

A role for the rice homeobox gene *Oshox1* in provascular cell fate commitment

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SUMMARY

The vascular tissues of plants form a network of interconnected cell files throughout the plant body. The transition from a genetically totipotent meristematic precursor to different stages of a committed procambial cell, and its subsequent differentiation into a mature vascular element, involves developmental events whose molecular nature is still mostly unknown. The rice protein *Oshox1* is a member of the homeodomain leucine zipper family of transcription factors. Here we show that the strikingly precise onset of *Oshox1* gene expression marks critical, early stages of provascular ontogenesis in which the developmental fate of procambial cells is specified but not yet stably determined. This suggests that the *Oshox1* gene may be involved in the establishment of the conditions

required to restrict the developmental potential of procambial cells. In support of this hypothesis, ectopic expression of *Oshox1* in provascular cells that normally do not yet express this gene results in anticipation of procambial cell fate commitment, eventually culminating in premature vascular differentiation. *Oshox1* represents the first example of a transcription factor whose function can be linked to specification events mediating provascular cell fate commitment.

Key words: Auxin transport, Cell fate specification/determination, Homeodomain leucine zipper (HD-Zip), *Oryza sativa*, *Oshox1*, Procambium, Transcription factor, Vascular system

INTRODUCTION

The vascular tissues of plants are organised in a network of interconnected cell files whose continuity throughout the plant body enables efficient long-distance transport and provides mechanical support (Howell, 1998). The vascular system is composed of two types of specialised conducting tissues: the phloem, through which photoassimilates are transported, and the xylem, which is the conduit for water and soil-derived nutrients. The vasculature originates from embryonic tissues, the vascular meristems, whose cells retain the ability to continually multiply (Aloni, 1987). New vascular strands develop in dynamic relationship to one another during a differentiation process that persists throughout plant life, ensuring flexibility to adapt to changes in the surrounding environmental pressure (Sachs, 1993; Aloni, 1995). Within the vascular meristems two stages are distinguished: the procambium, or provascular tissue, and the (vascular) cambium. The procambium is the apical meristem that gives rise to primary xylem and phloem in young parts of shoots and roots (Shininger, 1979). The cambium, rare in monocotyledons, is a lateral meristem that develops in older parts of the plant axis, where it produces secondary phloem and xylem. Finally, vascular elements can occasionally differentiate from parenchyma cells, to regenerate an interrupted vascular connection during adventitious root

development, or as a response to wounding or grafting (Sachs, 1981).

Most studies of vascular development have been mainly restricted to an anatomical approach, which has led to a strictly structural definition of procambium, according to which this tissue becomes recognisable as continuous strands of dense narrow cells, elongated parallel to the longitudinal axis of the organ (Nelson and Dengler, 1997). In older parts of the plant they become more vacuolated, but retain their elongated shape. Until overt cytodifferentiation occurs, procambial cells can only be described by operative criteria such as cell shape and vacuolation, which are variable and not necessarily correlated with their real developmental state. Indeed, we currently do not know whether such cytologically identifiable provascular cells have actually progressed towards differentiation into xylem or phloem, or whether they are simply uncommitted meristematic precursors.

It is widely accepted in the animal field to divide the process of cell fate commitment into two stages that are operationally defined, specification and determination (Slack, 1983). A cell or tissue explant is said to be specified to become a particular structure if it will develop autonomously into that structure when cultured in a neutral (non-inductive) environment. Determination involves a further restriction of developmental potential that is maintained in explants irrespective of the range of conditions present in the organism, and that persists stably

in the course of normal development. Nevertheless, this process is not necessarily irreversible, since alterations in the developmental state can occasionally occur even after differentiation is completed, wherever cells have retained their genetic totipotency (Maclean and Hall, 1987). The concepts of cell fate specification and determination are extendible to plants, even though it is often difficult to discriminate these stages experimentally. To date, no criteria have been set to distinguish different stages in the sequence of events occurring during provascular cell fate commitment. Furthermore, only a few reports have addressed the identification of molecular or biochemical markers to describe this process (Gahan, 1981; Demura and Fukuda, 1994), and little is known about the regulatory proteins involved.

Recent reports have indicated that certain transcription factors of the homeodomain leucine zipper (HD-Zip) class may fulfil control functions in provascular and vascular development. HD-Zip proteins are encoded by a class of homeobox genes apparently unique to plants (Ruberti et al., 1991). Characteristically, they have a leucine zipper dimerisation motif linked to their DNA-binding domain. The HD-Zip class of transcription factors has been grouped in families I to IV (Sessa et al., 1994). Whereas HD-Zip IV proteins appear to be involved in establishing epidermal cell fates (Rerie et al., 1994; Di Cristina et al., 1996; Lu et al., 1996), specific members of the other HD-Zip families have been associated with (pro)vascular development. The *Arabidopsis IFL1/REV* gene encodes an HD-Zip III protein that appears to be implicated in differentiation of interfascicular and xylary fibres, and of tracheary elements (Zhong and Ye, 1999; Ratcliffe et al., 2000). Furthermore, the *Arabidopsis Athb8* gene, which encodes another HD-Zip III protein, is expressed exclusively in procambial cells and during revascularisation following wounding (Baima et al., 1995). Finally, the tomato HD-Zip I protein *Vahox1* is encoded by a gene that is specifically expressed in the phloem during secondary growth (Tornero et al., 1996).

In the current report we provide experimental criteria to distinguish progressive stages of provascular cell fate commitment, and identify the rice HD-Zip II gene *Oshox1* (Meijer et al., 1997) as the first marker of a specification phase in procambium ontogenesis. Furthermore, we present evidence suggesting that this gene acts as a regulatory switch during the reversible transition between a totipotent meristematic ground state and an intermediate developmental condition at which procambial cell fate is specified but not yet stably determined.

MATERIALS AND METHODS

Isolation of *Oshox1* genomic sequence and vectors construction

Oryza sativa (L.) Indica cultivar IR58 total DNA, partially digested with *Sau3A*I, was cloned in λ GEM11 (Promega) *Bam*HI arms. Plaques of the amplified genomic library were lifted and hybridised with a radiolabelled *Oshox1* cDNA fragment (Memelink et al., 1994). From two purified positive clones, three overlapping fragments hybridising to different regions of the *Oshox1* cDNA were isolated, subcloned in pBluescript II SK(+) (Stratagene) and sequenced by the Double Strand sequencing service of Eurogentec. This revealed that in addition to the entire *Oshox1* open reading frame, the clones contained 1596 bp of upstream sequences. To allow fusion of the

Oshox1 promoter-leader region to the β -glucuronidase reporter gene (*gusA*), the sequence around the start codon (CAATGG) was modified to an *Nco*I site (CCATGG) by standard PCR techniques. Subsequently, the 1596 bp *Oshox1* promoter-leader sequence was fused to the *gusA* start codon in binary vector pCAMBIA1391z (CAMBIA; Roberts et al., 1997), to obtain the *Oshox1*-GUS reporter construct. The 35S-*Oshox1*-nos expression cassette was excised from pMOG-*Oshox1* (Meijer et al., 1997) and cloned between the *Eco*RI and *Hind*III sites of binary vector pMOG22 (Zeneca Mogen), to obtain the 35S-*Oshox1* construct.

Plant transformation and growth conditions

Embryonic calli were induced on scutella from germinated seeds of *Oryza sativa* (L.) Japonica cultivar Taipei 309 (Rueb, 1994) and transformed with *Agrobacterium tumefaciens* strain LBA1119 (C58 pTiBo542 Δ T-DNA) harbouring the desired binary vector, essentially as described by Hiei et al. (1994). Regenerated transgenic plantlets were transferred to the greenhouse and grown in hydroponic culture with a regime of 12 hours light, 28°C, 85% relative humidity and 12 hours dark, 21°C, 60% relative humidity. If not otherwise indicated, seeds were surface sterilised (Rueb et al., 1994) and germinated in the dark at 28°C for 4 days on filter paper moistened with water (wild type) or with a 75 mg/l hygromycin solution (transgenic). Germinated seeds were grown in a 12 hours light:12 hours dark cycle at 28°C. 35S-*Oshox1* transgenic rice lines were selected on the basis of their resistance to hygromycin and of their *Oshox1* overexpression at the RNA level (not shown). Analyses were performed on 9 independent 35S-*Oshox1* lines. Photographs of wild-type and transgenic plants were taken with an Olympus CAMEDIA Digital Camera C-1400 L and processed using Adobe Photoshop 5.0.

Histochemical localisation of β -glucuronidase activity

To exclude any influence of transgene position on promoter activity, analyses were performed on 10 independent *Oshox1*-GUS lines. To assay β -glucuronidase (GUS) activity, 100 μ m vibratome sections (Leica VT1000S) or freshly dissected plant organs were vacuum infiltrated for 5 minutes and subsequently incubated in 100 mM phosphate buffer pH 7.7 containing 2 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-gluc; Biosynth AG), 0.5 mM potassium ferricyanide, 10 mM EDTA and 0.1% Triton X-100 at 37°C, for 1 hour to overnight depending on staining intensity. Samples were depleted of chlorophyll in 70% ethanol and stored at 4°C. As a control for reliability of the GUS assay, CaMV 35S-GUS transgenic plants (pCAMBIA1301 binary vector; CAMBIA; Roberts et al., 1997) showed reporter gene activity in virtually all cell types of different organs, thereby demonstrating that substrate accessibility was not limiting GUS detection. GUS activity was never observed in non-transgenic rice tissues. To verify that the GUS staining patterns were not influenced by tissue metabolism or by leakage of GUS enzyme or 5-bromo-4-chloro-3-indole intermediate, control experiments were performed in which tissues were prefixed and/or in which detection was performed in different buffer systems. Prefixation in 2% glutaraldehyde in 100 mM cacodylate buffer pH 7.2 for 2 minutes on ice prior to GUS detection (Stomp, 1992) resulted in identical staining patterns as in standard GUS assays, although staining intensity was much reduced. The same was true when pretreatment with MS medium containing 1 mM spermidine was used as an alternative to fixation (De Block and Van Lijsebettens, 1998). Finally, a Tris-based reaction buffer (De Block and Van Lijsebettens, 1998) gave the same results as the standard buffer.

