Novel promoters that induce specific transgene expression during the green to ripening stages of tomato fruit development

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Abstract

Fruit-specific promoters have been used as genetic engineering tools for studies on molecular mechanism of fruit development and advance in fruit quality and additional value by increasing functional component. Especially fruit-ripening specific promoters have been well utilized and studied in tomato; however, few studies have reported the development of promoters that act at fruit developing stages such as immature green and mature green periods. In this study, we report novel promoters for gene expression during the green to ripening stages of tomato fruit development. Genes specifically expressed at tomato fruit were selected using microarray data. Subsequent to confirmation of the expression of the selected 12 genes, upstream DNA fragments of the genes LA22CD07, Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at which specifically expressed at fruit were isolated from tomato genomic DNA as promoter regions. Isolated promoter regions were fused with the *GUS* gene and the resultant constructs were introduced into tomato by agrobacterium-mediated transformation for evaluation of promoter activity in tomato fruit. The two promoters of LA22CD07, and LesAffx.6852.1.S1_at showed strong activity in the fruit, weak activity in the flower and undetectable activity in other tissues. Unlike well-known fruit-ripening specific promoters, such as the E8 promoter, these promoters exhibited strong activity in green fruit in addition to red-ripening fruit, indicating that the promoters are suitable for transgene expression during green to ripening stages of tomato fruit development.

Keywords: *fruit-specific promoter, tomato, green stage, red stage, fruit development*

Key Message

Novel fruit specific promoters have been identified and are suitable for transgene expression during green to ripening stages of tomato fruit development.

Abbreviations: GUS, beta-D-glucuronidase gene

Introduction

The tomato (*Solanum lycopersicum*) is one of the major Solanaceae crops and one of the most widely eaten fruits in the world. Genetic engineering has been used in an effort to improve the quality of the tomato fruit (Butelli et al. 2009; Dharmapuri et al. 2002; Le et al. 2006; Lewinsohn 2001; Mollet et al. 2008; Rosati et al. 2000; Schijlen et al. 2006, 2007; Wang et al. 2008).

The tomato also serves as a vehicle for the production of useful proteins. For example, we reported the overexpression of the miraculin gene and the production of miraculin protein in the tomato fruit (Hirai et al. 2010; Hiwasa-Tanase et al. 2012; Sun et al. 2007; Yano et al. 2010). Chen et al. (2009) reported the production of thymosin alpha1, an immune booster that plays a role in the maturation, differentiation and function of T-cells, in the tomato fruit. Zhang et al. (2007) described the expression of human coagulation Factor IX in the tomato fruit.

The cauliflower mosaic virus 35S promoter (35S promoter) is a constitutive promoter that is widely used for the expression of foreign genes in higher plants. However, in some cases the 35S promoter is not suitable for gene expression because of the possibility that 35S promoter-driven constitutive gene expression could be damaging to plant growth and development.

To overcome the problem of the 35S promoter, tissue-specific promoters have been isolated. Fruit-specific promoters have been isolated as tools for fruit-specific gene expression. In the tomato, promoters from ethylene response genes, such as E8 and E4, have been well studied as fruit-specific promoters (Cordes et al. 1989; Coupe and Deikman 1997; Deikman et al. 1992, 1998; Deikman and Fischer 1988; Kneissl and Deikman 1996; Lincoln et al. 1987; Montgomery et al. 1993a; Xu et al. 1996). Polygalacturonase (Montgomery et al. 1993b; Nicholass et al. 1995) and lipoxygenase promoters (Beaudoin and Rothstein 1997) have also

been reported as fruit specific in the tomato. These classical promoters have been reported to act during the late ripening stage of fruit development. On the other hand, information of promoters that act at fruit expanding stage (immature green), mature green stage and throughout the developmental stage are much less common than the fruit-ripening specific types, although recently Estornell et al. (2009) reported some promoters driving gene expression preferentially in the fruit with different activity ranges.

Many promoter variations expand the capability of intended use depending on the purpose. Therefore, in this study we attempted to isolate novel fruit-specific promoters with different activity from classical promoters. We selected 12 genes which showed high expression in fruit tissues using microarray data obtained from tomato cultivar 'Micro-Tom', which has become a model plant of the Solanaceae family (Matsukura et al. 2008). Upon confirmation of the expression of the selected genes, cloning of the promoter regions, and the promoter analysis using *GUS* gene, we finally identified two promoters with fruit-specific activity. Unlike some classical fruit-specific promoters, these promoters were driven *GUS* gene expression throughout the fruit development in the green to ripening stages.

Materials and methods

Identification of candidate genes from microarray data

Tomato genes which show fruit-specific expression were selected by using gene expression data from following three sources; (i) a dataset available in MiBASE (old version, http://www.kazusa.or.jp/jsol/microtom/) using 'Micro-Tom' cDNA array produced by Japan Solanaceae genomics consortium (Yano et al., 2006), (ii) a dataset GSE19326 available in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/gds) (Ozaki et al. 2010), and (iii) datasets 'Wild type

tomato fruit development (set 1 and set 2)' available in Tomato Functional Genomics Database (http://ted.bti.cornell.edu/cgi-bin/TFGD/miame/home.cgi) (Alba et al. 2005). Sequences of LA15CA04, LA22CD07, LC09AH08, LC04DC11, LA12AA05, LA14AD08 and FB14DB02 were obtained from MiBASE (http://www.pgb.kazusa.or.jp/mibase/). Consensus sequences of unigenes from which Les.331.1.S1_at, Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at probes were designed were obtained from Affymetrix website (http://www.affymetrix.com). Consensus sequences of TC115787 and TC116003 were obtained from Dana-Farber Cancer Institute Tomato Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=tomato).

RNA isolation and Real-time PCR (RT-PCR) analysis

Total RNA was isolated from the leaves, flowers, stems, roots, and green and red fruits of 3-month-old 'Micro-Tom' plants using TRIzol® (Invitrogen, USA) according to the manufacturer's instructions. One microgram of total RNA from each sample was treated with RQ1 RNase-Free DNase (Promega, USA) and was used for first-strand cDNA synthesis with a poly-T primer and SuperScript II Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions.

