The Arabidopsis RETARDED ROOT GROWTH Gene Encodes a Mitochondria-Localized Protein That Is Required for Cell Division in the Root Meristem

Xiaojing Zhou, Qiang Li, Xun Chen, Jianping Liu, Qinghua Zhang, Yajie Liu, Kede Liu*, and Jian Xu*

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China (X.Z., Q.L., X.C., J.L., Q.Z., Y.L., J.X., K.L.); and Department of Biological Sciences and Centre for Bioimaging Sciences, National University of Singapore, Singapore 117543 (J.X.)

To develop a growing root, cell division in the root meristem has to be properly regulated in order to generate or propagate new cells. How cell division is regulated in the root meristem remains largely unknown. Here, we report the identification and characterization of the Arabidopsis (Arabidopsis thaliana) RETARDED ROOT GROWTH (RRG) gene that plays a role in the regulation of root meristem cell division. In the root, RRG is predominantly expressed in the root meristem. Disruption of RRG function reduced numbers of dividing cells, the rate of cell production, and endoreduplication, and thus affected meristem size and root growth. Quantitative reverse transcription-polymerase chain reaction and marker-assisted analyses revealed that expression levels of several cell cycle genes were decreased in the mutant roots, indicating a defect in cell cycle progression. Mutations in RRG, however, did not affect the expression of key root-patterning genes and an auxin-responsive marker, suggesting that RRG is not essential for root patterning and auxin signaling. RRG is a mitochondria-localized protein conserved in plants and shares a DUF155 domain with proteins related to cell division in yeast, and rrg mutants displayed extensive vacuolization in mitochondria. We propose that Arabidopsis RRG is a conserved mitochondrial protein required for cell division in the root meristem.

In plants, postembryonic development is driven and sustained by cell proliferation in the meristems and meristematic regions, which are the principal sites of cell division (Lyndon and Cunningham, 1986; Donnelly et al., 1999; Traas and Bohn-Courseau, 2005; Fleming, 2006; Bennett and Scheres, 2010). Properly controlled cell division (both in orientation and time) is essential for the desired final forms of individual organs and overall plant architecture. In recent years, significant progress has been made in understanding the basic molecular machinery of cell division control in plants and animals (Meijer and Murray, 2001; Inzé and De Veylder, 2006; De Veylder et al., 2007). However, how cell division is coordinated during organogenesis and the development of multicellular organisms remains largely unknown.

The postembryonic root meristem of Arabidopsis (Arabidopsis thaliana) is responsible for postembryonic root growth (Scheres et al., 1996). Within the root meristem, a small group of mitotically less active quiescent center (QC) cells are centrally positioned in the stem cell reservoir of the root. The QC keeps the surrounding stem cells in an undifferentiated state and therefore defines the root stem cell niche (Dolan et al., 1993; van den Berg et al., 1997). Root stem cells divide to maintain various cell lineages that constitute the root by means of asymmetric cell division, whereas symmetrical transverse division of meristematic cells results in growth. Upon leaving the root meristem, cells start to expand and differentiate by switching from the mitotic cell cycle to the endoreduplication cycle (or endocycle; Inzé and De Veylder, 2006; De Veylder et al., 2007), resulting in cells with nuclear DNA content of 4C (C = haploid DNA content), 8C, 16C, and 32C (Galbraith et al., 1991).

