

Isolation of a citrus promoter specific for reproductive organs and its functional analysis in isolated juice sacs and tomato

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Abstract While searching for genes expressed in acid lemon but not in acidless lime pulp, we isolated clone *C1111* which showed the following expression phenotypes: (1) while it was expressed in the ovaries in both varieties, its mRNA was detected only in the pulp of the acid fruit, (2) no or very low expression of the gene was detected in vegetative organs. These expression patterns suggested that *C1111* is an ovary- and pulp-specific gene. The ability of ~2-kb fragments upstream of the transcription start site of the lemon and lime genes to confer reporter-gene activity was investigated by transient expression in isolated juice vesicles of both varieties. Whereas *C1111* promoter from lemon showed faint activity in lemon and lime juice

vesicles, no activity was evident with the lime promoter. The activities of the 2-kb fragments and their delimited fragments were further investigated in tomato. The results indicated that the promoters were active in a manner similar to that in acid lemon and acidless lime: the lemon promoter generated activity in the fruit endocarp, analogous to citrus fruit pulp. The delimitation analyses identified an expression-conferring region which, in the lemon promoter, contained a sequence homologous to a fruit-specific element of the melon *cucumisin* gene. Another region, which reduced promoter activity, contained an I-Box-like sequence, identified as a fruit-specific negative element. Taken together, *C1111* promoter was confirmed to be pulp- and flower-specific. Differences in the expression of *C1111* between the two varieties could be attributable to changes in the gene promoter region.

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Introduction

The precise control of transgene activity is of paramount importance in any plant gene-modification strategy. The promoters that drive transgene expression are key elements in controlling gene transcription. Over the years, a wide range of promoters of plant, viral and bacterial origin has been used to drive transgene expression in plant cells. Some promoters are constitutive, while others respond to different stimuli, such as environmental signals, biotic and abiotic stresses, plant growth regulators, etc. (reviewed in Potenza et al. 2004). Transgene expression can be beneficially adjusted by using promoters that are suitable for the plant's background and for the type of transgene. Moreover, spatial (specific expression at the organ, tissue and

cell levels) and temporal (developmental stage) expression of the transgene can minimize potential adverse effects (Potenza et al. 2004). The use of computational approaches to identify promoters in plant genomes is expected to further enhance the number of new promoters from different species (Rombauts et al. 2003).

To date, a number of fruit-specific promoters have been isolated, most of them belonging to ripening-related tomato genes. The *E4* and *E8* genes are coordinately regulated by ethylene during fruit ripening (Karaaslan and Hrazdina 2010; Kneissl and Deikman 1996; Xu et al. 1996); their 5' UTRs contain specific sequences which confer the ethylene response, in addition to other sequences responsive to fruit-development signals. The promoter of the tomato fruit-specific gene *2A11* includes strong positive regulatory elements which confer the gene's expression in the fruit and a strong negative regulatory element that inhibits its expression in the leaf (Vanhaaren and Houck 1991, 1993). Additional tomato fruit-specific promoters include that of the polygalacturonase gene (*PG*), which plays a role in cell-wall degradation during fruit ripening, the promoter of the T-proline-rich protein F1, which is specifically expressed in the ovary and young fruit, and the promoter of *ACC synthase* (Carmi et al. 2003; Fraser et al. 2002; Lau et al. 2009; Lin et al. 2007). Fruit-specific promoters have also been isolated from plant species other than tomato, such as that of the ripening-upregulated gene *ACC-oxidase* from apple and peach, that of *Expansin* from sour cherry, and the promoter of *PG* from apple (Atkinson et al. 1998; Rasori et al. 2003). Fruit specificity might be combined with other elements, which confer expression under specific conditions or timing. For instance, the above promoters may contain both ripening (ethylene)- and fruit-specific responsive regions; expression of the *GalUR* gene from strawberry, involved in vitamin C biosynthesis, is restricted to the fruit in a light-dependent manner, and thus its promoter has been shown to contain a few known light-responsive elements (Agius et al. 2005). Interestingly, ethylene-responsive elements are not restricted to promoters of genes related to the ripening of climacteric fruits, since they are also present in the promoters of the carotenoid-accumulation genes *capsanthin/capsorubin synthase* and *fibrillin* from pepper, a non-climacteric fruit (Kuntz et al. 1998).

The above-mentioned studies suggest the presence of fruit-specific *cis*-acting elements. Indeed, the promoter of the *cucumis* gene from melon contains a region with a fruit-specific enhancer element adjacent to a G-Box, and an I-Box-like sequence, which probably acts as a negative regulatory element (Yamagata et al. 2002). Different fruit-specific *cis*-acting elements are present in the promoters of the *ADP-glucose pyrophosphorylase* gene from melon

and in *metalothionein* from oil palm, where they probably act as negative elements and block expression of the genes in other tissues (Omidvar et al. 2010; Yin et al. 2009). In citrus, three promoters have been reported, but none of them is fruit-specific: (1) the glutathione peroxidase (*Gpx1*) promoter, which confers expression in vegetative tissues and cultured cells of tobacco plants, in particular upon salt, osmotic and oxidative stress (Avsian-Kretchmer et al. 2004), (2) the phenylalanine ammonia-lyase (*Pal*) promoter which confers expression in xylem tissues of tobacco and petioles of tobacco and citrus plants (de Azevedo et al. 2006), and (3) a type-3 metallothionein-like gene promoter which confers high transient expression in embryonic suspension calli and juice sacs, and low transient expression in young leaves (Endo et al. 2007). This promoter is also active in *Arabidopsis* seedlings, leaves, flowers, siliques and seeds.

As part of research aimed at studying citrus fruit acidity, we isolated a cDNA clone (*C1111*) that is highly expressed in the pulp of acid lemon [*Citrus limon* (L.) Burm cv. Eureka] but shows no apparent expression in the pulp of acidless lime (*C. limettioides* Tan.). The clone encodes a protein of unknown function. Expression analyses of *C1111* in the two varieties suggested that it is ovary- and pulp-specific. In the present study, we focus on the isolation and functional analysis of *C1111* promoter from the two varieties. A fragment of about 2 kb upstream of the transcription start site showed regions of varied homology between the two citrus varieties. While fused to a reporter gene, *uidA*, *C1111* promoter from lemon, but not from lime, showed activity in lemon and lime juice sacs. Stable transformation in tomato confirmed that these regions show activity similar to that observed for *C1111* expression in citrus. Delimitation analysis identified a region conferring expression. In this region, the lemon promoter contained a sequence homologous to a fruit-specific positive element of the *cucumis* gene from melon. Another sequence, homologous to the *cucumis* fruit-negative I-Box element was identified in a region, which reduced the activity of the promoters.

Materials and methods

Plant material

Different organs and fruits from acid lemon [*Citrus limon* (L.) Burm cv. Eureka] and acidless lime (*C. limettioides* Tan.) were collected from trees located in the central-coastal region of Israel, or from tomato seedlings and trees grown in pots in net houses (25-mesh transparent net) under natural environmental conditions.