The first-strand cDNA was subsequently used as a template for the expression analysis of the selected genes. RT-PCR reactions were performed with 25 to 30 cycles for the gene expression analysis using designed gene-specific primers (Table 1). After the PCR reaction, an equal volume of each amplified PCR product was subjected to electrophoresis on a 1% TAE agarose gel and was visualized using ethidium bromide.

Quantitative real-time PCR (qRT-PCR)

For the analysis of LA22CD07 and LesAffx.6852.1.S1_at expression

during fruit development and ripening, total RNA was isolated from the ovary, young (12, 15, and 18 days after flowering) and mature green fruits, orange fruits, and red fruits using the RNeasy plant mini kit (Qiagen, Japan) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 0.75 μg of total RNA using the Superscript VILO cDNA synthesis kit (Invitrogen, USA). A ten-fold dilution of thefirst -strand cDNA was used as a template for the qRT-PCR using SYBR Premix Ex Taq II (Takara-Bio Inc., Otsu, Japan) in a Thermal Cycler Dice Real-Time System TP800 (Takara-Bio Inc., Otsu, Japan) according to the manufacturer's instructions. The thermal cycling parameters were set at 95°C for 10 min to denature, followed by 40 cycles at 95°C for 5 sec and 68°C for 30 sec. The relative quantification of the target gene expression was calculated using the tomato *ubiquitin3* gene (X58253) as an internal control. The following primer sequences were used: LA22CD07 forward, 5'-GATCAAACTATTGCTGCCCAG-3', and reverse, 5'-CTCTTCCTTGCTTCCACTCCAA-3'; LesAffx.6852.1.S1_at forward, 5'-CTGAAATGTCCCGTGATGATGC-3' and reverse, 5'-CGCTTGCAGGTTCTCTGTTC-3'; *E8* forward, 5'-TGGAAAGCCCTAGAGTTGAGGA-3' and reverse, 5'-GAATCAACAAGTCCTTTAACAC-3'; and *ubiquitin3* forward, 5'-CACCAAGCCAAAGAAGATCA-3' and reverse, 5'-TCAGCATTAGGG CACTCCTT-3'.

Isolation of promoter regions

Genomic DNA was extracted from the tomato cultivar 'Moneymaker' using the CTAB method (Murray and Thompson 1980). Each 5′ flanking region of LA22CD07 and LesAffx.6852.1.S1_at was isolated from genomic DNA using the GenomeWalker™ Universal Kit (Clontech, USA) as the putative promoter regions. The promoter regions were obtained from a second PCR reaction using

the GenomeWalker™ Universal Kit, purified using the Wizard(R) SV Gel and PCR Clean-Up System (Promega, USA), and directly sequenced. The ATG start codons were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the sequences were compared with homologs of other plant species, such as *Arabidopsis*. Approximately 2 kb of 5′ upstream regions from the predicted ATG start site were re-amplified from the 'Moneymaker' genome using KOD Plus (TOYOBO, Japan). The amplified products were cloned into the pCR®-Blunt II-TOPO® Vector (Invitrogen, USA) and sequenced.

Transient promoter assay

The promoter region in the pCR®-Blunt II-TOPO® Vector was digested with restriction enzymes and ligated in front of the *GUS* gene in the pBI121 vector to replace the 35S promoter. The constructs containing the promoter region or pBI121 as a control was transformed into *Agrobacterium tumefaciens* strain GV3101 through electroporation and was used in a transient promoter assay. The assay was performed using green fruit of 'Micro-Tom' as previously described (Orzaez et al. 2006). The agrobacterium containing the construct was injected into green fruit and incubated 4 days at 25°C under long-day conditions (16 h light and 8 h dark). The total protein from the infected fruit was subjected to a quantitative GUS activity assay using 4-methylumbelliferyl-beta-D-glucuronide (4-MUG) as a substrate.

Production of transgenic tomato

The transformed *A. tumefaciens* was also used for the production of a transgenic tomato with 'Micro-Tom' cultivar. Transformants were produced according to Sun et al. (2006). The presence of the promoter-GUS fusions in the regenerated plants was confirmed by PCR using genomic DNA isolated from the

regenerated plants as templates.

GUS assay

For the quantitative analysis, GUS activity was assayed using the substrate 4-MUG according to Jefferson et al. (1987) with slight modifications (Moon and Callahan 2004). Tomato tissue was crushed using liquid nitrogen, and the protein was extracted in extraction buffer (Moon and Callahan 2004). The protein concentration was measured using the Bradford method (Bradford 1976). Approximately 100 µg of protein was used for the GUS assay. The reaction product 4-methylumbelliferone (4-MU) was measured with Safire (Tecan, Switzerland).

The histochemical GUS analysis was performed using 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-Gluc) according to Jefferson et al. (1987) with slight modifications to the assay buffer. To reduce the background from GUS staining, 100 mM phosphate (pH 8.0) was used instead of 50 mM phosphate (pH 7.0) in the assay buffer. For the analysis of the red fruit in Fig. 3B, 20% methanol (final volume) (Kosugi et al. 1990) was added to the assay buffer to further reduce the background staining. The tomato tissues were incubated in assay buffer at 37°C for 16 or 6 h. After staining, the sample was washed with 70% ethanol to terminate the reaction.

Results and Discussion

Identification of promotercandidate genes from microarray data for expression in green fruit

To obtain candidates for novel fruit-specific promoters with unique activities compared to classical promoters, such as the E8 promoter, which mainly acts in the fruit late-ripening stage, we employed two strategies. The first

strategy was to identify highly expressed genes in green fruit, and the second was to uncover novel fruit-specific genes.

Firstly we analyzed microarray data using mRNA from 'Micro-Tom' green fruit to identify genes that were highly expressed in green fruit and selected seven genes (LA15CA04, LA22CD07, LC09AH08, LC04DC11, LA12AA05, LA14AD08 and FB14DB02). Moreover microarray database of several 'Micro-Tom' tissues were available from the Kazusa DNA Research Institute and Cornell University due to obtain promoter candidate genes for fruit-specific expression. Consequently, five genes (Les.331.1.S1_at, Les.3122.2.A1_a_at, LesAffx.6852.1.S1_at, TC115787 and TC116003) were selected. In total, 12 promoter-candidate genes were identified (Table. 1).

