# The Procambium Specification Gene *Oshox1* Promotes Polar Auxin Transport Capacity and Reduces Its Sensitivity toward Inhibition[1]

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The auxin-inducible homeobox gene *Oshox1* of rice (*Oryza sativa*) is a positive regulator of procambial cell fate commitment, and its overexpression reduces the sensitivity of polar auxin transport (PAT) to the PAT inhibitor 1-*N*-naphthylphthalamic acid (NPA). Here, we show that wild-type rice leaves formed under conditions of PAT inhibition display vein hypertrophy, reduced distance between longitudinal veins, and increased distance between transverse veins, providing experimental evidence for a role of PAT in vascular patterning in a monocot species. Furthermore, we show that *Oshox1* overexpression confers insensitivity to these PAT inhibitor-induced vascular-patterning defects. Finally, we show that in the absence of any overt phenotypical change, *Oshox1* overexpression specifically reduces the affinity of the NPA-binding protein toward NPA and enhances PAT and its sensitivity toward auxin. These results are consistent with the hypothesis that *Oshox1* promotes fate commitment of procambial cells by increasing their auxin conductivity properties and stabilizing this state against modulations of PAT by an endogenous NPA-like molecule.

During development of multicellular organisms, most cells enter specific differentiation programs that overtly change their structure and specialize them toward a distinct function. Cell differentiation is preceded by the process of cell fate commitment, in which the developmental potential of the cell becomes restricted to a certain fate or subset of fates, but the explicit demonstration and realization of that developmental pathway is not yet apparent (Maclean and Hall, 1987). Cell fate commitment has been divided into the processes of specification and determination (Slack, 1991). However, the definitions of these processes are, by nature, operational, and knowledge of the molecular nature underlying the effective functional properties of specified or determined cell states in plants is still mostly lacking.

The vascular tissues of plants represent an attractive system for the study of cell fate commitment. In fact, vascular tissues consist of several distinct cell types arranged in a network of continuous strands to form a system of exquisitely complex topography (Steeves and Sussex, 1989). Yet, all types of highly specialized vascular cells derive from an anatomically homogeneous population of meristematic cells, the procambium, or provascular tissue. We have previously developed assays with which different stages of procambial cell fate commitment in rice (*Oryza sativa*) could be distinguished (Scarpella et al., 2000). However, despite the detailed studies available on different aspects of vascular development (for review, see Sachs, 1981, 2000; Fukuda, 1996, 1997; Nelson and Dengler, 1997; Berleth et al., 2000; Aloni, 2001; Dengler, 2001; Dengler and Kang, 2001; Kuriyama and Fukuda, 2001; Ye, 2002), the molecular mechanisms underlying procambial cell fate commitment remain elusive (e.g. Savidge, 2001). Nevertheless, it is possible that the plant hormone auxin may be involved in this process, because a number of studies have provided evidence for a role of this hormone in different aspects of vascular tissue development (for review, see Sachs, 1981; Berleth et al., 2000).

One key feature of auxin action is its translocation from cell to cell in a polar fashion, a process that is referred to as polar auxin transport (PAT; Goldsmith, 1977; Lomax et al., 1995; Muday and DeLong, 2001; Muday and Murphy, 2002). In the shoot, auxin moves unidirectionally through the vascular tissues from the apex to the base. In the root, two distinct polarities of PAT exist. Auxin moves acropetally through the central vascular cylinder and basipetally through the outer layers of root cells. Auxin entry into cells is facilitated by an auxin influx carrier that is thought to be encoded by *AUX1* (Marchant et al., 1999) and possibly by related genes (Parry et al., 2001). Auxin moves out of plant cells through an efflux carrier

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apparatus that is sensitive to synthetic PAT inhibitors (PATIs), such as 1-*N*-naphthylphthalamic acid (NPA), and requires the activity of at least two polypeptides (Morris, 2000; Muday and DeLong, 2001; Muday and Murphy, 2002). The first is an integral membrane transporter thought to be encoded by one of the members of the *PIN* gene family (Palme and Gälweiler, 1999). Both the AUX1 and the PIN proteins show an asymmetric localization in the plasma membrane that is consistent with a role in controlling the polarity of auxin movement (Gälweiler et al., 1998; Muller et al., 1998; Swarup et al., 2001; Friml et al., 2002). The second protein component of the auxin efflux carrier apparatus performs a regulatory function, and represents a high-affinity binding site for PATIs such as NPA (Rubery, 1990). Several studies indicate that this NPA-binding protein (NBP) is a peripheral membrane protein associated with the cytosolic face of the plasma membrane (Morris, 2000; Muday, 2000).

We have previously proposed a role for the auxin-inducible homeobox gene *Oshox1* of rice in the regulation of provascular cell fate specification (Scarpella et al., 2000). First, we have shown that *Oshox1* expression is switched on at a specific, critical stage of procambial development and that cells marked by *Oshox1* expression have been specified but not stably determined toward vascular differentiation. We have subsequently studied the effects of transgenic expression of *Oshox1* under control of the cauliflower mosaic virus 35S promoter on vascular development in the root. The 35S promoter drives *Oshox1* expression also in procambial cells close to the root tip, which in wild type do not yet express *Oshox1* and are still in an uncommitted stage of development. The result of ectopic *Oshox1* expression was that both cell fate specification and determination occurred closer to the root tip, as a consequence of which a premature vascular differentiation was observed. Also in the shoot, ectopic *Oshox1* expression similarly shifted vascular differentiation closer to the apex. Importantly, the premature vascular differentiation in the shoot and root appeared as an extremely subtle phenotype detected only by careful anatomical inspection, and occurred without any effect on morphology or growth characteristics. Therefore, the 35S-Oshox1 plants are particularly useful to investigate the mechanism underlying provascular cell fate commitment. A comparison of auxin physiology between wild type and 35S-Oshox1 showed that *Oshox1* overexpression is associated with a decreased sensitivity of PAT toward inhibition by NPA (Scarpella et al., 2000). We have proposed that this might represent an aspect of procambial cell fate commitment and that *Oshox1* might contribute to this process by stabilizing procambial cell fate toward endogenous modulations of PAT.

To test our hypothesis of *Oshox1* function, we have examined here the response of leaf vascular pattern formation under conditions of reduced PAT by inhibitors of three different classes in wild-type and 35S-Oshox1 seedlings, and found that both size and spacing of veins are under the control of PAT in wild-type rice and that *Oshox1* overexpression confers insensitivity to the vascular-patterning defects evoked by the different PATIs. Furthermore, here, we present evidence that direct effects of *Oshox1* overexpression are to reduce the affinity with which the NBP binds NPA and to enhance PAT and its sensitivity toward auxin. These results suggest that *Oshox1* might promote fate commitment in procambial cells by increasing their auxin conductivity properties and by simultaneously stabilizing this newly acquired state against modulations of PAT by an endogenous NPA-like molecule.

## RESULTS

### Effects of Different Classes of PATIs on Wild-Type Rice Seedling Development

It is possible to interfere with PAT through a number of chemically heterogeneous compounds collectively referred to as PATIs. Most of these PATIs belong to two groups, namely the phytotropins, exemplified by NPA, and the morphactins, typified by 2-chloro-9-hydroxyfluorene-9-carboxylic acid (HFCA). However, not all PATIs can be included in these two classes. The most common example is represented by 2,3,5-triiodobenzoic acid (TIBA).

We have previously studied rice seedlings overexpressing *Oshox1*, a proposed regulator of procambial cell fate commitment, and reported that both the processes of PAT and adventitious and lateral root development displayed reduced sensitivity toward the inhibitory effects of NPA (Scarpella et al., 2000). To test whether this property could be extended to other classes of PATIs or to other developmental processes, we decided to monitor the effects of different concentrations of the PATIs NPA, HFCA, and TIBA on wild-type and 35S-Oshox1 seedling development. Because the effects of these PATIs have not previously been studied in rice, we will first describe their influence on wild-type rice seedling development.

