Multiple Regulatory Elements Contribute to the Vascular-specific Expression of the Rice HD-Zip Gene Oshox1 in Arabidopsis

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The primary vascular tissues of plants differentiate from a single precursor tissue, the procambium. The role of upstream regulatory sequences in the transcriptional control of early vascular-specific gene expression is largely unknown. The onset of expression of the rice homeodomain-leucine zipper (HD-Zip) gene Oshox1 marks procambial cells that have acquired their distinctive anatomical features but do not yet display any overt signs of terminal vascular differentiation. The expression pattern of Oshox1 in rice appears to be mainly controlled by the activity of the 1.6 kb upstream promoter region. Here, we show that the Oshox1 promoter directs vascular, auxin- and sucrose-responsive reporter gene expression in Arabidopsis plants in a fashion comparable with that in rice. This is the case not only during normal development but also upon experimental manipulation, suggesting that the cis-acting regulatory elements that are instrumental in Oshox1 expression pattern are conserved between rice and Arabidopsis. Finally, through analysis of reporter gene expression profiles conferred by progressive 5' deletions of the Oshox1 promoter in transgenic Arabidopsis, we have identified upstream regulatory regions required for auxin and sucrose inducibility, and for cell type-, tissue- and organ-specific aspects of Oshox1 expression. Our study suggests that Oshox1 embryonic vascular expression is mainly achieved through suppression of expression in non-vascular tissues.

Keywords: Arabidopsis — Cis-elements — Procambium — Rice — Transcriptional regulation — Vascular marker.

Abbreviations: DAG, d after germination; GM, ground meristem; GUS, β-glucuronidase; HD-Zip, homeodomain-leucine zipper; NAA, naphthalene-1-acetic acid; β-NAA, naphthalene-2-acetic acid.

Introduction

create possibility to approach this issue experimentally. In all rice organs and at all developmental stages of the rice plant, *Oshox1* is first expressed in procambial cells that have already acquired their distinctive anatomical properties, but that have not yet entered terminal differentiation pathways (Scarpella et al. 2000). Additionally, *Oshox1* expression in rice is responsive to auxin and sucrose, and all these features are largely controlled by the activity of its own promoter (Scarpella et al. 2000).

In this study, we show that the *Oshox1* promoter directs reporter gene expression in *Arabidopsis* during the undisturbed course of development and under experimentally challenged conditions in a fashion comparable with that reported in rice. Further, we show that the *Oshox1* promoter confers auxin and sucrose responsiveness to reporter gene expression in *Arabidopsis*. Finally, by means of progressive 5′ deletions, we show that the *Oshox1* promoter has a complex and modular structure. Our study suggests that *Oshox1* embryonic vascular expression is mainly attained through suppression of expression in non-vascular tissues.

**Results**

*Oshox1* promoter-driven expression pattern in *Arabidopsis*

To investigate whether the cis-elements that are instrumental for the vascular-specific expression of the rice HD-Zip gene *Oshox1* are conserved in a dicot species, we analyzed in *Arabidopsis* the expression pattern of the β-glucuronidase (GUS) reporter gene when driven by the 1.6 kb *Oshox1* promoter fragment (*Oshox1*-GUS).

In post-embryonic root tips of *Arabidopsis* seedlings harboring the *Oshox1*-GUS transgene, reporter gene activity was detected in the central vascular cylinder with the exclusion of its most distal part (Fig. 1A, 6R). In mature regions of the root, *Oshox1*-GUS expression remained confined to the vascular cylinder, and expression was never detected in cortex or epidermis (Fig. 1B). *Oshox1*-GUS was expressed in all the cell types of the vascular cylinder, including the pericycle (Fig. 1C). In seedling hypocotyls, *Oshox1*-GUS expression was detected in all the cell types of the vascular cylinder, including the pericycle (Fig. 1D, E). In leaves, expression was detected in procambial strands before any detectable signs of cell wall lignification (Fig. 1I, J), and persisted in all the cell types of the completely differentiated vascular strands of mature leaves, including bundle sheath cells (Fig. 1K, L). Further, *Oshox1*-GUS expression was detected in leaf trichomes (Fig. 6X). In the basal part of the stems of mature plants, *Oshox1*-GUS expression was detected in the interfascicular and vascular cambium, and in the phloem (Fig. 1G, H). In hypocotyls of plants at the same stage, expression was detected in the cambial zone and in the phloem (Fig. 1F). *Oshox1*-GUS expression was detected in immature vascular strands of floral buds (Fig. 1M), and expression continued to be present in the completely differentiated vascular strands of mature flowers (Fig. 1N). In siliques, expression was restricted to vascular strands (Fig. 1O). Finally, in mature embryos, *Oshox1*-GUS expression was observed in procambial strands of the cotyledons, but not in the procambial cylinder of the embryonic axis (Fig. 1P).

