

University of Warwick institutional repository: <http://go.warwick.ac.uk/wrap>

This paper is made available online in accordance with publisher policies. Please scroll down to view the document itself. Please refer to the repository record for this item and our policy information available from the repository home page for further information.

To see the final version of this paper please visit the publisher's website. Access to the published version may require a subscription.

Author(s): Matthew O. Jones, Kenneth Manning, John Andrews , Carole Wright, Ian B. Taylor and Andrew J. Thompson

Article Title: Sex and Discipline Differences in Empathising, Systemising and Autistic Symptomatology: Evidence from a Student Population

Year of publication: 2008

Link to published version: <http://dx.doi.org/10.1071/FP08139>

Publisher statement: None

**The promoter from *SIREO*, a highly-expressed, root-specific *Solanum lycopersicum* gene, directs expression to cortex of mature roots**

**Matthew O. Jones<sup>1,2\*</sup>, Kenneth Manning<sup>2</sup>, John Andrews<sup>2</sup>, Carole Wright<sup>2</sup>, Ian B. Taylor<sup>1</sup>, and Andrew J. Thompson<sup>2</sup>**

\*Corresponding Author

1. Plant Science Division, School of Biosciences, University of Nottingham, Sutton  
10 Bonington, Loughborough, LE12 5RD
2. Warwick HRI, University of Warwick, Wellesbourne, Warwick, CV35 9EF

Email addresses:

[matthew.o.jones@nottingham.ac.uk](mailto:matthew.o.jones@nottingham.ac.uk)

15 [ian.taylor@nottingham.ac.uk](mailto:ian.taylor@nottingham.ac.uk)

[ken.manning@warwick.ac.uk](mailto:ken.manning@warwick.ac.uk)

[john.andrews@warwick.ac.uk](mailto:john.andrews@warwick.ac.uk)

[carole.wright@warwick.ac.uk](mailto:carole.wright@warwick.ac.uk)

[a.j.thompson@warwick.ac.uk](mailto:a.j.thompson@warwick.ac.uk)

20

## Abstract

Root-specific promoters are valuable tools for targeting transgene expression, but many of those already described have limitations to their general applicability. We present the expression characteristics of *SIREO*, a novel gene isolated from tomato (*Solanum lycopersicum* L.). This gene was highly expressed in roots but had a very low level of expression in aerial plant organs. A 2.4 kb region representing the *SIREO* promoter sequence was cloned upstream of the *uidA* GUS reporter gene and shown to direct expression in the root cortex. In mature, glasshouse-grown plants this strict root specificity was maintained. Furthermore, promoter activity was unaffected by dehydration or wounding stress but was somewhat suppressed by exposure to NaCl, salicylic acid and jasmonic acid. The predicted protein sequence of *SIREO* contains a domain found in enzymes of the 2-oxoglutarate and Fe(II)-dependent dioxygenase superfamily.

The novel *SIREO* promoter has properties ideal for applications requiring strong and specific gene expression in the bulk of tomato root tissue growing in soil, and is also likely to be useful in other Solanaceous crops.

## Introduction

It is often desirable to direct transgene expression only to root tissues to allow manipulation or investigation of root-specific functions. For example it may be desirable to engineer resistance to root pathogens (Okubara and Paulitz, 2005), to improve  
5 beneficial plant-microbe interactions in the rhizosphere (Cardon and Gage, 2006), to alter root-to-shoot signalling processes (Sobeih *et al.* 2004), or to manipulate root traits that influence capture of nutrients and water (White *et al.* 2005). In many such biotechnology applications it will be necessary to have a promoter that is highly active in the majority of cells in mature roots of field grown crops, such that transgenes are expressed where and  
10 when they are effective. However, only a few root-specific gene promoters have been identified (Bucher, 2002) and these often have activities that are restricted to early developmental stages (Suzuki, 1993), are limited to immature central cylinder regions (Yamamoto *et al.* 1991) or vascular tissues (Zhang *et al.* 2003) of the root cellular structure, are heavily regulated by biotic and abiotic factors (Mudge *et al.* 2002; Marin *et al.* 2006; Léon-Kloosterziel *et al.* 2005), or have been isolated because they confer root-specificity only in seedlings where roots are growing into sucrose-rich agar media (Marin  
15 *et al.* 2006).

Roots are the first and most critical plant organ to experience such stresses as osmotic and ionic stress arising from drought, soil salinization, heavy metal  
20 accumulation, nutrient deficiency, and the microorganisms of the rhizosphere. In response to these conditions, physiological and metabolic changes occur, requiring alterations in gene expression that control such processes as ion homeostasis, cellular protection and secondary metabolism (Fester *et al.* 2002; Giritch *et al.* 1998; Tirajoh *et al.* 2005; Yoshimoto *et al.* 2002). In some cases genes may exhibit root-specific

expression but they may also be regulated by environmental signals. For example, native expression of *LE $\alpha$ -DOXI*, an alpha dioxygenase involved in plant defence against oxidative damage in tomato roots, is induced by salt treatment, abscisic acid, wounding, pathogen challenge and ethylene exposure (Tirajoh *et al.* 2005). Environmental factors  
5 can also affect gene expression spatially: expression of the maize *LACI* gene, encoding a putative laccase spread from the distal zone into the root apex in response to salt stress (Liang *et al.* 2006), and promoters of the *Arabidopsis* genes *AtTPS12* and *AtTPS113*, encoding enzymes of terpenoid synthesis, were predominantly active in roots of uninfected plants, but tissue wounding and pathogen infection induced activity in leaves  
10 (Ro *et al.* 2006). Conversely, salicylic acid, jasmonic acid and pathogen infection induced expression of the soybean isoflavone synthase gene *IFS1* in both roots and shoots, whilst under normal conditions the gene was expressed at very low levels only in the shoot (Subramanian *et al.* 2004).

Numerous root-specific genes have been characterised that encode ion  
15 transporters whose expression is induced by depletion of the relevant ion in the plant or rhizosphere. These include *Arabidopsis* sulphur transporter genes (Yoshimoto *et al.* 2002) and phosphate transporter genes (Mudge *et al.* 2002; Koyama *et al.* 2005). Promoters of phosphate transporter genes induced under phosphate-starvation conditions have also been characterised in *Medicago* (Xiao *et al.* 2006). In tomato high activities of  
20 the promoter of the ribonuclease LX gene were induced in root tips in response to phosphate starvation (Köck *et al.* 2006) and expression of a root-specific gene encoding an lysyl-tRNA-synthetase-like protein is regulated by iron (Giritch *et al.* 1997). However, the strong inducibility of these nutrient-stress response genes and their localisation to the outermost cell layers of roots (Köck *et al.* 2006; Xiao *et al.* 2006) limits their use as  
25 general root promoters.

Tomato is a major global crop and a model crop for Solanaceous species including potato, pepper, eggplant and the more distantly related coffee. The only root-specific promoters from tomato that have been described to date are those of the phosphate-induced gene described above and the extensin genes with activity 5 predominantly in root hairs (Bucher *et al.* 1997; Bucher *et al.* 2002).

The aim of this study was to identify a promoter suitable for the expression of transgenes in a root-specific manner in major crops of the genus *Solanum*, such as tomato and potato. Here we use EST data to identify an abundant, root-specific transcript in 10 tomato, identify the promoter from this gene and then investigate tissue and cell specificity of this promoter in transgenic tomato under a range of environmental and hormonal treatments.

