Running title: Flexible tools for gene expression and silencing in tomato

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Flexible tools for gene expression and silencing in tomato

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[Wi] The online version of this article contains Web-only data.
ABSTRACT

As a genetic platform, tomato (*Solanum lycopersicum*) benefits from rich germplasm collection, ease of cultivation and transformation, and enables the analysis of biological processes impossible to investigate with other model species. To facilitate the assembly of an open genetic toolbox designed to study Solanaceae, we initiated a joint collection of publicly available gene manipulation tools. We focused on the characterization of promoters expressed at defined time windows during fruit development, for the regulated expression or silencing of genes of interest. Five promoter sequences were captured as entry clones compatible with the versatile MultiSite Gateway format: PPC2, PG, TPRP, and IMA from tomato, and CRC from *Arabidopsis thaliana*. Corresponding transcriptional fusions were made with the GUS gene, a nuclear localized GUS-GFP reporter, and the chimeric LhG4 transcription factor. The activity of the promoters during fruit development and in fruit tissues were confirmed in transgenic tomato lines. Novel Gateway destination vectors were generated for the transcription of artificial microRNA (amiRNA) precursors and hairpin RNAs under the control of these promoters, with schemes only involving Gateway BP and LR clonase reactions. Efficient silencing of the endogenous phytoene desaturase (*PDS*) gene was demonstrated in transgenic tomato lines producing a matching amiRNA under the CaMV 35S or PPC2 promoter. Lastly, taking advantage of the pOP/LhG4 two-component system, we found that well characterized flower-specific Arabidopsis promoters drive the expression of reporters in patterns generally compatible with heterologous expression. Tomato lines and plasmids will be distributed through a new NASC service unit dedicated to Solanaceae resources.
Solanaceae provide the world’s most important vegetable crops, including tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), and pepper (*Capsicum annuum*), and are a model eukaryote family for evolutionary, genetic and genomic studies. More than 3,000 Solanaceous species grow in habitats ranging from rain forests to deserts and mountains with regular snowfall. Thanks to conserved genome organization, this family is an excellent subject to explore the genetic and molecular basis of adaptation to diverse environments. Within the Solanaceae, tomato is a broadly used model system for studying plant-microbe interactions and the biology of fleshy fruits, owing to its simple diploid genetics, the short generation time, the routine protocols for production of transgenic plants and its exceptional publicly available genetic and genomic resources. These resources include large collections of single-gene mutations, an extensive EST database, a high-density genetic map, microarrays, an emerging genome sequence and well characterized populations designed for genetic mapping of simple and quantitative trait loci, and are used by a vibrant Solanaceae research community involving research laboratories in over 30 countries focused on both basic and strategic research (Knapp, 2002; Mueller et al., 2005; Wu et al., 2006).

The first draft sequence of the entire euchromatic portion of the tomato genome will soon be available (Mueller et al., 2009). As already witnessed for numerous other research communities focusing on key species, the availability of a well annotated genome will mark the onset of new lines of investigations and will greatly facilitate the characterization of genetic functions. Taking advantage of the EU-SOL European integrated project (http://www.eu-sol.net/), we initiated a collaborative effort for the construction of publicly available tools enabling gene manipulation at specific stages of tomato fruit development and in well defined tissues and cell types. Tomato is a berry with a thick pericarp that encloses many seeds. The fruit results from the fusion of a number of carpels, the ovary wall becoming the fleshy pericarp. Pollination triggers cell proliferation, followed by extensive cell expansion, resulting in a mature fruit that ranges from 1 to 1000 grams (Gillaspy et al., 1993; Lemaire-Chamley et al., 2005; Gonzalez et al., 2007). Fruit development and ripening are also characterized by dramatic metabolic shifts, including the conversion of starch to sugars, chloroplasts to chromoplasts and extensive cell wall disassembly, all under tight genetic control (Giovannoni, 2004). Changes in the spatial or temporal expression of regulatory genes or downstream effectors controlling these processes will substantially affect fruit tissue properties and the ripening process (Manning et al., 2006; Giovannoni, 2007). Indeed, the ability
to alter subtly the timing and location of expression of key regulators is an important
goal for crop biotechnology, but is often constrained by lack of suitable promoters.

To this end, a core set of versatile resources designed for temporal and tissue-specific
manipulation of gene expression in the tomato fruit has been created by independent
laboratories. All clones and vectors were constructed with the flexible Gateway recombinational
cloning technology (reviewed in Karimi et al., 2007a). Promoters that displayed tissue-specific
transcriptional activity have been captured as Gateway entry clones. These regulatory sequences
can be combined at will with any gene, hairpin RNA (hpRNA) expression cassette, or artificial
microRNA (amiRNA), via MultiSite Gateway protocols, for the creation of novel binary
T-DNA vectors. Expression of reporter genes in specific cell types illustrated the functionality
of such vectors. Silencing of the endogenous gene coding for phytoene desaturase
with amiRNA in stable transgenic tomato plants demonstrated that such vectors can be used to
efficiently control transcript abundance in fruit tissues. In addition, tomato
“driver” lines were generated for the transcriptional activation of any responder
transgene locus, based on the LhG4 two-component system. Expression of several
reporters and phenotypes induced with these lines facilitated their communal use for
genetic manipulations. The assembly of a Solanaceae genetic toolbox is an
important asset for future functional analysis in tomato and related crops.

RESULTS AND DISCUSSION

Cloning strategy and structure of the vector collection

The Gateway MultiSite recombinational cloning framework is ideally suited for
constructing versatile collections of genetic elements to be assembled in pre-
determined order, orientation, and open reading frame (ORF) registry (Karimi et al.,
2005, 2007a, 2007b). The overall strategy underlying our vector resources is
illustrated in Figure 1. Three types of elements have been considered so far: plant
promoters, to direct the transcription of any gene of interest in well-defined tomato
tissues; ORFs, to produce the encoded proteins; and gene-specific sequence tags
(GSTs) or amiRNA precursors to silence targeted genes.

