Journal of Experimental Botany, Vol. 56, No. 419, pp. 2433–2442, September 2005
doi:10.1093/jxb/eri236 Advance Access publication 25 July, 2005
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Journal of Experimental Botany

RESEARCH PAPER

GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in *Arabidopsis thaliana*

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Received 24 February 2005; Accepted 26 May 2005

Abstract

Lateral root development occurs throughout the life of the plant and is responsible for the plasticity of the root system. In *Arabidopsis thaliana*, lateral root founder cells originate from pericycle cells adjacent to xylem poles. In order to study the mechanisms of lateral root development, a population of *Arabidopsis* GAL4-GFP enhancer trap lines were screened and two lines were isolated with *GAL4* expression in root xylem-pole pericycle cells (J0121), i.e. in cells competent to become lateral root founder cells, and in young lateral root primordia (J0192). These two enhancer trap lines are very useful tools with which to study the molecular and cellular bases of lateral root development using targeted gene expression. These lines were used for genetic ablation experiments by targeting the expression of a toxin-encoding gene. Moreover, the molecular bases of the enhancer trap expression pattern were characterized. These results suggest that the lateral-root-specific *GAL4* expression pattern in J0192 is due to a strong enhancer in the promoter of the LOB-domain protein gene *LBD16*.

Key words: Genetic ablation, lateral root founder cells, lateral root primordia, patterning, pericycle.

Introduction

Plant roots are responsible for nutrient and water uptake and provide physical support to the plant. Most of the root system is made of lateral roots that originate post-embryonically. Lateral root development is controlled by different factors including nutrient concentration in the plant and the soil (Lopez-Bucio *et al*., 2003; Malamy, 2005, for a review). This plasticity allows adaptation of the root system to the soil, a very heterogeneous and changing environment, and is consequently very important for the survival of the plant (Grime *et al*., 1986; Hodge, 2004).

In *Arabidopsis thaliana*, lateral roots originate post-embryonically from a small number of differentiated cells situated in the root pericycle in front of xylem poles called pericycle founder cells (Casimiro *et al*., 2001; Dubrovsky *et al*., 2001). These cells undergo a defined program of oriented cell divisions and expansion to form a lateral root primordium (Malamy and Benfey, 1997; Dubrovsky *et al*., 2001; Casimiro *et al*., 2003). Unlike primary root formation that occurs during embryogenesis, lateral root formation is easily accessible to observation and experimentation. Moreover, lateral root formation can be initiated by the application of the plant hormone auxin. Nevertheless, molecular mechanisms of root branching are still poorly understood.

In order to study the molecular and cellular bases of lateral root development, an experimental system allowing targeted changes in gene expression would be useful. The GAL4/UAS two-component system was developed in *Drosophila* toward this goal (Brand and Perrimon, 1993). An enhancer trap strategy based on P elements was used to generate transgenic *Drosophila* expressing different patterns of a yeast transcription activator, GAL4. A chosen target gene can be placed under the control of a GAL4-activated promoter (Upstream Activation

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Sequences, UAS), transformed into *Drosophila* and maintained silently in the absence of GAL4. Genetic crosses between this single line and an enhancer trap line specifically activates the target gene in a chosen tissue or cell type. The phenotypic consequences of misexpression, including those lethal to the organism, can then be conveniently studied. GAL4-mediated transactivation is now widely used to elucidate the cell-specific functions of known genes, for targeted cell ablation and for conventional genetic approaches, as the basis of enhancer or suppressor screens in *Drosophila*. This system was adapted for *Arabidopsis* (Haseloff, 1999). The codon usage of a GAL4 derivative was altered to allow efficient expression in plants. *GAL4* can be expressed using characterized promoters to drive gene expression in chosen cells. This approach was used for instance to analyse the effects of cell ablation during *Arabidopsis* seed development (Weijers *et al*., 2003). However, the limited number of well-characterized promoters and corresponding expression patterns limits the cell types and developmental processes that can be targeted. Enhancer trap strategies can circumvent this problem. A GAL4 enhancer trap T-DNA vector was designed so that expression of the *GAL4* gene would be dependent upon the fortuitous proximity of an *Arabidopsis* enhancer element (Haseloff, 1999). The T-DNA also contains a GAL4-responsive green fluorescent protein (GFP) gene. GAL4 enhancer trap lines are useful markers to tag specifc cell types and reveal developmental transitions (Sabatini *et al*., 1999; Wysocka-Diller *et al*., 2000; Cary *et al*., 2002; Birnbaum *et al*., 2003). They have also been successfully used to study the developmental effects of targeted gene expression in different cell types or tissues in *Arabidopsis* (Sabatini *et al*., 2003; Gallois *et al*., 2004).

In order to obtain tools to study the molecular and cellular bases of lateral root development in *Arabidopsis*, a population of GAL4 enhancer trap lines was screened and two lines with GAL4 expression associated with lateral root development were isolated. It was shown that these lines can be used for gene transactivation experiments in lateral root founder cells and young lateral root primordia respectively. This strategy was used to conduct cell ablation experiments. Moreover, the molecular bases of the *GAL4* expression pattern in these enhancer trap lines was studied. The results suggest that a lateral root-specific enhancer present in the promoter of the *LOB-domain protein16* (*LBD16*) gene may be responsible for the *GAL4* expression pattern in the lateral-root-specific enhancer trap line J0192.

Materials and methods

Plant lines and growth conditions

C24 and Col-0 seeds were obtained from the Nottingham Arabidopsis Stock Center.

J0121 and J0192 (C24) were isolated from a collection of 401 GAL4-GFP ET lines generated by root transformation of C24 wild-type plants (J Haseloff, unpublished results) during a screen for lateral root expressed lines. Homozygous plants were generated by selfing.

UAS-GUS and UAS-DTA lines (ecotype C24) were provided by Jim Haseloff's laboratory (S Hodge and J Haseloff, unpublished results).