Germination of wild-type rice seedlings in the presence of PATIs resulted in pronounced and reproducible effects on their development. In all cases, the leaves of seedlings germinated on 5 or 10 $\mu$M of PATIs appeared shorter and narrower than the untreated ones. Furthermore, the leaves were frequently rolled and displayed different types and degrees of perturbations of the blade-sheath boundary, such as hypomorphism or complete absence of the ligule and displacement or absence of the auricles (e.g. Fig. 1, B and C). In addition to common effects of the different classes of PATIs, we could also recognize the presence of seemingly class-specific phenotypes. Seedlings germinated on 5 or 10 $\mu$M NPA showed dwarf and coiled shoots (Fig. 1, E and F).

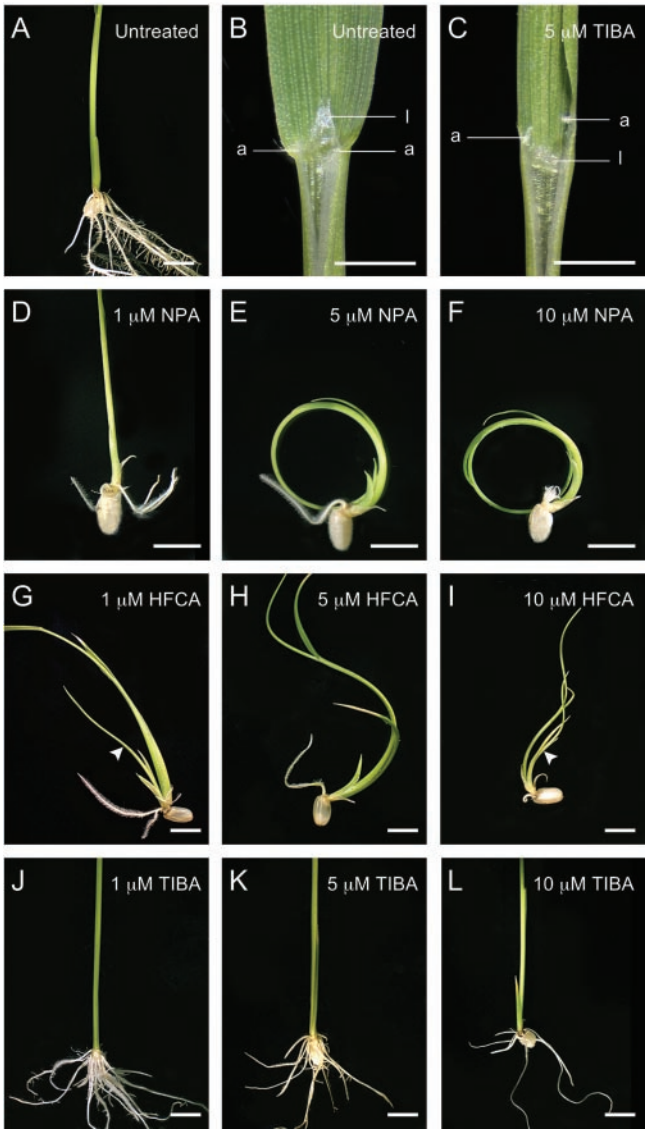


**Figure 1.** Effects of PATIs on wild-type rice seedling morphology. Seedlings germinated and grown for 2 weeks on medium without PATIs (A) or supplemented with different concentrations of PATIs (D–L). B and C, Detail of the fourth leaf of seedlings germinated and grown for 4 weeks on medium without PATIs (B) or supplemented with 5 $\mu$M TIBA (C). G and I, Arrowheads point at axillary shoots. a, Auricle; l, ligule. Scale bars in A and D through L = 5 mm; in B and C, scale bars = 1 mm.

Germination of wild-type seedlings on concentrations as low as 1 $\mu$M of HFCA induced shoot waving and premature development of axillary shoots (Fig. 1, G–I). The shoots of seedlings germinated on TIBA did not show obvious deviation from the untreated ones (Fig. 1, J–L). The roots of seedlings germinated on 1 or 5 $\mu$M NPA or HFCA were short and agravitropic (Fig. 1, D, E, G, and H), whereas those of seedlings germinated on 10 $\mu$M NPA or HFCA appeared stunted and swollen (Fig. 1, F and I). Furthermore, both of these PATIs caused a reduction of the number of adventitious and lateral roots (Fig. 2). Roots of seedlings germinated on TIBA showed only a slight agravitropism, and the effect on root elongation was less pronounced (Fig. 1, J–L). Furthermore, TIBA inhibited lateral root formation, but not adventitious root development (Fig. 2).

### Effects of PATIs on Vascular Pattern Formation in Wild-Type Rice Leaves

PATIs have been reported to have a strong impact on leaf vascular pattern formation in dicot plants (Mattsson et al., 1999; Sieburth, 1999). Therefore, we decided to investigate whether PATIs had a similar influence on leaf vascular tissue organization in rice, a monocot species. To address this question, the fourth leaf of untreated and PATI-germinated wild-

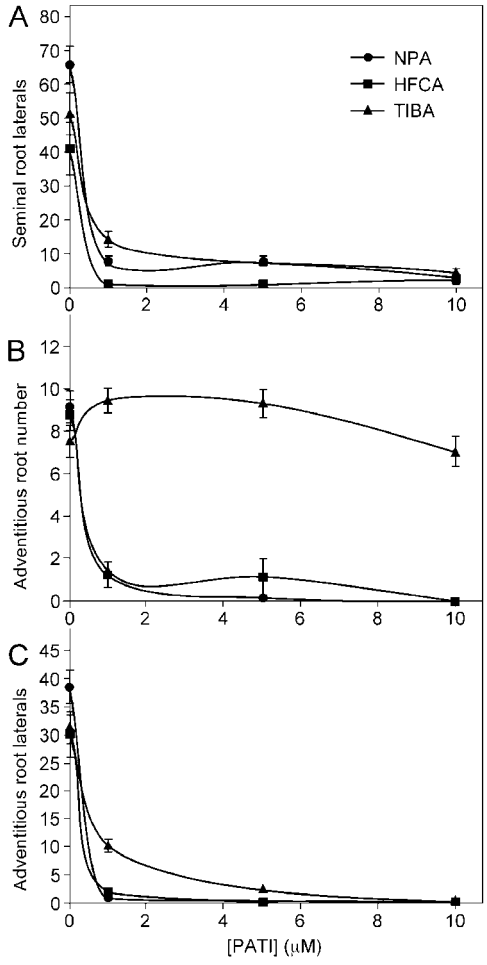


**Figure 2.** Effects of PATIs on root system complexity of wild-type rice. A, Number of lateral roots bore by the seminal root. B, Number of adventitious roots. C, Number of lateral roots bore by the adventitious roots. The results represent the mean ± SE of two separate experiments each performed on a population of $15 \leq n \leq 30$ seedlings per treatment. Difference between treated and untreated wild-type populations was determined by one-way ANOVA followed by Dunnett's test and was significant ($P < 0.001$) at all concentration points, except in B, where the TIBA-treated population was not significantly different from the untreated population.

type seedlings was cleared and inspected with dark-field illumination for the presence of vascular pattern alterations. In rice, the fourth leaf is the first one that is initiated postembryonically (Hoshikawa, 1993; E. Scarpella, S. Rueb, and A.H. Meijer, unpublished data). We chose this leaf for our analysis, because the position and width of procambial strands can only be affected before the emergence of these strands during leaf development, whereas the strands are insensitive to PATI-application once they are anatomically distinguishable (Mattsson et al., 1999). The fact that the fourth leaf of rice has not been initiated yet at the moment of PATI application excludes that our treatments might have been performed at a late and thus unresponsive stage of leaf development.