Each element was first captured in an entry clone, either via
restriction/ligation cloning in a plasmid carrying multiple restriction sites flanked by
attL and attR recombination sites, or via PCR amplification and addition of flanking
attB sites, followed by a BP clonase reaction. We chose to clone all promoters in
attL4-promoter-attR1 cassettes, and all other elements in attL1-sequence-attL2
cassettes. The resulting entry clones can be recombined with various Gateway
destination vectors in LR clonase reactions, to create expression vectors.
Interestingly, two or more elements available as entry clones can be assembled via a
single MultiSite LR clonase reaction in the order and orientation defined by the
position of the attL and attR sites flanking the recombined sequences (Cheo et al.,
2004; Karimi et al., 2007b). In our configuration, any promoter can be positioned
easily upstream of an ORF, a gene-specific sequence tag (GST), or an amiRNA
precursor and drive its tissue-specific transcription in transgenic plants. Lastly, novel
modular destination vectors can be constructed from any expression clone via
reverse BP clonase reactions, further expanding the possible cloning schemes
(Karimi et al., 2007b).

**Fruit-specific promoters**

Flowers and fruits are the main targets for genetic improvement of fruit crops.
However, these organs are the last to form and their manipulation with broadly
expressed promoters is therefore restricted to non-pleiotropic genes. Promoters
active solely in these organs bypass this limitation and permit the functional analysis
of all genes involved in fruit development. Such sequences facilitate the engineering
of fruit quality, either directly or providing proof of concept information from which
strategies can be developed to harness natural variation.

As an initial set of regulatory sequences of interest, we chose four promoters
from tomato genes shown previously to be transcribed specifically at different stages
of fruit growth and maturation. In addition, we included a well-characterized
Arabidopsis promoter of which the activity is restricted to specific carpel and nectary
tissues during flower development (Table I). The five promoter sequences are
documented in Supplemental Table S1. The anticipated temporal and spatial
transcriptional activity in the fruit for each cloned promoter is summarized below. The
size of each captured sequence is also indicated in parentheses. They all end in a
window of 1 to 34 nucleotides upstream of their cognate ORF.

**IMA** (0.5 kb). *INHIBITOR OF MERISTEM ACTIVITY* (accession no.
AM261628) encodes a 90 amino acid-long tomato protein that harbors a putative
central zinc finger domain similar to the recently characterized MINI ZINC FINGER
proteins (Hu and Ma, 2006; Sicard et al., 2008). While absent from vegetative
organs, **IMA** expression is detected in all floral organs, and to a greater extent in
carpels and petals. However, the highest expression was observed in the developing
fruit. **IMA** transcripts are detected as early as the pre-anthesis stage corresponding to
isolated carpels, accumulate gradually and strongly in fruit to reach a maximum at 10 days after anthesis (daa), then decrease to reach basal levels at the mature green stage. IMA acts as an inhibitor of cell proliferation during flower termination (Sicard et al., 2008). Its promoter sequence was identified via the Solanaceae Genome Network (SGN) database in a BAC clone encompassing the IMA gene sequence (accession no. AC122544).

**CRC** (3.7 kb). The Arabidopsis CRABS CLAW gene belongs to the YABBY transcription factor family (Bowman and Smyth, 1999). It is required for nectary development in Arabidopsis flowers and for abaxial-adaxial polarity specification of carpels. The CRC promoter is active throughout carpel primordia initiation, but becomes restricted to the valve abaxial domain upon anthesis. In addition, it is expressed in central placental domains and in nectaries throughout their development (Lee et al., 2005). This promoter was chosen because it marks the earliest stages of carpel initiation, a pattern not presently available from endogenous tomato promoters.

**TPRP** (2.6 kb). TPRP is a cell wall tomato proline-rich protein (Salts et al., 1991). TPRP expression levels are high in all tissues of young tomato fruits, corresponding with cell division phases II and III of fruit development (Gillaspy et al., 1993; unpublished data). Promoter activity is dramatically reduced in mature green and ripe fruits, and completely absent in all other parts of the plant (Salts et al., 1991; Carmi et al., 2003). The TPRP promoter has been used successfully to down-regulate the expression of the DE-ETIOLATED1 (DET1) gene resulting in an increase of the carotenoid and flavonoid content restricted to the fruit (Davuluri et al., 2005).

**PPC2** (2.0 kb). The PPC2 gene codes for a phosphoenolpyruvate (PEP) carboxylase isoform present in developing tomato fruits that is presumably involved in organic acid accumulation and CO₂ fixation. LYCes;PPC2 is highly and specifically expressed during the phase of rapid fruit growth, corresponding to cell expansion. Expression increases initially at the end of cell division and decreases at the beginning of ripening. In young growing fruits, LYCes;PPC2 mRNA has been specifically located by in situ hybridization in the pericarp and the gel surrounding the seeds (Guillet et al., 2002). Accordingly, the 2.0-kb promoter fragment has recently been shown to direct fruit-specific GUS expression during the cell expansion phase in the placenta, gel and later in the pericarp tissues in transgenic tomato plants (Guillet et al., 2009).

