Relationship between \textit{MdMADS11} Gene Expression and Juvenility in Apple

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Fruit trees have a long juvenile period after germination before flowering and fruit set. In spite of its horticultural importance, the physiological and molecular mechanisms of juvenility in fruit tree development are unknown; therefore, the relationship between the expression of the \textit{AGAMOUS-LIKE6} (\textit{AGL6}) homolog and juvenility was investigated in apple (\textit{Malus domestica} Borkh.). The results of sequence alignment, the phylogenetic tree, and Southern blot analysis suggested that \textit{MdMADS11} is an \textit{AGL6} ortholog in apple. An increase in the abundance of the mRNA of \textit{MdMADS11}, which promotes flowering, corresponded to changes in leaf area and serration with the transition from the juvenile to adult phase in apple seedlings. In contrast, the abundance of mRNA of \textit{MdJOINTLESS}, which may suppress flowering, decreased with the phase transition. Expression analysis of adult apple trees suggested that \textit{MdMADS11} also plays a role in seasonal flower initiation and floral organ formation in the adult phase. The results provide new insight into the molecular mechanism of the phase transition between juvenile and adult fruit trees.

Key Words: AGAMOUS-LIKE6, apple, flowering, juvenility, MdMADS11.

Introduction

Fruit trees, which are perennial woody plants, have a long juvenile period after germination before flowering and fruit set. Because fruit quality cannot be evaluated in the juvenile phase, the length of the juvenile period of fruit trees is a problem that needs to be addressed in fruit tree breeding. Juvenility is generally strong in young seedlings and becomes weaker with age. Parts of a single tree can show different levels of juvenility, that is, juvenility is strong in the proximal parts of trunks and branches, but weaker towards the distal parts (Poethig, 1990, 2003). There are some cultivation techniques that shorten the juvenile period, such as using dwarfing stocks and top-grafting, in fruit tree production; however, the physiological and molecular mechanisms of juvenility in fruit tree development are not known.

Homologs of some flowering-related genes from the model plant Arabidopsis have recently been studied in fruit trees. The overexpression of the apple homologs of \textit{LEAFY} and \textit{APETALA1} (\textit{API}), which are floral meristem identity genes in Arabidopsis, promotes flowering in Arabidopsis (Kotoda et al., 2002; Wada et al., 2002). The overexpression of Arabidopsis \textit{LEAFY} and \textit{API} promotes flowering in citrus (Pena et al., 2001). These observations suggest that the function of genes in the flowering pathway is conserved between Arabidopsis and fruit trees; therefore, other Arabidopsis flowering-related genes were studied in several tree species.

The overexpression of \textit{TERMINAL FLOWER1} (\textit{TFL1}) homologs from citrus, apple, and grapevine retard flowering in Arabidopsis (Carmona et al., 2007; Kotoda and Wada, 2005; Pillitteri et al., 2004). \textit{FLOWERING LOCUS T} (\textit{FT}) from Arabidopsis is a flowering timing gene and is well known as a candidate of florigen. \textit{FT} homologs have been isolated and studied from several tree species, including citrus, poplar, and grapevine (Carmona et al., 2007; Endo et al., 2009; Matsuda et al., 2009). Flowering is induced within a year in poplars that have been transformed with the poplar \textit{FT} homolog (Hsu et al., 2006). These findings are based on floral meristem identity-related genes and flowering timing genes that are downstream components of the flowering pathway. Juvenility genes are mostly located upstream of these genes that directly control flowering.

Carlsbecker et al. (2004) reported that \textit{DAL1} is related to juvenility in the conifer Norway spruce. \textit{DAL1} is...
considered to be the conifer homolog of Arabidopsis AGAMOUS-LIKE6 (AGL6). Because only a few reports have shown the role of AGL6 in Arabidopsis with respect to bract development and floral patterning (Koo et al., 2010), there is no comprehensive understanding of the developmental roles of AGL6. DAL1 expression increases with age in Norway spruce and promotes flowering in Arabidopsis (Carlsbecker et al., 2004). The overexpression of the orchid AGL6 homolog also promotes flowering and increases the expression of floral integrators FT and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) in Arabidopsis (Fan et al., 2007; Hsu et al., 2003). The results of these studies suggest that AGL6 is an upstream component of the flowering pathway; however, there is a lack of information on AGL6 homologs in fruit trees, even though their developmental roles need to be clarified to understand the molecular mechanism of juvenility and their contribution to fruit tree production. Therefore, we investigated the roles of the apple AGL6 homolog MdMADS11 (Yao et al., 1999).