Expression analysis of the promoter-candidate genes by RT-PCR

To examine whether the promoter-candidate genes uncovered from the microarray data are expressed in tomato fruit and the specificity, we performed RT-PCR analysis using the primer sets listed in Table 1.

We first examined the seven promoter-candidate genes predicted to have high expression levels in green fruit. As shown in Fig. 1, the expression was detected after 25 PCR reaction cycles and was clearly detectable at 27 and 30 cycles using cDNA template derived from green fruits. The expression levels were different among the promoter-candidate genes. Based on the expression levels at 27 and 30 cycles, we selected LA22CD07, LA12AA05 and LA14AD08, which were highly expressed in green fruit, for further studies.

Next, the organ-specific expression patterns were investigated for the five promoter candidate genes predicted fruit-specificity to understand which candidates displayed fruit-specific expression (Fig. 2). In this analysis, the expression of *E8* gene was also investigated to compare the expression of promoter-candidate genes with a well-known fruit-specific gene. As a result,

Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at exhibited fruit-specific expression. However, they also exhibited different expression patterns. Les.3122.2.A1_a_at showed specific and high expression in the both green and red fruit stages, whereas LesAffx.6852.1.S1_at was highly expressed in the green fruit but was only slightly expressed in the red fruit. Les.331.1.S1_at was also highly expressed in the green and red fruits; however, a low level of expression was detected in the flower. TC115787 was expressed in the flower, stem and root in addition to the green and red fruit. TC116003 was expressed throughout the examined organs except the red fruit. The *E8* gene was highly expressed in the red fruit but was almost undetectable in the green fruit. This result supports previous studies, which reported that the *E8* gene was expressed in a ripening-specific manner (Deikman and Fischer 1998; Kneissl and Deikman 1996; Lincoln et al. 1987).

We uncovered two promoter-candidate genes of Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at with fruit-specific expression and one gene of Les.331.1.S1_at with high expression in the fruit and low expression in the flower. Notably, these three candidates were highly expressed in the green fruit, in which E8 gene expression was almost undetectable. Moreover, the three candidates were also expressed in the red fruit. These results suggest that the promoters of the three candidate genes were active in fruit and have different activities than the E8 promoter.

From these results, six genes, LA22CD07, LA12AA05, LA14AD08, Les.331.1.S1_at, Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at, were selected for subsequent analysis.

BLASTN analysis of the candidates

To obtain functional information for the promoter-candidate genes, a BLASTN analysis was performed. The results were summarized in Table 2,

which listed the top hits of functionally annotated genes resulting from BLASTN analysis. The BLASTN analysis showed that LA14AD08 returned a hit for a clp-like energy-dependent protease from the tomato and stink bell (*Fritillaria agrestis*), indicating that LA14AD08 represents a family of Clp proteases. Although LA22CD07 and LA12AA05 hit to the tomato full-length cDNA sequences (Aoki et al. 2010), they did not hit to functionally annotated tomato gene. However, LA22CD07 and LA12AA05 returned hits for the erythroblast macrophage protein emp from *Ricinus communis* (XM_002525023) with an e-value of 5E-39 and the sufD protein from the *Ricinus communis* (XM_002534741) with an e-value of 2E-69, respectively. The result suggest that the two candidates are homologs of the erythroblast macrophage proteins emp or sufD.

Les.331.1.S1_at returned hits for the tomato LOX gene U13681 (Kausch and Handa 1995) and tomloxB (U09025) with e-values of 0 (Ferrie et al. 1994). Ferrie et al. (1994) reported the fruit-specific expression of the LOX gene. Beaudoin and Rothstein (1997) reported that the LOX gene promoter activity was active in tobacco and tomato fruits.

Les.3122.2.A1_a_at returned a hit for tomato gene S66607 (Pear et al. 1993), which has been described as a pectin methylesterase-like sequence, indicating that Les.3122.2.A1_a_at is a member of the pectin methylesterases. The expression pattern and promoter analysis of S66607 have not been analyzed; however, it has been reported that some members of the pectin methylesterases exhibited fruit-specific expression (Gaffe et al. 1997; Hall et a l. 1994).

LesAffx.6852.1.S1_at returned hits for tomato cDNAs with e-values of 0 whose functions have not been reported. LesAffx.6852.1.S1_at also returned a hit for a cysteine protease of *Gossypium hirsutum* (AY171099) with 69% identity, suggesting that the LesAffx.6852.1.S1_at is a member of the cysteine proteases.

Isolation and characterization of selected gene promoters

Because the Les.331.1.S1_at promoter had been analyzed previously (Beaudoin and Rothstein 1997), we decided to clone the promoter regions that have not been analyzed: LA22CD07, LA12AA05, LA14AD08, Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at.

To clone the promoter regions, we performed genome walking based on the sequence information of the candidates. In consideration of prospective practical use, the isolation of promoter regions were used genomic DNA from 'Moneymaker' which is cultivated variety. The PCR fragments obtained from genome walking were directly sequenced. The ATG start codons were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the sequences were compared with homologs of other plant species. Subsequently, the putative promoter regions, which were approximately 2 kb upstream from the predicted ATG start codon, were re-amplified and sequenced.

In order to analyze the activities of the isolated promoters, each promoter was cloned to replace the 35S promoter in vector pBI121. We first performed transient assays using 'Micro-Tom' green fruit. Significant GUS activity was obtained from the LA22CD07, Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at promoters (data not shown). The GUS activities of the LA12AA05 and LA14AD08 promoters were almost the same as that of uninfected green fruit, suggesting that the two promoter fragments do not function in green fruit.