Unlike in rice, *Oshox1*-GUS was not expressed in stomata or pollen of *Arabidopsis* (Fig. 1D, I–K, N).

In rice, *Oshox1* is ectopically induced in non-vascular cells of the root tip in response to auxin and sucrose (Scarpella et al. 2000). To test whether the cis-elements that are involved in auxin- and sucrose-regulated *Oshox1* gene expression are conserved in a dicot species, we tested *Oshox1*-GUS inducibility by auxin and sucrose in *Arabidopsis*. *Oshox1*-GUS was ectopically induced in non-vascular cells of the *Arabidopsis* root tip by naphthalene-1-acetic acid (NAA) (Fig. 1Q), IAA and 2,4-D, but not by naphthalene-2-acetic acid (β-NAA) (an inactive auxin analog). A similar *Oshox1*-GUS induction pattern was observed in the presence of 500 mM sucrose (Fig. 1R), but not upon a 500 mM mannitol treatment, thus excluding an induction due to osmotic stress.

In conclusion, the *Oshox1* promoter is able to drive vascular, auxin- and sucrose-responsive reporter gene expression in transgenic *Arabidopsis*.

*Oshox1*-GUS expression in *Arabidopsis* leaf development

To examine the dynamics of the *Oshox1*-GUS expression pattern, and to compare them with those of procambium formation, we simultaneously analyzed *Oshox1*-GUS and ET1335-GUS expression in *Arabidopsis* first leaf development. At all stages of leaf development, the onset of ET1335-GUS expression coincides precisely with overt procambium formation, and remains strong in fully differentiated vascular strands (Scarpella et al. 2004).

At 2 d after germination (DAG), the first two vegetative leaf primordia were recognizable as semi-spherical bulges flanking the shoot apical meristem (Fig. 2A, B). ET1335-GUS expression marking the formation of the primary procambial strand became visible in the elongated primordia at 3 DAG (Fig. 2C). At 4 DAG, lamina formation was initiated, and ET1335-GUS expression was additionally, but transiently, detected in ground meristem (GM) cells at the leaf tip (compare Fig. 2E and I). At this stage, *Oshox1*-GUS expression was first detected in GM cells at the leaf tip (Fig. 2F). ET1335-GUS expression labeling the appearance of the first loops of secondary procambial strands was detected at 5 DAG (Fig. 2G). *Oshox1*-GUS expression was detected in the distal portion of the primary procambial strand in 5 DAG primordia (Fig. 2H). At 6 DAG, ET1335-GUS expression marked the formation of the second loops of secondary procambial strands (Fig. 2I), and *Oshox1*-GUS expression in the primary procambial strand extended basally, and was additionally detected in the distal portion of the first loops of secondary procambial strands (Fig. 2J). At 7 DAG, ET1335-GUS expression labeled third order procambial strands in the first and second intercostal areas (Fig. 2K). At this stage, *Oshox1*-GUS expression in the first
Fig. 1 Histochemical localization of Oshox1-GUS activity in transgenic Arabidopsis. (A, C, E, H, K, L, N, P) Bright-field optics. (D, F, G, J, M, O) Dark-field illumination. (B, I, Q, R) DIC microscopy. (A) Apical region of the primary root of a 7-d-old seedling. (B) Basal region of the primary root of a 14-d-old seedling. (C) Detail of a transverse section through the basal region of the primary root of a 14-d-old seedling. (D) Hypocotyl of a 7-d-old seedling. (E) Detail of a transverse section through the hypocotyl of a 14-d-old seedling. (F) Transverse section through the hypocotyl of a plant at the green-silique stage (Altamura et al. 2001). (G) Transverse section through the basal part of the stem of a plant at the green-silique stage. (H) Detail of a tralverse section through the basal part of the stem of a plant at the green-silique stage. (I) Detail of the tip of the first leaf primordium of a 6-d-old seedling. (J) Abaxial view of the first leaf of a 8-d-old seedling. (K) Abaxial view of the fully differentiated third leaf of a 21-d-old seedling. (L) Detail of a secondary vein in a transverse section through a fully differentiated first leaf of a 14-d-old seedling. (M) Floral bud (stage 9 according to Smyth et al. 1990). (N) Mature flower (stage 15 according to Smyth et al. 1990). (O) Silique. (P) Mature embryo. (Q, R) Ectopic induction of Oshox1-GUS expression in the apical region of the primary root of a 7-d-old seedling in response to 1 μM NAA (Q) or 500 mM sucrose (R). Lignified cell walls appear white in (J) because of light reflection, and red in (H) because of phloroglucinol staining. 1, primary procambial strand; 2, secondary procambial strand; b, bundle sheath; c, vascular cambium; cz, cambial zone; e, endodermis; ic, interfascicular cambium; if, interfascicular fibres; ls, lignified vascular strands; p, phloem; pc, pericycle; ps, procambial strands; rh, root hair; vc, vascular cylinder; vs, vascular strands; x, xylem. Scale bars: (A, D, F, G, J) 200 μm (B, H, Q, R) 50 μm (C, E, I, L) 10 μm (K) 1 mm (M, P) 100 μm (N, O) 500 μm.

