(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
05 April 2018 (05.04.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/064058 A1

(51) International Patent Classification:
C12N 15/82 (2006.01) *C07K 14/415* (2006.01)

(21) International Application Number:
PCT/US2017/053487

(22) International Filing Date:
26 September 2017 (26.09.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/403,033 30 September 2016 (30.09.2016) US
15/703,434 13 September 2017 (13.09.2017) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,

(54) Title: *DRO1* RELATED GENES INFLUENCE LATERAL ROOT ORIENTATION AND GROWTH IN ARABIDOPSIS AND *PRUNUS* SPECIES

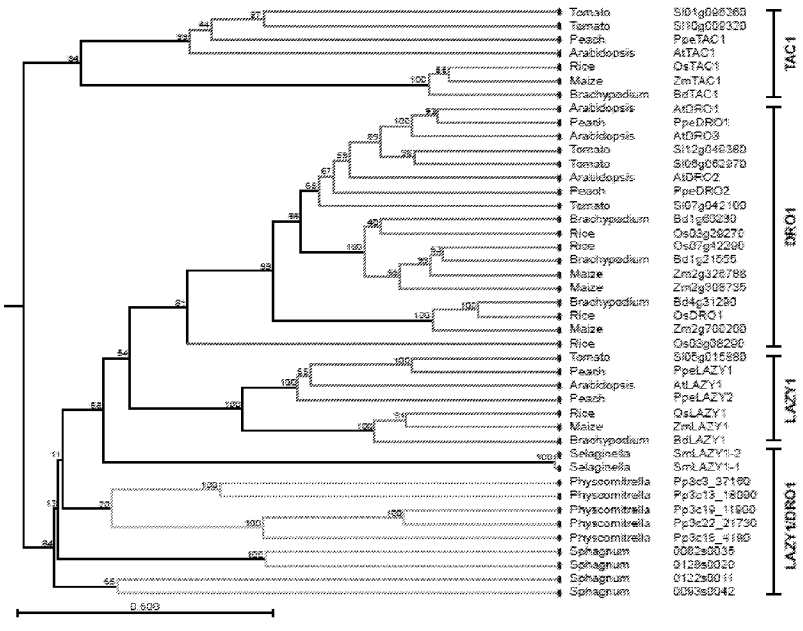

FIG. 1

(57) Abstract: Root orientation, or angle, is an important component of root architecture and depth of the root system. We have determined that *DRO1* and *DRO1*-related genes are present across diverse plant phyla and fall within the IGT gene family. *DRO1*, present in both Arabidopsis and peach, displayed root specific expression patterns. *AtDRO1* is predominantly expressed in both the root vasculature and root tips in a distinct developmental pattern. Mutation of *AtDRO1* led to more horizontal lateral root angles; over-expression of AtDRO1 under a constitutive promoter reduced lateral root angles and resulted in shoot phenotypes including upward leaf curling, shortened siliques, and narrow lateral branch angles. Over-expression of PpeDRO1 in plum (*Prunus domestica*) led to deeper rooting phenotypes. These data establish that *DRO1*-related genes serve a role in altering root architecture, providing a method for drought avoidance.

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CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

DRO1 Related Genes Influence Lateral Root Orientation and Growth in Arabidopsis and *Prunus* Species

CROSS-REFERENCE TO PRIOR FILED APPLICATIONS

[0001] This application claims priority to U.S. Patent Application 15/703,434 filed Sept. 13, 2017, which claims benefit of U.S. Patent Application 62/403,033 filed on September 30, 2016, the contents of which are incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The Sequence Listing submitted via EFS-Web as ASCII compliant text file format (.txt) and filed on September 26, 2017, named "SequenceListing_ST25", (created on August 28, 2017, 56 KB), is incorporated herein by reference. This Sequence Listing serves as paper copy of the Sequence Listing required by 37 C.F.R. § 1.821(c) and the Sequence Listing in computer-readable form (CRF) required by 37 C.F.R. § 1.821(e). A statement under 37 C.F.R. § 1.821(f) is not necessary.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] This invention relates to a gene *PpeDRO1* identified from peach and the role of *DEEPER ROOTING* genes in controlling root orientation and overall depth of the root system of two economically important Prunus tree species and to new methods of manipulating root system length by overexpression of *PpeDRO1.*

Description of the Relevant Art

[0004] Plant productivity can be greatly influenced by root architectural traits, which makes them an important target of agricultural improvement (Lopez-Arredondo *et al.* 2015. *F1000Researc*h 4:651; Kong *et al.* 2014. *Trends Biotech.* 32:597-598). Roots are essential for

uptake of water and nutrients, as well as stability within the soil. Changes to root architectural traits can alter access to different layers within the heterogeneous soil column, resulting in changes to nutrient and water availability or interaction with biotic factors. This is because resources such as water and nitrogen are found in deeper soil layers, while other nutrients such as phosphorous are more abundant in shallower strata (Lynch, J.P. 2011. *Plant Physiol.* 156:1041-1049; Lynch, J. P. 2013. *Ann. Bot.* 112:347-357). Thus, alterations in root system architecture can improve access to limiting resources.

