Mediation of plant-mycorrhizal interaction by a lectin receptor-like kinase

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Abstract

The molecular mechanisms underlying mycorrhizal symbioses, the most ubiquitous and impactful mutualistic plant-microbial interaction in nature, are largely unknown. Through genetic mapping, re-sequencing and molecular validation, we demonstrate that a G-type lectin receptor-like kinase mediates the symbiotic interaction between *Populus* and ectomycorrhizal fungus *Laccaria bicolor*. This finding uncovers an important molecular step in the establishment of symbiotic plant-fungal associations and provides a molecular target for engineering beneficial mycorrhizal relationships.

Keywords: Ectomycorrhizal fungus, G-type lectin receptor-like kinase, *Laccaria bicolor,* Mycorrhizal symbiosis, *Populus*

Populus species, as keystone members of boreal and temperate ecosystems[1], interact with a wide variety of microbes[2,3]. The *Populus-Laccaria bicolor* system has emerged as an excellent system of choice for studying plant-ectomycorrhizal interactions aided by the availability of both *Populus* and *Laccaria* reference genomes and genetic tools[4-5]. Modes of action and molecular mechanisms underlying ectomycorrhizal interactions are poorly understood in this and all other plant-mycorrhizal systems though progress has been made in recent years[6]. Active recruitment and acceptance of mycorrhization have been proposed to occur in a species-specific manner[7] and *L. bicolor* has been found to preferentially colonize *P. trichocarpa* over *P. deltoides*[8,9]. We therefore hypothesized the existence of distinct genetic loci that are present in *P. trichocarpa* but absent in *P. deltoides* and harbor high-fidelity recognition mechanisms for *L. bicolor.*

To test these hypotheses, we evaluated quantitative trait loci (QTL) previously identified in an interspecific *Populus* 54B F_1 pedigree for *L. bicolor* colonization[9]. Multiple phenotypic datasets from the QTL study exhibited bimodal distributions, suggestive of a major QTL segregating in the population, and included four genomic regions, ranging from 1.7 Mb to 15 Mb on linkage groups I, II, III and XI, reproducibly identified across three independent experiments. The QTL resolution was subsequently improved by genotyping the two parental lines and 299 progenies

from the 54B pedigree using the *Populus* 5K Illumina single nucleotide polymorphisms (SNP) array[10,11], which produced 1,744 segregating SNPs with less than 5% missing data. Of these, 677 SNPs were successfully incorporated into the genetic map, and on the basis of Multiple QTL Model mapping analyses, we confirmed two of the four previously identified QTLs[9]. The QTL on chromosome XI had the largest contribution, explaining up to 71% of the phenotypic variance across three different experiments. On the basis of marker segregation, we determined that the beneficial allele was derived from the *L. bicolor*-favored *P. trichocarpa* parent. Because the physical positions of SNPs were available in the *Populus* reference genome v2.2, we were able to resolve QTL on chromosome XI to a region harboring tandemly repeated receptor-like kinase genes (**Fig. 1a, b**).

We fine-mapped this QTL interval with 27 PCR probes targeting insertion/deletion (INDEL) polymorphisms in this region and tested each locus against 20 *P. trichocarpa* and 60 *P. deltoides* genotypes. Of the 27 loci, two INDELs occurred within a single locus, POPTR_0011s13000 v2.2 (a.k.a., Potri.T022200 v3.0) and exhibited strong species-specificity, such that they were consistently absent in *P. deltoides* but present in all *P. trichocarpa* genotypes (**Supplementary Figs. 1, 2 and 3**). We further assessed the extent of the structural variation in this locus by designing six additional primers spanning the coding and promoter regions and PCR confirmed that all 60 tested *P. deltoides* genotypes lacked this locus (**Supplementary Fig. 1)**. Subsequently, we established sequence conservation of this locus across 917 *P. trichocarpa* genotypes[12-14] (**Supplementary Fig. 2**). Segregation analysis in the 54B F_1 pedigree (**Supplementary Fig. 3**) also confirmed co-location of these INDELs with the QTL peak on LGXI and bulked-segregant analysis showed that progenies carrying this gene exhibited twice the colonization rate compared to ones lacking this gene (**Fig. 1c**). This unambiguous conservation suggests that this locus is likely under species-specific purifying selection and that POPTR_0011s13000 encodes a positive regulator of *Populus-L. bicolor* colonization. Our results are consistent with a previous report[15], where POPTR_0011s13000 expression was reduced due to overexpression of the enzyme 1-aminocyclopropane-1-carboxylic acid oxidase and was correlated with a drastic reduction in Hartig net depth, a structure formed by ectomycorrhizal fungi. In addition, the transcript of POPTR_0011s13000 was shown to be upregulated by *L.*