Plants were grown at 23 °C, 60% humidity in 45 μE constant light on vertical ½ MS 1.2% phytagel plates under long-day conditions (16/8 h light/dark). Seeds were surface-sterilized and cold-treated for 2 d at 4 °C in the dark before transfer to the growth chamber. Plants in soil were grown in a 1:1 (v/v) compost/vermiculite mix in a growth room at 21 °C in a 16/8 h light/dark cycle.

Root length was measured from digital images of the plates using the NIHimage 1.62 software. Emerged lateral roots were counted using a binocular microscope. Data were analysed using the Excel statistical package. Experiments were repeated at least two times independently.

Microscopy

Plants were screened for GFP expression using a Fluo III fluorescence microscope (Leica) with GFP1 (excitation filter 425 nm; emission filter 480 nm) and GFP3 filters (excitation 480 nm; emission 525 nm).

For confocal microscopy, seedlings were stained for 10 min in 5 mg l^{-1} propidium iodide (PI). Imaging was performed using a Leica DMRXA microscope and Leica TCS SP confocal software. A long pass 500 nm dichroic was used as the beam splitter. Emission maxima were 510 nm and 610 nm for GFP and PI, respectively. 20–40 nm bandwidths were used. Objectives used were Leica ×63 PlanApo NA 1.2, ×20 PlanApo NA 0.7, and ×10 PlanApo NA 0.4.

GUS activity was assayed by immersing seedlings in a staining solution (Svistoonoff *et al*., 2003) at 37 °C. 0.5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ were added to limit the diffusion of the blue staining. Tissues were cleared in 70% ethanol for 2 d. Tissues were then immersed in 50% (v/v) ethanol/10% (v/v) glycerol for 2 h, 30% (v/v) ethanol/30% (v/v) glycerol for 2 h, and in 50% (v/v) glycerol for 2 h. Seedlings were then mounted in 50% (v/v) glycerol and visualized on a DMRB microscope (Leica).

Root sections

Samples were fixed (Svistoonoff *et al*., 2003) and cleared in 70% ethanol for 2 d. Ethanol dehydration was performed (90%, 100% twice) at room temperature (15 min per step). Samples were then embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Sections (6 μm) were cut with a Historange 2218 LKB microtome. Sections were stained for 10 min in aqueous 0.05% ruthenium red solution and mounted in Clearium moutant (Surgipath, Peterborough, UK).

Genomic DNA blot analysis

Genomic DNA was extracted from homozygous J0121, J0192, and wild-type (C24) seedlings using the DNeasy kit (Qiagen) according to the manufacturer's instructions. 3 μg of genomic DNA was digested with *Apo*I and *Bgl*II (New Englands Biolabs). DNA fragments were separated on a 1% agarose gel and capillary blotted onto a Hybond N^+ membrane (Amersham). A 504 pb GAL4 probe was synthesized using primers mPPR1-5 (5′-CGGCAAGCTTGGATCCAACAATG-3′) and mPPR1-3 (5′-CCCGGAGCTCGTCCCCCAGGCTG-3′) and labelled with α-^{32}P dCTP by random priming. Hybridization was carried out in high stringency conditions. Filters were washed at 65 °C in 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS; 0.5× SSC, 0.1 SDS, and 0.1× SSC, 0.1%SDS (10 min each). Hybridization patterns were visualized using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics).

Mapping of the T-DNA insertion sites

Genomic DNA was isolated from J0121 and J0192 seedlings using the DNeasy kit (Qiagen) as recommended by the supplier. TAIL-PCR was performed as described by Liu *et al.* (1995) using the specific right border primers GAL4131REV (5′-GACACTTGGCGCACTTCGGCTTCTTC-3′), GAL499REV (5′-CACTTGAGTTCTTGAGGCGGGCAGAT-3′), and GAL445REV (5′-AGCTTCATTGTTGGATCCGGTTCTCT-3′). The insertion site was confirmed by PCR using primers J0121PreR (5′-CGGGTAAGTCTTGTTCGTTAGGTT-3′), J0121PostF (5′-CCTCCATTCCATCATTCTTTCG-3′), J0192RB (5′-GACGTGTCGACTGACAGGG-3′), and J0192LB (5′-CCCACAAACGAAAAATCCCC-3′) designed to anneal the genomic DNA flanking the insertion site in J0121 and J0192, respectively, and T-DNA primers GAL445REV and LB102FWD (5′-GGAACAACACTCAACCCTATCTCGGG-3′). PCR products were cloned into pGEM-T easy (Promega) and sequenced. Database searches were performed using the BLAST program through the NCBI server (www.ncbi.nlm.nih.gov).

Gene expression analyses

RNA was extracted from various tissues from wild-type Col-0 plants using the RNEasy kit (Qiagen). cDNA was synthesized from 1 µg of total RNA using oligo(dT) and AMV reverse transcriptase (Promega) in 25 µl according to the manufacturer's instructions. 1 µl of the reverse transcriptase reaction was used in the subsequent PCR amplification using gene specific primers LBD16RT5 (5′-CGGTACAACGGCGGGGACAG-3′), LBD16RT3 (5′-TGGTGGCCAGCTATCTGTGC-3′) within 35 cycles (95 °C, 30 s; 55 °C, 30 s; 72 °C 30 s). These primers were chosen in a variable region of the gene and on both sides of an intron so that there was a 749 bp difference between the amplification products from cDNA and genomic DNA. The PCR reactions were performed in 2 mM $MgCl_2$, 50 mM KCl, 10 mM TRIS–HCl (pH 9), 0.1% Triton X-100, 0.2 mM each dNTPs, 0.2 µM of each primer, and 1 unit of *Taq* polymerase (Promega) in a total volume of 20 µl. The *ACTIN2* transcript was used as an internal control. The same procedure and samples were used with primers actin2-5 (5′-CCATTCTTGCTTCCCTCA-3′) and actin2-3 (5′-GACGTAAGTAAAAACCCAG-3′) and an annealing temperature of 50 °C. 5 µl of the PCR reaction were separated by electrophoresis on a 1.4% agarose gel. Reactions were repeated at least twice.