Fig. 2 Expression of Oshox1-GUS, ET1335-GUS and GT5211-GUS in Arabidopsis first leaf development. (A–D) Lateral view. (E–R) Abaxial view. DIC microscopy. (N, P, R) Enlargements of the areas boxed in M, O and P, respectively. Upper right: marker line identity. Lower left: primordium age in d after germination (DAG). Numbers refer to vein orders (for clarity, higher-order veins have not been labeled). Arrows in E and F indicate marker expression in GM cells at the primordium tips. Scale bars: (A–H) 25 μm (I, J) 50 μm (K, L, N, P and R) 100 μm (M, O and Q) 1 mm.
loops of secondary procambial strands extended basally, and was additionally detected in the distal portion of the second loops of secondary procambial strands, and in third order procambial strands in the first intercostal areas (Fig. 2L). Throughout leaf development, Oshox1-GUS expression domains always appeared in continuity with pre-existing vasculature.

High levels of GT5211-GUS expression mark procambium formation (Scarpella et al. 2004). GT5211-GUS does not seem to be expressed in any type of mature vascular tissues, as it disappears completely from mature higher order veins, which consist exclusively of fully differentiated cells (Scarpella et al. 2004). Unlike GT5211-GUS expression (Fig. 2Q, R), both ET1335-GUS and Oshox1-GUS expression persisted in veins of all orders in fully differentiated leaves at 14 DAG (Fig. 2M–P).

In summary, at all stages of leaf development, Oshox1-GUS expression appeared in procambial strands after their overt formation was labeled by ET1335-GUS expression. Furthermore, Oshox1-GUS was transiently expressed in GM cells at the tip of 4–8 DAG primordia (Fig. 1J, 2F). Finally, Oshox1-GUS expression appeared to proceed continuously and progressively within individual procambial strands, and persisted in fully differentiated veins.

Oshox1-GUS expression during vascular dedifferentiation and redifferentiation in Arabidopsis

We next asked whether the association between Oshox1-GUS expression and vascular differentiation observed in Arabidopsis also persisted under perturbed experimental conditions. In Arabidopsis and other dicots, cell proliferation can be reinitiated in vascular cells of hypocotyl explants that are cultured in the presence of a suitable combination of hormones (Guzzo et al. 1994, Guzzo et al. 1995, Ozawa et al. 1998). The dedifferentiation of vascular cells in this culture system gives rise to callus tissue, within which new vascular tissues subsequently differentiate. As such, this system conveniently allows testing of the dynamics of Oshox1-GUS expression during experimentally induced vascular dedifferentiation and redifferentiation in Arabidopsis.

To this aim, hypocotyls of Oshox1-GUS Arabidopsis transgenic seedlings were explanted on medium containing the synthetic auxin 2,4-D and the cytokinin kinetin. After 3 d of culture, Oshox1-GUS expression was detected in all cell types of the swelling hypocotyls (Fig. 3A). After 7 d, the enlarged epidermal and cortical cells started to detach in layers from the central vascular cylinder, and small calli formed at both cut ends upon dedifferentiation of vascular cells (Fig. 3B). Oshox1-GUS expression was down-regulated in those vascular cells that had reacquired meristematic activity (Fig. 3B, D), and reappeared only in close association with new vascular cells within the callus tissue (Fig. 3B, E). After 10 d, epidermal and cortical cells were almost completely lost, and cell division was also resumed in vascular cells along the hypocotyl axis, giving rise to small calli lacking Oshox1-GUS expression (Fig. 3C). Also in these calli, Oshox1-GUS activity later reappeared in newly differentiated vascular elements (Fig. 3F).

In summary, the Arabidopsis vascular dedifferentiation and redifferentiation system, Oshox1-GUS expression was first ectopically induced following excision. Expression was then silenced in vascular cells that had reacquired meristematic properties, and reappeared in cells that were undergoing vascular differentiation. Therefore, Oshox1-GUS expression in Arabidopsis is associated with vascular differentiation during both undisturbed and experimentally challenged development.

