Development 130, 645-658
© 2003 The Company of Biologists Ltd
doi:10.1242/dev.00243

The *RADICLELESS1* gene is required for vascular pattern formation in rice

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Accepted 31 October 2002

SUMMARY

The molecular mechanisms through which the complex patterns of plant vascular tissues are established are largely unknown. The highly ordered, yet simple, striate array of veins of rice leaves represents an attractive system to study the dynamics underlying pattern formation. Here we show that mutation in the *RADICLELESS1* (*RAL1*) gene results in distinctive vascular pattern defects. In *ral1* embryonic scutella, secondary veins are absent and in the prematurely aborted and discontinuous primary veins, cells are misaligned to each other. In *ral1* leaves, longitudinal and commissural (transverse) veins display altered spacing and the commissural veins additionally show atypical branching and interruptions in their continuity. The vascular pattern alterations of *ral1* occur in the context of normally shaped leaf primordia. Anatomical inspection and analysis of the expression of the procambium specification marker Oshox1-GUS and of the auxin-inducible reporter DR5-GUS demonstrates that all the vascular patterning aberrations of *ral1* originate from defects in the procambium, which represents the earliest identifiable stage of vascular development. Furthermore, the *ral1* mutant is unique in that procambium formation in leaf primordium development is delayed. Finally, the *ral1* vascular patterning distortions are associated with a defective response to auxin and with an enhanced sensitivity to cytokinin. *ral1* is the first mutant impaired in both procambium development and vascular patterning to be isolated in a monocot species.

Key words: Auxin resistance, Commissural veins, Cytokinin hypersensitivity, DR5, Embryo mutant, *Oryza sativa*, *Oshox1*, Procambium, *RAL1*, Venation pattern

INTRODUCTION

The vascular system of plants consists of a network of continuous strands, the vascular bundles, which efficiently ensure long-distance transport of water and dissolved materials, and provide mechanical support (Esau, 1965a). Vascular bundles typically consist of the two kinds of conducting tissues, the phloem and the xylem, each comprising a variety of specialised cell types. All types of vascular cells differentiate from a common primary meristematic tissue, the procambium, or provascular tissue. The procambium becomes recognisable as files of dense, narrow cells, the major axis of which is parallel to that of the procambial strand. During vascular development, procambial cells undergo an anatomically undetectable restriction of their potential that progressively commits them towards a vascular differentiation fate (Scarpella et al., 2000). Two types of patterns can be identified within the vascular system (Esau, 1965b): the longitudinal pattern, deriving from the three-dimensional array of vascular bundles within individual organs, and the radial pattern, arising from the spatial arrangement of phloem and xylem within each vascular bundle. The successful use of leaf vascular patterns as a species-specific diagnostic feature (e.g. Klucking, 1995) indicates that the patterning processes must be strictly regulated. However, the molecular mechanisms underlying the different aspects of vascular tissue pattern formation are still largely unknown.

Although the influence of various plant hormones in promoting vascular differentiation has been reported (e.g. Aloni, 1987), the role of auxin is unique. Auxin application not only triggers vascular differentiation per se, but also induces the differentiation of a slender strip of cells into a continuous vascular strand that extends towards the basal pole of the plant (Sachs, 1981). Experimental evidence suggests that polar transport of auxin is directly responsible for the directionality of the vascular response (Sachs, 1981). However, proper auxin perception and response should nevertheless be essential for the relay of auxin signals in vascular differentiation. Consistently, vascular abnormalities have been reported for auxin response mutants with closely related primary defects. Mutations at three *Arabidopsis* loci, *MONOPTEROS* (*MP*), *BODENLOS* (*BDL*) and *AUXIN-RESISTANT6 (AXR6*), result in a complex phenotype characterised by an impaired auxin perception or response, a severely reduced vascular system, and defective embryo axis formation with consequent failure to produce an embryonic root (Berleth and Jürgens, 1993; Przemeck at al., 1996; Hamann et al., 1999; Hobbie at al., 2000). These common features suggest related primary defects in the molecular machinery underlying the alignment of cell differentiation with the axis of auxin flow at various developmental stages. Strong support for this also comes from the identification of the *MP* gene, which encodes a transcriptional regulator of the auxin response factor family

that is specifically expressed in the vasculature (Hardtke and Berleth, 1998). Importantly, the DNA-binding domain of the MP protein appears to interact with auxin response elements, short conserved sequences essential for the rapid auxin regulation of certain classes of auxin inducible genes (Ulmasov et al., 1997a). The recent finding that the *BDL* gene encodes a member of the Aux/IAA family of proteins that would interact with MP to provide the proper auxin response that is necessary for embryo patterning strengthens further the link between auxin response, embryo axialisation and vascular patterning (Hamann et al., 2002).

The striking association between impaired embryo axis formation, reduced vascularisation and defective auxin sensitivity in the dicot *Arabidopsis*, prompted us to investigate whether the same relationship could also underlie vascular pattern formation in monocots, despite the fact that dicot and monocot leaves have highly divergent vascular patterns (Nelson and Dengler, 1997). Most dicot leaves show a reticulate pattern of highly branched veins, whereas most monocot leaves show a typical striate venation pattern, in which major veins lie parallel along the proximodistal axis of the leaf, and largely lack major vein branching. Furthermore, vascular ontogeny in monocots and dicots shows fundamental differences. For example, in dicots the primary vein extends progressively from the stem vasculature into the leaf primordium, and secondary veins develop in continuity with the primary vein. In contrast, in monocot leaf primordia parallel veins arise isolated from each other and from the stem vasculature.

In a previous study, Nagato and co-workers classified 188 embryo mutants of rice (Hong et al., 1995). Using the recessive *radicleless1* (*ral1*) mutant from this collection, we provide genetic evidence that auxin sensitivity is associated with embryonic root development and vascular pattern formation in a monocot species. Furthermore, we show that these alterations are coupled to an altered sensitivity to cytokinin. Our investigations indicate that the *RAL1* gene has an early function in the establishment of vascular patterns during embryonic and post-embryonic development as well as an important role in the proper response to auxin and cytokinin.

MATERIALS AND METHODS

Vector construction

The heptadic repeat of the DR5 element (Ulmasov et al., 1997b) coupled to the CaMV 35S -47 minimal promoter was excised from the DR5-GusXX-47 plasmid (Benjamins et al., 2001) and fused as an *Nco*I *Hin*dIII fragment to the *gusA* start codon in the binary vector pCAMBIA1391z (AF234312) to obtain the DR5-GUS reporter construct.

Plants and growth conditions

Oryza sativa (L.) Japonica cultivar Taichung 65, in which background the *ral1* (*odm40*) mutant allele was induced (Hong et al., 1995), was used as a wild-type control strain in all studies. Upon outcrossing of the *ral1* homozygous mutant to wild type, the heterozygous F_1 population did not show any obvious morphological difference from wild type (data not shown). Furthermore, in the F_2 population, the radicleless phenotype behaved as a recessive trait, having frequencies of segregation significantly close to 25% (Hong et al., 1995) (our unpublished observations).

All seeds were surface sterilised (Rueb et al., 1994) and germinated in the dark at 28°C for 4 days on half-strength Murashige and Skoog (MS) medium in which MS vitamins were replaced with B5 vitamins and supplemented with 10 g/l sucrose and 7 g/l agarose (replaced by 2.5 g/l phytagel for seedlings that had to be transferred to the greenhouse). Germinated seeds were grown in a 12 hours light:12 hours dark cycle at 28°C. Embryonic calli induced on scutella from germinated seeds were transformed with *Agrobacterium tumefaciens* strain LBA4404 (Ach5 pTiAch5 ΔT-DNA) or LBA1119 (C58 pTiBo542 ΔT-DNA) harbouring the DR5-GUS binary vector as described previously (Scarpella et al., 2000). Seedlings and regenerated transgenic plantlets were transferred to the greenhouse and grown in hydroponic culture with a regime of 12 hours light, 28°C, 85% relative humidity and 12 hours dark, 21°C, 60% relative humidity. Genetic crosses were performed to introduce the Oshox1-GUS transgene (Scarpella et al., 2000) into the *ral1* mutant background. Flowers of *ral1* plants were emasculated by submerging whole inflorescences in a water bath at 42°C for 6 minutes. Inflorescences were subsequently blotted dry on filter paper and flowers that opened on either the day of the treatment or the following day were fertilised by applying pollen from flowers of Oshox1-GUS plants at anthesis. As a wild-type control for the expression of the transgene in the *ral1* mutant, the Oshox1-GUS expression pattern was analysed in the Taichung 65 background and found to correspond to the previously reported expression pattern in Taipei 309 (Scarpella et al., 2000).

Tissue culture assays

The ability of seedlings to form callus tissues was assayed by germinating seeds on callus-induction medium supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) as described previously (Rueb et al., 1994). Callus tissue growth properties were evaluated by transferring callus pieces of approximately 2-3 mm in diameter to new callus-induction medium supplemented with either 1 or 2 mg/l 2,4-D. In both callus induction and callus growth experiments the response was monitored weekly during a 1-month period. The capability of callus tissues to regenerate plantlets was assayed by transferring callus pieces of approximately 2-3 mm diameter to LS basal medium, to which 40 g/l sucrose and 7g/l agarose were added and supplemented or not with 0.3 mg/l N^6-benzyladenine. The response was evaluated monthly during a 3-month period. Calli were transferred to new medium after each monthly examination.