Materials and Methods

Plant materials

Leaves, buds, and flowers were sampled at 10:00 from 13-year-old apple trees (adult tree, Malus domestica
‘Fuji,’ grafted onto M.9, *M. prunifolia*) and 8-year-old apple seedlings of a cross between ‘Fuji’ and ‘Himekami’ that flowered for 3 seasons.

**Southern blot analysis**

Genomic DNA extraction from the leaves of the adult trees and Southern blot analysis were performed as described in Moriguchi et al. (2006). The cDNA fragment corresponding to the coding region of *MdMADS11* (Fig. 1a), except for the MADS domain, was used as a probe after labeling it using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Germany). Nucleotide sequences used for the primers were 5’-CGATACCAACGTTGCTCCTT-3’ and 5’-TTCAGAGACCATCCTTGG-3’.

**RNA analysis**

As shown in Figure 2a, an approximately 2 m long trunk of the seedling was divided into three equal parts and leaves were sampled from the branches in each part for expression analysis of the transition from juvenile to adult phase in June or July. Buds and leaves were sampled from the adult trees on May 22, June 5, 25, July 13, and August 2 to analyze seasonal changes in *MdMADS11* and *MdFT1* expressions, respectively. Apical buds from vegetative shoots and spurs were used as leaf buds and flower buds, respectively. Floral organs were sampled from the adult trees in early May for expression analysis.

RNA extractions were performed using the CTAB method (Moriguchi et al., 2006) or using an RNeasy Plant Mini Kit (Qiagen, Germany). Removal of genomic DNA from the RNA sample and reverse transcription were performed using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed by the method of Nishio et al. (2010) with the exception of using a QuantiTect SYBR Green PCR Kit (Qiagen). PCR was also performed with the cDNA, electrophoresed in
an agarose gel, and stained with ethidium bromide. The primer sets were designed as follows: 5'-CTTGGAGACCTCAACAAAGCA-3' and 5'-ACCAGTCAAGCTCCTTGGAA-3' for *MdMADS11*; 5'-GCACTGGATCTGTCTCTC-3' and 5'-ATATCGGCATCGCAGAGAAC-3' for *MdJOINTLESS*; 5'-CTAGAGCTGATATTGGTGGA-3' and 5'-ACGACAGACACCGGTAATCC-3' for *MdFT1*; and 5'-ACTCGCATTGCTAGGGTTTC-3' and 5'-ATACCACTGGAGACTG-3' for *MdACTIN*.

A sequence comparison of *MdJOINTLESS* and *MdJOINTLESS*-like proteins has not been published, although the nucleotide sequence of *MdJOINTLESS* cDNA is available (accession number DQ402055). The phylogenetic tree and sequence alignment are shown in Figures 1b and 3, respectively. The amino acid sequence of *MdJOINTLESS* showed 72% identity to that of *Arabidopsis* SVP and was included in the SVP family in the phylogenetic tree.

### Transformation of Arabidopsis with *MdMADS11* cDNA

The coding region of *MdMADS11* was amplified by PCR and cloned using Mighty Cloning Kit (Takara, Japan). The insert was replaced with *GUS* between a cauliflower mosaic virus 35S promoter and nopaline synthase terminator in pBI121. The following primers were used for the amplification of *MdMADS11* by PCR: 5'-GCGGATCCAAAAAGAAGGGAATGAG-3' and 5'-GGCGATATCTTATGATGCTGCAATAAG-3'.

3' *Arabidopsis* ecotype Columbia was transformed using *Agrobacterium tumefaciens* with pBI121 binary vector containing *MdMADS11* cDNA. Seeds (T2) were harvested from T1 plants grown on media containing kanamycin and were used for the observations. *Arabidopsis* plants were grown in vermiculite : perlite (1 : 1) at 25°C under an 18-h photoperiod. The introduction of *MdMADS11* cDNA into the *Arabidopsis* genome and its expression were tested by PCR using genomic DNA and RT-PCR, respectively.