Expression patterns conferred by progressive 5′-end deletions of the Oshox1 promoter

Because the 1.6 kb Oshox1 promoter region directs GUS reporter gene expression in transgenic Arabidopsis in a fashion that mimics the expression pattern of Oshox1 in rice (see Discussion), we took advantage of the short life cycle of Arabidopsis and its faster transformation procedure to map the cis-acting elements responsible for the Oshox1 expression pattern. To this aim, we generated a series of 5′ deletions of the Oshox1 promoter (Fig. 4). These fragments were fused to the GUS gene, and their expression patterns were analyzed in transgenic Arabidopsis.

Fig. 3 Histochemical localization of Oshox1-GUS activity during vascular dedifferentiation and redifferentiation in explanted transgenic Arabidopsis hypocotyls. (A–C) Hypocotyl explants after 3 (A), 7 (B) or 10 (C) d of culture. (D–F) Details of longitudinal sections through calli originated from the vascular cylinder of hypocotyl explants cultured for 7 (D, E) or 10 (F) d. (D) The callus does not show anatomical signs of vascular differentiation nor Oshox1-GUS expression. (E) Vascular tissues have started to differentiate within the callus. Note Oshox1-GUS expression in procambial-like cells (pc) and in differentiating tracheary elements (te). Arrows indicate epidermal and cortical cells detaching from the vascular cylinder of the hypocotyl explants. c, callus tissue originating from the vascular cylinder of the hypocotyl explants; e, epidermis; v, vascular tissues. Scale bars: (A–C) 250 µm (D, F) 25 µm (E) 50 µm.
In mature embryos, Oshox1-GUS expression was detected in primary and secondary procambial strands of cotyledons, with stronger expression in the apical region of the strands (Fig. 1L, 5F, L) and expression was absent from the embryonic axis (Fig. 1L, 5R). Reporter gene expression was never detected in the lines obtained from the transformation with the shortest promoter construct of the deletion series, HN-GUS (Fig. 5A, G, M). In cotyledons of the SN-GUS mature embryos, expression was detected in the basal region of primary procambial strands and in surrounding GM tissue (Fig. 5B, H). In MN-GUS mature embryonic cotyledons, expression was much stronger and was observed in both primary and secondary procambial strands and in neighboring GM tissue (Fig. 5C, I). In cotyledons of PN-GUS mature embryos, the expression pattern was very similar to that conferred by the full-length promoter, but expression was stronger in the basal region of procambial strands (Fig. 5D, J). Finally, in DN-GUS mature embryonic cotyledons, expression was observed in both primary and secondary procambial strands, with stronger expression in the apical region of the strands, as in Oshox1-GUS embryos (Fig. 5E, K). However, unlike in Oshox1-GUS and PN-GUS embryos, DN-GUS expression was also detected in GM cells surrounding the apical region of the procambial strands (Fig. 5E, K). Like the full-length promoter, none of the promoter deletion fragments could drive reporter gene expression in the axis of mature Arabidopsis embryos (Fig. 5M–Q).

As in embryos, expression of the shortest promoter construct, HN-GUS, was never detected at the seedling stage (Fig. 6A, G, M, S). In cotyledons of 1-week-old seedlings, expression of all other constructs was detected in correspondence with both primary and secondary vascular strands (Fig. 6B–F).

Similarly, expression of all constructs, except HN-GUS, was observed in the vascular cylinder of the basal, mature region of the root (Fig. 6G–L), although the level of expression varied among the different constructs. None of the Oshox1 promoter fragments, except for the full-length promoter, was able to drive reporter gene expression in the region of the vascular cylinder close to the root apex (Fig. 6M–R). In young developing first leaves, expression of all constructs, except HN-GUS, was detected at the apical hydathode (Fig. 6S–X). Furthermore, the PN-, DN- and Oshox1-GUS constructs were also expressed in trichomes (Fig. 6V–X), but only the DN-GUS construct could additionally confer the expression in differentiating vascular strands typical of the Oshox1-GUS construct (Fig. 6W, X). Finally, none of the Oshox1 promoter fragments, except for the full-length promoter (Fig. 1), was able to confer auxin or sucrose responsiveness to reporter gene expression.