[0005] Root system architecture refers to the spatial distribution of roots within the soil. Root system architecture changes are mediated by multiple processes, including growth rates and lengths of individual roots, the rate and extent of root branching, and the orientation, or angle, of those branches. The types of roots that contribute to root system architecture can differ between plant species. Dicots typically have taproot systems that consist of an embryonic primary root and a branching network of secondary lateral roots (LR). Monocots generally have a more complex fibrous root system. The fibrous systems also have an embryonic primary root and secondary lateral roots, but their contribution is smaller (McSteen, P. 2010. *Cold Spring Harb. Perspect. Biol.* 2:1-18; Smith and De Smet. 2012. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 367:1441-1452). Instead, the majority of monocot root mass and volume comes from seed-borne seminal roots and/or shoot-borne crown roots (McSteen, *supra*; Smith and De Smet, *supra*; Hochholdinger and Zimmerman. 2008. *Curr. Opin. Plant Biol.* 11:70-74). Many genetic and environmental factors influence root architectural traits. Work in *Arabidopsis thaliana*, maize, and rice has uncovered a great deal of information as to root structure, the genetic components regulating root system architecture, and the close relationship between root system architecture and the soil environment (Malekkpoor Mansoorkhani *et al.* 2014. *Biotechnol. Genet. Eng. Rev.*30:95-112; Wachsman *et al.* 2015. *New Phytol.* 208:26-38; Smith and De Smet, *supra*; Uga *et al.* 2015. *Breed Sci.* 65:111-119; Giehl *et al.* 2014. *J. Exp. Bot.* 65:769-778).

[0006] Past and current root system architecture research focusing on primary and lateral root growth and branching has revealed key roles for numerous genes and gene networks, multiple hormones including auxin, cytokinins, giberellins, brassinosteroids, abscisic acid (ABA), and ethylene, and soil nutrients including nitrogen and phosphorous (Satbhai *et al.* 2015. *J. Exp.*

Bot. 66:1099-1112; De Smet, I. 2012. *New Phytol.* 193:867-873; Vermeer and Geldner. 2015. *F1000Prime Rep.* 7:32; Wachsman *et al.*, *supra*). In contrast, relatively little is known about the factors that regulate root growth orientation, or angle. In Arabidopsis, a role for the hormone auxin was identified in influencing lateral root growth angle (Rosquete *et al.* 2013. *Curr. Biol.* 23: 817-822). In monocots, many quantitative trait loci have been identified for deep rooting and root angle (Uga *et al.*, *supra*), however few genes have been identified as regulators of these traits. Through a quantitative trait loci analysis in rice, Uga *et al.* identified DEEPER ROOTING 1 (DRO1) as a regulator of root system architecture depth by modulating crown root angles (Uga *et al. 2013a. Nat. Genet. 45:1097-1102)*. A rice variety containing a truncated copy of DRO1 exhibited shallow rooting, while another variety with a full-length copy had a deeper and narrower root system architecture. Transgenic introduction of an additional single or double copy of full length DRO1 resulted in incremental increases in root system depth. Importantly, the deeper rooting in rice conferred by DRO1 allowed drought avoidance, and thus increased grain yield and seed filling under drought conditions. This finding highlights the advantage for plant roots that can reach lower levels of the soil column under water-limited conditions. The ability to rapidly exploit lower soil layers is a beneficial trait in maize, as well, to optimize water capture (Lynch 2013, *supra*). Similarly, a recent study of tree species during a drought in Italy found deep rooting as a key trait associated with survival (Nardini *et al.* 2016. *Plant Cell Environ.* 39:618-627).

SUMMARY OF THE INVENTION

[0007] We have identified and isolated *PpeDRO1* cDNA (SEQ ID NO:1) as the causative nucleic acid molecule for the deep rooting phenotype in Arabidopsis and plum and confirmed that overexpression of *PpeDRO1* cDNA and increased production of PpeDRO1 result in the creation of altered root architectures such as narrow root angles in genetically altered Arabidopsis and deep rooting in genetically altered *Prunus* trees (and both narrow root angles and deep rooting in transgenic *Prunus*) compared to the root architecture (angles and depth) in wild-type Arabidopsis and *Prunus* trees.

[0008] In accordance with this discovery, it is an object of the invention to provide a method to

routinely control root architecture in *Prunus* trees by overexpression of *PpeDRO1* cDNA (SEQ ID NO: 1) or a DNA sequence encoding PpeDRO1 (SEQ ID NO: 2) in genetically altered *Prunus* trees or germplasm to obtain novel root architectures such as deep rooting in genetically altered *Prunus* trees while still retaining normal flower and fruit development. It is another object of the invention to provide an isolated or recombinant polypeptide (SEQ ID NO: 2) encoded by *PpeDRO1* cDNA (SEQ ID NO: 1).

[0009] It is an object of this invention to have an expression vector that contains a heterologous promoter operably linked to a polynucleotide that encodes PpeDRO1 which has the amino acid sequence of SEQ ID NO: 2. It is a further object of this invention that the polynucleotide has the DNA sequence of SEQ ID NO: 1. It is another object of this invention to have a genetically altered cell containing this expression vector. It is yet another object of this invention that the genetically altered cell is a genetically altered *Prunus* plant cells and genetically altered *Prunus* plants generated from this genetically altered *Prunus* plant cell produces PpeDRO1 in increased amounts than wild-type *Prunus* plants, and that the increased amount of PpeDRO1 causes the genetically altered *Prunus* plant to have a root architecture of narrower lateral root branch angles and longer root systems compared to the lateral root branch angles and depth of root systems in the root architecture of wild-type *Prunus* plants. It is a further object of this invention that the genetically altered *Prunus* cell and the genetically altered *Prunus* plant can be *Prunus persica* (peach), *Prunus domestica* (plum), *Prunus avium* (cherry), *Prunus salicina* (Japanese plum) and/or *Prunus armeniaca* (apricot).

