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

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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 *SlREO*, 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 *SlREO* 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 predicated protein sequence of *SlREO* contains a domain found in enzymes of the 2-oxoglutarate and Fe(II)-dependent dioxygenase superfamily.

The novel *SlREO* 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 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 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 *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 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 *LEα-DOX1*, 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 can also affect gene expression spatially: expression of the maize *LAC1* 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 (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 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 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 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 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 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, *SlREO*, by genome walking 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'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3') and Adapter 2 (PO_4-ACCTGCCC-NH_2) 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 SlREO coding sequence was performed using Hi-Fi Extensor DNA polymerase (ABgene Epsom, UK) and PCR primers Walk-1: 5′-CCCTCACGAATATGGTTCCACATCAGA-3′, Adapter-3: 5′-CTAATACGACTCACTATAGGGC-3'; Walk-2: 5'-CGGAACAGATTATGGGGGTTCAATGAT-3' and Adapter-4: 5'-TCGAGCGGCCGCCCGGGCAGGT-3′). For the primary PCR, 100 ng adapter-ligated genomic DNA was used as template in a reaction mixture (100 µl) containing 200 nM 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^{o}C for 2 min followed by 10 cycles of 94^{o}C for 10 seconds and 68^{o}C for 5 min then 20 cycles of 94^{o}C for 10 seconds and 68^{o}C for 5 min extending by 10 s/cycle and a final extension incubation of 68^{o}C for 10 min. The nested PCR was performed under similar conditions to the primary 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 ^{32}P-labelled RNA probe was prepared from the coding region of *SlREO* 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′-AGGGCAGCAGCACAGCATCGTAAAACTAGTTTGAACT-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.

Constructs for plant transformation

The *SlREO* promoter-GUS transgene (pSlREO::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′- *AAAAAGCAGGCT*TCCACAAGGCAACGGATGGATC-3′, adjacent to the start codon of *SlREO*, and the reverse primer 5′-GGTTCAAAGTAAAAACCCATTAATT*GACCCAGCTTTCT*-3′ for the first round. Primers for the second round were 5′-GGGGACAAGTTTGTACA*AAAAAGCAGGCT*-3′ and 5′- *ACCCAGCTTTCT*TGTACAAAGTGGTCCCC-3′ (italicised bases are common to primers used in both rounds of PCR). The amplified product was cloned into the 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

pTcEXP was transferred to *S. lycopersicum* L. cv Ailsa Craig Tm2^{a} (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

Histochemical staining was performed on T_1 plants, obtained from selfing of primary transformants. Sterilised T_1 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^{-1} 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 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.

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 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 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_1 plants that showed GUS expression in the roots (and so had not lost the transgene by segregation) were 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^{-3} ground limestone, 2.4 kg m^{-3} hoof and horn meal, 2.4 kg m^{-3} superphosphate and 1.2 kg m^{-3} potassium sulphate). Because the T_1 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 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 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 determined at a protein concentration of 50 μg ml^{-1}.

Hormone and NaCl treatments of root cultures

Sterilised T_1 seed from pTcEXP transformed lines were germinated on MS media in Magenta pots (Sigma-Aldrich, Dorset, UK) and at three weeks root sections were removed and stained for GUS activity to identify lines that contained the *SlREO::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^{-1} MS, 15 g l^{-1} 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 transferred to ½ MS media supplemented with either 50 μM indole acetic acid (IAA), 50 μM benzylaminopurine (BAP), 50 μM gibberellic acid, 100 μM jasmonic acid, 100 μM salicylic acid, 100 μM abscisic acid or 170 mM NaCl and incubated for 24 h before harvesting roots. Control cultures were incubated in ½ MS alone. Hormones were obtained from Sigma-Aldrich (Dorset, UK). All root material was frozen in liquid nitrogen upon harvest and stored at -80^{o}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).

Results

Identification of a putative root-specific gene in tomato

The tomato gene index (http://www.tigr.org, accessed 6th April, 2004) was searched for tentative consensus (TC) sequences based on two criteria: to be represented by the largest 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 15th April, 2008), that differ only by a single nucleotide substitution and a 101 nucleotide 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 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 14th 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 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 *SlREO* (*Solanum lycopersicum* root-expressed 2-ODD).

Isolation and structural analysis of the SlREO *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 rolD* 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 SlREO *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 *SlREO* is more highly expressed in roots than in other tissues.

