DOI: 10.1007/s10535-016-0637-z BIOLOGIA PLANTARUM 60 (4): 667-676, 2016

Functional analysis of the *Malus domestica MdHMGR2* gene promoter in transgenic *Arabidopsis thaliana*

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Abstract

The first rate-limiting enzyme of the mevalonate pathway during isoprenoid biosynthesis is 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR). In this study, the expression pattern of the *MdHMGR2* gene in *Malus domestica* suggests that *MdHMGR2* was expressed in a tissue-specific manner and was significantly induced by ethephon (ETH), indoleacetic acid (IAA), methyl jasmonate (MeJA), and salicylic acid (SA). The *MdHMGR2* promoter was isolated, sequenced, and analyzed through bioinformatics tools, and the results suggest the presence of various putative *cis*-acting elements responsive to different hormones. Activity of β-glucuronidase (GUS) driven by the full length *MdHMGR2* promoter and its 5′deletion fragments was detected in transgenic *Arabidopsis thaliana*. A strong GUS activity was observed in seedlings, roots, newly growing true leaves, anthers, and stigmas in transgenic *Arabidopsis* containing the full *MdHMGR2* promoter. The results indicate that a region from -1050 to -827 was crucial for promoter activity. In addition, the *MdHMGR2* promoter was induced in response to ETH, IAA, MeJA, and SA. The analysis suggests that an ethylene-responsive element in the region from -1050 to -1005 was required for the ethylene inducibility.

Additional key words: abscisic acid, apple, *cis*-acting element, ethylene, gibberellin, β-glucuronidase, indoleacetic acid, methyl jasmonate, salicylic acid.

Introduction

Isoprenoids are the largest and most structurally diverse group of natural products (Sacchettini *et al*. 1997, Verpoorte and Alfermann 2000). In higher plants, they play important roles as hormones - abscisic acid (ABA), gibberellins (GAs), cytokinins, and brassinosteroids, as quinones in electron carriers, as components of membrane architecture (sterols), as photosynthetic pigments (carotenoids and a side chain of chlorophyll), in subcellular targeting and regulation (prenylation of proteins), as plant defense and communication (monoterpenes, sesquiterpenes, and diterpenes), and as valuable commercial products (artemisinin, taxol, pigments, flavors, rubbers, and vitamins) (Harborne and Tomas-Barberan 1991, Cieza *et al*. 2003). In addition, many isoprenoids are necessary for quality in fruit crops (Nieuwenhuizen *et al*. 2013).

In higher plants, the mevalonate (MVA) pathway in the cytosol is one of the major pathways to generate isoprenoids, which provides precursors for sesquiterpenes (C_{15}), triterpenes (C_{30}), and polyterpenes (Ha *et al*. 2003). The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is known as rate-limiting enzyme of the MVA pathway in catalyzing conversion of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) to MVA. Plant HMGR is encoded by a small multigene family, where isoforms exhibit different spatial and temporal gene expression patterns. In ginseng plants, two isoforms of *HMGR* (*PgHMGR1* and *PgHMGR2*) have been identified and show distinct expression patterns with a high sequence identity. Expressions of *PgHMGR1* and *PgHMGR2*

Submitted 10 June 2015, *last revision* 8 December 2015, *accepted* 18 January 2016.

Abbreviations: ABA - abscisic acid; ERE - ethylene-responsive element; ETH - ethephon; GA - gibberellin; GUS - β-glucuronidase; HMGR - 3-hydroxy-3-methylglutaryl-CoA reductase; IAA - indoleacetic acid; MeJA - methyl jasmonate; MVA - mevalonate; qPCR - quantitative polymerase chain reaction; 5'-RACE - 5'-rapid amplification of cDNA ends; SA - salicylic acid.

Acknowledgments: This work was supported by the National Natural Science Foundation of China, Grant No. 31170255.

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are relatively low in seeds, leaves, stems, and flowers, and higher in petioles of seedlings and roots, whereas *PgHMGR1* and *PgHMGR2* show different expression patterns in roots of different ages (Kim *et al*. 2014). Moreover, HMGR has an important impact on plant development and growth. The *Hmg1* mutant in *Arabidopsis thaliana* exhibits dwarfism, early senescence, and male sterility, and the *hmg1 hmg2* double mutant is lethal during male gametophyte development (Suzuki *et al*. 2004, 2009).

Plant HMGR has a critical regulatory role in the MVA pathway not only for normal plant development, but also for responses to various physiological and environmental stimuli such as phytohormones, radiation, wounding, pathogen attack, feedback mechanisms, and endogenous protein factors (Stermer *et al*. 1994, Enjuto *et al*. 1995, Korth *et al*. 2000, Leivar *et al*. 2011). The *HMGR1* gene spatial expression pattern is modulated by irradiance in *Arabidopsis thaliana* (Learned and Conolly 1997). Potato *HMGR* isogenes are activated differentially by wounding or pathogens (Yang *et al*. 1991). *Medicago truncatula MtHMGR1* has been demonstrated to be essential for nodule development (Kevei *et al*. 2007).

In *Malus domestica*, three *HMGR* genes (*HMGR1*, *HMGR2*, and *HMGR3*) have been isolated to date. The *MdHMGR1* gene shows a constitutive expression pattern during low temperature storage of apples, whereas the *MdHMGR2* gene is more sensitive to developmental stimuli and ethylene, being suppressed completely by an ethylene action inhibitor 1-methylcyclopropene (Rupasinghe *et al*. 2001, Pechous and Whitaker 2002).