Northern analysis and isolation of *C1111*

RNA from different tissues of citrus was extracted using a phenol–chloroform method that was slightly modified as a function of the source tissue. With juice sacs and fruit peel, the extraction was performed essentially as described previously (Sadka et al. 2000). With other organs—flowers, ovaries, roots, stems, leaves and *flushing leaves*, the extraction was performed as described by Shlizerman et al. (2007). Northern blot analysis was carried out using a [α^{32} P]dCTP-radiolabeled probe for the full-length cDNA of *C1111* from lemon. The membranes were scanned with a Fujifilm BAS-1500 Phosphoimager (Fuji Photo Film Co., Tokyo, Japan). The probe was then stripped by washing with a solution containing $0.1\times$ SSC and 0.1% (w/v) SDS for 15 min at 95°C, and the membrane was rehybridized with [α^{32} P]dCTP-radiolabeled probe for 18S RNA.

Enrichment of poly-A-mRNA from total RNA was performed using a PolyATrack mRNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The mRNA was used for the construction of a cDNA subtraction library using PCR-Select cDNA Subtraction kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Acid lemon cDNA served as the tester while acidless lime cDNA served as the driver. When the final PCR products were separated by agarose gel, seven distinct bands could be detected (not shown). The bands were cloned separately into pGEM-T (Promega). The insert of each clone was used as a probe in a northern blot analysis in which RNA from the juice sacs of acid lemon was compared to that from acidless lime. Transcript levels of *C1111* displayed the most pronounced difference between the two citrus varieties. Isolation of the complete transcript was performed by 5'-RACE using CapFishing Full-Length cDNA Premix kit (Seegene, Rockville, MA, USA).

Table 1 Primers used for the cloning of delimited fragments. Li, acidless lime primers, Le, acid lemon primers. All primers marked with F are forward primers. Cfr is the reverse primer used for all constructs

Primer	Sequence (5' to 3')
Le1770 (F)	TGTCTAGTTCTCATCTCTCCCACAACACT
Li1771 (F)	TGTCTAGAGCTTGTACGTTACGTGTGTAT
Li1583 (F)	TGTCTAGAGTATTGCAGCCTTGTAGGTT
Le1229 (F)	TGTCTAGAGTTATATTACATCAGACATC
Le954/Li1262 (F)	TGTCTAGAATGATTTGCAGATGCACTATA
Le832 (F)	TGTCTAGACTTGATATTTAATCTTATTTTAAAC
Li1034 (F)	TGTCTAGATTTAATCTTCTTTAAACGAT
Le525/Li 738 (F)	TGTCTAGAATCTTGAACATTATACTTTAC
Le324/Li323 (F)	TGTCTAGAATGAAATATCTTCTATGTGGC
Cfr (R)	GACCCGGGCATTTTTTCTATTTTCATTCTTTCA

Isolation of promoter sequences of *C1111*

DNA was extracted from lemon or lime leaves using a previously described method (Sadka et al. 2000). Based on the sequence of *C1111*, reverse primer (5'-GGATCCC ACTTGCTAAGTTGTTTCT-3') and reverse nested primer (5'-CTGGTTCTAAATCCATGGAGGCATA-3') were designed for the isolation of *C1111* promoter sequence using Genome Walking by the Unpredictable Primed PCR technique described previously (Dominguez and Lopezlarrea 1994). Further upstream sequences were isolated using the Universal Genome Walker kit (Clontech) according to the manufacturer's instructions, using reverse primers designed based on the isolated sequence. Isolated fragments were cloned separately into pGEM-T Easy vector (Promega), sequenced and assembled into one sequence of about 1.8 kb in length. Sequences upstream of the transcription start site of *C1111* from acid lime were isolated and cloned according to the strategy used for promoter isolation from lemon. The longest fragments from lemon and lime were isolated by PCR from genomic DNA primer-adaptor containing a *Cfr*9I restriction site. The fragments were digested with *Cfr*9I and cloned into the *Sma*I site in *pGPTV* (Becker et al. 1992) in front of the *uidA* gene.

Construction of expression vectors with *C1111* promoter fragments

Sequence-specific primers containing *Xba*I and *Cfr*9I restriction sites were used to generate delimited promoter fragments by PCR (Table 1). The amplified sequences were then cloned into pGEM-T Easy vector and further into *pGPTV* in front of the *uidA* gene, as described above. Fragments were designated as follows: LepC1 (containing lemon *C1111* promoter, -1,770 to +30 bp) and LipC1 (containing lime *C1111* promoter, -1,771 to +30 bp), LepC2 (containing lemon *C1111* promoter, -1,229 to +30 bp) and LipC2 (containing lime *C1111* promoter,

–1,583 to +30 bp), LepC3 (containing lemon *C1111* promoter, –954 to +30 bp) and LipC3 (containing lime *C1111* promoter, –1,262 to +30 bp), LepC4 (containing lemon *C1111* promoter, –832 to +30 bp) and LipC4 (containing lime *C1111* promoter, –1,034 to +30 bp), LepC5 (containing lemon *C1111* promoter, –525 to +30 bp) and LipC5 (containing lime *C1111* promoter, –738 to +30 bp), LepC6 (containing lemon *C1111* promoter, –324 to +30 bp) and LipC6 (containing lime *C1111* promoter, –323 to +30 bp).

Transient expression assay in isolated juice sacs by particle bombardment

Juice sacs from green mature fruits of acid lemon and acidless lime were subjected to particle bombardment with vector plasmids containing the longest promoter fragment, LepC1 and LipC1, essentially as described, with modifications (Endo et al. 2007). Juice sacs were detached and placed separately on MS medium containing 0.2% Gelrite (Serva, Germany) in a Petri dish. Particle bombardment was carried on using the PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. The transformation efficiency was estimated by co-transformation of a control plasmid containing the *gfp* gene under the control of the CaMV 35S promoter. Gold particles (1.0 mm in diameter, 500 mg) were coated with 0.8 mg of the respective plasmids and control plasmid, and a helium pressure of 7.6 MPa was employed. The target distance between the stop screen and the Petri dish was set to 6 cm. Following bombardment, the juice sacs were kept at 25°C for at least 24 h under dark conditions. GFP was detected with an MZ FL III fluorescence stereomicroscope (Leica, Milton Keynes, UK) fitted with a filter set (Leica GFP-Plus). Following the detection of GFP expression, the bombarded tissues were subjected to histochemical staining of GUS activity for a few days using the X-Gluc method, as described below.

Plant transformation, regeneration, and growth

Constructs' transformation into *Agrobacterium tumefaciens*, strain EHA105, was carried out essentially as described by Hood et al. (1993). Tomato (*Solanum lycopersicon* cv. MP1) cotyledons were transformed following the method of McCormic (1991). Regenerated tomato plants were transferred to pots containing commercial soil (Shaham, Givaat Ada, Israel) and grown in a greenhouse under a constant temperature of 24–25°C and natural light conditions. Positive transgenic plantlets were confirmed by PCR analysis and then subjected to GUS staining.

GUS activity assay

GUS staining was carried out using the X-Gluc method (Kosugi et al. 1990), with some modifications. Tissue segments were incubated in a solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl β - δ -glucuronide, 50 mM sodium phosphate buffer pH 7 and 20% (v/v) methanol, followed by 5 min incubation under vacuum, and 2 h incubation in the dark at 37°C.