**PG** (4.8 kb). Polygalacturonase (PG) is a cell wall hydrolase abundantly secreted during fruit ripening and contributing to fruit softening. PG expression is
tightly related to the ripening process. Transcriptional activation at the onset of ripening results in high level of PG mRNA (Biggs and Handa, 1989; DellaPenna et al., 1989). Within the ripening fruit, a 1.4-kb promoter fragment has been shown to direct GUS expression mostly to the outer region of the pericarp and the columella tissue in transgenic tomato plants (Montgomery et al., 1993). But a longer (4.8 kb) PG promoter was preferred because it yields higher levels of chloramphenicol acetyl transferase reporter activity in ripening transgenic tomato fruits (Nicholass et al., 1995) and has been successfully used to engineer metabolite fruit content (Davidovich-Rikanati et al., 2007; Kovacs et al., 2007).

The PPC2, TPRP, and IMA promoters were amplified by PCR with sense and antisense primers including the attB4 and attB1 sequences, respectively. The amplified fragments were recombined with pDONRP4-R1 to produce the corresponding entry clones. The larger PG and CRC promoters were introduced by conventional restriction/ligation cloning into pEN-L4R1 in which a multicloning site is flanked by the attL4 and attR1 sequences.

Expression of the GUS reporter in tomato fruits

In the context of MultiSite Gateway expression constructs, the described promoters (pEN-L4promoterR1) were fused to the GUS gene (pEN-L1SIL2) (Karimi et al., 2007b) in the pK7m24GW destination vector via MultiSite LR clonase reaction (Karimi et al., 2005) (Table II). Initial validation of promoter activity was achieved with a previously described fruit transient expression assay (Orzaez et al., 2006). Briefly, the promoter-GUS binary expression vectors were transformed into Agrobacterium tumefaciens and injected into fruits of different developmental stages, from 20 daa to breaker + 11 days (Br+11). Several days after injection, fruits were sampled and thin sections were analyzed for GUS activity via classical histochemical assays (Supplemental Fig. S1 and Supplemental Materials and Methods). GUS staining consistent with previously published expression results were obtained for late stage promoters, such as in PGpro:GUS injected ripening fruits, but the assessment of the promoter activity in the transient assay was restricted to more than 20–daa-old fruits and concurrent with wounding and stress. These technical constraints probably explain why temporal and spatial expression might be partly artifactual in agroinjected fruits. For example, the early-stage IMA promoter was active in ripening fruits, and higher level of GUS staining was observed in placenta and gel, presumably because of a better penetration of Agrobacterium in these tissues. Nevertheless, transient expression data provided the initial evidence that the
Gateway cloning framework was compatible with gene expression in tomato, a prerequisite for in depth analysis of promoter activity in stable transgenic plants.

To this end, the MicroTom cultivar was agroinfected with an improved protocol yielding transformation frequency consistently >80% (see Materials and Methods). The ploidy of regenerated plantlets was measured with flow-cytometry and polyploid plants were discarded. Between 12 and 25 independently transformed plants were generated for each promoter-GUS transgene (Table II). Following GUS staining and selection of plants with one or two transgene copy, two representative lines were selected for each construct and the GUS staining pattern of 10 kanamycin-resistant T1 plants per original line were characterized at specific stages of development: flower, 6 mm buds (~stage 9 according to Baldet et al., 2006), and anthesis; fruit, 6 daa (cell division), 10 daa, 15 daa (cell expansion stage) (Lemaire-Chamley et al., 2005), mature green (MG, transition to ripening), and Br+7 (ripe fruit). GUS staining of T1 leaves and entire young T2 plantlets completed the analysis.

As expected, strong GUS activity was observed in all 35Spro-GUS tissues while no staining was detected in wild-type controls (Fig. 2). The five selected fruit promoters only displayed fruit-specific expression, with no GUS staining in vegetative organs (Fig. 2). Flower buds showed weak GUS staining only in the IMApro:GUS, TPRPpro:GUS, and PPC2pro:GUS lines.

In agreement with earlier reports (Sicard et al. 2008), IMApro:GUS fruits displayed weak but consistent GUS staining at early stages of development, particularly in placental and vascular tissues. Additional staining of seed tegument was observed in ripe fruit (Fig. 2). CRCpro:GUS fruits displayed a remarkable GUS staining restricted to the outer carpel wall of from early stages until MG (Fig. 2). This result is reminiscent of the CRC native expression that is largely restricted to the abaxial epidermis in the Arabidopsis carpel (from the base to the tip of the carpel, along its entire circumference), where it promotes abaxial cell fate (Eshed et al. 1999). In TPRPpro:GUS fruits, GUS staining peaked at the immature green stage (Fig. 2), consistent with earlier reports (Gillaspy et al., 1993). PPC2pro:GUS fruits displayed strong GUS staining limited to the fruit expansion phase (Fig. 2), in agreement with published data (Guillet et al., 2002, 2009). In PGpro:GUS fruits, GUS activity was not detected in early stages, was weak at the onset of ripening (MG) and high in the ripe fruit (Br+7) (Fig. 2), again as published previously (Montgomery et al., 1993).

In summary, the temporal regulation of the five promoters observed in stable transgenic tomato fruits was in agreement with the data reported in wild-type or stable transgenic plants. In addition to targeting selected stages of fruit development,
several tested promoters also offer the possibility to address transgene expression to specific fruit tissues. For example, the CRC promoter can specifically target genes expressed in the epidermis of developing tomato fruit and involved in cuticle or phenylpropanoid synthesis (Mintz-Oron et al., 2008).