**Discussion**

The role of upstream regulatory sequences in the transcriptional control of early vascular-specific genes, and the evolutionary conservation of such elements, is largely unknown. Studies in Arabidopsis and other species have identified a number of genes expressed at early stages of vascular development (Demura and Fukuda 1994, Baima et al. 1995, Hardtke and Berleth 1998, Hiwatashi and Fukuda 2000, Scarpella et al. 2000, Elge et al. 2001, Clay and Nelson 2002, Hamann et al. 2002, Kang and Dengler 2002, Ohashi-Ito et al. 2003, Groover et al. 2003, Ohashi-Ito and Fukuda 2003, Carland and Nelson 2004, Kang and Dengler 2004, Scarpella et al. 2004). Among them, the rice gene Oshox1 is one of the few for which promoter sequences have been shown to mimic the endogenous pattern of expression (Scarpella et al. 2000). In this study, we have analyzed the dynamics of Oshox1 promoter-driven reporter gene expression in Arabidopsis, and found them to be similar to those reported for rice. Taking advantage of this correlation, we have explored the role of upstream regulatory sequences of Oshox1 in the control of its early vascular gene expression in Arabidopsis.

As in rice, the onset of Oshox1-GUS expression in Arabidopsis seems to coincide with a specific stage of procambium development at which cells have already acquired anatomical conspicuousness, but do not yet display overt signs of terminal vascular differentiation. First, in both rice and Arabidopsis mature embryos, Oshox1-GUS activity was detected in procambial strands of the cotyledons, but not in those of the embryonic axis. Second, in both rice and Arabidopsis, procambial cells closest to the post-embryonic root apex did not express Oshox1-GUS, whereas expression appeared in procambial cells further away from the root tip. Third, Oshox1-GUS expression marked procambial strands before any signs of terminal vascular differentiation in both rice and Arabidopsis leaves. At all stages of Arabidopsis leaf development, expression of Oshox1-GUS was invariably
initiated after that of ET1335-GUS, which is expressed simultaneously with the appearance of procambial cell features (Scarpella et al. 2004). Therefore, we suggest that Oshox1-GUS expression, in combination with previously described reporter gene expression markers of early vascular development (e.g. Clay and Nelson 2002, Hamann et al. 2002, Groover et al. 2003, Ohashi-Ito and Fukuda 2003, Carland and Nelson 2004, Scarpella et al. 2004), can be used to visualize distinct stages of procambial development that cannot be identified by anatomical features. Fourth, as in rice, Oshox1-GUS expression in *Arabidopsis* persisted in completely differentiated vascular strands of all organs. Rice plants do not undergo secondary growth. However, Oshox1-GUS expression was detected in the interfascicular and vascular cambium of *Arabidopsis*, indicating that the regulatory sequences in the Oshox1 promoter are sufficient to direct
expression in secondary vascular meristems. Further, in rice, Oshox1-GUS expression was also detected in trichomes, stomata and pollen (Scarpella et al. 2000). In Arabidopsis, Oshox1-GUS was expressed in trichomes, but we never observed expression in stomata or pollen, probably reflecting differences in upstream regulatory events or in specific cell-type development. Finally, in both rice and Arabidopsis roots, Oshox1-GUS expression was ectopically induced by auxin and sucrose.

If the congruence of Oshox1-GUS expression in rice and Arabidopsis is more than a coincidence, it should also be observed under altered experimental conditions. In monocots, root tips can be regenerated after excision by dedifferentiation and subsequent redifferentiation of cells of the vascular cylinder (Feldman 1976). In rice, Oshox1-GUS expression was ectopically induced in epidermal and cortical cells of the root at the site of tip excision (Scarpella et al. 2000). Expression subsequently was rapidly down-regulated in the regenerated root tip, where Oshox1-GUS expression again became confined to the central vascular cylinder (Scarpella et al. 2000). Both Oshox1-GUS ectopic induction and root tip regeneration were found to be strictly dependent on polar auxin transport (Scarpella et al. 2000). In Arabidopsis and other dicots, vascular cells of hypocotyl explants can be induced to dedifferentiate through the action of a suitable combination of hormones (Guzzo et al. 1994, Guzzo et al. 1995, Ozawa et al. 1998). The dedifferentiation of vascular cells in this culture system gives rise to callus tissue, within which new vascular cells differentiate. We found that Oshox1-GUS expression was ectopically induced in epidermal and cortical cells of the cultured hypocotyl explants before vascular cells had resumed division. Oshox1-GUS expression was subsequently down-regulated in dedifferentiated, proliferating vascular cells. Expression of the transgene reappeared within the meristematic tissues in close association with newly differentiating vascular cells. Oshox1-GUS ectopic induction and vascular cell dedifferentiation were both found to be dependent on the presence of the synthetic auxin 2,4-D in the culture medium (not shown). Therefore, the expression dynamics of Oshox1-GUS in these experiments paralleled Oshox1 expression during root tip regeneration in rice (Scarpella et al. 2000).

