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RESEARCH ARTICLE

Multiple GUS expression patterns of a single *Arabidopsis* gene

Ekrem Dündar[1,2]

1 Balıkesir Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü, Balıkesir, Turkey
2 Physiological and Molecular Plant Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

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Correspondence
Ekrem Dundar, Balıkesir Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü, Balıkesir, Turkey.
Email: dundar@balikesir.edu.tr, ekremdundar1@yahoo.com

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Abstract

Ten independent transposant lines with gene or enhancer traps (ET) inserted into the same gene (At2g01170) were identified in *Arabidopsis thaliana*. Transposon insertions were confirmed for each line. Only three of five ET lines and only one of the five gene trap (GT) lines displayed uidA (GUS) staining. The GUS (β-glucuronidase) expression patterns of the ET lines were different in all three lines. In the GT line, the GUS expression was restricted to the vascular tissue under all conditions examined. The variation in ET GUS expression suggests that each ET was controlled by different enhancer elements or the different elements of the trapped locus may give rise to different GUS expression patterns. Of five GT lines, three have the *GUS* gene in the same orientation as the At2g01170 open reading frame, yet only one yielded GUS staining. Regardless of the insertion construct, only those transposants with an insertion at the 3′ end of the gene yielded GUS staining. Some transposants displayed a longer root phenotype in the presence of kanamycin that was also observed in 3′ insertion sites in At2g01170. Taken together, these data show that insertions in the 5′ end of the gene disrupted expression and emphasise the complexity encountered with ET and GT constructs to characterise the expression patterns of genes of interest based solely on GUS expression patterns.

Introduction

Transposon insertions have become very powerful tools for gene isolation and function analysis (Parinov & Sundaresan, 2000; Walbot 2000; Raina *et al.*, 2002; May & Martienssen, 2003). Transposons were initially identified in maize as sequences of DNA that excise and insert themselves in different locations in the same genome. These sequences need a transposase that cuts out the transposon and inserts it to another location in the genome (McClintock, 1950, 1987). The transposase is encoded by the transposon in autonomous but not in non-autonomous transposons. Sundaresan *et al.* (1995) developed an efficient insertional mutagenesis system in *Arabidopsis* using the maize Ac/Ds family of transposable elements (McClintock, 1987). They used an immobilised Ac as the source of transposase to mobilise the non-autonomous Ds element. They constructed an insertion cassette in which they engineered the *GUS* (β-glucuronidase or *uidA*) gene in Ds such that it expresses when the cassette inserts behind a native promoter in parallel orientation with the chromosomal gene [gene trap (GT)] or in the proximity of an enhancer that will drive the expression of a weak promoter in front of the *GUS* gene [enhancer trap (ET)]. β-glucuronidase is able to accommodate long N-terminal fusion proteins and still preserve its function (Jefferson *et al.*, 1987). When its substrate x-glucuronide (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide) is present, the enzyme generates a blue precipitant. Hence, it is possible to visualise the expression pattern of a gene that received the insertion cassette. Because the sequence of the insertion cassette is known, it is possible to use a variety of PCR approaches to obtain the sequence of the genomic DNA adjacent to the insertion site (Springer *et al.*, 1995). Thus, a collection of transposants has been generated in which plants

have been scored for GUS expression in addition to using PCR to identify the exact location of each insertion cassette in the *Arabidopsis* genome (Sundaresan *et al.*, 1995; Springer, 2000).

As part of our analysis of amino acid transporters in plants, we have screened the CSHL (Cold Spring Harbor Laboratories) transposant collection for GT and ET insertions in genes that have sequence similarity to previously described amino acid transporters. Our analysis of the CSHL collection identified 10 independent insertion events targeted to a single gene (At2g01170) that had been annotated as a putative GABA transporter. This gene is located in a region of chromosome 2 that appears to be a hot spot for transposon insertions (Parinov *et al.*, 1999), and has a ubiquitous mRNA expression pattern (Dundar, 2003; Dundar & Bush, 2008). The large number of insertions in this single gene allowed us to address some simple questions regarding the ET and GT technology. Specifically, we asked: (a) Do GTs always express when inserted in parallel orientation with the same gene? (b) Do ETs always express the same pattern when inserted within the same gene? and (c) Do ETs and GTs differ in GUS expression when inserted into the same gene?