Results

C1111 isolation and its expression patterns in acid lemon and acidless lime

In a search for genes playing a role in the regulation of citrus fruit juice acidity, a cDNA subtraction library was constructed from the pulp of acid lemon fruit, characterized by the accumulation of about 0.3 M citric acid. cDNAs common to those present in the pulp of acidless lime fruit (which accumulates less than 0.01 M citric acid) were subtracted. The taxonomic relationship between these two varieties is discussed elsewhere (Sadka et al. 2001). The mRNA for one of the isolated clones, *C1111*, displayed high and relatively constant expression in the pulp of the acid fruit throughout fruit development, but was below detection levels in the pulp of acidless lime (Fig. 1a). *C1111* expression was analyzed in vegetative and reproductive organs (Fig. 1b–d). While relatively high mRNA levels were detected in the juice sacs of 10- to 20-mm diameter acid lemons (Fig. 1b, left panel), it was below detection level in the outer (flavedo) and inner (albedo) peels of these fruits (Fig. 1b, left panel) and 40-mm fruits (Fig. 1b, middle panel). Similarly, flavedo and albedo fractions obtained from 20-mm diameter acidless lime fruits did not show the mRNA for *C1111* (Fig. 1b, right panel). These results clearly showed that *C1111* mRNA accumulates mostly in the juice sacs of the acid fruits. Citrus fruits develop from the ovary, and juice-sac primordia bud from the ovary wall even before fertilization. Therefore, the mRNA level of *C1111* was analyzed in the ovaries of closed and open flowers of acid lemon. While low levels of the mRNA could be detected in the whole flower, very high levels were detected in the ovary of the closed flowers (Fig. 1c, left panel). Following anthesis, a remarkable reduction in transcript level was detected (Fig. 1c, left panel). In the ovaries of the acidless variety, the mRNA level was lower than in the acid line, but showed similar post-anthesis reduction (Fig. 1c, right panel). The expression of *C1111* was also investigated in leaves, roots and green branches of the two varieties: mRNA was below detection levels in both the acid lemon (Fig. 1d, left panel) and acidless lime (Fig. 1d, middle panel). Some expression was detected in flushing leaves (smaller than 1 mm) from

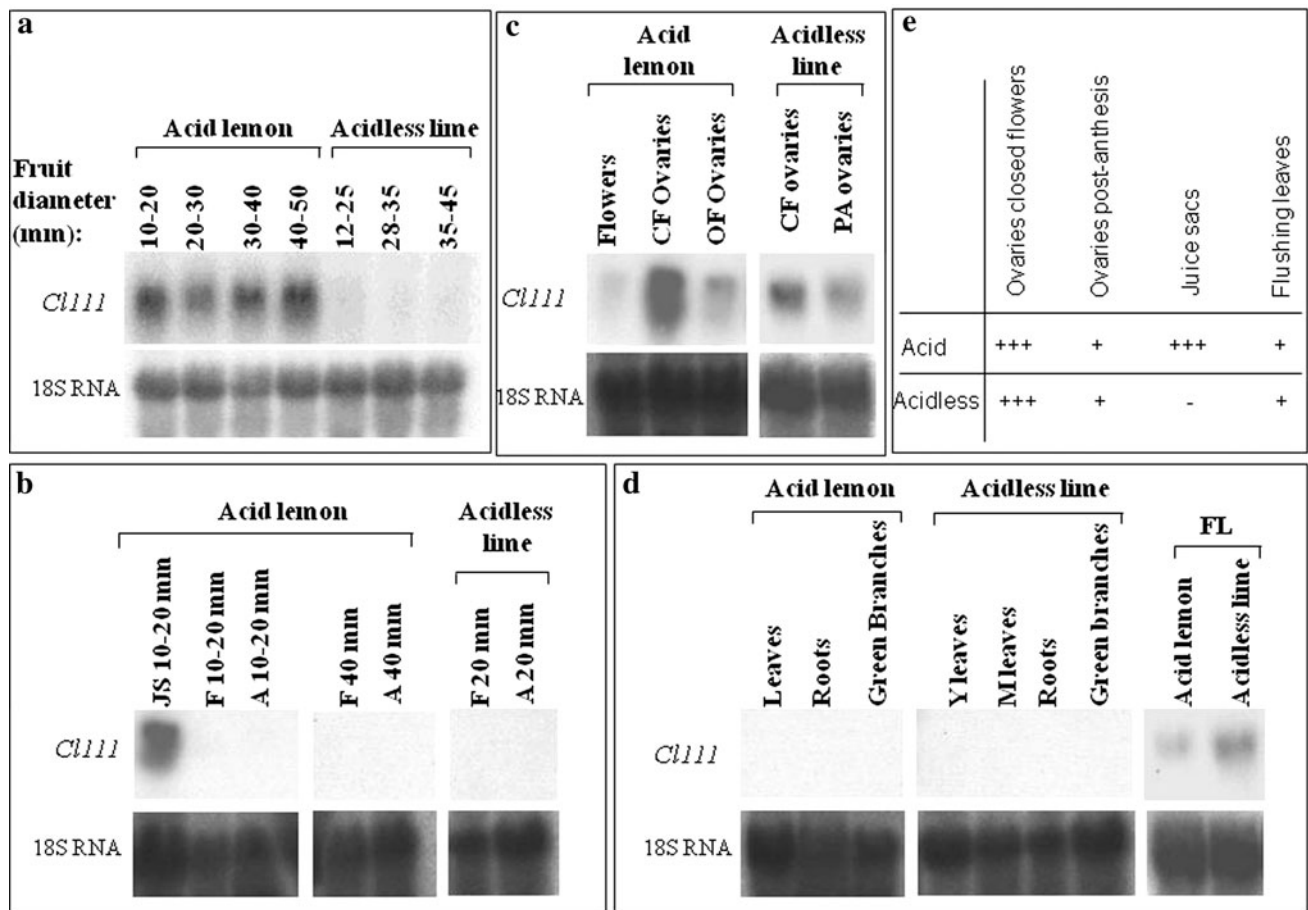


Fig. 1 mRNA levels of *Cl111* in acid lemon and acidless lime. Northern analyses were performed using RNA from different tissues: **a** Juice sac cells at various developmental stages, as indicated by fruit diameter. **b** Fruit peel tissues, flavedo (F) and albedo (A), in comparison to juice sacs (JS). **c** Flowers and ovaries of closed flowers (CF) and open flowers (OF). **d** Vegetative organs: leaves, roots, green

branches, young (Y) leaves, mature (M) leaves, roots and flushing leaves (FL). **e** Summary of the expression profile of *Cl111* in acid lemon and acidless lime. Samples in **b**, **c** and **d** were separated on one gel. Following hybridization with *Cl111* cDNA as the probe (upper panels), probes were stripped away and the membranes were rehybridized with 18S rRNA probe

both varieties, with the acidless lime displaying higher transcript levels than the acid lemon (Fig. 1d, right panel). The expression patterns of *Cl111* are summarized in Fig. 1e. The transcript levels were relatively high in the ovaries of closed flowers, and were remarkably reduced post-anthesis. They were re-induced in the juice sacs of the acid lemon but not in those of the acidless lime. Both varieties displayed some expression in *flushing leaves*. In most vegetative organs, the mRNA was below detection levels, or displayed only a very low signal.