Cytological techniques and microscopy

Fresh tissues or rehydrated GUS-stained samples were fixed overnight in 2% glutaraldehyde in 100 mM cacodylate buffer pH 7.2. Samples were then dehydrated in a graded ethanol series, embedded in Histo-resin (Leica) according to manufacturer's instructions, and sectioned with a LKB ultramicrotome. Sections (10 μ m) were dried

onto slides at 37°C and stained with 0.1% Toluidine Blue in 0.5 M acetate buffer pH 4.4 (root apices), or counterstained with 0.5% Safranin O in water (GUS-stained material), before mounting in epoxy resin for microscopic observation. Vibratome sections (100 µm) of 10-day-old dark-grown wild-type and 35S-Oshox1 seedlings were stained with Toluidine Blue as above, and mounted in 80% glycerol for microscopic observation. Samples were viewed using a Leica MZ12 stereomicroscope or a Leitz Diaplan microscope with bright-field optics settings. Images were acquired with a Sony 3CCD Digital Photo Camera DKC-5000 and processed using Adobe Photoshop 5.0.

In situ mRNA localisation

Seedlings (1- and 2-week-old) were transferred to the dark at 28°C 16-24 hours before harvesting to reduce their starch content. Root apices (1 cm) and seedling culm basal region (0.5 cm), containing the shoot apex, were fixed overnight in FAA (50% ethanol, 5% acetic acid, 3.7% p-formaldehyde). Samples were dehydrated in a graded ethanol series, after which ethanol was replaced stepwise with xylene. Infiltration and embedding in Paraplast Plus (Sigma) were performed as described by Sylvester and Ruzin (1994). Inflorescences (18 cm, approx. 8 days before heading) were fixed in 4% p-formaldehyde, 10 mM DTT, 1× PBS buffer. Dehydration, infiltration and embedding in butyl-methyl methacrylate (BMM) were performed as described by Baskin et al. (1992). Samples were sectioned on a Leitz rotary microtome (8 µm, Paraplast Plus) or on a LKB ultramicrotome (6 µm, BMM) and sections transferred onto poly-L-lysine coated slides. Linearised plasmid templates were used to synthesise digoxigenin-labelled antisense and sense riboprobes according to manufacturer's instructions (Boehringer Mannheim), except that 20 units of RNasin (Promega) were included in the reactions. In situ hybridisation with hydrolysed probes was performed as described by Langdale (1994) except that sections were treated with 1 mg/l of proteinase K for 30 minutes at 37°C and that RNasin (Promega) was added to the hybridisation mix (0.4 units/µl). After detection, sections were dehydrated through a graded ethanol series, transferred to xylene and mounted in Eukitt (O. Kindler GmbH & Co.). For whole-mount in situ hybridisation, mature embryos were prefixed as described by Engler et al. (1998), embedded in 5% agarose, bisected longitudinally through the median axis and processed according to the method of Engler et al. (1998). Identical hybridisation patterns were obtained with riboprobes of the full-length *Oshox1* cDNA and of the 0.4 kb 5' region lacking the conserved homeobox sequence. If not otherwise indicated, hybridisation signal was not observed with control sense RNA probes.

Wounding

The tissue inbetween two longitudinal vascular bundles of fully expanded leaves from greenhouse-grown wild-type and *Oshox1*-GUS plants was wounded using a needle. Attention was paid not to damage the vasculature, as this could lead to leakage of GUS and misinterpretation of the results. Wounded leaves were either left attached to the plant, or removed and incubated in 100 mM sodium phosphate buffer pH 7.7 in the greenhouse, which gave identical results. At different time points the wounded leaves were sampled and immediately subjected to GUS analysis (*Oshox1*-GUS) or longitudinally hand-sectioned and processed for histochemical detection of lignin with acidic phloroglucinol (wild type; Schneider, 1981).

Chemical treatments

Surface-sterilised seeds of *Oshox1*-GUS plants were sown on filter paper moistened with the following solutions: 5 µM indole-3-acetic acid (IAA), 1 µM each of naphthalene-1-acetic acid (NAA), naphthalene-2-acetic acid (β-NAA) and 1-N-naphthylphthalamic acid (NPA), 0.25 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 500 mM each of sucrose and mannitol, 0.01 and 2.5 mM p-chloromercuribenzenesulfonic acid (PCMBs), 10 µM N⁶-benzyladenine (BA), 4 µM 22(S),23(S)-

homobrassinolide and 6.8 µM uniconazole. All solutions were prepared in water and filter-sterilised, except for brassinolide and uniconazole, which were dissolved in dimethyl sulfoxide (DMSO). Control seeds were sown on water or DMSO at the final concentration as for chemical treatments. Seedlings were sampled after 1 week and subjected to GUS analysis.

Root tip growth and regeneration assays

Root apices (0.2, 0.5 and 1 mm, including the root cap) were excised aseptically from 2-week-old wild-type, *Oshox1*-GUS and 35S-*Oshox1* rice seedlings under a dissecting microscope fitted with a calibrated eyepiece micrometer. Excised tips were placed on filter paper moistened with modified MS medium (Philips and Dodds, 1977), containing 2% sucrose and no hormones. After 9 days of culture at 28°C in the dark, the length of the segments was recorded and GUS analysis performed. For root tip regeneration experiments, seedlings were returned to the dark at 28°C and GUS analysis was performed 1 week after surgery.

Polar auxin transport assays

Transport of [³H]IAA (25 Ci/mmol, Amersham) was measured in the most distal 2 cm of seminal roots (region in which lateral root primordia are absent) of 5-day-old vertically grown wild-type and 35S-*Oshox1* seedlings, and in the 2 cm of mesocotyl immediately below the coleoptilar node of 5- and 10-day-old wild-type and 35S-*Oshox1* seedlings. Excised tissues were placed in agar blocks containing 10⁻⁷ M [³H]IAA, in the presence or absence of 10⁻⁶ M NPA, and incubated for 2.5 (mesocotyls) or 3 (roots) hours in the dark at room temperature. To prevent drying, the tissues were overlaid with silicon oil. After incubation, the explants were cut in 3 segments: a basal part (5 mm), which was the one enclosed in the agar, a middle (5 mm) and a top (10 mm) part. Radioactivity in the segments was counted after overnight incubation in a scintillation liquid. As a control for simple diffusion of [³H]IAA in mesocotyls, transport in the acropetal direction was used. Since this could not be used for the roots where active transport occurs in both directions, a diffusion factor (DF) was calculated as the ratio between the radioactivity accumulated in the presence of NPA in the middle and basal segment. Subsequently, the amount of diffused [³H]IAA in the middle segment in the absence of NPA was calculated as the product of the DF by the radioactivity measured in the basal part in the absence of NPA. Finally, active transport was calculated as the difference between the radioactivity measured in the middle segment in the absence of NPA and the diffused [³H]IAA in the middle segment in the absence of NPA. The same calculations were done for the basipetal transport in the mesocotyl, resulting in an amount of auxin active transport comparable to that calculated using the acropetal transport as a measure of simple diffusion of [³H]IAA. Since 35S-*Oshox1* tissues showed a reduced sensitivity to NPA, wild-type DFs were also used for the transgenics. Incubation times were optimised in order to prevent accumulation of [³H]IAA in the top segment of the explants, which would eventually result in the achievement of a steady-state equilibrium in the diffusion process. This expedient enables the diffusion component occurring in the direction opposite to that of the polar auxin transport to be ignored, thus simplifying considerably the calculations and avoiding the introduction of further sources of error. The data obtained were tested for significance of the difference between wild-type and 35S-*Oshox1* populations by repeated-measures analysis of variance (two-factor ANOVA with replication).