[0010] It is a further object of the invention to have methods of controlling root architectures in plants by generating a genetically altered plant having the expression vector described above. It is a further object that the genetically altered plant can be *P. persica*, *P. domestica*, *P. avium*, *P. salicina*, and/or *P. armeniaca*.

[0011] It is another object of the invention to have a method of producing a genetically altered *Prunus* plant having the altered characteristics of deep rooting (narrower lateral root branch angles and longer root system compared to lateral root branch angles and root system depth of a wild-type *Prunus* plant) by (i) transforming at least one wild-type *Prunus* cell with an

expression vector that contains a heterologous promoter operably linked to a polynucleotide that encode PpeDRO1, such that PpeDRO1 has at least the amino acid sequence of SEQ ID NO: 2 to produce at least one transformed *Prunus* cell, (ii) selecting at least one transformed *Prunus* cell that produces an increased amount of PpeDRO1 compared to the amount of PpeDRO1 produced by a wild-type *Prunus* cell to produce a transgenic *Prunus* cell that produces an increased amount of PpeDRO1, and (iii) inducing the transgenic *Prunus* cell that produces increased amount of PpeDRO1 to grown into a genetically altered *Prunus* plant that produces increased amount of PpeDRO1 compared to the amount of PpeDRO1 produced by a wild-type *Prunus* plant, such that the increased amount of PpeDRO1 causes the genetically altered *Prunus* plant to have the altered root architecture of narrower lateral root branch angles and longer root system compared to the lateral root branch angles and root system depth of a wild-type *Prunus* plant. It is another object of this invention that the polynucleotide encoding PpeDRO1 has a DNA sequence of at least SEQ ID NO: 1. It is a further object of this invention to have a genetically altered *Prunus* plant produced by this method, as well as seed, stem, leaf, flower, pollen, fruit, cells, and progeny, so long as these genetically altered plant parts and progeny contain the heterologous promoter operably linked to the polynucleotide that encodes PpeDRO1 and can produce PpeDRO1 in increased amounts compared to the amount produced by wild-type *Prunus* plant or part or progeny.

[0012] It is an object of this invention to have a genetically altered *Prunus* cell containing an expression vector that contains a heterologous promoter operably linked to a polynucleotide that encodes PpeDRO1 which has the amino acid sequence of at least SEQ ID NO: 2. It is another object of this invention that the genetically altered *Prunus* cell can be induced to grow into a genetically altered *Prunus* plant which produces increased amount of PpeDRO1 compared to amount of PpeDRO1 produced by a wild-type *Prunus* plant, and that increased amount of PpeDRO1 causes the genetically altered *Prunus* plant to have an altered root architecture, namely narrower lateral root branch angles and longer root system, compared to the lateral root branch angles and root system depth of a wild-type *Prunus* plant. It is another object of this invention that the polynucleotide encoding PpeDRO1 has a sequence of at least SEQ ID NO: 1. Another object of this invention is a germplasm containing the genetically altered *Prunus* cell or its progeny.

[0013] Another object of the invention is a genetically altered *Prunus* plant having an altered root architecture of narrower lateral root branch angles and longer root system compared to the lateral root branch angles and root system depth of a wild-type *Prunus* plant. This genetically altered *Prunus* plant contains a heterologous promoter operably linked to a polynucleotide encoding PpeDRO1 which has the sequence of SEQ ID NO: 2, and the genetically altered *Prunus* plant produces increased amount of PpeDRO1 compared to the amount of PpeDRO1 produced by a wild-type *Prunus* plant, and the said increased amount of PpeDRO1 causes the genetically altered *Prunus* plant to have the altered root architecture of narrower lateral root branch angles and longer root system compared to the lateral root branch angles and root system depth of a wild-type *Prunus* plant. It is further object of this invention that polynucleotide (which is operably linked to the heterologous promoter) in the genetically altered *Prunus* plant has a DNA sequence of at least SEQ ID NO: 1. It is further object of this invention to have genetically altered *Prunus* plant parts such as, but not limited to, seeds, stems, leaves, flowers, pollen, fruits, cells, and germplasm, as well as progeny of the genetically altered *Prunus* plant, so long as these genetically altered plant parts and progeny contain the heterologous promoter operably linked to the polynucleotide that encodes PpeDRO1.

[0014] Other objects and advantages of this invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fee.

[0016] FIG. 1 depicts the phylogenetic tree of IGT genes across plant phyla. The phylogenetic tree was constructed using the UMPGA algorithm using CLC Workbench software. The alignment used to construct the tree was built using a combination of MUSCLE and manual refinement. TAC, DRO, and LAZY clades are indicated on the right. Species common names

and sequence IDs are listed. Blue, dicots; magenta, monocots; orange, lycophytes; green, bryophytes.

[0017] FIGs. 2A, 2B and 2C show that AtDRO1 control lateral root orientation in Arabidopsis: *atdro1* mutants exhibit wide branch angles and tip angles, as seen qualitatively (FIG. 2A) and quantitatively (FIG. 2B), as well as shorter primary roots (FIG. 2C). Asterisks indicate Student t-test values of $p < .05$, unless otherwise noted.