GFP markers specific to fruit cell types

Promoter-GUS transgenes were useful to report tissue-specific transcription. Yet higher spatial resolution can be achieved via the transcriptionally-regulated expression of nuclear localization signal (NLS) fused to fluorescent proteins. Such markers enable the selection of particular cell types, as well as the tracking of cell proliferation and enlargement in the course of fruit development. Therefore, we generated a promoter:NLS-GFP-GUS construct for each of the five selected promoters via LR recombination into the destination vector pK7S*Nm14GW (Karimi et al., 2007b) (Table II). Stable tomato (cv. MicroTom) reporter lines carrying each of the promoter:NLS-GFP-GUS transgenes (Table II) were generated, screened and analyzed as described above. In addition, GFP production was verified in fresh 150 µM-thick pericarp sections with epifluorescence microscopy. Promoter:GUS and promoter:NLS-GFP-GUS lines displayed similar GUS staining patterns (data not shown).

The NLS fused to GFP proved very effective because the fluorescence signal was detected in the nucleus of all cell types in the pericarp and gel of 35Spro:NLS-GFP-GUS fruits (Fig. 3, A and B). In the pericarp of PPC2pro:NLS-GFP-GUS fruits, GFP was absent from the fruit epidermis and from most underlying dividing cells, and was essentially limited to the large expanding mesocarpic cells (Fig. 3, C and D). This observation is consistent with the hypothesis that malate synthesized via PECase serves as a counterion for potassium that accumulates in the vacuole, thus providing the driving force for fruit cell enlargement (Guillet et al., 2002).

To conclude, in addition to the information provided by promoter-GUS transgenic lines about expression targeted at specific developmental stages and in particular tissues, promoter:NLS-GFP-GUS lines can be used to define the spatial distribution of a transgene driven by a selected promoter in tomato fruits with higher cellular resolution.

Vectors for silencing genes in fruits
RNA-mediated gene silencing -or RNA interference (RNAi)- is a method of choice to generate loss-of-function phenotypes caused by partial to complete gene knock-down. Artificial microRNAs (amiRNAs) have been shown to efficiently silence genes in multiple plant species, including tomato (Alvarez et al., 2006; Schwab et al., 2006; reviewed in Ossowski et al., 2008). This approach entails the expression of an endogenous plant microRNA precursor engineered to yield a 21-nucleotide double-stranded amiRNA that is chosen to silence a gene -or several genes- of interest according to experimentally defined target selection parameters. The DNA sequence coding for an amiRNA can easily be synthesized by overlapping PCR amplification (Schwab et al., 2006) with the addition of the attB1 and attB2 recombination sites at their 5’ and 3’ end, respectively, and subsequently captured as an amiRNA entry clone (attL1-amiRNA-attL2). Once in this format, any amiRNA can be cloned downstream of any promoter of interest, including those described above, in a simple MultiSite LR clonase reaction. The addition of attB-flanking sites to an amiRNA precursor (i.e. to the MIR319a backbone in this particular case) did not alter its ability to silence a target gene in Arabidopsis (Supplemental Fig. S2; Supplemental Materials and Methods).

To confirm silencing in tomato, we generated MicroTom lines in which the PDS gene was silenced via the transcription of a matching amiR-pds precursor under the control of the CaMV 35S or PPC2 promoter (Tables I and II). The PDS enzyme is involved in carotenoid biosynthesis and protects chlorophyll from photo-oxidation in developing tissues. PDS silencing causes photo-bleaching that can be readily recorded in the course of development. Bleaching was observed in all tissues of the 35Spro:amiR-pds tomato plants (Fig. 4, B and C). The most severely affected plantlets did not survive in the greenhouse (data not shown). In contrast, the 18 PPC2pro:amiR-pds primary transformants did not display any visual phenotype during vegetative growth. However, one third of these T0 plants produced fruits that remained light orange during ripening and only turned light red when left on the plant (Fig. 4, D and E). Our observations are consistent with the PPC2 profile documented through reporter expression and indicating that the promoter activity is the highest at the early stages of fruit development (Fig. 2) and in the mesocarp (Fig. 3C), i.e. at developmental stages and in fruit tissues less sensitive to light than the exocarp where the 35S-driven expression is very high (Fig. 3A) and the photobleaching more likely to occur. Our results further illustrates how fruit promoters and constructs developed herein can be used to target specific fruit tissues and alter expression of candidate genes in tomato.
Because amiRNA target recognition relies on a short 21-nucleotide sequence, amiRNAs yield more specific silencing than the numerous small interfering RNAs derived from the long (hundreds of bp) double-stranded hpRNA molecules. However, because many genes encoded in Solanaceae have yet to be identified, hpRNA silencing is still valuable: for example to avoid the degradation of a gene different from the target but serendipitously containing sequences matching a selected amiRNA, or to silence simultaneously multiple closely homologous transcripts.

Therefore, we constructed a new series of hpRNA expression vectors with the aim to restrict gene silencing to specific tissues or organs (Supplemental Table S5). For this purpose, we implemented novel cloning schemes involving only BP and LR clonase reactions to position any promoter available as an entry clone in front of a silencing transgene (Supplemental Fig. S3, S4 and S5; Supplemental Materials and Methods). The constructs generated were derived from the pHELLSGATE12 vector (Wesley et al., 2001; Helliwell and Waterhouse, 2003; Hilson et al., 2004) in which the sequence targeted for silencing (GST), originally captured in an entry clone (att\textsubscript{L1}-GST-att\textsubscript{L2}), was transferred simultaneously in two independent Gateway cassettes separated by an intron spacer (att\textsubscript{R1}-ccdB-att\textsubscript{R2}-intron_spacer-att\textsubscript{R2}-ccdB-att\textsubscript{R1}). This spacer consisted of two head-to-head introns that enabled splicing of the encoded transcript regardless of its orientation. In this configuration, the att\textsubscript{R1} and att\textsubscript{R2} recombination sites were inverted with respect to one another, so that the two copies of the target sequence were inserted as inverted repeats into the resulting hpRNA expression clone (Helliwell and Waterhouse, 2003).