RESULTS

The vasculature is the main location of *Oshox1* expression

Previous RNA blot analysis showed that the *Oshox1* gene is

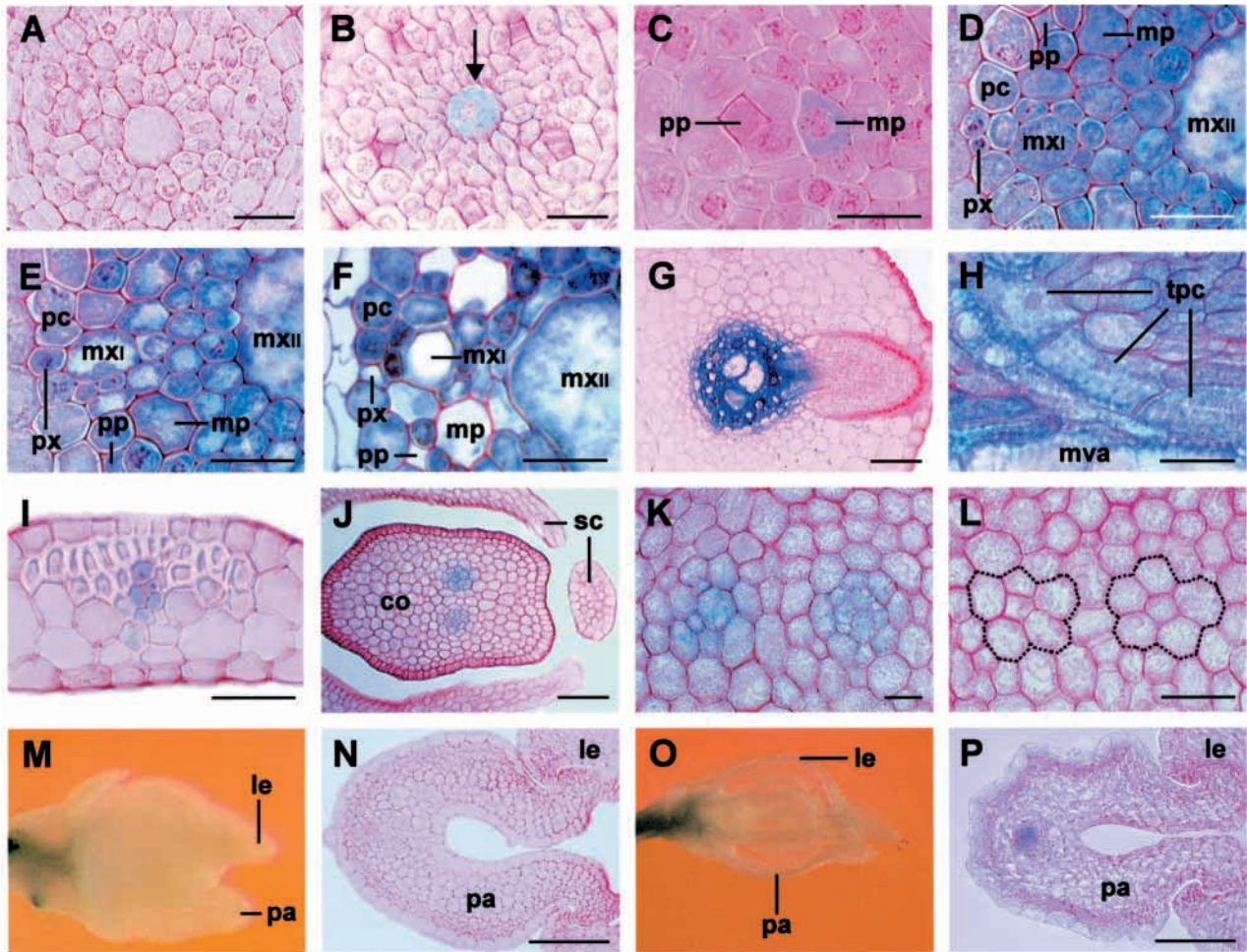


Fig. 2. *Oshox1*-GUS gene expression during (pro)vascular development in transgenic rice. (A-G) Details of the provascular cylinder in a transverse section through primary roots at different distances from the root tip. (A) 0.20 mm from the tip. (B) 0.25 mm from the tip. The arrow indicates the central late metaxylem precursor. (C) 0.3 mm from the tip. Note the presence of expression in the future metaphloem element (mp), but not in the adjacent protophloem precursor (pp), which will enter the differentiation pathway later, although reach maturity earlier than the mp. (D) 1.1 mm from the tip. Note that expression has not yet reached the pericycle (pc) completely and that the central late metaxylem precursor (mxII) has already started differentiation, whereas all the other cell types have not. (E) 1.2 mm from the tip. Note that the mp and early metaxylem (mxI) precursors have now started differentiation, whereas the pp and protoxylem (px) ones have not. (F) 28 mm from the tip. Note that expression has now fully reached the pc and that the pp, mp, px and mxI have already completed differentiation, whereas the mxII has not. (G) 33 mm from the tip in the region of an emerging secondary root primordium. (H) Detail of parenchyma cells transdifferentiating into vascular elements in the region where the adventitious root connects to the main axis of the stem. (I) Transverse section through the margin of a developing leaf. (J) Transverse section through the coleoptilar region of a 25-day-old embryo. (K) Detail of the provascular strands in J. (L) Detail of the provascular strands in a transverse section through the coleoptilar region of a late 7-day-old embryo. (M) Immature spikelet before the lemma and palea have sealed the inside. (N) Detail of a transverse section through the spikelet in M, 0.30 mm below its apex. (O) Immature spikelet after closure of the wings of the palea and lemma. (P) Detail of a transverse section through the spikelet in O, 0.27 mm below its apex. co, coleoptile; le, lemma; mva, main vascular axis; pa, palea; sc, scutellum; tpc, transdifferentiating parenchyma cells. Scale bars: (A-F,H,I,K,L), 30 μ m; (G,J,N,P), 150 μ m.

in mature embryos *Oshox1* was found to be expressed in provascular bundles of scutellum and embryonic axis (Fig. 1O,P).

***Oshox1* is expressed before any cytologically identifiable sign of vascular differentiation**

To determine the precise location and timing of the onset of *Oshox1* expression during (pro)vascular development, a selection of organs from *Oshox1*-GUS plants was examined in further detail. A series of root transverse sections revealed that *Oshox1* was not expressed in provascular cells in the most

distal part of the root apex, although a prostele was already identifiable (Fig. 2A), but first became detectable at approximately 0.25 mm from the root tip in provascular cells of the mitotic region. Expression started in the precursor of the central late metaxylem element (Fig. 2B), which is the first cell that will undergo vascular differentiation, as shown in Fig. 2D and as described by others (Kawata et al., 1978). Subsequently, expression spread basipetally and radially to the other prostelar elements, pericycle included, following the order in which they commence vascular differentiation (Fig. 2C-F and Kawata et al., 1978). In all cell types *Oshox1* expression started clearly

before any cytological sign of differentiation could be recognised (Fig. 2B-E). During lateral root formation, *Oshox1* was found to be downregulated in pericycle cells which, upon dedifferentiation, resume their meristematic activity to give rise to a lateral root primordium. Expression reappeared after a provascular strand was recognisable in the lateral root primordium (Fig. 2G). In rice, the vascular bundles interconnecting adventitious root primordia with the vasculature of the main axis differentiate from ground parenchyma cells (Kaufman, 1959). *Oshox1* expression was detected in such parenchyma cells that transdifferentiate into vascular elements (Fig. 2H). In the rest of the aerial part of the plant, *Oshox1* expression became detectable in provascular strands at an early stage of procambium development, before any sign of vascular differentiation could be observed (Fig. 2I). During spikelet development, *Oshox1* expression could be first detected in procambial strands of the palea and lemma as soon as they enclosed the underlying floral organ primordia (Fig. 2O). In agreement with the observation of others (Takeoka et al., 1993), no cytological evidence of an actual progression towards vascular differentiation could be observed at this stage of spikelet development (Fig. 2P). Furthermore, the procambial cells in the palea and lemma of these spikelets showed no cytological differences when compared to provascular cells in the palea and lemma of slightly younger spikelets that do not yet express *Oshox1* (Fig. 2M,N). Finally, although *Oshox1* was found to be expressed in provascular bundles of the mature rice embryo (Fig. 2J,K), no expression could be detected in 7-day-old embryos despite the fact that conspicuous provascular bundles are evidently present in the scutellum and embryonic axis (Fig. 2L and Jones and Rost, 1989). Only in the scutellum of the mature embryo, could occasional, partial differentiation into immature tracheary elements with secondary wall thickenings be observed (not shown). However, the majority of the *Oshox1*-expressing vascular strands in the mature embryo were still primarily procambial (Fig. 2J,K), as has been demonstrated also in previous studies where in most of the embryonic provascular tissue no evidence of secondary wall thickening could be detected by light or electron microscopy analysis (Bechtel and Pomeranz, 1978).

Taken together, analysis of provascular tissue in different rice organs revealed that *Oshox1* expression invariably starts in procambial cells that do not yet show any cytological progression towards vascular differentiation. This would suggest that such provascular cells are undergoing hidden changes in their developmental state, and that *Oshox1* expression might play a role in some of the regulatory aspects that such developmental process would imply.

Oshox1-GUS expression genuinely reflects *Oshox1* transcript accumulation

To confirm the spatial and temporal aspects of *Oshox1* gene expression as revealed by the reporter gene analysis, in situ mRNA hybridisation was performed on tissue sections and whole-mount preparations. In the root, *Oshox1* transcript was detected specifically in procambium derivatives of the central cylinder, beginning approximately 0.25 mm from the root apex (Fig. 3A-C). In the shoot apical region, *Oshox1* expression was found to be associated with provascular and vascular traces of the stem (Fig. 3H). *Oshox1* transcript was also detected in parenchyma cells that transdifferentiate into the vascular

elements that will connect the adventitious root to the main axis (Fig. 3I). In developing leaves, *Oshox1* expression was restricted to provascular and vascular strands at different developmental stages, where the signal was mainly localised in the central differentiating region of the bundle (Fig. 3D-G). In young immature spikelets, *Oshox1* transcript was detected in provascular and vascular strands of all organs (Fig. 3J). Finally, in the mature rice embryo *Oshox1* expression was limited to its provascular tissue (Fig. 3L). In situ mRNA localisation also revealed expression of *Oshox1* in developing trichomes of palea and lemma (Fig. 3K), again confirming what was observed during *Oshox1*-GUS analysis. It still remains unclear whether *Oshox1*-GUS expression in stomatal guard cells and pollen reflects bona fide *Oshox1* expression. In conclusion, in situ hybridisation largely confirmed that the *Oshox1*-GUS pattern mimics expression of the endogenous *Oshox1* gene.

***Oshox1* expression is promptly induced by wounding**

Mechanical wounding often induces transdifferentiation of parenchyma cells into vascular elements (Sachs, 1981). Although this effect is thought to be largely due to the interruption of auxin transport following severance of vascular bundles, the wounding event in itself might also be involved in induction of vascular elements differentiation (Church and Galston, 1988). The (pro)vascular-specific *Oshox1* expression pattern therefore prompted us to analyse the capacity of *Oshox1* to respond to mechanical injury of the plant. Upon wounding the tissue between two longitudinal vascular bundles of leaves from *Oshox1*-GUS plants, *Oshox1* was very rapidly, within minutes, induced ectopically in mesophyll and epidermis (Fig. 4A). As in most other monocotyledonous tissues, vascular regeneration does not occur in rice leaves, but cells do respond to injury by lignin deposition. The first indication of this event was detected approximately 6 hours following wounding. A massive induction of *Oshox1* was particularly evident in bundle sheath cells facing the wounded area. Interestingly, only these bundle sheath cells, and not those at the opposite side of the vascular bundles, later showed lignin deposition (Fig. 4B). In roots, no wound inducibility of *Oshox1* expression could be detected.