Cell division in the root meristem plays an important role in the maintenance of root meristem activity and root growth (Meijer and Murray, 2001; De Veylder et al., 2003; Gutierrez, 2005). Diverse developmental and signaling pathways are known to regulate cell division in the root meristem (Scheres et al., 1996; Meyerowitz, 1997; Schiefelbein et al., 1997; Gutierrez, 2005; De Veylder et al., 2007), including but not limited to those associated with root patterning and hormonal homeostasis. For instance, studies on the GRAS family transcription factors SHORTROOT (SHR) and SCARECROW (SCR) have revealed that both SHR and SCR regulate asymmetric cell division of the cortex/endodermis stem cell daughters and thus control radial patterning in the Arabidopsis root (Di Laurenizio et al., 2005).
1996; Helariutta et al., 2000; Nakajima et al., 2001). SHR regulates the spatiotemporal activation of specific genes involved in cell division and, together with SCR, directly activates a D-type cyclin, providing a direct link between key root-patterning factors, specific components of the cell cycle machinery, and root patterning (Sozzani et al., 2010). On the other hand, the auxin-inducible AP2 domain transcription factors PLETHORA1 (PLT1) and PLT2 are known to be dose-dependent master regulators of root stem cell and meristem activity. High levels of PLT activity promote stem cell division and lower levels promote the mitotic activity of stem cell daughters; further reduction in levels is required for cell elongation and cell differentiation (Aida et al., 2004; Galinha et al., 2007). PLT1 and PLT2 function upstream of HIGH PLOIDY2, a nucleus-localized SUMO E3 ligase required for the regulation of meristem activity and endocycle onset, substantiating a role for auxin and PLTs in the regulation of cell division and meristem development (Ishida et al., 2009). Conversely, auxin signaling is also regulated by cell division-related factors. Mutations in the HOBBIT gene, encoding a homolog of the CDC27 subunit of the anaphase-promoting complex and required for cell cycle progression in the Arabidopsis root, lead to a reduction in DR5;GUS auxin reporter gene expression and accumulation of the AXR3/IAA17 repressor of auxin responses (Bilou et al., 2002).

In both unicellular and multicellular organisms, recent studies have indicated that the mitochondrion has a fundamental role in the regulation of cell division. In yeast, an increase in mitochondrial DNA in cells overexpressing the conserved mitochondrial DNA maintenance protein Abf2p actively promotes the initiation of cell division and reduces the fraction of cells in G1 (Blank et al., 2008). Similarly, the suppression of human tumor cell proliferation through depolarizing respiration-dependent mitochondrial membrane potential can reduce the rate of cell proliferation and arrest cell division at the G0/G1 phase (Holmuhamedov et al., 2002). These data suggest that mitochondria, which generate more than 90% of the cell’s energy, determine when cells initiate division and how fast cells divide.

Plant mitochondria have functions both different from and possibly more elaborate than their mammalian and fungal counterparts (Vanlerberghe and McIntosh, 1997; Mackenzie and McIntosh, 1999), thus posing a more complex challenge to the identification of molecular factors regulating their behaviors and functions. Recently, AtPHB3, encoding an evolutionarily conserved mitochondrial type I prohibitin in Arabidopsis, was shown to be present in mitochondrial multimeric complexes of the root meristem and necessary for mitochondrial morphology and cell division in the root meristem (Van Aken et al., 2007), providing evidence for plant mitochondria in the regulation of cell division in the root meristem. In addition, modifications of mitochondrial function were postulated to be central to the establishment and maintenance of the maize (Zea mays) root QC (Jiang et al., 2006). Despite these advances, however, our understanding of the molecular mechanisms regulating mitochondrial behaviors and functions in the root meristem is still at an early stage.

Here, we report the identification and characterization of the Arabidopsis RETARDED ROOT GROWTH (RRG) gene. We show that, in the root, RRG is predominantly expressed in the root meristem and is required for cell division in the root meristem. RRG encodes a mitochondria-localized protein and may have an evolutionarily conserved cell division-related function in both unicellular and multicellular organisms.

RESULTS

Mutations in the RRG Gene Result in Retarded Root Growth

To identify genes regulating root development in Arabidopsis, we screened our ethylmethane sulfonate (EMS)-mutagenized Columbia-0 (Col-0) collection for mutants exhibiting longer or shorter root length compared with the wild-type control and isolated a short-root mutant whose root growth was significantly retarded over time (Fig. 1A). We named this mutant rrg-1.