Sequences of the regions upstream of the transcription start sites of *Cl111* from acid lemon and acidless lime

The transcription start site of *Cl111* was determined by cloning the full transcript with 5'RACE PCR using Cap Fishing technology. Fragments of about 1.8 kb upstream of the transcription start site of *Cl111* were isolated from acid lemon (LepC1) and acidless lime (LipC1) and

sequenced (Fig. 2). The two sequences contained a TATA-box and a C-rich region about 50 bp upstream of the putative transcription start site, in addition to a -CAT box at approx. position -170 bp. The two sequences could be divided into four regions according to their similarity. The first region, which included the 5'-UTR and about 300 bp upstream of the transcription start site, contained nearly identical sequences. The second region, -303 to -462 (160 bp) in lemon and -304 to -675 (372 bp) in lime, contained sequences with no apparent similarity. The third region, -463 to -1,235 (773 bp) in lemon and -676 to -1,559 (884 bp) in lime, contained sequences with about 70–80% identity between the two varieties. The fourth region, -1,236 to -1,831 bp (596 bp) in lemon and -1,560 to -1,816 (257 bp) in lime, contained regions with no apparent similarity. Overall, in comparison to the lime sequence and based on homologous regions, the lemon sequence contained internal deletions amounting to about 320 bp.

Fig. 2 continued

Lemon	TTATTTTCTCTTGAT-----ATTTAATCTTATTTTAAACAATAAAAAAAAAG	-768
lime	CTATTTTCTCTGGATGTAAAAAGGATATTTAACTCTTTAAACGAT---AAAAAG	-977
	***** * * *	
Lemon	ATTCATTTGTAGTTGTTGGGATTAATAACCATTAATAAACTAACCATGTAACTCATT	-704
lime	ATTCATCTTGCAGTTGTTGGGATTAATAATAATTACAAAATTAATAAACTCATC	-917
	***** * * *	
Lemon	AAATGCGTATAAAAATCAAAAATTACCAAAAATAGCACCTAGCTAAATTTGTACCTT	-644
lime	AAATTACGTATAAAAATCAAAAATTACCAAAAATAGCACCTAACTAAATTTGTAGCTT	-857
	***** * * *	
Lemon	ATTTACAATATAAATCAAGAATCTATACAGTATAATTTATCTTAAATAAAATGTTTTTG	-584
lime	ATTTACAACATAAATCAAGAATCATGCCTATATAAATCATCTTAAATGACACGCTTTTG	-797
	***** * * *	
Lemon	TCACAGTAACAACCTCAACAGCAATAG-CATAAATGATCATAGTTATCTAAAATGCAT	-525
lime	TCACAGTAACAACCTCAACAGCAATAAATAGAAATTCATAGTT-TCFAAAATGCAT	-737
	***** * * *	
Lemon	TCGAAATAATAAAAATATTTATGAGAAATCTTGAACATTATATTTTACTTTCATCAA	-465
lime	TCGAAATAATCAAAAATCATTATGAGAAATCTTGAACATTATCTTACTTCCATCAA	-678
	***** * * *	
Lemon	TATTAGA-----GTTTTTTTTATTTAATAAAGTAATATA-----	-429
lime	TAAAAAATAAATAGTATAAAACTAGTTATCCCATCAATCCAAAAATAATAATACATCTG	-618
	** * *	
Lemon	-----ACTATTTTATTTA-----AATTTT	-408
lime	GAAGCATTGAAAATAAATCAATGAATAATCTTTTATATTAGGGATAAATAATTAT	-558
	* * * * * * * * * * * *	
Lemon	TTTTT-----TAAAAAATAATATA	-387
lime	TTTTTAACATGCTGTTAACCTCTAATGGTGCTAATTAATAAATAAATAAATAAAT	-498
	***** * * *	
Lemon	TTTCATCTTCT-----TTTTGTATCACACA	-363
lime	TTATAAATTCACATGAAAAGCTGTAATAAATAAATAATTTAATAATAAATAAATA	-438
	** * * * * * * * * * * *	
lime	TAAAGTATTAGATGATAATACAAGACAGTAATTATAATGGAGTGTGCTTCACTATAAAT	-387
	** * * * * * * * * * * *	
Lemon	-----ATCTTTTTGA-----GATGTT-----	-307
lime	TACAAATTTAATATTATTCTTATAATTATACAGTCGTTAATAAATGCTGCATCGTAAAC	-318
	** * * * * * * * * * * *	
Lemon	-----CGATTAGATTTTA--ATGAAATATCTTCTATGTGGCTCCAATAGAGTAAT	-259
lime	AGTTCTAACATGGTTAGATTCTAGTATGAAATATCTTCTATGTGGCTCCAATAGAGTAAT	-258
	* * * * * * * * * * * *	
Lemon	GACAGCCACCCTTCGGGAGAAAAAGCCGAGAGCGGACGATTTCGAATCTTGACATCGTGT	-199
lime	GACAGCCACCCTTCGGGAGAAAAAGCGAGAGCGGACGATTTCGAATCTTGACATCTTGT	-198
	***** * * *	
Lemon	TGGCGACTGGAGTAGGGACGTGTAACAATGTCATCAACTCGTCAAACCAAACCTTCATT	-139
lime	TGGCGACGGGAGTAGGGACGTGTAACAATGTCATCAACTCGTCAAACCAAACCTTCATT	-138
	***** * * *	
Lemon	AAATAAATTAATTACATGGTAGTTTGTAGCCTTAAAGTCTTTGTGGTAAGTAGGAACTA	-79
lime	AAATCAATTAATTACATGGTAGTTTGTAGCCTTAAAGTCTTTGTGGTAAGTAGGAACTA	-78
	***** * * *	
Lemon	CCTACCAACTCTTCCCCCATGTTTTATAAGAACAATAAGAACAGCATGCGCCAGTGTG	-19
lime	CCAACCAACTCTTCCCCCATGTTTTATAAGAACAATAAGAACAGCATGCGCCAGTGTG	-19
	** * * * * * * * * * * *	
	+1	
Lemon	CTCTTCTTACTTCTGCTTAAAAATCTGAAAGAATGAAATAGAAAAAATG 30	
lime	CTCTTCTTACTTCTGCTTAAAAATCTGAAAGAATGAAATAGAAAAAATG 30	
	***** * * *	

Activity analysis of the promoters by transient expression in isolated juice sacs

The ability of the full-length promoter fragments from lemon and lime, LepC1 and LipC1, to confer gene activity was investigated by fusing them to the reporter gene *uidA*

followed by transient transformation into isolated juice vesicles of lemon and lime. The efficiency of the transformation was assessed by co-transformation with a construct containing 35S promoter fused to *gfp*. Typical results of the transformations are shown in Fig. 3. As expected, GFP fluorescence was detected in both lemon and lime