Microtechniques and microscopy

Dissected samples or 100-μm vibratome sections were fixed overnight in 2% glutaraldehyde and embedded in glycol methacrylate as described (Scarpella et al., 2000). Sections (10 μm) were dried onto slides and stained with 0.05% Toluidine Blue O in 50 mM citrate buffer pH 4.4 before mounting in epoxy resin for microscopic observation using bright-field optics. Whole-mount cleared preparations were obtained by autoclaving dissected samples in 80% lactic acid for 20 minutes at 121°C. Samples were mounted in fresh 80% lactic acid and viewed with dark-field optics. Histochemical detection of β-glucuronidase (GUS) activity was performed on freshly dissected plant organs or 100 μm vibratome sections. Samples were permeabilised in 90% acetone for 1 hour at –20°C, washed twice under vacuum for 5 minutes with 100 mM phosphate buffer pH 7.5-7.7, 5 mM potassium ferricyanide, and incubated at 37°C in 100 mM sodium phosphate buffer pH 7.5-7.7, 10 mM sodium EDTA, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM 5-bromo-4-chloro-3-indolyl-β–D-glucuronic acid (X-gluc; Biosynth AG). Reaction was stopped in 70% ethanol after 30 minutes (DR5-GUS roots), 2 (DR5-GUS shoots) or 16 (Oshox1-GUS) hours. Samples were either viewed immediately or fixed in ethanol:acetic acid 3:1 and mounted in chloral hydrate:glycerol:water 8:3:1 before observation with bright-field or differential interference contrast optics. All samples were observed with a Zeiss Axioplan 2 Imaging microscope or with a Leica MZ12 stereoscopic microscope. Images

were acquired with a Sony 3CCD digital photo camera DKC-5000. All images were processed using Adobe Photoshop 5.0.

RESULTS

Procambium pattern formation is altered in the *ral1* embryo

In the embryo of the recessive *ral1* mutant, apical structures such as the scutellum (cotyledon) and the plumule (embryonic shoot) are present, whereas basal structures, such as the mesocotyl and the radicle, are missing (Hong et al., 1995) (Fig. 1A,E,F,J, Fig. 2A,G). The plumule, which comprises the shoot apical meristem (SAM) and three leaf primordia, appears normal in *ral1* (Fig. 1B,G), except that it is often oriented in a direction similar to that of the radicle in wild type and it has ectopic starch granules (Fig. 1A,E,F,J). In the scutellum of the *ral1* embryo, the provascular system differs from that of the wild-type. In wild type, a primary procambial bundle arises from the shoot apical region and extends along the median proximodistal axis of the scutellum (Fig. 1C,E). Upon reaching the tip, it branches into smaller secondary bundles that develop basipetally (Fig. 1D,E). In *ral1*, a normal primary procambial bundle arises from the shoot apical region, but the scutellar provascular system is reduced to a narrow strand that ends prematurely and without apical branching (Fig. 1H,I,J). Furthermore, serial sectioning shows that this procambial strand is discontinuous (Fig. 1H,J) and, within each file, procambial cells are improperly aligned (not shown).

In conclusion, the *RAL1* gene is required in the embryo for the formation of the basal pattern elements and for the orderly development of continuous procambial strands of all orders.

The *ral1* mutation affects different aspects of plant vegetative and reproductive development

To assess possible post-embryonic functions of the *RAL1* gene, we generated adult mutant plants, exploiting the capacity that mutant seedlings share with wild type of spontaneously producing adventitious roots (Fig. 2B,H). However, the *ral1* mutant develops fewer adventitious and lateral roots than the wild type (Fig. 2C,I; Table 1). Roots of *ral1* seedlings are more slender than either wild-type seminal or adventitious roots, because of a reduction in the number of xylem and phloem poles in the central vascular cylinder, and in the number of cortical cell layers (Fig. 1U-X; Table 1). Furthermore, the diameter of the metaxylem elements is reduced, whereas that of the cortical cell is increased (Fig. 1W,X; Table 1). Finally, in *ral1* roots, obvious deviations from the wild-type pattern of alignment of vascular elements, or interruptions in their files were never observed, when examined at the procambial stage or after differentiation (not shown).

At maturity, *ral1* plants are smaller, show increased apical dominance and have shorter leaves (Fig. 2D,J). Internodes of *ral1* plants are thinner, but there are significantly more vascular bundles that are closer to each other than in wild type (Fig. 1P,T, Table 1). Inflorescences of *ral1* plants produce normal looking, fertile spikelets (flowers) together with abnormal spikelets in an approximate ratio of one to three (Table 1). Abnormal spikelets in *ral1* appear narrower (Fig. 2E,K), because of the reduced development of the palea, the smaller of the two bracts enclosing the floral organs. Instead of the normal boat shape, in *ral1* this bract shows a flat triangular shape and is completely devoid of any vasculature (not shown). Finally, the abnormal spikelets differentiate four or five stamens, instead of the invariable six of wild type (Fig. 2F,L). The inflorescences of *ral1* do not differ with respect to the length of their axes or the number of primary branches, whereas the number of secondary branches per primary branch is significantly reduced (Table 1).

In summary, the *RAL1* gene acts on different aspects of post-embryonic organ development during both the vegetative and the reproductive phases. With regard to vascular development, we observed a reduction in the size of the vascular cylinder in the root, an alteration in the spatial arrangement of vascular bundles in the stem, and the absence of veins in one of the two floral bracts.

Leaf venation pattern is altered in the *ral1* mutant

The leaves of *ral1* plants appear normal in shape, but are smaller than in wild type (Table 1). Wild-type rice leaves show the typical striate venation pattern, in which major longitudinal veins of three orders, the midvein and the large and small veins, lie parallel along the proximodistal axis of the leaf, and are connected transversely by minor commissural veins (Kaufman, 1959). 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, as indicated in Table 1. A comparison between mature wild-type and *ral1* leaves revealed that all the venation pattern parameters are altered in the mutant (Table 1). In fact, the number of both the large veins and the small veins between two large veins is reduced (Fig. 1K,L). Furthermore, the distance between two adjacent longitudinal veins is reduced. Conversely, the distance between two adjacent commissural veins is increased, as is the area enclosed by two adjacent longitudinal veins and two adjacent commissural veins. Finally, four of the seven small veins normally present in the wild-type midrib region (Fig. 1M,N) are absent in *ral1* (Fig. 1Q,R). The alterations in vascular pattern parameters observed in *ral1* might result from a premature arrest in leaf development. According to this interpretation, mature *ral1* leaves would simply represent immature stages of wild-type leaves. To test this hypothesis, we examined the distance between adjacent longitudinal vascular bundles in a representative wild-type immature leaf population of either the same length or width as mature *ral1* leaves. The fact that there is no wild-type leaf population with both the same length and width as the *ral1* leaves suggests that the hypothesis of prematurely arrested development is not valid. Furthermore, in both cases the distance between longitudinal veins in *ral1* leaves was significantly smaller than that in wild-type (Table 1), indicating a fundamental alteration of their normal spatial regularity.

When analysed in transverse sections, all vascular bundles in *ral1* leaves showed the typical radial organisation of vascular tissues, with xylem towards the adaxial surface and phloem oriented towards the abaxial one. Furthermore, as in the root vascular cylinder, the diameter of (late) metaxylem elements was reduced in all leaf vascular bundles (Fig. 1O,S; Table 1). Finally, a smaller bundle sheath extension and subepidermal sclerenchyma was consistently observed in association with longitudinal veins of all orders (Fig. 1K-O,Q-S).