## Materials and methods

### *Isolation of the promoter sequence*

Promoter sequence was obtained for the gene of interest, *SIREO*, by genome walking  
5 upstream of the Sol Genomics Network tentative unigene SGN-U315518 open reading  
frame by PCR using a method adapted from Diatchenko *et al.* (1996) and Zhang and Gurr  
(2000). Genomic DNA from *S. lycopersicum* L. cv Ailsa Craig was digested separately  
with the restriction enzymes *Dra*I; *Eco*RV; *Fsp*I; *Hpa*I; *Nru*I; *Pml*I; *Pvu*I; *Sca*I; *Sma*I;  
*Stu*I and *Swa*I. An adapter prepared by annealing the oligomers Adapter 1 (5'-  
10 CTAATACGACTCACTATAGGGCTCGAGCGGCCCGCCCGGGCAGGT-3') and  
Adapter 2 (PO<sub>4</sub>-ACCTGCCC-NH<sub>2</sub>) was ligated to the blunt ended DNA. Nested PCR  
amplification between forward primers that anneal to the adapter and reverse primers that  
anneal to the *SIREO* coding sequence was performed using Hi-Fi Extensor DNA  
polymerase (ABgene Epsom, UK) and PCR primers Walk-1: 5'-  
15 CCCTCACGAATATGGTTCCACATCAGA-3', Adapter-3: 5'-  
CTAATACGACTCACTATAGGGC-3'; Walk-2: 5'-  
CGGAACAGATTATGGGGTTCAATGAT-3' and Adapter-4: 5'-  
TCGAGCGGCCCGCCCGGGCAGGT-3'). For the primary PCR, 100 ng adapter-ligated  
genomic DNA was used as template in a reaction mixture (100 µl) containing 200 nM  
20 each of the primers Walk-1 and Adapter-3, 200 µM dNTPs and 2.5 U Extensor Hi  
Fidelity PCR enzyme mix (ABgene). Reaction conditions were 94°C for 2 min followed  
by 10 cycles of 94°C for 10 seconds and 68°C for 5 min then 20 cycles of 94°C for 10  
seconds and 68°C for 5 min extending by 10 s/cycle and a final extension incubation of  
68°C for 10 min. The nested PCR was performed under similar conditions to the primary  
25 PCR except 0.02 µl of the primary PCR reaction from the *Nru*I digest was used as

template and the primers were Walk-2 and Adapter-4. PCR products purified from an agarose gel were sequenced using an Applied Biosystems 3130xl DNA Analyser (Applied Biosystems, Foster City, CA, USA).

Potential *cis*-regulatory elements in the promoter were analysed using the PLACE database (Higo *et al.* 1999, <http://www.dna.affrc.go.jp/PLACE/index.html>, accessed 27 September, 2006).

#### *RNA extraction and analyses*

RNA was extracted from leaf, stem and roots and analysed on northern blots as described in Thompson and Corlett (1995). A <sup>32</sup>P-labelled RNA probe was prepared from the coding region of *SIREO* by reverse-transcription of total RNA from roots using oligo(dT) primer (SuperScript II, Invitrogen, Carlsbad, CA, USA). The cDNA was amplified by two rounds of PCR using a single forward primer (5'-CCTCTTCACGAAAGCTTTGG-3') and the reverse primers: 5'-AGGGCAGCAGCACAGCATCGTAAACTAGTTTGA-3', incorporating a T7 binding site, in the first round and 5'-GAGAATTCTAATACGACTCACTATAGGGCAGCAGCACA-3' in the second following the manufacturer's instructions. Blots were exposed to PhosphorImager screens and an image of the hybridisation signal was captured using a PhosphorImager SI (Molecular Dynamics). To quantify the signal from each band, ImageQuant v5.1 software (Molecular Dynamics) was used to position a grid over each array of bands and then pixel volume was integrated for each grid cell. The background signal, determined in an identical way from an area of the blot that was free from any hybridisation signal, was then subtracted.

25



### *Constructs for plant transformation*

The *SIREO* promoter-GUS transgene (pSIREO::GUS) was constructed from 2.4 kb of promoter sequence obtained from the gene walk. This was amplified by nested PCR using the forward primer 5'-AAAAAGCAGGCTTCCACAAGGCAACGGATGGATC-3',  
5 adjacent to the start codon of *SIREO*, and the reverse primer 5'-GGTTCAAAGTAAAAACCCATTAATTGACCCAGCTTTCT-3' for the first round. Primers for the second round were 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3' and 5'-ACCCAGCTTTCTTGTACAAAGTGGTCCCC-3' (italicised bases are common to primers used in both rounds of PCR). The amplified product was cloned into the  
10 Gateway® donor vector pDONOR221 (Invitrogen, Paisley, UK) and then moved by recombination (LR reaction) into the pKGWFS7 destination vector (Karimi *et al.* 2002). The resulting plasmid was named pTcEXP and was confirmed by sequence analysis.

### *Plant transformation*

15 pTcEXP was transferred to *S. lycopersicum* L. cv Ailsa Craig Tm2<sup>a</sup> (a near-isogenic line containing a tobacco mosaic virus resistance gene) by *Agrobacterium*-mediated transformation according to Bird *et al.* (1988) using the *A. tumefaciens* strain LBA4404.

### *Histochemical localisation of GUS activity*

20 Histochemical staining was performed on T<sub>1</sub> plants, obtained from selfing of primary transformants. Sterilised T<sub>1</sub> seeds were germinated on moistened filter paper and then transferred to MS media. Tissues from whole plants or seedlings were immersed in a solution containing GUS buffer (1 mM 5-bromo-5-chloro-3-indolyl-β-D-glucuronide, 50 mM sodium phosphate pH 7.0, 0.1% (v/v) Triton X-100, 4 mM potassium ferricyanide

and 100 µg ml<sup>-1</sup> chloramphenicol) and then incubated at 37°C overnight (Jefferson *et al.*, 1987). Leaf tissue was cleared (Leidl *et al.* 1993) for one hour and then rinsed in water.

To prepare sections, roots of six week old plants grown on MS media were stained and then fixed for 3 h in 50 mM sodium phosphate pH 7.0 containing 2.5% (v/v) *para*-formaldehyde and 2% (v/v) glutaraldehyde. Tissue was rinsed three times (15 min  
5 each) in 5 mM sodium phosphate pH 7.0 and then dehydrated in a series of ethanol washes. Fixed tissue was embedded in LR White resin (London Resin Company, Theale, Berkshire, UK) and 10 µm sections cut by microtome (Reichert Ultracut E ultramicrotome) before viewing by light microscopy.

10

#### *GUS activity assay*

For the fluorometric GUS assay, protein extracts were prepared from shoot and root tissues frozen in liquid nitrogen and stored at -80°C. Ground tissue was added to GUS extraction buffer (50 mM sodium phosphate pH 7.0, 1 mM β-mercaptoethanol, 10 mM  
15 EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium lauryl sarcosine and centrifuged for 10 min at 18,000 x g. The protein concentration of the supernatants was determined against bovine serum albumin (BSA) standards according to Bradford (1976). GUS activity assays were performed in triplicate on each extract as described (Jefferson *et al.* 1987) and quantified at 365 nm excitation and 455 nm emission wavelengths, using a  
20 standard curve constructed from dilutions of 4-methylumbelliferone.

#### *Analysis of GUS expression during leaf and root development*

To investigate GUS expression during development, four 641-1 T<sub>1</sub> plants that showed GUS expression in the roots (and so had not lost the transgene by segregation) were  
25 grown in a glasshouse in John Innes number 2 compost (7:3:2 ratio of loam:peat:coarse

sand and grit, plus 0.6 kg m<sup>-3</sup> ground limestone, 2.4 kg m<sup>-3</sup> hoof and horn meal, 2.4 kg m<sup>-3</sup> superphosphate and 1.2 kg m<sup>-3</sup> potassium sulphate). Because the T<sub>1</sub> plants could have been heterozygous or homozygous for the transgene, there could have been plant-to-plant variation in GUS activity simply due to zygosity. For this reason the same four  
5 plants were sampled non-destructively at three different growth stages. Thus any variation observed between growth stages could not be due genetic variation. At three growth stages (stage 1, 4-true leaves; stage 2, 10-11 true leaves and first trusses; stage 3, 12-18 true leaves and two or more trusses with set fruit) leaflets were sampled from the youngest fully expanded leaf, and, to obtain root tissue, the root ball was removed from  
10 the pots and several main roots (1 - 5 g FW) were excised where they emerged close to the hypocotyl. The remaining root system was repotted and the plants resumed growth prior to the next sampling. Root samples were briefly washed free of soil and all tissue samples were frozen and stored at -80°C. Equal weights of tissue from the four plants were powdered in liquid nitrogen and combined for protein extractions. GUS activity was  
15 determined at a protein concentration of 50 µg ml<sup>-1</sup>.