***Oshox1* expression is modulated by auxin and sucrose**

The role of auxin as the main regulatory factor in vascular development is well established and has been demonstrated in different experimental systems (Aloni, 1995; Fukuda, 1996). Furthermore, sucrose, in combination with auxin, is required to induce a balanced phloem and xylem differentiation (Aloni, 1987; Warren Wilson et al., 1994). Therefore, we tested the effect of auxin and sucrose on *Oshox1*-GUS expression. *Oshox1* was induced ectopically in cortex and epidermis cells located at the boundary between the mitotic and the transition zone of the root and in the root cap by IAA, 2,4-D and NAA (Fig. 5A), but not by β -NAA (an inactive auxin analogue) or NPA (an auxin transport inhibitor). The same induction pattern was observed in the presence of 500 mM sucrose (Fig. 5B), but not upon a 500 mM mannitol treatment, thus excluding an induction due to osmotic stress.

Brassinosteroids induce entry into the final stage of

tracheary element differentiation in cultured *Zinnia elegans* cells (Iwasaki and Shibaoka, 1991). Accordingly, inhibition of brassinosteroid biosynthesis by uniconazole prevents tracheary element differentiation, without affecting the expression of genes induced during procambium development and early stages of vascular differentiation (Yamamoto et al., 1997). Brassinosteroids and uniconazole, alone or in combination, were tested for their effect on tracheary element differentiation in rice calli, confirming what was observed in the *Zinnia elegans* xylogenesis system (unpublished results). No effect of brassinosteroids and uniconazole on Oshox1-GUS expression could be detected.

The finding that *Oshox1* is affected by auxin and sucrose, but not by brassinosteroids suggests a relation of *Oshox1* to procambial cell fate commitment rather than to subsequent vascular differentiation events.

The fate of provascular cells expressing *Oshox1* is specified but not stably determined

In the root, the column of procambium develops uninterruptedly as a single axial entity behind the growing apical meristem. The resulting correlation between cell position along the main axis of the root and the degree of cell-type differentiation makes it possible to accurately predict the developmental history and fate of each of the cells from their original location (van den Berg et al., 1998). Therefore, we decided to use roots from Oshox1-GUS rice plants to establish the developmental fate of the provascular cells expressing *Oshox1*.

Oshox1 expression in the root could first be detected approximately 0.25 mm from the root apex in provascular derivatives of the central prostele (Fig. 2B). Root apices of 0.2, 0.5 and 1 mm in length, showing no indication of mature tracheary elements, were excised from Oshox1-GUS rice seedlings and placed on a non-inductive, hormone-free medium. After 9 days of culture in this neutral environment, the root apices either had not further developed (0.2 mm apices) or had grown to a length of about 6 (0.5 mm apices) and 3 (1 mm apices) mm and contained mature tracheary elements (Table 1 and Fig. 6A). This would suggest that the developmental fate of procambial initials and their immediate most distal derivatives (0.2 mm apices) is not yet specified towards vascular differentiation, whereas provascular cells located at the boundary between the division and the transition zone of the root (0.5 mm apices) have become specified towards vascular cell identity. Since *Oshox1* is already expressed in procambial cells at 0.5 mm from the root tip, but not in 0.2 mm apices, this suggests that *Oshox1* is expressed at a stage of provascular development at which procambial cells are at least specified to become vascular elements. To understand whether provascular cells expressing *Oshox1* were also stably determined to become vascular elements, 0.5 and 1 mm root tips were removed from the seedlings, which were thereafter cultured on water for root tip regeneration. When 1 mm root tips were excised, the only response was an enhanced lateral root production around the site of root tip excision detectable in 40% of the cases (Table 1 and Fig. 6B). Regeneration of the root tip could never be observed, suggesting that procambial cells entering the elongation zone of the root (1 mm from the tip) appear stably determined towards vascular differentiation. When 0.5 mm root tips were

Table 1. Responses of different regions of the root of Oshox1-GUS plants in root tip growth and regeneration assays

	Root tip growth assay	Root tip regeneration assay		
		Root tip regeneration	Enhanced lateral root formation	No response
1.0*	98.5±0.5 (2.9±0.3)‡	0.0±0.0	38.5±0.5	61.5±0.5
0.5*	99.0±1.0 (6.5±0.6)‡	40.5±2.5	59.0±2.0	0.5±0.5
0.2*	0.0±0.0		Not determined	

The results represent the mean number of roots responding to the assay ± s.e.m. of 2 separate experiments each performed on 100 root tips.

*Length (mm) of the excised (root tip growth assay) or removed (root tip regeneration assay) region of the root tip.

‡Mean length (mm) ± s.e.m. of the root tips that had grown after 9 days in culture.

cut off, root tip regeneration was detected in about 40% of the cases, while in 60% enhanced lateral root formation was again observed (Table 1 and Fig. 6B). *Oshox1* appears, therefore, to be expressed at a stage of procambium development at which provascular cells are already specified but not yet stably determined to become vascular elements, since their broader prospective potential enables these cells to promptly switch to a different developmental program, in order to achieve formation of a new root apical meristem. Unexpectedly, in the roots where the tip had regenerated, a massive ectopic induction of *Oshox1* was detectable at the site of root tip excision, involving cells of protoderm, ground meristem and root cap, in addition to provascular cells (Fig. 6B). Since this pattern resembled the induction of *Oshox1* expression observed in the presence of sucrose or auxin (Fig. 5A,B), the same root tip regeneration experiment was done culturing the seedlings, after root tip excision, in the presence of PCMBs or NPA, inhibitors of sucrose and auxin transport, respectively. Neither root tip regeneration, nor *Oshox1* induction could ever be observed under these conditions, suggesting that auxin and sucrose are both required for proper root tip regeneration. Since *Oshox1* expression is not wound-inducible in roots, its ectopic expression in this case seems related to the process of root tip regeneration and reprogramming of a specified developmental fate. Such ectopic expression was subsequently rapidly silenced in the regenerating root tip. Here *Oshox1* expression became again confined to the prostele, marking the region where we showed that the developmental fate of procambial cells has been specified but not stably determined.

Ectopic *Oshox1* expression anticipates procambial cell fate commitment and vascular differentiation

To gain insight into the biological role of *Oshox1* during (pro)vascular development, we attempted to overcome the regulatory mechanisms controlling its expression. Since the CaMV 35S promoter drives strong reporter gene expression in all rice organs, including the most distal region of the root and shoot apex (Fig. 7), this promoter is suited to ectopically express *Oshox1* in provascular cells that normally do not yet express this gene.

35S-Oshox1 transgenic rice plants were indistinguishable from the wild type with respect to overall size and morphology, and to growth of the aerial part and root system (Fig. 8A,D).