Materials and methods

Arabidopsis growth conditions

Arabidopsis thaliana ecotype Landsberg erecta was acquired from the Arabidopsis Biological Resource Center at The Ohio State University. Transposon insertion mutants came from the Cold Spring Harbor collection (Sundaresan *et al.*, 1995). *Arabidopsis* seeds were sterilised and grown based on Sundaresan *et al.* (1995). The seeds were soaked in 95% alcohol for 10 min, 20% Clorox and 0.1% Tween 20 for 5 min, washed with sterile water for 2 min twice and added 0.1% agar (top agar). The sterilised seeds were stratified 1–3 days and then transferred onto MS plates (Murashige & Skoog, 1962) containing 1% sucrose, and 4.5 g L^{-1} Agar gel (Sigma-Aldrich, St Louis, MO, USA) or 6 g L^{-1} Bacto™ Agar (BD Biosciences, Boston, MA, USA) and kanamycin (50 μg mL^{-1}). After stratification, plates were transferred into a controlled growth chamber that was set to 21°C with a 10-h light (150 μ*E* m^{-2} s^{-1}) and 14-h dark cycle. Seedlings were transferred into well-watered Sunshine Mix soil (Wetsel Seed Co., Harrisonburg, VA, USA) when needed, and were grown in controlled growth chambers set to 21°C with an 8-h light (200 μE m^{-2} s^{-1}), 16-h dark cycle. For root growth observation, vertical plates were prepared exactly the same as regular plates and they were kept vertical during growth. GT1236 is a GT transposant of *AAP6* (*Amino Acid Permease 6*; Rentsch *et al.*, 1996) and was used as control for the root growth experiments. Vertical growth experiments were conducted three times. Average, standard deviation and standard error values for each root length were calculated using common spreadsheet software.

Polymerase chain reactions and sequencing

Tail-PCR was performed as described (Liu & Whittier, 1995) in a DNA-Engine PTC-200 (MJ Research, Inc., Watertown, MA, USA). The PCR protocols and nested primers were as described by Tsugeki *et al.* (1996), and the arbitrary degenerate (AD) primers were from two reports: AD1 [5′-NTC GA(G/C) T(A/T)T (G/C)G(A/T) GTT-3′], AD2 [5′-NGT CGA (G/C)(A/T)G ANA (A/T)GAA-3′] and AD3 [5′-(A/T)GT G NA G(A/T)A NCA NAG A-3′] were from Liu *et al.* (1995), while AD2n [5′-(G/C)TT GNT A(G/C)T NCT NTG C-3′] and AD5 [5′-(A/T)CA GNT G(A/T)T NGT NCT G-3′] were from Tsugeki *et al.* (1996). Standard PCR reactions were performed as described (Sambrook *et al.*, 1989). TAIL-PCR products were gel extracted using a gel extraction kit (Qiagen Inc., Valencia, CA, USA), and sequenced by the Keck Center at the University of Illinois at Urbana-Champaign.

Southern blotting

Plant genomic DNA was isolated as described in Dellaporta *et al.* (1983). Genomic DNA was digested with *Eco*RI and the restriction products were separated on a 0.8% agarose gel in TAE buffer. *Eco*RI cuts once in the transposon insertion cassette. As the probe was designed to amplify a 400 bp part of GUS (left primer sequence was 5′-cccttacgctgaagagatgc-3′ and the right primer sequence was 5′-ggcacagcacatcaaagaga-3′), each insertion will yield a single band with this set up. DNA was depurinated in 0.25 N HCl, denatured in 0.5 N NaOH/1.5 M NaCl and neutralised in 1 M Tris/1.5 M NaCl (pH 8). DNA fragments were transferred onto a Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 10× standard saline citrate (SSC; pH 7) for 16–24 h and cross-linked to the membrane using a ultraviolet cross-linker (Stratagene, La Jolla, CA, USA). ^{32}P-labelled probes were synthesised with random hexamer primers using a Mega Prime DNA labelling kit (Amersham Pharmacia Biotech). Probes were cleaned up from unincorporated nucleotides with Bio-Spin columns (Bio-Rad, Hercules, CA, USA) and probe-specific activity was measured using liquid scintillation spectroscopy. Prehybridisation, hybridisation and washing the membranes were carried out as described in Sambrook *et al.* (1989). For hybridisation, 106 cpm mL^{-1} per hybridisation was used. Blots were washed twice in 50 mL 2× sodium saline citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) for

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15 min each time at 42°C and twice in 50 mL 0.1× SSC/0.1% SDS for 15 min each time at 62°C. The blots were then exposed to an X-ray film for 1–7 days in an X-ray cassette at −80°C freezer.