Tissue-specificity of SlREO *promoter activity*

To analyse the activity of the *SlREO* 5′ flanking region, a 2.4 kb fragment was fused to the *E.coli* reporter gene *uidA*, encoding β-glucuronidase (GUS), to create the *SlREO::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 *SlREO* promoter showed a similar tissue specificity to that observed for the mRNA of the endogenous *SlREO* gene (Fig. 1), suggesting that the 2.4 kb promoter was sufficient to confer the observed root specificity.

Cellular localisation of SlREO *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_1 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 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 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 SlREO *promoter in mature plants*

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 harvest stages ($P < 0.001$), but not further by the third harvest ($P > 0.05$; Fig. 4).

SlREO *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 μM 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 μM): while no response to abscisic acid was observed both jasmonic acid and salicylic acid reduced the activity of the *SlREO* 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 SlREO

The presence of the 2-OG Fe(II) domain in *SlREO* places this gene in the 2-oxoglutarate (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 of 2-oxoglutarate to CO_2 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 *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-1* gene from apple (*Malus domestica*) which is up-regulated in adventitious and primary 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 *SlREO* expression in the roots was down regulated by approximately 95%. The roots appeared morphologically normal (data not shown) and so the function of *SlREO* 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.

A comparison of the SlREO *promoter to other root-specific promoters*

SlREO 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 25th August, 2008). To provide an indication of the SlREO 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 SlREO::GUS lines was 226 pmol 4-MU μg protein^{-1} min^{-1}, and in Figure 4 the average for one line at different developmental stages was 43 pmol 4-MU μg protein^{-1} min^{-1}. In comparison, the GUS activity in tomato roots containing the enhanced *mas*35s::GUS construct was 50 pmol 4-MU μg protein^{-1} min^{-1} (Bassett et al, 2007; mean of 10 independent lines), a 35s::GUS construct gave 33 pmol 4-MU μg protein^{-1} min^{-1} in tomato seedlings (Garoosi et al, 2005; one line), and a 35s::GUS construct with a translational enhancer gave 100 and 800 pmol 4-MU μg protein^{-1} min^{-1} in tomato leaf and fruit, respectively (Krasnyanski et al, 2001; mean of 7 independent lines). We conclude that the strength of the SlREO 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 *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 *SlREO* promoter is likely to be well suited to such applications because of its activity specifically 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 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 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 *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 heterologous host, and so it is important to have available root-specific promoters from a range of crop types; the *SlREO* promoter is most likely to be of use in the economically important and closely related crops tomato and potato.

Generally the *SlREO* promoter showed robust and easily detectable activity in roots, either grown in culture, or from glasshouse-grown plants, and it was particularly 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 *SlREO* 2-ODD gene is predicted to function in secondary 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 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).