The *HMGR* promoter has been studied in several plant species. In *Arabidopsis*, an alternative promoter is used by the *HMGR1* gene to generate two mRNAs (*HMGR1L* and *HMGR1S*; Lumbreras *et al*. 1995). Spatial and temporal expression patterns driven by the *AtHMGR2* promoter in transgenic tobacco are restricted to meristematic (root tip and shoot apex) and floral tissues (Enjuto *et al*. 1995). Beta-glucuronidase (GUS) activity driven by the *MaHMGR1* promoter in transgenic tobacco is increased by ABA treatment and in dark-grown seedlings (Jain *et al*. 2000). The GATA and SORLIP motifs were identified as being involved in regulation of radiation-mediated expression of *PkHMGR* in *Picrorhiza kurroa* (Kawoosa *et al*. 2013). However, studies related to regulatory stimuli of the *HMGR* promoter have mainly concentrated on radiation, pathogens, and wounding, with little work focusing on responses to phytohormones.

In this study, the *MdHMGR2* gene promoter was isolated from *Malus domestica* and analyzed by *PlantCARE* and *PLACE* databases. Spatial and temporal expression patterns of *GUS* driven by the *MdHMGR2* promoter and its deletion fragments were identified in transgenic *Arabidopsis*. With the aim to elucidate the regulation of *MdHMGR2* by phytohormones, *MdHMGR2* expression in apple fruits and GUS activity in transgenic *Arabidopsis* were examined in response to various hormone treatments.

Materials and methods

Different tissues used in this study were obtained from five-year-old apple (*Malus domestica* Borkh. cv. White winter pearmain) trees. *Arabidopsis thaliana* L. ecotype Col-0 was grown in a growth chamber maintained at a temperature of 22 °C, an air humidity of 65 %, a 16-h photoperiod, and an irradiance of 100 μmol m^{-2} s^{-1}. For hormone treatment, mature apple fruits were first used to make fruit discs of a consistent size and uniform thickness by using a hole punch. Then the fruit discs were placed on filter papers soaked with carrier solvents, which were 0.1 % (v/v) ethanol (control) with 100 μM ABA, 500 mg dm^{-3} ethephon (ETH), 400 μM gibberellin (GA), 100 μM indoleacetic acid (IAA), 100 μM methyl jasmonate (MeJA), or 500 μM salicylic acid (SA) solutions. For control and hormone treatments, samples were taken after 0, 1, 2, 4, and 6 h to analyze *MdHMGR2* gene expression.

Expression of *MdHMGR2* was measured by real time quantitative polymerase chain reaction (qPCR). The total RNA was prepared using an RNA isolation kit (*RNAplant Plus* reagent, *TianGen*, Beijing, China). Primers used for amplification of the *MdHMGR2* gene and endogenous reference *Actin* gene are shown in Table 1. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to calculate relative gene expression levels.

The promoter region of the *MdHMGR2* gene was amplified from the genomic DNA of apple leaves using the specific primers pHMGR2F/pHMGR2R (Table 1). The primers pHMGR2F and pHMGR2R were designed based on the sequence upstream of the *MdHMGR2* encoding region from the sequence MDC015298.78 in the apple genome database (http://www.rosaceae.org/). The PCR products were cloned into the pMD-18T vector and sequenced. The nucleotide sequence of the *MdHMGR2* promoter has been deposited in *GenBank* under accession number KR908782. Putative functional *cis*-acting elements of the *MdHMGR2* promoter were identified by the *PlantCARE* database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al*. 2002) and *PLACE* database (http://www.dna.affrc.go.jp/PLACE/signalup.html) (Higo *et al*. 1999).

The transcription start site of the *MdHMGR2* gene was identified by 5′-rapid amplification of cDNA ends (5′ RACE) using a *SMART RACE* cDNA amplification kit (*Clontech*, USA). The total RNA was isolated from apple leaves. Gene-specific primers *HMGR2GSP* and *HMGR2NGSP* listed in Table 1 were designed according

to the *MdHMGR2* cDNA sequence (*GenBank*: EF580921.1). The second round PCR products were cloned into the pMD-18T vector and 15 positive clones were sequenced to determine the transcription start site.

A series of promoter fragments of *MdHMGR2* were generated by PCR amplification using primers (six forward primers F1 - F6 and a common reverse primer R1) listed in Table 1. The amplified promoter fragments of 1 180 bp (-1 050/+130, with the transcription start site designated as +1), 1 135 bp (-1005/+130), 957 bp (-827/+130), 726 bp (-596/+130), 305 bp (-175/+130), and 231 bp (–101/+130) were digested with *Hind*III and *Xba*I and directly cloned into the corresponding sites of the plant binary vector pBI121-GUS replacing the CaMV35S promoter. The several recombinant plasmids were designated as *MdHMGR2P-D1*, *MdHMGR2P-D2*, *MdHMGR2P-D3*, *MdHMGR2P-D4*, *MdHMGR2P-D5*, and *MdHMGR2P-D6*. All constructs were verified by sequencing and then transferred into the *Agrobacterium tumefaciens* L. strain GV3101 by using the freeze-thaw method.

Agrobacterium-mediated transformation of *Arabidopsis* was performed using the floral dip method (Clough and Bent 1998). The T3 homozygous transformants of three independent lines for each construct were used for promoter analysis.

To explore the response of the transgenic *Arabidopsis* plants to hormones, the 10-d-old *MdHMGR2P-D1*, *MdHMGR2P-D2*, *MdHMGR2P-D3*, *MdHMGR2P-D4*, *MdHMGR2P-D5*, and *MdHMGR2P-D6* seedlings were transferred into six-well plates containing half strength Murashige and Skoog medium supplemented with 500 mg dm^{-3} ETH, 100 μM IAA, 100 μM MeJA, or 500 μM SA for 3 h. The control treatment consisted of the half strength MS medium alone. Beta-glucuronidase activity in the transgenic plants was analyzed by histochemical staining and fluorometric assays (Jefferson *et al*. 1987). For histochemical staining, tissues of *Arabidopsis* plants at different stages of development were incubated in a GUS staining solution at 37 °C for 12 - 18 h in the dark followed by removing chlorophyll with 95 % (v/v) ethanol. At least six independent transgenic plants were observed by histochemical staining assay for each construct. For fluorometric assays, frozen tissue samples were ground in an extraction buffer. The supernatant was collected after centrifugation at 12 000 *g* and 4 °C for 10 min. The total protein content was determined using the Bradford method with bovine serum albumin as standard. Fluorescence was detected based on a standard curve for 4-methylumbelliferone using a fluorescence spectrophotometer *F-4500* (*Hitachi*, Tokyo, Japan).