#### *Hormone and NaCl treatments of root cultures*

Sterilised T<sub>1</sub> seed from pTcEXP transformed lines were germinated on MS media in Magenta pots (Sigma-Aldrich, Dorset, UK) and at three weeks root sections were  
20 removed and stained for GUS activity to identify lines that contained the *SIREO::GUS* transgene. Healthy root tissues (2-5 cm) from one positive plant of each line were transferred to Petri dishes containing 15 ml ½ MS media (2.2 g l<sup>-1</sup> MS, 15 g l<sup>-1</sup> sucrose, pH 5.6-5.7). After one week the root cultures were sub-divided into 250 ml flasks containing 50 ml ½ MS media. After a further 21 days three separate cultures were  
25 transferred to ½ MS media supplemented with either 50 µM indole acetic acid (IAA), 50

5  $\mu\text{M}$  benzylaminopurine (BAP), 50  $\mu\text{M}$  gibberellic acid, 100  $\mu\text{M}$  jasmonic acid, 100  $\mu\text{M}$  salicylic acid, 100  $\mu\text{M}$  abscisic acid or 170 mM NaCl and incubated for 24 h before harvesting roots. Control cultures were incubated in  $\frac{1}{2}$  MS alone. Hormones were obtained from Sigma-Aldrich (Dorset, UK). All root material was frozen in liquid nitrogen upon harvest and stored at  $-80^{\circ}\text{C}$  until extraction of protein for GUS assays.

#### *Statistical analysis*

GUS activity levels were calculated using weighted linear regression and pairwise comparisons made using one-tailed t-tests (performed in Microsoft Excel).

10

## Results

### *Identification of a putative root-specific gene in tomato*

The tomato gene index (<http://www.tigr.org>, accessed 6<sup>th</sup> April, 2004) was searched for tentative consensus (TC) sequences based on two criteria: to be represented by the largest  
5 number of expressed sequence tags (ESTs) but only from libraries prepared from root tissues. This search identified TC124822, made up of 21 ESTs. This TC, at the time of writing, is now represented by SGN-U315518 and SGN-U315519, two UniGenes in the Sol Genomics Network (SGN) database (<http://www.sgn.cornell.edu>, accessed 15<sup>th</sup> April, 2008), that differ only by a single nucleotide substitution and a 101 nucleotide  
10 insertion/deletion, apparently due to a splice site variation. It is therefore likely that these ESTs and the two UniGenes represent a single tomato gene that is highly expressed in a root-specific manner.

BLAST searches with the open reading frame of this gene, revealed 81% amino acid identity to the tomato UniGene, SGN-U315520, and weaker homology to 32 other  
15 tomato genes (ranging from 42 to 22% amino acid identity). These tomato genes are of unknown function but, have a common PF03171 domain named 2OG-Fe(II) (<http://pfam.janelia.org>, accessed 14<sup>th</sup> April, 2008). Genes containing this domain are members of the superfamily known as the 2-oxoglutarate (2OG) and Fe(II)-dependent dioxygenases (2-ODDs). The 2OG-Fe(II) domain is found in 256 *Arabidopsis* genes that  
20 encode enzymes catalysing a range of reactions including hydroxylation, desaturation and epoxidation (Prescott and John, 1996; Prescott and Lloyd, 2000). On this basis we named the root-specific gene *SIREO* (*Solanum lycopersicum* root-expressed 2-ODD).

### *Isolation and structural analysis of the SIREO promoter*

Using genome walking we obtained 2.4 kb of promoter sequence upstream of the putative transcription start site (GenBank accession EU591493). This sequence was analysed by PLACE (data not shown) and contained putative *cis*-element sequences for hormone responses (auxin response element, AuxRE, Hagen and Guilfoyle, 2002; gibberellin responsive MYB factor binding site, MYB GA, Gubler and Jacobsen, 1995 and ethylene responsive enhancer element, ERE, Ithzaki *et al.* 1994) and binding sites for organ and tissue specific transcription factors (ASF1, L1 box, Abe *et al.* 2001). The promoter sequence did not contain the *cis*-elements associated with other root-specific genes including the bean *GRP1.8* gene (Keller and Baumbgartner, 1991), the repeated ATATTs present in the promoters of the *Agrobacterium rhizogenes roID* gene (Elmayan and Tepfer, 1995) and a root-specific peroxidase gene (Hertig *et al.* 1991). However, it did contain as-1, which binds activation sequence factor 1 (ASF-1) and is found in domain A, the root-specific domain of the CaMV 35S promoter (Benfey *et al.* 1990; Klinedinst *et al.* 2000), and also a sequence shown to be over-represented in genes which are repressed by phytochrome A and so are commonly expressed in the dark (Hudson and Quail, 2003).

### *Tissue-specificity of SIREO mRNA levels in wild-type tomato plants*

Expression was very strong in root tissues; taking a mean over two experiments it was 49 and 16-fold greater in roots than in leaves or stems, respectively (Fig. 1). Expression in flowers was intermediate between leaf and stem. This analysis confirmed that *SIREO* is more highly expressed in roots than in other tissues.

### *Tissue-specificity of SIREO promoter activity*

To analyse the activity of the *SIREO* 5' flanking region, a 2.4 kb fragment was fused to the *E.coli* reporter gene *uidA*, encoding  $\beta$ -glucuronidase (GUS), to create the *SIREO::GUS* transgene. This was introduced into *S. lycopersicum* by *Agrobacterium*-mediated transformation. Five independently-transformed tomato lines (named 641-1 through to 641-5) were regenerated and expression of *GUS* mRNA was determined in leaves, roots and stems by northern analysis (Fig. 2A). In addition, GUS activity was measured for leaves and roots of six-week old plants (Fig. 2B). *GUS* mRNA was much more abundant in root tissue compared to leaf and stem, and the mean GUS activity in root tissue averaged across the five transformants was 118-fold greater than the mean activity in leaf tissue ( $P < 0.001$ ). Thus both GUS activity and mRNA levels directed by the *SIREO* promoter showed a similar tissue specificity to that observed for the mRNA of the endogenous *SIREO* gene (Fig. 1), suggesting that the 2.4 kb promoter was sufficient to confer the observed root specificity.

### *Cellular localisation of SIREO promoter activity in roots*

The localisation of GUS activity in roots, from radicle emergence to full establishment of the root system was determined in T<sub>1</sub> generation "641" plants. GUS staining was absent from the emerging radicle and could first be detected in root tissue two days after germination (Fig. 3A). GUS staining was not observed in developing cells at the primary root tip but was concentrated at the distal end of the differentiation zone (Fig. 3B and C). This pattern was maintained in lateral roots (Fig. 3D). The primordia of lateral roots

were clearly marked as dense collections of unstained cells but the GUS staining occurred only towards the basal region of each lateral root (Fig. 3H, I). Within more mature roots, greater spatial variation in GUS staining was observed; in some cases expression covered the entire root system and in others expression was apparently absent  
5 from some entire branches (Fig. 3E, F, G). This variation was observed in each of three independent transgenic lines but the cause is unknown.

In transverse section (Fig. 3J), staining for GUS activity was revealed to be greatest in the cortex, particularly in the layer of cortical cells immediately below the epidermis. GUS staining was not apparent in the epidermis. There was also no staining in  
10 the endodermis or vascular tissue, although we cannot exclude the possibility that this was due to lack of penetration of the substrate through the endodermis in intact roots. GUS staining was not detectable in leaves or flowers (data not shown).

#### *Activity of the SIREO promoter in mature plants*

15 GUS expression was determined in leaves and roots from glasshouse-grown plants during their development from young plants (approximately 10 cm high) to fruiting plants (approximately 0.9 m high). GUS activity in leaves remained very low (never significantly different from the WT leaves that lack the GUS transgene;  $P > 0.05$ ; Fig. 4), whilst in roots activity was very high and increased significantly between the first two  
20 harvest stages ( $P < 0.001$ ), but not further by the third harvest ( $P > 0.05$ ; Fig. 4).