Staining for GUS expression

Staining for GUS expression was performed as reported (Sundaresan et al., 1995). GUS staining solution consisted of 100 mM Na phosphate at pH 7, 10 mM ethylene diamine tetra acetic acid (EDTA), 0.1% Triton X-100 (Bio-Rad), 1 mg mL^{-1} X-glucuronide (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide; Rose Scientific Ltd, Edmonton, Alberta, Canada), 100 μg mL^{-1} chloramphenicol, 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide (potassium ferricyanide and potassium ferrocyanide were excluded when seedlings were tested to visualise a possible weak GUS staining). Seedlings were placed into the GUS solution, vacuum infiltrated for 30 min and transferred into a 37°C incubator. After incubating for 1–3 days at 37°C, the seedlings were placed in multiple changes of 70% ethanol to extract chlorophyll to better visualise the blue product of X-glucuronide.

RNA extraction

Total RNA was isolated based on a described protocol (Logemann *et al.*, 1987). Half a gram (per extraction) *Arabidopsis* tissue was ground in a mortar with liquid nitrogen. About 1 mL extraction buffer [25 mM Tris–HCl pH 8, 25 mM EDTA, 75 mM NaCl, 1% SDS and 1 M β-mercaptoethanol] was added as soon as the frozen powder started to melt. An equal volume (1 mL) of phenol : chloroform : isoamyl alcohol (25:24:1) was added and mixed by vortexing or inverting. The solution was transferred into 2 × 2 mL Eppendorf tubes, centrifuged 10 min at 13 400 *g* at 4°C, extracted the aqueous phase with phenol–chloroform and then with chloroform. The aqueous phase was incubated at 4°C in 2 M LiCl overnight and centrifuged 20 min at 4°C at 13 400 *g* The pellet was rinsed with 2 M LiCl, resuspended in 500 μL TE (Tris–EDTA pH 8) after air drying and re-precipitated with 3 M sodium acetate pH 5.2 (0.1 volume) and 100% ice-cold ethanol (2.5 volume) by centrifuging 10 min at 13 400 *g* at 4°C, after incubating 15–45 min at −80°C. The pellet was washed with ice-cold 70% ethanol (centrifuged 1 min at 17 000 *g*) and resuspended in 30–50 μL DEPC (diethylpyrocarbonate) water after air or vacuum drying. All solutions used were prepared with DEPC-treated water and autoclaved. The mortar and pestle were treated with RNaseZap (Ambion, Inc., Austin, TX, USA) before use.

Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction was performed using Retroscript RT-PCR kit (Ambion, Inc.) and manufacturer's manual was followed. About 2 μg total RNA was run on a 1% agarose gel to quantify and check the quality and 2 μg of total RNA template was used for reverse transcription. For amplification of target cDNAs, PCR was performed using manufacturer's manual (Ambion, Inc.) in a DNA-Engine PTC-200 (MJ Research, Inc.). The PCR primers for At2g01170 cDNA were GabaStart (5′-GCG GCG ATC AAT CCT TTG TTC CCG TC-3′) and GabaStop (5′-TTC AGC TAA GAA TGT TGG AGA TGG GA-3′) and they were designed so as to amplify the full-length cDNA, which is 1.7 kb long.

Results

GUS staining patterns

Ten independent transposant lines (ET187, ET5279, ET2687, ET6395, ET7061, GT1713, GT1250, GT4841, GT5812 and GT7321) were identified that have transposon insertions in the At2g01170 gene of *A. thaliana* (Fig. 1). The mutant lines were obtained from the Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, USA). Insertion sites of all the transposants (noted in the CSHL databank) were confirmed with TAIL-PCR (data not shown).

Analysis of the GUS staining patterns for each line showed that only a few of them exhibited GUS staining and the staining patterns were not identical among those lines (Fig. 2). To make sure any weak GUS expression is also detected, potassium ferricyanide and potassium

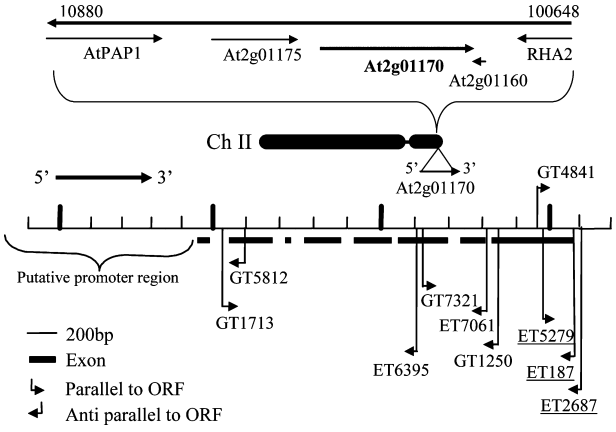


Figure 1 Insertion mutants, their point of insertions in At2g01170, and the relative position of At2g01170 with respect to neighbouring genes. Underlined names indicate the GUS expressing lines. ET, enhancer trap; GT, gene trap; ORF, open reading frame.