The three promoters from LA22CD07, Les.3122.2.A1_a_at and LesAffx.6852.1.S1_at that exhibited GUS activity in the transient assay were further analyzed using stable transgenic tomatoes. We conducted a GUS histochemical assay of leaves, roots, stems, flowers, green fruits and red fruits in regenerated T_0 plants. At least three independent T_0 plants per construct were assayed. The GUS staining pattern was almost identical among the tested plants containing the same construct, although the staining intensity varied (data not shown). Fig. 3 shows the results of a typical GUS staining of the various tissues

of transgenic plants containing promoter-GUS fusion constructs. Unlike the transgenic plants containing the 35S promoter, tissue-specific GUS staining patterns were observed among the transgenic plants containing the LA22CD07 or LesAffx.6852.1.S1_at foreign promoter regions. Fig. 3a shows the results from a 16h GUS staining experiment. The transgenic plants containing the LA22CD07 promoter exhibited strong GUS staining in the green and red fruits, weak staining in the flowers and undetectable staining in the leaves and roots. The transgenic plants containing the LesAffx.6852.1.S1_at promoter also displayed strong staining in the green and red fruits, but the flower staining was stronger than that of LA22CD07. No staining was detected in the tissues from the transgenic plants containing the Les.3122.2.A1_a_at promoter (data not shown). In the case *GUS* gene driven by 35S promoter, the GUS staining was detected everywhere in tomato plant and the staining levels were relatively high. However in the green fruit the GUS staining levels were almost same between LA22CD07, LesAffx.6852.1.S1_at and 35S promoters. In the red fruit the staining levels were also high in these promoters but non-specific staining was observed in the non-transgenic plants. Therefore the red fruits were further treated with assay buffer containing methanol for 6 h. As shown in Fig. 3b, GUS staining was almost no detected in the wild-type plants and was observed in red fruits of the transgenic plants containing the LA22CD07 and LesAffx.6852.1.S1_at promoters. Moreover the staining levels were relatively high especially in LesAffx.6852.1.S1_at promoter compared with 35S promoter. These results indicated that these promoters were active in both green and red fruits.

Quantitative real-time PCR analysis of LA22CD07 and LesAffx.6852.1.S1_at were performed to investigate the detail of the promoter activities during fruit development and to compare the activity of E8 promoter as known fruit-ripening specific (Fig. 4). The expression of *E8* gene was slightly detected in mature green stage and rapidly increased from orange stage. On the

other hand, the expression level of LA22CD07 was gradually increased from 12 days after flowering and reached the highest in the red stage. In the LesAffx.6852.1.S1_at the expression was already detected in the ovary and then gradually increased as described at LA22CD07. The result suggested that the novel two promoters had different activation pattern from E8 promoter and were active from small green fruit or ovary stages. Although we have not examined the GUS staining between flowers and green fruits, it might be possible that the two promoters are active at early stages of fruit development (flower to green fruit) because the GUS staining was also observed in the both flowers.

Conclusions

In this study, we isolated novel two fruit-specific promoters from the tomato. These promoters exhibited activities that were different from classical fruit ripening-specific promoters, such as the E8 promoter. The activities are detected throughout during fruit development from ovary to red-ripe fruit. Therefore, the identified two promoters might outperform some fruit-specific promoters that act only fruit-ripening stage depending on the intended purpose. The two promoters will supply us tools to express genes of interest in fruit regardless of the developmental stage. In this study, we examined only tomato promoters. However, it might be possible to use these promoters in the fruits of other plants because BLAST analysis revealed homologs of LA22CD07 and LesAffx.6852.1.S1_at from many plant species.

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References

Alba R, Payton P, Fei Z, McQuinn R, Debbie P, Martin GB, Tanksley SD, Giovannoni JJ (2005) Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. Plant Cell 17: 2954-2965. doi:10.1105/tpc.105.036053

Aoki K, Yano K, Suzuki A, Kawamura S, Sakurai N, Suda K, Kurabayashi A, Suzuki T, Tsugane T, Watanabe M, Ooga K, Torii M, Narita T, Shin-I T, Kohara Y, Yamamoto N, Takahashi H, Watanabe Y, Egusa M, Kodama M, Ichinose Y, Kikuchi M, Fukushima S, Okabe A, Arie T, Sato Y, Yazawa K, Satoh S, Omura T, Ezura H, Shibata D (2010) Large-scale analysis of full-length cDNAs from the tomato (Solanum lycopersicum) cultivar Micro-Tom, a reference system for the Solanaceae genomics. BMC Genomics 11:210. doi:10.1186/1471-2164-11-210

Beaudoin N, Rothstein SJ (1997) Developmental regulation of two tomato lipoxygenase promoters in transgenic tobacco and tomato. Plant Mol Biol 33:835-46. doi: 10.1023/A:1005773722657

Bradford MM (1976) A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Analytical Biochem 72:248-254. doi:10.1016/0003-2697(76)90527-3

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J, Martin C (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 26:1301-1308. doi:10.1038/nbt.1506

Chen Y, Wang A, Zhao L, Shen G, Cui L, Tang K (2009) Expression of thymosin alpha1 concatemer in transgenic tomato (*Solanum lycopersicum*) fruits. Biotechnol Appl Biochem 52:303-312. doi:10.1042/BA20080054

Cordes S, Deikman J, Margossian LJ, Fischer RL (1989) Interaction of a developmentally regulated DNA-binding factor with sites flanking two different fruit-ripening genes from tomato. Plant Cell 1:1025-1034. doi: 10.1105/tpc.1.10.1025

Coupe SA, Deikman J (1997) Characterization of a DNA-binding protein that interacts with 5' flanking regions of two fruit-ripening genes. Plant J 11:1207-1218. doi: 10.1046/j.1365-313X.1997.11061207.x

Deikman J, Fischer RL (1988) Interaction of a DNA binding factor with the 5'-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J 7:3315-3320.

Deikman J, Kline R, Fischer RL (1992) Organization of ripening and ethylene regulatory regions in a fruit-specific promoter from tomato (*Lycopersicon esculentum*). Plant Physiol 100:2013-2017. doi:10.1104/pp.100.4.2013

Deikman J, Xu R, Kneissl ML, Ciardi JA, Kim KN, Pelah D (1998) Separation of cis elements responsive to ethylene, fruit development, and ripening in the 5'-

flanking region of the ripening-related E8 gene. Plant Mol Biol 37:1001-1011. doi: 10.1023/A:1006091928367

Dharmapuri S, Rosati C, Pallara P, Aquilani R, Bouvier F, Camara B, Giuliano G (2002) Metabolic engineering of xanthophyll content in tomato fruits. FEBS Lett 22:30-34. doi:10.1016/S0014-5793(02)02699-6

Estornell LH, Orzáez D, López-Peña L, Pineda B, Antón MT, Moreno V, Granell A (2009) A multisite gateway-based toolkit for targeted gene expression and hairpin RNA silencing in tomato fruits. Plant Biotechnol J 7:298-309. doi: 10.1111/j.1467-7652.2009.00402.x