Constructs and generation of transgenic plants

A 1.5 kb *LBD16* promoter fragment was amplified from genomic DNA from Col-0 plants using primers LBD16prom5 (5′-CCAAAGCTTCCTAAGCCACCTAAGCAGA-3′) and LBD16prom3 (5′-TGGGATCCGCGAAACGAACAAAAAAG-3′) containing a *Hin*dIII and a *Bam*HI site, respectively. The digested fragment was then cloned in the binary vector pBI101.3 (Clontech) just upstream of the *uidA* gene to create the p*LOB16:GUS* plasmid. Proper cloning was checked both by restriction digest and sequencing. p*LOB16:GUS* was introduced into *Agrobacterium tumefaciens* PMP90 by electroporation. Wild-type Col-0 *Arabidopsis* plants were transformed using the floral dip method (Clough and Bent, 1998).

Results

A screen for lateral root development related enhancer trap lines

A collection of 401 GAL4-GFP enhancer trap (ET) lines generated by root transformation of wild-type *Arabidopsis* plants ecotype C24 (J Haseloff and S Hodge, unpublished results) was screened for *GFP* expression in lateral root founder cells or during lateral root development. Plants were grown on vertical agar plates and scored at 7 d and 10 d after germination. Four lines showing *GFP* expression in lateral root founder cells or primordia were selected and *GFP* expression was studied throughout development (Table 1). *GFP* expression in J0121 root tissues occurs in mature xylem-pole pericycle cells, i.e. in the pericycle cells competent to make lateral root primordia (LRP). The three other lines had *GFP* expression in LRP, but only line J0192 showed *GFP* expression limited to lateral root development. Lines J0121 and J0192 were selected for further studies on the basis of their *GFP* expression related to lateral root development. Homozygous plants were generated by selfing.

Table 1. *Lateral root enhancer trap lines*

Line	No. of T-DNA insertions[a]	*GFP* expression pattern
J0121	1	Root xylem-pole pericycle cells
J0192	1	Lateral root primordia
J2772	1	Root cap, lateral root primordia, base of lateral roots
Q850	3	Root cap, lateral root primordia

[a] The number of T-DNA insertions was studied by Southern blot hybridization using a *GAL4* probe.

Confocal microscopy studies of *GFP* expression were conducted at different stages of development. In J0121 plants, *GFP* expression starts in the elongation zone of the root (Fig. 1A). No expression was detected in the root apical meristem (Fig. 1A). *GFP* fluorescence was limited to pericycle cells adjacent to xylem poles. *GFP* expression was turned off in LRP as early as stage I (data not shown). Some weak *GFP* expression was found in the hypocotyl epidermis (Fig. 1C) particularly toward the base. No *GFP* expression was found in the shoot, the leaves, the flowers, or the siliques (data not shown).

In J0192 plants, no *GFP* expression was found in the primary root. GFP could only be detected in lateral root primordia as early as stage I, after the asymmetric division responsible for the formation of the lateral root founder cells (Fig. 1D). *GFP* was expressed in all of the cells of young LRP (Fig. 1E, F). At later developmental stages, GFP fluorescence was limited to the base and flanks of the primordia (Fig. 1G) and disappeared upon emergence. No *GFP* expression was found in the main root, in the mature lateral roots, in the shoot, the leaves, the flowers, or the siliques (data not shown).

In summary, J0121 and J0192 plants showed complementary *GFP* expression patterns. In J0121, *GFP* was expressed in cells competent to form LRP but was switched off in LRP while in J0192 it was expressed specifically in young LRP.

J0121 and J0192 driven gene transactivation

In order to confirm the pattern of gene transactivation, homozygous ET plants were crossed with transgenic C24

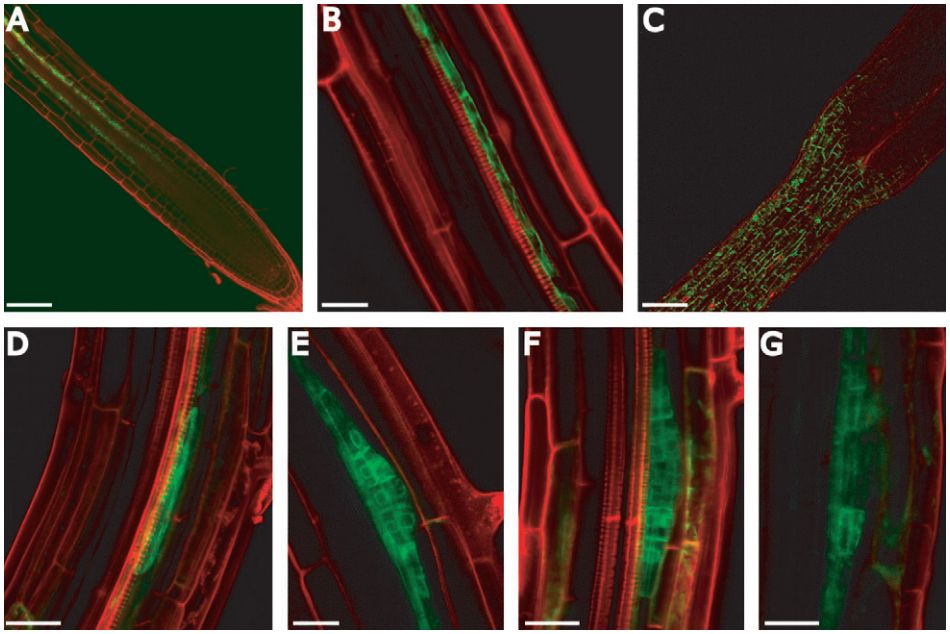


Fig. 1. *GFP* expression in the enhancer trap lines J0121 (A–C) and J0192 (D–G). (A, B, D, E, F, G) Longitudinal confocal sections of roots and (C) projected confocal view of the top part of a hypocotyl of living plants showing GFP fluorescence (green) and counterstained with propidium iodide (red). (A) *GFP* expression starts in the pericycle cells of the elongation zone. No GFP fluorescence is visible in the root meristem. (B) Expression in pericycle cells adjacent to a xylem strand. (C) Expression in the hypocotyl epidermis. (D) Expression in a stage I primordium. (E) Expression in a stage II primordium. (F) Expression in a stage III primordium. (G) Expression in a stage V primordium. Stages are defined according to Malamy and Benfey (1997).

lines containing a β-glucuronidase (GUS) encoding gene (*uidA*) under the control of a synthetic GAL4-dependent promoter (UAS). *UAS-uidA* plants showed no GUS activity in the absence of GAL4 (data not shown). The F_1 plants were grown on vertical agar plates together with control plants (J0121 and J0192) and the pattern of GUS activity was analysed at different developmental stages.