ferrocyanide were omitted from the GUS staining solution but no difference was detected. Different patterns of GUS staining among the ET lines was an unexpected result that might be because of enhancers associated by different genes or different enhancer elements associated with unique regions of the At2g01170. Among the five GT lines, three have the *GUS* gene in the same orientation as the At2g01170 open reading frame, yet only one yielded GUS staining. Initial results showed that this GT line (GT4841) displayed multiple, unique patterns of GUS staining among seedlings on the same plate. Subsequent analysis showed we had a mixture of transposants in our GT4841 seed collection (data not shown) and a screen of individual plants yielded a stable line (GT4841-A) displaying consistent GUS staining and mapping to At2g01170. Significantly, GUS staining in GT4841-A exhibited increased intensity when the plants were exposed to various stress conditions (Fig. 3) although mRNA levels at the same conditions did not change (Dundar, 2003; Dundar & Bush, 2008). Southern blot analysis showed that there was only one transposon insertion in GT4841-A (which was isolated from a single seed and a pool of F2 plants generated from this single seed were used to extract genomic DNA), and only one insertion in each GT and ET lines were investigated (Fig. 4). Genomic DNA from all other insertion lines was also isolated using a pool of plants in case there might be multiple copies in some seeds of the line.

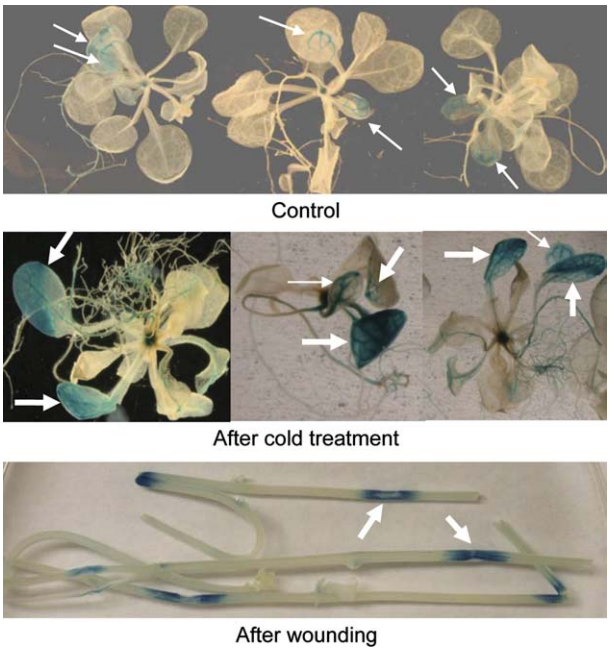
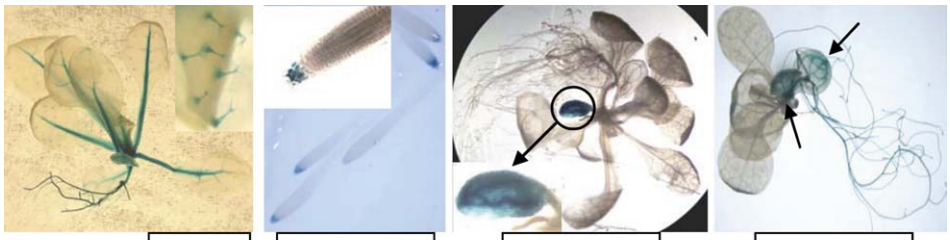


Figure 3 GUS expression of GT4841-A is enhanced by various stress conditions. Thin arrows point the GUS-stained areas (cotyledons) in control seedlings and the thick arrows point the stress-induced GUS staining at rosette leaves (some cotyledons of the stressed plants are not seen because they are hindered by the rosette leaves). Three-week-old plants were placed into 4°C for 16–24 h for cold treatment. Stems of 4- to 6-week-old plants were crushed with a pair of forceps where staining shows, and let 16- to 24-h stay (growing continued) before GUS staining.

A root phenotype in At2g01170 transposants is also insertion site dependent

The roots of *Arabidopsis* seedlings expressing the kanamycin resistance gene (*NPT II*) are shorter when grown in the presence of kanamycin than they are when grown on kanamycin-free media. When GT4841-A seedlings were grown on vertical plates containing kanamycin, root growth was not inhibited and its roots were longer than the control (GT1236), which is also expressing the *NPT II* gene (Fig. 5).