Ferrie BJ, Beaudoin N, Burkhart W, Bowsher CG, Rothstein SJ (1994) The cloning of two tomato lipoxygenase genes and their differential expression during fruit ripening. Plant Physiol 106:109-118. doi:10.1104/pp.106.1.109

Gaffe J, Tiznado ME, Handa AK (1997) Characterization and functional expression of a ubiquitously expressed tomato pectin methylesterase. Plant Physiol 114:1547-1556. doi: http://dx.doi.org/10.1104/pp.114.4.1547

Hall LN, Bird CR, Picton S, Tucker GA, Seymour GB, Grierson D (1994) Molecular characterisation of cDNA clones representing pectinesterase isozymes from tomato. Plant Mol Biol 25:313-318. doi: 10.1007/BF00039542

Hirai T, Fukukawa G, Kakuta H, Fukuda N, Ezura H (2010) Production of recombinant miraculin using transgenic tomato in a closed-cultivation system. J Agric Food Chem 58: 6096-6101. doi:10.1021/jf100414v

Hiwasa-Tanase K, Hirai T, Kato K, Duhita N, Ezura H (2012) From miracle fruit to transgenic tomato: mass production of the taste-modifying protein miraculin in transgenic plants. Plant Cell Rep 31:513-525. doi:10.1007/s00299-011-1197-5

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907.

Kausch KD, Handa AK (1995) Molecular cloning and nucleotide sequence of a lipoxygenase cDNA from ripening tomato fruit. Plant Physiol 107:669-670. doi:10.1104/pp.107.2.669

Kneissl ML, Deikman J (1996) The tomato E8 gene influences ethylene biosynthesis in fruit but not in flowers. Plant Physiol 112:537-547. doi: http://dx.doi.org/10.1104/pp.112.2.537

Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay forß-glucuronidase (GUS) in transformed cells: methanol almost suppresses a putative endogenous GUS activity. Plant Sci 70:133-140. doi:10.1016/0168-9452(90)90042-M

Le LQ, Lorenz Y, Scheurer S, Fötisch K, Enrique E, Bartra J, Biemelt S, Vieths S, Sonnewald U (2006) Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression. Plant Biotechnol J 4:231-242. doi: 10.1111/j.1467-7652.2005.00175.x

Lewinsohn E, Schalechet F, Wilkinson J, Matsui K, Tadmor Y, Nam KH, Amar O, Lastochkin E, Larkov O, Ravid U, Hiatt W, Gepstein S, Pichersky E (2001)

Enhanced levels of the aroma and flavor compound S-linalool by metabolic engineering of the terpenoid pathway in tomato fruits. Plant Physiol 127:1256-1265. doi: http://dx.doi.org/10.1104/pp.010293

Lincoln JE, Cordes S, Read E, Fischer RL (1987) Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc Natl Acad Sci USA 84:2793-2797.

Matsukura C, Aoki K, Fukuda N, Mizoguchi T, Asamizu E, Saito T, Shibata D, Ezura H (2008) Comprehensive resources for tomato functional genomics based on the miniature model tomato Micro-Tom. Curr Genom 9:436-443. doi: 10.2174/138920208786241225

Mollet B, Niederberger P, Pétiard V (2008) Novel tomato flavours introduced by plastidial terpenoid pathway engineering. Trends Biotechnol 26:4-6. doi: 10.1016/j.tibtech.2007.10.004

Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL (1993a) Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. Proc Natl Acad Sci USA 90:5939-5943.

Montgomery J, Pollard V, Deikman J, Fischer RL (1993b) Positive and negative regulatory regions control the spatial distribution of polygalacturonase transcription in tomato fruit pericarp. Plant Cell 5:1049-1062. doi:10.1105/tpc.5.9.1049

Moon H, Callahan AM (2004) Developmental regulation of peach ACC oxidase promoter-GUS fusions in transgenic tomato fruits. J Exp Bot 55:1519-1528. doi:

10.1093/jxb/erh162

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 10:4321-4325. doi:10.1093/nar/8.19.4321

Nicholass FJ, Smith CJ, Schuch W, Bird CR, Grierson D (1995) High levels of ripening-specific reporter gene expression directed by tomato fruit polygalacturonase gene-flanking regions. Plant Mol Biol 28:423-435. doi: 10.1007/BF00020391

Ozaki S, Ogata Y, Suda K, Kurabayashi A, Suzuki T, Yamamoto N, Iijima Y, Tsugane T, Fujii T, Konishi C, Inai S, Bunsupa S, Yamazaki M, Shibata D, Aoki K (2010) Coexpression analysis of tomato genes and experimental verification of coordinated expression of genes found in a functionally enriched coexpression module. DNA Res. 17: 105-116. doi:10.1093/dnares/dsq002

Orzaez D, Mirabel S, Wieland WH, Granell A (2006) Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. Plant Physiol 140:3-11. doi: http://dx.doi.org/10.1104/pp.105.068221

Pear JR, Sanders RA, Summerfelt KR, Martineau B, Hiatt WR (1993) Simultaneous inhibition of two tomato fruit cell wall hydrolases, pectinmethylesterase and polygalacturonase, with antisense gene constructs. Antisense Res Dev 3:181-190.

Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. Plant J 24:413-419. doi: http://dx.doi.org/10.

1104/pp.105.068221

Schijlen EG, de Vos CH, Martens S, Jonker HH, Rosin FM, Molthoff JW, Tikunov YM, Angenent GC, van Tunen AJ, Bovy AG (2007) RNA interference silencing of chalcone synthase, the first step in the flavonoid biosynthesis pathway, leads to parthenocarpic tomato fruits. Plant Physiol 144:1520-1530. doi: http://dx.doi.org/10.1104/pp.107.100305

Schijlen E, Ric de Vos CH, Jonker H, van den Broeck H, Molthoff J, van Tunen A, Martens S, Bovy A (2006) Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit. Plant Biotechnol J 4:433-444. doi: 10.1111/j.1467-7652.2006.00192.x

Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol 47:426-431. doi: 10.1093/pcp/pci251

Sun HJ, Kataoka H, Yano M, Ezura H (2007) Genetically stable expression of functional miraculin, a new type of alternative sweetener, in transgenic tomato plants. Plant Biotechnol J 5:768-777. doi: 10.1111/j.1467-7652.2007.00283.x

Wang S, Liu J, Feng Y, Niu X, Giovannoni J, Liu Y (2008) Altered plastid levels and potential for improved fruit nutrient content by downregulation of the tomato DDB1-interacting protein CUL4. Plant J 55:89-103. doi: 10.1111/j.1365-313X.2008.03489.x

Xu R, Goldman S, Coupe S, Deikman J (1996) Ethylene control of E4 transcription during tomato fruit ripening involves two cooperative *cis* elements.