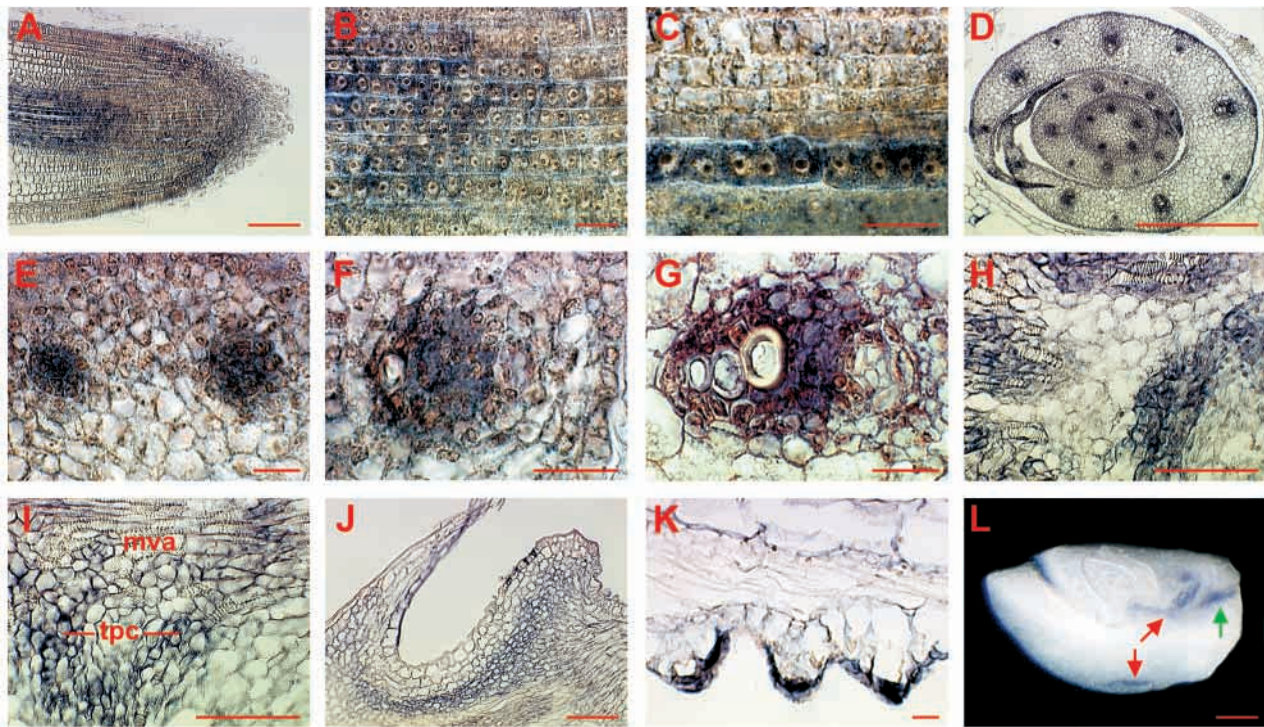


Fig. 3. In situ localisation of *Oshox1* transcript in wild-type rice. (A) Longitudinal section through the apical region of a primary root. (B) Detail of the prostele in A. (C) Detail of pericycle cells in a longitudinal section through the apical region of a primary root. (D) Transverse section through a 1-week-old seedling 0.1 mm below the shoot apex. (E-G) Details of D showing progressive stages of (pro)vascular development: early (E, left) and late (E, right) procambial strands, first protoxylem element differentiation stage (F), third protoxylem element differentiation stage (G). (H) Detail of the subapical region of a longitudinal section through a 1-week-old seedling showing the nodal (pro)vascular anastomosis. (I) Detail of a longitudinal section through a 1-week-old seedling showing parenchyma cells transdifferentiating into vascular elements in the region of the developing connection between an adventitious root and the main axis of the stem. (J) Detail of a longitudinal section through an immature spikelet showing a provascular strand in the developing basal part of the palea. (K) Details of developing trichomes in the lemma of an immature spikelet. (L) Whole-mount embryo preparation. Red arrows indicate signal in the vascular bundles of the embryonic axis and scutellum. The signal in the area where the embryo was attached to the suspensor (green arrow) was also observed with the control sense probe and interpreted as non-specific. mva, main vascular axis; tpc, transdifferentiating parenchyma cells. Scale bars: (A,H-J), 150 μm ; (B,C,E-G,K), 300 μm ; (D,L), 650 μm .

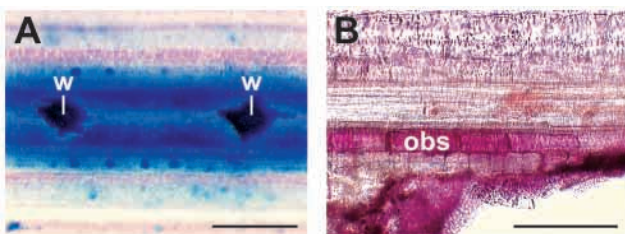


Fig. 4. Effect of wounding on *Oshox1*-GUS expression and lignin deposition. (A) Detail of a transgenic rice leaf blade harbouring the *Oshox1*-GUS construct, subjected to GUS assay 30 minutes after wounding. (B) Detail of a longitudinal section through a wild-type rice leaf blade stained with acidic phloroglucinol to detect lignin deposition (red staining) 6 hours after wounding. The wounded area is situated in the lower right corner of the section. obs, outer bundle sheath cells facing the damaged area; w, wound. Scale bars: 300 μm .

Flowering time was unaffected, and the structure of the flowers displayed no obvious morphological alterations (not shown). The size and shape of leaves of the transgenic plants were comparable to those of wild type, and their vascular pattern did not show any discontinuity (not shown). The cellular anatomy

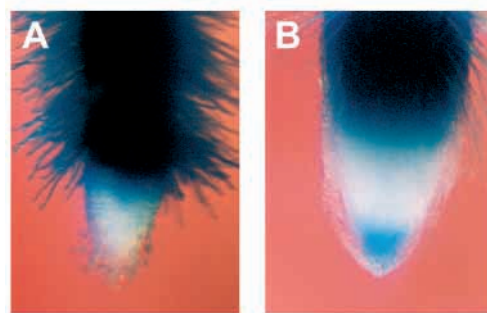


Fig. 5. Modulation of *Oshox1*-GUS expression by auxin and sucrose in transgenic rice. (A, B) Ectopic activation of *Oshox1*-GUS in the apical region of a primary root upon treatment with 10^{-6} M NAA (A) or 0.5 M sucrose (B).

of the leaf and root was examined in transverse sections, revealing that the cells of both transgenic and wild-type plants were arranged similarly and were of comparable size and shape (Fig. 8B,C,E,F). However, a subtle but remarkable phenotype was observed when the anatomy of differentiating vascular tissues was examined in further detail. In the root apical region

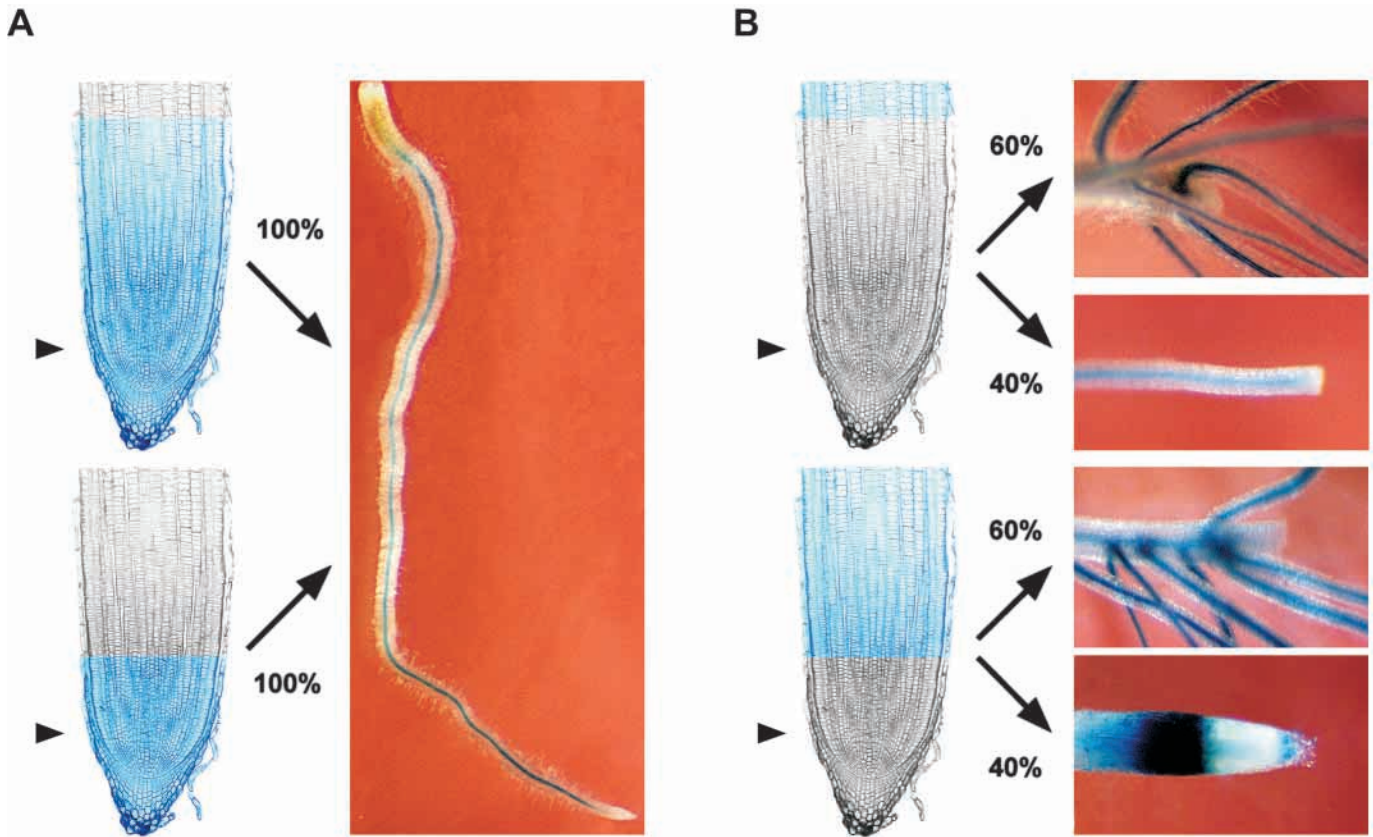
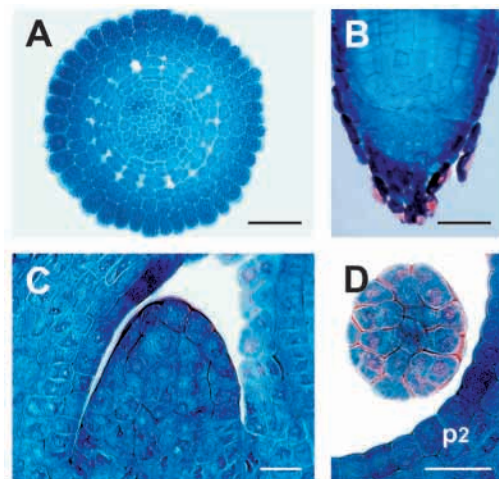


Fig. 6. Developmental fate of provascular cells expressing *Oshox1*. (A) Root tip growth and (B) regeneration assays. On the left-hand side of A and B, median longitudinal sections of a wild-type rice root are shown, in which the grey region indicates the part that was cut off and discarded during the different experiments. The arrowhead indicates the approximate onset of *Oshox1* expression. On the right-hand side of A and B, the growth and regeneration responses of the roots to the surgical treatments and their effect on *Oshox1*-GUS expression are represented.