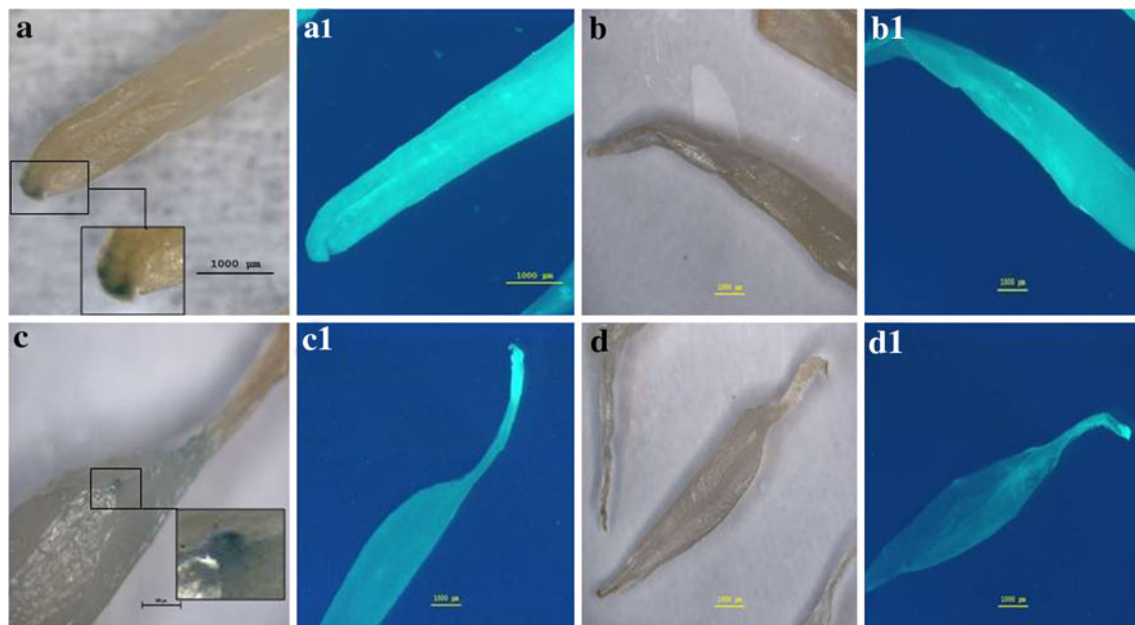


Fig. 3 Promoter activity in acidless lime (**a, b**) and acid lemon (**c, d**) juice sacs by transient expression analysis using lemon promoter (LepC1, **a, c**) and lime promoter (LipC1, **b, d**). GUS-stained

spots are magnified in **a** and **c**. GFP fluorescence can be observed on each juice sac (a1, b1, c1 and d1) by bombardment with the control plasmid (CaMV 35S-GFP)

juice sacs (Fig. 3 a1, b1, c1 and d1). Following a few days of incubation, relatively faint GUS spots were detected in lemon and lime juice sacs transformed with LepC1 (Fig. 3a, c), but not with LipC1 (Fig. 3b, d).

Functional analysis of *C1111* promoter in tomato flower organs

Regardless of the faint GUS staining detected in transformed juice sacs, the above results suggested that the cloned lemon fragment contains active promoter, and the varied expression of *C1111* between the pulps of lemon and lime (Fig. 1a) might be due to sequence differences in the two promoters, or to a missing *trans*-acting factor in lime juice sac cells. Due to inefficient transient transformation of the juice sacs, and because of the complexity of stable transformation in citrus and the long juvenile period of the transformants, the activities of the above constructs and their delimited fragments were examined in tomato which, as discussed below, has been shown to be a promising system to analyze fruit-specific and other promoters from various species. Therefore, we constructed a series of promoter deletions fused to the *uidA* gene and transformed them into tomato. To compare the lime and lemon promoters, the design of the delimitation was based on the sequence homology between them. Most constructs generated 8–10 independent transgenic lines, which were analyzed by GUS staining. The results of all transformed

lines are presented in Supplementary Presentation (SP), while Figs. 4, 5, and 6 present the results of three representative lines. The description of the results, given below, is for all transformed lines.

Most of the lines transformed with the full-length promoter fragment from lemon (LepC1) displayed relatively strong GUS activity in the ovaries, as well as in the anthers (Fig. 4 and SP). In the ovaries, staining was detected in the walls, as well as in the inner parts, including the ovules. In the anthers, GUS activity was detected in the inner part, including the tapetum and pollen grains. In contrast to the lemon fragment, lines transformed with the full-length promoter of the acidless lime (LipC1) showed faint or no GUS activity in the ovaries and anthers. Control plants, as well as plants transformed with the smallest promoter fragments of both varieties (LepC6 and LipC6) did not display any GUS staining in the flower organs. Addition of 200 upstream bases to the lemon promoter (LepC5) generated faint staining in five lines, while two lines showed no staining. No GUS activity was detected in most of the lines transformed with the parallel fragment from lime (LipC5). A further increase in the length of the lemon promoter (LepC4) resulted in a significant increase in GUS activity in most transformed lines, while most of the lines transformed with LipC4 did not show any GUS staining. The lemon fragment containing an additional 120 bp of the upstream sequence (LepC3) generated GUS activity in

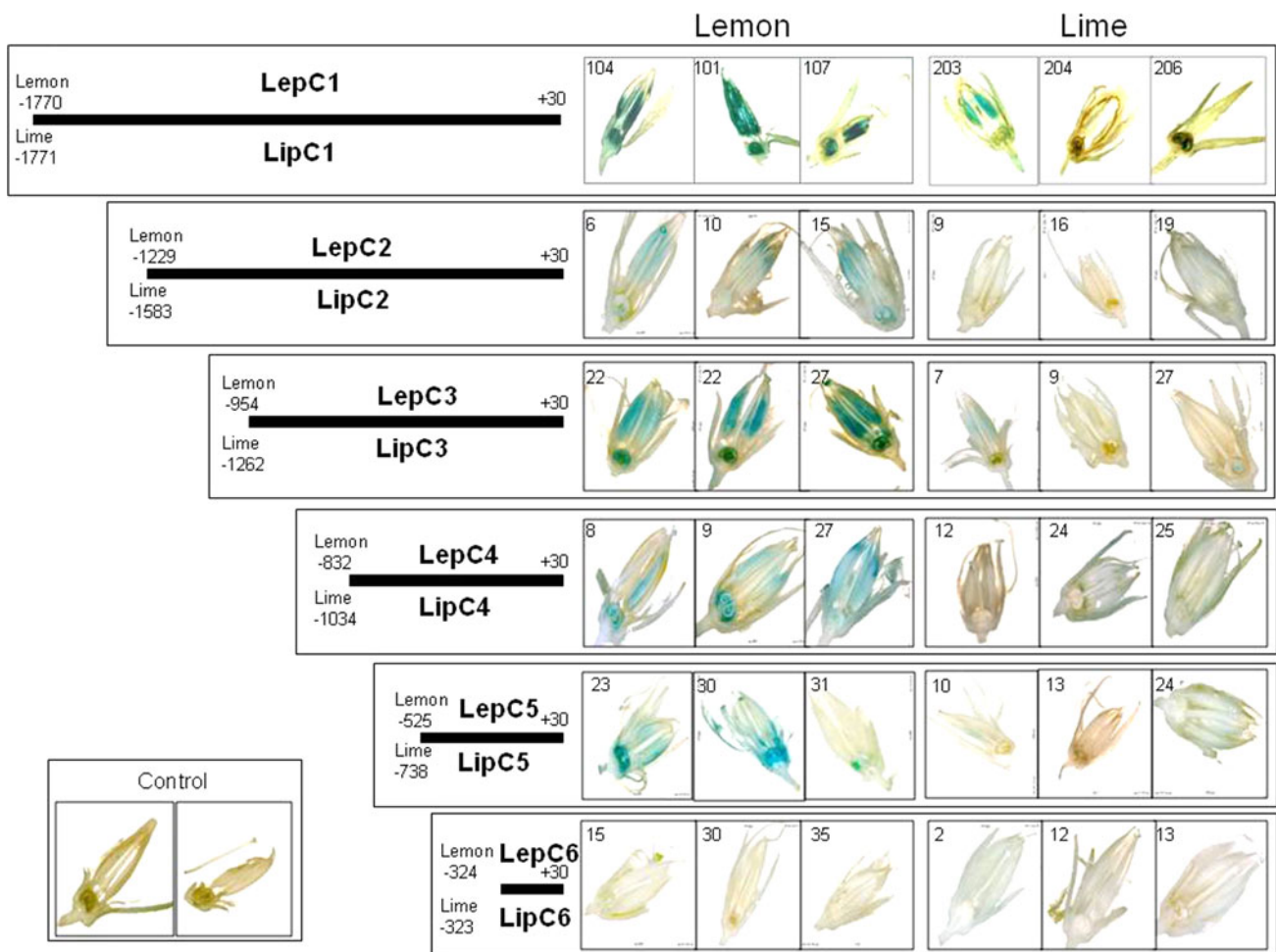


Fig. 4 *C1111* promoter of from acid lemon and acidless lime is active in tomato flowers. GUS staining of flowers was performed in tomato lines transformed with the acid lemon, acidless lime or control construct, as indicated. The positions of the lemon and lime promoter