[0018] FIG. 3A and FIG. 3B depict *atdro1* mutant responds normally to gravity stimulus. After a 90 degree gravistimulus at 5 days post germination, images were taken of WT and *atdro1* seedlings. Primary root tip angle in relation to the root-shoot junction was measured at each time point for *atdro1* mutant (FIG. 3A, inset). FIG. 3A shows graphs of the changes in tip angles over time for wild-type and *atdro1* mutant. FIG. 3B shows representative images for wild-type and *atdro1* mutant at the indicated time points.

[0019] FIG. 4A depicts qPCR data comparing AtDRO1 expression in dissected roots and shoots and shows that *AtDRO1* expression is largely root specific. FIGs. 4B, 4C, 4D, 4E, 4F, and 4G depict pAtDRO1::GUS staining in primary root and lateral root (LR) and shoot organs across 2 week old seedlings. FIG. 4H, the cartoon on the left, indicates the regions of the root where different staining patterns were found; 2-3 examples of each region are represented.

[0020] FIG. 5A and FIG. 5B depict representative full-root images of roots from two pAtDRO1::GUS seedling lines. FIG. 5A shows Arabidopsis pAtDRO1::GUS transgenic line 2; FIG. 5B shows Arabidopsis pAtDRO1::GUS transgenic line 7. Asterisks indicate stage of staining pattern, referring to FIGs. 4B – 4G. Black, oldest lateral roots; green, middle lateral roots; red, young lateral roots; blue, newly emerged lateral roots.

[0021] FIG. 6A presents qPCR data testing two T2 AtDRO1 overexpression (OE) lines (Lines 3 and 8) for AtDRO1 overexpression. FIGs. 6B, 6C and 6D show that AtDRO1 overexpression results in narrower lateral root angles. Lines 3 and 8 were selected for subsequent experiments. Images (FIG. 6B) and graphs (FIG. 6C) show the significant decrease in lateral root branch

angles of Lines 3 and 8 with respect to gravity and compared to wild-type Arabidopsis. FIG. 6D shows that primary root length was shorter in Line 8, but not Line 3. Asterisks indicate Student t-test values of $p < .05$, unless otherwise noted.

[0022] FIGs. 7A, 7B, 7C, and 7D show that AtDRO1 OE Line 1 is a silenced line exhibiting a similar phenotype to *atdro1* mutants. FIG. 7A shows qPCR data demonstrating that AtDRO1 OE Line 1 has higher relative express DRO1 expression compared to wild-type Arabidopsis. FIG. 7B shows qPCR data measuring native 3' UTR expression and indicating native gene silencing. FIG. 7C and FIG. 7D show that Line 1 exhibits wide lateral root branch angles, both quantitatively (FIG. 7C) and qualitatively (FIG. 7D).

[0023] FIGs. 8A, 8B, 8C, and 8D show differences in Arabidopsis shoot architecture depending on AtDRO1 expression. FIG. 8A shows wild-type Arabidopsis (WT) shoot architecture. FIG. 8B show AtDRO1 OE plants with altered shoot architecture by exhibited narrower shoot branch angles, upward cauline leaf curling, shorter and "shrink-wrapped" siliques, and upward rosette leaf curling compared to WT. FIG. 8C shows that this altered shoot and leaf phenotypes were not seen in Arabidopsis plants overexpressing AtDRO1 lacking the C-terminal EAR-like motif (AtDRO1ΔEAR OE). FIG. 8D shows that shoot branch angles were statistically narrower in Arabidopsis AtDRO1 OE plants Lines 3 and 8 compared to WT. P-values from a Student t-test are indicated on the graph.

[0024] FIGs. 9A, 9B, and 9C depicts IGT family protein alignment showing conserved domains. Alignment of rice, Arabidopsis and peach TAC1, LAZY1 and DRO1 related genes highlights the 5 conserved domains shared among IGT family members. The fifth domain contains an EAR motif (LxLxL) in LAZY1 genes, and a similar motif in DRO1 and DRO1-related genes.

[0025] FIG. 10A and FIG. 10B show that C-terminal EAR-like motif is required for AtDRO1 OE lines to have altered root architect phenotypes. No statistical quantitative difference (FIG. 10A) or qualitative difference (FIG. 10B) was found in lateral root branch angle in Arabidopsis lines overexpressing AtDRO1ΔEAR, compared with WT. A Student's t-test was used to evaluate significance.

[0026] FIG. 11A shows relative expression of PpeDRO1 in the indicated plum tissue from plum plant lines overexpressing PpeDRO1 using qPCR. Highest PpeDRO1 expression occurred in roots. FIG. 11B shows that leaf curling was seen in young transformed plum plants overexpressing PpeDRO1, similar to the upward leaf-curling phenotype in Arabidopsis AtDRO1 OE plants. FIG. 11C and FIG. 11E show that transformed plums overexpressing PpeDRO1 in tissue culture grow roots normally on rooting medium. FIG. 11D and FIG. 11F show that transformed PpeDRO1 OE plums grow long roots on shoot multiplication medium containing cytokinin (BA), which is not seen in wild-type, control plum plants.

[0027] FIG. 12 depicts overexpression of PpeDRO1 in individual genetically altered plum lines. qPCR data confirm overexpression across multiple genetically altered plum lines transformed with a PpeDRO1 OE construct.