Taken together, these observations indicate that the *ral1*

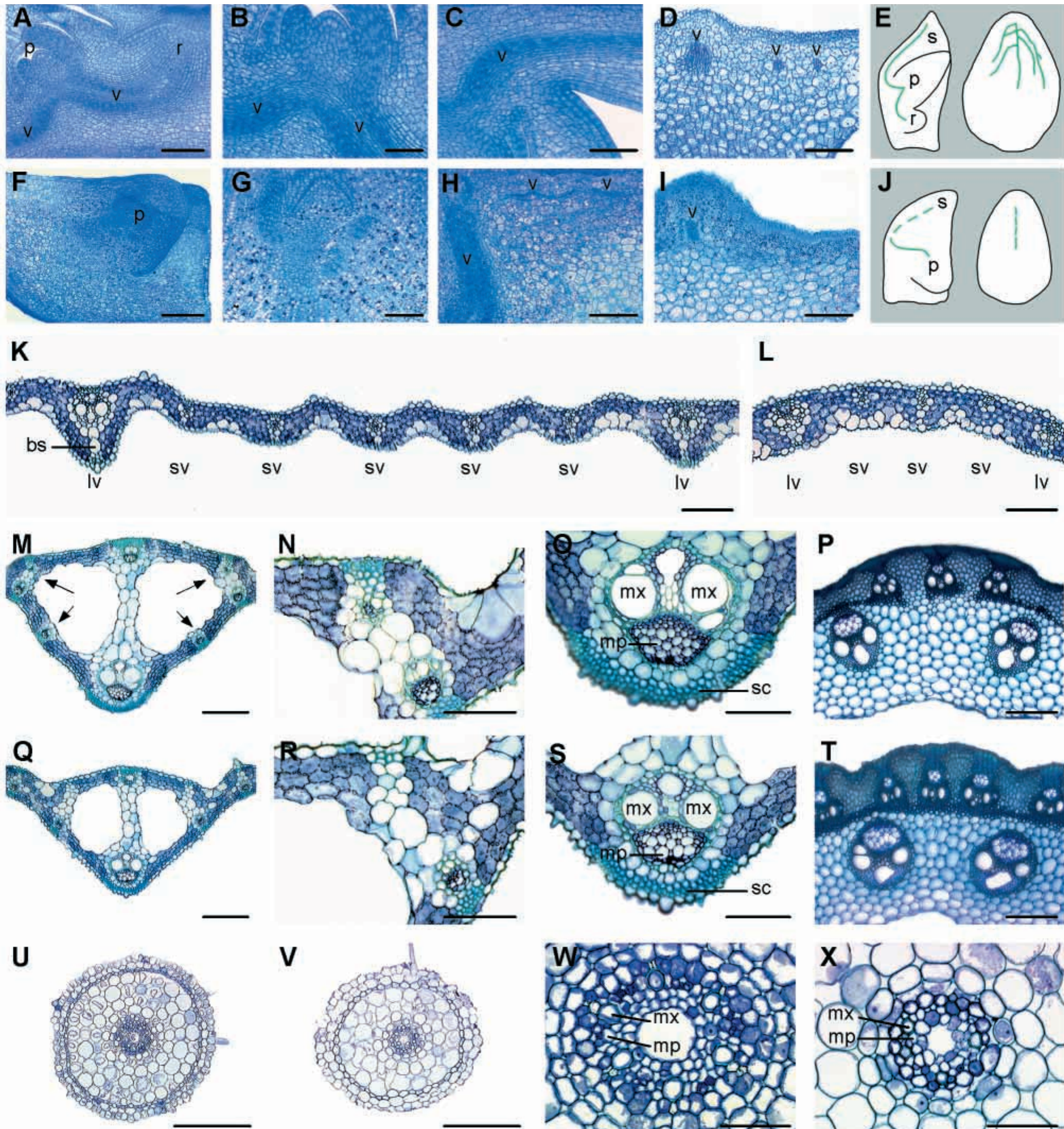

Fig. 1. Anatomy of wild-type and *ral1* mature embryos and plants. (A-E,K,M-P,U,W) Wild type. (F-J,L,Q-T,V,X) *ral1*. (A-C,F-H) Longitudinal sections through a mature embryo showing details of the embryonic axis (A,F), the shoot apical region (B,G) and the basal region of the scutellum (C,H). Note that the absence of vasculature in F and G, as compared with A and B, is due to the fact that in *ral1* the plumule lies slightly off the median plane. (D,I) Detail of the dorsal region of the scutellum in a transverse section through a mature embryo 180 μm below the scutellum tip. (E,J) Schematic representation of a median longitudinal section through a mature embryo (left) and of a dorsal view of a mature embryo (right). The embryonic provascular system is shown in green. (K,L) Region between two large veins in a transverse section through the middle of a mature leaf blade. (M,Q) Midrib region in a transverse section through the middle of a mature leaf blade. Arrows in M indicate the veins of the wild type that are missing in *ral1*. (N,R) Detail of the upper right-hand corner in M and Q, respectively. (O,S) Detail of the large vascular bundle of the midrib in M and Q, respectively. (P,T) Detail of transverse sections through the apical region of the first internode showing details of the internode wall. (U,V) Transverse section through an adventitious root 12 mm from the root tip. (W,X) Detail of the vascular cylinder in U and V, respectively. Toluidine Blue-stained granules in F,G,I showed typical blue-brown staining with iodine solution (not shown), revealing ectopic starch formation. bs, bundle sheath extension; lv, large vein; mp, metaphloem; mx, late metaxylem element; p, plumule; r, radicle; s, scutellum; sc, sclerenchyma; sv, small vein; v, provascular tissue. Scale bars: (A,F) 150 μm (B,D,G,I,K,L,N,O,R,S) 50 μm (C,H,M,Q,P,T,U,V) 100 μm (W,X) 25 μm.

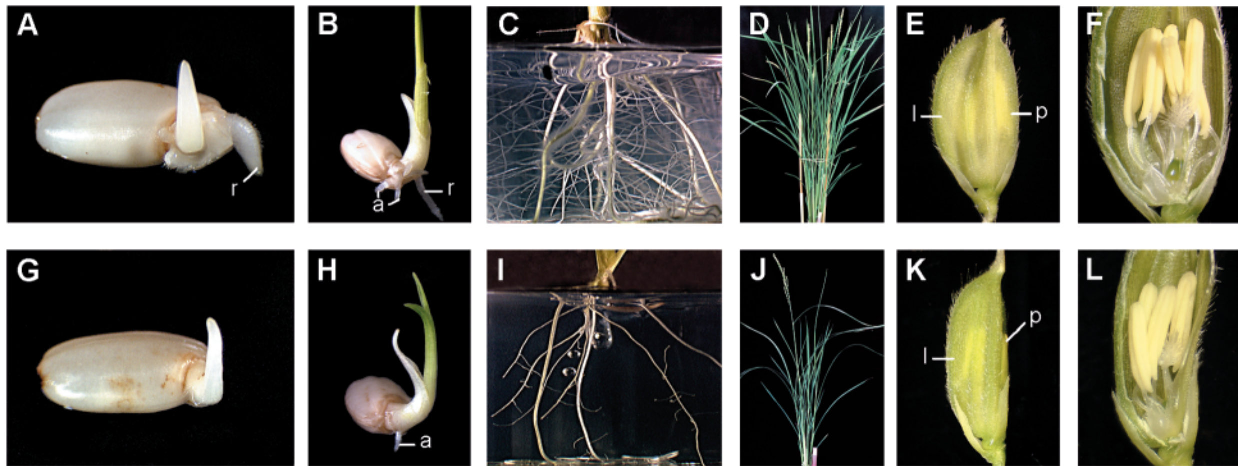

Fig. 2. Morphology of wild-type and *ral1* seedlings and mature plants. (A-F) Wild type. (G-L) *ral1*. (A,G) 3-day-old seedling. (B,H) 6-day-old seedling. (C,I) Root system of a 3-week-old seedling. (D,J) 6-month-old plant. (E,K) Mature spikelet. (F,L) Floral organs in a bisected spikelet. a, adventitious root; l, lemma; p, palea; r, radicle (seminal root). Corresponding *ral1* and wild-type images are at the same magnification.

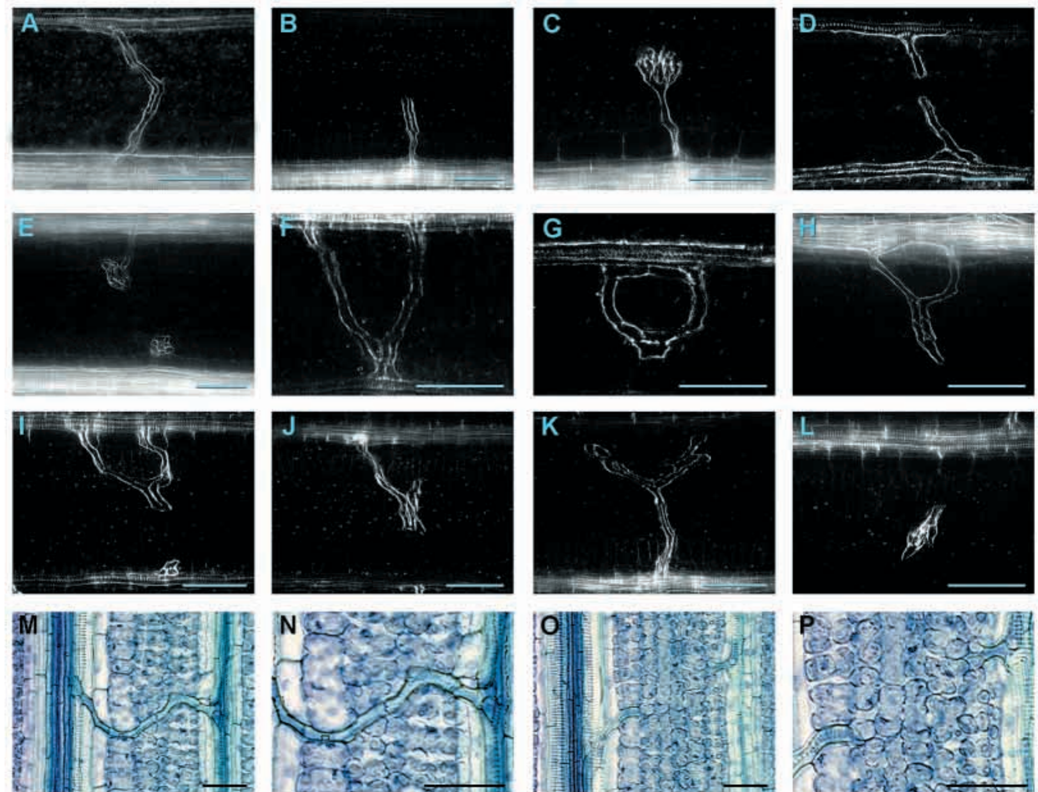

Fig. 3. Morphology and anatomy of wild-type and *ral1* leaf blade commissural veins. (A,M,N) Wild type. (B-L,O,P) *ral1*. (A-L) Dark-field images of cleared leaves. (A) Uninterrupted commissural vein connecting two longitudinal veins. (B,C) Interrupted simple (B) or compound (C) commissural vein associated with one longitudinal vein. (D,E) Interrupted simple (D) or compound (E) commissural vein associated with two longitudinal veins. (F) Uninterrupted 'Y' vein forming two connections with one longitudinal vein and one with the other. (G-K) Y veins showing interruptions at different positions. (L) Isolated patch of xylem elements (vascular island). (M-P) Paradermal sections through mature leaf blades. (M) Uninterrupted commissural vein connecting two longitudinal veins. (N) Detail of M. (O) Interrupted simple commissural vein associated with two longitudinal veins. (P) Detail of O. Scale bars: (A-L) 50 μm (M-P) 25 μm.

mutation affects the normal spatial arrangement of both longitudinal and commissural veins in the leaf, without altering their radial patterning. Moreover, the *RAL1* gene seems to be required for the correct development of non-vascular cell types organised around the veins.

