lateral root by accumulating auxin directly in lateral root (Sun et al. 2018). The MADS-box gene, *AGL21* regulates auxin accumulation in lateral root primordia and lateral root by enhancing local auxin biosynthesis and stimulates lateral root initiation and growth in *Arabidopsis* (Yu et al. 2014).

Apple MADS-box genes, expressed in pedicel AZ, *MdJ*, *MdMC*, and *MdSEP* homologs reported from Nakano et al. (2015) in different apple cultivar, were validated in this study. To confirm the function of *MdMADS* in the apple, transgenic apples are necessary with antisense suppression and sense complementation of *MdJ, MdMC*, and *MdSEP* genes to wild type and *j* mutant tomato. *MdJ* has the potential to be involved in formation of the AZ and auxin transport in step 1 of abscission process. The self-abscission might result in hierarchical ranking of auxin transport between pedicels according to the order of flowering and location of pedicels. The phase transition to vegetative growth occurred in accordance with the ranks based on the expression degree

of *MdJ* between fruit pedicels. *MdJ* is thought to induce rapid growth of the shoot as well as vegetative phase transition. It seems that *MdJ* not only contributes to auxin gradation but also influences auxin signaling and transport in fruit pedicel of self-abscising apple. In future studies, the interaction between *AUX/IAA* TFs and MADS-box genes, including *MdJ*, needs to be investigated. Furthermore, additional experiments are needed to determine if the MdMADS proteins which are homologous with J, MC, and SlMBP21 are physically interact with each other, resulting in forming heterodimer.

Acknowledgements This work was supported by the education, research and student guidance grant funded by Jeju National University in 2019.

Compliance with ethical standards

Conflict of interest Seong Heo and Yong Suk Chung declare that they have no conflict of interest.

Research involving human and animal participants This article does not contain any studies with human subjects or animals performed by any of the authors.

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