Plant Mol Biol 31:1117-1127. doi: 10.1007/BF00040829

Yano K, Watanabe M, Yamamoto N, Tsugane T, Aoki K, Sakurai N, Shibata D (2006) MiBASE: A database of a miniature tomato cultivar Micro-Tom. Plant Biotechnol. 23: 195-198. doi:10.5511/plantbiotechnology.23.195

Yano M, Hirai T, Kato K, Hiwasa-Tanase K, Fukuda N, Ezura H (2010) Tomato is a suitable material for producing recombinant miraculin protein in genetically stable manner. Plant Sci 178: 469-473. doi: 10.1016/j.plantsci.2010.02.016

Zhang H, Zhao L, Chen Y, Cui L, Ren W, Tang K (2007) Expression of human coagulation Factor IX in transgenic tomato (*Lycopersicon esculentum*). Biotechnol Appl Biochem 48:101-107. doi: 10.1042/BA20060224

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Table 1. Selected genes found from microarray data and summary of their expression

Category	Database	ID	Result of RT-PCR	Forward primer for RT-PCR	Revers primer for RT-PCR
High expression in green fruit	MiBASE[a]	LA15CA04	Low expression	5'-TCACTCACCAAGCCCTTTCTCTC-3'	5'-TCCTGAGAAGCAGCCTTAGGAAC-3'
	MiBASE[a]	LA22CD07	High expression	5'-CGATCCGCGCTAATCATCGT-3'	5'-AGCCGTGCTCTGCATCTTTG-3'
	MiBASE[a]	LC09AH08	Low expression	5'-TGGTGGTGAGGCTGTTGAGC-3'	5'-CCATGAGTCGGAACCTGTGC-3'
	MiBASE[a]	LC04DC11	Low expression	5'-TGGCGTTTTCTTCATCCTCCA-3'	5'-CAGCTGCCCTTATCCTGAACTGA-3'
	MiBASE[a]	LA12AA05	High expression	5'-CGGGGTGTTGATGCTGAAAC-3'	5'-GAGGGGCTTCCATTCATTATCAGA-3'
	MiBASE[a]	LA14AD08	High expression	5'-AACCCTCGCCGGAGCATCAA-3'	5'-TTTAATGGGATCCCCAACTTCTTG-3'
	MiBASE[a]	FB14DB02	Low expression	5'-GCAATAGCTGGTCGGCTAGAACA-3'	5'-ATCGATTCGCTGCGGCCTTA-3'
Fruit specific expression	GEO[b]	Les.331.1.S1_at	Fruit specific expression	5'-ATGTCTTTGGGTGGAATTGTGGATGCC-3'	5'-CATCTCCTCGCAAAGCTACCAGTTC-3'
	GEO[b]	Les.3122.2.A1_a_at	Fruit specific expression	5'-ATGTATGCTACGACCATTACTGGTAGCC-3'	5'-CAACCCGCTGGATTAATGAGACCAC-3'
	GEO[b]	LesAffx.6852.1.S1_at	High expression in fruit, Low expression in flower	5'-GAAAGACCAACTGAGCCTCTTTCAGAAG-3'	5'-ATGCCGCCGTTGTTTATCACCCATTC-3'
	TFGD[c]	TC115787	No fruit specific expression	5'-CCACTTGTGGAATTGGATGGATGTTG-3'	5'-GATCACTTGGAGGAGCTGTATAGCC-3'
	TFGD[c]	TC116003	No fruit specific expression	5'-ATGCCGCCGTTGTTATCACCCATTC-3'	5'-GAAAGACCAACTGAGCCTCTTTCAGAAG-3'

2 [a] URL: http://www.pgb.kazusa.or.jp/mibase/.
3 [b] Gene Expression Omnibus, URL: http://www.ncbi.nlm.nih.gov/gds, dataset GSE19326.
4 [c] Tomato Functional Genomics Database, URL: http://ted.bti.cornell.edu/cgi-
5 bin/TFGD/miame/home.cgi.
6

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Table 2. Summary of BLAST analysis.

ID of genes	Category	Organism	Accession	Definition	E-value
LA22CD08	Top hit	*Solanum lycopersicum*	L38581	Lycopersicon esculentum clp-like energy-dependent protease mRNAcomplete cds.	0
	Top hit of functionally annotated genes	*Fritillaria agrestis*	AF037459	Fritillaria agrestis clp-like energy-dependent protease (clpP) mRNA, complete cds.	1.00E-35
LA12AA07	Top hit	*Solanum lycopersicum*	AK322312	Solanum lycopersicum cDNA, clone: LEFL1036AH12, HTC in leaf.	0
	Top hit of functionally annotated genes	*Ricinus communis*	XM_002525023	Ricinus communis erythroblast macrophage protein emp, putative, mRNA.	5.00E-39
LA14AD05	Top hit	*Solanum lycopersicum*	AK322226	Solanum lycopersicum cDNA, clone: LEFL1035AG05, HTC in leaf.	0
	Top hit of functionally annotated genes	*Ricinus communis*	XM_002534741	Ricinus communis Protein sufD, putative, mRNA.	2.00E-69
Les.331.1. S1_at	Top hit	*Solanum lycopersicum*	AK326139	Lycopersicon esculentum lipoxygenase (LOX) mRNA, complete cds.	0
	Top hit of functionally annotated genes	*Solanum lycopersicum*	U13681	Lycopersicon esculentum lipoxygenase (LOX) mRNA, complete cds.	0
Les.3122.2. A1_a_at	Top hit	*Solanum lycopersicum*	S66607	Lycopersicon esculentum pectinmethylesterase-like sequence.	0
	Top hit of functionally annotated genes	*Solanum lycopersicum*	S66607	Lycopersicon esculentum pectinmethylesterase-like sequence.	0
LesAffx.6852.1.S1_at	Top hit	*Solanum lycopersicum*	AK326008	Solanum lycopersicum cDNA, clone: LEFL2001CF07, HTC in fruit.	0
	Top hit of functionally annotated genes	*Gossypium hirsutum*	AY171099	Gossypium hirsutum cysteine protease mRNA, complete cds.	2.00E-119

2

3

4

Figure captions

Fig. 1

Real-time PCR analysis of the promoter-candidate genes for high expression levels in green fruits. The expression levels of the genes in green fruits were analyzed at 25, 27 and 30 cycles of RT-PCR.