Wild-type rice leaves show the typical striate venation pattern, in which longitudinal veins of three orders, the midvein and the large and small veins, lie parallel along the proximo-distal axis of the leaf and are connected transversely by minor (commissural) veins (Kaufman, 1959; Fig. 3A). The distribution and arrangement of these classes of veins follow a highly regular pattern, which can be described by a series of venation pattern parameters, such as the distance between longitudinal or transverse veins and the number of large veins and that of small veins in between two adjacent large ones. Vein morphology in PATI-treated leaves deviated from that of the untreated ones in that longitudinal veins appeared thicker (Fig. 3B). This broadening of the vascular bundles often made it very difficult to unambiguously distinguish between small and large veins in the leaf. As a consequence, it became impossible to determine with certainty the number of large veins and that of small veins in between two adjacent large ones. However, PATI-treated rice leaves did show vascular tissue-patterning defects, in that at rising concentrations of PATIs, the distance between transverse veins decreased and that between longitudinal veins increased (Fig. 3, A and B). All three classes of PATIs showed qualitatively similar defects, but quantitative analysis of the leaf venation pattern parameters was restricted to NPA-treated leaves (Fig. 3, C and D).

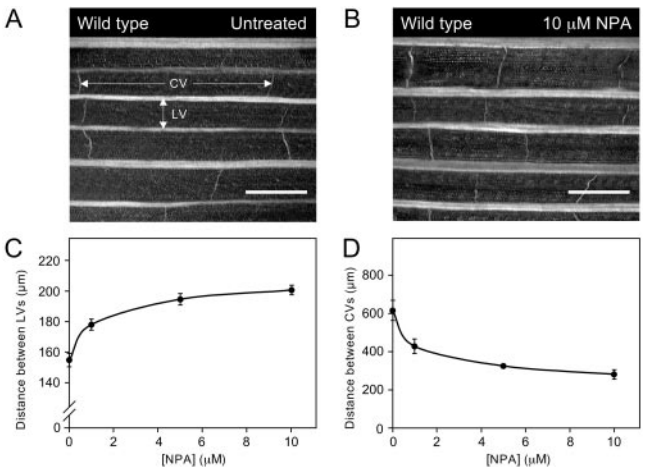


**Figure 3.** Effects of PATIs on the leaf vascular pattern of wild-type rice. A and B, Detail of the vascular pattern of the fourth leaf of seedlings germinated and grown for 4 weeks on medium without PATIs (A) or containing 10 $\mu$M NPA (B), as viewed with dark-field illumination after clearing. C and D, Effect of NPA concentration on the distance between longitudinal veins (LV; C) and on that between transverse commissural veins (CV; D). Arrows in A exemplify how the distance between each adjacent pair of LVs or CVs was measured in all experiments. The results represent the mean ± SE of two separate experiments each performed on a population of $15 \leq n \leq 30$ seedlings per treatment. Difference between treated and untreated wild-type populations was determined by one-way ANOVA followed by Dunnett's test and was significant ($P < 0.001$) at all concentration points. Scale bars = 200 $\mu$m.

### Effects of PATIs on Root Development and Leaf Vascular Patterning in 35S-Oshox1 Rice Seedlings

When germinated under the same conditions of PAT inhibition as for wild type, 35S-Oshox1 seedlings did not show any appreciable deviation from wild-type behavior as to shoot morphology, root elongation, and gravitropism (data not shown). However, significant differences between 35S-Oshox1 and wild type were observed when the effects of PATIs on root system complexity were evaluated. In all cases, adventitious as well as lateral root development in 35S-Oshox1 seedlings appeared more resistant to low concentrations of PATIs (Fig. 4). The venation pattern parameters of the fourth leaf of wild-type and 35S-Oshox1 seedlings germinated in the absence of PATIs were not significantly different (Fig. 5, C and D). Furthermore, when we assessed the effect of PATIs on vein morphology in 35S-Oshox1 seedlings, we could observe, similarly as in wild type, hypertrophy of longitudinal veins of all orders (Fig. 5, A and B). However, in contrast to wild type, the distance between longitudinal or transverse veins was not significantly altered in 35S-Oshox1 by germination on PATIs. Again, all three classes of PATIs induced qualitatively similar defects, but quantitative analysis of the leaf venation pattern parameters was restricted to NPA-treated leaves (Fig. 5, C and D).

### NPA-Binding Constants in 35S-Oshox1 Rice Seedlings and Suspension-Cultured Cells

PATIs of the phytotropin and morphactin classes have been reported to interfere with PAT through the binding to a membrane-localized NBP (Rubery, 1990). To determine whether the reduced NPA sensitivity displayed by 35S-Oshox1 seedlings was associated with changes in NBP-binding constants, NPA binding to microsomal membranes from wild-type and 35S-Oshox1 seedlings was assayed. Analysis of the NPA-binding data revealed that the affinity of the NBP toward NPA, described by the affinity constant

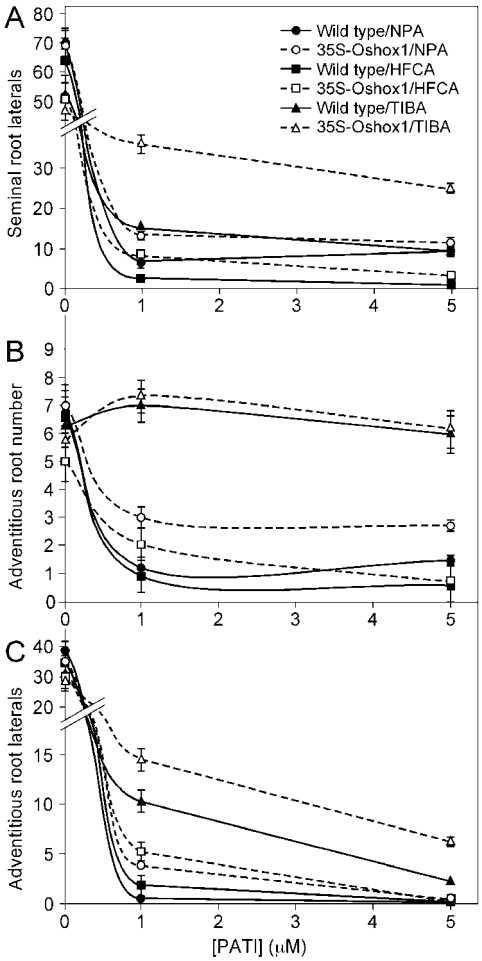


**Figure 4.** Effects of PATIs on root system complexity of wild-type and 35S-Oshox1 rice. A, Number of lateral roots bore by the seminal root. B, Number of adventitious roots. C, Number of lateral roots bore by the adventitious roots. Three independent 35S-Oshox1 lines were used in all experiments and considered as different genotypes in the subsequent statistical analysis. The results represent the mean ± SE of two separate experiments each performed on a population of $15 \leq n \leq 30$ seedlings per genotype and per treatment. Difference between wild-type and the three independent 35S-Oshox1 populations was determined by two-way ANOVA followed by Bonferroni's test. No significant difference in the behavior of the three different 35S-Oshox1 lines used was detected. Untreated wild-type and 35S-Oshox1 populations were not significantly different. Difference between PATI-treated wild-type and 35S-Oshox1 populations at the 1 μM concentration point was significant at $P < 0.001$ in all cases, except for TIBA treatment in B. At the 5 μM concentration point, difference was significant only for NPA treatment in B and for TIBA treatment in A and C.