Table 1. Primer sequences used in this study.

Primer name	Primer sequence (5′-3′)
qRTHMGR2F	CCCACGATCCCTGGAAAGACG
qRTHMGR2R	GCGTGGTTCCGACGACGACA
ActinF	CAGTGGTCGTACAACTGGTAT
ActinR	AGGTAGCTCATAGCTCTTCTC
pHMGR2F	CTCTCTTCCTCACTCCTTCTATTT
pHMGR2R	GGACTGGACGAAGTCAATGC
HMGR2GSP	TCCAGCGGGAGCCCCACCAGCGAACT
HMGR2NGSP	ATGACGGCGTGGTTCCGACGACGACAG
F1	CCCAAGCTTTCACTCCTTCTATTTCAAAAACAA
F2	CCCAAGCTTTACTTTGTGTGTGGGCA
F3	CCCAAGCTTTGGTTCTTTTCGTTAACTCCG
F4	CCCAAGCTTTGACAGAGTTGATGGAAAAGATT
F5	CCCAAGCTTGACAAGCCCAGACA
F6	CCCAAGCTTCCAACTCACCCCGCACTC
R1	GCTCTAGAAGAAAGGGGGGAATTAGGTAAT

Results

To examine *MdHMGR2* expression patterns in different apple tissues, the total RNA was isolated from young tender leaves, old leaves, stems, fruits, and roots of apple. The highest expression of the *MdHMGR2* gene was exhibited in roots followed by fruits and stems, and the lowest expression in old leaves. The expression of *MdHMGR2* in old leaves was much lower compared to tender leaves (Fig. 1).

In order to examine whether the *MdHMGR2* gene was induced by phytohormones, 0.1 % (v/v) ethanol (control), ABA, ETH, GA, IAA, MeJA, and SA were applied to fruit discs and samples were collected at different time points (Fig. 2). The treatments with ETH, IAA, MeJA, and SA significantly increased the amount of the

MdHMGR2 mRNA with a maximum at 4 h (7.5-, 6.7-, 20-, and 3.6-fold, respectively). However, *MdHMGR2* expression was only slightly enhanced by ABA and GA with a maximum again at 4 h. Thus, *MdHMGR2* was expressed in a tissue-specific manner and was significantly induced by ETH, IAA, MeJA, and SA.

The *MdHMGR2* was further studied by promoter analysis to understand the mechanism of its regulation. Initially, a 1 509 bp upstream DNA sequence including the putative promoter of the *MdHMGR2* gene was obtained from the apple genomic DNA. An adenine 156 bp upstream of the translation initiation codon ATG was identified as the transcription start site by 5′ RACE. The *MdHMGR2* promoter sequence was analyzed for putative *cis*-acting regulatory elements using the *PlantCARE* and *PLACE* databases. A putative TATA box (TATAAAA) was detected at a position -35 to -29, which is consistent with the previous data for a TATA box 32 ± 7 upstream from the transcription start site (Joshi 1987). A number of potential *cis*-acting elements associated with hormone-related responses were identified in the *MdHMGR2* promoter. Several light-responsive elements and tissue-specific elements were also found in the *MdHMGR2* promoter (Table 2).

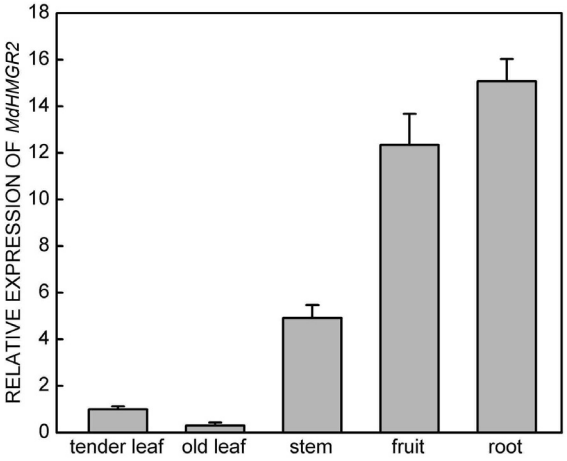

Fig. 1. Relative expression of the *MdHMGR2* gene in different apple tissues (tender leaves, old leaves, stems, fruits, and roots) determined by real time quantitative PCR analysis. The *error bars* represent standard deviations (SDs) of three replicates.

Table 2. Putative *cis*-acting elements in the *MdHMGR2* promoter.

Regulatory element	Sequence	Location	Function
ABRE	ACGT	-145	abscisic acid-responsive element
CE3	CGCGTGTC	-163	abscisic acid-responsive element
AuxRE	TGTCTC	-689, -177	auxin-responsive element
NTBBF1ARROLB	ACTTTA	-673	auxin-responsive element
ERE	AWTTCAAA	-1039	ethylene-responsive element
P-box	CCTTTT	-30, -10	gibberellin-responsive element
T/G-box	AACGTG	-574	methyl jasmonate-responsive motif
WBOXATNPR1	TTGAC	-795, -605, -232, -561,	binding sites for salicylic acid-induced WRKY transcription factors
GATABOX	GATA	-807, -312, -265	light-responsive element
SORLIP1AT	GCCAC	-778, -623, -358	light-responsive element
POLLEN1LELAT52	AGAAA	-730, -479, -413, -369, -353	pollen specific expression element
ROOTMOTIFTAPOX1	ATATT	-748	root-specific motif

To identify significant regulatory regions in the *MdHMGR2* promoter, the full *MdHMGR2* promoter and its five 5′ deletion promoter fragments were fused to the GUS reporter gene. Since the period to generate transgenic apples was too long, the constructs were introduced into *Arabidopsis thaliana*. A schematic representation of the *MdHMGR2* promoter-GUS constructs is shown in Fig. 3.