Commissural vein development is altered in *ral1* leaves

In *ral1* leaves, the majority of the commissural veins can be classified as normal, in that, as in wild type (Fig. 3A,M,N), they develop a single uninterrupted connection with each of the two adjacent longitudinal veins. However, in approximately 40% of the commissural veins in the mutant leaves we could observe a range of aberrations that were tentatively grouped in three classes. The first class comprises interrupted commissural veins associated with one (Fig. 3B,C) or two (Fig. 3D,E) longitudinal veins. The interruptions can end with either a single (Fig. 3B,D) or a bunch of xylem elements (Fig. 3C,E).

Table 1. Morphometric analysis of wild-type and *ral1* plants

	Wild type		
	Seminal (root)	Adventitious (root)	*ral1*
Root			
Cortical cell size – radial (μm)	28.4±0.8 (40)	23.0±0.3 (107)	31.8±0.5 (101)***
Cortical cell size – tangential (μm)	33.2±1.0 (39)	26.8±0.5 (104)	37.1±0.6 (97)***
Metaxylem element size – radial (μm)	12.3±0.3 (38)	13.1±0.2 (37)	7.2±0.2 (28)***
Metaxylem element size – tangential (μm)	10.3±0.1 (36)	11.0±0.2 (37)	7.9±0.1 (30)***
Number of cortical cell layers	5.0±0.0 (10)	5.1±0.1 (15)	4.0±0.0 (20)***
Number of xylary poles	6.0±0.0 (10)	6.0±0.0 (14)	4.7±0.1 (20)***
Number of adventitious roots	5.2±0.3 (10)		3.0±0.2 (10)***
Number of lateral roots	77.8±6.3 (33)	48.9±5.0 (52)	8.8±2.4 (27)***
Root elongation (mm)	16.0±0.8 (20)	12.1±0.6 (82)	9.8±0.7 (35)***

	Wild type	*ral1*
Leaf		
Blade length (cm)	60.7±1.9 (40)	47.3±2.2 (46)***
Blade width (cm)	1.2±0.0 (40)	0.9±0.0 (46)***
Blade thickness (μm)	49.3±0.9 (48)	53.5±0.7 (71)***
Mesophyll cell size – longitudinal (μm)	9.4±0.1 (123)	9.7±0.1 (105)
Mesophyll cell size – radial (μm)	10.2±0.2 (100)	11.3±0.2 (102)***
Mesophyll cell size – tangential (μm)	14.5±0.4 (100)	16.2±0.4 (101)**
Metaxylem element size – radial (μm)	66.6±1.8 (24)	43.0±0.5 (24)***
Metaxylem element size – tangential (μm)	52.2±0.7 (24)	41.1±0.3 (24)***
Number of mesophyll cells	3.0±0.1 (45)	3.0±0.0 (57)
Number of LVs	9.2±0.2 (40)	7.2±0.2 (46)***
Number of SVs in between two adjacent LVs	5.0±0.4 (20)	3.1±0.1 (38)***
Distance between two adjacent LVs (μm):		
mature leaves	119.9±2.1 (70)	109.5±1.4 (73)***
immature wt leaves of same length/width as mature *ral1*	120.7±2.1 (21)/127.7±2.2 (22)	109.3±2.1 (31)**/***
Distance between two adjacent CVs (μm)	618.8±9.8 (66)	760.9±12.8 (74)***
Area enclosed by two CVs and two lVs (μm^2)	71866.0±2826.0 (40)	83274.6±2309.9 (48)**
Stem		
Number of vascular bundles in the outer ring	24.9±0.1 (11)	32.1±0.2 (14)***
Number of vascular bundles in the inner ring	13.2±0.1 (11)	14.1±0.1 (14)***
Flower		
Number of spikelets per panicle	152.1±8.9 (10)	126.6±7.3 (13)*
Number of fertile spikelets per panicle	143.9±7.9 (10)	33.8±2.4 (13)***
Number of primary branches per panicle	12.6±0.3 (10)	11.9±0.3 (13)
Number of secondary branches per primary branch	2.0±0.1 (125)	1.6±0.1 (155)***
Length of the panicle axis (cm)	26.6±0.6 (8)	27.2±0.3 (13)

Outer cortical cell and metaxylem element size, and number of cortical cell layers and of xylary poles were determined in digital microscope images of transverse sections taken 12 mm from the root tip. Number of adventitious and of lateral roots were determined in 2-week-old seedlings. Root elongation in 24 hours was monitored daily during a 1-month period. Leaf morphometric analyses were done on mature leaves of 6-month-old plants, unless otherwise indicated. Blade width and thickness, mesophyll cell size and number, metaxylem element size and vascular pattern parameters were measured through the middle region of the leaf blade. Blade thickness and mesophyll cell size and number (between the adaxial and the abaxial epidermis) were determined in the interveinal region using digital microscope images of transverse or longitudinal sections. Vascular pattern parameters were measured in dark-field microscopic digital pictures of cleared leaf preparations. The region of the leaf blade between the two most marginal adjacent large veins was excluded from all the measurements. Wild-type immature leaf populations were not significantly different in their length ($0.75<P\leq0.90$) or width ($0.90<P\leq0.95$) from the *ral1* mature leaf population. Number of stem vascular bundles in the outer and inner ring were determined in digital microscope images of transverse sections through the apical part of the first internode of plants at the ripen-inflorescence stage. Morphometry of flowers was performed on ripe inflorescences after harvesting. Morphometric analysis using digital images was performed with the ImageJ 1.21 software. Results represent the mean±s.e.m. of populations of the size indicated in parenthesis. Asterisks indicate the significance of the difference between wild-type and *ral1* populations as determined by single-factor ANOVA (root morphometry except for number of adventitious roots) or Student's *t*-test analysis (number of adventitious roots, leaf and flower morphometry): *$0.01\leq P<0.05$, **$0.001\leq P<0.01$, ***$P<0.001$. CVs, commissural veins, lVs, longitudinal veins; LVs, large veins; SVs, small veins.

In the second class, we grouped together commissural veins that develop two connections with one of the two longitudinal veins, and that therefore we refer to as 'Y' veins (Fig. 3F-K). Such Y veins can develop without any interruptions (Fig. 3F), or show discontinuities at different locations (Fig. 3G-K). Finally, the third class consists of isolated patches of xylem elements that form in the interveinal region, named vascular islands (Fig. 3L). The commissural vein defects in *ral1* were observed by dark-field illumination of cleared intact tissues, which reveals the presence of xylem elements, but not of procambial cells or other vascular cell types, such as phloem elements. Therefore, we examined the ends of the interrupted commissural veins in paradermal tissue serial sections of mature leaves and confirmed that these are not connected by any (pro)vascular cell file (Fig. 3O,P). Additionally, when analysed in transverse section, even the most aberrant commissural veins showed the typical radial organisation of xylem and phloem within the strand (not shown).

In conclusion, these results indicate that the *ral1* mutation affects the continuity of commissural veins and induces atypical branching in these veins without altering their radial tissue organisation.

Procambium formation during leaf development is delayed in *ral1*

To identify the earliest differences between wild-type and *ral1* vascular development, we decided to follow this process during leaf primordium formation. To this aim, we compared wild-type and *ral1* primordia close to their insertion onto the shoot apex, where differentiating vascular strands are in their most advanced stage of development. In wild type, a median procambial strand could be identified in the first primordium (Fig. 4A). In the second primordium, the median strand started to undergo vascular differentiation, as shown by the presence of protophloem elements (Fig. 4B). Finally, in the third primordium, the first two protoxylem elements had differentiated (Fig. 4C). In *ral1*, no anatomical sign of a median procambial strand could be detected in the first primordium (Fig. 4D), but a median strand, anatomically indistinguishable from that in wild type, could be detected in the second primordium (Fig. 4E). Therefore, in *ral1*, procambial strand formation during leaf primordium development is delayed compared with wild type. However, the median strand in the third primordium of *ral1* appears to be at the same differentiation stage as in wild type, judging from the presence of protophloem and two protoxylem elements (Fig. 4F). This suggests that vascular differentiation occurs more rapidly in *ral1* than in wild type. In fact, whereas in wild type two plastochrons divided the formation of a procambial strand from the stage where protophloem and two protoxylem elements could be distinguished, in *ral1* the same process required one plastochron only. Furthermore, in *ral1* leaf primordia, procambial strands arise significantly ($P<0.001$) closer to each other (126.0 μm±3.5, n=10) than in wild type (182.3 μm±6.9, n=10).

In summary, the *RAL1* gene is required for the initiation of procambial strands at the correct stage of leaf primordium development. However, the delayed procambial formation in *ral1* seems to be compensated for by faster vascular differentiation.

Procambial expression of the auxin-responsive DR5-GUS marker is absent in *ral1*

To reveal possible other developmental differences in the (pro)vascular strands of wild type and *ral1*, we monitored DR5-GUS expression. The endogenous and inducible pattern of expression of this marker has been used to monitor auxin responses at the cellular level (Sabatini et al., 1999). In wild type, DR5-GUS expression was clearly observed in the median procambial strand of the first primordium, and from that stage it marked the presence of all strands as soon as they could be anatomically identified (Fig. 4G). In *ral1*, procambial DR5-GUS expression was only ever observed in the two procambial strands next to the differentiating midvein in the third leaf primordium (Fig. 4H). Furthermore, DR5-GUS expression during vascular differentiation is also altered in *ral1*. In fact, in vascular strands of the fourth leaf primordium, DR5-GUS is expressed in differentiating xylem and metaphloem in wild type (Fig. 4O), whereas in *ral1*, expression is restricted to the differentiating third protoxylem element (Fig. 4S). In vascular bundles of the fifth leaf primordium, DR5-GUS expression is restricted to differentiating metaphloem in wild type (Fig. 4P), whereas in *ral1*, expression is additionally detected in protoxylem parenchyma (Fig. 4T).