fragments are marked by *numbers*, and they are similar to those in Fig. 2. *Minus* and *plus signs* mark the position of the nucleotide relative to the putative transcription start site. The *number* in each photograph represents the number of the presented line

both tissues that was comparable to that of the full-length promoter in most transformed lines. The parallel fragment from lime (LipC3) generated no or faint GUS activity in the transformed lines. The addition of a further 300 bp of upstream sequence to the lemon promoter (LepC2) generated a significant reduction in GUS activity, i.e., the GUS staining of most lines was reduced to a faint or very faint level in the ovary and anther (Fig. 4), whereas most transformed lines containing the parallel lime fragment (LipC2) showed no GUS activity.

Variable GUS activity was detected in flower stigmas of about half of the lines transformed with the lemon full-length promoter (LepC1). The same activity emerged in plants transformed with delimited fragments LepC5 to LepC2. No similar staining appeared in lines transformed with the lime full-length promoter (LipC1). However, two lines in plants transformed with LipC2 and LipC3 showed very faint GUS activity in their stigmas.

Functional analysis of *C1111* promoter in tomato fruit and vegetative organs

In most of the lines, transformed with either the lemon or lime promoters or their delimited fragments, variable GUS staining was detected in the seed coat (Fig. 5, seed). In some cases, staining was also evident in the vascular bundles (Fig. 5 and SP). However, variable staining of these tissues was also evident in the control plants, suggesting non-specific staining of the seed coat and fruit transport system.

The full-length fragment from lemon (LepC1) generated staining of a single cell layer in the endocarp in most of the lines (Fig. 5, marked by arrows) which, as explained in the “Discussion”, is analogous to the pulp in citrus fruit. GUS activity in the endocarp appeared to be unique to the lemon promoter, as none of the lines transformed with the lime full-length promoter (LipC1) or its delimited fragments

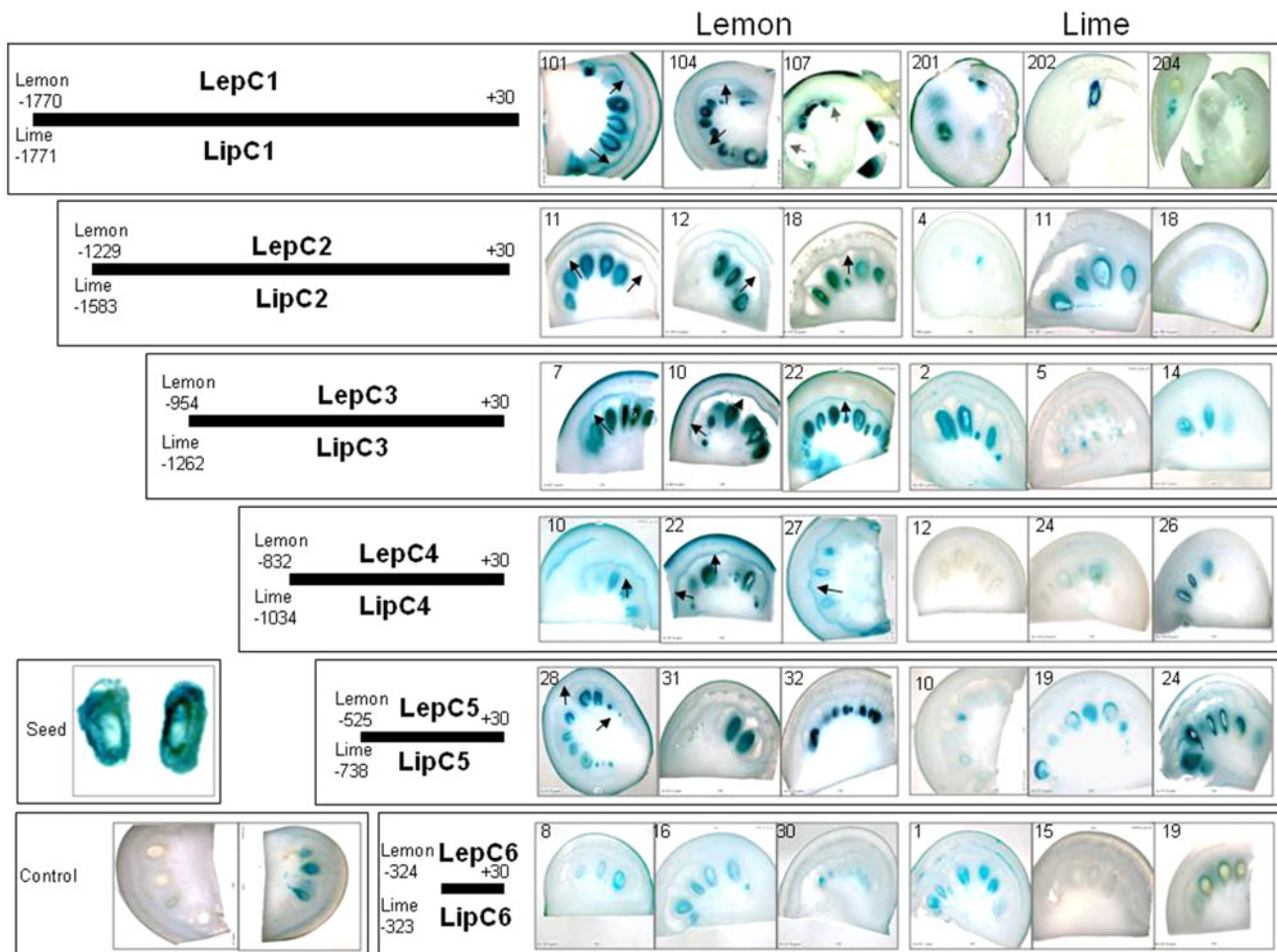


Fig. 5 *C1111* promoter from acid lemon and acidless lime is active in green tomato fruit. GUS staining of green fruits of about 1 cm in diameter was performed in tomato lines transformed with the acid lemon, acidless lime or control construct, as indicated. The positions of the lemon and lime promoter fragments are marked by *numbers*,

and they are similar to those in Fig. 2. *Minus* and *plus signs* mark the position of the nucleotide relative to the putative transcription start site. The *number* in each photograph represents the number of the presented line

(LepC5 to LipC2) showed any activity. The pattern of GUS activity in the endocarp of green fruit with the delimited lemon promoter fragments was generally similar to that in flower organs. The smallest fragment (LepC6) did not generate GUS staining, while LepC5 generated staining in five out of seven lines. Most lines (eight out of nine) transformed with the two larger lemon fragments, LepC4 and LepC3, showed a gradual increase in staining with increasing fragment length. As in the flowers, lemon fragment LepC2 generated remarkably decreased GUS staining in the endocarp of transformed plants.