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**Fig. 2.** Changes in leaf morphology with the phase transition. (a) Putative phases in apple seedlings. A (juvenile phase), B, and C (transition phase) have no flower. D (adult phase) is a mixture of branches with flowers and without flowers, while E and F (adult phase) have flowers. Typical leaves (b), leaf serration (c), leaf area (d), and SPAD value (e) in each part (A–F) of apple seedlings in early July. Error bars indicate the standard error of the mean (n = 10). Values in (d) and (e) are significantly different between positions at P < 0.05 after ANOVA.

**Fig. 3.** Amino acid sequence alignment of *MdJOINTLESS* and SVP homologs from various species. Species and their accession numbers are described in the legend of Figure 1.
Results

Characterization of MdMADS11 cDNA

The MdMADS11 cDNA used in this study was cloned by PCR using primers designed from the nucleotide sequence of MdMADS11 reported by Yao et al. (1999). Our deduced amino acid sequence of MdMADS11 was the same as that of Yao et al. (1999), although two nucleotides were found to be different between the two sequences. A phylogenetic tree based on the amino acid sequences of AGL6, SEPALLATA (SEP), AP1, FLOWERING LOCUS C (FLC), and SHORT VEGETATIVE PHASE (SVP)-like proteins showed that MdMADS11 is included in the AGL6 family (Fig. 1b). The amino acid sequence of MdMADS11 showed 63% and 65% identity to those of Arabidopsis AGL6 and Norway spruce DAL1, respectively (Fig. 1a). AGL6-I and AGL6-II motifs for transcription activation were conserved in MdMADS11 (Fig. 1a).

The DNA probes containing the MADS domain can detect several bands in Southern blot analysis because of its high sequence identity. Thus, the coding region, except for the MADS domain, was used as a probe for Southern blot analysis. A single band was observed in each digestion, although a weak band was observed in EcoRI digestion (Fig. 1c). An EcoRI site was found in the genomic DNA sequence corresponding to the probe region (data not shown). The AGL6 homologs in orchids and most Poaceae grasses are reportedly single-copy genes (Hsu et al., 2003; Reinheimer and Kellogg, 2009). An AGL6 gene family has not been reported in other similar studies (Fan et al., 2007; Ma et al., 1990). Therefore, it is likely that MdMADS11 exists in the apple genome as a single-copy gene.

The timing of flowering was assessed by examining the number of rosette leaves at flowering in the T2 generation of 35S::MdMADS11 lines (homozygous and heterozygous) that were selected by PCR (data not shown). Although there were 8 or more rosette leaves at flowering in the control line, there were 7 or less in some plants of all 35S::MdMADS11 lines (Fig. 1d).

Several abnormal and sterile flowers were observed in 35S::MdMADS11 plants showing the early flowering phenotype (Fig. 1e). Although the early- and normal-flowering phenotypes in 35S::MdMADS11 lines were considered to result from homozygous and heterozygous genotypes, respectively, it was difficult to identify the homozygous plants because of their extremely early-flowering phenotype and sterility.

Changes in leaf morphology and MdMADS11 gene expression with the phase transition

Leaves from position A to position F in Figure 2a were used along with the transition from the juvenile to adult phase. Leaf area gradually increased with the transition while serration decreased (Fig. 2b, c, d). The chlorophyll content expressed as the Soil & Plant Analyzer Development (SPAD) value measured by SPAD-502 (Minolta, Japan) also increased with the transition (Fig. 2e). The abundance of MdMADS11 mRNA gradually increased with the phase transition, while that of MdJOINTLESS mRNA gradually decreased (Fig. 4a). The abundance of MdMADS11 mRNA was higher in adult trees than in 3-year-old seedlings (Fig. 4b).