In conclusion, the lack of DR5-GUS expression in early procambial development indicates that in leaves the *RAL1* gene is required for the procambial subdomain of DR5-GUS expression, and suggests that in *ral1*, (pro)vascular strands that are anatomically indistinguishable from wild-type ones have a reduced endogenous response to auxin.

Expression pattern of the procambium specification marker Oshox1-GUS is altered in *ral1*

To further analyse the nature of the vascular defects of *ral1*, we monitored the expression of a second marker, the Oshox1-GUS gene reporter. The onset of Oshox1-GUS expression marks a stage in procambium development at which cell fate has been specified, but not stably determined, towards vascular differentiation (Scarpella et al., 2000). Oshox1-GUS expression therefore can visualise differences in developmental potential of procambial cells, even in the absence of any anatomical difference. In wild type, Oshox1-GUS expression could be first detected in the median strand of the second primordium approximately 100 μm above its insertion onto the SAM (103.8 μm±14.6, n=5). Therefore, it was clearly visible in this strand in a section taken at the level of the SAM, that is one plastochrone after procambium formation (Fig. 4I). In *ral1*, we could not detect significant differences in the onset of Oshox1-GUS expression. In fact, in *ral1* Oshox1-GUS expression could be first detected in all strands of the third leaf primordium, that is again one plastochrone after their emergence (Fig. 4J). However, Oshox1-GUS expression during vascular differentiation is altered in *ral1*. In wild type, Oshox1-GUS expression remains present in all vascular cells, eventually disappearing only in the specific elements that (selectively) lose their cellular contents upon terminal differentiation (xylem tracheary and phloem sieve elements; Fig. 4Q,R). In contrast to wild type, Oshox1-GUS expression in *ral1* is absent from the mature phloem in the fourth and fifth leaf primordium, and the level of expression is much lower in all vascular cell types (Fig. 4U,V). Therefore, the *RAL1* gene is required for the correct Oshox1-GUS expression pattern in different subpopulations of differentiating and differentiated vascular cells, although it seems not to be required for the onset of Oshox1-GUS expression in the procambium.

Finally, in *ral1* embryos Oshox1-GUS expression was absent from the aberrant provasculature of the scutellum (Fig. 4X,Z), whereas expression marked the complete procambial system of wild-type embryos (Fig. 4W,Y). Furthermore, in *ral1*, Oshox1-GUS expression was much reduced in the procambial bundle that arises from the shoot apical region and that was anatomically indistinguishable from wild type. These observations may either point to an impaired procambium specification in the *ral1* embryo or be a reflection of a delay in procambium initiation during embryogenesis.

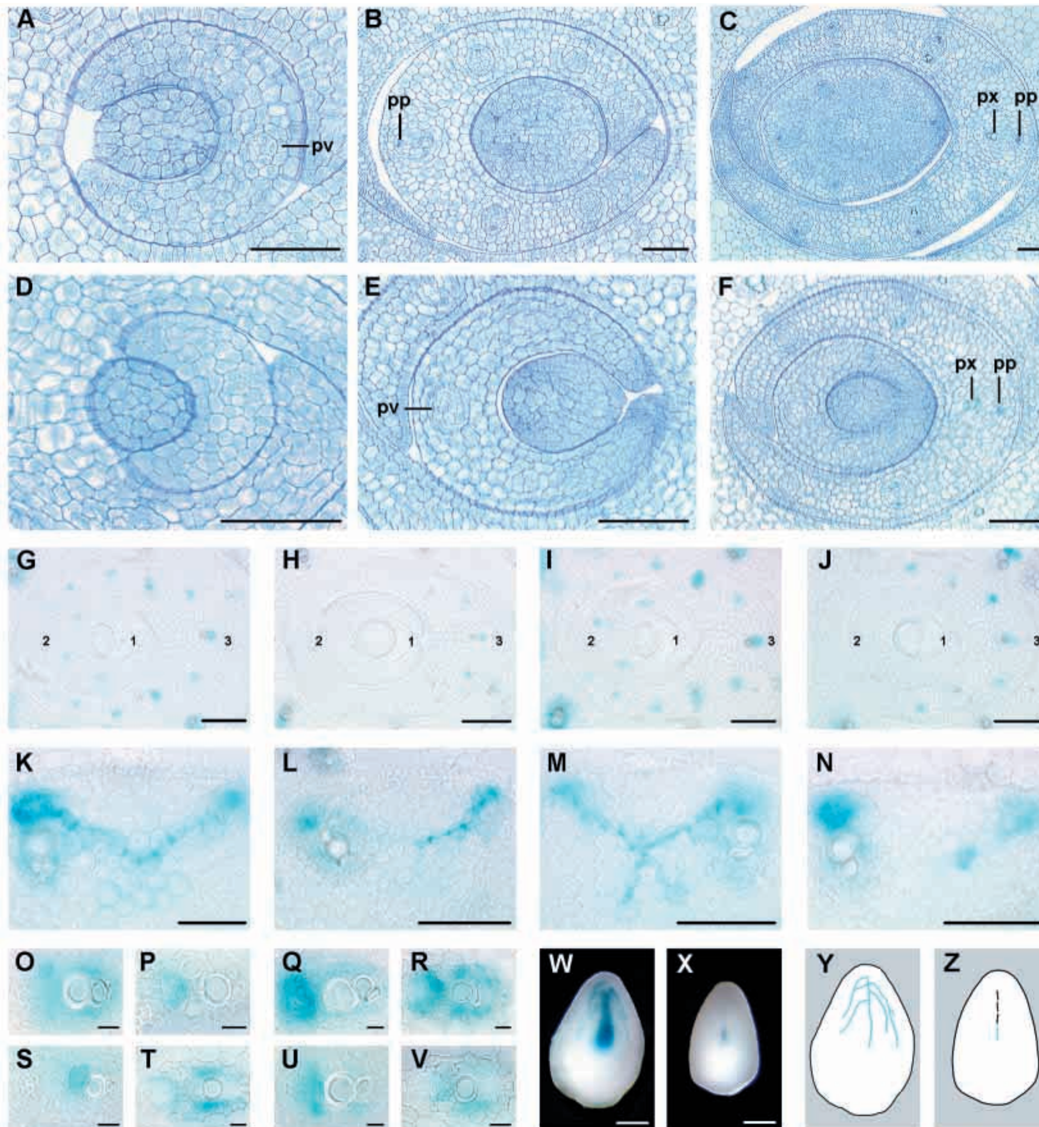

Fig. 4. Vascular development in wild type and *ral1*. (A-C,G,I,K,O-R,W,Y) Wild type. (D-F,H,J,L-N,S-V,X,Z) *ral1*. (A-F) Transverse sections through 2-week-old seedlings, 10 µm above the insertion of the first (A,D), second (B,E) and third (C,F) leaf primordium on the shoot apex (sixth, fifth and fourth leaf, respectively). (G-J) DR5-GUS (G,H) or Oshox1-GUS (I,J) expression in transverse vibratome sections (100 µm) through the shoot apex of 2-week-old seedlings. (K-N) Transverse section 200 µm above the shoot apex of 2-week-old seedlings showing Oshox1-GUS expression that identifies commissural veins developing in the fourth leaf primordium (third leaf). (O-V) DR5-GUS (O,P,S,T) or Oshox1-GUS (Q,R,U,V) expression in vascular bundles at a comparable stage of differentiation in the fourth (second protoxylem element stage; O,S,Q,U) and fifth (late metaxylem element stage; P,T,R,V) leaf primordium (third and second leaf, respectively). Xylem is oriented to the right. (W,X) Oshox1-GUS expression in mature embryos (dorsal view). (Y,Z) Schematic representation of the dorsal view of mature embryos showing the vascular system of the scutellum expressing (in blue) or not (in black) Oshox1-GUS. 1, 2, 3, first, second, and third leaf primordium, respectively; pp, protophloem; pv, provascular strand; px, protoxylem. Scale bars: (A-F) 100 µm (G-N) 50 µm (O-V) 25 µm (W,X) 350 µm.

