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Identification of a short putative 5´ regulatory sequence from transgenic hairy root of tomato-regulating specific expression pattern

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Abstract

The *Solanum lycopersicum* cultivar MT1 was transformed by the A4 strain of *Agrobacterium rhizogenes* harboring the promoter trapping binary vector pPRF120. Transformed hairy root lines generated from the transformation were verified for stable expression of the inserted *gus* (β-glucuronidase) transgene in the genomic DNA. The 68i line was found to be stably transformed by pPRF120 *via* PCR analysis. This positive line was used for further study to detect promoter sequences that control expression of the integrated T-DNA, which contains a promoterless *gus* reporter gene. By using a 'DNA walking' approach, a single unknown flanking sequence was successfully amplified by two rounds of adaptor ligation PCR (AL-PCR). The AL-PCR patterns obtained were specific and reproducible for a given genomic library. AL-PCR products from the genomic libraries digested with either *Dra*I (clone 1B) or *Hpa*I (clone 5B) contain ~350 bp and ~1400 bp of upstream region respectively. The alignment of the sequences from clones 1B and 5B showed a very high percentage of similarity (97%). Resulting BLASTn analysis of the 5B clone indicated that 183 bp was 97% similar to chromosome 11 of *Solanum lycopersicum,* whereas 1105 bp was 97% similar to integrated Ri plasmid agropine of *A. rhizogenes* strain A4 for complete T_L-DNA. The bioinformatics-based characterization of the short 256 bp putative novel promoter region directly upstream of T-DNA insertion *via* PlantCARE revealed the presence of several motifs for plant transcription factors such as circadian, TGA-element and motifs involved in light responsive control including CATT, ATCT, Sp1 and G-box.

Keywords*: Agrobacterium rhizogenes; cis*-acting elements; insertional mutagenesis; *Solanum lycopersicum;* promoter trapping; T-DNA tagging

Abbreviations: BLAST_Basic Local Alignment Search Tool; *gus*_ β-glucuronidase; MT1_MARDI Tomato 1; NCBI_The National Center for Biotechnology Information; *npt*II_ neomycin phosphotransferase II; PCR_poymerase chain reaction; T-DNA_transferred DNA; X-gluc_ 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Introduction

The tomato (*Solanum lycopersicum* L.), a member of the family *Solanaceae*, is one of the most popular vegetables in the world (Arie et al., 2007) and is selected as a model plant in many areas of research, including plant-pathogen interactions (Meissner et al., 1997; Meissner et al., 2000; Emmanuel and Levy, 2002), promoter studies and genetic studies. Many constitutive promoters have been successfully cloned

Table 1. Sequences of primers used in the experiments

Primer	Sequence 5' – 3'
GUS-F	CGC CGA TGC AGA TAT TCG TA
GUS-R	ATT AAT GCG TGG TGG TGC AC
rol B-F	TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC
rol B-R	ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA
GSP1	CCA CAG TTT TCG CGA TCC AGA CTG AAT
AP1	GTA ATA CGA CTC ACT ATA GGG C
GSP2	GGT TGG GGT TTC TAC AGG ACG TAA CAT
AP2	ACT ATA GGG CAC GCG TGG T
M13-F	GTA AAA CGA CGG CCA GT
M13-R	GGA AAC AGC TAT GAC CAT G

and are used to drive gene expression in transformed plants (Dobrowolska and Kononowicz, 2004; Squires et al., 2007).

Unlike constitutive promoters, cryptic promoters do not normally drive gene expression and are activated by insertion of a T-DNA. These cryptic promoters have been identified in the 5'-UTR, introns and intergenic sequences in both plants and animals (Fobert et al., 1994; Terrinoni et al., 2001; Han and Zhang, 2002; Wu et al., 2003). Promoter traps are tools that are used to tag fragments of genomic DNA in order to discover regions that are influenced by endogenous promoters (Wei et al., 1997; Alvarado et al., 2004). This approach has been used to trap both constitutive and cryptic promoters. The advantages of using T-DNA tagging as a tool for functional genomics in plants have been reviewed (Bade et al., 2003; Radhamony et al., 2005).