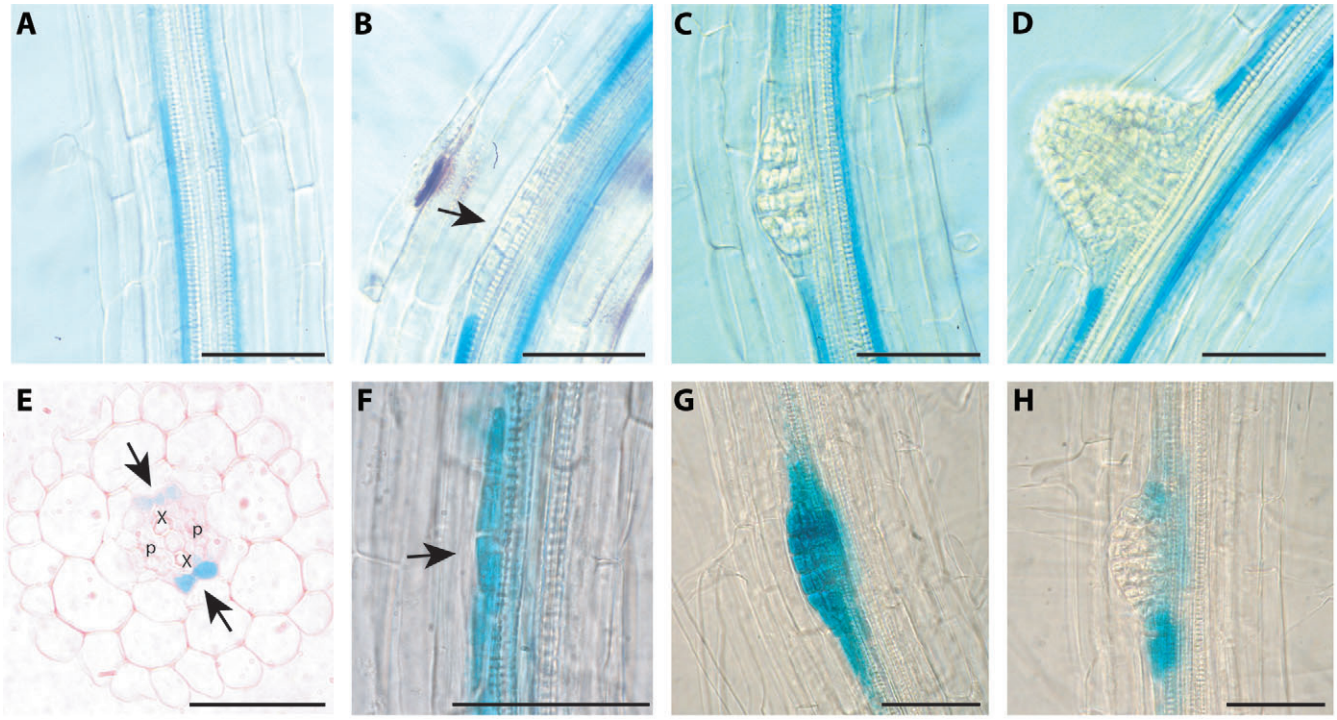
J0121≫GUS plants showed GUS activity in 2–3 files of root pericycle cells in front of the xylem pole (Fig. 2A, E). Expression started in the elongation zone of the root. No expression was found close to the root tip. No GUS activity was detected in lateral root primordia from stage I to emergence (Fig. 2B–D). After emergence, the pattern of GUS activity in the lateral root was similar to the pattern of expression in the primary root. Weak *GUS* expression was found in the epidermis at the base of the hypocotyl (data not shown). No GUS activity was detected in the rest of the plant or during embryogenesis (data not shown). This indicates that the *GFP* expression pattern in J121 is due to *cis*-activation by GAL4. Consequently, J0121 directs GAL4-mediated gene transactivation in xylem-pole pericycle cells but not in lateral root primordia.

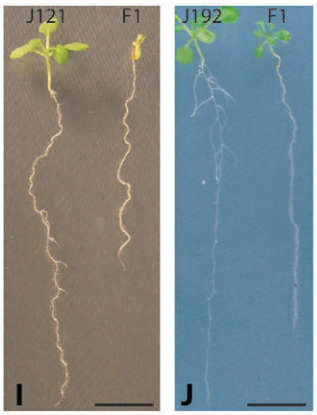
Discrete patches of GUS activity were detected along the root of J0192≫GUS plants. Closer examination revealed that GUS activity was localized in LRP. Figure 2F–H shows GUS activity at different stages of lateral root development. It was found in all the cells from stage I–IV primordia (Fig. 2F, G), but was restricted to the flanks of the primordia at later stages (Fig. 2H). GUS activity disappeared at the onset of lateral root emergence. No GUS activity was detected in the aerial parts or during embryogenesis (data not shown). This pattern is consistent with the pattern of *GFP* expression in the J0192 line and indicates GAL4-mediated gene transactivation in young LRP from stage I to stage IV and at the base of older LRP.