To clone the gene that was disrupted in the rrg-1 mutant, the rrg-1 mutant was crossed to ecotype Landsberg erecta (Ler) for mapping. All F1 plants displayed a wild-type phenotype, whereas the F2 progeny segregated for 2,491 wild-type and 781 mutant seedlings, indicating that the rrg-1 mutation is recessive in a single nuclear gene ($\chi^2 = 2.17 < \chi^2_{0.05(1)} = 3.84$). Map-based cloning further showed that the RRG locus is located in a 25.3-kb region flanked by markers CER473407 and CER481032 on bacterial artificial chromosome clones F23010 and F10D13, respectively (Fig. 1B). Sequencing of the genomic DNA amplified from this region revealed a single G-to-A transition in the root-1 mutant background, which led to a missense mutation from Glu to Lys at amino acid 346 of the encoded protein of the At1g69380 gene (Fig. 1C).

To verify whether the G-to-A mutation in At1g69380 caused the short-root phenotype in rrg-1, we next performed a complementation test by introducing a 3.8-kb genomic DNA fragment containing the upstream sequence, the full-length coding region, and the downstream sequence of At1g69380 into rrg-1. We found that all transgenic plants obtained were phenotypically indistinguishable from wild-type plants, confirming that the root growth phenotype observed in rrg-1 was caused by the disruption of At1g69380 (Fig. 1D). In agreement with this result, a T-DNA insertion mutant in which the T-DNA fragment is located in the seventh exon of At1g69380 showed a similar short-root phenotype to rrg-1 (Fig. 1A). We
thus renamed At1g69380 as RRG and designated the T-DNA mutant rr-g-2. Notably, reverse transcription (RT)-PCR analysis of mRNA transcript levels of RRG in rr-g-1, rr-g-2, and wild-type plants revealed that RRG transcript was absent in rr-g-2 but still present in rr-g-1, although lower than that in the wild type, suggesting that rr-g-1 is a knockdown allele whereas rr-g-2 is null for the RRG gene (Fig. 1E). No obvious phenotypic abnormality was observed in the embryonic root meristem of both mutant alleles when compared with the wild-type control (Supplemental Fig. S1), indicating that the role of RRG is confined to postembryonic root development.

**The RRG Gene Encodes a Novel Protein Localized to Mitochondria**

The RRG gene consists of seven exons and six introns (Fig. 1C) and encodes a novel protein with 373 amino acids. More detailed protein sequence analysis revealed a mitochondrial signal sequence at the N terminus of the RRG protein, a conserved DUF155 domain, and a C-terminal transmembrane domain (Fig. 2A). Sequence search against the National Center for Biotechnology Information protein database with the RRG protein identified putative orthologs of RRG in plant and nonplant organisms such as bacteria, fungi, and other eukaryotes. All of these orthologs contain the DUF155 domain (Supplemental Fig. S2), whose function is not yet known. Interestingly, two putative yeast orthologs of RRG, Sad1-Interacting Factor2 (SIF2; Miki et al., 2004) and Required for Meiotic Nuclear Division1 (RMND1; Enyenihi and Saunders, 2003), have been associated with cell division. This suggests a role for RRG in meristematic cell division in the Arabidopsis root.

To examine the subcellular localization of RRG in living Arabidopsis cells, we generated transgenic Arabidopsis plants with the 35S::RRG-GFP construct and analyzed the expression of RRG-GFP in vivo with confocal laser scanning microscopy. We found that RRG-GFP expression was confined to small punctate structures in root cells (Fig. 2B), indicating that RRG is localized to certain organelles, possibly mitochondria, as suggested by protein sequence analysis. Indeed, we confirmed this possibility by showing that the mitochondrial dye MitoTracker Orange (Fig. 2C) and the RRG-GFP fusion protein colocalize in the mitochondria (Fig. 2D). To further reveal the nature of the structures marked by RRG-GFP, we performed in tobacco (Nicotiana tabacum) leaf epidermal cells colocalization experiments of RRG-GFP with mt-rk CD3-991 (Nelson et al., 2007), a well-known mitochondria marker generated with the red fluorescent protein mcherry (Shaner et al., 2004). We observed that RRG-GFP (Fig. 2E) colocalized with mt-rk CD3-991 (Fig. 2F) at mitochondria (Fig. 2, E–G) and thus concluded that RRG is localized to mitochondria.