For both hpRNA and amiRNA silencing, software is available that selects the most likely specific target sites taking into consideration entire genome sequences: for example, SPADS for the design of GSTs cloned into hpRNA expression vectors (Thareau et al., 2003; Sclipe et al., 2007); and Web MicroRNA Designer for the selection of amiRNA sequences introduced into microRNA precursor backbone by overlapping PCR (http://wmd3.weigelworld.org/). Nevertheless, some amiRNAs and hpRNAs fail to yield efficient silencing and current algorithms cannot accurately predict silencing efficiency.

**Driver constructs and tomato lines**

In tomato, as in most crop plants, transformation protocols require tissue culture. However, because many tissue- and cell type-specific promoters display extensive expression in callus, transgenes with deleterious effects might be selected against during tissue culture. A solution to this problem is to dissociate the target
gene expression locus from its transcriptional regulator. For this purpose, we established reference tomato driver lines facilitating both gene misexpression and silencing. These lines were constructed with elements of the pOp/LhG4 system engineered for transcriptional activation into plant species (Moore et al., 1998; Rutherford et al., 2005; Wielopolska et al., 2005; Ori et al., 2007). Its main components are the LhG4 chimeric transcription factor, consisting of the bacterial LacI DNA-binding and yeast GAL4 activation domains, and the synthetic pOp promoters containing multiple copies of the lac operator bound by LhG4. The driver locus codes for the LhG4 gene under the control of a promoter with a particular spatio-temporal pattern. The responder locus contains a synthetic pOp promoter driving the transcription of a gene, hpRNA or amiRNA of interest, and is only active in the presence of LhG4. Both loci were combined in the same plant either by crossing or by transformation.

Firstly, we created an array of reporter lines to validate the use of the pOp/LhG4 system in tomato, including pOP:GUS, pOP:ER-GFP, pOP:dsRFP, and pOP:KAN1. The first three reporters were useful for the detailed examination of promoter activity. The pOP:KAN1 reporter drove the potent tomato KANADI1 gene that imposes abaxial identity and severe growth arrest whenever expressed ectopically outside of its normal domain (Eshed et al., 2001). This reporter provided a simple and sensitive test for spatial activity of any examined driver line because even low level of expression –possibly escaping detection with the other reporters– might result in clearly visible phenotypes. The KAN1 reporter is particularly relevant to reveal background activity within large organs, such as the tomato fruits, that are difficult to characterize via classical histological staining or microscopical analysis.

Secondly, we crossed these reporter lines with tomato driver lines in which the LhG4 transgene was under the control of one of five Arabidopsis flower-specific promoters (described in Pekker et al., 2005). Significantly, the Arabidopsis FIL, AP1, AP3, and CRC promoters maintained their specific expression domains in F1 tomato plants expressing both a driver and reporter construct. The FIL promoter stimulated expression in all organ primordia, but not in the shoot apical meristem proper (Fig. 5, A and B). The AP1 promoter drove expression in the sepals, petals, and flower meristems, because all were strongly inhibited upon transactivation of KAN1 (Fig. 5, C and D), and the AP3 promoter only in the petals and stamens, of which the petals became radialized upon transactivation of KAN1 (Fig. 5, E and F). The CRC promoter strongly inhibited carpel development upon KAN1 transactivation, but nowhere else (Fig. 5, G and H). Only one promoter, FUL, failed to show the expected early expression in the pericarp (data not shown). The proportion of observed vs.
expected patterns was in the same range as that obtained when attempting to systematically recapitulate transcription pattern with cloned promoter sequences (Lee et al., 2006). To conclude, defined Arabidopsis promoters are a useful resource to misexpress or silence genes in other species, such as tomato.

Lastly, we have expanded the repertoire of driver constructs by generating via MultiSite LR recombination all of the transcriptional fusions between each of the tomato fruit promoters described above and the LhG4AtO ORF, optimized for expression in plants (pEN-L1LHATOG4L2) (Karimi et al., 2007b) (Table II).

**Perspectives**

All plasmids and seed stocks described here are made available via the Nottingham Arabidopsis Stock Centre (NASC) (http://Arabidopsis.info) to ensure long-term distribution and appropriate documentation of the materials. The centralization of resources and related information at the SGN website (Mueller et al., 2005) and complemented at NASC will also facilitate their integration into reference genome browsers as well as genotype and phenotype databases. As demonstrated by the Arabidopsis community, shared genetic tools promote rapid and significant scientific achievements, which is particularly relevant for a plant family of high commercial value. For example, the tomato promoters available as Gateway entry clones might be used to characterize genes involved in key processes taking place during the successive phases of fruit development, namely cell proliferation (TPRP and CRC; mitotic cycle), cell expansion (PPC2; endoreduplication, starch synthesis and storage, cell wall synthesis) and ripening (PG; cell wall and starch breakdown, soluble sugar and carotenoid accumulation), or in specific fruit tissues (IMA, vascular tissues; CRC, cuticle formation and secondary metabolism). Promoters only -or mainly- active in flower or fruit should avoid some of the common pitfalls associated with strong constitutive promoters (such as CaMV 35S) that also affect vegetative plant parts and therefore hamper the analysis of gene function, as clearly illustrated for PDS amiRNA silencing in tomato.

Finally, it is worth noting that the Gateway clones described here and in a recent companion paper (Estornell et al., 2009) are compatible with a wide range of complementary entry clones (coding for alternative terminators, reporters or activators) and destination vectors designed for various functional assays in plants or in other heterologous systems (reviewed in Karimi et al., 2007a).
MATERIALS AND METHODS

Bacterial strains

Host *Escherichia coli* strains were either DH5α (with entry and expression clones) or DB3.1 (with destination vectors) (Invitrogen, Carlsbad, CA, USA). Bacterial cultures were grown at 37°C in Luria broth (LB) medium with appropriate antibiotics.