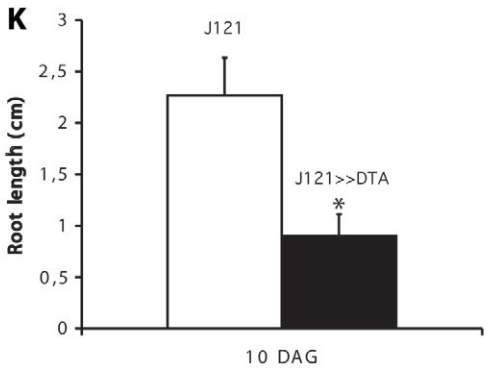
Genetic ablation using J0121 and J0192 enhancer trap lines

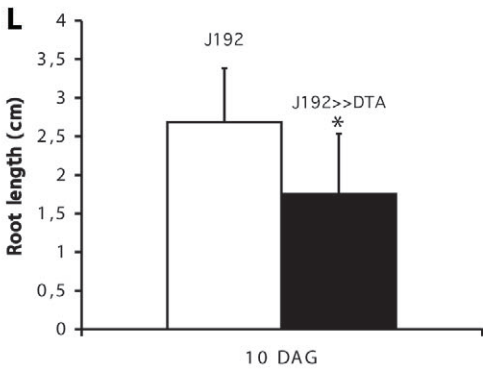
J0121 and J0192 were used in genetic ablation experiments using the Diphtheria toxin chain A (DTA) gene. DTA is a highly active ADP-ribosylase specific for eukaryotic elongation factor 2, and inhibits translation in a cell autonomous manner in *Arabidopsis* (Czako *et al*., 1992). This gene is silent in the absence of GAL4 and the UAS-DTA homozygous line has a wild-type phenotype (data not shown). F_1 plants from crosses between homozygous ET lines and transgenic C24 lines containing a UAS-DTA gene construct were grown on vertical agar plates together with control (J0121, J0192, UAS-DTA) plants and scored for root growth and lateral root emergence 10 d after germination (10 DAG).

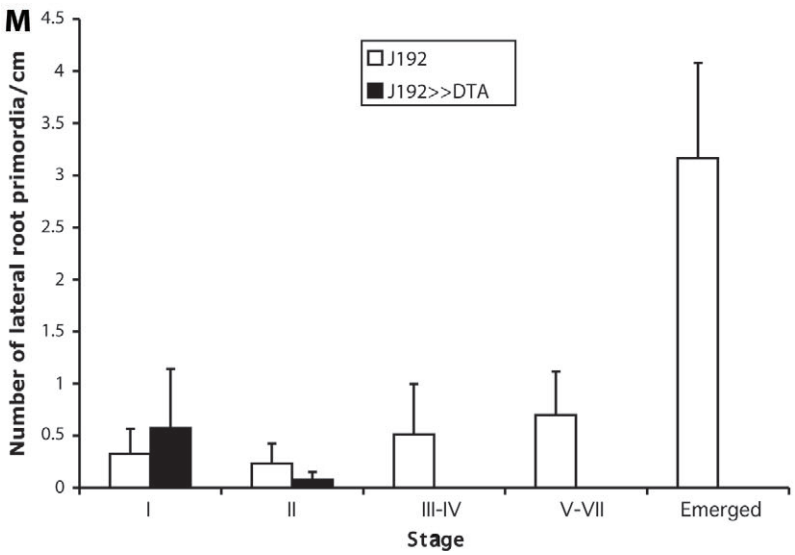
Germinating J0121≫DTA F_1 plants were morphologically normal thus confirming the absence of any *GAL4* expression during embryogenesis. As shown in Fig. 2I, J0121≫DTA plants exhibited reduced growth. Root length


A
B
C
D
E
X
p
p
X
F
G
H


J121
F1
I
J192
F1
J


K
Root length (cm)
3
2,5
2
1,5
1
0,5
0
J121
J121>>DTA
*
10 DAG


L
Root length (cm)
4
3,5
3
2,5
2
1,5
1
0,5
0
J192
J192>>DTA
*
10 DAG


M
Number of lateral root primordia/cm
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
J192
J192>>DTA
I
II
III-IV
V-VII
Emerged
Stage

was reduced by 58% in J0121≫DTA compared with control plants (Fig. 2K) and no lateral root primordia were formed. The absence of any lateral root primordia in J0121≫DTA plants confirms that the *GAL4* expressing cells in J0121, i.e. the xylem-pole pericycle cells, are necessary for lateral root development. J0121≫DTA plants did not survive more than 15 d on plates. The hypocotyl surface looked irregular and leaves failed to develop properly probably because of the low level of *GAL4* epidermal expression.

The germination rate and phenotype of germinating J0192≫DTA plants were similar to the control showing that *GAL4* is not expressed during embryogenesis. Figure 2J shows 10-d-old J0192≫DTA and J0192 plants grown on the same plate. Toxin expressing plants have reduced root growth compared with the control (Fig. 2L). Of the 61 10-d-old J0192≫DTA plants analysed, only two showed one adventitious and one lateral root, respectively. No emerged lateral root was found in the other J0192≫DTA plants, compared with an average of 5.39±3.33 lateral roots per plant for the J0192 plants grown in the same conditions (n=54). 10-d-old J0192≫DTA (n=15) and control plants (n=8) were fixed and analysed to determine at what stage lateral root development aborted. The overall longitudinal and radial structure of the root was normal and no cell tier was missing (data not shown). J0192≫DTA plants had a similar number of stage I primordia to that found in control plants, 2 stage II primordia were found in one plant out of 15, and no primordia corresponding to later stages of lateral root development were observed (Fig. 2M). These results indicate that ablation of lateral root primordia occurs at the end of stage I in J0192≫DTA plants. This result is in agreement with the pattern of gene transactivation described previously with *GAL4* expression commencing in late stage I primordia. J0192≫DTA plants managed to survive, but developed slower than control plants when transferred to soil. Floral development was normal and the plants were fertile (data not shown).