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**Figure 1.** Isolation of rr-g mutants and map-based cloning of the RRG gene. A, Seven-day-old seedlings of the wild type (WT), rr-g-1, and rr-g-2 grown vertically on Murashige and Skoog agar medium. B, Physical mapping of the RRG locus. Vertical lines on the top of the image indicate the positions of markers (from left to right: nga80, CER451198, CER461580, CER473407, CER473866, CER481032, CER480837, CER469950, CER450582, and nga111). The numbers below the vertical lines correspond to the recombinants that have a recombination break point between the marker and the RRG locus. BACS, Bacterial artificial chromosomes. C, Structure of the RRG gene and schematic representation of the point mutation in rr-g-1 and the T-DNA insertion in rr-g-2 in the RRG gene. The black boxes represent the exons, and lines indicate introns. D, Complementation of the rr-g-1 mutant by the RRG gene. rr-g-1/RRG gDNA indicates an rr-g-1 mutant transformed with a vector containing RRG genomic DNA. E, RT-PCR analysis of RRG expression in wild-type and rr-g mutant plants.
The RRG Gene Is Preferentially Expressed in the Root Meristem, Where It Positively Regulates Cell Proliferation

To understand the role of RRG in postembryonic root development, we next investigated the expression pattern of the RRG gene in Arabidopsis seedling roots. To this end, we fused a 2.4-kb promoter fragment of RRG to the GUS reporter gene and transformed the resulting construct (RRG::GUS) into wild-type Col-0 plants. Fourteen independent T0 transgenic plants were generated, and all of them displayed identical GUS expression patterns in the primary and lateral root tips (Fig. 3A; data not shown). More detailed GUS expression analysis in primary and lateral roots of 7-d-old seedlings of T1 plants revealed that the RRG gene is preferentially expressed in QC cells, cortex/endodermis stem cells and their daughter cells, and endodermal and stele cells in the root meristem (Fig. 3, B and C), suggesting a role for RRG in these cells. Moreover, GUS staining could also be seen in leaves and pollen (Fig. 3, A and D).

To unravel whether the observed expression pattern of RRG is correlated with its function in the root meristem, we next examined and analyzed the sizes of root meristem of wild-type and rrg mutant plants. We found that the average size of the root meristem was significantly reduced from 260.1 ± 21.3 μm in the wild type to 184.7 ± 26.5 μm in rrg-1 and 197.6 ± 26.1 μm in rrg-2 (Fig. 4, A–D), suggesting that RRG positively regulates root meristem size in Arabidopsis seedlings. The average cortex cell length in the differentiation zone, however, was unchanged between the wild type (170.2 ± 10 μm) and rrg mutants (173.4 ± 11.8 μm in rrg-1 and 174.2 ± 11.2 μm in rrg-2; Fig. 4E), indicating that RRG functions specifically in the root meristem.

To maintain a certain meristem size, cell proliferation and cell expansion in the root meristem have to be properly controlled and coordinated. We thus investigated whether RRG plays a role in the control of cell proliferation and cell expansion by analyzing the average cortex cell number and cell length in the root meristem of wild-type and rrg mutant plants. We found that the average cortex cell numbers in the root meristem of rrg-1 and rrg-2 were 18.8 ± 4.3 (n = 31) and 20.1 ± 2.9 (n = 38), respectively, which were approximately 55% of that in the wild type (34.2 ± 2.8 [n = 35]; Fig. 4F), indicating a reduction in cell proliferation in rrg mutants. Consistently, the increasing of cortex cells in rrg mutant meristem was slower than in the wild-type control (Fig. 4, H and I), suggesting that the rate of cell proliferation was decreased in rrg mutants. In contrast, the cortex cell length in root meristem of rrg mutants was significantly (P < 0.01) increased compared with the wild-type control (wild type, 7.7 ± 0.8 μm; rrg-1, 9.8 ± 1.0 μm; rrg-2, 9.7 ± 1.1 μm; Fig. 4G), suggesting that cell elongation in the meristematic cells was
accelerated in the rr g mutant background. Together, our data provide evidence that RRG positively promotes cell proliferation and negatively regulates cell expansion in the root meristem.