[0028] FIGs. 13A, 13B, 13C and 13D depict genetically altered plums overexpressing PpeDRO1 (PpeDRO1 OE plums) exhibit altered phenotypes from wild-type plum. FIG. 13A shows that for a fixed shoot height, PpeDRO1 OE plums had significantly longer roots than controls (wild-type plums). FIG. 13B shows that PpeDRO1 OE plums exhibited greater shoot dry weight, and greater root dry weight as a population, compared to controls. FIG. 13C shows that the number of roots growing through the lower mesh grid in pots was significantly higher in PpeDRO1 OE plum plants when considered as a population compared to control plum plants. FIG. 13D shows a pictorial comparison between control plum and PpeDRO1 OE plum Line 2. Asterisks indicate Student t-test values of $p < .05$.

DETAILED DESCRIPTION OF THE INVENTION

[0029] Currently, most fruit and nut trees are grown as scions grafted to rootstocks. Rootstock varieties are generally chosen for resistance to soil pathogens and their ability to dwarf the scion. Root architectures are only selected as a general trait, as there is substantial variation. Root orientation, or angle, is an important component of the overall architecture and depth of the root system, however little is known about the genetic control of this trait. Numerous studies

have been conducted in plants to understand the genetics underlying root structure. Recent reports in rice identified a role for *DEEPER ROOTING 1(DRO1)* in controlling the orientation of the root system, leading to positive changes in grain yields under drought conditions.

[0030] We previously proposed that DRO1 was a member of the IGT gene family (named for a conserved amino acid motif), which plays a major role in controlling lateral organ orientation (Hollender and Dardick. 2015. *New Phytol*. 206:541-556). Other IGT family members include TILLER ANGLE CONTROL 1 (TAC1) and LAZY1 which have been shown in both monocot, including rice and maize, and dicot species, including Arabidopsis and *Prunus persica* (peach), to control the orientation of various lateral shoot organs, including tillers, pedicels, petioles, and branches (Roychoudhry and Kepinski. 2015. *Curr. Opin. Plant Biol*. 23:124-131). Loss of TAC1 results in narrower angles in branch, tiller, leaf and flower angles compared to wild-type controls in both monocots and dicots (Yu *et al*. 2007. *Plant J*. 52:891-898; Ku *et al*. 2011. *PLoS One* 6:1-7; Dardick *et al*. 2013. *Plant J*. 75:618-630). In contrast, loss of LAZY1 leads to wider branch angles of these lateral organs compared to controls (Yoshihara *et al*. 2013. *Plant J*. 74:267-279; Li *et al*. 2007. *Cell Res*. 17:402-410; Dong *et al*. 2013. *Plant Physiol*.163:1306-1322; Yoshihara and Lino. 2007. *Plant Cell Physiol*. 48:678-688). While the functions of these IGT genes appear to function in the same processes, the genes show a very low level of sequence similarity having only 4 or 5 conserved short protein motifs. Due to the lack of sequence conservation, the authors of the DRO1 manuscript (Uga *et al*., *supra*) did not identify the gene as an IGT family member or that counterparts exist in dicot species. This lack of sequence identity across the IGT family makes their identification in plant genomes sometimes difficult. Conservation of TAC1 and LAZY1 function across angiosperms led us to hypothesize that DRO1 has a similar function in monocots and dicots, despite having different root types and little sequence similarity.

[0031] Here we used phylogenetic analysis to evaluate DRO1-like genes across the plant kingdom and found that they form a distinct clade of the IGT gene family. Using genetic and molecular tools, we evaluated the presence, expression, and functionality of DRO1-like genes in dicots, demonstrating that DRO1-related genes in Arabidopsis, peach, and *Prunus domestica* (plum) influence root system architecture via changes in primary root length and lateral root

angle and define the spatial expression in Arabidopsis. However, our data suggests that the role of *DRO1* in gravity sensing in the primary root tip may not be conserved from rice to Arabidopsis. *DRO1*, present in both Arabidopsis and peach, displayed root-specific expression patterns. Promoter-reporter constructs revealed that *AtDRO1* is predominantly expressed in both the root vasculature and root tips in a distinct developmental pattern. The lack of staining in the youngest lateral roots suggests that *AtDRO1* does not affect growth orientation until roots have grown ~200-250µm. This appears to correlate with the stage at which the lateral roots cease strict horizontal growth and can begin to explore the soil and respond to gravity (Rosquete *et al.*, *supra*). The loss of staining in the oldest lateral root tips suggests that they are released from AtDRO1 control after a certain period of growth. The strong root tip staining is consistent with a role in directing growth orientation, however the normal gravitropic response in *atdro1* primary roots suggests that, unlike rice, AtDRO1 may not be directly connected to gravity in Arabidopsis primary roots, but may function transiently to orient lateral roots to gravity. Alternatively, these genes may be involved in modulating responses to smaller changes in gravity, and a 90 degree rotation assay is above a set response threshold. Mutation of *DRO1* led to more horizontal lateral root angles.

[0032] While loss of AtDRO1 led to dramatic changes in lateral root angles, overexpression had a relatively minor effect despite the high level of overexpression. This may be due to physical constraints of the root cells. For example, cells on the lower side of the emerging lateral root may not be able to compress or decrease in size enough to allow for a much more downward angle. In contrast, because lateral roots emerge and grow at a downward angle already in wild type plants, there is more space to explore above the root than below. Despite the relatively minor decrease in lateral root angle in plants with overexpressed DRO1, these small changes could lead to a large cumulative angle change over the life of the plant, particularly in perennial species.