Genetic ablation experiments using the GAL4 enhancer trap lines J0121 and J0192 were consistent with the *GAL4* expression patterns as revealed by GFP fluorescence and *uidA* transactivation experiments. Ablation of the root pericycle cells facing the xylem poles (J0121≫DTA) prevented lateral root development consistent with their role as pericycle founder cells. Ablation of young lateral root primordia using J0192 led to the formation of viable plants devoid of lateral roots. No effect on the cells of the root meristem was observed as expected from the lack of GUS staining in the *uidA* transactivation experiments for these two enhancer trap lines. The reduction of primary root growth observed in both J0121≫DTA and J0192≫DTA plants might be due to a reduction in nutrient acquisition linked to the absence of lateral roots and/or the stress of cell ablation in mature root inner tissues. The authors are currently trying to analyse how the cells close to ablated cells react using confocal imaging and 3D-reconstruction techniques.

T-DNA insertions have no effect on J0121 and J0192 root development phenotype

Use of transactivation strategies to study lateral root development depends on the absence of any defects due to insertion of the enhancer trap T-DNA. Homozygous J0121 and J0192 were grown on vertical plates together with control (C24) plants and scored for root growth and lateral root emergence 10 d after germination. No change in root development was observed in homozygous J0121 and J0192 plants (data not shown).

The molecular bases of the enhancer trap GAL4 expression were characterized. Southern hybridization was used to determine the number of T-DNA inserted in the enhancer trap lines. A *GAL4* probe was used to hybridize genomic DNA from WT (C24) and homozygous J0121 and J0192 plants. Southern blot experiments showed that a single enhancer trap T-DNA was inserted in lines J0121 and J0192 (Fig. 3A).

The T-DNA insertion sites were mapped. Thermic Asymmetric Interlaced-PCR (Liu *et al*., 1995) was used to clone the genomic DNA flanking the right border of the T-DNA inserts. TAIL-PCR products (370 bp and 300 bp for J0121 and J0192, respectively) were cloned and sequenced. The corresponding genomic DNA sequence matched BAC clone T20L15 (ch. V) and MHK10 (ch. II) for J0121 and J0192, respectively. These insertion sites were confirmed by sequencing PCR products obtained using primers corresponding to (i) genomic sequences located on both sides of the putative T-DNA location, and (ii) both ends of the T-DNA.

Fig. 2. Specific transactivation in pericycle cells and lateral root primordia. GUS activity in J0121≫*uidA* (A–E) and J0192≫*uidA* (F–H) F_1 roots and the phenotype of J0121≫DTA (I, K) and J0192≫DTA F_1 (J, L, M) plants. (A) GUS activity in the pericycle cells in front of the xylem poles. (B) GUS activity is excluded from a stage I primordium (arrow). (C) GUS activity in pericycle cells in front of the xylem poles but not in a stage V primordium. (D) GUS activity is not visible in a lateral root just after emergence. (E) Transverse section (6 μm thick) showing GUS activity in pericycle cells files (arrows) adjacent to the xylem poles (x), (p) phloem poles. (F) GUS activity in a stage I primordium. (G) GUS activity in a stage IV primordium. (H) GUS activity in the flanks of a stage VI lateral root primordium. (I) 10-d-old control (J0121) and J0121≫DTA (F_1) plants. (J) 10-d-old control (J0192) and J0192≫DTA (F_1) plants. (K) J0121≫DTA plants display reduced primary root growth. Control (J0121, n=47) and J0121≫DTA (n=53) plants were grown on vertical agar plates. Root length was measured 10 d after germination (10 DAG). The values shown are means ±SD. Significance was analysed by ANOVA test. *, P <0.05 compared with control plants. (L) J0192≫DTA (n=61) plants display reduced root growth compared to control (J0192, n=54) plants. The values shown are means ±SD. Significance was analysed by ANOVA test. *, P <0.05 compared with control plants. (M) Lateral root developmental stage distribution in control (J0192, n=8) and J0192≫DTA (n=15) plants. Plants were cleared and the number and stages (Malamy and Benfey, 1997) of lateral root primordia were recorded. Bars are 50 μm (A–H), and 1 cm (I, J).

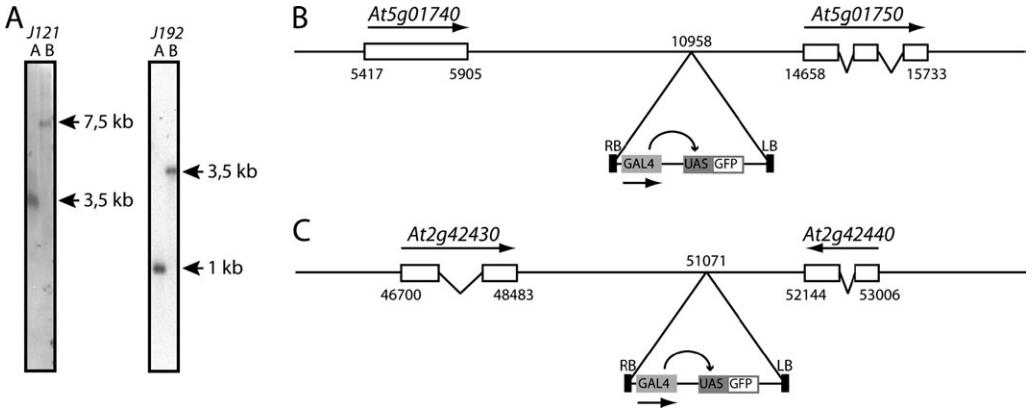


Fig. 3. Molecular characterization of the enhancer trap lines J0121 and J0192. (A) Southern blot hybridization of genomic DNA isolated from J0121 and J0192 plants. 3 μg of genomic DNA was digested with *Apo*I and *Bgl*II and hybridized with a ^{32}P-labelled *GAL4* probe. (B) Enhancer trap T-DNA insertion site in J0121. Positions are relative to BAC clone T20L15. (C) Enhancer trap T-DNA insertion site in J0192. Positions are relative to BAC clone MHK10.