$K_a$, was reduced in 35S-Oshox1 seedlings, whereas the number of NPA-binding sites (i.e. the amount of NBP), estimated by the $B_{max}$, was not significantly altered (Table I). However, it was possible that the reduced $K_a$ might have been, at least partly, a consequence of other aspects of the 35S-Oshox1 phenotype. In fact, 35S-Oshox1 seedlings display a premature vascular differentiation associated with reduced PAT capacity (Scarpella et al., 2000), both of which features might affect the NBP-binding constants (Suttle, 1991; Ruegger et al., 1997; Zhong and Ye, 2001). Therefore, to exclude any influence of overt vascular differentiation in our studies, we decided to study the effect of *Oshox1* overexpression in a rice cell suspension culture, where vascular differentiation does not occur (data not shown). Overexpression of *Oshox1* in a cell suspension system had no appreciable effect on cell morphology, nor induced vascular differentiation (data not shown), suggesting that *Oshox1* expression per se is not sufficient for vascular differentiation to occur. Although not affecting cell morphology, overexpression of *Oshox1* did alter functional properties of the suspension-cultured cells. Like in seedlings, *Oshox1* overexpression in a cell suspension system resulted in a reduced sensitivity toward NPA, as measured by the reduced amount of auxin that 35S-Oshox1 cells accumulate in the presence of NPA, when compared with the wild type (Fig. 6B). Furthermore, NPA-binding assays performed on microsomal membrane preparations from wild-type and 35S-Oshox1 cells confirmed that, as in

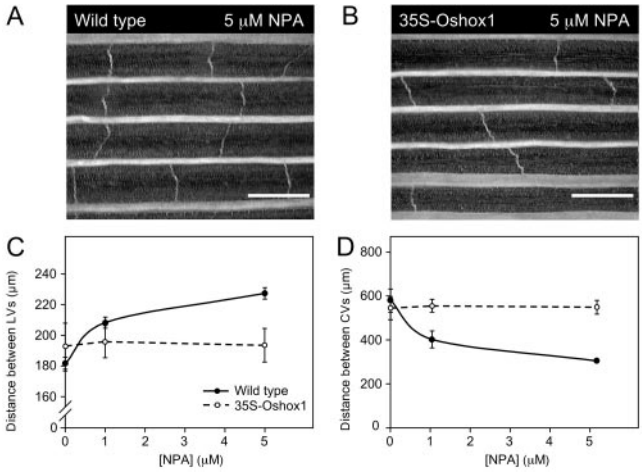


**Figure 5.** Effects of PATIs on the leaf vascular pattern of wild-type and 35S-Oshox1 rice. A and B, Detail of the vascular pattern of the fourth leaf of wild-type (A) and 35S-Oshox1 (B) seedlings germinated and grown for 4 weeks on medium containing 5 μM NPA, as viewed with dark-field illumination after clearing. C and D, Effect of NPA concentration on the distance between longitudinal veins (LV; C) and on that between transverse commissural veins (CV; D) in wild type and 35S-Oshox1. In all experiments, the distance between each adjacent pair of LVs or CVs was measured as illustrated in Figure 3A. Three independent 35S-Oshox1 lines were used in all experiments and were considered as different genotypes in the subsequent statistical analysis. Data represent the mean ± SE of two separate experiments each performed on a population of $15 \leq n \leq 30$ seedlings per genotype and per treatment. Difference between treated wild-type and 35S-Oshox1 populations was determined by two-way ANOVA followed by Bonferroni's test and was significant at $0.01 \leq P < 0.05$ at the 1 μM concentration point and at $0.001 \leq P < 0.01$ at the 5 μM concentration point in A, and significant at $P < 0.001$ at all concentration points in B. No significant difference in the behavior of the three different 35S-Oshox1 lines used was detected. Untreated wild-type and 35S-Oshox1 populations were not significantly different. Scale bars = 200 μm.

**Table I.** *Binding constants of the NBP in wild type and 35S-Oshox1*

The affinity constant $K_a$ corresponds to the inverse of the dissociation constant $K_d$ ($K_a = 1/K_d$). Three (seedling) or two (cell suspension) independent 35S-Oshox1 lines were used in all experiments and were considered as different genotypes in the subsequent statistical analysis. Data represent the mean ± SE of three experiments, each of which performed in duplicate. Asterisks indicate the significance of the difference between wild-type and 35S-Oshox1 populations as determined by two-way ANOVA followed by Bonferroni's test. No significant difference in the behavior of the two (cell suspension) or three (seedling) different 35S-Oshox1 lines used was detected. **, $0.001 \leq P < 0.01$.

| | Seedling | | Cell Suspension | |
|---|---|---|---|---|
| | Wild type | 35S-Oshox1 | Wild type | 35S-Oshox1 |
| $B_{max}$ (pmol mg$^{-1}$) | 2.88 ± 0.14 | 3.26 ± 0.16 | 0.55 ± 0.07 | 0.45 ± 0.06 |
| $K_a$ (nmol$^{-1}$ L) | 0.29 ± 0.01 | 0.08 ± 0.01** | 0.37 ± 0.04 | 0.19 ± 0.02** |

seedlings, the $K_a$ for NPA binding was reduced in 35S-Oshox1 cells, whereas the $B_{max}$ was not significantly affected (Table I).

### PAT Capacity in 35S-Oshox1 Rice Seedlings and Suspension-Cultured Cells

Because the cell suspension system conveniently allows evaluating the direct effects of *Oshox1* by uncoupling them from any secondary overt phenotypical effect, we decided to investigate whether other aspects of auxin transport physiology were altered by *Oshox1* overexpression in the rice cell suspension system. As in 35S-Oshox1 seedlings (Fig. 8A), no significant changes in auxin influx capacity were observed in 35S-Oshox1 cells, when compared with wild type (Fig. 6A). We previously reported that in roots of 10-d-old 35S-Oshox1 seedlings, PAT capacity was reduced and vascular differentiation occurred closer to the root tip (Scarpella et al., 2000). However, it was not possible to discriminate between cause and effect. Therefore, we decided to monitor auxin efflux capacity in wild-type and 35S-Oshox1 cells, where vascular differentiation does not occur. We unexpectedly observed a clear enhancement of auxin efflux in 35S-Oshox1 cells (Fig. 6C), suggesting that this is likely to be a direct effect of *Oshox1* and that the reduced PAT capacity that we previously measured in roots of 10-d-old 35S-Oshox1 seedlings is likely to be a consequence, rather than a cause, of the premature vascular differentiation. To confirm this interpretation in planta, we carefully monitored the course of vascular development in wild-type and 35S-Oshox1 roots during the first 10 d post-germination (dpg), to determine when exactly the premature vascular differentiation induced by *Oshox1* overexpression could be first anatomically detected. By means of confocal microscopy, we monitored the elongation of the procambial precursor of the central late metaxylem element, which is the first procambial cell in the root vascular cylinder that shows overt anatomical signs of an ongoing vascular differentiation process (Kawata et al., 1978; Scarpella et al., 2000). As inferred by the elongation of this xylem procambial precursor, we could detect a clear premature vascular differentiation in roots of 7-d-old 35S-Oshox1 seedlings (Fig. 7, C and D), whereas roots of 3-d-old 35S-Oshox1 seedlings were anatomically indistinguishable from wild type (Fig. 7, A and B). Because the 35S promoter is equally active in 3- and 7-d-old root tip regions (data not shown), we conclude that the premature vascular differentiation observed in 10-d-old 35S-Oshox1 roots cannot be interpreted as a direct consequence of the *Oshox1* ectopic expression. To evaluate the primary effect of *Oshox1* on PAT capacity and its sensitivity toward NPA inhibition, we therefore decided to measure these physiological parameters in the anatomically indistinguishable wild-type and 35S-Oshox1 3-d-old roots. Consistent with what observed in the cell suspension system, 35S-Oshox1 3-d-old roots showed an increased PAT capacity and a reduced sensitivity to the inhibitory effects of NPA, compared with wild type (Fig. 8, B and C).