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Figures

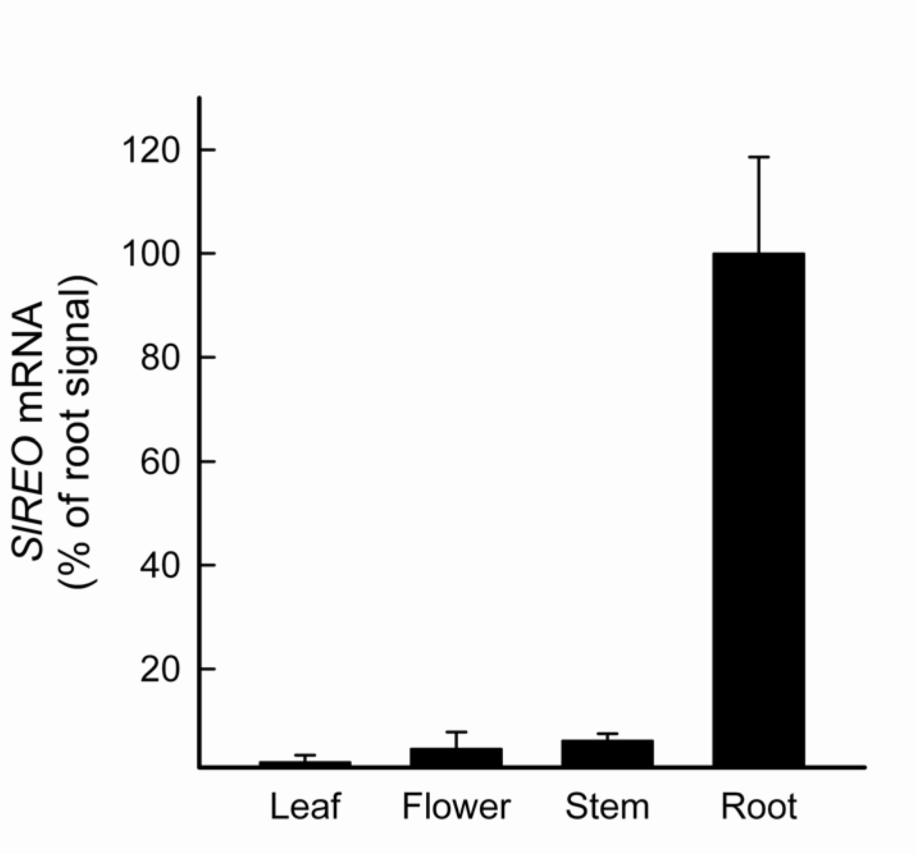
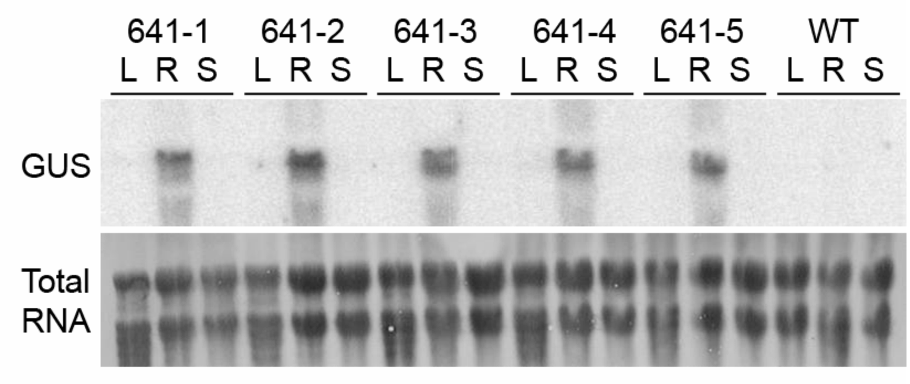
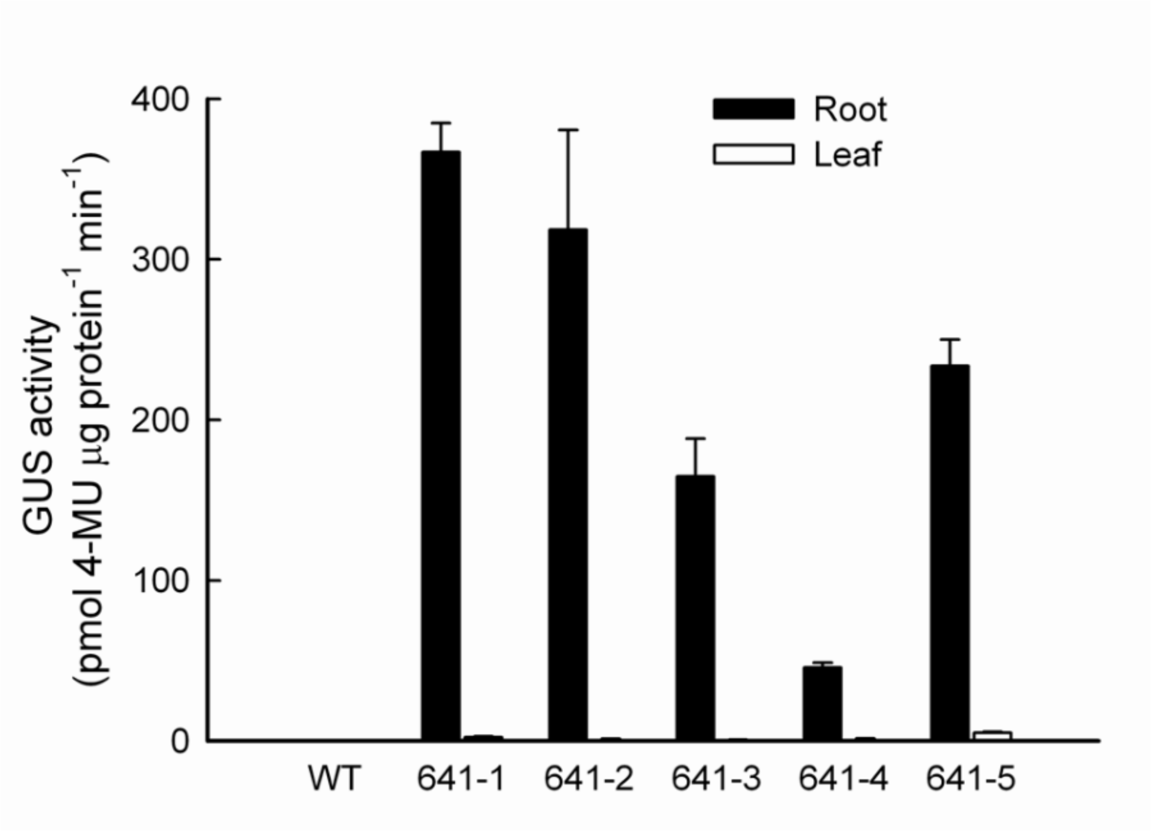