The conservation of Oshox1-GUS expression profiles both during unchallenged development and after experimental interference in rice and Arabidopsis prompted us to exploit the shorter life cycle of Arabidopsis and its faster transformation procedure to investigate the role of cis-acting regulatory sequences in the control of early vascular gene expression. Our analysis of different truncated versions of the Oshox1 promoter in transgenic Arabidopsis plants implicates a series of cell type-, tissue- and organ-specific regulatory elements in the control of the Oshox1 expression profile (Fig. 7). Further, our study suggests that Oshox1 embryonic vascular expression is mainly achieved through suppression of expression in non-vascular tissues. No expression was ever detected when the reporter gene was solely under the control of the sequence of the Oshox1 promoter located between position –118 and the start codon of the Oshox1 open reading frame (position +1; HN fragment). This region probably represents a minimal promoter unable to direct transcription per se at detectable levels. The sequence of the Oshox1 promoter between position –244 and +1 (SN fragment) was sufficient to direct reporter gene expression in primary procambial strands and neighboring GM tissue of mature embryonic cotyledons. Since the region from –118 to +1 was not able to drive any reporter gene expression, the elements responsible for the embryonic expression of the SN-GUS construct are likely to be located in the region between position –244 and –118. Furthermore, in this region, one or more elements capable of driving expression in vascular strands of seedling cotyledons and root also seem to be present. Finally, this region also seems to contain a regulatory element capable of driving expression in leaf hydathodes. Promoter functional studies have implicated CCA/TGG repeats and CCCCC stretches in positive control of vascular gene expression (Hauffe et al. 1993, Hatton et al. 1995, Torres-Schumann et al. 1996, Yin et al. 1997, Lacombe et al. 2000). Consistent with a function in vascular expression, the SH fragment of the Oshox1 promoter contains three CCA/TGG repeats and four CCCCC stretches with respect to the slightly shorter SN promoter fragment, the region of the Oshox1 promoter from position –290 to +1 (MN fragment) seems to contain an element that, in mature embryonic cotyledons, directs additional expression in secondary procambial strands and in surrounding GM tissue. In comparison with the shorter MN promoter fragment, the Oshox1 promoter region that spans position –528 to +1 (PN fragment) seems to contain a regulatory element that suppresses the expression in GM tissue of mature embryonic cotyledons that is conferred by the elements present in the region between –290 and –118. Furthermore, the region of the Oshox1 promoter from –528 to –244 seems to contain an element capable of directing reporter gene expression in leaf trichomes. Deletion of the AACA negative regulatory element in a number of vascular promoters results in ectopic activity in cell types other than the vascular ones (Keller and
Baumgartner 1991, Hauffe et al. 1993, Hatton et al. 1995, Hatton et al. 1996, Lacombe et al. 2000, Liu et al. 2003). Consistent with a role in suppressing expression in non-vascular cells, the PM fragment of the Oshox1 promoter contains two AACCA motifs. Further, this region contains 12 CCA/TTG repeats and four CCCCC stretches, both of which have been implicated in positive control of vascular expression (Hauffe et al. 1993, Hatton et al. 1995, Torres-Schumann et al. 1996, Yin et al. 1997, Lacombe et al. 2000). The Oshox1 promoter sequence between position –898 and +1 (DN fragment) drives expression in GM tissue of cotyledons of mature embryos. This suggests either that the sequence between –898 and –528 contains a positive regulatory element that directs expression in this tissue or that it contains an element that counteracts the negative effects on GM tissue expression that the element located between position –528 and –290 seems to have. Furthermore, the sequence of the Oshox1 promoter located between –898 and –528 additionally contains an element capable of directing expression in differentiating vascular strands of developing leaves. In agreement with a role in vascular expression, the DP region of the Oshox1 promoter contains 13 repeats of the CCA/TTG positive vascular regulatory element. Finally, the full-length Oshox1 promoter confers procambial expression in mature embryonic cotyledons. The sequence of the Oshox1 promoter from position –1,621 to –898 is thus likely to contain an element that suppresses the GM tissue-specific element present in the region between position –898 and –528. Furthermore, the sequence from position –1,621 to –898 seems also to contain an element capable of driving expression in the vascular cylinder close to the post-embryonic root apex. Consistent with a role in driving vascular expression, and in suppressing expression in non-vascular cells, this region of the Oshox1 promoter contains 35 repeats of the CCA/TTG positive vascular regulatory element, and three copies of the AACCA negative regulatory element of non-vascular expression.