bicolor at 4-week post inoculation in a transcriptomics study[16], and RT-PCR analysis confirmed the induction of POPTR_0011s13000 transcript by *L. bicolor* inoculation (**Supplementary Fig. 4**).

Functional annotation of POPTR_0011s13000 revealed a G-type lectin receptor-like kinase, hereafter designated as PtLecRLK1, a member of receptor-like kinase proteins implicated in the regulation of plant development, stress responses and innate immunity[17,18]. In a canonical manner, PtLecRLK1 is predicted to contain an extracellular domain consisting of a G-lectin domain, an S-locus glycoprotein domain, a plasminogen/apple/nematode protein domain, a transmembrane domain and an intracellular serine/threonine protein kinase domain (**Supplementary Fig. 5**). In addition, at the N-terminus, the protein carries a hydrophobic segment of 22 amino acids, potentially acting as a signal peptide (**Supplementary Fig. 5**). Given these cumulative observations, we sought to validate PtLecRLK1's role in root colonization via heterologous expression in Arabidopsis, a non-host species for *L. bicolor*.

By using Arabidopsis transgenic plants expressing 35S:PtLecRLK1-GFP, we found that GFP fluorescence signal was detected at the cell periphery (**Supplementary Fig. 6a)**. Upon plasmolysis of root epidermal cells of *35S:PtLecRLK1-GFP* transgenic plants, GFP fluorescence signal was mostly detected in the cytosol (**Supplementary Fig. 6b**), implying a potential induced-endocytic process of PtLecRLK1 protein under osmotic conditions given that PtLecRLK1 protein was predicted to contain transmembrane motif (**Supplementary Fig. 5**). Morphologically, Arabidopsis transgenic lines constitutively expressing *PtLecRLK1* were indistinguishable from wild-type Columbia-0 (Col-0) (**Fig. 2a** and **Supplementary Fig. 7)**. However, in the presence of *L. bicolor*, *L. bicolor* completely enveloped the root tips of the transgenic plants, forming a fungal sheath (mantle) indicative of ectomycorrhiza formation (**Fig. 2b**). In contrast, no root mantle was observed in the roots of control plants (**Fig. 2b**), though seedlings were co-cultivated with *L. bicolor* on sugar-reduced medium favoring mutualistic interactions[19,20]. These results indicate that the transgene *PtLecRLK1* can mediate colonization of the non-host species Arabidopsis by *L. bicolor*. In terms of lateral root formation in response to *L. bicolor* inoculation, a process that is known to be independent of root colonization and

functional symbiosis[21], the transgenic plants behaved similarly as the wild type (**Supplementary Fig. 8**).

To anatomically characterize root colonization and its associated fungal structures, we sectioned and stained the mycorrhizal roots in Arabidopsis *35S:PtLecRLK1* transgenic plants and roots of the wild-type Col-0 co-cultivated with *L. bicolor*. Microscopic observations confirmed the formation of a fungal mantle surrounding the root and interstitial penetration of hyphae between cortical root cells forming a shallow Hartig net-like structure in *35S:PtLecRLK1* transgenics but not in the wild-type Col-0 (**Fig. 2c**).