Fig. 1. Levels of *SlREO* mRNA in different organs of WT plants. RNA was isolated from leaf, stem, root, and flower tissues of mature unstressed tomato plants. mRNA levels were quantified from two independent northern blots, n = 7 for leaf, flower and stem, n = 5 for roots. Error bars represent standard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


641-1
641-2
641-3
641-4
641-5
WT
L R S
GUS
Total RNA

A.


Root
Leaf
GUS activity
(pmol 4-MU μg protein^{-1} min^{-1})
400
300
200
100
0
WT
641-1
641-2
641-3
641-4
641-5

B.

5

Fig. 2. Organ specificity of GUS expression driven by the *SlREO* promoter. Independent primary (T_0) 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.

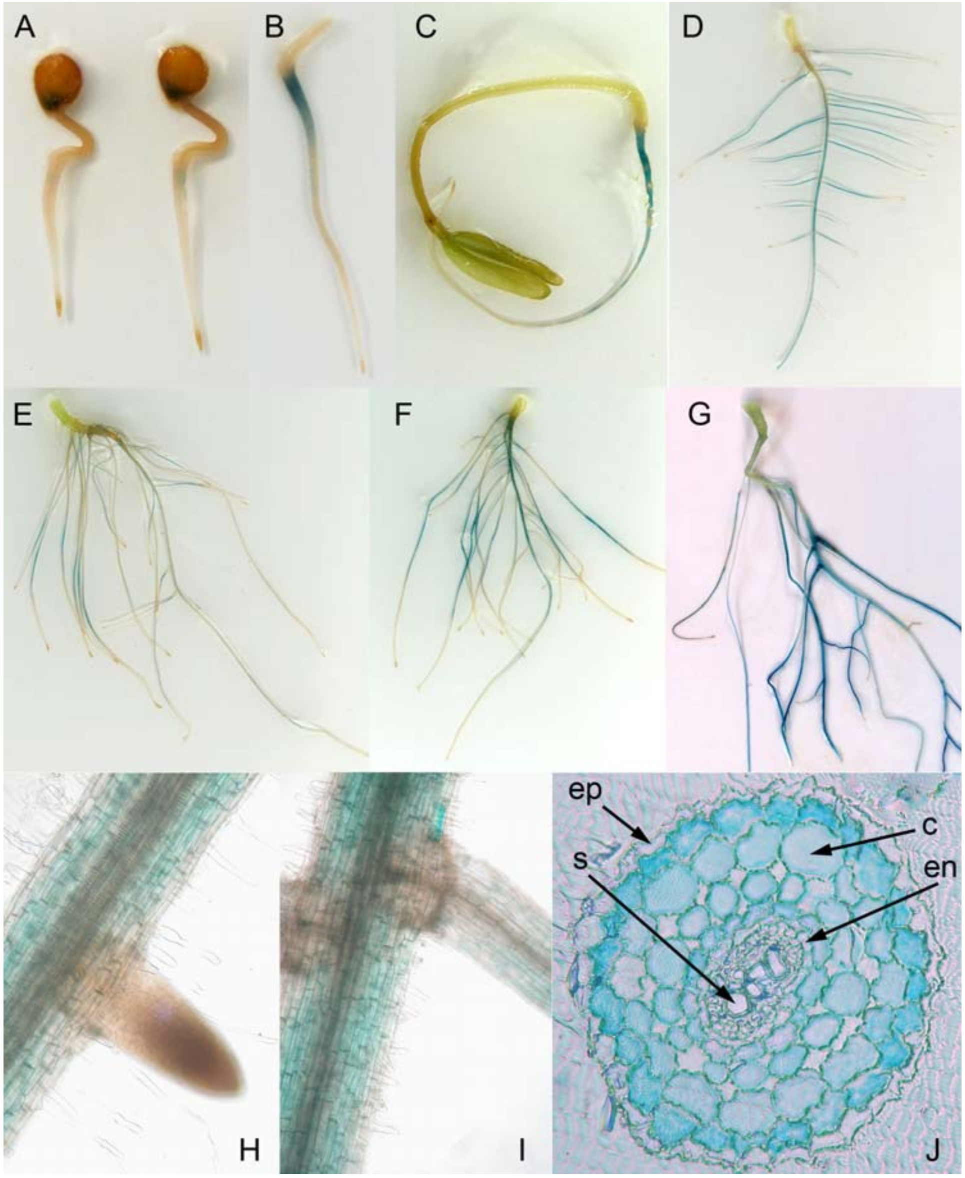


Fig. 3. Histochemical localisation of GUS activity in T_1 seedlings. A, 2-day-old seedlings (WT on left, 641 on right); B, 3-day-old seedling; C, 5-day-old seedling; D, 8-day-old seedlings;. E and F, 11-day-old seedlings with "patchy" expression; G, roots of 21-day-old plant propagated from the same 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.