BP and LR clonase reactions

Additional information about the basic Gateway site-specific recombinational cloning protocols is provided on line by the manufacturer (http://www.invitrogen.com/). Entry clones were created in BP clonase reactions with 50-100 ng of PCR product and 50 ng of donor vector. Expression clones were created in LR clonase reactions with 30 ng of each entry clones and 50-80 ng of destination vector. Reactions were done in 10 μl total volume containing 2 μl of BP or LR clonase, incubated at 25°C overnight. The reaction was inactivated by addition of 1 μl of proteinase K and 5 μl from each reaction was used to transform *E. coli* competent cells as described.

Promoter entry clones

Specific oligonucleotides were designed to amplify the *PPC2*, *TPRP*, and *IMA* promoters, including the attB4 and attB1 sites upstream of the 5’ and 3’ gene-specific priming sequences, respectively (Supplemental Table S2). Promoters were amplified by PCR with either tomato genomic or plasmid DNA as template. PCR mix contained 0.5 μM of each primer, 25 ng template DNA, 200 μM of each dNTP, 2.5 U of Platinum HiFi DNA polymerase (Invitrogen), 1 x PCR buffer and 1 mM MgSO4. Programmed cycles were as follows: 5 min initial denaturing step at 94°C; 30 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 50°C, 1 to 3 min extension (1 min per kb) at 68°C, and 5 min termination at 68°C. PCR products were purified with the High Pure PCR Purification Kit (Roche Diagnostics, Brussels, Belgium). BP clonase recombination reactions were done with the purified PCR products and pDONRP4-P1R to generate the entry clones. After transformation into DH5α competent cells, colonies were screened by PCR for the correct insert size and clones were sequence validated.
Large DNA fragments are more difficult to capture into entry clones. Because the PG and CRC promoters were 4.8 kb and 3.7 kb, respectively, we chose conventional restriction/ligation techniques to build the corresponding entry clones. The PG promoter originally cloned into pBIN19 (Nicholass et al., 1995) was isolated as a fragment obtained by BamHI and partial HindIII digestion. The isolated fragment was ligated to pBlueScript (Clontech, Mountain View, CA, USA) plasmid linearized by BamHI-HindIII digestion, to generate an intermediate clone. Two complementary PG fragments were ligated at once into SalI-XhoI-linearized pEN-L4R1: from 5' to 3', a XhoI-XbaI fragment isolated from the PG intermediate clone, and an XbaI-XhoI fragment isolated from the original PG pBIN19 derivative. The plasmid pEN-L4R1 carried a multiple cloning site flanked by the attL4 and attR1 recombination sites (attL4-Xmnl-SalI-BamHI-Kpnl-cdcb-XhoI-attR1; O. Nyabi, M. Karimi, P. Hilson, and J. Haigh, unpublished results). The CRC promoter originally cloned into pBS (Lee et al., 2005) was isolated as a SalI-XhoI fragment and cloned into the same sites within pEntrL4-R1. Digested fragment were separated by electrophoresis in agarose gels, extracted and purified with the High Pure PCR Purification Kit (Roche Diagnostics). After transformation into DH5α competent cells, clones were screened by restriction analysis to identify plasmids with the expected insert in the correct orientation and subsequently sequence validated.

Creation of amiRNA vectors

According to Schwab et al. (2006), the plasmid pRS300 was used as template to introduce an amiRNA sequence targeting the tomato and Arabidopsis PDS gene (5'-TCCATGCAGCTACCTTTCCAC -3') into the miR319a precursor by site-directed mutagenesis. Overlapping PCR amplification steps were done as described in the original cloning protocol except that, in the final PCR reaction, the oligonucleotides A and B (based on the pBSK backbone) were replaced by two alternative oligonucleotides, attB1-amiRNA-fw and attB2-amiRNA-rev, carrying the attB1 and attB2 sites, respectively. These two primers can be used for the amplification of any amiRNA derived from the miR319a precursor backbone (see Supplemental Materials and Methods for details). The resulting PCR product containing the amiR-pds precursor was cloned into pDONR221 by BP clonase reaction resulting in the pEN-L1-miRpds-L2 entry clone (Table I), then subcloned into the pK7WG2 or pK7m24GW destination vectors by LR clonase reaction, to yield the expression vectors driving amiR-pds silencing under the control of the CaMV 35S, PPC2, PG, TPRP, IMA or CRC promoters (Table II).
Transactivation clones

Reporter OP clones were sequence verified after restriction-ligation subcloning downstream of an OP array (10xOP-TATA-MCS-3’OCS in BJ36) and transferred into the NotI site of the pART27 binary vector. \( p_{OP}:GUS \) and \( p_{OP}:RFP \) were described previously (Lifschitz et al., 2006). \( p_{OP}:KAN1 \) was generated by PCR amplification of the ORF of the tomato KAN1 ortholog, designated SGN-U321055. Driver lines were generated by transcriptional fusion of promoters in front of the chimeric \( LhG4 \) (MCS-LhG4-3’OCS in BJ36) (Moore et al., 1998) and transferred into the NotI site of pART27. The Arabidopsis LhG4 promoter lines were described before (Pekker et al., 2005), but briefly, a 6.1-kb \textit{FILAMENTOUS FLOWER}, a 1.7-kb \textit{APETALA1}, a 0.5-kb \textit{APETALA3} and a 3.5-kb \textit{CRABS CLAW} Arabidopsis promoter fragments were subcloned in front of \( LhG4 \) in pART27.