The establishment of an ectomycorrhizal symbiosis requires numerous coordinated processes leading to the development of new fungal or host structures. Such changes require an orchestrated co-response between the host and its symbiont through the exchange of various signaling molecules. Several hundreds of symbiosis-regulated genes have been identified in ectomycorrhizal fungi in association with their hosts[22]. Through transcriptomics analysis of differentially expressed genes in Col-0 wild type and *35S:PtLecRLK1* transgenic lines with and without *L. bicolor* inoculation, we found that *L. bicolor* inoculation resulted in upregulation of nine defense-related genes in Col-0 but only two in the *PtLecRLK1* transgenic lines (**Fig. 2d; Supplementary Table 1**). More importantly, *L. bicolor* inoculation resulted in downregulation of 24 defense-related genes in *35S:PtLecRLK1* transgenic plants (vs one in Col-0) (**Fig. 2d**). The relative low number of defense-related genes detected in this study is likely due to the inoculation method (i.e., no direct contact between the *L. bicolor* hyphae and the root in the beginning of inoculation) and the relatively short inoculation time (i.e., 6 days).

Inoculation of wild-type Col-0 with *L. bicolor* also triggered alterations in a number of metabolites (**Supplementary Table 2**). However, many of these metabolic changes were diminished in the transgenic plants expressing *PtLecRLK1* (**Supplementary Table 2**). The large-scale *L. bicolor*-induced, up-regulated metabolite defense responses in wild-type Arabidopsis (i.e., histidine, sinapoyl malate, sinapoyl-4-O-glucoside, syringin, coniferin, hexacosanoic acid, kaempferol, cholesterol and other sinapoyl conjugates) were significantly reduced in the

transgenic plants, a trend that is similar to that observed between *L. bicolor*-incompatible host *P. deltoides* and –compatible host *P. trichocarpa* though the exact defense-related metabolites differed between Arabidopsis and *Populus*[16]. Furthermore, the down-regulation of amino acids, fatty acids and organic acids in wild-type plants in response to *L. bicolor*, which are often attributed to symbiont utilization and are likely due to altered host carbon partitioning to up-regulated defense responses[16], were also muted in the transgenic plants (**Supplementary Table 2**).

In both the *Populus*-*L. bicolor* and now the transgenic Arabidopsis-*L. bicolor* systems, we found that plant-fungus interaction is mediated by a G-type lectin receptor-like kinase. Receptor-like kinases have been shown to govern perception of pathogens[23-25]. Here we discovered PtLecRLK1 as a key regulator of fungal root colonization. This discovery reveals a role of plant lectin receptor-like kinase in the mycorrhizal symbiosis and demonstrates the importance of this protein in plant-microbial interactions. One implication of this work is that the design of host-microbe symbioses into non-host organisms is feasible. In particular, PtLecRLK1 provides an immediate target for rational design of mycorrhizal symbiosis in economically important crops to enhance water and nutrient acquisition in marginal lands.

Methods

Identification of INDELs and design of primers. We evaluated one *P. trichocarpa* (93-968) and two *P. deltoides* (ILL-101 and D124) resequenced genomes to identify and validate INDEL polymorphisms on chromosomes I and XI and to design PCR primers (**Supplementary Table 3**). Primers amplifying the expected sequence present in *P. trichocarpa* and absent in *P. deltoides* genotypes were subsequently used to screen 60 *P. deltoides* genotypes collected from the Yadkin and Carney Fork river systems in North Carolina and Tennessee[3], as well as from the University of Florida Association Mapping population. In addition, 2 parental lines and 299 progenies from the 54B pedigree were similarly screened. Twenty-seven primer pairs (**Supplementary Table 3**) were selected and designed to target INDELs within 5', coding,

intronic and 3' regions for PCR validation (**Supplementary Methods**), with product sizes ranging from 100 to 1000 bp.

Genotyping and genetic mapping. The *Populus* Illumina 5K array[10,11] was used to genotype two parental lines and 299 progenies from the 54B pedigree[9]. For INDEL genotyping, primers described above were used to amplify DNA from target genotypes. Samples were resolved at 80V for 30 min on 1.5-2.0% agarose gel. After validating 60 individuals of *P. deltoides*, the positive PCR products were sequenced. All sequences of the same locus were aligned with Mega 4.1[ref 26]. JoinMap 3.0[ref 27] was used to construct the genetic linkage map using the species-specific INDELs and 1,744 segregating SNPs from the above assay. The Kosambi mapping function[28] was used to convert recombination frequencies to centiMorgans. A step-wise reduction of the LOD score from 7 to 3 with a maximum recombination of 45% was used to assign markers to linkage groups. Only markers showing the highest congruency were used in map construction. QTLs were detected as described previously[11].