of the 35S-*Oshox1* plants, we could detect abundant secretory vesicles contributing to the growth of secondary cell wall thickenings in the cytoplasm of the precursors of the central late metaxylem elements approximately 0.7 mm from the root tip (0.67 ± 0.06 mm, $n=10$; Fig. 9B). At the same distance from the root apex of wild-type plants ($n=10$), these cells still show the dense cytoplasm and small vacuole typical of undifferentiated cells of the procambium or other meristematic tissues (Fig. 9A). The first signs of secretory vesicle formation marking the onset of vascular differentiation could be detected in wild-type roots at approximately 1.2 mm from the apex (1.24 ± 0.08 mm, $n=10$; Fig. 9C). At this distance from the root tip in 35S-*Oshox1* plants ($n=10$), the precursors of the late metaxylem elements have already started elongation and show advanced stages of cytoplasmic degradation and cell wall modification (Fig. 9D). The situation appears to be analogous in the shoot, where at 1 mm below the apical meristem of wild-type plants (0.95 ± 0.02 mm, $n=10$), there is no detectable

presence of a peripheral cylinder of vascular bundles (PCVB; Fig. 10A), and where in the vascular bundles that are in the most advanced stage of differentiation the protophloem is hardly visible and the precursor of the second protoxylem element has just begun radial enlargement (Fig. 10C). At the same distance from the shoot apical meristem (SAM) in 35S-*Oshox1* plants ($n=10$), a PCVB is already clearly recognisable (Fig. 10B), and in the vascular bundles the protophloem is easily identifiable, up to four protoxylem vessels have already

Fig. 7. Histochemical localisation of CaMV 35S-GUS gene expression in transgenic rice. (A) Transverse section through a primary root 200 μ m from the root tip. (B) Detail of the root apical meristem region in a median longitudinal section through a primary root. (C) Detail of the shoot apical meristem region in a median longitudinal section through a 1-week-old seedling. (D) Transverse section through a 1-week-old seedling 10 μ m below the shoot apex. p2, second leaf primordium. Scale bars: (A,B), 80 μ m; (C,D), 30 μ m.



differentiated and the future late metaxylem elements can be clearly distinguished (Fig. 10D). The same developmental state could be observed in wild-type plants at 2.2 mm below the SAM (2.20 ± 0.03 mm; Fig. 10E,G), a distance at which the vascular bundles in 35S-Oshox1 plants ($n=10$) have reached complete maturity, with the recognisable presence of a metaphloem, the stretching of protoxylem vessels into a lacuna and the full differentiation of late metaxylem elements (Fig. 10F,H). Taken together, this would suggest that expressing *Oshox1* ectopically in procambial cells that do not yet express this gene is sufficient to anticipate their entrance into a vascular differentiation pathway.

Since the onset of *Oshox1* expression marks procambial cells whose developmental fate has already been specified but that do not yet manifest any obvious sign of vascular differentiation, it was of particular interest to determine whether ectopic expression of *Oshox1* in the most distal region of the root alters the developmental state of provascular cells even in the absence of any evident cytological sign of vascular differentiation. To address this question, root tips of 0.5 mm in length were excised from wild-type and 35S-Oshox1 roots, and the seedlings then cultured for root tip regeneration. As in the previous experiments, wild-type roots regenerated their apices in 40% of the cases, while 60% displayed enhanced lateral root formation around the site of excision. Root tip regeneration was detected in only 10% of the 35S-Oshox1 roots, while 30% showed increased lateral root production and 60% did not react in any appreciable fashion to the treatment (Table 2). Hence, provascular cells situated 0.5 mm from the root tip of 35S-Oshox1 plants showed a response resembling that of determined procambial cells, which in wild-type roots are situated 1 mm from the root apex. Similarly, when 0.2 mm root tips were cultured for root tip growth on a neutral medium, the wild-type tips did not develop any further, exactly as expected, whereas the transgenic ones grew considerably (up to 20 mm after 9 days in culture, with an average length of 12 mm; Table 2), showing a characteristic response of specified provascular cells, which are located at 0.5 mm from the tip in wild-type plants. Taken together, these observations would suggest that ectopic expression of *Oshox1* in procambial cells that normally do not yet express this gene results in anticipation of their cell fate commitment, ultimately leading to premature vascular differentiation.

Aspects of polar auxin transport are affected upon *Oshox1* ectopic expression

As described above, *Oshox1* gene expression was found to be inducible by auxin. The critical role of auxin in (pro)vascular development results particularly from its polar transport through plant tissues (Lomax et al., 1995). The pathways of such polar auxin transport (PAT) are complex and not fully understood, but it is clear that there is a major stream of auxin in the vascular tissues running from the shoot apex to its base where it joins the root. Into the root itself, auxin travels predominantly down through the central stele, then upon reaching the root tip is distributed back upwards along the root in the epidermis and subtending cortical cells, towards the elongation zone (Jones, 1998). To determine whether *Oshox1* ectopic expression could result in alterations of PAT, we monitored the transport of ^3H -labelled IAA through excised mesocotyls and root apices of wild-type and 35S-

Table 2. Responses of different regions of the root of wild-type and 35S-Oshox1 plants in root tip growth and regeneration assays

	Root tip growth (0.2 mm)*	Root tip regeneration (0.5 mm)*		
		Root tip regeneration	Enhanced lateral root formation	No response
Wild type	0.0±0.0	39.5±1.5	59.5±1.5	1.0±0.0
35S-Oshox1	74.5±0.5 (12.8±1.2)‡	10.5±0.5	31.0±3.0	58.5±2.5

The results represent the mean number of roots responding to the assay \pm s.e.m. of 2 separate experiments each performed on 100 root tips.

*Length of the excised (root tip growth assay) or removed (root tip regeneration assay) region of the root tip.

‡Mean length (mm) \pm s.e.m. of the root tips that had grown after 9 days in culture.

Oshox1 plants. Whereas basipetal auxin transport was not significantly different in wild-type and 35S-Oshox1 mesocotyls (Fig. 11A), in 35S-Oshox1 root tips the acropetal transport was reduced approximately 60% when compared with the wild type (Fig. 11B). An important difference between these tissues is that vascular differentiation is still in progress in root apices (Fig. 2), whereas mesocotyls contain two fully mature vascular bundles. Hence, cytological differences resulting from the precocious vascular differentiation in 35S-Oshox1 plants are found in root tips but not in mesocotyls, where PAT appears to be identical in wild type and 35S-Oshox1.

In addition to (pro)vascular development, the co-ordinated movement of auxin within the plant has been implicated in a variety of vectorial developmental processes, including root elongation, lateral and adventitious root formation, and gravitropic response (Aloni, 1995; Lomax, 1995). Because of the difference in PAT capacity between wild-type and 35S-Oshox1 roots, we investigated whether this could result in a difference in those developmental processes in which auxin is known to play a major role. As the data in Table 3 show, we could not detect any significant difference between wild-type and 35S-Oshox1 plants concerning these parameters.

While wild-type and 35S-Oshox1 mesocotyls showed identical PAT capacity, we noticed that 35S-Oshox1 mesocotyls were significantly less sensitive to NPA, accumulating approximately 50% less auxin than the wild type in basal segments of explants subjected to PAT assays in the presence of such PAT inhibitor (Fig. 11C). Moreover, in 35S-Oshox1 root apices, the NPA-induced auxin accumulation was reduced to a higher extent (Fig. 11D) than expected based only on the reduced auxin transport capacity observed in these tissues (Fig. 11B), indicating also in root tips the presence of a decreased NPA sensitivity. Therefore, we decided to examine whether this difference in sensitivity to NPA also resulted in an altered phenotypic response to exogenously supplied auxin or NPA. The data in Table 4 show that whereas root elongation and gravitropism in 35S-Oshox1 were indistinguishable from the wild type, 35S-Oshox1 plants showed increased lateral and adventitious root formation, compared to the wild type, when cultured in the presence of auxin or NPA. In conclusion, lateral and adventitious root development in 35S-Oshox1 plants appear to be more sensitive to auxin and less sensitive to the inhibitory effect of NPA.

Table 3. Morphometric analysis of wild-type and 35S-Oshox1 intact plants and excised root tips (ex planta)

	Wild type	35S-Oshox1
Mesocotyl elongation (mm)	15.3±3.3	15.1±1.3
Root elongation (mm)	11.0±0.7	14.4±0.8
Number of lateral roots	86.8±4.0	88.5±6.0
Gravitropic response (degrees)	82.6±2.3	83.9±1.4
Root elongation ex planta (mm)	6.8±0.4	6.4±0.3
Number of lateral roots ex planta	0.6±0.6	0.4±0.3
Gravitropic response ex planta (degrees)	83.9±3.1	84.1±2.5
Number of vascular bundles	14.7±0.6	14.5±0.8
Radial size of metaxylem elements (µm)	26.8±0.6	24.7±0.4
Tangential size of metaxylem elements (µm)	26.9±0.6	25.0±0.4

Seedlings for root elongation and gravitropic response and root tips for gravitropic response ex planta were grown on solid half-strength MS medium supplemented with 10 g/l sucrose. Seedlings for determination of all the other parameters were grown on moistened filter paper. In all cases temperature and light:dark cycle conditions were as described in Materials and Methods.

Mesocotyl elongation was measured in 20 dark-grown 10-day-old seedlings. Root elongation in 24 hours was measured in 15 vertically grown 4-day-old seedlings. Number of lateral and adventitious roots was determined in 7 cm seminal roots of 20 2-week-old seedlings. Gravitropic response angles were measured in 15 vertically grown 4-day-old seedlings 24 hours after turning seedlings containers 90°. Root elongation ex planta and number of lateral roots ex planta were determined in 30 1 mm root tips after 9 days of growth. Gravitropic response angles ex planta were measured in 30 vertically grown 1 mm root tips. Number of vascular bundles and size of metaxylem elements were determined in overlapping 250× microphotographs of transverse sections obtained from the basal part of the internode immediately above the coleoptilar node of 10 10-day-old seedlings. All results represent the mean ± s.e.m. of 2 separate experiments each performed on a population of above-mentioned size.

The data obtained were tested for significance of the difference between wild-type and 35S-Oshox1 populations by repeated-measures analysis of variance (two-factor ANOVA with replication). The probabilities that the data would arise from identical populations were in all cases $P>0.05$ (i.e. not significantly different from wild type).