rrg Mutants Display Aberrant Mitochondrial Structure

To learn if the disruption of RRG affected mitochondria, we examined the number and structure of mitochondria in the root meristem cells of wild-type and mutant plants by using transmission electron microscopy. We analyzed the section plane of mitochondria in multiple sections and found that the number of mitochondria per unit of cell area in rr g-2 (0.45 ± 0.09 per μm²) was comparable with that of the wild type (0.46 ± 0.08 per μm²), but a vast majority (82%) of mitochondria in mutant cells displayed extensive internal vacuolization compared with wild-type controls (Fig. 4, J and K). These results suggest that RRG is required for the maintenance of mitochondrial structure.

Mutations in the RRG Gene Lead to Alterations in Cell Division and Endoreduplication

Because the rate of cell production was reduced in the rr g mutants, we speculated that rr g might have a defect in the progression of cell division. To test this, we first estimated the average cell cycle duration in the root meristem of rr g mutants and wild-type plants. We found that the average values for estimated cell cycle duration in rr g-1 (20.9 h) and rr g-2 (24 h) were markedly longer than in the wild type (16.8 h; Fig. 5A), indicating that mutations in RRG led to increased cell cycle duration in the root meristem. We then examined the expression levels of several cell cycle-related genes in the wild type and rr g-2, including CYC D4;1, HISTONE H4, CYCB1;1, CDKB1;1, E2Fa, DP a, DEL1, and WEE1. Quantitative (q)RT-PCR results showed that the expression levels of CYC D4;1, E2Fa, DP a, and DEL1 did not change significantly in rr g-2, but the expression levels of HISTONE H4, CYCB1;1, CDKB1;1, and WEE1 were significantly down-regulated (Fig. 5B), implying that cell cycle progression in rr g-2 was disturbed. In addition, we found that the number of root meristem cells at the G2/M phase, revealed by the CYCB1;1-GFP marker (Colón-Carmona et al., 1999; Fig. 5C), was decreased from 32.3 ± 4.2 (n = 40) in wild-type roots to 15.5 ± 3.2 (n = 35) in rr g-2 roots (Fig. 5D). Since the size of the root meristem was smaller in rr g mutants, we estimated the number of GFP-positive cells per unit root meristem area and found that it was also significantly reduced in rr g-2 than in the wild type (Fig. 5D).

During the cell cycle, a cell can follow two different paths, namely mitosis (cell division) and endoreduplication (Inze and De Veylder, 2006). Mutants affecting cell division often display altered endoreduplication levels (Cebolla et al., 1999; Boudolf et al., 2004; Dhondt et al., 2010). To investigate whether RRG also plays a role in endoreduplication, the ploidy levels of wild-type and rr g
mutant roots were measured and compared. We found that at 9 d after germination, the proportions of root cells with nuclear contents of 4C, 8C, and 16C were lower in rrg mutants than in the wild type (Fig. 5D), indicating that endoreduplication was severely compromised in rrg mutants. By contrast, the proportion of 2C cells was increased in the rrg-2 null mutant (Fig. 5, E and F), further confirming that cell division was impaired.

Expression of Root-Patterning Genes and an Auxin-Responsive Marker Is Not Affected in rrg Mutants

The expression of RRG overlaps with key root-patterning genes such as SHR, SCR, and WOX5 (Di Laurenzio et al., 1996; Helariutta et al., 2000; Wysocka-Diller et al., 2000; Sabatini et al., 2003; Sarkar et al., 2007), raising the intriguing possibility that RRG may function together with these genes to regulate the identity and mitotic activity of QC and proximal root stem cells. To address this possibility, we analyzed the expression levels and patterns of SHR::SHR-GFP (Fig. 6, A and B; Nakajima et al., 2001), SCR::H2B-YFP (Fig. 6, C and D; Heidstra et al., 2004), and WOX5::ERGFP (Fig. 6, E and F; Blilou et al., 2005) in the rrg mutant background. We found that the expression levels and patterns of these markers in the root meristem were not obviously affected by the rrg mutations, suggesting that RRG is not involved in the regulation of QC and proximal root stem cell identity and acts independently of known patterning
genes. Intriguingly, while in our experimental conditions all the wild-type roots had four tiers of columella root cap cells that could be stained with Lugol’s solution (Supplemental Fig. S3A), approximately 20% of rrg-2 seedlings had only three tiers of Lugol-stained columella root cap cells, suggesting that columella stem cell division was affected in rrg-2 mutants. The expression levels of RBR, CLE40, and ACR4, which have previously been shown to regulate columella stem cell division (Wildwater et al., 2005; De Smet et al., 2008; Stahl et al., 2009), however, were comparable in wild-type and rrg-2 roots (Supplemental Fig. S3B), indicating that RRG is likely involved in the control of columella stem cell division through a yet unknown mechanism.