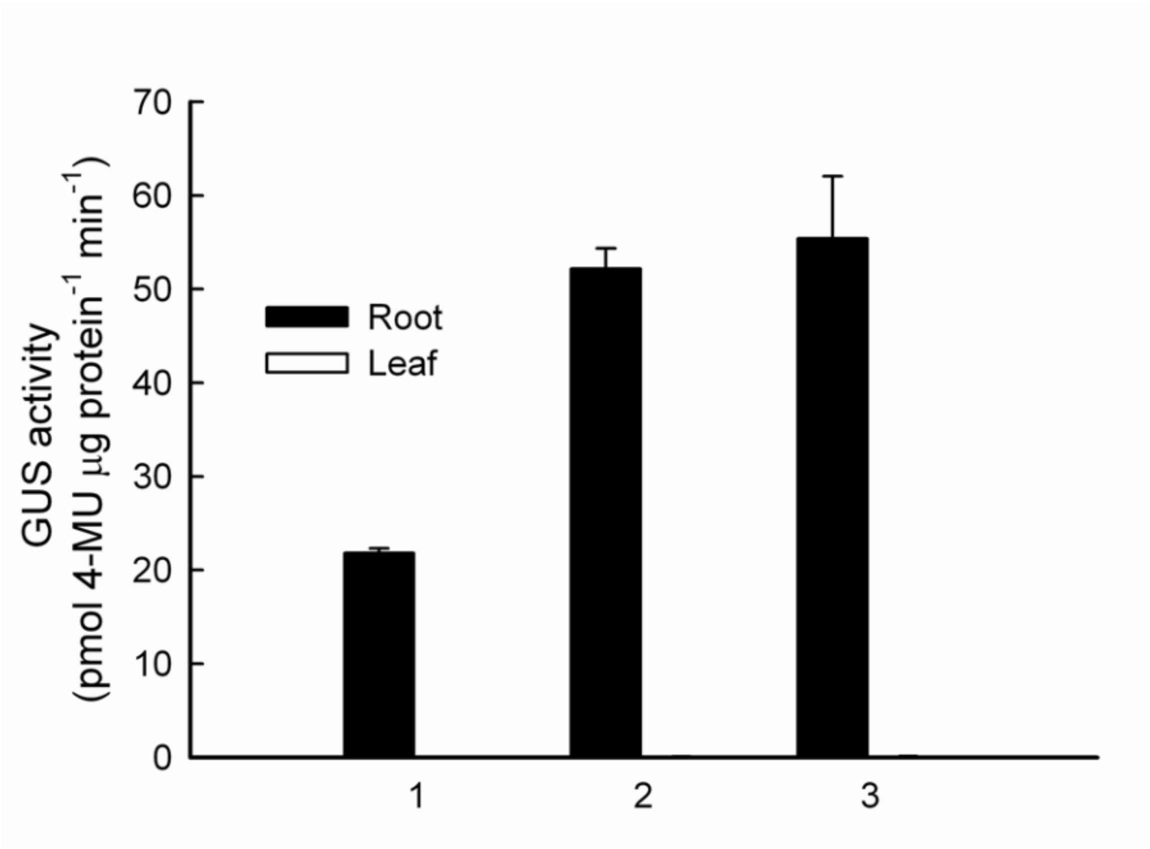


Fig. 4. GUS activity determined in glasshouse-grown plants. Four T_1 plants originating from the line 641-1 were grown in compost. Leaf and root samples were collected from each plant at three developmental stages (see materials and methods). Tissues from the four plant replicates were pooled and then four extracts prepared from samples of each pool; each extract was assayed in triplicate. Error bars show standard error of the mean, 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.