PCR genotyping *PtLecRLK1*. Six primers (**Supplementary Table 3**) spanning the promoter, coding or intronic regions of the *PtLecRLK1* gene were used in PCR reactions to examine its presence or absence in 20 *P. trichocarpa* and 60 *P. deltoides* genotypes (**Supplementary Methods**).

Generation of Arabidopsis transgenic plants. A 2,220 bp Nisqually-1 Potri.T022200 v3.0 (*PtLecRLK1*) coding sequence was synthesized and cloned into pUC57 by GenScript USA Inc. (Piscataway, NJ). Twenty pg of the synthesized DNA was used as template to amplify Potri.T022200 CDS with and without the stop-codon with Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA). PCR products were subcloned into the pENTR™/D-TOPO® vector (Thermo Fisher Scientific Inc.) and resulting plasmid DNAs were verified by sequencing. Subsequently, *PtLecRLK1*-coding region in pENTR vector was transferred by LR reaction into the binary plant destination vectors pGWB402Ω[ref 29] ($P_{2\times35S\Omega}$-*att*R1-*att*R2-T_{NOS})) and pGWB405 (P_{35S}-*att*R1-*att*R2-sGFP-T_{NOS}) using the manufacturer's protocol (Thermo Fisher Scientific Inc.). *Agrobacterium tumefaciens* strain GV3101 (pMP90)

was transformed with the binary plant expression plasmids and used to transform *Arabidopsis thaliana* ecotype Col-0 by floral dipping. Transformed T1 individuals were selected by plating surface sterilized and cold-treated (2 d at 4°C) seeds on ½ MS-medium solidified with 0.8% (w/v) agar and supplemented with 1% (w/v) sucrose, 100 µg mL^{-1} kanamycin and 100 µg mL^{-1} cefotaxime. Two independent lines (#8, #10; without GFP tag) with single T-DNA insertion (based on $Kanamycin^{+/-}$ 3:1 ratio) were selected for further studies. The expression of *PtLecRLK1* transgene was validated by RT-PCR. Total RNA was extracted from leaves of 14-day-old transgenic Arabidopsis lines using the Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Santa Clara, CA) according to the manufacturer's instructions. Five-hundred ng of total RNA were reverse transcribed using $oligo(dT)_{18}$ and RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). RT-PCR was performed using 2x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.) in a total volume of 25 µL including cDNA template corresponding to 25 ng total RNA and 1 µM of each primer. The following cycling conditions were applied: initial denaturation for 2 min at 95°C, followed by 30 cycles of 20 s 95°C, 20 s 57°C and 2.5 min at 72°C with a final elongation step for 7 min at 72°C. Amplification of *AtACTIN8* (At1g49240) served as a control.

Plant-fungal co-culture. Free-living mycelia of *L. bicolor* isolate S238N were grown for ten days on cellophane-covered agar (12 g L^{-1}) plates containing sugar-reduced Pachlewski medium $P20^{ref19}$. For fungal co-cultivation, six-day-old Arabidopsis seedlings of wild-type Col-0 or transgenic lines expressing *PtLecRLK1* were transferred from ½ MS plates to P20 plates with or without fungal inoculum. All seedlings were grown at 23±1°C with 12 h photoperiod (~125 µmol photons m^{-2} s^{-1}). A minimum of 20 plants per genotype were used for the morphological and anatomical assays. In the lateral root formation assay, *L. bicolor*-containing agar blocks were placed ~1cm away from the root tip on both sides of root. The number of lateral roots was counted six days after co-cultivation.