Fig. 4. Changes in MdMADS11 and MdJOINTLESS gene expression with the phase transition. (a) Gene expression was analyzed by RT-PCR in A–F described in Figure 2a. Total RNA was prepared for the expression analysis of MdMADS11 and MdJOINTLESS in late June and early July, respectively. Relative expression was determined in triplicate measurements in three independent biological replicates. The relative expression levels are normalized against MdACTIN with standard errors and shown by taking the maximum level of the transcripts as 1.0. The values for each gene are significantly different between positions at P < 0.05 after ANOVA. (b) Expression of MdMADS11 in leaves of 3-year-old seedlings (Juvenile) and adult trees (Adult) in late June. PCR was performed with cDNA prepared from total RNA extracted from the leaves, electrophoresed in an agarose gel, and stained with ethidium bromide. The number of PCR cycles was 42 for MdMADS11 and 26 for MdACTIN as a control.
MdMADS11 gene expression in buds and floral organs

To investigate the role of MdMADS11 in the adult phase, seasonal changes in the abundance of MdMADS11 mRNA were determined in the buds of adult trees (Fig. 5a). The abundance in leaf buds was high from early May to late June and subsequently decreased in mid-July, whereas that in flower buds did not decrease. Although MdFT expression was previously described by Hättasch et al. (2008) and Kotoda et al. (2010), it was determined in this study to provide a reference for flowering induction and initiation (Fig. 5c). Because FT protein may not be a mobile signal in apple (Tränkner et al., 2010), MdFT1 expression was shown in flower buds. MdFT1 expression increased in mid-July, suggesting that flowering induction and initiation occur between late June and mid-July, as described by Kotoda et al. (2010). The abundance of MdMADS11 mRNA was found to be high in sepals, petals, and ovaries with receptacles, but low and not detectable in stamens and styles, respectively (Fig. 5b).

Discussion

A sequence comparison has not been performed on the amino acid sequences of MdMADS11, although Yao et al. (1999) have cloned MdMADS11 cDNA. MdMADS11 contains MADS-, I-, K-, and C-domains, which is similar to that observed in several other MADS-box proteins in plants (De Bodt et al., 2003). Whereas MADS- and K-domains are generally well conserved among MADS-box proteins, the C-domain is a highly variable region and may be important for functional specificity (Kaufmann et al., 2005). AGL6 contains AGL6-I and AGL6-II motifs, which may play a key role in the transcription activation of target genes (Ohmori et al., 2009). These AGL6-specific motifs were well conserved in MdMADS11. The results from sequence alignment, the phylogenetic tree, and Southern blot analysis suggest that MdMADS11 is an AGL6 ortholog in apple. MdMADS11 promoted flowering in the transgenic Arabidopsis.

The transition from the juvenile to adult phase causes morphological changes other than flower bud formation. In rosaceous fruit trees, leaves are deeply serrated in the juvenile phase and this is reduced during the transition to the adult phase (Hirata, 1983; Katoh and Ooishi, 2002). Leaf area increases with age in tree species (Greenwood et al., 2008; Poethig, 2003) and this increase is independent of environmental factors such as light conditions (Day et al., 2001). Leaf area is also larger in the distal parts in the adult phase than in the proximal parts in the juvenile phase in herbaceous plants (Poethig, 1988). Leaf thickness and chlorophyll content appear to increase during the transition from the juvenile to adult phase in tree species (Greenwood, 1995; Greenwood et al., 1989; Poethig, 1990). These changes may increase the photosynthetic rate per leaf during the phase transition, resulting in higher assimilated productivity per tree in adult trees (Apple et al., 2002). In this study, leaf area and the SPAD value increased from A to F in Figure 2d and e, suggesting an increase in the photosynthetic rate per leaf. The reduction of leaf serration in this study also corresponded to that found in previous studies (Hirata, 1983; Katoh and Ooishi,