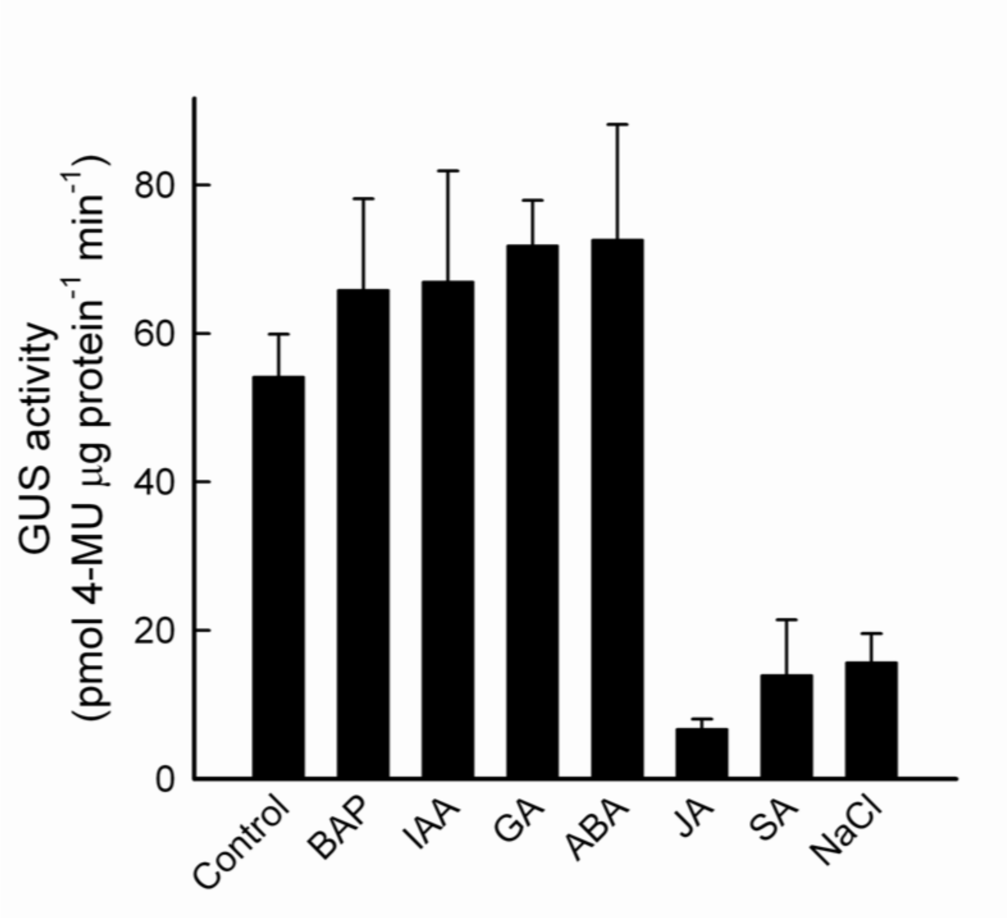


Fig. 5. GUS activity in isolated root cultures from 641-1 T_1 plants. Roots were incubated for 24 h in either ½ MS media alone (Control), or ½ MS media supplemented with 50 µM benzylaminopurine (BAP), 50 µM gibberellic acid (GA), 50 µM indole acetic acid (IAA), 100 µM abscisic acid (ABA), 100 µM jasmonic acid (JA), 100 µM salicylic acid (SA), or 170 mM NaCl. n = 3 for treatments, and n = 7 for control. Error bars represent standard error of the mean.