The Mechanism Forming the Cell Surface of Tip-Growing Rooting Cells Is Conserved among Land Plants

Highlights
- 336,000 T-DNA lines and a genome assembly were generated in Marchantia polymorpha
- 33 genes required for rhizoid growth were identified
- Six of the 33 genes were functionally characterized in plants for the first time
- Genes belonging to these orthogroups were active in the first land plant roots

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In Brief
Honkanen et al. identify 33 genes required for the growth of rhizoid rooting cells in the liverwort Marchantia polymorpha in a screen of 336,000 T-DNA-mutagenized lines and using a de novo genome assembly. Related genes were active during the development of the first plant rooting structures sometime before 460 million years ago.
The Mechanism Forming the Cell Surface of Tip-Growing Rooting Cells Is Conserved among Land Plants

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SUMMARY

To discover mechanisms that controlled the growth of the rooting system in the earliest land plants, we identified genes that control the development of rhizoids in the liverwort Marchantia polymorpha. 336,000 T-DNA transformed lines were screened for mutants with defects in rhizoid growth, and a de novo genome assembly was generated to identify the mutant genes. We report the identification of 33 genes required for rhizoid growth, of which 6 had not previously been functionally characterized in green plants. We demonstrate that members of the same orthogroup are active in cell wall synthesis, cell wall integrity sensing, and vesicle trafficking during M. polymorpha rhizoid and Arabidopsis thaliana root hair growth. This indicates that the mechanism for constructing the cell surface of tip-growing rooting cells is conserved among land plants and was active in the earliest land plants that existed sometime more than 470 million years ago [1, 2].

RESULTS AND DISCUSSION

The first land plant rooting structures comprised systems of tip-growing filamentous cells (rhizoids). Comparing the genetic mechanism that controls the development of filamentous rooting cells (rhizoids and root hairs) among different groups of land plants allows us to reconstruct the mechanism that controlled the development of these first land plant rooting systems. To identify genes required for the growth of filamentous rooting cells, we sequenced Marchantia polymorpha spores with the pCAMBIA1300 T-DNA vector and screened for plants with defective rhizoid morphology (DRM). 301 DRM mutants were isolated (Table S1); 165 mutants were crossed to wild-type and the mutant phenotype was inherited in the F1 generation, whereas 136 were not successfully crossed (Table S1). The approximate 1:1 segregation of wild-type to DRM mutant rhizoid phenotypes in the F1 generation for each of the 165 inherited mutants indicates that the DRM phenotypes were caused by single nuclear mutations (Table S2). The DRM mutant rhizoid phenotype co-segregated with the hygromycin resistance encoded by the hygromycin phosphotransferase gene on the T-DNA in 62 of the 165 inherited mutant lines (Table S2). This is consistent with the hypothesis that the insertion of a T-DNA carrying a functional hygromycin resistance gene caused the mutation that resulted in defective rhizoid growth in 37% of the inherited mutants (Table S2).

To identify T-DNA insertion sites, we first generated a draft assembly of the M. polymorpha genome. Because the plants used in the mutant screen grew from spores generated in a cross between wild-type male (Takaragaike-1 [Tak-1]) and female (Tak-2) accessions, DNA was isolated from Tak-1 and Tak-2 plants, pooled, and sequenced. Illumina HiSeq technology was used to generate 84,554,420 short-insert paired-end reads and 32,963,957 long-insert paired-end reads. The draft genome comprised 4,137 scaffolds with a total scaffold length of 206 Mb (Data S1), scaffold N50 length of 376 kb, and estimated coverage of 64 x (Data S1). To identify protein-coding genes in this draft genome, we sequenced, assembled, and mapped an M. polymorpha gametophyte transcriptome onto the genome assembly. The transcriptome was generated using pooled RNA isolated from mature dorsal thallus epidermis (excluding midrib region and gemma cups), the meristematic zone, rhizoids, and 0- and 1-day-old gemmae. RNA was sequenced using Illumina HiSeq in 183,475,609 short-insert paired-end reads and assembled into contigs (Data S1); 29,453 gametophyte-expressed contigs were mapped to the genome assembly. The whole-genome shotgun assembly (DDBJ: LVLJ00000000) and transcriptome shotgun assembly (ENA: GEFO00000000 and GenBank: GEF001000000) have been deposited at the DNA Data Bank of Japan, European Nucleotide Archive, and GenBank.