The full-length promoter fragment from lemon (LepC1) generated strong GUS activity in the fruit peel of five of the nine transformed lines. In contrast, only two of the lines transformed with the lime full-length fragment (LipC1) displayed staining in the green fruit peel (Fig. 5, SP). As in the other organs, the smallest fragments from both varieties

(LepC6 and LipC6) did not generate GUS staining in the peel. Lemon fragment LepC5 generated staining in the fruit peel in five of the transformed lines, while the parallel lime fragment generated relatively strong activity in one line, and faint activity in another (Fig. 5, SP). As in the endocarp, lines transformed with the two larger lemon fragments, LepC4 and LepC3, showed a gradual increase in staining with increasing fragment length: most lines (eight out of nine for both constructs) showed GUS activity in the fruit peel. No remarkable change in GUS staining was evident in lines transformed with LipC4 or LipC3 relative to lines transformed with the smaller fragments. As in the other organs, lemon fragment LepC2 generated a remarkable decrease in GUS staining. The parallel lime fragment generated staining comparable to that of the smaller lime fragment.

As expected from the citrus *C1111* expression data, no GUS activity was detected in tomato stems or roots, or in

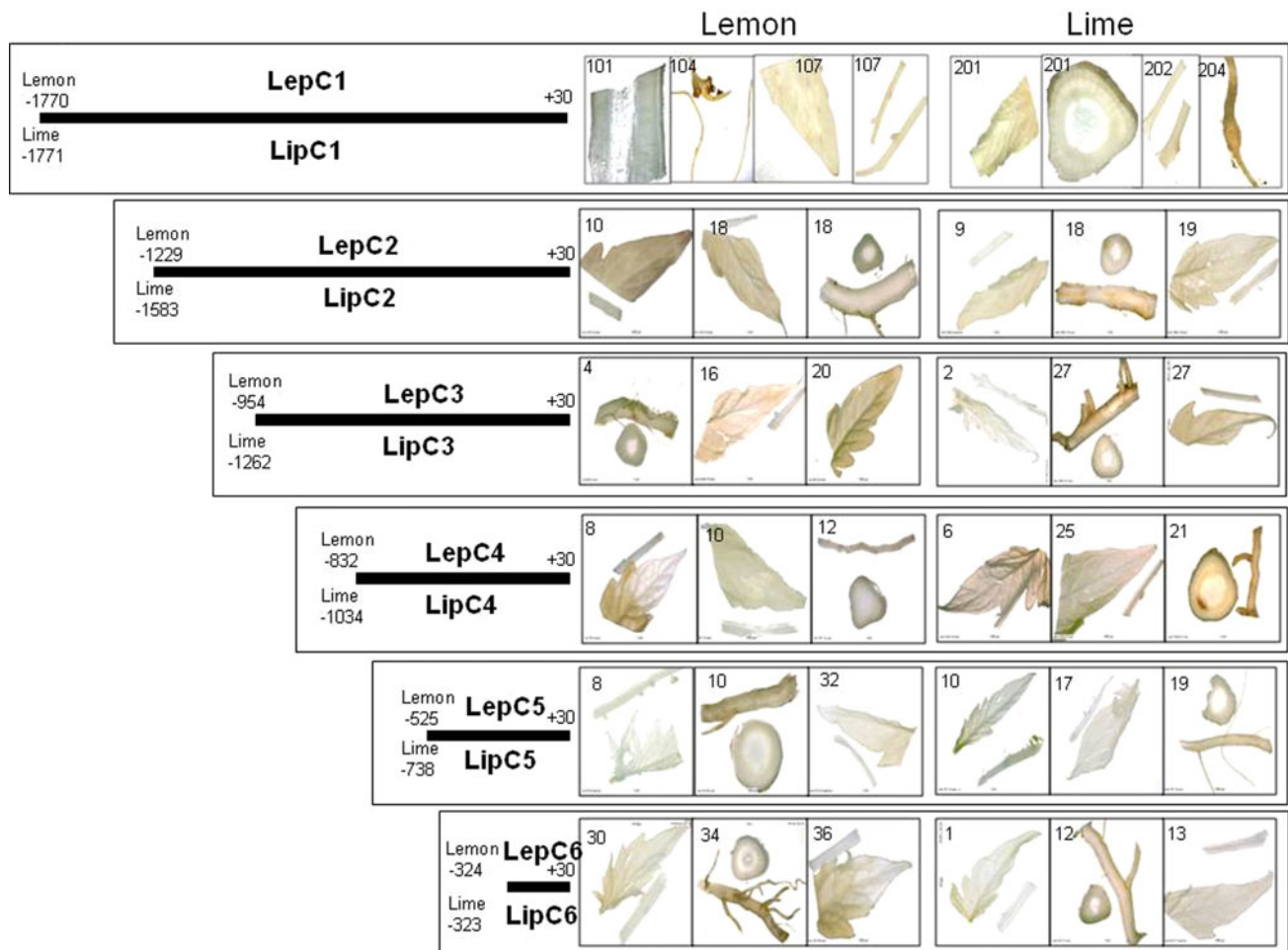


Fig. 6 *CIII* promoter from acid lemon and acidless lime is not active in vegetative organs of tomato. GUS staining of shoots, roots and leaves was performed in tomato lines transformed with the acid lemon or acidless lime construct, as indicated. The positions of the lemon and lime promoter fragments are marked by *numbers*, and they

are similar to those in Fig. 2. *Minus* and *plus signs* mark the position of the nucleotide relative to the putative transcription start site. The *number* in each photograph represents the number of the presented line

petioles or leaves transformed with the lime or lemon full-length promoters (Fig. 6, SP).

Discussion

The cDNA of *CIII* shows significant homology to a number of clones in a few plant species, including two genes in *Arabidopsis*. However, the function of its product is not yet known, and is currently under investigation. Although *CIII* expression levels were higher in acid lemon than in acidless lime (Fig. 1e), its pattern of expression was similar in the ovaries of both varieties, while a remarkable difference in mRNA levels between the two varieties was evident in the juice sacs. Moreover, in most vegetative tissues and the fruit peel, the expression levels of *CIII* were very low or below detection. Based on

these results, *CIII* might be considered an ovary- and pulp-specific gene. To examine this hypothesis, *CIII* promoter was isolated from acid lemon and acidless lime. Sequence comparison showed that the two promoters were nearly identical in the most proximal 300 bp, while more upstream sequences showed varied degrees of homology. Transient expression assay carried out in lemon and lime juice sacs showed faint activity, but it was interesting to note that only the promoter from lemon was active in the juice sacs of both varieties. Hence, although other modes of gene regulation, such as mRNA stability, cannot be excluded, the obvious difference in *CIII* expression profiles between the pulps of the two cultivars could be attributed to the sequence variability between the two promoters.