Tomato transformation and transactivation

MicroTom tomato lines were transformed with the \textit{Agrobacterium tumefaciens} strain GV3101. Briefly, 8 day-old MicroTom cotyledons were cut at both extremities and in the middle, placed on solid KCMS in Petri dishes, incubated at 25°C for 24 h, soaked for 30 min with shaking in \textit{Agrobacterium} suspension (\textit{Agrobacterium} was grown in LB to OD = 1, pelleted and resuspended in liquid KCMS to OD = 0.05 to 0.08), blotted dry on sterile Whatman paper, and incubated on solid KCMS in Petri dishes in the dark for 48 h at 25°C. For regeneration, the cotyledons were laid on 2Z medium with 250 mg L\(^{-1}\) timentin (ticarcillin and clavulanate; GlaxoSmithKline) and 100 mg L\(^{-1}\) kanamycin, and incubated during 15 days at 25°C in the light. Regeneration medium was changed every 15 days when necessary, with timentin concentration reduced to 150 mg L\(^{-1}\). In case of \textit{Agrobacterium} overgrowth, timentin concentration was kept at 250 mg L\(^{-1}\). Regenerated plantlets were transferred to rooting medium supplemented with 100 mg L\(^{-1}\) kanamycin and 75 mg L\(^{-1}\) timentin. With this protocol and the vectors described herein, transformation frequencies observed using MicroTom cotyledons were consistently >80% and up to 90%. Less than 15% of the transgenic plantlets were polyploid according to flow cytometry analysis. Cultivation from cotyledon explants to transfer of transgenic plants into the greenhouse lasted 2 to 3 months, and 2 to 3 additional months were required to collect transgenic seeds. The composition of in vitro culture media is provided in Supplemental Table S3 and S4.
Cotyledon transformation of M82 tomato and leaf discs were carried out according to McCormick (1991) with the *Agrobacterium tumefaciens* strain GV3101. promoter:LhG4 lines were crossed to 10xe lines to generate the F1 promoter>>reporter plants.

Upon request, all novel materials described will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

**GUS histochemical staining**

Tissues from stably transformed tomato plants were soaked, vacuum-infiltrated and incubated for 30 min (PG), 1 h (3SS, PPC2, TPRP), 2 h (CRC), or overnight (IMA) at 37°C in GUS staining solution (0.5 mM X-gluc, 0.15 M NaH₂PO₄, pH7, 2 mM K₃Fe(CN)₆, 2 mM K₄Fe(CN)₆, 0.05% Triton X100). GUS-stained tissues were cleared overnight in 100% ethanol and stored in 70% ethanol.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Materials and Methods**

**Supplemental Fig. S1.** GUS activity in tomato fruits (cv. Micro Tom) agroinjected with promoter:GUS transgenes generated via MultiSite Gateway cloning.

**Supplemental Fig. S2.** Albino phenotype resulting from PDS silencing in Arabidopsis.

**Supplemental Fig. S3.** Creation of novel hpRNA destination vectors.

**Supplemental Fig. S4.** Construction of the intron-spacer donor vector pDONRP1-R2-I-R2-P1.

**Supplemental Fig. S5.** Alternative scheme to generate specific hpRNA destination vectors.

**Supplemental Table S1.** Theoretical promoter sequences

**Supplemental Table S2.** Primers designed for PCR amplification of genetic elements and for validation of recombined plasmids

**Supplemental Table S3.** Composition of MicroTom transformation media (per liter)

**Supplemental Table S4.** Vitamins for MicroTom transformation media

**Supplemental Table S5.** Specific hpRNA destination vectors
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### Table I. Genetic elements in entry clones

| Class            | Element(s) | Recipient pDONR | Entry clone      | att sites    |
|------------------|------------|-----------------|------------------|--------------|
| Promoter         | PPC2       | pDONR P4-P1R    | pEN-L4-PPC2-R1   | attL4–attR1  |
| Promoter         | TPRP       | pDONR P4-P1R    | pEN-L4-TPRP-R1   | attL4–attR1  |
| Promoter         | IMA        | pDONR P4-P1R    | pEN-L4-IMA-R1    | attL4–attR1  |
| Promoter         | CRC        | pEN-L4R1        | pEN-L4-CRC-R1    | attL4–attR1  |
| Promoter         | PG         | pEN-L4R1        | pEN-L4-PG-R1     | attL4–attR1  |
| Reporter enzyme  | gus, intron| pDONR221        | pEN-L1SIL2       | attL1–attL2  |
| amiRNA           | PDS        | pDONR221        | pEN-L1-miRpdL2   | attL1–attL2  |
| Two-component    | LHG4AtO    | pDONR221        | pEN-L1LhG4AtOL2  | attL1–attL2  |
### Table II. Expression vectors