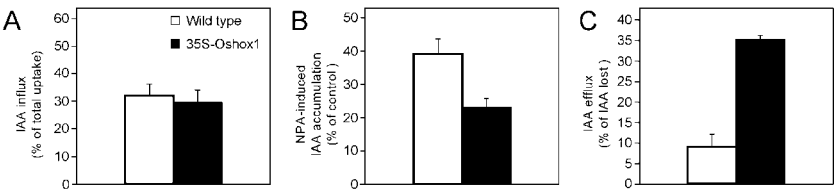


**Figure 6.** Auxin influx and efflux physiology in wild-type and 35S-Oshox1 rice suspension-cultured cells. A, Auxin influx. B, NPA-induced auxin accumulation. C, Auxin efflux. Two independent 35S-Oshox1 lines were used in all experiments and considered as different genotypes in the subsequent statistical analysis. Data represent the mean ± SE of six (A), five (B), or three (C) separate experiments. Difference between treated wild-type and 35S-Oshox1 populations was determined by two-way ANOVA followed by Bonferroni's test and was significant at $P < 0.001$ in B and C and not significant in A. No significant difference in the behavior of the two different 35S-Oshox1 lines used was detected.

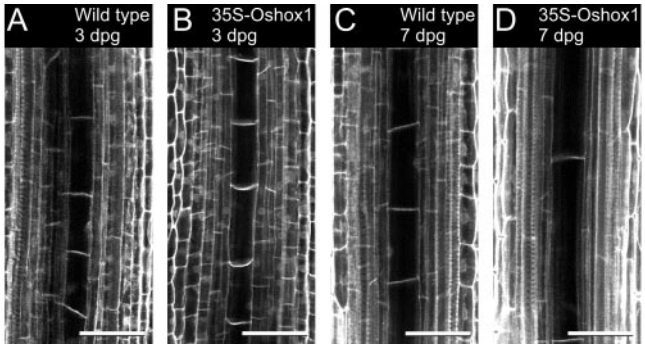


**Figure 7.** Vascular differentiation in the root of wild-type and 35S-Oshox1 seedlings 3 or 7 dpg. A through D, Detail of the vascular cylinder in the region between 261 and 488 $\mu$m below the root tip (root cap excluded) in a longitudinal confocal laser scanning microscopic optical section. In all panels, the cell file of the central late metaxylem precursor is positioned at the middle of the section. Three independent 35S-Oshox1 lines were used for anatomical inspection. Scale bar = 50 $\mu$m.

### Sensitivity of PAT toward Auxin in 35S-Oshox1 Rice Seedlings

PAT is autoregulated by the endogenous auxin level through its influence on the auxin efflux carrier (Hertel and Flory, 1968; Rayle et al., 1969; Goldsmith, 1977; Yoon and Kang, 1992). We have previously shown that adventitious and lateral root development in 35S-Oshox1 seedlings displayed increased auxin sensitivity, compared with wild type (Scarpella et al., 2000). To test whether *Oshox1* overexpression led to changes in the autoregulatory properties of PAT, we monitored the increase in PAT capacity that is induced by exogenous auxin in the anatomically indistinguishable wild-type and 35S-Oshox1 3-d-old roots. The data reported in Figure 8D show that whereas in wild-type, PAT was enhanced of approximately 40% by the presence of auxin, in 35S-Oshox1, the increase in PAT induced by auxin was of approximately 100%.

## DISCUSSION

### PAT Inhibition Evokes Specific Vascular Pattern Defects in Wild-Type Rice Leaves

A large number of classical studies (for review, see Sachs, 1981) together with more recent genetic and novel experimental evidence (for review, see Berleth et al., 2000) have associated the plant hormone auxin with vascular tissue development in dicot plants. However, the existence of such a link in monocot plants has always been rather speculative. Tsiantis et al. (1999) have recently shown that PAT inhibition induced hypertrophic vascular bundles in maize leaves, supporting the possible interplay between PAT and vascular development in a monocot species. Confirming and extending these findings, we have shown here that NPA, HFCA, and TIBA, PATIs of three different classes, not only induce vein hypertrophy in rice leaves but also evoke highly reproducible alterations in the pattern of vein spacing, including an increased distance between longitudinal veins and a reduced distance between transverse veins. These results indicate a role for PAT in restricting vascular tissue differentiation to narrow regions and regulating the distance between longitudinal and transverse veins.

Two recent studies have addressed the critical role of PAT in vascular patterning in dicot plants (Mattsson et al., 1999; Sieburth, 1999). Like we observed in rice, veins of PATI-treated dicot leaves also appeared hypertrophic. However, in rice, alterations in vein spacing were the only pattern defects, whereas in leaves of dicot seedlings, PATIs also induced vein interruptions or bifurcations, anomalously shaped veins, and regions of increased or ectopic vascularization along the margins (Mattsson et al., 1999; Sieburth, 1999). The differences between the response of dicot and rice leaf vascular patterns toward PAT inhibition could have several explanations. The reticulate venation patterns of dicot leaves and the striate patterns of monocot leaves are fundamentally different, both as to their eventual appearance in mature leaves and as to their ontogeny during leaf development (Nelson and Dengler, 1997). Therefore, it is possible that inherent differences in the mechanisms underlying vascular pattern formation in dicot and monocot leaves may lead to different phenotypical

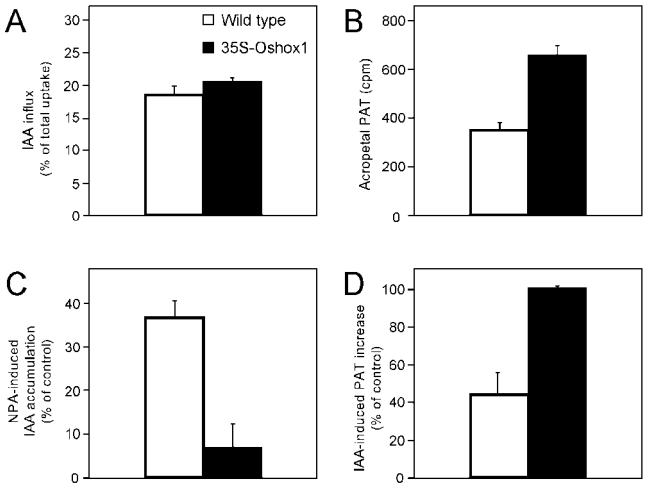


**Figure 8.** Auxin influx and PAT physiology in wild-type and 35S-Oshox1 roots of 3-d-old seedlings. A, Auxin influx. B, Acropetal PAT. C, NPA-induced auxin accumulation. D, Auxin-induced PAT increase. Three independent 35S-Oshox1 lines were used in all experiments and considered as different genotypes in the subsequent statistical analysis. Data represent the mean ± SE of two (A and D) or four (B and C) separate experiments each performed on $11 \leq n \leq 24$ (A–C) or $19 \leq n \leq 24$ (D) seedlings per genotype. Difference between treated wild-type and 35S-Oshox1 populations was determined by two-way ANOVA followed by Bonferroni's test and was significant at $P < 0.001$ in B through D and not significant in A. No significant difference in the behavior of the three different 35S-Oshox1 lines used was detected.

outcomes when leaves are elicited with similar treatments. Adding further to the relation between auxin and vein spacing in monocot leaves, we have recently identified an auxin-insensitive mutant of rice that displays opposite effects on the spacing of veins as those evoked by PATI treatment (E. Scarpella, S. Rueb, and A.H. Meijer, unpublished data).