Fig. 2

Real-time PCR analysis of the promoter-candidate genes for fruit-specific expression. The tissue-specific expression levels of the candidate, *E8* and *actin* genes were analyzed using RT-PCR with first-strand cDNAs from the leaves, flowers, stems, roots, and green and red fruits. L, leaves; F, flowers; S, stems; R, roots; G, green fruits; R, red fruits.

Fig. 3

Histochemical GUS assay of the transgenic plants. The leaves, flowers, roots, and green and red fruits of T_0 plants were used for the GUS assay. The blue staining represents GUS activity. (**a**) Results of the 16h GUS staining of various tissues (**b**) Results of the 6h GUS staining of red fruits with buffer containing methanol. L, leaves; R, roots; F, flowers; G, green fruits; R, red fruits.

Fig. 4

Quantitative real-time PCR analysis of LA22CD07 and LesAffx.6852.1.S1_at. **a** The developmental stages of the fruits used for these experiments. Bar = 1 mm. Relative expression levels of LA22CD07 (**b**) and LesAffx.6852.1.S1_at (**c**) during fruit development and ripening. The expression level of the E8 gene was analyzed as a control (**d**). The fruits were harvested at 12, 15, and 18 days after flowering and at the fruit developmental stages as follows: ovary (OV), mature green stage

(MG), orange stage (OR), and red ripening stage (RE). The mean values of three independent experiments are shown. The error bars represent the standard error.

Fig. 1

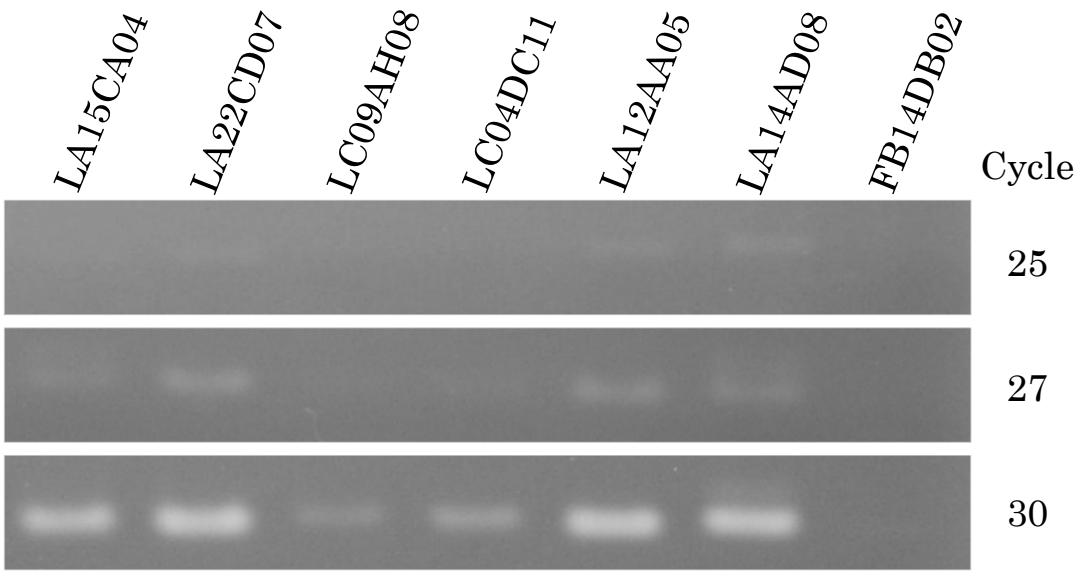
LA15CA04
LA22CD07
LC09AH08
LC04DC11
LA12AA05
LA14AD08
FB14DB02
Cycle
25
27
30

Fig. 2

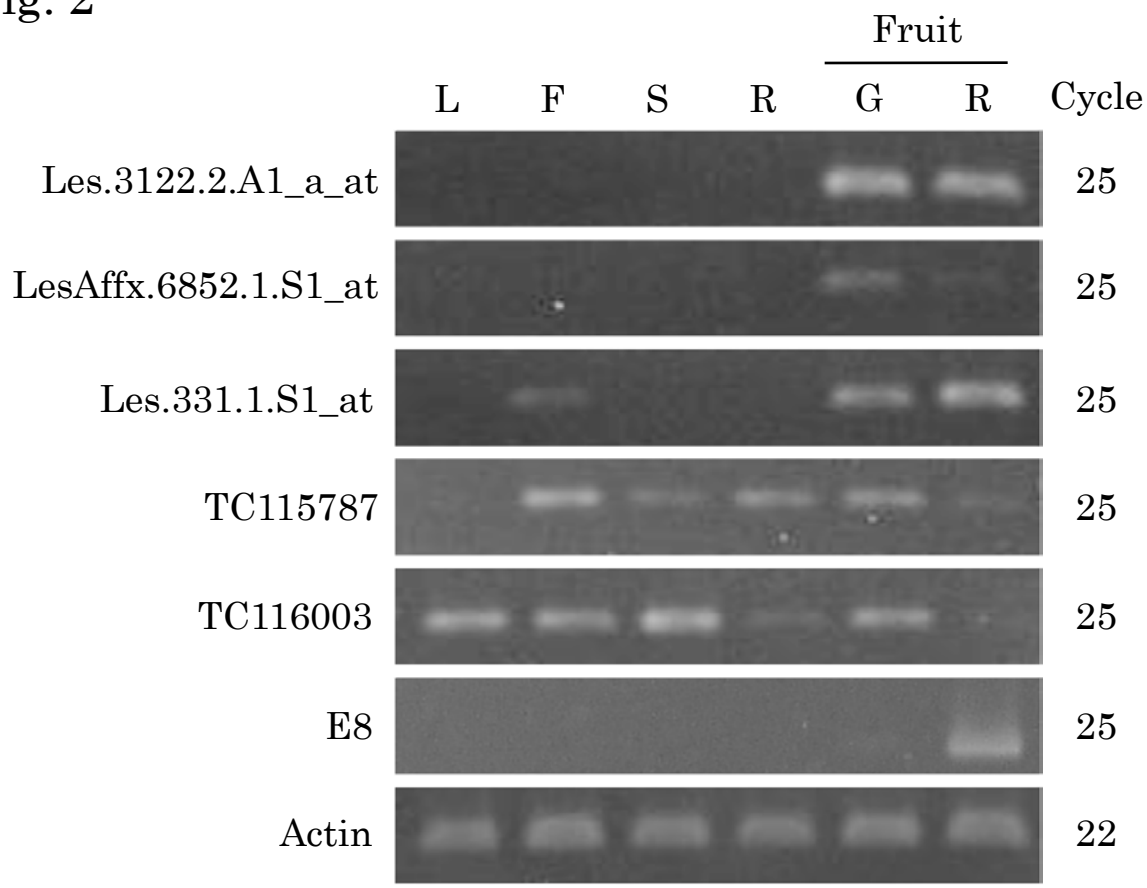
Fruit
L
F
S
R
G
R
Cycle
Les.3122.2.A1_a_at
25
LesAffx.6852.1.S1_at
25
Les.331.1.S1_at
25
TC115787
25
TC116003
25
E8
25
Actin
22