| Destination vector | Recombined entry clones | Expression vector |
|---------------------|-------------------------|-------------------|
| pK7m24GW            | pEN-L4-PPC2-R1 x pEN-L1SIL2 | pK7-B4-PPC2-B1-GUSintron-B2 |
| pK7m24GW            | pEN-L4-PG-R1 x pEN-L1SIL2 | pK7-B4-PG-B1-GUSintron-B2 |
| pK7m24GW            | pEN-L4-TPRP-R1 x pEN-L1SIL2 | pK7-B4-TPRP-B1-GUSintron-B2 |
| pK7m24GW            | pEN-IMA-R1 x pEN-L1SIL2 | pK7B4-IMA-B1-GUSintron-B2 |
| pK7m24GW            | pEN-L4-CRC-R1 x pEN-L1SIL2 | pK7-B4-CRC-B1-GUSintron-B2 |
| pDesR4-cdbB-L1-NF-B2-S*-B3-7K | pEN-L4-PPC2-R1 | PExB4-PPC2-B1-NF-B2-S*-B3-7K |
| pDesR4-cdbB-L1-NF-B2-S*-B3-7K | pEN-L4-PG-R1 | PExB4-PG-B1-NF-B2-S*-B3-7K |
| pDesR4-cdbB-L1-NF-B2-S*-B3-7K | pEN-L4-TPRP-R1 | PExB4-TPRP-B1-NF-B2-S*-B3-7K |
| pDesR4-cdbB-L1-NF-B2-S*-B3-7K | pEN-IMA-R1 | PExB4-IMA-B1-NF-B2-S*-B3-7K |
| pDesR4-cdbB-L1-NF-B2-S*-B3-7K | pEN-L4-CRC-R1 | PExB4-CRC-B1-NF-B2-S*-B3-7K |
| pK7GW2              | pEN-L1miR-pdsL2 | pK7-B1-miRpdps-B2 |
| pK7m24GW            | pEN-L4-PPC2-R1 x pEN-L1miRpdps-L2 | pK7-B4-PPC2-B1-miRpdps-B2 |
| pK7m24GW            | pEN-L4-PG-R1 x pEN-L1miRpdps-L2 | pK7-B4-PG-B1-miRpdps-B2 |
| pK7m24GW            | pEN-L4-TPRP-R1 x pEN-L1miRpdps-L2 | pK7-B4-TPRP-B1-miRpdps-B2 |
| pK7m24GW            | pEN-IMA-R1 x pEN-L1miRpdps-L2 | pK7B4-IMA-B1-miRpdps-B2 |
| pK7m24GW            | pEN-L4-CRC-R1 x pEN-L1miRpdps-L2 | pK7-B4-CRC-B1-miRpdps-B2 |
| pK7m24GW            | pEN-L4-PPC2-R1 x pEN-LHGATO4 | pK7-B4-PPC2-B1-LHGATO4-B2 |
| pK7m24GW            | pEN-L4-PG-R1 x pEN-LHGATO4 | pK7-B4-PG-B1-LHGATO4-B2 |
| pK7m24GW            | pEN-L4-TPRP-R1 x pEN-LHGATO4 | pK7-B4-TPRP-B1-LHGATO4-B2 |
| pK7m24GW            | pEN-IMA-R1 x pEN-LHGATO4 | pK7B4-IMA-B1-LHGATO4-B2 |
| pK7m24GW            | pEN-L4-CRC-R1 x pEN-LHGATO4 | pK7-B4-CRC-B1-LHGATO4-B2 |
FIGURE LEGENDS

**Fig. 1.** Assemblage of different genetic elements captured into entry clones via LR reaction to facilitate genetic studies in tomato. B1, B2, B4, L1, L2, L4, and R1: corresponding att Gateway recombination sites. Sp, spectinomycin; Sm, streptomycin; Km, and Kan, kanamycin bacterial and plant selectable markers, respectively; LB, left T-DNA border; RB, right T-DNA border; Prom, promoter; ORF, open reading frame; amiRNA, artificial microRNA; GST, gene-specific sequence tag; pdk-cat intron, pHELLSGATE12-derived intron spacer; ccdB, counter-selectable bacterial marker; and 3´OCS, octopine synthase terminator.

**Fig. 2.** GUS activity in tomato (cv. MicroTom) stably transformed with promoter:GUS transgenes generated via MultiSite Gateway cloning. MG, Mature Green; Br, Breaker. Size bar = 10 mm.

**Fig. 3.** Detection of nuclear GFP in cells of tomato fruit pericarp. Sections were prepared from 8 daa fruits. A and B, 35Spro:NLS:GUS-GFP tissues showing GFP signal in all fruit tissues. B, Magnified image showing localization of GFP in the nucleus. C and D, Fruit pericarp cross section from daa tomato fruits transformed with PPC2pro:NLS:GUS-GFP showing preferential GFP localization in expanding cells from fruit mesocarp. D, Magnified image of mesocarp showing localization of GFP in polyploid nucleus from large cell. Size bar = 400 μm (A, C) and 100 μm (B, D).

**Fig. 4.** Tissue-specific amiRNA silencing in tomato fruits. Flowers at anthesis (top), mature leaves (middle) and ripe fruits (Breaker +7) (bottom). A, Wild type (MicroTom). B and C, 35Spro:amiR-pds plants with strong to extreme photobleaching in all organs. D and E, PPC2pro:amiR-pds plants with moderate and transient photobleaching restricted to the fruit. Size bar = 10 mm.

**Fig. 5.** Tissue-specific transactivation in tomato with selected Arabidopsis promoters. A and B, Expression of dsRED or GUS, activated in trans by the organ primordia and abaxial domain pFIL:LhG4 driver. Abaxial expression is evident after leaf primordium has expanded. C-D, Flower-specific growth arrest stimulated by specific transactivation of a pOP:KAN1 reporter, with the pAP1:LhG4 driver. E-F, Petal-only radialization stimulated by specific transactivation of a pOP:KAN1 reporter, with the
pAP3:LhG4 driver. G-H, Carpel-specific growth arrest stimulated by transactivation of the pOP:KAN1 reporter with the pCRC:LhG4 driver. WT, wild type.
Transcriptional regulation
- TPRP
- PG
- CRC
- Your promoter

Protein expression
- GUS
- NLS:GFP:GUS
- Your gene

Gene silencing
- amiRNA designer software

Entry clones

Expression clones

Novel destination vectors

BP clonase or restriction/ligation reaction

LR clonase reaction

Reverse BP clonase reaction

promoter::ccdB hp

promoter::att R1-ccdB-attR2

attL 4-ccdB-attR1::ORF

attL 4-ccdB-attR1::amiRNA