DISCUSSION

To date no information has been available concerning the actual developmental state of procambial cells, and as to when their commitment to differentiation into the various vascular cell types takes place. The operational distinction between a specification and a determination stage during the process of procambial cell fate commitment is an important result of our efforts to put (pro)vascular ontogenesis in a broader developmental perspective. Using the rice root as experimental system, we have shown that the developmental potential of procambial initials and their immediate derivatives has not yet been restricted towards vascular differentiation, and therefore they must be regarded as uncommitted meristematic precursors. Instead, the developmental fate of procambial cells located at the boundary between the mitotic and transition zone of the root has become specified towards a vascular cell identity. Finally, we have shown that procambial cells entering the elongation zone of the root appear to be stably determined towards vascular differentiation.

In addition to defining the developmental stages of provascular cell fate commitment, we have identified a transcriptional regulator that is expressed during the specification events occurring during the restriction of developmental potential of procambial cells. The sharp onset of the *Oshox1* gene expression during this particularly dynamic period of provascular development provides evidence for a

Table 4. Responses of wild-type and 35S-Oshox1 plants to exogenous auxin or auxin transport inhibitor

	NAA		NPA	
	Wild type	35S-Oshox1	Wild type	35S-Oshox1
Root elongation (mm)	10.9±1.3	9.7±0.9	19.1±1.9	23.1±3.7
Number of lateral and adventitious roots	30.4±5.9	46.1±3.1*	13.0±4.3	30.3±3.6*
Gravitropic response (degrees)	0.0±0.0	0.0±0.0	65.8±4.9	64.4±5.3

Seedlings were grown on solid half-strength MS medium supplemented with 10 g/l sucrose and in the presence of either 1 µM NAA or 1 µM NPA. Temperature and light:dark cycle conditions were as described in Materials and Methods.

Root elongation and gravitropic response angles (the angle between the root axis and the surface of the medium) were measured in seminal roots of 10 vertically grown 9-day-old seedlings. Number of lateral and adventitious roots was determined on a second group of 10 vertically grown 9-day-old seedlings. For each parameter the mean ± s.e.m. is indicated.

Asterisks indicate the significance of difference between wild-type and 35S-Oshox1 populations as determined by repeated-measures analysis of variance (two-factor ANOVA with replication; $P<0.05$).

discrete regulatory event occurring at the beginning of this phase. Indeed, this is when multiple vascular-lineage-specific effector genes, which might represent potential targets of *Oshox1* regulatory function, are upregulated or begin to be expressed (Demura and Fukuda, 1994). Interestingly, the regulatory mechanisms in charge of procambial cell fate specification are likely to be strictly conserved amongst plant species, as we could observe the same precise timing of *Oshox1* promoter-driven reporter gene expression during provascular development in rice and *Arabidopsis* (unpublished results).

Ectopic expression of *Oshox1* in provascular cells where normally this gene is not yet expressed, is not only sufficient to anticipate vascular differentiation events, but also to alter the timing of procambial cell fate commitment. This observation, together with the precise onset of *Oshox1* expression during provascular cell fate specification, suggests a relevance for this gene as a timely molecular switch in the transition to a developmentally restricted phase of procambial cell ontogenesis. The responsiveness of *Oshox1* expression to signals known to affect (pro)vascular development, such as wounding, sucrose and auxin, provides further support for the involvement of this gene in procambial cell fate commitment.

Auxin has been shown to exert its vast influence on (pro)vascular development, principally as a consequence of its polarised flow through plant tissues. The directionality of its transport is thought to result from the polar distribution of specialised carrier molecules in the plasma membranes. According to the chemiosmotic hypothesis, auxin is assumed to leave transport competent cells only through the activity of specific carrier proteins that are localised at the basal (basipetal PAT in the shoot and acropetal PAT in the root) or apical (basipetal PAT in the root) extremity of these cells (Jones, 1998). A large body of biochemical and physiological evidence suggests that at least three polypeptides are essential elements of the auxin efflux machinery: a plasma membrane-localised carrier protein; an NPA-binding protein; and a labile, probably cytosolic, component (Bennett et al., 1998). We have found that 35S-Oshox1 root apices, where an anticipation of vascular differentiation could be cytologically detected, showed a reduced PAT when compared with the wild type, while a

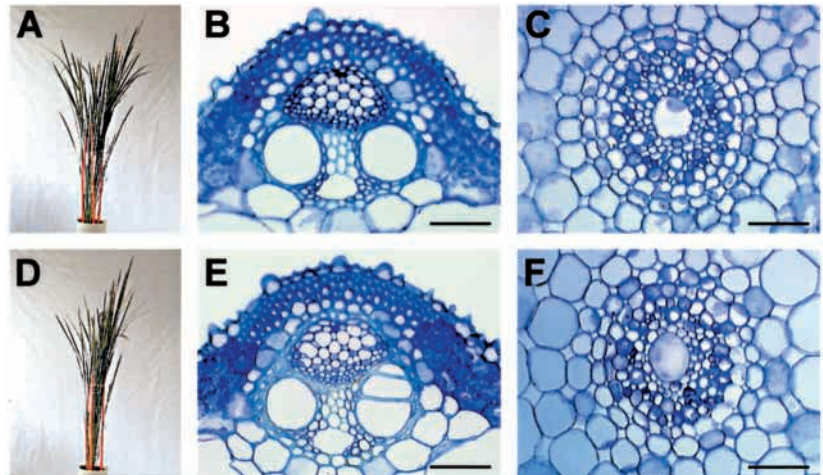


Fig. 8. Morphology and anatomy of wild-type and 35S-Oshox1 rice plants. (A-C) Wild type. (D-F) 35S-Oshox1. (A,D) Side view of 4-month-old plants. (B,E) Details of the midrib large vascular bundles in transverse sections through the middle region of fully expanded leaf blades. (C,F) Details of the vascular cylinders in transverse sections through primary roots 1 cm from the tip. Scale bars: 90 μm .

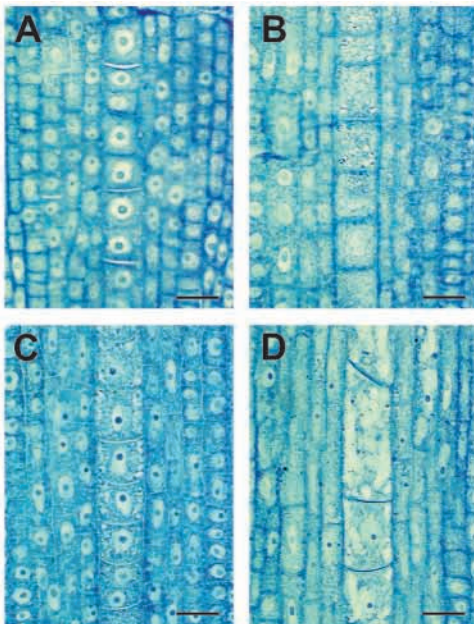


Fig. 9. Effect of *Oshox1* ectopic expression on vascular differentiation in the distal region of the rice root apex. (A,C) Wild type. (B,D) 35S-Oshox1. (A,B) Details of the regions between 0.7 and 0.9 mm from the root tip in median longitudinal sections through primary roots. (C,D) Details of the regions between 1.2 and 1.4 mm from the root tip in median longitudinal sections through primary roots. The cell file of the central late metaxylem precursors is positioned at the middle of all the sections. Scale bars: 30 μm .

difference in PAT could not be detected in mesocotyls, where the vasculature of wild-type and 35S-Oshox1 plants is cytologically indistinguishable. This would strongly suggest that the premature vascular differentiation and the reduced PAT observed in 35S-Oshox1 root apices are two intrinsically correlated phenomena, representing different aspects of the same developmental event. Interestingly, the putative plasma membrane-associated component of the auxin efflux carrier has been shown to be localised in provascular and xylem parenchyma cells (Gälweiler et al., 1998; Steinmann et al., 1999). Progression through the differentiation process

necessarily implies a reduction in the amount of procambial cells (all of which may transport auxin), in favour of an increase in that of mature vascular cells, of which only a limited number (the parenchymatic cells of the xylem) would be able to efficiently transport auxin.

The reduction in PAT observed in 35S-Oshox1 plants was not phenotypically associated with any difference in auxin-dependent developmental responses, other than (pro)vascular development. However, 35S-Oshox1 plants did respond differently from the wild type to exogenous application of auxin or NPA in lateral root formation, a process strongly dependent on the acropetal component of PAT in the root (Reed et al., 1998; Table 3, which shows that lateral root formation, dependent on auxin flow from the shoot, was completely suppressed ex planta, whereas gravitropism and root elongation were not). In contrast, 35S-Oshox1 and wild-type plants did not show any significant difference in their response to exogenously applied auxin or NPA with regard to root elongation and gravitropic response, which are dependent on the basipetal component of PAT in the root (Rashotte et al., 2000; Table 3). Lateral root formation is believed to be preceded by inhibition of the acropetal PAT immediately downstream of the future site of primordia emergence (Mathesius et al., 2000). This effect would result from the action of an endogenous NPA-like molecule, partially inhibiting the acropetal PAT. This would give rise to a local enhancement of auxin concentration required for pericycle cells to enter division. The different responses of wild-type and 35S-Oshox1 roots to exogenously applied auxin can be explained by the precocious vascular differentiation and associated PAT reduction in 35S-Oshox1 root tips. In 35S-Oshox1 plants, the less efficient drainage of auxin away from the pericycle cells would result in an increased number of lateral and adventitious roots than in the wild type, leading to an apparently enhanced sensitivity to auxin. Exogenous application of NPA would inhibit the efflux carriers in wild-type plants, resulting in a depletion of auxin flowing from the shoot into the root, which would consequently give rise to a reduced lateral root production. The precocious vascular differentiation, and its concurrent PAT reduction, does not explain the observation that NPA-treated 35S-Oshox1 plants produce more lateral roots than the wild type. However, this