All 10 At2g01170 transposant lines were screened on kanamycin plates for the presence or absence of the



	ET187	ET2687	ET5279	GT4841-A
Roots	strong	strong (at tips only)	–	medium
Cotyledones	strong	–	strong	medium
Rosette Leaves	weak	–	–	weak
Midribs	strong	–	–	weak
Veins	weak	–	–	weak
Trichomes	strong	–	–	–

Figure 2 Three of five enhancer trap (ET) lines, and one of five gene trap (GT) lines displayed GUS staining. Plants were 3 weeks old when tested for GUS staining. Inserts in ET187, ET2687, and ET5279 show trichome, root tip and cotyledon staining, respectively. A dash (–) represents no staining.

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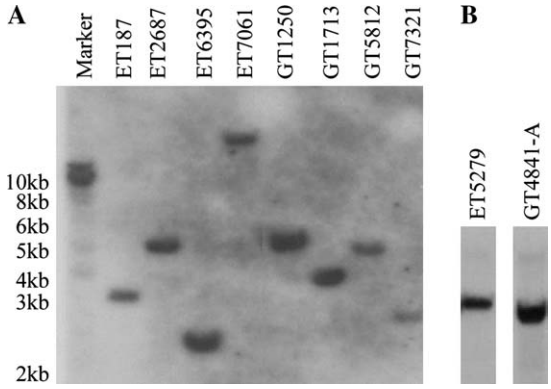

Figure 4 Southern blot analysis of At2g01170 ET and GT insertion mutants. About 20-µg genomic DNA was digested with *Eco*RI. A and B are results from independent experiments. The probes were prepared using primers that amplify a 400 bp part of *GUS* segment in the transposon cassette. The left primer sequence was 5′-cccttacgctgaagagatgc-3′ and the right primer sequence was 5′-ggcacagcacatcaaagaga-3′.

kanamycin-dependent short root phenotype. Only transposants with insertions in the 3′ end of At2g01170 exhibited longer roots, while insertions in the middle of the gene caused partial phenotype (Fig. 6). The root phenotype was not affected by the stress conditions that increased GUS expression or was an increase of GUS expression detected in roots under the stress (data not shown).

Genotype analysis of At2g01170

To determine if homozygous or heterozygous deletions of At2g01170 was causing the longer root phenotype, an RT-PCR analysis was run using primers that amplify a full length cDNA of this gene. Results of the analysis displayed all the lines contained heterozygous seedlings despite consecutive selections against kanamycin sensitivity (Fig. 7). Progeny analysis displayed around 95% kanamycin resistance from all lines. According to Hardy–Weinberg equation ($p^2 + 2pq + q^2 = 1$), this suggests around 35% heterozygous seedlings (which confirm the RT-PCR result for the existence of the wild-type copy in all lines; Fig. 7) and 60% homozygous seedlings for kanamycin resistance. We were unable, however, to detect fusion transcripts despite multiple attempts. The designed primers did amplify the chimaeric sequence from the genomic DNA (data not shown) but were unable to do so using the same cDNA template that successfully yielded the amplification of the wild-type At2g01170 message.

Discussion

Transposon mutagenesis has become a powerful tool for gene identification and characterisation (Ramachandran & Sundaresan, 2001; May & Martienssen, 2003). The *GUS* gene as a visual marker is an essential part of this system for analysing gene expression pattern (Ito *et al.*, 1999; Wu *et al.*, 2003). When inserted in parallel orientation with the chromosomal gene, the GUS of GT constructs can serve as a visual marker for the expression pattern of the target gene. For weak promoters, the *GUS* gene is preferred over other reporters because of its stronger visibility (Mantis & Tague, 2000). Using transposon-GUS enhancer/GT system, Sundaresan *et al.* (1995) generated thousands of transposants that have been very useful to identify and characterise many genes. As the gene identification is based on GUS expression, a mutant phenotype is not required to isolate genes. This allows researchers to identify genes that are not easily detected by classic genetic approaches because of their abundant expression or expression at multiple developmental stages (Springer, 2000). The trap systems are also very useful for the transactivation and control of genes (Engineer *et al.*, 2005; Jia *et al.*, 2007). It is important, however, to confirm that the GUS expression pattern compares to the actual expression pattern of the wild-type gene (Shaul *et al.*, 1999) in both ET and GT transposants. In ET lines, the active enhancer element can be associated with a gene that is several kilobases away from the ET insertion. Even with GT lines, cryptic promoter elements may drive expression outside the context of the nearest gene to the insertion site.