Fluorometric analysis reveals that the *MdHMGR2P-D1* plants showed the highest GUS activity whereas GUS expressions in the *MdHMGR2P-D2* and *MdHMGR2P-D3* plants were 33 and 79 % lower, respectively, compared to those in the *MdHMGR2P-D1* plants (Fig. 6). The loss of a region from -1050 to -827 induced a sharp decrease in GUS activity. In addition, GUS expression was undetectable in the *MdHMGR2P-D4*, *MdHMGR2P-D5*, and *MdHMGR2P-D6* transgenic plants in any tissue and at any developmental stage. Taken together, the results illustrate that the region from -1050 to -827 was critical for promoter activity.

As mentioned above, the *MdHMGR2* gene was strongly induced by the treatments with ETH, IAA, MeJA, and SA. The treatment with ETH, IAA, MeJA, and SA also increased GUS activity in the *MdHMGR2P-D1* transgenic plants. To identify a regulatory region for hormone-responsive expression in the *MdHMGR2* promoter, the *MdHMGR2P-D1*, *MdHMGR2P-D2*, *MdHMGR2P-D3*, *MdHMGR2P-D4*, *MdHMGR2P-D5*, and *MdHMGR2P-D6* transgenic plants were treated with ETH, IAA, MeJA, and SA. There was no GUS signal detected by histochemical staining in the *MdHMGR2P-D4*, *MdHMGR2P-D5*, and *MdHMGR2P-D6* transgenic plants

treated with the hormones. Further, fluorometric analyses were performed in the *MdHMGR2P-D1*, *MdHMGR2P-D2*, and *MdHMGR2P-D3* transgenic plants treated with the hormones. The *MdHMGR2P-D1* plants treated with ETH showed a 2.5-fold increase in GUS activity whereas no GUS activity induction was found in the *MdHMGR2P-D2* and *MdHMGR2P-D3* plants treated with ETH (Fig. 7*A*). The *MdHMGR2P-D1*, *MdHMGR2P-D2*, and *MdHMGR2P-D3* plants treated with IAA increased GUS activity 3.2-, 2.8-, and 2.6-fold, respectively (Fig. 7*B*). Methyl jasmonate induced 3.4-, 3.2-, and 2.8-fold increases in GUS activity in the *MdHMGR2P-D1*, *MdHMGR2P-D2*, and *MdHMGR2P-D3* plants, respectively (Fig. 7*C*). The *MdHMGR2P-D1*,