In this study, a promoter trapping binary vector pPRF120 (Fobert et al., 1991) containing the promoterless *gus* gene and the *npt*II gene, which confers resistance to kanamycin, was transformed to tomato cultures using the A4 strain of *A. rhizogenes*. Following infection with *A. rhizogenes,* the T-DNA was integrated randomly into the tomato genome. GUS expression would then indicate that the T-DNA was inserted into the tomato genome at a location that placed it under the control of a plant promoter sequence. This genomic promoter would then drive the expression of the *gus* gene. The expression pattern of GUS reporter genes can be complex and may be used to demonstrate idiosyncratic gene expression for the organisms being studied (Geisler et al., 2002; Buzas et al., 2005). In this paper, the tagging of a genomic promoter from a stably transformed tomato hairy root line that exhibits GUS expression in the nodular and root tips regions was reported.

Materials and methods

Plant materials and preparation of explants

The seeds of tomato cultivar MT1 were provided by the Malaysian Agricultural Research and Development Institute (MARDI, Klang, Malaysia) and initially used as plant material for transgenic plants. Tomato seeds were surface-sterilized and inoculated on Petri dishes containing Murashige and Skoog (1962) medium with 3% (w/v) sucrose, 4.405 gL^{-1} inositol, MS vitamins, 0.3% (w/v) Phytagel® and a pH value adjusted to 5.8. The cultures were maintained under 27±1°C air temperature in the dark for 2 days and followed by a 16 hd^{-1} photoperiod with a light intensity of 50 µmol $m^{-2}s^{-1}$ photosynthetic flux density (PPFD).

Genetic transformation, maintenance of hairy root culture and detection of gus expression

Plants were transformed by dipping week-old germinated aseptic seedlings, slightly cut in the cotyledons, into a solution containing *A. rhizogenes* strain A4 harboring promoter trapping vector, pPRF120 for 3 min. The seedlings were dried and placed on the MS media for 3 days co-cultivation period. Co-cultivated seedlings were grown on new MS media supplemented with 400 μgmL^{-1} cefotaxime and were assessed for the growth of transgenic hairy roots from the cut sites of cotyledons. The transgenic hairy roots were maintained by subculturing the young roots on MS media supplemented with 100 μgmL^{-1} kanamycin. Individual transformed lines of hairy root were assayed for GUS expression. GUS expression was detected with the standard destructive method using X-gluc as a substrate (Jefferson et al., 1987).

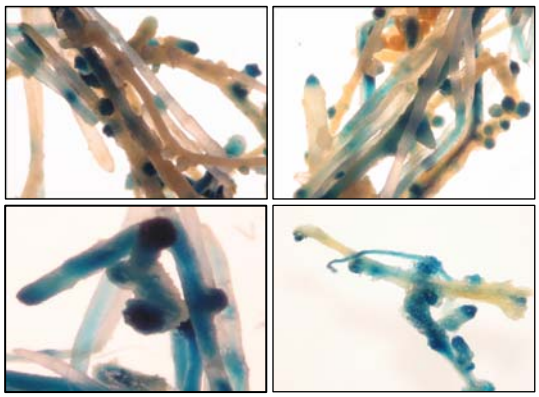
Genomic analysis of transgenic hairy roots

Genomic DNA was extracted from the 68i line hairy root samples using the method described by Doyle and Doyle (1990) and quantified spectrophotometerically and by agarose gel electrophoresis. PCR was performed using a set of GUS and rol B primers (Table 1) designed to amplify regions from the *gus* gene and the *A. rhizogenes*-specific *rol* B gene respectively as described by Hamill et al. (1991). The expected band sizes for the PCR amplifications were 789 bp for *gus* and 780 bp for *rol* B.