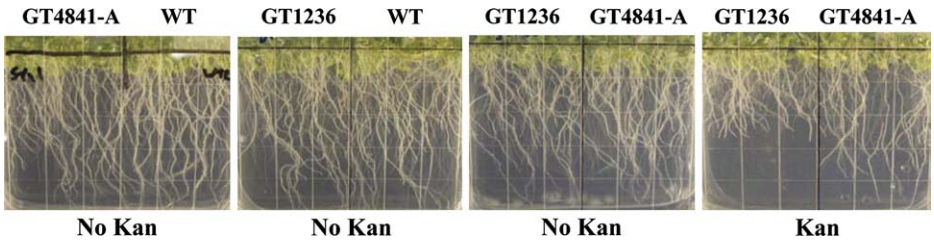

Figure 5 Root growth in GT4841-A is not sensitive to kanamycin. GT1236 is a transposon mutant for *AAP6* (Rentsch *et al.*, 1996) and used as a positive control. Plants were 2 weeks old when pictured.

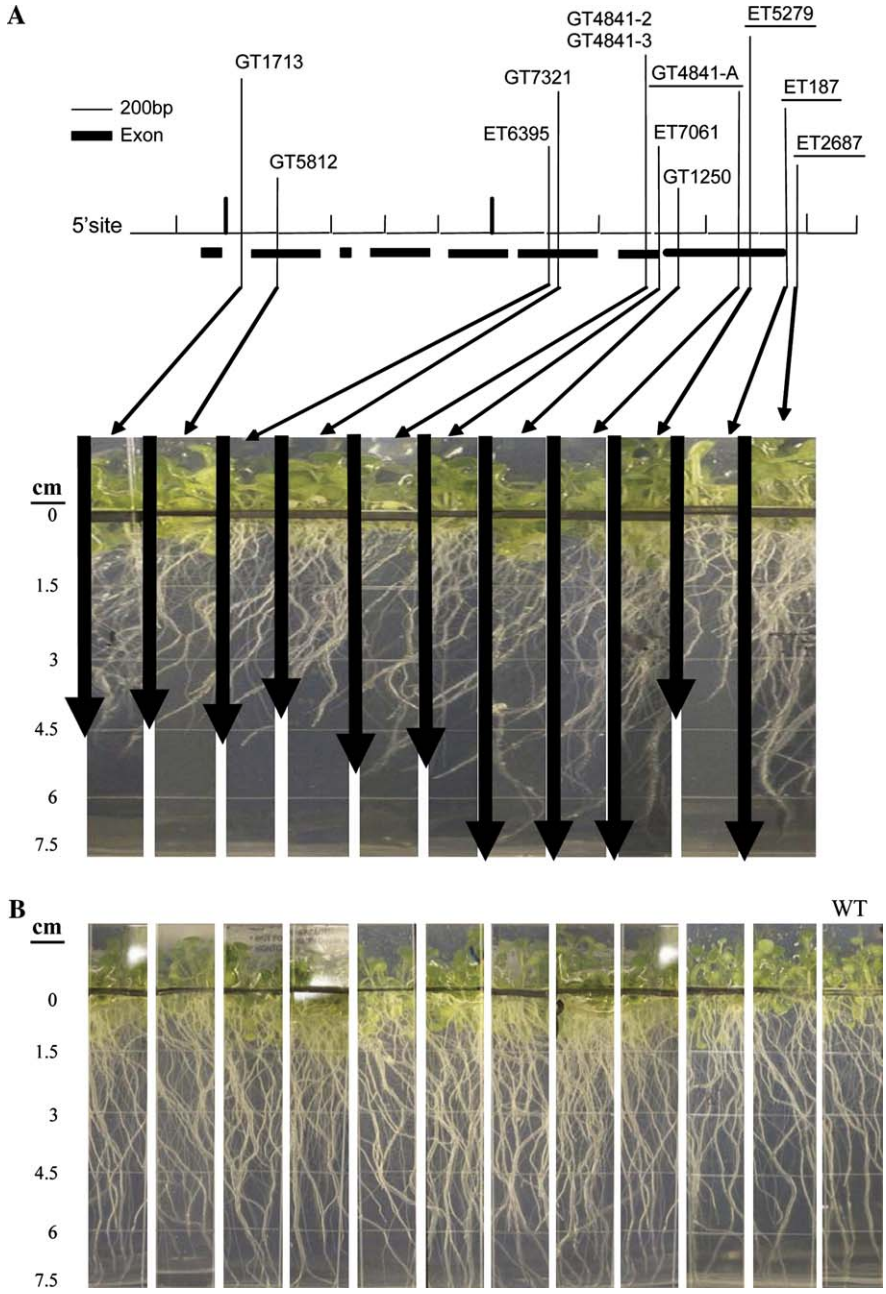

Figure 6 Growth of transposant lines on kanamycin containing plates with respect to their insertion sites on At2g01170. (A) Upper panel shows point of insertions for each transposant. Lower panel shows the root pictures aligned with the respective point of insertions of the mutant lines. Arrows on the left side of the roots show their average lengths at 2-week-old stage based on three independent vertical growth experiments. The average lengths with standard errors are as follows: GT1713: 4.53 ± 0.27 cm; GT5812: 4.23 ± 0.36 cm, ET6395: 4.57 ± 0.38 cm, GT7321: 4.3 ± 0.35 cm, GT4841-2,3: 5.62 ± 0.22 cm, ET7061: 5.4 ± 0.29 cm, GT1250: 7.23 ± 0.12 cm, GT4841-A: 7.08 ± 0.22 cm, ET5279: 7.19 ± 0.32 cm, ET187: 4.08 ± 0.22 cm, ET2687: 7.07 ± 0.24 cm. (B) Controls grown without kanamycin are shown with the same order as in (A) with wild type at the right side. The seeds used for all the lines were harvested at the same time for each experiment.

The 10 independent ET and GT insertions in the same gene (At2g01170) of *Arabidopsis* were examined here to understand better the response of ETs and GTs to different insertion sites in the same gene. This gene was determined to have a ubiquitous mRNA expression pattern (Dundar, 2003; Dundar & Bush, 2008). Five transposants were ET lines in which the *GUS* gene is placed in front of a weak promoter that can drive GUS expression

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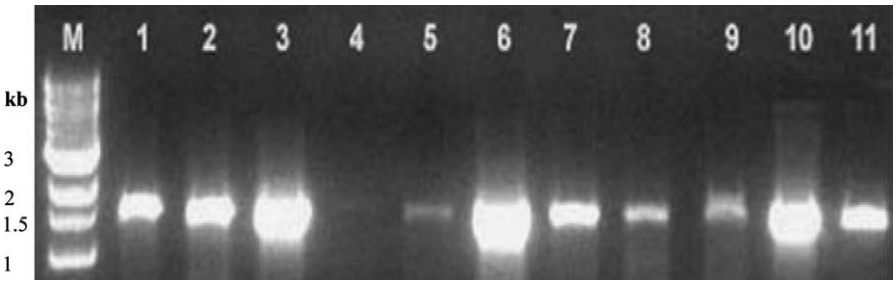