[0033] In addition, we demonstrate that ectopic *AtDRO1* expression gives architectural phenotypes in shoot organs as well as roots. Overexpression of AtDRO1 under a constitutive promoter reduced lateral root angles and resulted in shoot phenotypes including upward leaf curling, shortened siliques, and narrow lateral branch angles. A conserved C-terminal EAR-like

motif found in IGT genes was required for these ectopic phenotypes. Overexpression of *PpeDRO1* in plum (*Prunus domestica*) led to deeper rooting phenotypes. Collectively, these data indicate a potential application for DRO1 related genes to alter root architecture for drought avoidance and improved resource utilization.

[0034] Experiments in plums suggest that DRO1 will be a useful tool in controlling rooting capacity and depth in crops. In addition, the ability of PpeDRO1 OE plums to root on shoot-multiplication media may prove useful in developing tools for the vegetative propagation of fruit trees. Deep rooting fruit/nut trees created by DRO1 overexpression would be useful as rootstocks or as ungrafted scions for high density fruit/nut production, as well as the potential to mitigate the effects of drought. Drought avoidance strategies may be very advantageous for designing rootstocks to be grown in moisture-poor soils. Such trees would produce roots that explore deeper into the soil subsurface allowing them to tap into additional water and nutrients. Having narrower root structures would also allow the trees to be planted more closely together, as their roots would not directly compete for the same resources. Deeper roots may also provide trees with enhanced stability particularly in loose or wet soils. The implications of using DRO1 to generate designer crops and rootstocks could have a great impact on agriculture as the population increases and the climate changes.

[0035] "Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere *et al*. 1987. *Meth. Enzymol*. 143:277) and particle-accelerated or "gene gun" transformation technology (Klein *et al.* 1987. *Nature (London)* 327:70-73; U.S. Pat. No. 4,945,050, incorporated herein by reference). Additional transformation methods are disclosed below. Thus, isolated polynucleotides of the present invention can be incorporated into recombinant constructs, typically DNA constructs, capable of introduction into and replication in a host cell. Such a construct can be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic

plants have been described in, *e.g.*, Pouwels *et al.* 1985. Supp. 1987. *Cloning Vectors: A Laboratory Manual*; Weissbach and Weissbach. 1989. *Methods for Plant Molecular Biology,* Academic Press, New York; and Flevin *et al.* 1990. *Plant Molecular Biology Manual*, Kluwer Academic Publishers, Boston. Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (*e.g.*, a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

[0036] As used herein, the terms “nucleic acid molecule”, "nucleic acid sequence", "polynucleotide", "polynucleotide sequence”, "nucleic acid fragment", "isolated nucleic acid fragment" are used interchangeably herein. These terms encompass nucleotide sequences and the like.

[0037] The term "isolated" polynucleotide refers to a polynucleotide that is substantially free from other nucleic acid sequences, such as other chromosomal and extrachromosomal DNA and RNA, that normally accompany or interact with it as found in its naturally occurring environment. However, isolated polynucleotides may contain polynucleotide sequences which may have originally existed as extrachromosomal DNA but exist as a nucleotide insertion within the isolated polynucleotide. Isolated polynucleotides may be purified from a host cell in which they naturally occur. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also embraces recombinant polynucleotides and chemically synthesized polynucleotides.

[0038] As used herein, "recombinant" refers to a nucleic acid molecule which has been obtained by manipulation of genetic material using restriction enzymes, ligases, and similar genetic engineering techniques as described by, for example, Sambrook *et al.* 1989. Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY or DNA Cloning: A Practical Approach, Vol. I and II (Ed. D. N. Glover), IRL

Press, Oxford, 1985.

[0039] A “construct” or “chimeric gene construct” refers to a nucleic acid sequence encoding a protein, here the DRO1 protein, operably linked to a promoter and/or other regulatory sequences.

[0040] As used herein, the term "express" or "expression" is defined to mean transcription alone. The regulatory elements are operably linked to the coding sequence of the DRO1 gene such that the regulatory element is capable of controlling expression of the DRO1 genes. "Altered levels" or "altered expression" refers to the production of gene product(s) in transgenic organisms (or genetically altered organisms) in amounts or proportions that differ from that of normal or non-transformed organisms.

[0041] As used herein, the terms "encoding", “coding”, or "encoded" when used in the context of a specified nucleic acid mean that the nucleic acid comprises the requisite information to guide translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (*e.g.*, introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (*e.g.*, as in cDNA).

[0042] The term "operably linked" refers to the association of two or more nucleic acid fragments on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0043] "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns,

and polyadenylation recognition sequences.

[0044] "Promoter" refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. The tissue-specificity of a promoter, for example, is exemplified by the promoter sequence (described above) which specifically induces gene expression in root tips. Promoters that cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg. 1989. *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity. A “heterologous promoter” is a promoter that is operably linked to a polynucleotide to which the promoter is not normally operably linked. That is, if the polynucleotide is usually operably linked to an inducible promoter, then, when the polynucleotide is linked to a constitutive promoter, that constitutive promoter is “heterologous” to that polynucleotide. The polynucleotide could be usually operably linked to a particular inducible promoter; but when it is operably linked to a different inducible promoter, that different inducible promoter is “heterologous” to that polynucleotide.