GenomeWalker library construction

In order to create uncloned, adaptor-ligated genomic libraries as described by Siebert et al. (1995), the DNA was initially digested with seven different blunt end restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Stu*I, *Hpa*I, *Ssp*I and *Alu*I) to produce blunt ends for adaptor ligation. The digested products were purified and the GenomeWalker adaptors were ligated to the

Fig 1. Histochemical assay for GUS expression in the transgenic hairy roots of line 68i. Expression was detected only in specific parts of the hairy root cultures



blunt ends of the DNA fragments, as recommend by the manufacturer (Clontech).

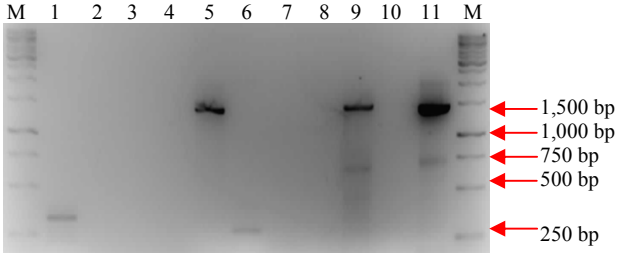
DNA walking and cloning of PCR products

A pair of gene-specific primers was designed using the *gus* sequence as a template. The first gene-specific primer (GSP1) was designed to complement near the 5' end of the first *gus* exon and the second (GSP2) was designed to be nested within the sequence amplified by GSP1. Primary PCR was performed using GSP1 and adaptor primer 1 (AP1). The product for primary PCR was diluted 20-fold and used as a template for the nested PCR as described in the GenomeWalker Universal Kit (Clontech). Products were visualized by agarose gel electrophoresis and gel purified using the QIAquick Gel Extraction kit (Qiagen). PCR fragments were cloned into pGEM-T Easy vector (Promega) and transformed into competent *Escherichia coli* JM109 (Promega). Transformed colonies were detected by blue-white colony screening and were tested with PCR using the M13-F and M13-R primers (Table 1) to confirm the insertion of the products. Recombinant plasmid DNA was isolated from positive colonies by the QIAprep Spin Miniprep kit (Qiagen).

Analysis of product

The recombinant plasmids containing inserts of the correct sizes were sequenced and the sequences analyzed using the ClustalW sequence alignment tool and BLAST. Sequences were also compared to the database of Plant *Cis*-Acting Regulatory Elements, PlantCARE (Lescot et al., 2002) in order to detect promoter related motifs.

Fig 2. Nested-PCR amplification of the seven uncloned genomic libraries from the transgenic hairy root line 68i. M: 1kb DNA ladder. Wells 1-7 are PCR amplification of the restriction libraries DraI, EcoRV, PvuII, StuI, HpaI, SspI, AluI (in order). Well 8: Negative control for libraries in well 1-7; Well 9: Human genomic library control (Self-constructed). Well 10: Negative control for libraries in well 9 and 11; Well 11: Human genomic library control (Pre-constructed)



Results

Detection of gus expression and its presence in the genomic DNA of transgenic hairy roots

A total of 368 independent transgenic root lines were generated and about 87 independent kanamycin-resistant hairy root lines were tested for GUS expression. Four hairy root lines were found to reproducibly contain GUS activity after several generations of subculture. The 68i line was chosen for analysis due to the specific pattern of the GUS expression where only the nodule-like structure and the tip of the nodes show expression of the gene (Fig 1). To further verify the insertion of the *gus* gene, DNA was extracted from three samples of the 68i line and PCR was performed using *gus*- and *rol* B-specific primers. PCR products were visualized by agarose gel electrophoresis and a ~789 bp amplicon for the *gus* primers and a ~780 bp amplicon for the *rol* B primers, which both correspond to the expected band sizes, were detected (data not shown).