Figure 7 Genotype analyses of the transposants by RT-PCR analysis. Wild-type copy of At2g01170 amplified from all the mutant lines suggested all were heterozygous (lanes are not equal loads). Primers used were GabaStart (5′-gcggcgatcaatcctttgttcccgtc-3′) and GabaStop (5′-ttcagctaagaatgttggagatggga-3′). M, size marker; 1, GT4841-A; 2,ET187; 3, ET2687; 4, ET5279; 5, ET6395; 6, GT7321; 7, GT1250; 8, GT1713; 9, ET7061; 10, GT5812; 11, wild type.

when activated by nearby chromosomal enhancers. The distance to the enhancer stimulating the expression, however, can be tens of thousands of bases away in animal systems (Mautner *et al.*, 1996; Bulens *et al.*, 1997), and several kilobases in plant systems (Ahn *et al.*, 2007). Therefore, it is not unusual to get different GUS expression patterns for an ET compared with that of gene the ET is inserted into (Sundaresan *et al.*, 1995). It was unexpected, however, to observe three distinct GUS expression patterns for the three ET lines described here that are inserted into the same gene (At2g01170) on chromosome II. As opposed to the average gene density of chromosome 2 (5115 genes/19700kb = about 1 gene per 4 kb), the region of chromosome 2 that At2g01170 is located has five genes in 10 kb area, that is twice more dense than that of chromosome 2 (NCBI web site). The proximity of these genes to At2g01170 (Fig. 1) when combined with insertion disruption, may give rise to their enhancers to affect GUS expression pattern. As enhancers can act only several kilobases away from the gene they belong to in plant systems (Ahn *et al.*, 2007) however, a more likely hypothesis is that different elements of the trapped locus may give rise to different GUS expression patterns, as they get disrupted and/or affected by the insertion. These data suggest how small differences in chromosome location (even within 100 bp) can have a significant impact on which enhancer elements play a dominant role in controlling *GUS* gene expression.