### *SIREO promoter activity under hormone and stress treatments*

To establish if promoter activity responded to hormones or salinity stress, cultured roots from line 641-1, were exposed to six classes of phytohormones and NaCl (Figure 5). Isolated root cultures were used so that direct root responses to the treatments could be observed. If whole plants had been used the treatments could potentially have generated secondary signals in the leaves that might have influenced root gene expression. No significant induction or reduction in GUS activity could be measured following 24 h exposure to the auxin IAA or the cytokinin BAP at physiologically relevant concentrations (50  $\mu$ M each, Xu *et al.* 1995) whilst gibberellic acid at the same concentration caused a 33% increase in promoter activity ( $P = 0.04$ ; Fig. 5). Root cultures were also exposed to the stress-related hormones (all at 100  $\mu$ M): while no response to abscisic acid was observed both jasmonic acid and salicylic acid reduced the activity of the *SIREO* promoter compared to untreated roots by 88% ( $P < 0.01$ ) and 74% ( $P < 0.01$ ), respectively. The SA treatment was repeated for line 641-2 and a similar reduction in GUS activity was observed (data not shown). Treatment of root cultures with NaCl at a concentration previously shown to affect expression of salt-inducible genes in tomato roots (Tirajoh *et al.* 2005) reduced GUS activity by 71% ( $P < 0.01$ ; Fig. 5). Wounding of roots did not have a significant effect on GUS activity in two lines tested (641-1, 641-2; data not shown), and rapid dehydration of roots to 50% of initial fresh weight did not affect GUS activity ( $P > 0.05$ , data not shown).

## Discussion

### *Possible functions of SIREO*

The presence of the 2-OG Fe(II) domain in *SIREO* places this gene in the 2-oxoglutarate  
5 (2OG) and Fe(II)-dependent dioxygenase (2-ODD) superfamily (EC1.14.11.2). The 2-  
ODDs catalyse a range of substrate conversions that result in protein modifications, lipid  
metabolism, biosynthesis of secondary metabolites and repair of alkylated DNA and  
RNA (reviewed by Hausinger, 2004). These reactions involve oxidative decomposition  
10 of 2-oxoglutarate to CO<sub>2</sub> and succinate, with the production of highly oxidising Fe(IV)  
oxo- or other activated oxygen species that hydroxylate the substrate. Sequence analysis  
reveals little sequence similarity between known 2-ODDs beyond the conserved domain.

The functions performed by some plant 2-ODD are encoded by multigene  
families, such is the case with 1-aminocyclopropane-1-carboxylate oxidases, responsible  
for the last step in ethylene biosynthesis (Tang *et al.* 1993) and GA 20 oxidases in  
15 *Arabidopsis*, which catalyze sequential steps in gibberellin biosynthesis (Prescott and  
John, 1996). The expression of the different GA20 oxidase genes shows differential  
spatial distribution, although this is limited to the aerial plant parts (Phillips *et al.* 1995).  
Other 2-ODD have been reported to exhibit root-specific expression including the *ARRO-  
I* gene from apple (*Malus domestica*) which is up-regulated in adventitious and primary  
20 roots in a response to auxin (Butler and Gallagher, 2000) and a gene from the  
Solanaceous plant *Hyoscyamus niger* that is involved in the biosynthesis of the tropane  
alkaloid scopolamine (Matsuda *et al.* 1991).

We have produced tomato RNAi lines in which *SIREO* expression in the roots  
was down regulated by approximately 95%. The roots appeared morphologically normal  
25 (data not shown) and so the function of *SIREO* remains unknown, although is likely to be

involved in some aspect of secondary metabolism that is specific to roots. Our data on the localisation and developmental timing of expression in roots suggest a function that is not related to cell growth and expansion, but rather differentiation and maturation.

##### 5 *A comparison of the SIREO promoter to other root-specific promoters*

SIREO is apparently highly expressed in roots because it is highly represented in tomato root EST libraries (24 out of 13,115 ESTs in five root libraries; <http://compbio.dfci.harvard.edu/tgi/>, accessed 25<sup>th</sup> August, 2008). To provide an indication of the SIREO promoter strength we compared our GUS activity data to other published work in tomato. In Figure 2, the average GUS activity in the roots of five independent SIREO::GUS lines was 226 pmol 4-MU  $\mu\text{g protein}^{-1} \text{ min}^{-1}$ , and in Figure 4 the average for one line at different developmental stages was 43 pmol 4-MU  $\mu\text{g protein}^{-1} \text{ min}^{-1}$ . In comparison, the GUS activity in tomato roots containing the enhanced *mas35s*::GUS construct was 50 pmol 4-MU  $\mu\text{g protein}^{-1} \text{ min}^{-1}$  (Bassett et al, 2007; mean of 10 independent lines), a *35s*::GUS construct gave 33 pmol 4-MU  $\mu\text{g protein}^{-1} \text{ min}^{-1}$  in tomato seedlings (Garosi et al, 2005; one line), and a *35s*::GUS construct with a translational enhancer gave 100 and 800 pmol 4-MU  $\mu\text{g protein}^{-1} \text{ min}^{-1}$  in tomato leaf and fruit, respectively (Krasnyanski et al, 2001; mean of 7 independent lines). We conclude that the strength of the SIREO promoter in tomato roots is of a similar order of magnitude to that which can be achieved with strong constitutive promoters.

Promoters showing strong activity in a strict root-specific manner have potential benefits over constitutive promoters in a wide range of applications (Bucher, 2002). Von Schweinichen and Büttner (2005) used the *Arabidopsis Pyk10* promoter to over-express a plant cell wall invertase in *Arabidopsis* roots; expression was not detected in leaves whilst invertase expression in the roots was able to increase rates of phloem unloading

and increase root development. Grichko and Glick (2001) introduced the bacterial enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase into tomato to catabolise the immediate precursor of ethylene to improve response to flooding. Plants transformed with ACC deaminase under the transcriptional control of the root-specific  
5 *rolD* promoter from *Agrobacterium rhizogenes* were more tolerant to flooding than untransformed plants. In contrast, plants constitutively over-expressing this gene are proposed to have negative effects due to an increased metabolic burden (Grichko and Glick, 2001). The above are examples of metabolic engineering in roots, and the *SIREO* promoter is likely to be well suited to such applications because of its activity specifically  
10 in the cortex. However, this promoter is unlikely to be well suited to applications that require transgene expression in the epidermis, e.g. for modifications of ion uptake or secretion of citrate or phytases to improve uptake of phosphorous (Bucher, 2002; Mudge *et al.* 2003). When considering application of the promoter it should also be noted that we observed some unexplained variation whereby some branches of the root system did not  
15 appear to stain for GUS (e.g. Figure 3F). Such variation may be explained by unknown environmental variables, or possibly gene silencing effects.

A further application is to engineer resistance to root pathogens such as nematodes, fungi and parasitic plants. Transgenic plants over expressing *sarcotoxin IA*, a gene encoding an antimicrobial protein, in a root-specific manner under the control of the  
20 tobacco *TobRB7* promoter were reported to be more resistant to a root parasitic weed (Radi *et al.* 2006). However, although the *TobRB7* promoter showed strong root-specificity in tobacco (Yamamoto *et al.* 1991), when transformed into tomato it directed approximately equal gene expression in leaves and roots (Chan *et al.* 2005). A strawberry homolog of this gene, *FaRB7*, is expressed predominantly in roots (Vaughan  
25 *et al.* 2006). However, when the promoter of this gene was introduced into tobacco it

conferred constitutive expression (Vaughan *et al.* 2006). Gittins and co-workers (2001) reported different spatial and temporal activities of a tomato *rbcS* promoter depending on whether it was transformed into tomato or into a heterologous host. These examples demonstrate that the tissue specificity of a promoter cannot be guaranteed in a  
5 heterologous host, and so it is important to have available root-specific promoters from a range of crop types; the *SIREO* promoter is most likely to be of use in the economically important and closely related crops tomato and potato.

Generally the *SIREO* promoter showed robust and easily detectable activity in roots, either grown in culture, or from glasshouse-grown plants, and it was particularly  
10 active in mature roots. The promoter was relatively insensitive to the environmental treatments tested including dehydration, wounding and abscisic acid, and exhibited only small decreases in response to SA, JA and NaCl in comparison to the differences between roots and leaves in mature plants.