Fig. 5. MdMADS11 gene expression in buds (a) and floral organs (b) and MdFT1 gene expression in flower buds (c). (a) Seasonal changes in the abundance of MdMADS11 mRNA were determined by RT-PCR in the leaf buds (closed circles) and flower buds (open circles) of adult trees. The values are significantly different and not significantly different between dates in leaf buds and flower buds, respectively, at P < 0.05 after ANOVA. (b) The abundance of MdMADS11 mRNA was also determined in sepals, petals, stamens, styles, and ovaries with receptacles (OvRe) in the adult trees. The values are significantly different between organs at P < 0.05 after ANOVA. In (a) and (b), relative expression was determined in triplicate measurements in three independent biological replicates. The relative expression levels are normalized against MdACTIN with standard errors and shown by taking the maximum level of the transcripts as 1.0. (c) A seasonal change in MdFT1 gene expression was determined by RT-PCR using gene specific primers. PCR was performed with cDNA prepared from total RNA extracted from the flower buds, electrophoresed in an agarose gel, and stained with ethidium bromide. The number of PCR cycles was 27 for MdFT1 and 23 for MdACTIN as a control.
an increase in the abundance of \textit{MdMADS11} mRNA corresponded well to that in the leaf area, suggesting that \textit{MdMADS11} expression increases with the transition from the juvenile to adult phase (Fig. 4a). This observation is further supported by the fact that the expression of \textit{MdMADS11} in adult trees is higher than that in young seedlings without flowers (Fig. 4b). Carlsbecker et al. (2004) reported that \textit{DAL1} might have a regulatory role in the juvenile-to-adult transition in Norway spruce because of an increase in \textit{DAL1} expression with age; therefore, \textit{AGL6} homologs could be related to the phase transition in tree species. A decrease in the abundance of \textit{MdMADS11} mRNA also corresponded to the juvenile-to-adult transition in the opposite way, because \textit{MdMADS11} is the apple homolog of \textit{SVP}, which suppresses flowering (Jang et al., 2009). Although \textit{MdMADS11} expression is shown for comparison to \textit{MdMADS11} expression, \textit{MdMADS11} may also be important for phase transition, and therefore future studies should investigate its gene family and molecular function.

\textit{FT} homologs may play a role in seasonal flower initiation in the adult phase of poplar, citrus, and apple, because they are highly expressed during the seasonal flowering period (Hättasch et al., 2008; Hsu et al., 2006; Kotoda et al., 2010; Nishikawa et al., 2007). Because there is a lack of information on the relationship between \textit{AGL6} homologs and seasonal flower initiation, seasonal changes in \textit{MdMADS11} gene expression were analyzed in adult apple trees. A rapid decrease in the abundance of \textit{MdMADS11} mRNA in leaf buds with putative seasonal flower initiation is interesting because \textit{MdMADS11} may promote flowering; however, unlike that described for \textit{MdFT} (Hättasch et al., 2008; Kotoda et al., 2010) no peak in flower buds was detected in \textit{MdMADS11} expression, suggesting that other factors are involved in the control of seasonal flower initiation. In addition, \textit{AGL6} homologs may play a role in floral organ formation, as described in orchids, petunia, and rice (Hsu et al., 2006; Ohmori et al., 2009; Rijpkema et al., 2009). The abundance of \textit{MdMADS11} mRNA in some floral organs was high compared to that in leaves and buds, and abnormal flowers were found in \textit{Arabidopsis} transformed with \textit{MdMADS11}, suggesting that \textit{MdMADS11} may also play a role in floral organ formation. \textit{MdMADS11} mRNA was not detected in floral organs (data not shown), suggesting that \textit{MdMADS11} and \textit{MdMADS11} have contrasting roles.

The expression patterns of the \textit{AGL6} homolog, \textit{MdMADS11}, and \textit{SVP} homolog, \textit{MdMADS11}, corresponded to the juvenile-to-adult transition in apple seedlings. Because \textit{AGL6} and \textit{SVP} are located upstream of floral integrators, \textit{FT}, \textit{TWIN SISTER OF FT}, and \textit{SOC1} (Hsu et al., 2003; Jung et al., 2009), \textit{MdMADS11} and \textit{MdMADS11}, which may promote and repress the transition of vegetative to reproductive growth, respectively, are likely to be involved in the regulation of juvenility upstream of the floral integrators in apple. Although the floral meristem identity-related genes \textit{API}, \textit{LEAFY}, and \textit{TFL1} homologs, and the downstream components of the flowering pathway \textit{FT} and \textit{SOC1} homologs have been studied in fruit trees, the central component of the regulation of juvenility could be located upstream of those genes; therefore, our results provide new insight into the molecular mechanism of phase transition from juvenile to adult in fruit trees.

\textbf{Literature Cited}


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