An alternative explanation for the differences between the responses of leaf vascular pattern to PAT inhibition in dicots and in rice could be searched for in the reduced auxin sensitivity displayed by monocot leaves compared with dicot leaves (e.g. Wenicke et al., 1981; Schmidt and Willmitzer, 1988). As a consequence, ectopic accumulation of auxin attributable to treatment with PATI inhibitors would not induce similar responses in monocot and dicot tissues because of fundamental differences in auxin sensitivity. However, we do not favor this hypothesis, because the reduced auxin sensitivity displayed by rice leaves seems to be restricted to their mature stages. In fact, immature rice leaves can be induced to callus formation by the very same auxin treatments that prove to be ineffective on mature leaves (Wenicke et al., 1981). In our study, embryos were directly germinated in the presence of PATIs and our evaluation was based on analysis of a leaf that is initiated completely postembryonically. Therefore, in our experimental design PATIs are expected to be able to affect any process in leaf development that is sensitive to inhibition of PAT.

In addition to vascular tissue development, PAT has been shown to be essential for other aspects of dicot plant development, such as adventitious shoot and root formation, lateral root development, root elongation, and gravitropic response (e.g. Lomax et al., 1995). We have confirmed here that in rice, these developmental processes also are dependent on PAT. However, not all of these processes seem to be equally sensitive to different classes of PATIs. Variable effects were observed on adventitious shoot development, adventitious root development, and shoot morphogenesis. On the other hand, lateral root development, leaf proximo-distal polarity acquisition, and vascular tissue pattern formation seem to be equally affected by all classes of PATIs. This suggests that these latter developmental parameters, because they are independent of the chemical nature of the PATI, might represent more bona fide phenotypical indicators of specific PAT alterations.

### *Oshox1* Confers Resistance toward the Effects of PAT Inhibition on Lateral and Adventitious Root Development and on Leaf Vascular Pattern Formation

We previously reported that seedlings overexpressing the rice homeobox gene *Oshox1*, a proposed regulator of procambial cell fate commitment, display reduced sensitivity to the inhibitory effects of NPA on the acropetal component of PAT in the root and on lateral root development (Scarpella et al., 2000), which itself specifically depends on acropetal PAT (Reed et al., 1998). Root elongation and gravitropic response, both dependent on basipetal PAT (Rashotte et al., 2000), conversely displayed wild-type NPA sensitivity in the 35S-Oshox1 seedlings. We have here extended our previous observations showing that in 35S-Oshox1 seedlings lateral and adventitious root development is less sensitive to inhibition by different classes of PATIs. Furthermore, we have shown that in 35S-Oshox1 seedlings, leaf vascular pattern is insensitive to PATI treatment. PAT-competent cells are localized in the vascular tissues of the shoot, and the cells of the vascular cylinder of the root are those responsible for the acropetal component of PAT (Lomax et al., 1995, and refs. therein). Taken together, these observations suggest that *Oshox1* overexpression seems to confer resistance to PAT toward inhibition only as to the specific component of it that takes place in the vascular tissues.

### *Oshox1* Reduces the Affinity of the NBP toward NPA

We have shown here for the first time, to our knowledge, that alterations in the expression level of a gene, *Oshox1*, can affect the affinity with which the NBP binds NPA in seedlings, without any effect on the abundance of the NBP itself. Genetic evidence from the Arabidopsis mutants *tir3* and *ifl1* has associated aberrant vascular tissue development and reduced PAT capacity with reduced number of NPA-binding sites (Ruegger et al., 1997; Zhong and Ye, 2001). Furthermore, physiological studies have also provided evidence for an association among vascular tissue development, PAT capacity, and NPA-binding activity (Suttle, 1991). To exclude that the reduced NPA-binding affinity measured in 35S-Oshox1 seedlings was not, at least partly, a consequence of the premature vascular differentiation and associated decreased PAT capacity that we observed in 10-d-old roots of these seedlings (Scarpella et al., 2000), we analyzed the effect of *Oshox1* overexpression in suspension-cultured cells and found that also here, NPA-binding affinity was reduced without any effect on the number of NPA-binding sites. This suggests that the reduced affinity of the NBP toward NPA displayed by 35S-Oshox1 seedlings and isolated cells is a direct effect of the *Oshox1* overexpression and not a consequence of the altered vascular development and PAT capacity observed in seedlings.

The reduced affinity of the NBP toward NPA is the likely cause of the PATI-resistance of *Oshox1*-overexpressing seedlings and suspension-cultured cells. However, the NBP has been reported to specifically interact with PATIs of the phytotropin and morphactin classes but not with TIBA (Rubery, 1990; E. Scarpella, K.J.M. Boot, S. Rueb, and A.H. Meijer, unpublished data). The present model for the action of TIBA on PAT inhibition predicts that this PATI binds directly to the efflux carrier rather than to the NBP (Rubery, 1990). In view of this, it is difficult to

explain how a reduced affinity of the NBP toward NPA may account for the increased resistance to TIBA that the 35S-Oshox1 seedlings display. However, a change in the number of NPA-binding sites as well as in their affinity to NPA has been previously reported to increase resistance also to TIBA (Suttle, 1991).

Specific inhibition of PAT correlates with the high-affinity binding of phytotropins and morphactins to the NBP (for review, see Rubery, 1990; Lomax et al., 1995; Muday, 2000). However, additional lower affinity NPA-binding activities have been found in Arabidopsis seedlings (Jensen et al., 1998; Murphy and Taiz, 1999; Murphy et al., 2000). Several proteins have been recently purified by NPA-affinity chromatography from membrane fractions derived from Arabidopsis seedlings (Murphy et al., 2000, 2002; Noh et al., 2001). These proteins fall into the lower affinity category, and some of them show some similarities with proteins involved in mammalian actin-dependent vesicular cycling (Muday and Murphy, 2002). Two other recent studies strongly suggest interplay between PATIs and actin-dependent vesicular cycling of the putative auxin efflux carrier protein PIN1 in the regulation of PAT in Arabidopsis (Geldner et al., 2001; Gil et al., 2001; for review, see Muday and Murphy, 2002). Treatments with brefeldin A (BFA), an inhibitor of intracellular vesicle movement, result in mislocalization of the PIN1 protein, and high concentrations of PATIs prevent the BFA-induced PIN1 mislocalization or the recovery of PIN1 polar subcellular localization after BFA removal (Geldner et al., 2001). *Oshox1* seems not to be an integrated component of such a regulatory pathway, in that its overexpression does not alter the wild-type sensitivity toward the inhibitory effects of BFA on lateral and adventitious root development (data not shown). Furthermore, the differences in sensitivity to PAT inhibition that we observed between wild type and 35S-Oshox1 were evident at concentrations of NPA that are not sufficient to prevent the BFA-induced mislocalization of the PIN1 protein or the recovery of its localization after BFA removal. Therefore, consistently with the measured $K_a$ values for NPA binding in wild-type and 35S-Oshox1, both falling in the high-affinity range, we conclude that *Oshox1* specifically interferes with the action of a high-affinity NBP, the identity of which at present remains still unknown.

### *Oshox1* Enhances PAT and Its Sensitivity toward Auxin

We previously reported that 10-d-old 35S-Oshox1 roots showed reduced PAT capacity associated with premature vascular differentiation, but we were unable to discriminate between cause and effect (Scarpella et al., 2000). To address this question, we have studied here the effect of *Oshox1* on auxin efflux in suspension-cultured rice cells, where *Oshox1* overexpression does not lead to any overt morphological changes. In 35S-Oshox1 cells, auxin efflux was unexpectedly enhanced compared with wild-type. To solve the apparent contradiction with our previous report, we monitored the course of vascular tissue differentiation in wild-type and 35S-Oshox1 roots and observed that the premature vascular differentiation could be unambiguously observed in 7-d-old 35S-Oshox1 roots but not in 3-d-old roots. Direct PAT measurements on 3-d-old roots confirmed the conclusion from the cell suspension experiments that *Oshox1* overexpression confers enhanced PAT capacity in the absence of anatomical differences with wild type. Furthermore, as already observed in older seedlings, PAT was more resistant toward NPA inhibition in 3-d-old 35S-Oshox1 roots.