Promoter functional analysis of auxin-regulated genes has identified a number of cis-elements involved in auxin responsiveness: the (G/T)GTCCCAT and TGCTCT elements (reviewed in Guilfoyle et al. 1998), the related GTTCCCAT sequence (Sakai et al. 1996), and the AAGG and AAGG DoF elements (Kim et al. 1994, Liu and Lam 1994, Qin et al. 1994, Ulmasov et al. 1994, Zhang and Singh 1994, van der Zaal et al. 1996, Kisu et al. 1998, Baumann et al. 1999, Kang and Singh 2000). The sequence of the Oshox1 promoter between position –1,621 and –898 is necessary for auxin responsiveness. In agreement with such a role, this region contains 11 DoF elements and one GGTCCT element. In addition, these motifs might contribute to the procambial expression conferred by the full-length Oshox1 promoter. In fact, mutations in DoF elements abolish both auxin responsiveness and procambial expression (Baumann et al. 1999), and specific DoF transcription factors are expressed in the procambium (Baumann et al. 1999, Gualberti et al. 2002).

To date, five different types of cis-elements have been implicated in sucrose-regulated gene expression: the G-box (Giuliano et al. 1988), the B-box (Grierson et al. 1994, Zourelioudou et al. 2002), the SURE (Grierson et al. 1994), the SP8 element (Ishiguro and Nakamura 1994) and the TGGACGG sequence (Maeo et al. 2001). The sequence of the Oshox1 promoter from position –1,621 to –898 is necessary to confer sucrose responsiveness. Consistent with such function, this region contains four SP8 elements (TACTATT, TCACTATT and TACTAT) and one B-box (CTAAC).

A growing body of evidence is highlighting the importance of combinatorial control of transcriptional regulation in plants (Singh 1998). The mosaic of regulatory elements present in the promoter of Oshox1 implies that the control of its expression is a complex process involving both multiple DNA–protein and protein–protein interactions. It is conceivable that distinct transcription factor binding to the different regulatory elements of the Oshox1 promoter may operate in a combinatorial fashion to specify the temporal and spatial aspects of Oshox1 gene expression. The isolation and analysis of these proteins will help to elucidate the molecular mechanisms by which gene expression at early stages of vascular development is regulated.

Materials and Methods

Vector construction

The Oshox1-GUS construct containing the 1.6-kb region upstream of the start codon of the Oshox1 open reading frame fused to the GUS coding sequence in pCAMBIA-1391z (AF234312) has been described previously (Scarpella et al. 2000). The HN-GUS plasmid was constructed by digesting Oshox1-GUS with HindIII and by subsequently religating the vector thus obtained. The SN promoter fragment was excised from Oshox1-GUS with Sphi and NcoI and subcloned into the corresponding sites of pUC21 (AF223641). The promoter fragment was subsequently excised with BgiII and NcoI, and cloned into the BamHI and NcoI sites of pCAMBIA-1391z to give rise to the SN-GUS construct. The MN promoter fragment was excised from the pUC21-Oshox1 promoter (Scarpella et al. 2000) with MluI and NcoI, subcloned into the corresponding sites of pUC21, re-excised as an Ncol–BamHI fragment and cloned into the Ncol and BamHI sites of pCAMBIA-1391Z to give rise to the MN-GUS construct. The PN promoter fragment was excised from the pUC21-Oshox1 promoter (Scarpella et al. 2000) with MluI and NcoI, subcloned into the corresponding sites of pUC21, re-excised as an Ncol–BamHI fragment and cloned into the Ncol and BamHI sites of pCAMBIA-1391Z to give rise to the MN-GUS construct. The PN promoter fragment was excised from the pUC21-Oshox1 promoter with SpeI and NcoI, and cloned into the Smal and Ncol sites of pCAMBIA-1391Z to give rise to the SM-GUS construct. Finally, the DN promoter fragment was excised from the pUC21-Oshox1 promoter with DraI and NcoI, and cloned into the Smal and Ncol sites of pCAMBIA-1391Z to give rise to the DN-GUS construct.

Plant transformation and growth conditions

Arabidopsis thaliana (L.) Heynh ecotypes C24 (Oshox1-GUS) and Col-0 (Oshox1-, DN-, PN-, MN-, SN- and HN-GUS) were transformed using the vacuum infiltration method (Clough and Bent 1998). The progeny of 20 independent primary transformants per ecotype per transgene were selected for preliminary expression pattern analysis. The GUS expression profile was identical in all tested lines, except for two Oshox1-GUS lines, one of which showed additional expression in...
the root cap, while the other showed patchy expression not restricted to vascular tissues. Except for the HN-GUS lines, which never showed reporter gene expression, 2–4 lines per ecotype per transgene were either considerably weaker or stronger than the remaining lines. More detailed analyses were performed on the progeny of three (Oshox1-GUS) or five (DN-, PN-, MN-, SN- and HN-GUS) representative, independent primary transformants per ecotype per transgene. Representative lines were selected based on medium level of expression (except for HN-GUS lines, in which reporter expression was never detected), and insertion of one copy of the transgene. Seeds were surface sterilized (McCourt and Keith 1998) and plated on MA medium (Masson and Paszkowski 1992) containing 25 mg l–1 hygromycin (Duchefa Biochemie, Amsterdam, The Netherlands). Plates were incubated in the dark at 4°C for 4 d and then moved to a 16 h light : 8 h dark cycle at 21°C. Induction with auxins (1 µM IAA; 1 µM NAA; 1 µM β-NAA; 1 µM 2,4-D) and sugars (500 mM sucrose; 500 mM mannitol) in MA medium was performed as described (Scarpella et al. 2000).