DNA walking in the GenomeWalker library

DNA walking was performed on all seven uncloned genomic libraries and the product of the nested PCR was visualized by agarose gel electrophoresis (Fig 2). Three uncloned genomic libraries exhibited PCR amplification bands. The *Dra*I library amplified a band of ~350 bp, the *Hpa*I library amplified a band of ~1,400 bp and the *Ssp*I library at ~250 bp. Sequencing of the 1B clone (from the *Dra*I library) and the 5B clone (from the *Hpa*I library) produced a

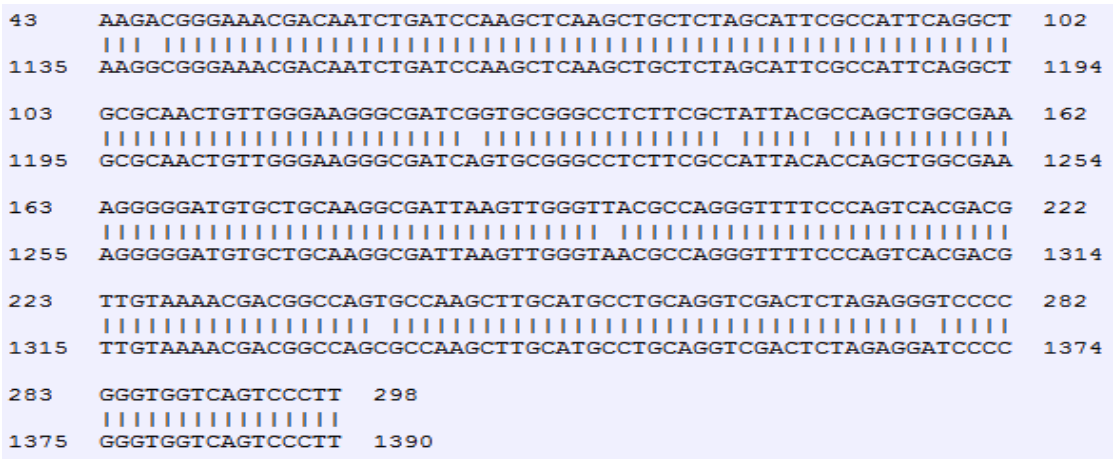


Fig 3. Aligment between clone 1B and 5B sequences

Fig 4. BlastN analysis between sequence of clone 5B with the NCBI genbank database

325 bp sequence and a 1,417 bp sequence respectively (data not shown).

DNA Sequence Analysis

DNA sequences from clone 1B (325 bp) and clone 5B (1,417 bp) were aligned with ClustalW to detect overlapping regions. Overlapping and non-overlapping regions were analyzed for similarities with other DNA regions in the database at NCBI with BLAST. In the sequence from the 1B clone, the region from +43-298 (numbered from the 5' end of the adaptor primer sequence) was 97% similar to the +1135-1390 region in the sequence from the 5B clone (Fig 3). By searching the NCBI database with BLAST, the sequences derived from the integrated promoterless *gus* sequence were identified. The left border region of the agropine Ri plasmid from the *Agrobacterium rhizogenes* strain A4 was maintained for co-transformation (Fig 4).

Further analysis of the sequence from 5B clone with NCBI database of *Lycopersicon*, has shown the homology to the complete clone (C11HBa0054I23) for part of chromosome 11 of *Solanum lycopersicum*. About 179 bp sequence from the position 1178 to 1360 of the clone 5B are 97% identical to chromoso-