In Arabidopsis root development, a distal auxin maximum and its associated gradient have been shown to be essential for the control and coordination of cell division and cell differentiation (Grieneisen et al., 2007). To learn whether the phenotypes observed in rrg mutants were caused by changes in auxin level and response, we examined the expression of the auxin-responsive marker DR5rev::GFP (Fig. 6, G and H; Friml et al., 2003) in rrg mutants. No obvious differences between rrg mutant and wild-type seedlings were detected with regard to the expression level and pattern of DR5rev::GFP in the root tip, suggesting that the RRG mutations did not cause changes in auxin level and response. Consistently, we found that rrg-2 mutants exhibited a wild-type-like root growth response to naphthylacetic acid, indole-3-acetic acid, indole-butyric acid, or 2,4-dichlorophenoxyacetic acid at all concentrations examined (Supplemental Fig. 5).
S4, A–D), indicating that rrg-2 mutants are not defective in auxin response or sensitivity. Moreover, we found that the expression level and pattern of RRG::GUS were not obviously affected by the application of 1 μM 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid for 3 h (data not shown), suggesting that RRG function is not primarily regulated by auxin.

**DISCUSSION**

Because of its simple structure, easily traceable cell lineage, distinctly separate zones of growth activities, and transparency, the Arabidopsis root has emerged as a powerful model for studying cell division, cell elongation, and cell differentiation in plants (Scheres and Wolkenfelt, 1998). Over the last two decades, studies using the Arabidopsis root system have resulted in the identification of many key regulators of cell division and contributed significantly to our understanding of the cell division machinery in plants (Hemerly et al., 1995; Doerner et al., 1996; De Veylder et al., 2001b; Masubelele et al., 2005). Here, taking advantage of the Arabidopsis root system, we identified and characterized a novel gene, RRG, in Arabidopsis. We show that in the root, RRG is predominantly expressed in the root meristem and that disruption of RRG function results in reduced root meristem size and retarded root growth, thus revealing an important role for RRG in root development.

Interestingly, while meristematic cortex cell number was significantly reduced in rrg mutants, the length of cortex cells was mildly increased in the root meristem and unaltered in the root differentiation zone when compared with the wild-type control. These results indicated that RRG acts specifically in the root meristem to regulate cell division and cell expansion. Growth kinematics analysis further indicated that cortex cell number in the root meristem and the rate of cell proliferation decreased in the mutant, possibly because of a defect in the progression of cell division. In agreement with this hypothesis, we found that the smaller population of dividing cells per root meristem area in the rrg mutant visualized by the CYCB1;1-GFP marker suggested that mutation in RRG affected cell proliferation in the root meristem. Moreover, the expression of some cell cycle-related genes was inhibited in the rrg-2 mutants, suggesting that RRG was correlated with the cell cycle progression.

When they exit from mitotic cell division, cells engage into an endoreduplication cycle, which involves chromosomal DNA replication without intervening mitosis or cytokinesis, leading to an increase in the ploidy level. Usually, mutants with reduced cell division have more endocycles to enlarge cell size to compensate for the reduction of cell number (De Veylder et al., 2001a; Dewitte et al., 2007; Ramirez-Parra and Gutierrez, 2007). In contrast, the rrg mutants have less endocycling (Fig. 5, E and F), implying that the entry into S phase was reduced or delayed in both mitotic cycles and endocycles. Moreover, accompanied by the decrease of the polyploid nucleus, the 2C population, which reflects the accumulation of G1 cells, increased accordingly, suggesting that perhaps there is a specific cell cycle effect on the G1 phase. Taken together, our results suggest that RRG is required for the proper progression of both mitotic cycles and endocycles in the postembryonic root meristem.