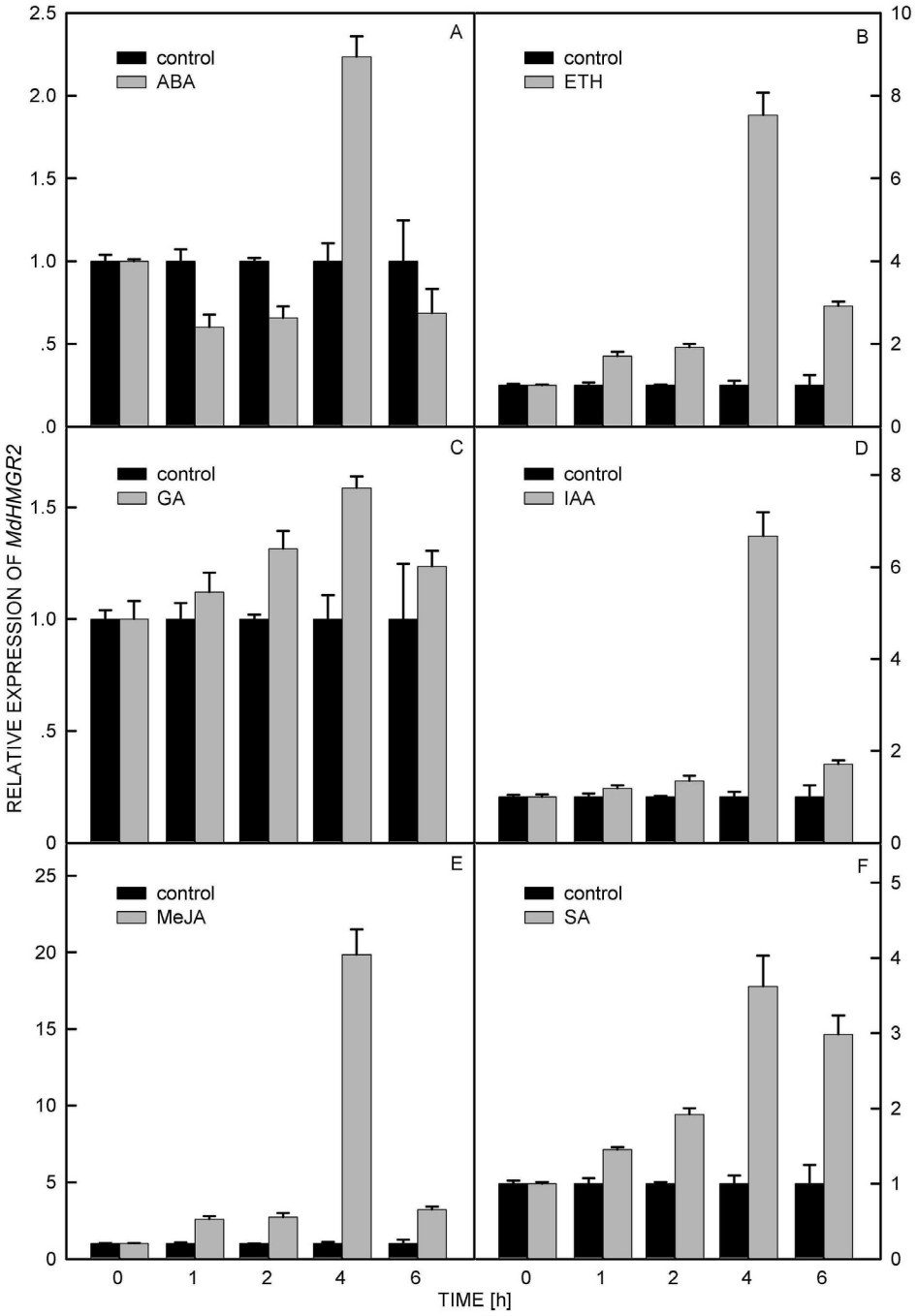

Fig. 2. Relative expressions of the *MdHMGR2* gene in apple fruit discs treated with hormones abscisic acid (ABA), ethephon (ETH), gibberellin (GA), indoleacetic acid (IAA), methyl jasmonate (MeJA), and salicylic acid (SA) and collected at 0, 1, 2, 4, and 6 h after the start of the treatments. The expression of the control at different times was set to 1. The *error bars* represent SDs of three replicates.

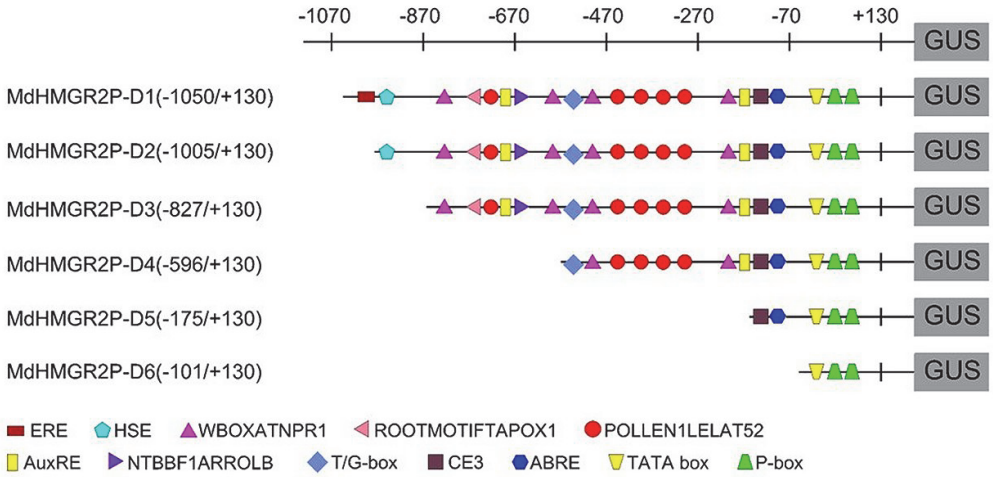

Fig. 3. A schematic representation of *MdHMGR2* promoter-*GUS* constructs. The *MdHMGR2* promoter fragments of different lengths were inserted into the pBI121 vector containing the *GUS* reporter gene. Putative functional *cis*-acting elements of the *MdHMGR2* promoter were identified by the *PlantCARE* and *PLACE* databases. The putative *cis*-acting elements are represented by different symbols. The numbers indicate the nucleotide position of each *MdHMGR2* promoter fragment.