Microscopic observation. To evaluate the Hartig net formation and detect fungal structures, roots were sectioned and stained with UVitex 2B (Polysciences, Inc., Warrington, PA) or Alexafluor 488-WGA (Molecular Probes, Inc., Eugene, OR) and propidium iodide (Thermo

Fisher Scientific Inc.). Approximately 1-cm long root tips from Arabidopsis seedlings, which were grown with or without fungi for two weeks, were fixed in 4% (w/v) para-formaldehyde in phosphate-buffered saline (PBS; pH 7) overnight at 4˚C. Roots were washed in PBS and embedded in 3% (w/v) agarose. Twenty-micrometer transverse sections were prepared using a microtome RM2245 (Leica Biosystems, Wetzlar, Germany). Sections were stained in 1% (w/v) UVitex 2B or Alexafluor 488-WGA and 20mM propidium iodide in PBS for 2 min and then washed. All samples were observed using Zeiss confocal microscopy imaging.

Date availability. Data that support the findings of this study have been deposited into public repositories. The transcriptomics data has been deposited by JGI at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the accession code SRP117109 and is available before publication.

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Author Contributions

J.L., W.M., J.-G.C. and G.A.T. initiated the project and coordinated data analysis and manuscript preparation; J.L., W.M., J.W. and O.C. analyzed the QTLs and performed genotyping; O.C., X.W., A.B., K.Z., Y.Y., M.X., D.W., H.W., J.L.M.-F., K.R.C., L.M., J.-M.A., R.M., S.S.J. and J.-G.C. performed Arabidopsis-related experiments; Z.Z., P.M., T.J.T. and D.A.J. performed metabolite profiling; W.S., J.M. and J.S. analyzed the *Populus* resequencing data; L.G. and F.L.T. assisted with QTLs and plant-fungal experiments; J.Z., T.L., P.R., E.L., X.Y. and K.B. performed transcriptomics analysis; J.L., W.M., J.-G.C. and G.A.T. drafted the manuscript and all authors critically reviewed and approved the final version of the manuscript for publication.

Additional information

Supplementary information is available for this paper. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.-G.C.

Competing financial interests

The strategy for genetic engineering of host-microbe symbiosis into non-host organisms based on the discovery described in this paper has been included in a patent application filed by UT-Battelle, LLC.