[0045] "RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be an RNA sequence

derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into polypeptides by the cell. "cDNA" refers to a DNA that is complementary to and derived from an mRNA template. The cDNA can be single-stranded or converted to double stranded form using, for example, the Klenow fragment of DNA polymerase I. "Sense" RNA refers to an RNA transcript that includes the mRNA and so can be translated into a polypeptide by the cell. "Antisense", when used in the context of a particular nucleotide sequence, refers to the complementary strand of the reference transcription product. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene. The complementarity of an antisense RNA may be with any part of the specific nucleotide sequence, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

[0046] A "protein" or "polypeptide" is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide. Each protein or polypeptide has a unique function.

[0047] It is to be understood that as used herein the term "transgenic" includes any cell, cell line, callus, tissue, plant part, or plant the genotype of which has been altered by the presence of a heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation. “Genetically altered” is a synonym of transgenic.

[0048] As used herein, the term "plant" includes reference to whole plants, plant organs (*e.g.*, leaves, stems, roots, *etc.*), seeds, plant cells, and progeny of same. Parts of transgenic plants

are to be understood within the scope of the invention to comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves, roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells, are also an object of the present invention.

[0049] As used herein, the term "plant cell" includes, without limitation, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

[0050] The successful transformation of *Prunus* with PpeDRO1 is a major step in manipulating root system length by overexpression of PpeDRO1, thus ensuring the development of improved varieties of *Prunus*.

[0051] The creation of deep rooting *Prunus* trees DRO1 overexpression would be useful as rootstocks or as ungrafted scions for high density fruit production and also provides drought avoidance strategies may be very advantageous for designing rootstocks to be grown in moisture-poor soils.

[0052] Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

EXAMPLE 1 Phylogeny

[0053] Phylogenetic analyses revealed that the Arabidopsis genome harbors three potential DRO1-like genes: *AtDRO1* (At1g72490), *AtDRO2* (At1g19115) and *AtDRO3* (At1g17400). Based on protein sequence similarity, we placed rice DRO1 and its Arabidopsis homolog (At1g72490) within the IGT gene family, named for a conserved motif (FIG. 1, Uga *et al.* 2013b.

Nat. Genet. 45:1097-1102; Hollender and Dardick, *supra*). This gene family shows relatively low levels of sequence conservation, but is marked by five short motifs as well as a conserved intron-exon structure (Dardick *et al.*, *supra*). Members of the IGT family can be found throughout moss, lycophyte, monocot, and dicot species (FIG. 1, Hollender and Dardick, *supra*). Our phylogenetic analysis revealed the presence of multiple *DRO1*-related genes in both monocots and dicots. To further define the evolutionary relationships of IGT family members, we performed BLAST and conserved motif searches to identify DRO1-like genes in *Arabidopsis*, maize, rice, tomato, peach, brachypodium, selaginella, physcomitrella and sphagnum. Maxiumum likelihood analyses revealed that DRO1 formed a distinct IGT gene clade that was more closely related to LAZY1 than TAC1 (FIG. 1). In addition, the DRO1 clade contained two sub-clades; one consisting of monocot DRO1 genes and another that contained unknown monocot and dicot DRO1-like genes. Arabidopsis, peach, and tomato DRO1-like genes were more closely related to the uncharacterized family of DRO1-like genes than to rice DRO1. Consistent with previous reports, TAC1 genes were not found in primitive plant species (Dardick *et al.*, *supra*). Primitive plant IGT genes formed outgroups from the LAZY and DRO clades, suggesting these clades split off from an ancient ancestor.

[0054] While the closest Arabidopsis protein to OsDRO1 is At1g72490 (which we named AtDRO1 here), AtDRO1 was found to be more closely related to a separate clade of uncharacterized monocot DRO1-related genes, including three rice genes (FIG. 1). The dicot *DRO1*-related genes group together into a single clade, however, some *DRO1*-related family members may have independently arisen through duplication. For example, in addition to *DRO1*, Arabidopsis contained two other *DRO*-related genes while peach contained only one other. These genes formed independent out-groups from *AtDRO1* and *PpeDRO1*.

[0055] Alignments and trees were constructed using CLC genomics workbench (CLC bio). Sequences were obtained from the most recent versions of sequenced genomes available on the Phytozome web tool. Amino acid alignment was generated using MUSCLE and then manually refined. A minimum likelihood tree was constructed using the UPGMA algorithm.

EXAMPLE 2 Arabidopsis Mutants

[0056] To determine if *AtDRO1* plays a role in root growth orientation, we evaluated the phenotypic effects of putative *AtDRO1* loss-of-function mutations. The Columbia (Col-0) ecotype was used as the wild-type (WT) line in all experiments. The T-DNA insertional line *atdro1* mutant (SALK_201221C) and seed were obtained from Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). For phenotyping, seed from homozygous lines were grown on large, vertically oriented plates. Seeds were surface sterilized and sown on plates containing 0.5xMS and 0.8% bactoagar. To observe root growth, seeds were sown on square plates and grown vertically. Both standard size (100 x 15 mm) and large (245 x 25 mm) growth plates were used. Once sown, seedlings were stratified at 4℃ in the dark for 2 days, then placed in growth chambers at 20℃, 16L: 8D, and ~100µmol m-2 sec-1. Plates were imaged weekly for 2-4 weeks using a Canon EOS Rebel T3 camera, and lateral root branch and tip angles were manually calculated from these images using ImageJ. For gravity experiments, plates were rotated 90 degrees on the 5th day after germination, then imaged every 10 minutes. Primary root tip angles were measures with respect to the root-shoot junction. For shoot branch angles, seedlings were grown for 2 weeks on plates, then transplanted into 4-inch pots containing Metromix 360 soil, (Sun-Gro Horticulture) and grown until bolting (~6-7inches in height). Bolts were then photographed and pressed. Angles were manually calculated by measuring the tangent of each lateral branch point.