Postembryonic root growth and development depend heavily on the structure of embryonic root (radicle) formed during embryogenesis. After seed germination, the elongated radicle develops into the primary root, which starts to produce cells through
RRG Is Required for Root Meristem Cell Division

RRG Is Required for Root Meristem Cell Division


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MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Col-0 ecotype was used as the wild type unless otherwise noted. T-DNA insertion mutant line rrg-2 (N39410) was obtained from the Nottingham Arabidopsis Stock Centre. Previously published lines used in this study include SHR::SHR-GFP (Nakajima et al., 2001), SCR::H2B-YFP (Heidstra et al., 2004), WOX5::ERGFP (Bilou et al., 2005), DR5rev::GFP (Friml et al., 2003), and D box CYCB1;1-GFP (Colón-Carmona et al., 1999). These marker lines were crossed to the rrg-2 mutant.

EMS Mutagenization and Map-Based Cloning

For the EMS mutagenesis experiment, about 15,000 Col-0 seeds were mutagenized with freshly made EMS solution for 16 h as described previously (Weigel and Glazebrook, 2002). Seeds were sown on soil and grown in a plant room at 21°C (22°C under long-day conditions). Plants were grown in a soil:vermiculite (3:1, v/v) mixture under long-day greenhouse conditions with a controlled temperature range of 21°C to 28°C and a humidity range of 50% to 60%.

RT-PCR and qRT-PCR Analyses

Total RNAs were isolated from roots of 7-d-old seedlings with the Trizol reagent (Invitrogen). Equal amounts of total RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Fermentas). Primers RRG-F and RRG-R were used to amplify a 495-bp fragment of RRG (Bilou et al., 2005). Reactions were run and analyzed on the CFX Manager (Bio-Rad). The primer RRG-F and RRG-R were used to amplify a 495-bp fragment of RRG (Bilou et al., 2005). Reactions were run and analyzed on the CFX Manager (Bio-Rad).
Vector Construction

For complement testing, a 3.8 kb fragment containing upstream, coding, and downstream regions of the RRG gene was amplified from Col-0 genomic DNA using primers ATG69380-BamHI and ATG69380-BamHI. The PCR product was cloned into the pcCAMBIA 3301 vector, and the resulting construct was introduced into rrg-1 mutant plants using the floral dipping method (Clough and Bent, 1998).

For the RRG::GUS promoter fusion construct, a 2.4 kb upstream sequence from the RRG (At1g69380) gene was amplified from Col-0 wild-type genomic DNA using primers AtB1-RRGpro and AtB2-RRGpro. The PCR product was then cloned into pCR2.1 vector (Invitrogen) to create pCR-RRGp. To introduce the recombination site sequences for Gateway cloning, the adaptor primers AtB1-ad and AtB2-ad (the same primers used in the GUS assay) were utilized to amplify the pCR-RRGp vector. The PCR product was then inserted into the modified pHellsgate vector and finally cloned into the pMDC163 binary vector (Curtis and Grossniklaus, 2003) via BP and LR reaction to create RRG::GUS.

For construction of the 35S::RRG-GFP reporter plasmid, the RRG coding sequence was amplified from Col-0 cDNA using primers AtB1-RRG and AtB2-RRG. The PCR products were then cloned into pCR2.1 vector to create pCR-RRG. To introduce the recombination site sequences for Gateway cloning, the adaptor primers AtB1-ad and AtB2-ad (the same primers used in the GUS assay) were utilized to amplify the pCR-RRG vector. The PCR fragment was first cloned into the pHellsgate vector and then integrated into the pMDC163 binary vector (Curtis and Grossniklaus, 2003) via BP and LR reaction. The resulting plasmid was further transformed into Agrobacterium tumefaciens strain GV3101 and introduced into Arabidopsis Col-0 using the floral dipping method (Clough and Bent, 1998).