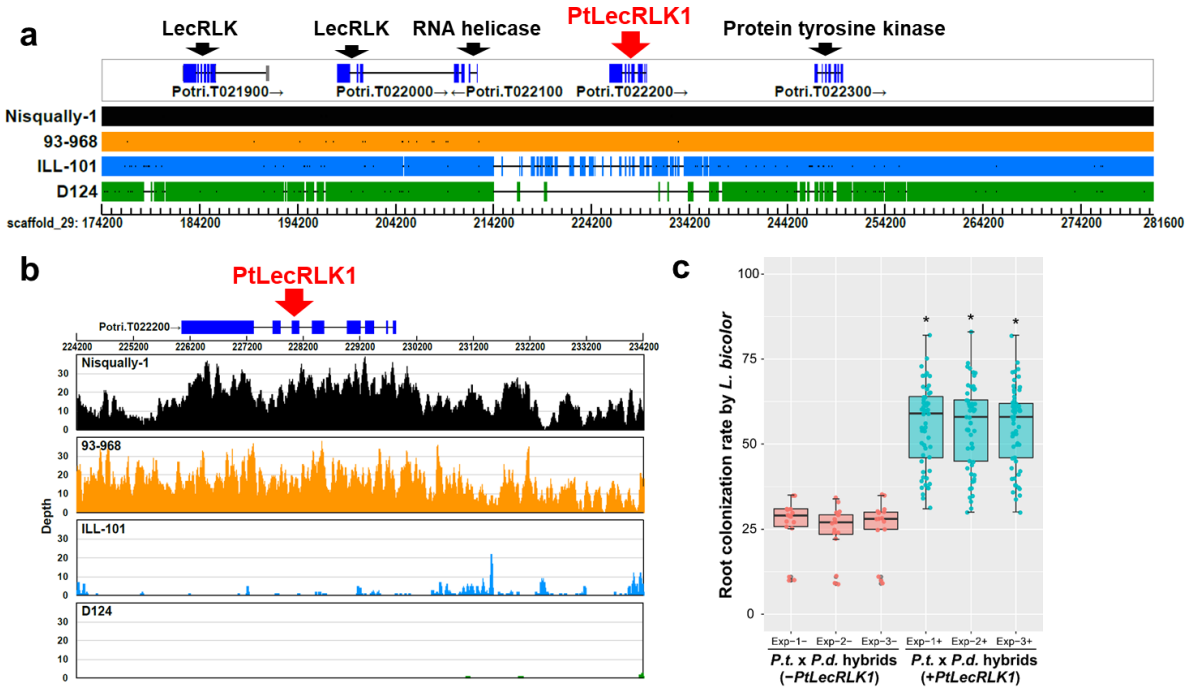
Figure Legends

Figure 1. Identification of *PtLecRLK1* locus as a *Populus-Laccaria* QTL. (a) A genomic deletion event harboring *PtLecRLK1* locus. The genomic region containing *PtLecRLK1* locus is present in *P. trichocarpa* genotypes (Nisqually-1 and 93-968) but absent in *P. deltoides* (ILL-101 and D124). The arrows indicate genes in the QTL region and their annotations. LecRLK, lectin receptor-like kinase. **(b)** A close view, represented by sequence depth, of the genomic region around *PtLecRLK1*. **(c)** Root colonization rate by *L. bicolor* evaluated among *P. trichocarpa* (*P.t.*) × *P. deltoides* (*P.d.*) hybrids with (-) or without deletion (+) of the *PtLecRLK1* locus. Shown are box plot analysis of root colonization rate in *P. trichocarpa* (*P.t.*) × *P. deltoides* (*P.d.*) hybrids with (-) (n=16) or without deletion (+) (n=57) of the *PtLecRLK1* locus. In each box plot, the dot denotes the mean value of root colonization rate from one individual genotype with eight biological replicates, the central rectangle spans the first quartile to the third quartile, the line inside the rectangle shows the median, and the whiskers denote 1.5 interquartile ranges from the box and outlying values plotted beyond the whiskers. "Exp-", three independent experiments. *, significantly different from genotypes with *PtLecRLK1* deletion as determined by ANOVA ($p<0.05$).

Figure 2. Co-cultures of Arabidopsis transgenic plants expressing *PtLecRLK1* with *L. bicolor*. (a) Plant morphology. Shown are wild type plant Col-0 (left) and *35S:PtLecRLK1* transgenic line #8 (right) grown under the same conditions. Scale bar, 1cm. **(b)** Root of wild type plant Col-0 (left) and *35S:PtLecRLK1* transgenic line #8 (right) co-cultivated with *L. bicolor*. Scale bar, 2mm. **(c)** Microscopic observation of a transversal section of a root of wild type plant Col-0 (left) and *35S:PtLecRLK1* transgenic line #8 (right) co-cultivated with *L. bicolor*. Propidium iodide (red) was used to stain root cell walls and UVitex 2B (green) was used to stain fungal cell walls. H, hyphae. CC, cortical root cell. PH, penetrating hyphae. M, mycelium. Experiments in panel (a) to (c) were repeated five time independently with similar results. **(d)** Transcriptomics analysis of defense-related genes in the wild-type Col-0 and *35S:PtLecRLK1* transgenic plants (line #8) co-cultivated with *L. bicolor* with three biological replicates.

DESeq2 (version 1.8.1) was used to determine differentially expressed genes (DEG) between pairs of conditions (p-value < 0.05).