Vascular dedifferentiation and redifferentiation assay

Hypocotyls from 2-week-old seedlings of three independent Oshox1-GUS C24 lines were aseptically excised and cultured up to 14 d at a 16 h light : 8 h dark cycle at 25°C on callus induction medium (Vergunst et al. 1998), in which 2% glucose was replaced by 2% sucrose to enhance the frequency of callus formation (Iwami and Goto 1990). This concentration of sucrose (~50 mM) has no effect on Oshox1-GUS expression (not shown). Material was harvested for GUS analysis at 3, 7, 10 and 14 d after transfer to callus induction medium. Results are representative of three independent experiments, each performed on 10 hypocotyls per transgenic line per time point.

Microtechniques and microscopy

Histochemical detection of Oshox1-GUS activity was performed on whole seedlings, freshly dissected plant organs or hand sections as described (Scarpella et al. 2004). Briefly, samples were permeabilized in 90% acetone for 1 h at –20°C, washed twice for 5 min with 100 mM phosphate buffer pH 7.5–7.7, and incubated at 37°C for 16 h in 100 mM sodium phosphate buffer pH 7.3–7.7, 10 mM sodium EDTA, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Biosynth AG, Staad, Switzerland), 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide. High concentrations of potassium ferrocyanide and potassium ferricyanide reduce diffusion of the intermediate of the GUS staining reaction at the expense of sensitivity of detection (Lojda 1990). This concentration of succrose (~50 mM) has no effect on Oshox1-GUS expression in certain organs at specific developmental stages, such as distal root tips, young leaf primordia, immature embryos and embryo axis of mature embryos, could thus be due to high stringency of staining conditions. Therefore, for those organs and stages, we performed additional stainings, in which the concentrations of potassium ferrocyanide and potassium ferricyanide were reduced to 0.5 mM. No additional GUS expression domains were detected under those conditions, thereby confirming the genuine absence of expression in those organs and stages. Histochemical detection of ET1335-GUS and GT5211-GUS activities was performed on whole seedlings as described (Scarpella et al. 2004). The reaction was stopped by fixing the samples in ethanol : acetic acid 3 : 1 for 1–1.6 h at room temperature, depending on the age of the seedlings. Samples were stored in 70% ethanol at 4°C. Rehydrated samples were cleared in chloral hydrate : glycerol : water 8 : 3 : 1 before microscopic observation. For histological analysis, rehydrated GUS-stained samples were fixed overnight in 2% glutaraldehyde and embedded in glycol methacrylate as described (Scarpella et al. 2000). Sections (10 µm) were dried onto slides at 37°C and counterstained with 0.5% Safranin O in water before mounting in DPX for microscopic observation. Samples were viewed with a Leica MZ12 stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a Sony 3CCD Digital Photo Camera DKC-5000 (Sony Corporation, Tokyo, Japan); with a Wild TYPE 376788 stereomicroscope (Wild, Heerbrug, Switzerland) equipped with a Nikon DXM1200 digital camera (Nikon, Tokyo, Japan); with a Zeiss Axiosplan 2 Imaging microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Sony 3CCD Digital Photo Camera DKC-5000 (Sony Corporation, Tokyo, Japan); or with an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) equipped with a Photometric CoolSnap fx digital camera (Roper Scientific, Trenton, NJ, USA) and a MicroColor liquid crystal tunable RGB filter (Cambridge Research and Instrumentation, Inc., Woburn, MA, USA). Images were assembled using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA), and figures were labeled using Canvas 8 (ACD Systems Ltd, Saanichton, BC, Canada).

Computational analysis

Promoter sequence analysis was performed by using the Plant CARE (http://oberon.fvms.ugent.be:8080/PlantCARE/index.html) (Lescot et al. 2002) and PLACE (http://www.dna.afrc.go.jp/PLACE/) (Higo et al. 1999) databases of plant regulatory elements.

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References


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