In conclusion, the *SIREO* 2-ODD gene is predicted to function in secondary  
15 metabolic pathways in roots, and its promoter is likely to be particularly suited to applications that require high level expression of transgenes in the bulk of cells of the mature root, but not those applications that require epidermal expression. Importantly, the promoter also offers root-specificity that is stable throughout plant development and maintained under a range of environmental conditions. One clear application may be the  
20 root-specific manipulation of metabolic pathways known to be active in the cortex, such as flavonoid and isoprenoid biosynthesis (Chen *et al.*, 2004; Hans *et al.*, 2004; Saslowsky and Winkel-Shirley, 2001).

## Acknowledgements

We thank Carol Evered for assistance in microscopy. We are grateful to Angela Hambidge and Linda Brown for technical assistance. This work was funded by the Department for Environment, Food and Rural Affairs, UK, project HP0218.

5

## References

Abe M, Takahashi T, Komeda Y (2001) Identification of a *cis*-regulatory element for L1 layer-specific gene expression, which is targeted by an L1-specific homeodomain protein. *The Plant Journal* **26**, 487-494.

- 10 Bassett, CL, Callahan, AM, Artlip, TS, Scorza, R and Srinivasan, C (2007) A minimal peach type II chlorophyll a/b-binding protein promoter retains tissue-specificity and light regulation in tomato. *BMC Biotechnology* **7**, 47

- Benfey PN, Takatsuji H, Ren L, Shah DM, Chua NH (1990) Sequence requirements of the 5-enolpyruvylshikimate-3-phosphate synthase 5'-upstream region for tissue-specific  
15 expression in flowers and seedlings. *Plant Cell* **2**, 849-856.

Bird CR, Smith CJS, Ray JA, Moureau P, Bevan MW, Bird AS, Hughes S, Morris PC, Grierson D, Schuch W (1988) The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. *Plant Molecular Biology* **11**, 651-662.

- Bradford, M (1976) A rapid and sensitive method for the quantitation of microgram  
20 quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**, 248-254.

- Bucher M (2002) Molecular Root Bioengineering. In Y Waisel, A Eshel, U Kafkafi, eds, *Plant Roots. The Hidden Half*, Third Edition pp 279-294 (Marcel Dekke, Inc.: New York)
- Bucher M, Brunner S, Zimmermann P, Zardi GI, Amrhein N, Willmitzer L, Riesmeier  
5 JW (2002) The expression of an extensin-like protein correlates with cellular tip growth in tomato. *Plant Physiology* **128**, 911-923.
- Bucher M, Schroerer B, Willmitzer L, Riesmeier JW (1997) Two genes encoding extensin-like proteins are predominantly expressed in tomato root hair cells. *Plant Molecular Biology* **35**, 497-508.
- 10 Butler ED, Gallagher TF (2000) Characterization of auxin-induced *ARRO-1* expression in the primary root of *Malus domestica*. *Journal of Experimental Botany* **51**, 1765 - 1766
- Cardon ZG, Gage DJ (2006) Resource exchange in the rhizosphere: Molecular tools and the microbial perspective. *Annual Review of Ecology, Evolution and Systematics* **37**, 459-488.
- 15 Chan YL, Prasad V, Sanjaya, Chn KH, Liu PC, Chan MT, Cheng CP (2005) Transgenic tomato plants expressing an *Arabidopsis* thionin (*Thi2.1*) driven by fruit-inactive promoter battle against phytopathogenic attack. *Planta* **221**, 386-393.
- Chen F, Ro D-K, Petri J, Gershenzon J, Bohlmann J, Pichersky E, Tholl D (2004) Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the  
20 formation of the volatile monoterpene 1,8-cineole. *Plant Physiology* **135**, 1956-1966
- Diatchenko L, Lau YC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive

hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Science USA* **93**, 6025-6030

5 Elmayan T, Tepfer M (1995) Evaluation in tobacco of the organ specificity and strength of the *rolD* promoter, domain A of the 35S promoter and the 35S<sup>2</sup> promoter. *Transgenic Research* **4**, 388-396

Fester T, Schmidt D, Lohse S, Walter MH, Giuliano G, Bramley PM, Fraser PD, Hause B, Strack D (2002) Stimulation of carotenoid metabolism in arbuscular mycorrhizal roots. *Planta* **216**, 148-154.

10 Garoosi GA, Salter MG, Caddick MX and Tomsett AB (2005) Characterisation of the ethanol-inducible *alc* gene expression system in tomato. *Journal of Experimental Botany* **56**, 1635-1642.

Giritch A, Ganal M, Stephan UW, Baumlein H (1998) Structure, expression and chromosomal localisation of the metallothionein-like gene family of tomato. *Plant*  
15 *Molecular Biology* **37**, 701-714.

Giritch A, Herbik A, Balzer HJ, Ganal M, (1997). A root-specific iron-regulated gene of tomato encodes a lysyl-tRNA-synthetase-like protein. *European Journal of Biochemistry* **244**, 310-317.

Gittins JR, Hiles ER, Pellny TK, Biricolti S, James DJ (2001) The *Brassica napus extA*  
20 promoter: a novel alternative promoter to CaMV 35S for directing transgene expression to young stem tissues and load bearing regions of transgenic apple trees (*Malus pumila* Mill). *Molecular Breeding* **7**, 51–62.



- Grichko VP, Glick BR (2001) Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35S, *rolD* or *PRB-1b* promoter. *Plant Physiology and Biochemistry* **39**, 19-25.
- 5 Gubler F, Jacobsen JV (1995) Gibberellin-responsive elements in the promoter of a barley high pI-  $\alpha$ -amylase gene. *Plant Cell* **4**, 1435-1441.
- Hagen G, Guilfoyle T (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Molecular Biology* **49**, 373-385.
- Hans J, Hause, B, Strack D, Walter MH (2004) Cloning, characterization, and immunolocalization of a mycorrhiza-inducible 1-deoxy-D-xylulose 5-phosphate  
10 reductoisomerase in arbuscule-containing cells of maize. *Plant Physiology* **134**, 614-624.
- Hausinger RP (2004). Fe(II)/ $\alpha$ -ketoglutarate dependent hydroxylases and related enzymes. *Critical Reviews in Biochemistry and Molecular Biology* **39**, 21-68.
- Hertig C, Rebmann G, Bull J, Mauch F, Dudler R (1991) Sequence and tissue-specific expression of a putative peroxidase gene from wheat (*Triticum aestivum* L.). *Plant  
15 Molecular Biology* **16**, 171-174.
- Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database 1999. *Nucleic Acids Research* **27**, 297 - 300.
- Hudson ME, Quail PH (2003) identification of promoter motifs involved in the network of phytochrome A-regulated gene expression by combined analysis of genomic sequence  
20 and microarray data. *Plant Physiology* **133**, 1605-1616.

Itzhaki H, Maxson JM, Woodson WR (1994) An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-S-transferase (GST1) gene. *Proceedings of the National Academy of Sciences USA* **91**, 8925–8929

5

Jefferson RA, Kavanagh TA, Bevan, MW (1987) GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**, 3901–3907.

Karimi M, Inze D Depicker A (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends in Plant Science* **7**, 193-195.

10 Keller B, Baumgartner C (1991) Vascular-specific expression of the bean grp 1.8 gene is negatively regulated. *Plant Cell* **3**, 1051-1061.

Klinedinst S, Pascuzzi P, Redman J, Desai M, Arias J (2000) A xenobiotic-stress-activated transcription factor and its cognate target genes are preferentially expressed in root tip meristems. *Plant Molecular Biology* **42**, 679-688.

15 Koyama T, Ono T, Shimizu M, Jinbo T, Mizuno R, Tomita K, Mitsukawa N, Kawazu T, Kimura T, Ohmiya K, Sakka K (2005) Promoter of *Arabidopsis thaliana* phosphate transporter gene drives root-specific expression of transgene in rice. *Journal of Bioscience and Bioengineering* **99**, 38-42.

Köck M, Stenzel I, Zimmer A (2006) Tissue-specific expression of tomato ribonuclease  
20 LX during phosphate starvation-induced root growth. *Journal of Experimental Botany* **57**, 3717-3726.