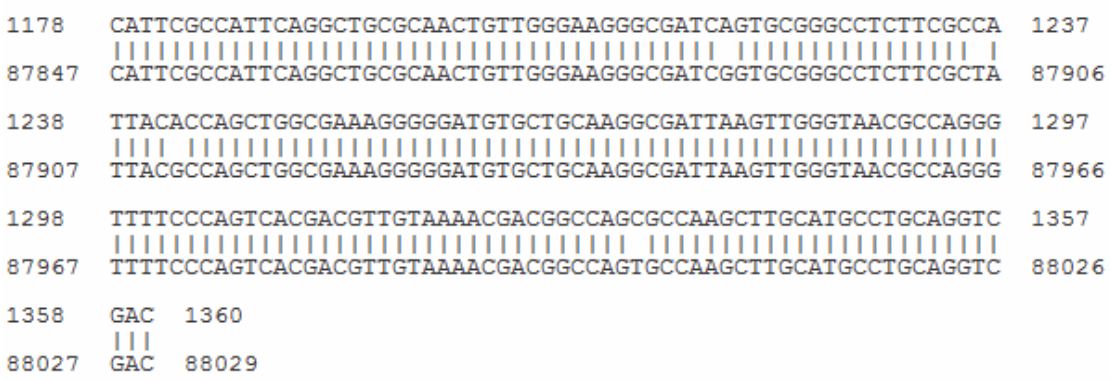


Fig 5. BlastN analysis between sequence of clone 5B with the NCBI genbank database for lycopersicon

me 11 *Solanum lycopersicum* (Fig 5). A graphical representation of the combined data from both the sequencing and the BLAST searches in the NCBI database of the sequences of 1B and 5B was demonstrated (Fig 6). The 256 bp region directly upstream of the T-DNA insertion region that is flanked by the promoterless *gus* gene and the agropine Ri plasmid was analyzed using PlantCARE (Lescot et al., 2002) and the results are as shown in Table 2.

Discussion

Only 256 bp of the plant genomic DNA was isolated for analysis even though the DNA walking was successful. The reason for this is that the insertion of the agropine Ri left border T-DNA occurred at a region between the plant genome DNA and the inserted pPRF120 T-DNA during the *Agrobacterium*-mediated co-transformation event of the 68i line (Fig 6). It is interesting to note that since the insertion of the agropine Ri fragment is believed to happen randomly (Vergunst et al., 1998), the chances of insertion of the T-DNA carrying the promoter trap occurring at nearly the same location should be extremely low. Given the size of the genome (Bennett and Smith, 1976; Van der Hoeven et al., 2002), it is unlikely that the insertion took place randomly and ended up in a region so close to the promoter trap T-DNA insertion.

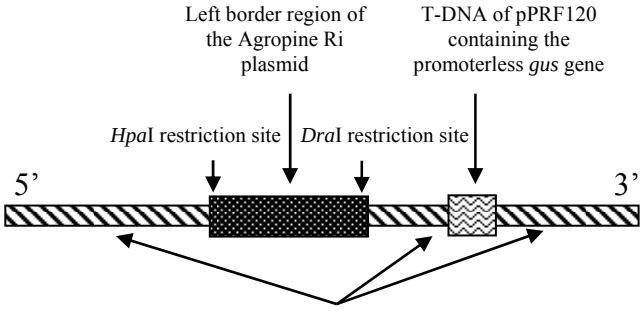
Some researchers believe that T-DNA insertion may not be entirely random and could be influenced by other factors (Szabados et al., 2002). One possibility is that the level of the DNA condensation may play a role in determining where the T-DNA will insert: tightly packed DNA will not favor the insertion of T-DNA while loosely packed DNA is more accessible. Loosely packed DNA tends to have more open replication forks because of DNA unwinding. This allows the T-DNA to slip in easily and may facilitate T-DNA insertion. Therefore, instead of being randomly integrated into the genomic DNA, T-DNA may preferentially insert into actively transcribed regions. This agrees with the previous observations that T-DNA insertions are rarely detected in interspersed, centromeric, telomeric and rDNA repeats but are enriched in chromosomal domains with a high gene density (Szabados et al., 2002).