The genomic locations of 57 of the 62 T-DNAs linked to DRM mutations were identified by thermal asymmetric interlaced (TAIL) PCR (Data S2). The T-DNA insertion sites of the 57 DRM mutants were distributed among 31 different genes (Figure S1). TAIL PCR was also carried out on DRM mutants that were sterile and could not be crossed, and this resulted in identification of the three alleles of MpCSLD1 and two alleles of MpSCD. Therefore, in total, 33 genes were identified in the mutant screen. Additional mutant alleles in eight of the 33 genes—Mpalba-3, Mpemb2756-2, Mpexi-1, Mppl4ka1-5, Mppl-2, Mppl1-1,
| Gene      | Predicted Function of Encoded Protein                                                                 | Closest Arabidopsis Homolog | Mutant Phenotype       | No. of Mutant Alleles | In Arabidopsis Expression Enriched in Root Hairs | Role in Root Hair Development |
|-----------|------------------------------------------------------------------------------------------------------|-----------------------------|------------------------|-----------------------|--------------------------------------------------|--------------------------------|
| **Cell Wall Biosynthesis and Integrity Sensing**                                                                                                                                       |                             |                        |                       |                                                   |                                  |
| *MpCSLD1* | cellulose synthase-like class D protein                                                             | AT3G03050                   | very short rhizoids    | 3                     | yes                                              | yes                            |
| *MpCSLD2* | cellulose synthase-like class D protein                                                             | AT3G03050                   | short rhizoids         | 5                     | yes                                              | yes                            |
| *MpPTI*   | PTI-like serine/threonine kinase                                                                    | AT2G30740                   | short rhizoids         | 2                     | yes                                              | yes                            |
| *MpXUT1*  | xyloglucan-specific galacturonosyltransferase                                                        | AT5G41250                   | very short rhizoids    | 3                     | yes                                              | yes                            |
| *MpGMP*   | GDP-mannose pyrophosphorylase                                                                      | AT2G39770                   | very short rhizoids    | 1                     | embryo lethal                                    |                                |
| *MpRHM*   | rhamnose biosynthesis                                                                             | AT1G78570                   | short rhizoids         | 1                     | yes                                              | yes                            |
| *MpTHE*   | CrRLK1L family receptor-like kinase                                                                | AT5G54380                   | very short rhizoids    | 1                     | yes                                              | yes                            |
| **Vesicle Transport and Cytoskeleton**                                                                                                                                                |                             |                        |                       |                                                   |                                  |
| *MpP4Ka1* | 1-phosphatidylinositol 4-kinase alpha                                                                | AT1G49340                   | very short rhizoids    | 6                     |                                                  |                                |
| *MpSCD*   | Rab guanine nucleotide exchange factor                                                               | AT1G49040                   | short rhizoids         | 2                     | yes                                              | yes                            |
| *MpSPI*   | WD-40 repeat protein                                                                               | AT1G03060                   | short rhizoids         | 3                     | yes                                              | yes                            |
| *MpSRI1*  | Rab guanine nucleotide exchange factor, similar to *S. cerevisiae* RIC1                           | AT3G61480                   | short rhizoids         | 3                     | yes                                              |                                |
| *MpWDL*   | microtubule-binding protein/TPX2 domain-containing protein                                         | AT2G35880                   | curly rhizoids         | 3                     | yes                                              |                                |
| *MpXI*    | class XI myosin                                                                                    | AT3G12130                   | short rhizoids         | 5                     | yes                                              | yes                            |
| *MpAP5M*  | AP-5 complex subunit mu                                                                            | AT2G20790                   | short rhizoids         | 1                     | yes                                              |                                |
| *MpREN*   | pleckstrin homology domain/RhoGAP domain-containing protein                                       | AT5G12150                   | curly rhizoids         | 1                     | yes                                              |                                |
| *MpSRI2*  | calcium-binding EF-hand family protein, similar to *S. cerevisiae* PAN1                          | AT1G21630                   | very short rhizoids    | 1                     | yes                                              |                                |
| *MpZWI*   | calmodulin-binding/microtubule motor                                                                | AT5G65930                   | short rhizoids         | 1                     | yes                                              |                                |
| **Others/Unknown Function**                                                                                                                                                    |                             |                        |                       |                                                   |                                  |
| *MpALBA*  | alba-like DNA/RNA-binding protein                                                                  | AT1G76010                   | short rhizoids         | 5                     |                                                  |                                |
| *MpEMB2756* | DUF616-containing protein, ceramidase                                                               | AT1G34550                   | short/few rhizoids     | 2                     |                                                  |                                |
| *MpEXL1*  | EXORDIUM-like                                                                                      | AT4G08950                   | short rhizoids         | 2                     |                                                  |                                |
| *MpBA1*   | fructose-bisphosphate aldolase                                                                    | AT4G38970                   | short rhizoids         | 4                     |                                                  |                                |
| *MpGATA1* | class A GATA zinc-finger transcription factor                                                        | AT5G25830                   | short rhizoids         | 2                     | yes                                              |                                |
| *MpIRE*   | AGC kinase                                                                                         | AT5G62310                   | very short rhizoids    | 1<sup>a</sup>         | yes                                              | yes                            |
| *MpSRI3*  | unknown protein, ceramide metabolic process                                                         | AT5G42660                   | short rhizoids         | 2                     |                                                  |                                |
| *MpTMT*   | tonoplast monosaccharide transporter                                                               | AT3G51490                   | short rhizoids         | 2                     | yes                                              |                                |
| *MpACL2*  | ATP citrate lyase subunit B                                                                       | AT5G49460                   | short rhizoids         | 1                     |                                                  |                                |
| *MpCPR*   | regulator of expression of pathogenesis-related (PR) genes                                        | AT5G64930                   | short rhizoids         | 1                     |                                                  |                                |