- Krasnyanski SF, Sandu J, Domier LL, Buetow DE and Korbani SS (2001) Effect of an enhanced CAMV 35S promoter and a fruit-specific promoter on *UIDA* gene expression in transgenic tomato plants. *In Vitro Cellular & Developmental Biology - Plant* **37**, 427-433.
- 5 Leidl BE, McCormick S, Mutschler MA (1993) A clearing technique for histochemical location of GUS activity in pollen tubes and ovules of *Lycopersicon*. *Plant Molecular Biology Reports* **11**, 194-201.
- Léon-Kloosterziel KM, Verhagen BW, Keurentjes JJ, Van Pelt JA, Rep M, Van Loon LC, Pieterse CM (2005) Colonization of the *Arabidopsis* rhizosphere by fluorescent  
10 *Pseudomonas* spp. activates a root-specific, ethylene-responsive *PR-5* gene in the vascular bundle. *Plant Molecular Biology* **57**, 731-748.
- Liang M, Haraldsen V, Cai X, Wu Y (2006) Expression of a putative laccase gene, *ZmLAC1*, in maize primary roots under stress. *Plant, Cell and Environment* **29**, 746-753.
- Marin E, Divol F, Bechtold N, Vavasseur A, Nussaume L, Forestier C (2006) Molecular  
15 characterization of three *Arabidopsis* soluble ABC proteins which expression is induced by sugars. *Plant Science* **171**, 84-90.
- Matsuda J, Okabe S, Hashimoto T, Yamada Y (1991) Molecular cloning of hyoscyamine 6  $\beta$ -hydroxylase, a 2-oxoglutarate-dependent dioxygenase, from cultured roots of *Hyoscyamus niger*. *Journal of Biological Chemistry* **266**: 9460 – 9464.
- 20 Mudge SR, Rae AL, Diatloff E, Smith FW (2002) Expression analysis suggests novel roles for members of the *Pht1* family of phosphate transporters in *Arabidopsis*. *The Plant Journal* **31**, 341-353.

- Mudge SR, Smith FW, Richardson AE (2003) Root-specific and phosphate regulated expression of phytase under the control of a phosphate transporter promoter enables *Arabidopsis* to grow on phytate as a sole P source. *Plant Science* **165**, 871-878.
- Okubara PA, Paulitz TC (2005) Root defense responses to fungal pathogens: A molecular  
5 perspective. *Plant and Soil* **274**, 215-226
- Phillips AL, Ward DA, Uknes S, Appleford N, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P (1995) Isolation and expression of three gibberellin 20-oxidase cDNA clones from *Arabidopsis*. *Plant Physiology* **108**: 1049-1057.
- Prescott, AG, John, P (1996). Dioxygenases: molecular structure and role in plant  
10 metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 245-271.
- Prescott AG, Lloyd MD (2000) The iron(II) and 2-oxoacid-dependent dioxygenases and their role in metabolism. *Natural Product Reports* **17**, 367-383.
- Radi A, Dina P, Guy A (2006) Expression of *sarcotoxin IA* gene via a root-specific *tob*  
15 promoter enhanced host resistance against parasitic weeds in tomato plants. *Plant Cell Reports* **25**, 297-303.
- Ro D, Ehling J, Keeling CI, Lin R, Mattheus N, Bohlmann J (2006) Microarray expression profiling and functional characterization of *AtTPS* genes: Duplicated *Arabidopsis thaliana* sesquiterpene synthase genes *At4g13280* and *At4g13300* encode  
20 root-specific and wound-inducible (Z)- $\gamma$ -bisabolene synthases. *Archives of Biochemistry and Biophysics* **448**, 104-116.

- Saslowsky D, Winkel-Shirley, B (2001) Localisation of flavonoid enzymes in *Arabidopsis* roots. *The Plant Journal* **27**, 37-48.
- Sobeih WY, Dodd IC, Bacon MA, Grierson D, Davies WJ (2004) Long-distance signals regulating stomatal conductance and leaf growth in tomato (*Lycopersicon esculentum*) plants subjected to partial root-zone drying. *Journal of Experimental Botany* **55**, 2353-2363.
- Subramanian S, Hu X, Lu G, Odelland JT, Yu O (2004) The promoters of two isoflavone synthase genes respond differentially to nodulation and defence signals in transgenic soybean roots. *Plant Molecular Biology* **54**, 623-639.
- 10 Suzuki H, Fowler TJ, Tierney ML (1993) Deletion analysis and localization of *SbPRP1*, a soybean cell wall protein gene, in roots of transgenic tobacco and cowpea. *Plant Molecular Biology* **21**, 109-119.
- Tang X, Wang H, Brandt AS, Woodson WR (1993) Organisation and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from *Petunia hybrida*. *Plant* 15 *Molecular Biology* **23**, 1151-1164.
- Thompson AJ, Corlett JE (1995). mRNA levels of four tomato (*Lycopersicon esculentum* Mill.L) genes related to fluctuating plant and soil water status. *Plant, Cell and Environment* **18**, 773-80.
- Tirajoh A, Aung TST, Byun McKay A, Plant AL (2005) Stress-responsive  $\alpha$ -dioxxygenase expression in tomato roots. *Journal of Experimental Botany* **56**, 713-723.
- 20 Vaughan SP, James DJ, Lindsey K, Massiah AJ (2006) Characterization of *FaRB7*, a near root-specific gene from strawberry (*Fragaria x ananassa* Duch.) and promoter

activity analysis in homologous and heterologous hosts. *Journal of Experimental Botany* **57**, 3901-3910.

5 von Schweinichen C, Büttner M (2005) Expression of a plant cell wall invertase in roots of *Arabidopsis* leads to early flowering and an increase in whole plant biomass. *Plant Biology* **7**, 469-475.

White PJ, Broadley MR, Hammond JP, Thompson AJ (2005) Optimising the potato root system for phosphorous and water acquisition in low-input growing systems. *Aspects of Applied Biology* **73**, 111-118.

10 Xiao K, Liu J, Dewbre G, Harrison M, Wang ZY (2006) Isolation and characterization of root-specific phosphate transporter promoters from *Medicago truncatula*. *Plant Biology* **8**, 439-449.

Xu W, Purugganan MM, Polisensky DH, Antiosiewicz, Fry SC, Braam J (1995). *Arabidopsis TCH4*, regulated by hormones and the environment, encodes a xyloglucan endotransglycolase. *The Plant Cell* **7**, 1555-1567.

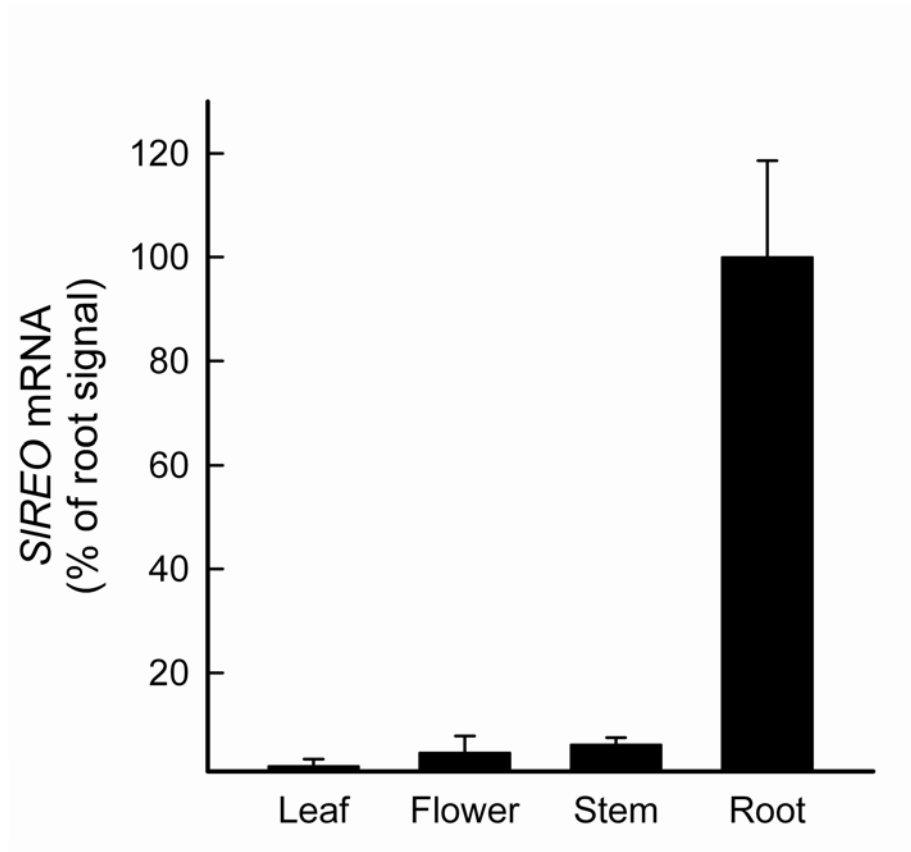
15 Yamamoto YT, Taylor CG, Acedo GN, Cheng C, Conkling MA (1991) Characterization of *cis*-acting sequences regulating root-specific gene expression in tobacco. *Plant Cell* **3**, 371-382.