Flavonoids have long been proposed as endogenous negative regulators of PAT (Jacobs and Rubery, 1988; Rubery, 1990), a hypothesis recently substantiated by observations that the *transparent testa4* (*tt4*) mutant of Arabidopsis, which is completely devoid of flavonoids, displays enhanced PAT while retaining wild-type NPA-binding constants (Brown et al., 2001). Because *Oshox1* overexpression directly affects the affinity of the NBP toward NPA, it is conceivable that this gene might reduce sensitivity of the NBP toward an endogenous NPA-like molecule, which could be the primary cause of PAT elevation in 35S-Oshox1 3-d-old roots. However, it still remains to be explained how a transcription factor, such as *Oshox1*, may modify the affinity of a receptor for a ligand without directly affecting the abundance of such receptor. A tentative explanation could be that *Oshox1* regulates the transcription of a protein that modulates the binding properties of the NBP. Physiological and genetic evidence support a role for reversible protein phosphorylation in the regulation of PAT and its sensitivity toward NPA (for review, see Muday and DeLong, 2001; DeLong et al., 2002). The transcriptional regulation of genes encoding phosphatase or kinase activities might thus represent a possible mechanism through which *Oshox1* could induce changes in the affinity of the NBP toward NPA.

We previously reported that *Oshox1* overexpression confers enhanced sensitivity to the effect of exogenous auxin on adventitious and lateral root development (Scarpella et al., 2000). It is possible that this hypersensitivity may be directly attributable to an increased sensitivity of PAT toward auxin, because low concentrations of polarly transported auxins have been reported to have a positive effect on PAT (Hertel and Flory, 1968; Rayle et al., 1969; Goldsmith, 1977; Yoon and Kang, 1992). In the 3-d-old 35S-Oshox1 roots, which are anatomically indistinguishable from wild type, PAT shows increased sensitivity to exogenous auxin. Therefore, *Oshox1* seems to increase the sensitivity of the PAT machinery toward auxin signals. It is possible that this increased sensitivity of PAT toward auxin might represent one of the primary causes for the increased PAT capacity

of these roots. Furthermore, it is conceivable that the same pathway that leads to the increase in sensitivity of PAT toward auxin signals might be interconnected at the biochemical level with that eventually resulting in the reduction of the affinity of the NPB toward NPA or an endogenous PATI.

### A Model for the Action of *Oshox1* in Procambial Cell Fate Commitment

One direct consequence of the altered expression of *Oshox1* seems to be the reinforcement of the auxin conductivity properties of procambial cells, possibly accomplished through an increased sensitivity of the PAT machinery toward auxin signals. The possibility that changes in auxin conductivity may underlie procambial cell fate commitment is consistent with the fact that one of the earliest detectable aspects of vascular tissue development is the enhanced PAT capacity of incipient vascular cells, which is measurable before any overt anatomical sign of vascular differentiation (Gersani and Sachs, 1984). An additional direct consequence of *Oshox1* overexpression is the decreased sensitivity of PAT toward inhibition. This effect can be conceivably ascribed to a reduced affinity of the NBP toward an endogenous NPA-like molecule. The existence of an NPA-insensitive auxin efflux carrier was previously postulated by Mattsson et al. (1999) to tentatively explain the unresponsiveness of anatomically recognizable procambial strands to PAT inhibition. Taken together, these observations prompt us to propose here that *Oshox1* would mediate the acquisition of a cell state in procambium commitment that is associated with an increase in auxin conductivity properties. Through its effect on the affinity with which the NBP binds an endogenous PATI, *Oshox1* would simultaneously stabilize this newly acquired cell state by reducing the sensitivity of the PAT machinery toward inhibition.

The importance of the proposed role of *Oshox1* in stabilizing a particular cell state acquired during procambial cell fate commitment can be appreciated when considering the high degree of flexibility of vascular tissues under both experimentally manipulated conditions and during the undisturbed course of development (e.g. Sachs, 1975, 1981). Reorientation of the course of vascular strands has been shown to be preceded by changes in the polarity of auxin transport (Gersani and Sachs, 1984). Because of the relative ease with which a previously established polarity of auxin transport can be reoriented in response to external stimuli or internal signals, it thus seems that once a certain polarity of auxin transport has been established, this needs to be stably maintained (at least until irreversible differentiation has been achieved) to eventually originate a coherent and consistent pattern of vascular tissues. The stable maintenance is particularly important during procambium early development, which displays features of high sensitivity to PAT inhibition (Mattsson et al., 1999).

From our hypothesis for the action of *Oshox1* proposed above, it follows that in 35S-Oshox1 seedlings, as a consequence of enhanced PAT and reduced NPA sensitivity, procambial cells would enter a phase of cell fate stabilization (commitment) earlier (i.e. closer to the meristem) than in wild type. This would eventually result in a premature vascular differentiation, which would consequently reduce the PAT capacity of the tissues, as we previously explained (Scarpella et al., 2000). However, altered *Oshox1* expression per se is not sufficient to result in premature or ectopic vascular differentiation, as shown by the 35S-Oshox1 cell suspension system discussed above and by analysis of 35S-Oshox1 embryos (E. Scarpella, S. Rueb, and A.H. Meijer, unpublished data). The premature vascular differentiation in 35S-Oshox1 seedlings could therefore more likely be attributable to the action of genes that are specifically expressed in the seedling vasculature and not in suspension-cultured cells, and the expression of which is not under the direct control of *Oshox1* gene activity. These vascular differentiation-specific genes, one example of which is the *TED3* gene of zinnia (*Zinnia elegans*; Demura and Fukuda, 1994) are plausibly active only postembryonically, in that vascular differentiation does not take place during wild-type embryogenesis.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Rice (*Oryza sativa* L. Japonica cv Taipei 309), in which the 35S-Oshox1 transgene was introduced (Scarpella et al., 2000), was used as a wild-type control strain in all studies. As an additional control, a 35S-$\beta$-glucuronidase transgenic line (Scarpella et al., 2000) was used in all experiments to monitor the possible effects of hygromycin, which was used to select the 35S-Oshox1 transgenics, on the measured parameters. Because, in all cases, we could not find any significant difference between wild-type and 35S-$\beta$-glucuronidase populations (data not shown), only the data from the comparison between wild-type and 35S-Oshox1 populations are presented. We have previously reported the characterization of nine independent 35S-Oshox1 transgenic lines and shown that we could detect identical phenotypes in all of them. Among these nine lines, we have selected two (cell suspension studies) or three (all other analyses) of them for the detailed characterization described here. All seeds were surface sterilized (Rueb et al., 1994) and germinated in the dark at 28°C for 4 d on described medium (Scarpella et al., 2000) supplemented with 75 mg $L^{-1}$ hygromycin in the case of transgenics and with or without different aliquots of filter-sterilized aqueous stocks of NPA (Supelco, Bellefonte, PA), HFCA (Riedel-De Haën, Seelze, Germany) or TIBA (Sigma-Aldrich, St. Louis). Germinated seeds were subsequently grown at a 12-h-light/12-h-dark cycle at 28°C for 2 weeks, or 4 weeks in the case of vascular pattern parameters measurements, after which the responses to the different growth conditions were evaluated. Containers were placed vertically under an angle of approximately 30° during both the germination and growth phases. All suspension-cultured cells were initiated simultaneously from scutellum-derived calli (Rueb et al., 1994) and grown on AA medium (Muller and Grafe, 1978) containing 4 mg $L^{-1}$ naphthalene-1-acetic acid (BDH, Poole, Dorset, UK) and 2 mg $L^{-1}$ kinetin (Research Organics, Cleveland) at 28°C in the dark on a gyratory shaker at 120 rpm. Stock suspensions were subcultured every 7 d at an initial density of approximately 30 mg $mL^{-1}$. For all experiments, 7-d-old suspensions at an approximate density of 100 mg $mL^{-1}$ were used.