Fig. 3

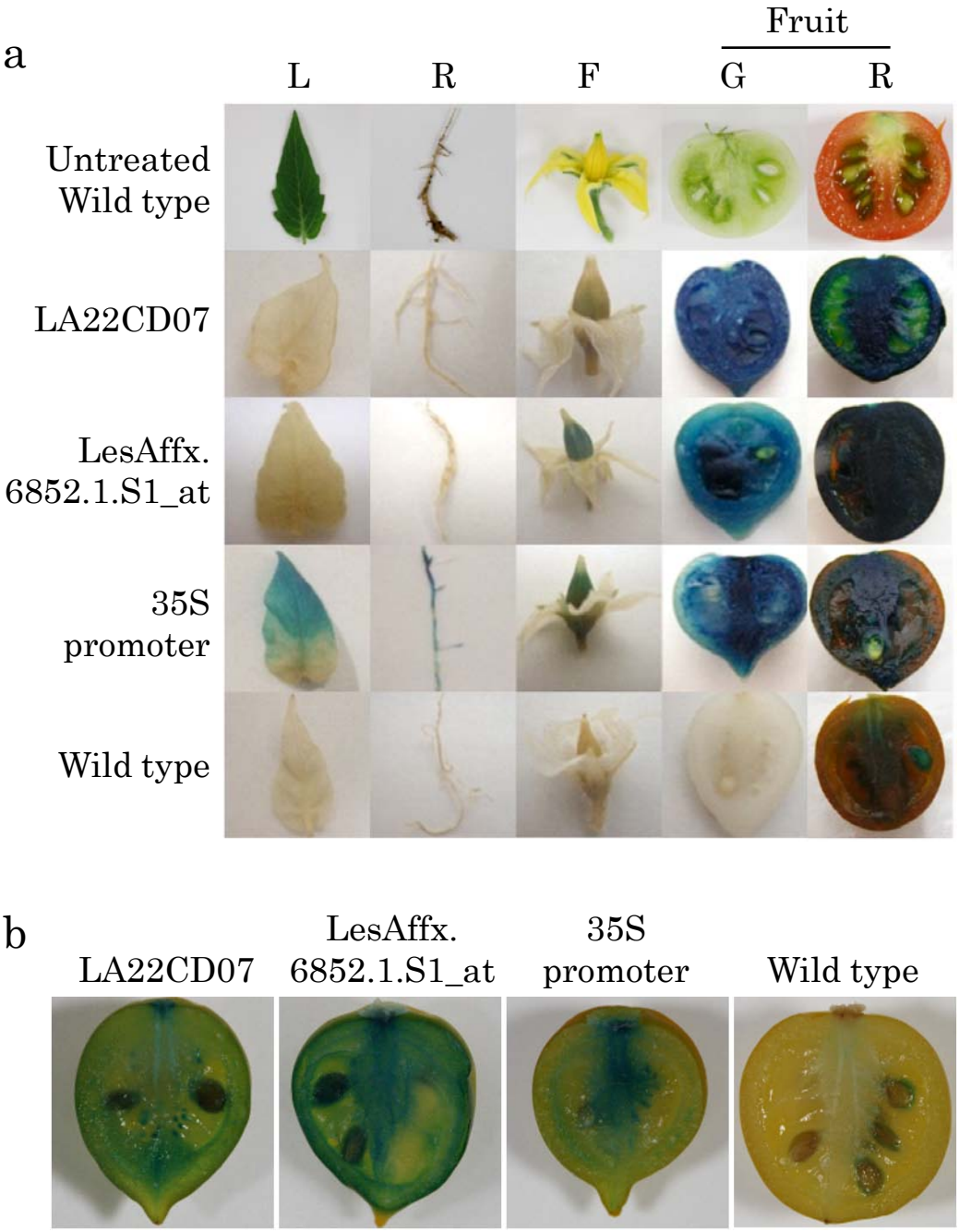
a
Fruit
L
R
F
G
R
Untreated Wild type
LA22CD07
LesAffx. 6852.1.S1_at
35S promoter
Wild type
b
LA22CD07
LesAffx. 6852.1.S1_at
35S promoter
Wild type

Fig. 4


a
12 15 18 MG OR RE
Days after flowering
b
LA22CD07
Relative expression
(LA22CD07/Ubiquitin3)
c
LesAffx.6852.1.S1_at
Relative expression
(LesAffx.6852.1.S1_at
/Ubiquitin3)
d
E8
Relative expression
(E8/Ubiquitin3)
0
0.2
0.4
0.6
0.8
1
1.2
OV 12 15 18 MG OR RE
Days after flowering