20 Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. *Plant Journal* **29**, 465-473.

Zhang P, Bohl-Zenger S, Puonti-Kaerlas J, Potrykus I, Grisse W (2003) Two cassava promoters related to vascular expression and storage root formation. *Planta* **218**, 192-203.

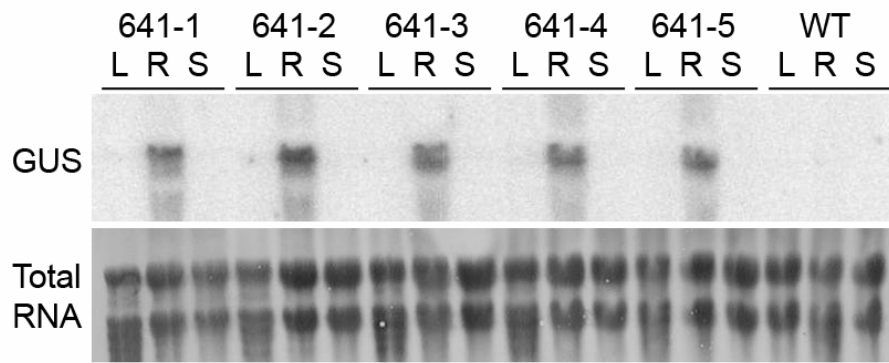
Zhang Z, Gurr SJ (2000) Walking into the unknown: a 'step down' PCR-based technique leading to the direct sequence analysis of flanking genomic DNA. *Gene* **253**, 145-150.

## Figures

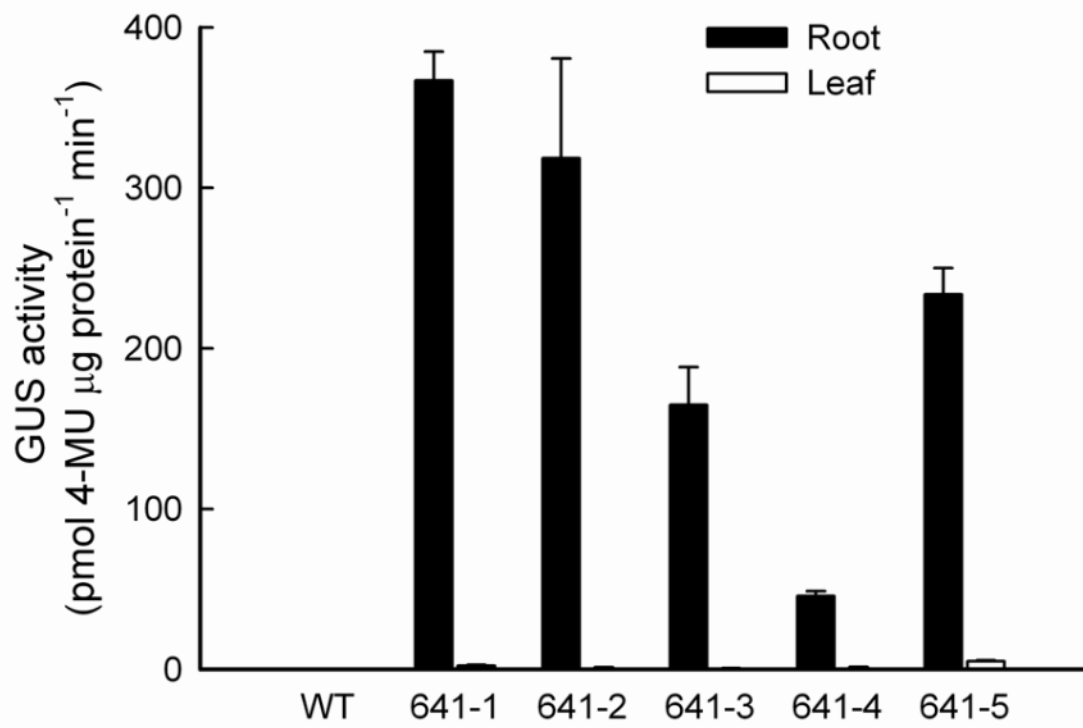


- 5 **Fig. 1.** Levels of *SIREO* mRNA in different organs of WT plants. RNA was isolated from leaf, stem, root, and flower tissues of mature unstressed tomato plants. mRNA levels were quantified from two independent northern blots,  $n = 7$  for leaf, flower and stem,  $n = 5$  for roots. Error bars represent standard error of the mean.



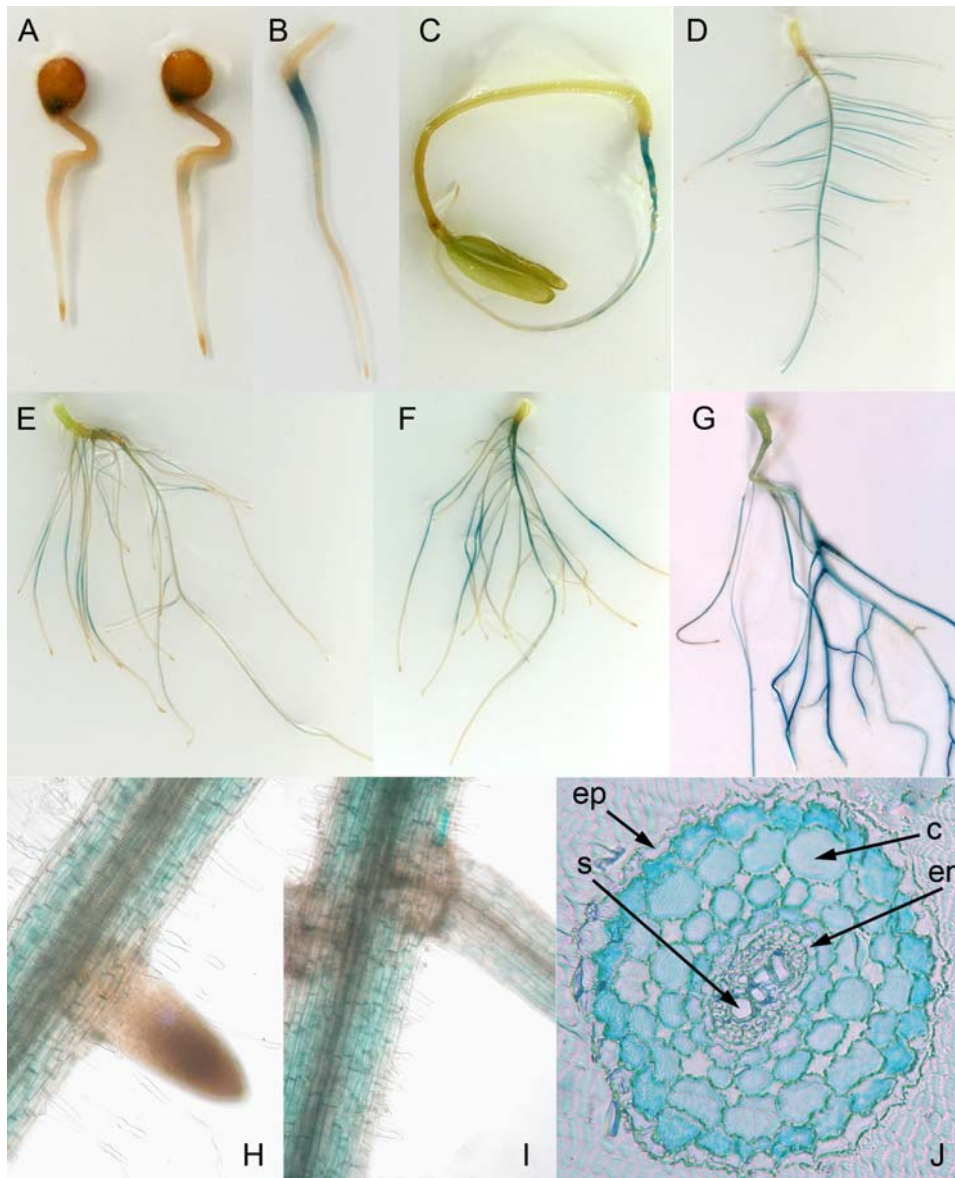


A.