Several studies have investigated the activity of plant promoters from one species in other species. Tomato is

the preferred system for heterologous expression in most cases, especially when fruit promoters are being analyzed; regardless of taxonomic relationships, promoters from potato, pepper, peach, strawberry, melon and apple showed pronounced activity in tomato, usually similar to the pattern detected in the source plant (Agius et al. 2005; Atkinson et al. 1998; duJardin et al. 1997; Karaaslan and Hrazdina 2010; Kuntz et al. 1998; Rasori et al. 2003; Yin et al. 2009). Therefore, in the present study, tomato was also employed for the functional analysis of *C1111* promoter from citrus. The activity of the *C1111* promoter in tomato generally paralleled the gene's expression in citrus, in terms of both tissue localization and the differences in expression between acid lemon and acidless lime. No expression of the gene in citrus (Fig. 1d) or activity of its promoter in tomato (Fig. 6) were detected in vegetative organs, as demonstrated for other fruit-specific promoters such as *E8* from tomato and *ACC-oxidase* and *PG* from apple (Atkinson et al. 1998; Deikman et al. 1992). The gene was expressed in both varieties in the ovaries, but expression in the acidless lime was lower than that in the acid lemon (Fig. 1c). In tomato, activity of the lime promoter in the ovaries was considerably lower than that of the acid lemon promoter (Fig. 4). The activity in the ovary walls and ovule was similar to that observed for the peach *ACC oxidase* promoter expressed in tomato (Rasori et al. 2003). Similar to the ovaries, there was also a remarkable difference between the activities of the two promoters in the anthers, with the lemon promoter being more active. The gene's expression was not analyzed in the anthers. However, the *C1111* product was localized to the anthers of lemon and lime flowers, with a higher level in the latter, by western blot using antibodies raised against C1111 (results not shown). Activity of the *C1111* promoter generally paralleled the expression of its gene in the fruit as well. GUS staining was evident in four fruit tissues. In plants transformed with lemon and lime promoters, it was detected in the seed coat, the fruit peel, and the vascular bundles. In plants transformed with the lemon promoter, it was also detected in the fruit endocarp (Fig. 5, SP). As already mentioned, fruits of control plants also exhibited variable non-specific staining in the seed coat and vascular bundles, as reported by others (Kosugi et al. 1990; R. Barg, ARO, personal communication). In tomato, as in most berry-type fruits, the endocarp consists of a single cell layer (Almeida and Huber 2001; Ho and Hewitt 1986). In lemon (but not in lime), *C1111* was expressed in the juice vesicles that stem from the endocarp, which is composed of a few cell layers (Cutter 1971; Fahn 1974; Roth 1977). Since the juice vesicles are considered part of the endocarp (Schneider 1968), it might be concluded

that the activity of the promoter in the endocarp of tomato paralleled *C1111* expression in citrus juice vesicles. The only exception to the parallel nature of the gene's expression and its promoter's activity was evident in the peel: while no expression of the gene was detected in citrus fruit peels (flavedo and albedo), promoter activity was detected in the peel of tomato fruit. To the best of our knowledge, the activity pattern of the promoter of *C1111*, particularly in the fruit endocarp, is quite unique in comparison to other fruit promoters, which usually show various levels of activity throughout fruit tissues, pericarp, placenta, locule and collumella (Atkinson et al. 1998, Rasori et al. 2003).

The results suggested the presence of *cis*-acting elements conferring expression in the anther (including pollen), ovary, fruit endocarp and fruit peel. There appeared to be a gradual increase in the activity of the lemon promoter in the above organs from LepC5 to LepC3. The additional upstream sequences in LepC2 markedly reduced the activity, which was fully restored in the full-length fragment. A search for promoter elements using Place (<http://www.dna.affrc.go.jp/PLACE/>) and PlantCare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) revealed many putative regulatory elements—over 400 in each of the promoters. Both promoters contained a few pollen- and anther-specific elements (Jeon et al. 1999; Koltunow et al. 1990; Twell et al. 1990; Wakeley et al. 1998). However, their numbers and distribution in the lemon and lime promoters did not lead to any conclusions on their possible involvement in the activities of the different fragments. To the best of our knowledge, no fruit peel-specific element has been identified to date, nor has a plant ovary-specific element, although an ovule-specific promoter has been reported (Nain et al. 2008). In contrast, a fruit-specific element has been identified in the promoter of the *cucumis* gene from melon (Yamagata et al. 2002). This element contains seven bases, TGTCACA, and the two most critical nucleotides for its activity were identified by *in vitro* analysis (Fig. 7a, marked with asterisks). A homologous element was identified between nucleotides –363 and –371 of the lemon promoter, located in LepC5. As shown in Fig. 7a, the lemon and melon elements can be aligned in two ways, both of which preserve the active nucleotides. LepC5 was the most proximal fragment to confer activity in tomato: most of the plants transformed with this fragment showed GUS activity in the ovaries and in the fruit endocarp. No similar element was found in the lime promoter. We suggest that this element is responsible, at least in part, for the expression of *C1111* in fruit pulp. Plants transformed with LepC2 and LipC2 showed a reduction in GUS activity as compared to those transformed with the shorter fragments and with the full-

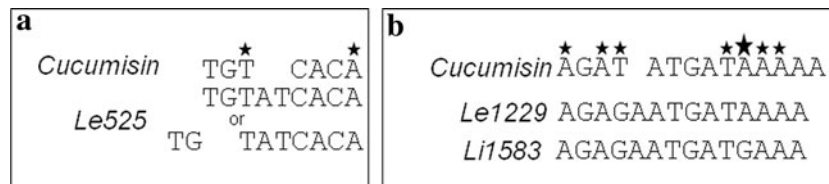


Fig. 7 *Cl111* promoter contains sequences homologous to regulatory elements from the promoter of the *cucumis* gene. Positive fruit-specific regulatory element **a** is aligned with a sequence found between bases –363 and –371 of the lemon promoter. Negative regulatory element, I-Box, from the *cucumis* gene is aligned with

length promoter, suggesting that a negative element(s) exists in between –954 and –1,229 bp in the lemon promoter and between –1,262 and –1,583 bp in the lime promoter. Indeed, these fragments contain a sequence homologous to a 14-base element of the *cucumis* promoter, termed I-Box (Yamagata et al. 2002), between bases –1,064 and –1,077 of the lemon promoter and bases –1,387 and –1,400 of the lime promoter (Fig. 7b). I-Box has been shown to play a role in downregulating the *cucumis* gene in melon fruit, and *in-vitro* analysis has identified nucleotides which are important for its activity (Fig. 7b, marked with asterisks). We suggest that the I-Box-like sequences are negative regulatory elements which reduce the expression of *Cl111*, at least in the fruit. However, the full-length promoters must contain other sequences which suppress the action of the I-Box. Moreover, the presence of fruit-specific and I-Box elements, similar to those found in the *cucumis* gene, can only partially explain the activity of the lemon promoter, and the differences between it and the lime promoter. The increase in GUS staining in LepC4 and LepC3 relative to LepC5 suggests the presence of further *cis*-acting elements with enhancer activity.

Gene transfer technology is enabling the metabolic engineering of plant organs, thus providing a better understanding of the regulation of complex metabolic processes. The identification of organ- and tissue-specific promoters is a long-term goal of the citrus research community. The promoter of *Cl111*, described here, is confirmed to be a pulp-, and flower organ-specific promoter which might be used in the future for citrus metabolic engineering. Although further analysis of this promoter is required to define specific sequences conferring expression in the pulp, ovary and anther, the results demonstrate that the region between bases –324 and –954 in the lemon promoter contains elements conferring expression in the above organs.

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homologous sequences found between bases –1,064 and –1,075 of the lemon promoter and bases –1,387 and –1,398 of the lime promoter. **b** Small asterisks denote nucleotides important for the activity of the *cucumis* promoter elements, while the large asterisks denotes the most important nucleotide for promoter activity

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