(Continued on next page)
Mpsr3-2, and Mpxut-3—were identified by sequencing DNA flanking T-DNA insertions in sterile DRM mutants. The Mpire mutation was complemented with a transgene expressing the wild-type MpIRE-coding sequence (Figure S3). Phylogenetic analysis was conducted to assign putative functions and identify related genes in Arabidopsis thaliana (Table 1). Trees were constructed with maximum-likelihood statistics using protein sequences predicted from the M. polymorpha transcriptome assembly and published A. thaliana genome (Figure S2; Data S3 and S4). In total, we identified between one and five alleles in 33 genes; multiple independent mutant alleles were identified for 17 genes, and single alleles were identified for 16 genes.

Of the 33 characterized DRM genes, five—MpCELLULOSE SYNTHASE-LIKE CLASS D 1 (MpCSLD1), MpCSLD2,
MpXYLOGLUCAN-SPECIFIC GALACTURONOSYLTRANSFERASE 1 (MpXUT1), MpGDP-MANNOSE PYRIDOSPHORYLASE (MpGMP), and MpRHAMNOSE BIOSYNTHESIS 1 (MpRH1)—encode proteins that are predicted to function in the synthesis of cell wall polysaccharides. Consistent with the assigned functions, each of these DRM mutants—Mpcsld1, Mpcsld2, Mpxut1, MpGmp, and MpRhm1—develops shorter rhizoids than wild-type, and Mpcsld1 and Mpxut1 mutant rhizoids also burst at their tips (Figure 1; Table 1). Closely related A. thaliana orthogroup members—AtCSLD3, AtXUT1, and AtRHM1—are expressed in root hairs and required for root hair growth because Atcsld3, Atxut1, and Atrhm1 mutants develop short root hairs [3–7]. A role for AtGMP1 (AT2G39770) in root hair development has not yet been defined. This is most likely because loss of AtGMP1 function is lethal and mutants do not survive to the stage where root hairs develop [8]. Taken together, these data demonstrate that the same molecular mechanism for wall synthesis operates in M. polymorpha rhizoids and A. thaliana root hairs.

The sensing of cell wall integrity requires a signal transduction cascade that has been defined in A. thaliana. Receptor kinases in the Catharanthus roseus RECEPTOR KINASE 1-LIKE (CrRLK1L) subclass are required for cell wall integrity sensing in tip-growing cells [9–11]. Maximum-likelihood phylogenetic trees were constructed using CrRLK1L protein sequences from A. thaliana, M. polymorpha, and Catharanthus roseus. This approach reveals that the same molecular mechanism for wall synthesis operates in M. polymorpha and A. thaliana root hairs.

Ten of the 33 genes identified in this screen encode proteins involved in vesicle transport or cytoskeleton function (Table 1; Figure 2) [14–23]. Close homologs of nine are highly expressed in root hairs, and three of these are required for root hair growth in A. thaliana. Mutations in the gene encoding the plant-specific class XI myosin, MpXI, result in the development of short rhizoids (Table 1; Figure 2), just as mutants that lack AtMRL1 activity develop short root hairs in A. thaliana. Taken together, these data suggest that at least some of the components associated with cell wall integrity sensing—RLCK and CrRLK1L proteins—have been conserved since M. polymorpha and A. thaliana last shared a common ancestor.

Figure 2. Phenotypes of Mutants with Defects in Cytoskeleton Function and Membrane Trafficking
Rhizoid elongation is defective in mutants with defects in cytoskeleton organization and function (Mpret, Mpscd, Mpxi, Mpxzi, Mpwdl) and membrane trafficking (Mpat5m, Mpspi4ka1, Mpspi, Mpxi1, Mpxi2); 21-day-old gemmalings. Scale bar, 5 mm. See also Figure S4.
enriched in root hairs (Arabidopsis eFP Browser 2.0 [29, 30]) compared to other root cells, suggesting that they are active during root hair growth.