Commissural vein defects in *ral1* leaves originate at the procambial stage

The interruptions in commissural veins of *ral1* could originate either directly from a discontinuous procambium formation or from a subsequent reversion of procambial cell identity within a continuous procambial strand. All anatomical studies of commissural vein development in monocot leaves strongly suggest that in each of these veins all procambial cells appear simultaneously, such that the commissural procambial strand is formed at once in a continuous fashion between the longitudinal veins (Kaufman, 1959; Blackman, 1971; Dannenhoffer and Evert, 1994; Dengler et al., 1997) (our own observations). However, unlike all other procambial strands, the early stages of commissural procambial strand formation cannot always be unambiguously distinguished in tissue sections of developing leaf primordia. Because of the absence of procambial DR5-GUS expression in *ral1*, Oshox1-GUS is currently the earliest available marker of procambial identity in rice. In wild type, Oshox1-GUS expression appeared simultaneously in all the procambial cells connecting two adjacent longitudinal veins (Fig. 4K). Oshox1-GUS expression also appeared simultaneously in the developing commissural

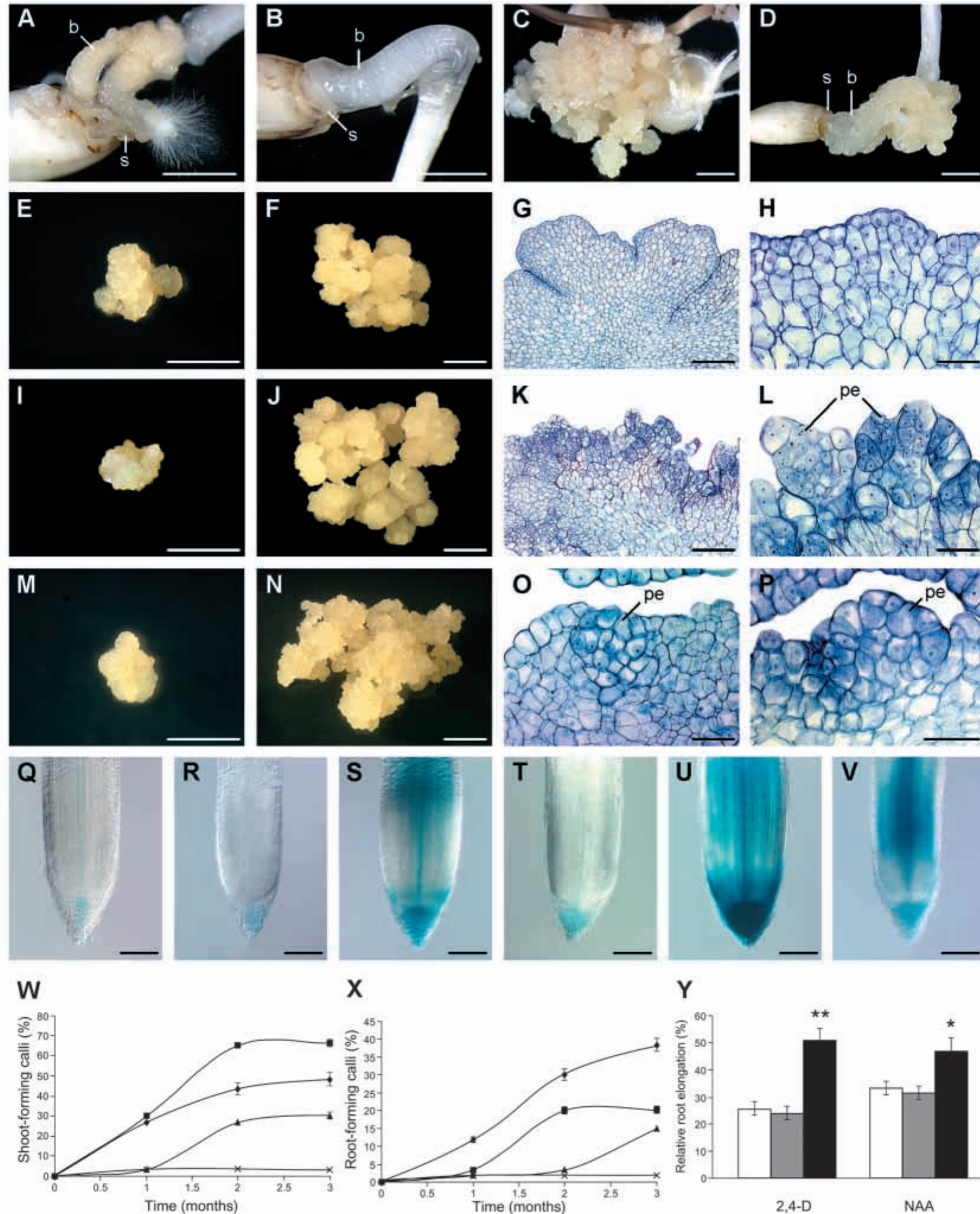

Fig. 5. Hormonal responses of wild type and *ral1*. (A,C,E-H,M-P,Q,S,U) Wild type. (B,D,I-L,R,T,V) *ral1*. (A-D) Seedlings 1 week (A,B) or 3 weeks (C,D) after callus induction on 2 mg/l 2,4-D. (E,F,I,J,M,N) Calli at the stage of transfer to the induction medium (E,I,M) and 3 weeks after (F,J,N) the transfer. Medium contained either 2 mg/l 2,4-D (E,F,I,J) or 1 mg/l 2,4-D (M,N). (G,H,K,L,O,P) Sections through the calli in F (G,H), in J (K,L), or in N (O,P). (Q-V) DR5-GUS expression in the root of 1-week-old seedlings grown for 24 hours on filter paper moistened with water (Q,R), with 0.1 (S,T) or 1 (U,V) μM NAA. (W,X) Frequency of shoot (W) or root (X) regeneration via somatic organogenesis in callus tissues grown on hormone-free medium (black diamond, wild type; black triangle, *ral1*) or on medium supplemented with cytokinin (black square, wild type; cross, *ral1*). The results represent the mean±s.e.m. of two separate experiments each performed on a population of 80-100 calli per genotype and per treatment. Difference between wild-type and *ral1* populations as determined by repeated-measures analysis of variance (single-factor ANOVA) was significant ($P<0.001$) at all time points. (Y) Relative elongation over 24 hours of wild-type seminal (white boxes) and adventitious (grey boxes) roots and *ral1* (black boxes) roots in the presence of 0.05 μM 2,4-D or 0.1 μM NAA. The results represent the mean±s.e.m. of two separate experiments each performed on a population of 20-35 seedlings per genotype and per treatment. Asterisks indicate the significance of difference between wild-type and *ral1* populations as determined by repeated-measures analysis of variance (single-factor ANOVA). *$0.01\leq P<0.05$, **$0.001\leq P<0.01$. b, shoot base; pe, proembryonic structure; s, scutellum. Scale bars: (A-D,E,F,I,J,M,N) 2 mm (G,K) 100 μm (H,L,O,P) 25 μm (Q-V) 50 μm.

veins of *ral1*, but almost invariably there were interruptions in continuity (Fig. 4L), ectopic expression (Fig. 4M) or isolated patches of expression in the interveinal regions (Fig. 4N). Each of these aberrations could be related to one of the classes of defects observed in mature commissural veins, namely interrupted veins, Y veins and vascular islands. Since the continuous expression of the Oshox1-GUS marker correctly predicts the differentiation of both the uninterrupted veins in wild type and of the uninterrupted Y veins in *ral1*, it is likely that the interrupted commissural veins and the vascular islands in *ral1* may result from discontinuities in procambium formation, with consequent fragmented Oshox1-GUS expression. Furthermore, the fact that procambium interruptions could indeed be detected in *ral1* embryos also argues in favour of this hypothesis.

Taken together, these observations indicate that the aberrations in commissural vein development in *ral1* leaves derive from defects occurring at the procambium stage. However, we cannot discriminate between whether they arise during procambium formation or cell fate specification.

The *ral1* mutant displays reduced sensitivity towards auxin

Because of the importance of auxin in various aspects of vascular development, and because of the phenotypic similarities between *ral1* and the auxin-resistant *mp*, *bdl* and *axr6 Arabidopsis* mutants, it is conceivable that the *ral1* phenotype may be related to alterations in the perception or response to auxin. The first indication of an impaired auxin response in *ral1* was the absence of DR5-GUS expression in procambial strands of mutant leaf primordia, as described above. We therefore tested this hypothesis by examining the capacity of *ral1* seedlings to form callus tissues in response to the auxin analogue 2,4-D. In wild type, the first signs of callus formation were detected at the level of the scutellum and at the base of the shoot 1 week after germination of the seeds in the dark on a medium containing 2 mg/l 2,4-D (Fig. 5A), and after 3 weeks massive callus production was observed (Fig. 5C). Callus induction in *ral1* was delayed over a week (Fig. 5B,D), and the response to 2,4-D was spatially restricted, in that the scutellum showed complete insensitivity towards 2,4-D-induced callus formation (Fig. 5D). When calli were explanted to new medium, growth in *ral1* appeared slightly enhanced compared with wild type (Fig. 5E,F,I,J). This can be explained by the fact that in rice the optimum concentration of 2,4-D for callus induction is higher (≥2 mg/l 2,4-D) than for callus growth (1 mg/l 2,4-D) (Yatazawa et al., 1967). Indeed, the enhanced growth of *ral1* calli could be phenocopied by growing wild-type calli with a lower concentration of 2,4-D (1 mg/l; Fig. 5M,N). This suggests that, like callus induction, callus growth in *ral1* is less sensitive to 2,4-D. In addition to the growth pattern, callus tissue organisation is also altered in *ral1*, the most obvious difference being the proliferation of somatic proembryonic structures at the periphery of the callus. This characteristic is absent from wild-type calli grown on 2 mg/l 2,4-D, but can be induced in wild-type calli grown on 1 mg/l 2,4-D (Fig. 5O,P), although to a lesser degree than in *ral1* calli grown on 2 mg/l 2,4-D.