J0121 enhancer trap T-DNA was inserted in an intergenic region between *At5g01740* and *At5g01750* that code for proteins of unknown function. The *GAL4* gene is about 5 kbp downstream and about 4 kbp upstream of *At5g01740* and *At5g01750*, respectively (Fig. 3B). Both *At5g01740* and *At5g01750* are expressed in the root stele (including the pericycle) as shown by micro-array experiments (Birnbaum *et al*., 2003) and may, therefore, both be responsible for *GAL4* expression pattern in J0121.

J0192 enhancer trap T-DNA was inserted between *At2g42430* and *At2g42440* at about 2.5 and 1 kbp, respectively. *At2g42430* and *At2g42440* encode the LOB-Domain proteins LBD16 and LBD17, respectively (Shuai *et al*., 2002). The *GAL4* gene was inserted downstream of and oriented in the same direction as *LBD16* (Fig. 3C). *LBD16* is specifically expressed in roots while *LBD17* is expressed throughout the plant except in roots. Moreover, the *LOB* gene is expressed at the base of lateral root primordia (Shuai *et al*., 2002) thus suggesting that *GAL4* expression pattern in J0192 might be due to an enhancer element regulating *LBD16* expression. For both ET lines, the intergenic position of their T-DNA insertion is consistent with the lack of developmental defect.

LBD16 *is expressed in young lateral root primordia*

In order to test whether the lateral root specific *GAL4* expression pattern of the J0192 enhancer trap line was due to a regulatory DNA sequence within *LBD16* promoter region, the *LBD16* expression pattern was studied. First, *LBD16* expression was analysed by RT-PCR (Fig. 4A). *LBD16* transcripts were detected in the root and the flower samples. No expression was found in siliques, leaves, or stem. These results are in agreement with those of Shuai *et al*. (2002).

A 1.5 kbp DNA fragment upstream of *LBD16* start codon was cloned and fused to the *uidA* reporter gene. This construct was introduced by floral dip (Clough and Bent, 1998) transformation into *Arabidopsis* ecotype Col-0. GUS activity was analysed in 19 independent transgenic T_2 lines. A similar expression pattern was observed with all the lines. In 3-d-old *LBD16-GUS* seedlings, GUS activity was detected in the root tip and near the base of the root at a site of lateral root initiation (Fig. 4B). This activity was strong, as 30 min of incubation were sufficient to reveal it. Longer incubation times (⩾2 h) showed *GUS* activity in the root vasculature too. In older seedlings, GUS activity was found in young LRPs but not in the root tip (Fig. 4C–E). Long incubation times revealed GUS activity in the root vasculature. This indicates that *LBD16* is not specifically expressed during lateral root development. However, these results suggest that a strong regulatory element responsible for gene expression in young lateral root primordia is present in the 1.5 kbp DNA sequence upstream of *LBD16* start codon.

Discussion

In this study, two GAL4 enhancer trap lines showing *GAL4* expression associated with lateral root development were isolated and characterized. In the enhancer trap line J0121, *GAL4* expression is specific to 2–3 root pericycle cell files adjacent to the xylem poles. It has been demonstrated that these cells continue to cycle after leaving the root apical meristem, whereas the rest of the pericycle cells differentiate, thus creating an 'extended meristem' (Beeckman *et al*., 2001) competent for lateral root formation (Dubrovsky *et al*., 2001; Casimiro *et al*., 2003). Lateral root founder cells arise from these three pericycle cell files in contact with the protoxylem and most of the cells in the LRP derive from the central file of founder cells (Kurup *et al*., 2005). These results are consistent with our ablation experiments. J0121-directed transactivation of a toxin gene

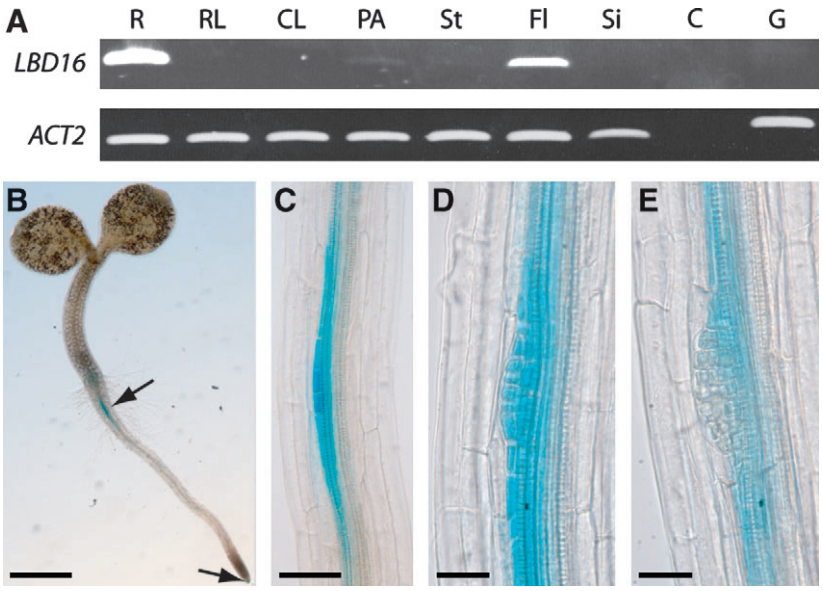


Fig. 4. *LBD16* expression pattern. (A) RT-PCR analysis of *LBD16* expression in 10-d-old root tissues (R), rosette leaves (RL), cauline leaves (CL), 10-d-old shoot apex (PA), inflorescence stem (St), flowers (Fl), and siliques (Si). A control without cDNA (C) and a genomic DNA control (G) were also included. *LBD16* primers are situated on both sides of an intron so that the genomic DNA amplification is 749 bp bigger that the cDNA amplification product. Amplification of the constitutive *ACTIN2* gene was used as a positive control. (B) 3-d-old *LBD16-GUS Arabidopsis* seedling showing blue GUS staining in the columella at the root tip and in a lateral root primordium (arrows). (C) GUS activity in a stage I lateral root primordium from a 5-d-old *LBD16-GUS* plant. (D) GUS staining in a stage III LRP from a 7-d-old *LBD16-GUS* seedling. (E) GUS activity in a stage IV LRP from a 7-d-old *LBD16-GUS* plant. Bars are 0.5 mm (B), 50 μm (C), and 25 μm (D, E).