The location of GT insertions in At2g01170 also had a significant impact on the presence or absence of GUS expression. Of three GT *GUS* transposants that were in parallel orientation with the At2g01170 open reading frame, only one displayed GUS staining. The absence of GUS staining in the two silent lines could be the result of a variety of factors. As these constructs are dependent on successful splicing of the gene in frame with the native transcript, splice site recognition and/or competition can easily account for the absence of GUS expression. Nussaume *et al.* (1995) have shown that a higher stringency in *Arabidopsis* was observed for the utilisation of certain splice donor and acceptor sites of the artificial intron before the *GUS* gene. They also suggested that a relatively low (62%) level of AT might be the reason for the poor splicing efficiency of this intron in *Arabidopsis*. Our analysis of the At2g01170 introns (GenBank accession number. AC006200) showed that the first five introns from the 5′ end have significantly lower (61–63%) AT than that of the remaining three introns, two of which have 70% AT. This suggests a lower efficiency for *GUS* splicing for the 5′ insertions not expressing GUS although they are in the correct orientation. Even with successful splicing, some chimaeric transcripts may be targeted for rapid degradation or translation may be blocked. Likewise, some chimaeric proteins may not fold properly and they could be targeted for immediate degradation.

Significance of the 5′ end of At2g01170

It is noteworthy that the only GUS-expressing ET and GT lines in At2g01170 were inserted in the 3′ end of the gene. For GTs, the absence of GUS staining for 5′ insertion lines could be the instability of the chimaeric message, a problem with translation or rapid protein degradation. The absence of GUS staining for 5′ ET insertions is probably an issue of proximity to required regulatory elements. Interestingly, At2g01170 transposant lines with the longer root phenotype also had insertions at the 3′ end of the gene. Presumably, these insertions yield a stable but smaller native protein as part of a chimaeric peptide with GUS that has a positive impact on root sensitivity to kanamycin. Although the nature of this response was beyond the scope of this study, we did some additional experiments to be able to come up with some hypotheses to explain these observations. Our genotype analysis

revealed all lines contained around 35% heterozygous seeds, and we confirmed the existence of the wild-type copy by RT-PCR (Fig. 7). As all the kanamycin-resistant seedlings (of any phenotype displaying transposant line) uniformly displayed the phenotype, it appears that the homozygous or heterozygous deletions of At2g01170 do not make any difference in terms of the longer root phenotype. Our failure to detect the chimaeric message despite multiple attempts suggests that the chimaeric message might have a very short half-life although apparently long enough to yield the chimaeric protein. The translational control of At2g1170 explains the high level expression of the GUS protein despite low level of its message (Dundar, 2003; Dundar & Bush, 2008). Combined data suggest that an abnormal copy (as part of the chimaeric protein) of At2g01170 might cause the longer root phenotype regardless of the presence of the wild-type copy. The 5′ insertions probably do not generate an effective abnormal copy because the mutated allele has a very small part of the wild-type gene left. The lines that have insertion in 3′ site and display the phenotype despite the lack of GUS expression (because of various reasons discussed above) could produce the abnormal copy of At2g01170, which could cause the phenotype as well. The observation that the longer root phenotype was not affected by the stress conditions that affect the GUS expression also suggests that the phenotype and the GUS expression are independent. Methylation is another factor that may involve this complication. Zilberman *et al.* (2007) has recently reported that longer genes tend to be methylated more often than shorter genes, and moderately expressed genes are also more likely to get methylated compared with highly expressed genes and poorly expressed genes. As the length of the native message is increased for the 3′ insertions and as At2g01170 can be considered a moderately expressed gene (Dundar, 2003; Dundar & Bush, 2008), methylation can also be one of the reasons for the varied GUS expression and the root phenotype, although Zilberman *et al.* (2007) determined the methylation status of At2g01170 as 'ambiguous'.

The results presented here clearly shown that ET-dependent and GT-dependent GUS expression is very sensitive depending on the insertion site of the expression cassette. Of the three GTs inserted into At2g01170 in the proper orientation, only one expressed GUS. Thus, GT lines not only must insert in the proper orientation, they must also insert in a place that yields a stable chimaeric protein. Quite surprisingly, the three ET lines inserted into At2g01170 each yielded a different pattern of GUS expression. These data showed that each ET appears to be affected by enhancers controlling other genes. Because these ET insertion sites are very close together, this observation serves to emphasise the need to confirm ET expression results by examining directly the expression pattern of putative tagged gene. While ET and GT GUS expression can be useful tools for describing the expression pattern of an undescribed gene, identifying genes that cannot be detected by classic genetic approaches because of their abundance in the genome or because of expression at more than one developmental stages, or because of their undetectable phenotype, these results clearly show how important it is to verify GUS expression data with direct measurements of tagged-gene expression.

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