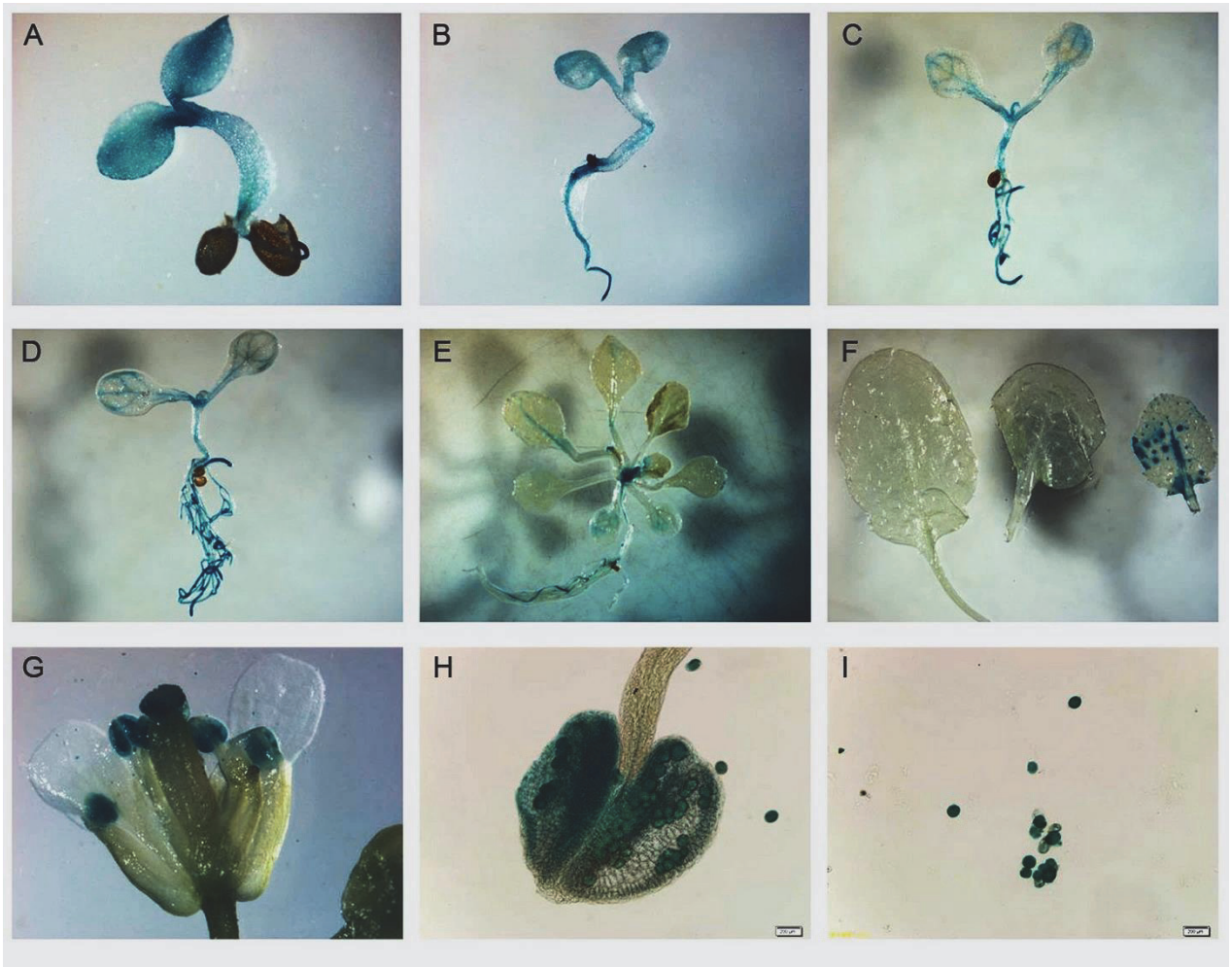

Fig. 4. A histochemical analysis of GUS activity in *MdHMGR2P-D1* transgenic *Arabidopsis* plants. *A* - 3-d-old seedling, *B* - 5-d-old seedling, *C* - 10-d-old seedling, *D* - 15-d-old seedling, *E* - 30-d-old plant, *F* - different developmental stages of leaves from a 50-d-old plant (*from left to right*: mature leaf, unexpanded leaf, newly growing true leaf), *G* - flower, *H* - anther, *I* - pollens.

MdHMGR2P-D2, and *MdHMGR2P-D3* plants were all responsive to the SA treatment, resulting in 2.4-, 2.3-, and 2.1-fold increases of GUS activity, respectively (Fig. 7*D*). These results suggest that the ethylene responsive elements (ERFs) were likely present in the *MdHMGR2* promoter region from -1050 to -1005.

In order to investigate the expression pattern of the *MdHMGR2* gene during the entire life cycle, the GUS expression pattern driven by the full *MdHMGR2* promoter was analyzed in the *MdHMGR2P-D1* transgenic *Arabidopsis* plants, and a strong GUS expression was detected in roots, hypocotyls, cotyledons, and newly growing true leaves of the 3-, 5-, 10-, and 15-d-old seedlings (Fig. 4*A-D*). A low GUS activity was observed in roots and newly growing true leaves of the 30-d-old transgenic plants (Fig. 4*E*). In the 50-d-old *Arabidopsis*, GUS activity was located in newly growing true leaves, whereas no GUS staining was observed in mature leaf tissue (Fig. 4*F*). During the stage of inflorescence development, the GUS signal was found only in anthers and stigmas (Fig. 4*G*). Moreover, pollens and anther walls had GUS staining in anthers (Fig. 4*H*,*I*). There was no GUS expression in petals, pistils, and siliques.

The results indicate that the *MdHMGR2P-D2* transgenic plants shared similar temporal and spatial GUS expression profiles as the *MdHMGR2P-D1* transgenic plants, whereas in the *MdHMGR2P-D3* plants, a faint GUS activity was located in cotyledons, newly growing true leaves, and a lower part of roots of the 3-, 5-, 10-, and 15-d-old seedlings (Fig. 5*A-D*). In the 30-and 50-d-old transgenic plants, newly growing true leaves had unique GUS staining (Fig. 5*E*,*F*). Expression of GUS was also detectable in anther walls, pollens, and stigmas of flowers (Fig. 5*G-I*).