In order to obtain further independent evidence of the defects in auxin perception or response in *ral1*, we compared the effectiveness of exogenous auxins to inhibit root elongation in wild-type and *ral1* seedlings. Roots of *ral1* seedlings elongate less than both wild-type seminal and adventitious roots (Table 1). However, *ral1* roots elongate approximately 2-fold more than wild-type roots in the presence of 0.05 μM 2,4-D and 1.5-fold more than wild-type roots in the presence of 0.1 μM of the auxin analogue NAA (Fig. 5Y). This reduced sensitivity of *ral1* roots to auxin analogues could also be shown using the auxin-responsive DR5-GUS reporter. In wild-type seminal and adventitious roots, we could detect a peak of DR5-GUS expression in all columella cells of the root cap and in the quiescent centre (Fig. 5Q). Additionally, we could detect a fainter procambial expression. In *ral1* roots, DR5-GUS expression was restricted to the mature columella cells (Fig. 5R), suggesting that in roots, as in leaves, the *RAL1* gene is required for DR5-GUS expression in the procambium. After treatment with 0.1 μM NAA or with 0.05 μM 2,4-D, DR5-GUS expression was ectopically induced in wild-type roots, but not in *ral1* (Fig. 5S,T). This defect in DR5-GUS inducibility could be largely rescued by increasing the concentration of auxin analogues to 1 μM (Fig. 5U,V). Consistently, the root elongation response in *ral1* was indistinguishable from wild type at concentration of auxin analogues of 1 μM (not shown).

In conclusion, multiple and independent lines of evidence indicate that the *RAL1* gene is required for different spatial and temporal aspects of a proper auxin perception or response.

The *ral1* mutant displays enhanced sensitivity towards cytokinin

Auxin and cytokinin interact in a complex fashion to control many aspects of plant development. More specifically, a large number of studies suggest a role for these two plant hormones in vascular tissue differentiation (Sachs, 1981; Aloni, 1995; Fukuda, 1996; Berleth et al., 2000; Mähönen et al., 2000; Inoue et al., 2001). Therefore, we decided to investigate whether the *ral1* mutation interferes with cytokinin perception or response. Shoot regeneration via direct organogenesis from callus tissues is a convenient system to test this hypothesis, in that regeneration can be stimulated by cytokinin application (Skoog and Miller, 1957; Sugiyama, 1999; Sugiyama, 2000). Addition of the cytokinin N^6-benzyladenine (BA) to the regeneration medium increased the number of shoots that differentiated from wild-type rice calli (Fig. 5W,X). Surprisingly, in spite of their enhanced embryogenicity (Fig. 5K,L), *ral1* calli produced fewer shoots than wild type calli (Fig. 5W). Furthermore, when BA was added to the medium, shoot regeneration from *ral1* calli was virtually abolished (Fig. 5X). Even prolonged culture of *ral1* calli on medium with or without BA did not improve shoot organogenesis, suggesting that the reduced regeneration capabilities were not simply due to a delay in the onset of the developmental programme that leads to shoot organogenesis. Unlike shoot regeneration, root formation was inhibited in wild-type calli by the presence of cytokinin (approximately 47.4% inhibition; Fig. 5W,X). Consistently with their enhanced sensitivity to cytokinin-induced shoot formation, root organogenesis in *ral1* calli was more inhibited in the presence of BA compared with wild type (approximately 86.7% inhibition; Fig. 5W,X).

In summary, these observations indicate that shoot and root development via direct organogenesis in *ral1* calli are hypersensitive to cytokinin.

DISCUSSION

We have described here that the *ral1* mutant of rice displays distinctive embryonic and post-embryonic vascular pattern defects, including altered spacing of parallel veins, interruptions in vein continuity, anomalous presence of branching veins and altered timing of pattern formation. Furthermore, we have shown that all the vascular patterning defects of *ral1* arise at the earliest recognisable stage of vascular development, the procambium. Additionally, we have demonstrated by means of multiple independent assays that the *ral1* mutant is defective in auxin perception or response. Finally, we have shown that mutation in the *RAL1* gene is associated with enhanced sensitivity to cytokinin. Although attempts to identify mutants in vascular pattern formation in monocot species have been made (Fladung, 1994) (Timothy Nelson, personal communication), to our knowledge this represents the first report of a monocot mutant genuinely impaired in procambium development and vascular patterning.

The *ral1* embryo displays specific and reproducible alterations of the spatial arrangement of the procambium that point to a role for the *RAL1* gene in controlling cell axialisation in this tissue. In fact, in the scutellum of the *ral1* embryo, the primary procambial bundle is reduced to a short, narrow and discontinuous strand. Furthermore, within this prematurely aborted strand, procambial cells are misaligned with respect to each other. Finally, secondary procambial strands are completely absent. In *Arabidopsis*, it has been observed that mutations at the *MONOPTEROS* (*MP*), *COTYLEDON VASCULAR PATTERN* (*CVP*) *1* and *2*, *SCARFACE* (*SFC*) and *VASCULAR NETWORK3* (*VAN3*) loci show largely intact primary procambial veins in the cotyledons, whereas secondary procambial veins are discontinuous or missing (Berleth and Jürgens, 1993; Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000). It has been proposed therefore that primary vein formation might be under the control of a different pathway than that specifying patterning of veins of higher order (Deyholos et al., 2000), or that the regulatory systems controlling the formation of different classes of veins display a different genetic robustness (Koizumi et al., 2000). The *ral1* mutant is unique in that the embryonic scutella, which are homologous to the cotyledons of dicot embryos, are also defective in the continuity of primary procambial veins. Mutation in a single gene is thus sufficient to affect the patterning of both the primary vein and higher-order veins. This suggests that the pathways that specify the orderly formation of different orders of procambial strands are not necessarily genetically separated in monocots, at least during embryogenesis.

At post-embryonic stages, mutation in the *RAL1* gene affects the overall leaf venation patterning. Opposite effects were observed on longitudinal vein spacing (decreased) versus commissural vein spacing (increased). This might be related to differences between the patterning processes of these two types of veins and/or the separation of these processes in time (Blackman, 1971; Dannenhoffer et al., 1990; Dannenhoffer and Evert, 1994; Dengler et al., 1997). The venation pattern alterations in *ral1* represent a genuine effect that is not a consequence of prematurely arrested leaf development or a defect in leaf morphogenesis. Early leaf development coincides with major vein appearance, and many of the *Arabidopsis* and maize leaf shape mutants display vascular patterning aberrations, suggesting that the same factors may play a regulatory role in both processes, or that one influences the other (Dengler and Kang, 2001; Schneeberger et al., 1995; Semiarti et al., 2001; Scanlon et al., 2002). Similarly to the *mp*, *cvp2*, *sfc*, *van3* and the *hemivenata* (*hve*) mutants of *Arabidopsis* (Przemeck et al., 1996; Candela et al., 1999; Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000), mutation in the *RAL1* gene specifically affects the vascular pattern of the leaf without causing any major alteration of leaf shape, thus arguing for a specific role of the *RAL1* gene in the regulation of vascular pattern formation. In further support of this is the observation that the earliest detectable defect in *ral1* leaf histogenesis is the delayed formation of procambial strands. Such delay in procambial strand formation has never been reported before for other mutants and provides evidence that procambium initiation and leaf primordium development can be genetically uncoupled. We have shown here that in wild-type rice, the onset of the expression of the auxin-inducible reporter DR5-GUS presages the sites of vascular differentiation. The procambial strands that are eventually formed in *ral1* leaf primordia, although anatomically indistinguishable from wild type, display a reduced endogenous response to auxin, as shown by the lack of DR5-GUS expression. This might indicate that, in *ral1*, procambium is formed through an alternative pathway that would compensate for the reduced or lost *RAL1* gene function. This rescue mechanism would involve genes able to take over at least part of the *RAL1* gene function in procambium formation. This interpretation might also explain the absence of defects in continuity of longitudinal veins in *ral1* leaves, and fit with the idea that functionally redundant mechanisms would control the formation of lower order of veins in dicot leaves (Koizumi et al., 2000).

After delayed procambial strand formation, vascular differentiation seems to occur more rapidly in *ral1* than in wild type. This might explain why we invariably observed a reduction in xylem element diameter. In fact, a narrow element could result from a rapid secondary wall differentiation, which would allow only limited time for cell expansion (Aloni and Zimmermann, 1983). Furthermore, analysis of the spatial and temporal aspects of the expression of the DR5-GUS and Oshox1-GUS markers during vascular development suggests that different subpopulations of procambial cells within one strand undergo vascular differentiation at different time points than in wild type. This could indicate that the *RAL1* gene has a function in the coordinated entrance of different, but anatomically indistinguishable, subsets of procambial cells into the vascular differentiation pathway. However, the relevance of such hypothetical synchronised process is not clear, in that in *ral1*, even in the most aberrant veins, all vascular cell types seem to be present at maturity.

Whereas the continuity of longitudinal veins in *ral1* is not affected, severe defects are present in commissural vein development, which eventually result in strand discontinuities, formation of aberrantly branching veins and development of isolated patches of vascular cells. Using the Oshox1-GUS reporter construct as a marker for procambial cell fate specification, we showed that, similar to the defects in global patterning of different orders of veins, the aberrations in commissural vein development originate at the procambial

stage. The alterations in the earliest signs of Oshox1-GUS expression perfectly simulate the range of commissural vein phenotypes that can be detected in mature *ral1* leaves. However, although virtually all developing commissural procambial strands in *ral1* displayed such aberrations in Oshox1-GUS expression, when *ral1* leaves were analysed at maturity, no more than 40% of all commissural veins displayed any detectable defect. This could suggest that early defects occurring at the procambial stage can somehow be rescued during vascular differentiation, as discussed above. This observation is in perfect agreement with the high level of flexibility that vascular tissues have been reported to display under different experimental conditions (e.g., Sachs, 1981; Sachs, 1989; Mattsson et al., 1999; Sieburth, 1999).