### Microtechniques and Microscopy

Seedling morphology was monitored with a stereoscopic microscope (MZ12, Leica, Wetzlar, Germany). Whole-mount cleared preparations of the

fourth leaf, which in rice is the first leaf that is initiated postembryonically, were obtained by autoclaving dissected samples in 80% (w/w) lactic acid for 20 min at 121°C. Samples were mounted in fresh 80% (w/w) lactic acid and viewed with an Axioplan 2 Imaging microscope (Zeiss, Welwyn Garden City, UK) using dark-field optics settings. Roots of wild-type and 35S-Oshox1 etiolated seedlings at 3 or 7 dpg were dissected and treated according to a modified version of the procedure described by Braselton et al. (1996). Roots were fixed for 1 h in ethanol:acetic acid (3:1, v/v) and, after rehydration, carbohydrates were hydrolyzed by a 15-min treatment with 5 N HCl and followed by an incubation of 2.5 h with Schiff's reagent (Sigma-Aldrich). After two washing steps with cold water of 15 min each, samples were dehydrated, infiltrated with LR White resin (London Resin, Theale, Berkshire, UK) and embedded directly on microscope slides. Samples were observed with a Zeiss Axioplan microscope equipped with a confocal laser scanning unit (MRC 1024 ES, Bio-Rad, Hercules, CA) using a 488- and a 512-nm excitation line and a 585-nm LP barrier emission filter. Microscopic images were acquired with a 3CCD digital photo camera (DKC-5000, Sony, Tokyo). All images were processed using Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA). Morphometric analysis of vascular pattern parameters was performed on digital pictures using the ImageJ 1.21 software.

## NPA-Binding Assays

Microsomal membranes were isolated from 10-d-old etiolated wild-type and 35S-Oshox1 seedlings or from cells at 7 d after subculturing. Cells were harvested by filtration under vacuum over a 30-mesh nylon cloth. Approximately 2 to 4 g of cells or whole seedlings were ground in liquid nitrogen and resuspended in cold microsome-isolation buffer (50 mM Tris-HCl, pH 8.0, 250 mM Suc, 0.1 mM $MgCl_2$, and 1 mM EDTA) to which 10 mM ascorbic acid and 1 mM dithiothreitol were freshly added. Samples were homogenized in a Potter S homogenizer (B. Braun, Melsungen, Germany) with 10 strokes at 1,500 rpm, and the homogenate was filtered over four layers of cheesecloth. The filtrate was centrifuged at 10,000 rpm for 20 min, and the supernatant was centrifuged at 35,000 rpm for 45 min. The pellet was resuspended in NPA-binding buffer (10 mM citrate-acetic acid buffer, pH 5.5, 250 mM Suc, and 5 mM $MgCl_2$), homogenized as described above and directly used for protein quantification. The whole isolation procedure was performed at 4°C. Binding assays were performed in 1 mL total volume of NPA-binding buffer with a final protein concentration of 0.1 to 0.2 mg $mL^{-1}$. Microsomes were incubated on ice in the dark for 1 h with $10^{-9}$ M [2,3,4,5(*n*)-$^3$H]NPA (58 Ci $mmol^{-1}$; Moravek Biochemicals, Brea, CA) and unlabeled NPA concentrations ranging from $10^{-10}$ to $10^{-3}$ M. Samples were subsequently filtrated under vacuum over GF/C glass microfiber filters (Whatman, Maidstone, Kent, UK) with a 1225 sampling manifold (Millipore, Bedford, MA) apparatus. Filters were washed with cold water and radioactivity was measured with a liquid scintillation counter (1214 Rackbeta, LKB-Wallac, Turku, Finland). Initial estimations for the $K_a$ and $B_{max}$ were done on Scatchard plots (Scatchard, 1949) obtained from the transformation of the displacement plots and used in the Ligand program (Munson and Rodbard, 1980) to determine the precise binding constants.

## Auxin Influx and Efflux Assays

For auxin influx experiments, contribution of carrier-mediated indole-3-acetic acid (IAA) uptake ($IAA_{carrier}$) was deduced by subtracting the accumulation of [5(n)-$^3$H]IAA ([$^3$H]IAA; Amersham, Little Chalfont, Buckinghamshire, UK) measured in the presence of $10^{-4}$ M unlabeled IAA (Sigma), i.e. the non-saturable IAA uptake ($IAA_{diffusion}$), from that measured in the absence of unlabeled IAA (total IAA uptake: $IAA_{total}$): $IAA_{carrier} = IAA_{total} - IAA_{diffusion}$. For simplification, we assume that the non-saturable component of the IAA uptake corresponds to the diffusion of IAA through the membrane, although it probably also includes the [$^3$H]IAA accumulated in the small volume of medium entrapped inside the cell pellet (Delbarre et al., 1996). Values were normalized to the total IAA uptake: $IAA_{influx} = (IAA_{carrier}/IAA_{total}) \times 100\%$. Auxin influx experiments in roots of 3-dpg wild-type and 35S-Oshox1 etiolated seedlings were performed essentially as described for the PAT measurements (Scarpella et al., 2000). The most distal 2 cm of the seminal roots were excised and the most basal part of them (approximately 5 mm) was placed in agar blocks containing $10^{-7}$ M [$^3$H]IAA, in the presence or absence of $10^{-4}$ M unlabeled IAA, and incubated in the dark at room temperature for 30 min. To prevent the tissues from drying, they were overlaid with silicon oil. After incubation, roots were cut into two segments: a basal segment (5 mm), which was that enclosed in the agar block, and an apical one (15 mm). Radioactivity in the basal segment was measured with a liquid scintillation counter. To determine auxin influx in cells, these were filtered over a 30-mesh nylon cloth, washed twice with cold water, resuspended in the same volume of hormone-free AA medium supplemented with 50 mM MES (pH 5.5), and incubated at 28°C for 20 min. [$^3$H]IAA ($10^{-8}$ M) was subsequently added in the presence or absence of $10^{-4}$ M unlabeled IAA or $10^{-6}$ M NPA. Duplicate samples (0.25 mL) were taken 15 min after the addition of [$^3$H]IAA, when equilibrium had been reached. Samples were filtered over GF/C filters, and radioactivity in the cells was measured with a liquid scintillation counter. NPA-induced IAA accumulation was expressed as the relative increase in total [$^3$H]IAA uptake in the presence of $10^{-6}$ M NPA at equilibrium. For auxin efflux experiments, cells were harvested, resuspended, and incubated in hormone-free AA medium supplemented with 50 mM MES (pH 5.5) as described above. [$^3$H]IAA ($10^{-8}$ M) was subsequently added, and cells were incubated at 28°C for 15 min. Duplicate samples (0.5 mL each) were taken at this point (time 0) and filtered over GF/C filters, and radioactivity in the cells was measured with a liquid scintillation counter ($IAA_{t=0}$). The remaining cells were filtered over GF/C filters and resuspended in 50 mM MES-buffered medium (pH 5.5) without [$^3$H]IAA. Duplicate samples were taken 3 min after the transfer to this medium and filtered over GF/C filters, and radioactivity in the cells was measured with a liquid scintillation counter ($IAA_{t=3}$). Auxin efflux was expressed as the percentage of radioactivity lost by the cells at the 3-min sampling point with respect to the radioactivity present in the cells at time 0: $([IAA_{t=0} - IAA_{t=3}]/IAA_{t=0}) \times 100\%$.

## PAT Assays

Acropetal PAT and NPA-induced IAA accumulation were measured in roots of 3-dpg wild-type and 35S-Oshox1 etiolated seedlings, as described (Scarpella et al., 2000). IAA-induced stimulation of PAT was measured by performing the PAT measurements in the presence or absence of $10^{-6}$ M unlabeled IAA and reducing the incubation time to 30 min and was expressed as the relative increase in PAT measured in the presence of the unlabeled IAA.

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