Three genes were identified that encode proteins predicted to be involved in endocytosis but that have not been functionally characterized in green plants to date. Their predicted function is based on the roles of similar proteins in yeast and mammals (Table 1; Figure 2). MpSRI2 (Mp SHORT RHIZOIDS2) encodes an EF-hand-containing protein that is similar to S. cerevisiae PAN1. PAN1 is required for association of the ARP-actin polymerization complex with clathrin-coated vesicles during endocytosis in yeast [19]. Mp SHORT RHIZOIDS1 (MpSRI1) encodes a protein similar to S. cerevisiae RIC1, which is a guanine exchange factor involved in activating Ypt6p GTPase and required for trafficking from early endosomes to the Golgi late in the endocytosis pathway [23]. MpAP5M is predicted to encode the subunit mu of the adaptor protein 5 (AP5) complex. AP5 is a tetrameric protein complex that coats vesicles acting as a cargo adaptor complex and is likely to be involved in endocytosis, but its precise function remains to be defined in any organism [20].

Figure 3. ALBA Proteins Are Required for Rhizoid and Root Hair Elongation in M. polymorpha and A. thaliana, Respectively

Three further genes were identified with no demonstrated function in green plants. MpSRI3 encodes a protein found in green plants but whose function has not been defined; it is similar to AtEMB2756, which mutates to an embryo lethal phenotype (Table 1; Figure S4) [31, 32]. MpSRI4 is similar to the yeast EFR3 gene that codes for a protein required for the localization of phosphatidylinositol-4-phosphate (PI4) kinase alpha at the plasma membrane, but no similar genes have been functionally characterized in green plants (Table 1; Figure S4) [33]. Consistent with this hypothetical role is the observation that rhizoid development is also defective in mutants in which the PI4 kinase alpha is defective (Table 1). ALBA proteins are nucleic-acid-binding proteins that form chromatin in archaea and bind RNA in a number of animal parasites [34–36]. Not only is MpALBA required for rhizoid development because Mpalba mutants develop short rhizoids but we discovered that loss-of-function alba mutants in A. thaliana develop shorter root hairs than wild-type (Col-0) (Table 1; Figure 3). This indicates that ALBA proteins are required for tip growth in both M. polymorpha and A. thaliana, and therefore are likely to be required for tip growth in rhizoids or root hairs throughout the land plants.

These data demonstrate that genes in the same orthogroups control the synthesis of new cell surface in liverwort rhizoids and angiosperm root hairs. This conservation suggests that this mechanism acted during the growth of the first land plant rooting structures at or soon after the colonization of the land by streptophytes. These data also indicate that some of these genes—such as THE and PTI—were co-opted during the evolution of pollen tubes, one of a suite of traits that evolved during the
evolution of the seed plant life cycle. Some genes previously shown to be involved in root hair growth have not been identified in this screen. This may be because the screen was not carried out to saturation and other rhizoid development genes remain to be discovered. Furthermore, many Arabidopsis gene families or subfamilies that contain genes implicated in root hair growth were present as a single-copy gene in M. polymorpha. Therefore, the M. polymorpha homologs of some genes involved in root hair growth are likely to have more general developmental roles than their Arabidopsis counterparts, and consequently result in severe growth defects or lethality when mutated. Moreover, it is likely that the function of some genes involved in root hair growth diversified in the lineage leading to the tracheophytes after the divergence of the last common ancestor of liverworts and angiosperms. Such divergence of function is supported by the observation that the phenotypes of some loss-of-function mutants in genes from the same orthogroup are different in M. polymorpha and A. thaliana. These data are consistent with the hypothesis that the evolution of the land plant body and life cycle involved a core set of genes with conserved functions that were active in the earliest land plants and underwent duplication followed by neo-functionalization. These novel functions programmed the development of novel structures and contributed to increased life cycle diversity during the subsequent radiation of land plants.

ACCESSION NUMBERS

The accession numbers for the whole-genome and transcriptome shotgun assembly data reported in this paper are DDBJ: LVLJ00000000, ENA: GEFO00000000, and GenBank: GEFO01000000.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, and four datasets and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.09.062.

AUTHOR CONTRIBUTIONS

S.H. screened 150,000 T-DNA lines and identified 38 tagged mutants; V.A.S.J. screened 105,000 lines and identified 8 tagged mutants; G.M. screened 81,000 lines and identified 15 tagged mutants; G.M. and H. Proust isolated DNA for genome sequencing and RNA for RNA sequencing; C.C. and A.J.H. assembled the genome under the guidance of S.K.; D.S.-M. helped with the DNA for genome sequencing and RNA for RNA sequencing; C.C. and A.J.H. worked with G.M. to determine co-segregation of 4 mutants and carried out TAIL-PCR; H. Prescott established all M. polymorpha growth and transformation protocols; S.H., V.A.S.J., and L.D. wrote the paper with much input from G.M. and comments from other authors; genes were grouped according to the classification established by S.H.; and L.D. conceived and designed the project.

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