(J0121≫DTA) completely abolished lateral root development, indicating that only xylem-pole pericycle cells can form lateral roots. The positioning of lateral root primordia in the vertical axis is less well known, but lateral root initiation never occurs in the meristematic region. Accordingly, *GAL4* expression in J0121 starts in the elongation zone of the root and is excluded from the root tip. Therefore, the root pericycle is a heterogeneous tissue containing at least two different cell populations with different fates and J0121 is a good marker of the cells competent to form lateral roots. One can speculate that intercellular signals possibly coming from the xylem are responsible for the specific characteristics of the adjacent pericycle cells. In legumes, an elegant set of experiments showed that the initiation of nitrogen-fixing nodule primordia in the root cortex in front of xylem poles is negatively controlled by ethylene (Heidstra *et al*., 1997). It was found that neither cytokinin nor the ethylene response pathway inhibitor Ag^+ changed the J0121 expression pattern, thus suggesting that none of these hormones are involved in pericycle patterning (L Laplaze, unpublished results). No GAL4 expression was found in J0121 during embryogenesis suggesting that specification of pericycle cells in front of the xylem pole occurs only post-embryonically and that pericycle cells in the embryo are not competent to make lateral roots. J0121 is currently being used as a marker in a genetic screen to try to identify mutants perturbed in the pathway responsible for the specific differentiation of xylem-pole pericycle cells. This will allow a better understanding of what makes these cells competent to develop lateral roots.

Line J0192 has lateral root-primordia-specific *GAL4* expression. No expression was found in other organs throughout the life of the plant including embryogenesis. This indicates that a very specific lateral root gene enhancer is present in the vicinity of the enhancer trap T-DNA insertion site. Molecular characterization of J0192 showed that a single T-DNA insertion is present between LOB-domain genes *LBD16* and *LBD17*. It was previously reported that *LBD16* transcripts are found specifically in roots while *LBD17* is expressed in rosette and cauline leaves, shoot, floral buds, and flower tissues, but not in roots (Shuai *et al*., 2002). Since *LBD16* and *LBD17* are not included in the 4608 cDNAs microarray that was used by Himanen *et al*. (2004), there is no information about expression in the early stages of lateral root initiation. Transgenic *Arabidopsis* plants carrying a 1.5 kbp *LBD16* promoter-*GUS* fusion have strong GUS activity in young lateral root primordia resembling *GFP* expression in J0192. This suggests that some regulatory element in *LBD16* promoter might be responsible for the *GFP* expression pattern in J0192. This element would be acting at least 4.3 kbp away from the minimal promoter in the GAL4 enhancer trap T-DNA. However, it cannot be ruled out that an enhancer outside of the *LBD16* promoter is responsible for *GAL4* expression in J0192. Careful analysis of the *LBD16* promoter using deletions and site-directed

mutagenesis should help to clarify this point. Nevertheless, this study's results indicate that GAL4 ET lines can be used to identify interesting genes and/or regulatory elements on the basis of the *GFP* expression pattern.

The LOB-domain proteins were defined by their homology to the LOB (Lateral Organ Boundaries) protein from *A. thaliana* (Shuai *et al*., 2002). These proteins do not show any similarity to any previously described protein domain and are only present in plants. The role of LOB-domain proteins during lateral root formation is still unknown. The corresponding gene family contains 43 genes in the *Arabidopsis* genome (Shuai *et al*., 2002) including the *ASYMMETRIC LEAVES2 (AS2*) gene that controls leaf development. Interestingly, *AS2* seems to regulate the expression of *KNAT* genes negatively (Lin *et al*., 2003) and some *KNAT* genes seem to be involved in root cell differentiation and are excluded from lateral root primordia (E Truernit, Siemering, Grbic, S Hodge, J Haseloff, personal communication). Moreover, down-regulation of *KNAT6* results in an increase in lateral root formation, therefore suggesting that *KNAT6* negatively regulates lateral root development (Dean *et al*., 2004). It is therefore possible that LOB-domain protein gene expression in young lateral root primordia is necessary to switch off some *KNAT* genes in a similar way to what happens during lateral organ formation in the shoot apical meristem. In later stages, they could be involved in the definition of boundaries as suggested by expression at the flanks of older LRPs (Shuai *et al*., 2002). Unfortunately, the phenotype of *LOB* overexpressing plants was not reported (Shuai *et al*., 2002). Functional analyses of LOB-domain protein genes expressed in the root should help to clarify this point.

Targeted gene expression using GAL4 is a powerful tool to analyse the molecular and cellular bases of development. However, it depends on the isolation of suitable *GAL4* expressing lines (enhancer trap or promoter). The isolation of two enhancer trap lines with *GAL4* expression patterns that are useful for studying lateral root development is reported here. These lines were characterized and it was shown that insertion of the enhancer trap T-DNA has no impact on root development in these conditions. This result is in agreement with the mapping of the insertion sites. Interestingly, lines J0121 and J0192 are complementary because one (J0121) tags cells competent to make lateral roots before lateral root initiation, and the other marks young lateral root primordia (J0192) after lateral root initiation. They will be very helpful to probe the mechanisms of this important developmental transition. For instance, these two lines are currently being used to study the role of plant hormones during the initiation and early development of lateral root primordia by targeted expression of hormone biosynthesis or hormone perception genes. Transactivation experiments together with other approaches should help us to gain a better understanding of lateral root development.

Acknowledgements

This work was partly funded by the EMBO (ALTF 110-1999 to LL), the Gatsby Charitable Foundation, and the Institut de Recherche pour le Développement. We thank Dr D Autran, Pr M Bennett, and Dr E Truernit for critical reading of the manuscript.

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