The previous studies that demonstrated the random and unbiased insertion of T-DNA were performed in *Arabidopsis thaliana,* which is a simple plant model with a genome containing mostly coding regions and very few non-coding regions (Radhamony 2005). Therefore, it is not surprising that the studies using this model did not demonstrate any bias in the T-DNA insertion sites. However, if the theory that the level of DNA condensation affects T-DNA insertion is true, then the closeness of the insertion of the binary T-DNA reporter gene into the DNA near where the agropine Ri T-DNA inserted would be less surprising. As the plant tissues used in the transformation were from hairy root cultures, it makes sense that most other genomic regions would be tightly condensed and that only the DNA encoding loci important in root production would be actively transcribed and open. This, in turn, would mean that there would be a greater likelihood that the binary T-DNA insertion and the agropine Ri T-DNA insertion would occur at the same loci due to a lack of open spaces in the condensed DNA. Importantly, if the T-DNA preferentially inserts into the loosely packed coding regions as opposed to non-coding condensed DNA, then a lower threshold of T-DNA tagging will be required in order to achieve saturation in whole genome promoter tagging. As such, the calculations on the level of tagging that needs to be done in order to saturate an entire genome for promoter isolation will have to be revised and a lower number can be adopted, thereby making large-scale promoter tagging more feasible.

Table 2. Putative motifs discovered in the 256 bp region upstream of the inserted T-DNA using PlantCARE

Motif	Organism	Matrix score	Sequence	Function
TATA-Box	*L. esculentum*	5	tttta	Core promoter element (-30 from transcription initiation site)
circadian	*L. esculentum*	6	caatctgatcctaattcaac	Regulatory element for circadian control.
CAAT-Box	*H. vulgare*	4	caat	Common *cis* element in promoters and enhancers.
CATT	*Z. mays*	6	gcattc	Light responsive element (partial).
G-Box	*Z. mays*	6	cacgac	Light responsive *cis* regulatory element
ATCT	*P. sativum*	9	aatctaatcc	Conserved module of DNA involved in light response. (Partial)
Sp1	*Z. mays*	5.5	cc(g/a)ccc	Light responsive element.
TGA-element	*B. oleracea*	6	aacgac	Auxin responsive element.
MBS	*A. thaliana*	6	caactg	MYB binding site involved in drought-inducibility

Fig 6. Graphical representation (not to scale) of the region of plant genomic DNA elucidated by GenomeWalking and the arrangement of the tagging T-DNA from pPRF120, the agropine Ri plasmid region integrated into the plant genome and the restriction sites for both HpaI and DraI.



L. esculentum genomic DNA from the 68i line

From the isolated sequences, we postulate that the long stretch of agropine Ri T-DNA co-transformed into the genomic region of 68i line along with the T-DNA for promoter trap in a close proximity may not play a significant role in regulating the expression of *gus* gene. Ri T-DNA insertion in such position would probably acts as interference towards the activity of the putative novel promoter sequence that was flanked by these inserted T-DNAs. In addition, we were fascinated on how this short sequence of 256 bp located upstream of the *gus* gene and was interrupted by the Ri T-DNA at the 5' end can possibly drive the expression of GUS in the 68i line. This piece of short fragment might be similar to those pseudo-promoters described by Buzas et al. (2005) and require more in-depth investigations on the molecular mechanisms that will define its function as a promoter. Furthermore, very short promoter sequences of 159 bp and 194 bp have been previously shown to drive expression of genes in *Arabidopsis* plant (Stangeland et al., 2005).

Based on the PlantCARE analysis, the region isolated seems to carry elements related to the light response and this may be related to the expression pattern seen in the transgenic line. GUS expression was seen in the nodule-like structures and root tips, and these locations may have promoters controlling the light response. Other than core promoter elements (TATA and CAAT) found in the sequence, motif for circadian cycle regulation responsible for biochemical and physiological processes in plant is also present. In addition to that, there are also motifs involve in light response regulation such as CATT, ATCT, G Box and SPI which act through induction and repression system of gen expression based on the light intensity. The sequence analysis also shows the presence of TGA elements involved in auxin response regulation important for root induction in plant. Future experiments aim to identify and elucidate full-length promoter sequence specifically active in the nodule-like structures and root tips based on the putative novel promoter sequence isolated from this study. A further dissemination on the short putative promoter sequence might reveal more details on the functionality of this fragment and will enlighten our understanding of short promoter sequences.

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