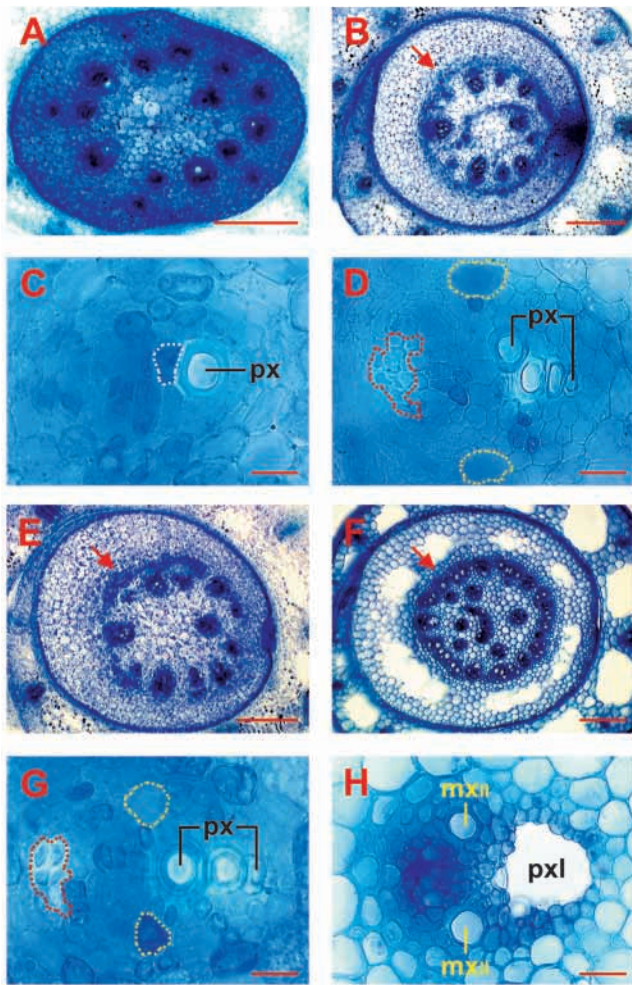


Fig. 10. Effect of *Oshox1* ectopic expression on vascular differentiation in the distal region of the rice shoot apex. (A,C,E,G) Wild type. (B,D,F,H) 35S-*Oshox1*. (A,B) Transverse sections through 10-day-old dark-grown seedlings 1.0 mm below the shoot apex. (C,D) Details of the vascular bundles in the most advanced stage of differentiation in A and B, respectively. (E,F) Transverse sections through 10-day-old dark-grown seedlings 2.1 mm below the shoot apex. (G,H) Details of the vascular bundles in the most advanced stage of differentiation in E and F, respectively. Red arrow, peripheral cylinder of vascular bundles; red dotted line, protophloem; white dotted line, precursor of the second protoxylem element; yellow dotted line, late metaxylem element precursor; mxII, late metaxylem element; px, protoxylem elements; pxl, protoxylem lacuna. Scale bars: (A,B,E,F) 300 μ m; (C,D,G,H) 30 μ m.

would be consistent with the presence of NPA-insensitive contra NPA-sensitive efflux carriers in 35S-*Oshox1* plants, which would allow more auxin than in the wild type to be transported from the shoot into the root. This assumption is supported by the fact that a reduced, but not completely abolished, NPA sensitivity of 35S-*Oshox1* was indeed observed during monitoring of PAT. Taken together, the differences in PAT and auxin/NPA responses between wild-type and 35S-*Oshox1* root apices can be consistently explained assuming that precocious and enhanced *Oshox1* expression leads to a reduction in the total amount of functional efflux

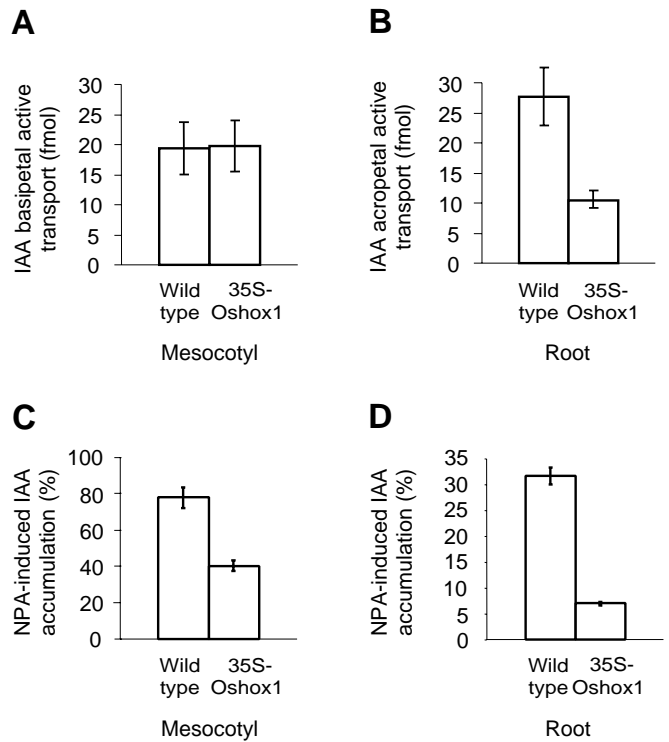


Fig. 11. Polar active transport and NPA-induced accumulation of [3 H]IAA in excised seminal root apices and mesocotyls of wild-type and 35S-*Oshox1* plants. (A) Basipetal active auxin transport in wild-type and 35S-*Oshox1* mesocotyls. (B) Acropetal active auxin transport in wild-type and 35S-*Oshox1* root apices. (C) NPA-induced auxin accumulation in wild-type and 35S-*Oshox1* mesocotyls. (D) NPA-induced auxin accumulation in wild-type and 35S-*Oshox1* root apices. The results represent the mean \pm s.e.m. of 2 (A,C) or 5 (B,D) separate experiments each performed on 20 plants. The differences between wild type and 35S-*Oshox1* apparent from B-D were reproducibly observed in all separate experiments. Auxin accumulation due to the presence of NPA (C,D) was measured in the basal segments (5 mm) of explants placed in agar blocks containing [3 H]IAA, and is shown as a percentage of the accumulation in the absence of NPA. Error bars indicate s.e.m.

carrier proteins, part of which have now become insensitive to the PAT inhibitor NPA. The putative NPA-insensitive auxin efflux carriers in 35S-*Oshox1* would be expressed independently of the differentiation status of vascular tissues, as a decreased NPA sensitivity was measured in roots as well as mesocotyls. The existence of an NPA-insensitive efflux carrier was previously postulated by Mattsson et al. (1999), who tentatively explained the unresponsiveness of anatomically recognisable provascular strands to experimental manipulations of PAT with the possible expression of NPA-insensitive auxin carrier proteins in provascular strands. Furthermore, in sunflower hypocotyls a loss of functional integrity of the auxin efflux system relative to the influx carriers was shown to occur with advancing tissue differentiation, due to a net loss of functional binding sites, as well as a reduction in the apparent affinity of the remaining sites for NPA (Süttle, 1991). Changes in the total amount of functional efflux carriers and ratio of NPA-sensitive versus NPA-insensitive carriers are thus likely to occur during (pro)vascular development, and may represent aspects of

procambial cell fate commitment. The altered PAT capacity and NPA sensitivity upon *Oshox1* ectopic expression suggest that this gene would act upstream of these events during normal development.

While a role for other regulatory proteins is by no means excluded, our results and those of others suggest that transcription factors of the HD-Zip type are involved at all different levels of (pro)vascular ontogenesis, from procambial cell identity acquisition to vascular differentiation. While some HD-Zip genes appear to play a role in development of specific vascular cell types (Tornero et al., 1996; Zhong and Ye, 1999; Ratcliffe et al., 2000), the HD-Zip gene *Athb8* (Baima et al., 1995) is expressed in procambium at an earlier stage than *Oshox1*, and thus active in uncommitted meristematic vascular precursors. We propose here that *Oshox1* represents a paradigm for a class of selector genes, the expression of which leads to a process that narrows the developmental potential of the totipotent meristematic precursors to culminate in provascular cell fate specification. After the checkpoint of provascular cell fate commitment, no significant changes in the expression of *Oshox1* could be observed. The fact that many plant cells are able to dedifferentiate to a meristematic state suggests that their identity, once established, must be actively maintained throughout plant life. *Oshox1* permanent expression in vascular tissues might therefore suggest a possible vascular tissue identity function for this gene. An additional or alternative possibility is that the permanent vascular expression of *Oshox1* points to a function in environmental adaptation. Such a role has been proposed for certain other members of the HD-Zip class (e.g., Frank et al., 1998; Söderman et al., 1999), and possible mechanisms by which these factors could regulate a response to environmental changes have been discussed (Steindler et al., 1999). Consistent with a similar function of *Oshox1* is the importance of the vascular system in ensuring morphological adaptability to environmental pressure, and the observation that *Oshox1* is also expressed in developing trichomes and stomatal guard cells, which play a role in water availability, temperature control and defence responses. Moreover, outer bundle sheath cells, which also express *Oshox1*, are more susceptible to death-inducing signals than are mesophyll cells during hypersensitive response in plant-pathogen interactions. Localised death of bundle sheath cells would prevent pathogens gaining entry to the vascular system and spreading systemically (Pennell and Lamb, 1997).

In future work, combined approaches integrating upstream regulators of *Oshox1* expression and downstream targets of its function should throw more light on the transcription factor network that establishes and maintains vascular identity, as well as on the role played by *Oshox1* in provascular cell fate specification.

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Note added in proof

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