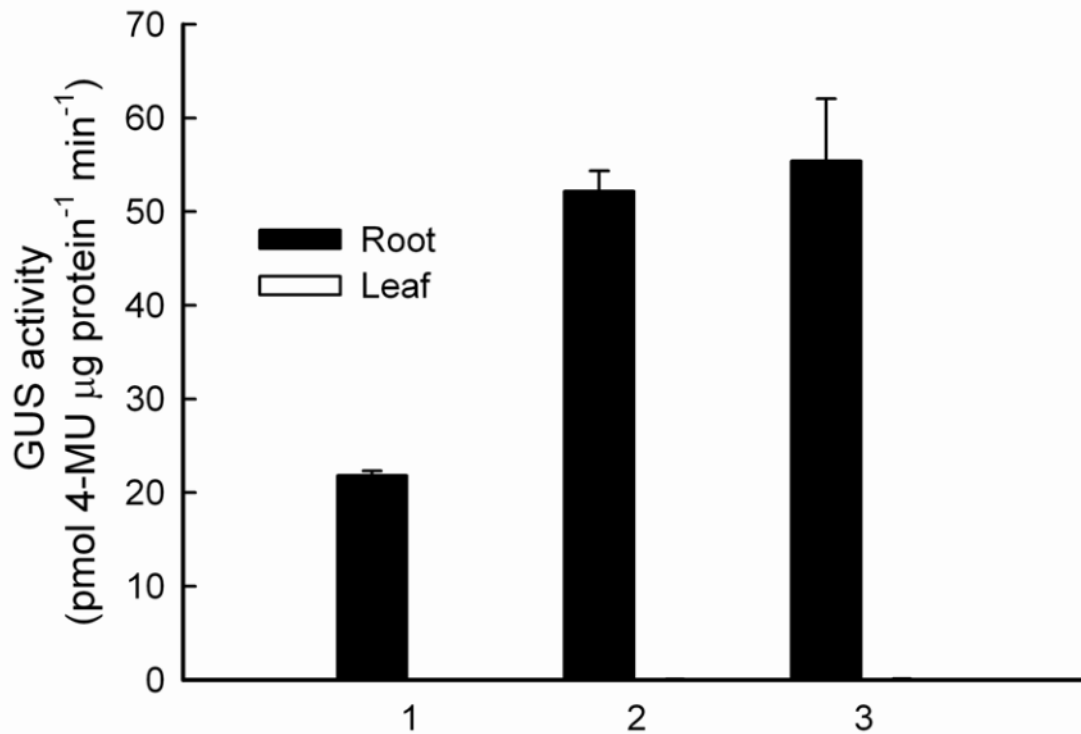


B.

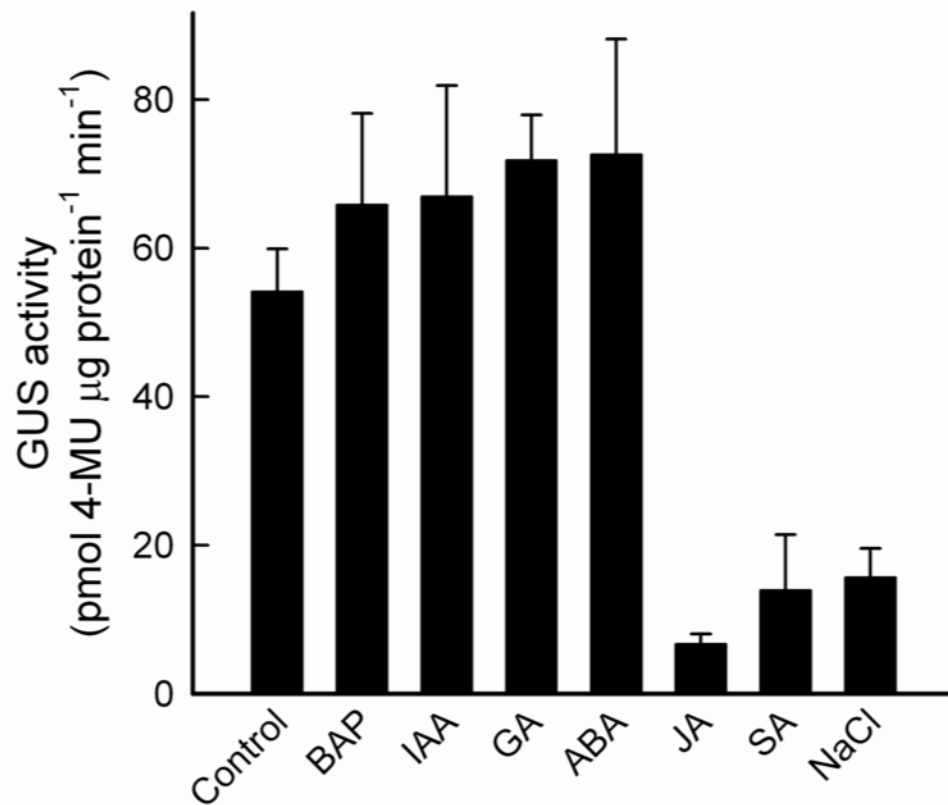
**Fig. 2.** Organ specificity of GUS expression driven by the *SIREO* promoter. Independent primary (T<sub>0</sub>) transformants (641-1 to 641-5) and WT were propagated as cuttings and then grown for six weeks in a glasshouse. A: Northern blot of leaf (L), root (R) and stem (S) total RNA probed with a GUS probe (GUS) and stained with methylene blue (Total RNA). B: GUS activity in leaf and root tissue. Bars for WT and some 641 leaf samples are too small to register on the plots. Error bars represent standard error of the mean for replicate plants, n = 3. GUS activity was not detectable in WT tissues.



**Fig. 3.** Histochemical localisation of GUS activity in T<sub>1</sub> seedlings. A, 2-day-old seedlings (WT on left, 641 on right); B, 3-day-old seedling; C, 5-day-old seedling; D, 8-day-old seedlings;. E and F, 11-day-old seedlings with “patchy” expression; G, roots of 21-day-old plant propagated from the same transgenic line as in E and F; H and I, lateral roots of 11 days old seedling. J, cross section of 6-week old root; c, cortex; en, endodermis; ep, epidermis; s, stellar tissue; the blue colouration in the stele and outside the epidermal layer was due to optical refraction. Histochemical analysis was performed on three independent “641” lines and representative images are shown.



5 **Fig. 4.** GUS activity determined in glasshouse-grown plants. Four T<sub>1</sub> plants originating from the line 641-1 were grown in compost. Leaf and root samples were collected from each plant at three developmental stages (see materials and methods). Tissues from the four plant replicates were pooled and then four extracts prepared from samples of each pool; each extract was assayed in triplicate. Error bars show standard error of the mean, 10 approximately equal to 95% confidence limits for the variation between extracts. GUS activity in leaf samples was zero or not significantly different from zero ( $P > 0.05$ ) and was too small to register on this plot.



**Fig. 5.** GUS activity in isolated root cultures from 641-1 T<sub>1</sub> plants. Roots were incubated for 24 h in either ½ MS media alone (Control), or ½ MS media supplemented with 50  $\mu\text{M}$  benzylaminopurine (BAP), 50  $\mu\text{M}$  gibberellic acid (GA), 50  $\mu\text{M}$  indole acetic acid (IAA), 100  $\mu\text{M}$  abscisic acid (ABA), 100  $\mu\text{M}$  jasmonic acid (JA), 100  $\mu\text{M}$  salicylic acid (SA), or 170 mM NaCl.  $n = 3$  for treatments, and  $n = 7$  for control. Error bars represent standard error of the mean.