Transmission Electron Microscopy

For ultrastructural analysis of root meristem cells with transmission electron microscopy, Arabidopsis root tips were excised from 7-d-old seedlings, fixed immediately in 5% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.4), and rinsed in the same buffer. These samples were then postfixed in 1% osmium tetroxide for 2.5 h at 5°C, dehydrated in a graded acetone series, and polymerized at 65°C for 3 d. Ultrathin sections were cut using a Reicert Ultracut S ultramicrotome and collected on mesh copper. Sections were further stained with uranyl acetate and observed with an H-7650 transmission electron microscope (Hitachi).

Flow Cytometric Assay

To measure the ploidy levels of root cells, root tips of Col-0 wild-type and rrg-2 seedlings at 9 d were chopped with a razor blade and suspended in 1 mL of cold nuclear extraction buffer (Doležel et al., 1989). Crude samples were filtered through a 60-μm nylon filter to remove cell debris and stained with 20 μL of 1 mg mL⁻¹ propidium iodide (Sigma-Aldrich). The nuclei were analyzed with the BD FACSCalibur flow cytometer using CellQuestPro software. Each sample was processed for four independent replicates.

Microscopy and Phenotypic Analyses

For Nomarski differential interference contrast (DIC) images, seedlings were cleared and mounted in a chloral hydrate clearing solution (chloral hydrate:water:glycerol, 8:2:1, w/v/v) and imaged using a Zeiss Axioskop 2 scanning microscope equipped with a Nikon DXM1200 camera and DIC optics. Confocal imaging was performed using the Leica TCS SP2 confocal laser scanning microscope and accompanying software. Propidium iodide (Sigma-Aldrich; 10 μg mL⁻¹ in distilled water) was used to stain the cell walls of living root cells (red signal). For photodynamic analyses, seedlings were imaged with a Coolpix 990 digital camera (Nikon) and primary root length was analyzed using ImageJ software (National Institutes of Health). Cortex cell number in the root meristem was determined as one file of cortex cells extending from the QC to where the cells started to elongate. Also, root meristem size was determined by this range.

Histochemical Analysis of GUS Activity

Histochemical analysis of GUS activity was performed according to a previously described protocol (Xu and Scheres, 2005). Stained samples were cleared for 24 h in 70% ethanol to remove chlorophyll. Nomarski DIC images of GUS expression were taken as mentioned above.

Subcellular Localization Analysis

To confirm that the RRG protein is indeed targeted in mitochondria, roots of 35S::RRG-GFP transgenic Arabidopsis plants were stained with the mitochondria-specific fluorescent dye MitoTracker Orange (CMTMRos; Invitrogen) and analyzed for colocalization as described previously (Jiang et al., 2006). The subcellular localization of RRG was further analyzed by cotransforming the mitochondrial marker m-rk CD3-991 and RRG-GFP into epidermal cells of tobacco (Nicotiana tabacum) leaves for transient expression according to Sparkes et al. (2006). Subcellular localization analysis with confocal laser scanning microscopy was performed as mentioned above.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number: RRG (At1g69380).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Structures of embryonic root meristems in wild-type and rrg mutant plants.

Supplemental Figure S2. Alignment of DUF155 domains.

Supplemental Figure S3. Root cap phenotype and expression of RRR, CLE40, and ACR4 in wild-type and rrg-2 roots.

Supplemental Figure S4. Responses of rrg-2 to exogenous auxins.

Supplemental Table S1. List of primers used.

ACKNOWLEDGMENTS

We thank Jianfeng Zhang, Huan Zhu, Lin Chen, Shu Xia, and Xiaoju Qian for their help in establishing the EMS mutant pool and screening for the mutants and Meixia Hu and Qiang Xu for assistance as trainee students. We are grateful to the Arabidopsis Biological Resource Center and the Nottingham Arabidopsis Stock Centre for providing the m-rk CD3-991 plasmid and T-DNA insertion lines.

Received August 19, 2011; accepted October 5, 2011; published October 7, 2011.

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