Figure 1


a
LecRLK
LecRLK
RNA helicase
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Protein tyrosine kinase
Potri.T021900→
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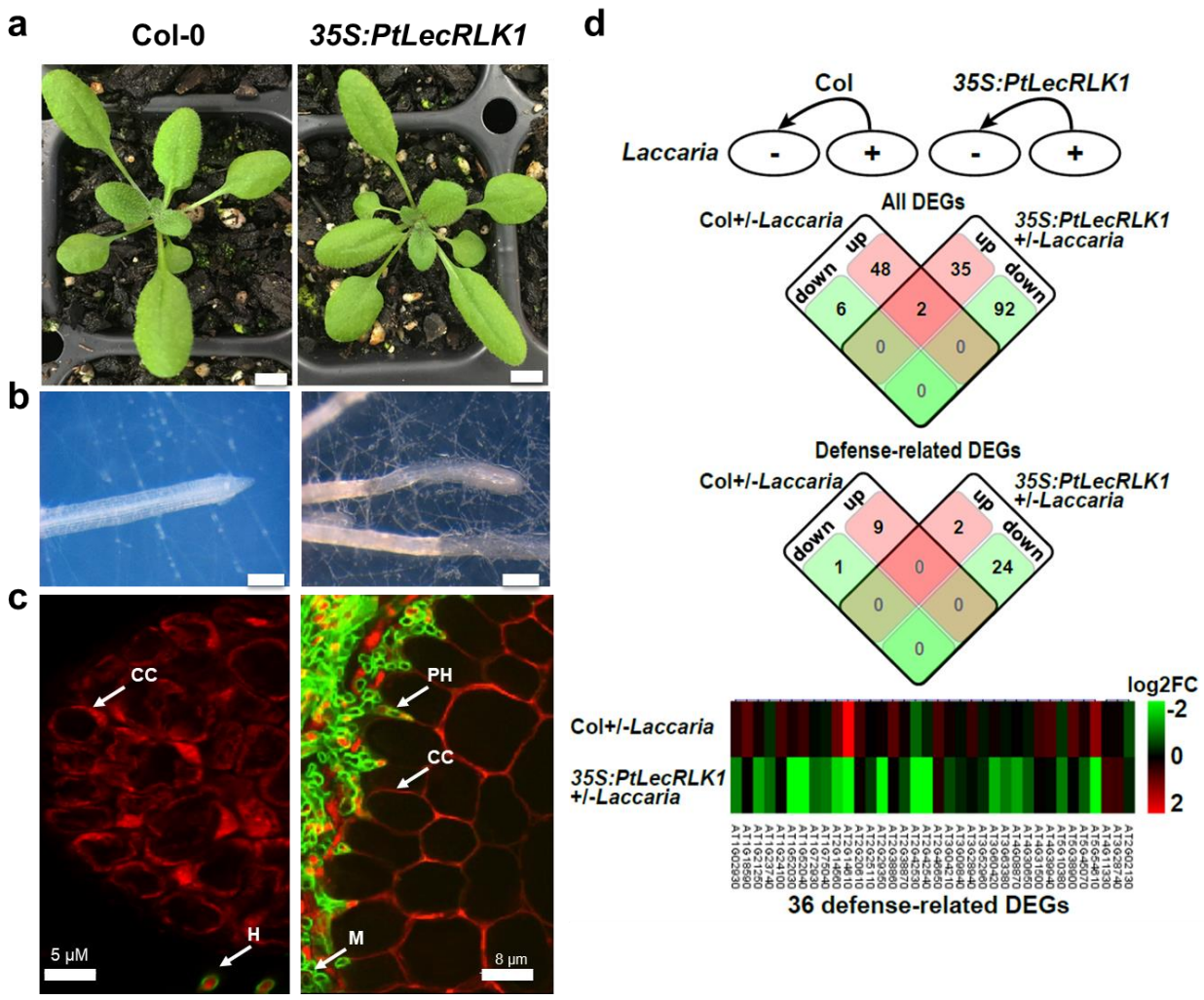
Potri.T022200→
Potri.T022300→
Nisqually-1
93-968
ILL-101
D124
scaffold_29: 174200
184200
194200
204200
214200
224200
234200
244200
254200
264200
274200
281600
b
PtLecRLK1
Potri.T022200→
Depth
c
Root colonization rate by L. bicolor
P.t. x P.d. hybrids (−PtLecRLK1)
P.t. x P.d. hybrids (+PtLecRLK1)

Figure 2


a
Col-0
35S:PtLecRLK1
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5 µM
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35S:PtLecRLK1
Laccaria
-
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-
+
All DEGs
Col+/-Laccaria
35S:PtLecRLK1 +/-Laccaria
up
down
48
35
6
2
92
0
0
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Defense-related DEGs
Col+/-Laccaria
35S:PtLecRLK1 +/-Laccaria
9
2
1
0
24
0
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log2FC
-2
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2
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36 defense-related DEGs