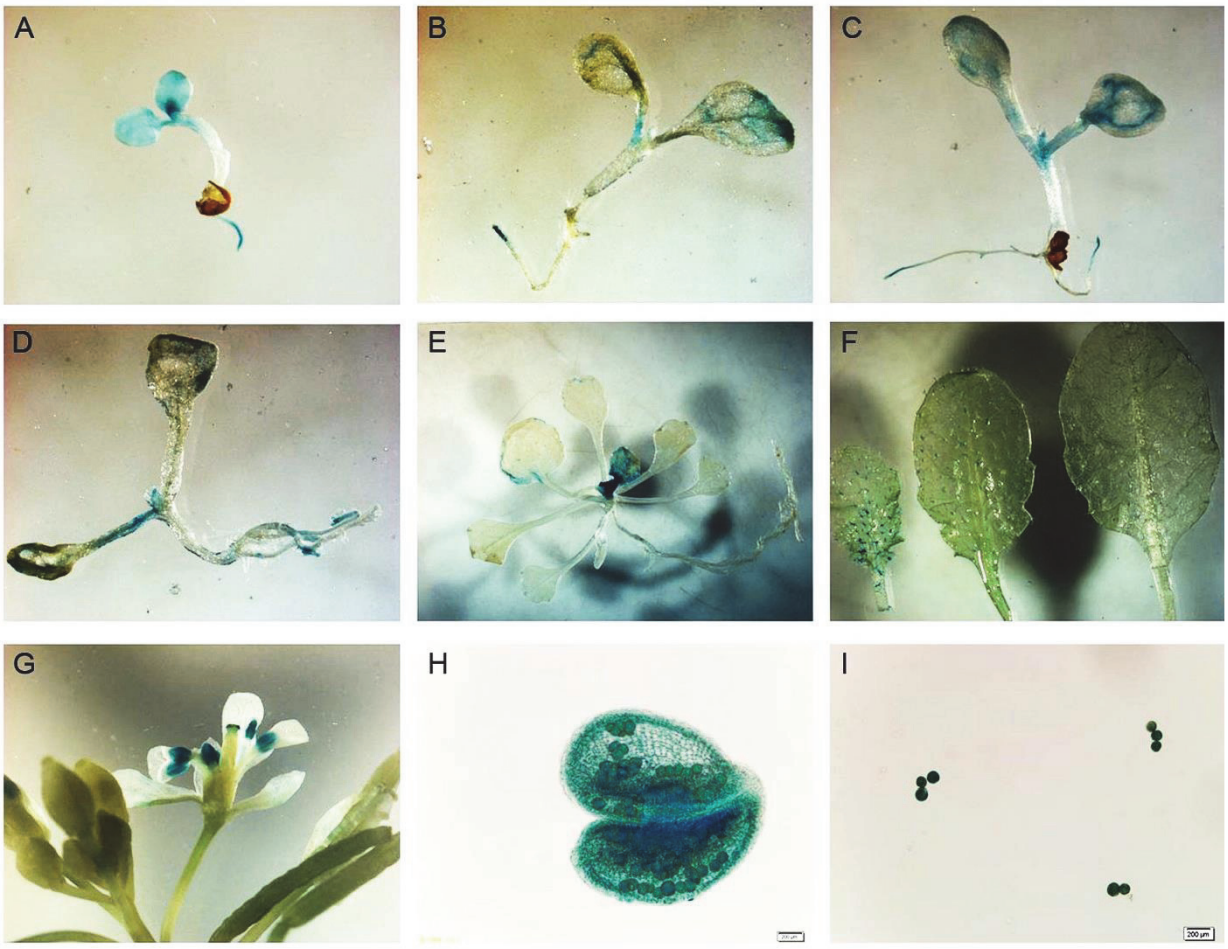

Fig. 5. A histochemical analysis of GUS activity in *MdHMGR2P-D3* transgenic *Arabidopsis* plants. *A* - 3-d-old seedling, *B* - 5-d-old seedling, *C* - 10-d-old seedling, *D* - 15-d-old seedling, *E* - 30-d-old plant, *F* - different developmental stages of leaves from a 50-d-old plant (*from left to right*: newly growing true leaf, unexpanded leaf, mature leaf), *G* - flower, *H* - anther, *I* - pollens.

Discussion

The real time qPCR results suggest that expression of *MdHMGR2* occurred in a tissue-specific manner with high levels in roots and fruits, and lower in stems and leaves. Furthermore, analysis of the *MdHMGR2* promoter in the transgenic *Arabidopsis* shows that GUS activity was detected in whole seedlings, roots, and newly growing true leaves, indicating that GUS expression was observed during the first stages of development especially in actively dividing tissues. It is worth noting that GUS activity was also found in anthers and stigmas. Consistent with these results, various root-specific motifs, pollen-specific expression elements, and fruit-specific enhancer elements were found to be present in the *MdHMGR2* promoter region by using bioinformatics analysis.

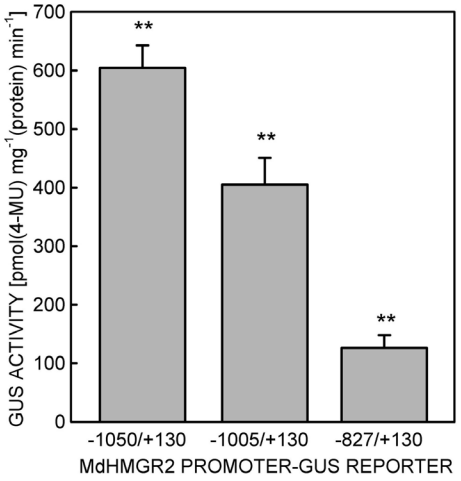

Fig. 6. Activity of GUS in 10-d-old *MdHMGR2P-D1* (-1050/+130), *MdHMGR2P-D2* (-1005/+130), and *MdHMGR2P-D3* (-827/+130) transgenic *Arabidopsis* seedlings analyzed fluorometrically. The *error bars* represent SDs of three replicates. Significant differences were assessed by Fisher's least significant difference test (**$P < 0.01$, *$P < 0.05$).

Although many reports have revealed that a minimal promoter should provide activity, the *HMGR* promoter is unusual. Previous studies have reported that deletion to nucleotide -503 results in an almost complete loss of expression in the *AtHMGR2* promoter (Enjuto *et al*. 1995) whereas in the *PkHMGR* promoter, smaller fragments starting from -651 cannot drive a detectable level of GUS expression in any of the tissues of *Arabidopsis* (Kawoosa *et al*. 2013). Consistent with these researches, deletion to nucleotide -827 resulted in the sharp decrease in GUS activity whereas deletion to nucleotide -596 led to the complete loss of expression in the *MdHMGR2* promoter. These results indicate that the promoter region from -1050 to -827 was important in maintaining maximal promoter activity and possessed important regulatory elements.

MdHMGR2 expression in apple fruits was dramatically induced after the treatments with ETH, IAA, MeJA, and SA. Previous studies have reported that *WsHMGR* expression is significantly elevated in response to SA and MeJA, similarly to expression of the *SmHMGR* gene (Liao 2009, Akhtar *et al*. 2013). The full *MdHMGR2* promoter was strongly induced in response to ETH, IAA, MeJA, and SA in accordance with *MdHMGR2* gene expression in apple fruits. No GUS signal was detected in the *MdHMGR2P-D4*, *MdHMGR2P-D5*, and *MdHMGR2P-D6* transgenic plants treated with the hormones, which further indicates that the promoter region from -1050 to -827 was necessary for promoter activity. GUS activity was induced by ETH in the *MdHMGR2P-D1* plants**.** In contrast, no GUS activity was responded to ETH in the *MdHMGR2P-D2* and *MdHMGR2P-D3* plants. The results indicate that *cis*-acting elements required for ethylene induction may be located in the region from -1050 to -1005. It is worth noting that there was only one ERE element in this region that has been identified as an ethylene-responsive element in the promoter region of ethylene-induced genes (Itzhaki *et al*. 1994, Tapia *et al*. 2005). The presence of this ERE element may be responsible for ethylene induction in the *MdHMGR2* promoter.