In the embryo, mutation in the *RAL1* gene seems to have a more dramatic effect on vascular development than in post-embryonic stages. In fact, in *ral1* embryos all orders of veins are affected in their development and display altered levels of Oshox1-GUS expression. This could suggest that the proposed rescue mechanism may play a role in the normalisation of the early vascular defects in the *ral1* mutant by partially replacing *RAL1* gene function in postembryonic vascular development, but not during embryogenesis. Alternatively, the function of the *RAL1* gene could be predominantly embryonic, and its role during post-embryonic stages may become restricted to a subset of functions in vascular development. Unlike the *ral1* mutant, *mp*, *cvp2*, *sfc* and *van3 Arabidopsis* mutants show defects in leaves similar to those in cotyledons (Przemeck et al., 1996; Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000). In this regard, it is interesting to notice that scutella show a vascular pattern that is more similar to that of dicot cotyledons than that of monocot leaves, in that the primary vein shows apical branching. Furthermore, auxin-induced callus formation readily occurs in embryonic or post-embryonic foliar organs with a branched venation pattern [monocot scutella and dicot cotyledons and leaves (Schmidt and Willmitzer, 1988; Rueb et al., 1994)], whereas foliar organs with a striate venation pattern (monocot leaves) do not form callus in response to auxin (Wernicke et al., 1981). Therefore, factors upstream of *RAL1* gene function, such as organ-specific auxin sensitivity and growth pattern, could be involved in determining the type of vascular pattern that will be eventually formed in leaves or scutella, possibly by a differential regulation of *RAL1* gene expression in these organs. These upstream factors could thus be responsible for the organ-specific appearance of the vascular pattern defects in *ral1*.

In association with the aberrant vascular pattern formation, we observed in the *ral1* mutant a reduced auxin response. A similar situation holds true for the *mp*, *bdl* and *axr6* mutants (Berleth and Jürgens, 1993; Przemeck et al., 1996; Hardtke and Berleth, 1998; Hamann et al., 1999; Hobbie et al., 2000). However, other vascular development mutants do not show altered auxin responses (Zhong et al., 1999; Carland et al., 1999; Candela et al., 2001; Zhong and Ye, 2001) and the *sfc* mutant shows an enhanced response to auxin (Deyholos et al., 2000). Furthermore, some of the mutants originally isolated because of an altered response to exogenously administrated auxin also display vascular development aberrations (e.g., Lincoln et al., 1990; Hobbie et al., 2000). Currently, we cannot determine any causal relationship between the defects in vascular development and the altered auxin response of the *ral1* mutant. It is possible that the reduced auxin sensitivity could be a consequence of the altered vascular development, since vascular tissues represent the preferential pathway through which auxin is transported (Lomax et al., 1995). Alternatively, primary defects in auxin perception or response could give rise to the vascular defects of *ral1*. Treatments of wild-type rice leaves with increasing concentrations of polar auxin transport inhibitors increase the distance between longitudinal veins and decrease that between commissural veins (Scarpella et al., 2002). Therefore, ectopic accumulation of auxin near source regions in the wild-type rice leaf results in vascular pattern alterations opposite to those induced by the *ral1* mutation. This is consistent with the possibility that the *ral1* vascular patterning defects might originate from a reduced sensitivity to vascular-inducing auxin signals. Similarities between the additional phenotypes of *ral1*, such as defective embryonic axis establishment, impaired adventitious and lateral root formation, increased apical dominance and abnormal flower development, and phenotypes of the *mp*, *bdl*, *axr6* and other primary auxin response mutants of *Arabidopsis* (e.g., Lincoln et al., 1990; Liscum and Reed, 2002), also suggest this possibility. Furthermore, the presence of these phenotypes in the *ral1* mutant seems to indicate that the *RAL1* gene, just like *MP*, *BDL* and *AXR6*, possesses patterning functions beyond the vascular system. This observation raises the issue of how patterning of the vascular tissues is coherently integrated with that of the surrounding tissues and organs in these mutants. Two main scenarios seem possible (Berleth et al., 2000). In the first, vascular patterning genes would act exclusively in incipient vascular tissues to control vascular differentiation in response to a polarising signal. Vascular tissues, in turn, would provide a scaffold system, in reference to which numerous morphological features would be organised. Alternatively, vascular patterning genes could be part of a more general cell polarisation mechanism that would mediate oriented cell differentiation in embryos, organ primordia and, most critically, in vascular strands. Currently available evidence seems to support both interpretations (Berleth and Jürgens, 1993; Przemeck et al., 1996; Hamann et al., 1999; Hamann et al., 2002; Sabatini et al., 1999; Hobbie et al., 2000; Nakajima et al., 2001). In any case, it is of particular significance that in both monocots and dicots, which display radically different embryo and vascular pattern formation and auxin sensitivity properties, mutation in single genes can result in defects in these processes that are essentially comparable. This suggests that, regardless of the ultimate phenotypical outcomes, the molecular mechanisms underlying these developmental processes are likely to be conserved in monocots and dicots.

It is more difficult to reconcile the *ral1* vascular patterning defects with the increased response towards cytokinin measured in the mutant. Cytokinin has long been known for its role in promoting procambial cell division and vascular differentiation in cultured tissues or in plants engineered to overproduce this hormone (Shininger, 1979; Aloni, 1995), and the recent cloning of the *WOODEN LEG*/*CYTOKININ RESPONSE1* (*WOL*/*CRE1*) gene has provided novel evidence of a role for cytokinin in vascular development. The *WOL*/*CRE1* gene encodes a cytokinin receptor, and is expressed in the procambium of the embryonic axis (Mähönen

et al., 2000; Inue et al., 2001). Mutation in the *WOL*/*CRE1* gene leads to differentiation of all procambial cells in the root and the basal part of the hypocotyl into protoxylem, a defect that has been associated with a reduced division activity of procambial cells (Scheres et al., 1995; Mähönen et al., 2000). A similar reduction in procambial cell division activity might be responsible for the reduced vascular cylinder in *ral1* roots. However, unlike *wol*/*cre1* mutants, the *ral1* defect does not affect the differentiation of any vascular cell type in particular within the root vascular cylinder. Furthermore, *wol*/*cre1* mutants display a reduced sensitivity to cytokinin, whereas the *ral1* mutant shows a hypersensitive response to this hormone. Alternatively, defects in cytokinin perception or response in the *ral1* mutant could be a consequence of the altered auxin sensitivity. In fact, these two hormones interact in a complex manner in plant development, and certain processes are regulated in an antagonistic fashion by them (Coenen and Lomax, 1997; Swarup et al., 2002). Furthermore, genetic analysis in *Arabidopsis* seems to suggest that the response to these two hormones is integrated at the molecular level (Swarup et al., 2002). Like *ral1*, mutation in the *POLARIS* (*PLS*) gene of *Arabidopsis* has also recently been associated with reduced vascularisation in the leaf, auxin resistance and cytokinin hypersensitivity (Casson et al., 2002). However, the *pls* mutant does not display any embryo defect. Therefore, the *RAL1* gene is unlikely to be molecularly identical to *PLS*.

Although alternative interpretations have been suggested (e.g. Kull and Herbig, 1995; Aloni, 2001), mainly two, not mutually exclusive, hypotheses have been proposed to explain the different aspects of vascular pattern formation: the signal-flow canalisation hypothesis (Sachs, 1981; Sachs, 1989), and the reaction-diffusion hypothesis (Meinhardt, 1982; Meinhardt, 1989). Whereas the former accounts for the formation of complex patterns of vasculature in response to a polarised flow of auxin, the latter explains the formation of orderly structures by the coupling of a short-range autocatalytic reaction with a long-range inhibitory process. It has been argued that the generation of the highly ordered and reproducible wild-type pattern of veins in monocot leaves and its coherent integration into leaf growth and morphogenesis are more directly reconcilable with a reaction-diffusion mechanism (Dengler et al., 1997; Nelson and Dengler, 1997). In agreement, all of the vascular phenotypes of the *ral1* mutant, which include an altered spacing of veins and the presence of interruptions, Y-shaped branches and vascular islands in the commissural vein pattern, resemble defects predicted by models of mutations in reaction-diffusion systems (Meinhardt, 1982; Meinhardt, 1989), while they are difficult to explain in terms of the canalisation hypothesis. Previously, the observation of interrupted veins and vascular islands in the *sfc* and *van* mutants of *Arabidopsis* provided support for the reaction-diffusion mechanism in leaf vascular patterning (Deyholos et al., 2000; Koizumi et al., 2000). However, certain aspects of wild-type vascular patterning in dicots are still more readily explained by the canalisation hypothesis (Nelson and Dengler, 1997). As reflected in recent reviews (Dengler and Kang, 2001; Ye, 2002), because of the absence of mutants, our understanding of the process of vascular pattern formation in monocot species is far inferior to that in dicots. In this contest, our study on the radically different leaf venation pattern of a monocot species provides the basis for the indispensable genetic analysis that will allow a more thorough investigation of one of the most intriguing elements of leaf architecture.

We are grateful to Prof Yasuo Nagato for the generous gift of *ral1* seeds. We thank Raoul Latib for invaluable help in morphometric analysis, Elly Schrijnemakers for plant care, René Benjamins and Dr Remko Offringa for the DR5-GUS precursor, Peter Hock for the graph and embryo drawings, Dr Gurdev S. Khush for information on compatibility of rice cultivars, Prof. Thomas Berleth and Prof. Hans Meinhardt for useful discussion and suggestions during the preparation of this manuscript, and Prof. Nancy Dengler, Prof. Timothy Nelson and Dr Steven Chatfield for critically reading the manuscript. E. S. was supported by a European Commission TMR Marie Curie Research Training Grant (ERBFMBICT972716).

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