GUS activity was always markedly induced by the IAA, MeJA, and SA treatments in the *MdHMGR2P-D1*, *MdHMGR2P-D2*, and *MdHMGR2P-D3* plants. These results indicate that *cis*-acting elements that respond to IAA, MeJA, and SA may be located in the region from -827 to +130. Two AuxRE elements (-689 to -684 and -177 to -172), both known as *cis*-acting elements related to auxin induction, were found in the region from -827 to +1. Previous research has indicated that a single copy of an AuxRE element is sufficient to confer auxin responsiveness to reporter genes (Inukai *et al*. 2005). Moreover, one NTBBF1ARROLB element was located at the position from -673 to -668. The NTBBF1ARROLB elements are identified as the target sequence of the protein NtBBF1, which is essential for expression of *rolB* in apical meristems and is involved in a response to auxin (Baumann *et al*. 1999). There is a T/G-box at the position from -574 to -569, which is found in the *LAP* promoter for JA induction and is specially recognized by JAMYC2 and JAMYC10 proteins (Boter *et al*. 2004). The promoter region from -827 to +1 possessed 4 WBOXATNPR1 elements at the positions -795 to -791, -605 to -601, -561 to -557, and -232 to -228. A number of WRKY proteins have been shown to bind to the TTGACC/T sequence (W-box) related to pathogen and SA induction (Yu *et al*. 2001). Above all, the region from -827 to +130 was essential for responses to IAA, MeJA, and SA.

In summary, the comparative analysis of the *MdHMGR2* gene and its promoter demonstrates that *MdHMGR2* expression occurred in a spatio-temporal manner and was induced by ETH, IAA, MeJA, and SA.

Furthermore, the *MdHMGR2* promoter region from -1050 to -827 was found to contribute to promoter activity. The ERE element in the *MdHMGR2* promoter region from -1050 to -1005 was important for ethylene induction. Further experiments will be performed to obtain the exact elements involved in tissue specific expression and response to IAA, MeJA, and SA in the *MdHMGR2* promoter.

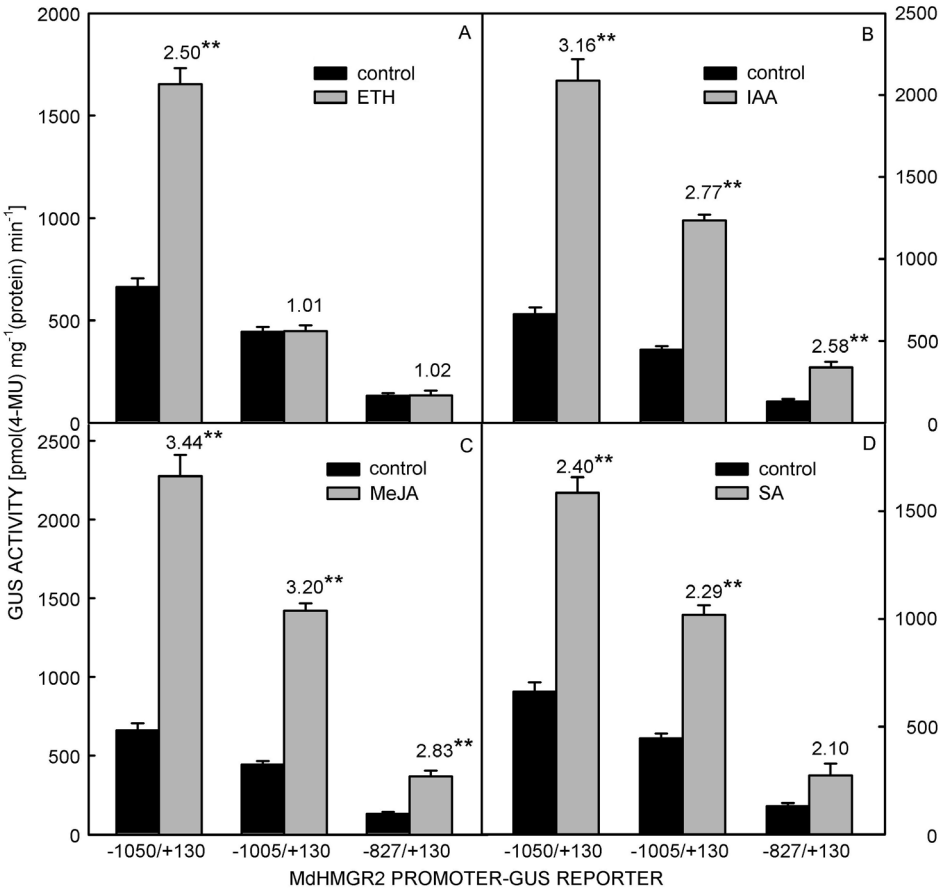

Fig. 7. Activity of GUS in 10-d-old *MdHMGR2P-D1* (-1050/+130), *MdHMGR2P-D2* (-1005/+130), and *MdHMGR2P-D3* (-827/+130) transgenic *Arabidopsis* seedlings treated with with 500 mg dm^{-3} ethephon (ETH, *A*), 100 μM indoleacetic acid (IAA, *B*), 100 μM methyl jasmonate (MeJA, *C*), or 500 μM salicylic acid (SA, *D*) for 3 h. The control treatment consisted of half strength Murashige and Skoog medium alone. The numbers over the *bars* represent fold increases of GUS activity in response to the hormone treatments *versus* the control treatment. The *error bars* represent SDs of three replicates. Significant differences between the control and treatment were assessed by the two-tailed paired *t*-test (**$P < 0.01$, *$P < 0.05$).

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