[0057] Lateral root branch angles, tip angles, and primary root lengths were measured in 14 dpg plants. The *atdro1* mutants exhibited wide lateral root growth angles with respect to gravity (FIG. 2A). Branch and tip angles were significantly increased, by an average of 18 and 25 degrees, respectively, and primary root length was 11 mm shorter on average compared to WT plants (FIGs. 2B and 2C). The wide lateral root angle phenotype led to wider root systems overall.

[0058] In rice, primary root tips of plants containing the truncated version of *OsDRO1* were reported to display a delayed response to gravity stimulation (Uga *et al.* 2013b, *supra*). To assess whether *atdro1* mutants display similar gravitropic defects, we performed gravity response assays. Mutants and wild-type control plants were grown on vertically oriented plates

for 5 days, and then rotated 90 degrees. As the root grew downward toward the gravity vector, we recorded the angle of the primary root tip with respect to the root-shoot junction and gravity (FIG. 3A, inset). Neither the rate nor the degree of tip reorientation was significantly different from wild-type, however *atdro1* mutants showed a non-significant difference toward the end of the experiment (FIG. 3B).

EXAMPLE 3 Arabidopsis Transgenic Lines

[0059] Arabidopsis pAtDRO1::GUS transgenic lines were constructed by cloning a 2kb fragment of the AtDRO1 promoter sequence, including the 5' UTR, upstream of the GUS (beta-glucuronidase) coding sequence in the pBI101 vectors, using SalI and SmaI restriction sites. AtDRO1 overexpression lines were made by amplifying the coding sequence of AtDRO1 (At1g72490) from Arabidopsis cDNA, and cloning downstream of the 35S promoter in our in-house pBIN-AFRS overexpression vector, using SalI and BamHI restriction sites (SEQ ID NO: 3). pBIN-AFRS was created by replacing the T-DNA region of pBIN-ARS with an artificially synthesized T-DNA that removes unwanted plasmid sequences and includes several multiple cloning sites (unpublished). Similarly, ΔEAR constructs were made by amplifying the AtDRO1 coding sequence, using a reverse primer that removed the final 15bp (the EAR-like motif) from the C-terminal end of the protein. The resulting fragment was also cloned into the 35S pBIN-AFRS vector using SalI and BamHI restriction sites. Constructs were transformed into Col-0 plants using the floral dip method, and transformants were subsequently selected on 0.5xMS plates containing kanamycin.

[0060] Seedlings were grown on 0.5x MS plates and collected at 14 days past germination (dpg) for analysis. Localization of pAtDRO1gene activity in cells and tissues of the transformed seedlings was demonstrated by histological staining. The staining is very sensitive. Beta-glucuronidase (GUS) converts 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to a blue product. Seedlings were immersed in cold 90% acetone for 20 minutes, washed in GUS reaction buffer without X-gluc, and then immersed in GUS reaction buffer containing X-gluc. Samples were then vacuum-infiltrated for 20 minutes on ice, and subsequently placed at 37°C in the dark for 4 hours. Seedlings were dehydrated through an ethanol series, and fixed in FAA

fixative (50% Ethanol, 5% Formaldehyde, 10% Acetic acid, rest water). Light microscopy was performed using a Zeiss Axiozoom microscope.

[0061] In 14 dpg seedlings, pAtDRO1::GUS staining was strongest in primary and lateral root tips, as well as root vasculature nearest the tips, with faint expression visible in the shoot vasculature of the strongest lines (FIGs. 4B-4G, FIGs. 5A and 5B). In some lines, a gradient of expression could be observed along the longitudinal axis of the youngest part of the primary root (FIGs. 5A and 5B). In the oldest part of the root system, nearest the root-shoot junction, lateral roots exhibited a gradient of staining along their longitudinal axes (FIGs. 4C and 4D). Within each plant, staining was excluded from the root tips of a subset of these oldest lateral roots (FIG. 4C). In younger lateral roots, staining was restricted to the root tips (FIG. 4E). This tip-specific expression only became apparent when lateral roots reached ~200-250 µm in length, as newly emerged lateral roots lacked staining all together (FIGs. 4F and 4G). In roots with staining along the primary root access, expression was restricted from the region surrounding a new, developing lateral root. This overall pattern was consistent with a potential role for DRO1 in root growth and root tip *in situ* data shown in rice (Uga *et al.*, *supra*).

[0062] Next, we tested if overexpression of AtDRO1 is sufficient to decrease lateral root angles and increase primary root length. We generated and tested expression levels in whole seedlings of transgenic T2 overexpression (OE) lines transformed with 35S::AtDRO1 and selected representative homozygous OE lines from two lines (Line 3 and Line 4). See FIG. 6A for qPCR data. qPCR data was also obtained from other AtDRO1 OE lines (data not shown). AtDRO1 OE lines displayed a significant decrease in the average angle of lateral root branching, by 5 degrees in OE Line 3 and 9 degrees in OE Line 8 (FIGs. 6B and 6C), thus giving narrower lateral root branch angles. Primary root lengths in OE Line 3 were not significantly different from the wild-type; however, they were significantly shorter in OE Line 8 (FIG. 6D).

[0063] A single OE line (Line 1) exhibited horizontal roots, similar to the *atdro1* T-DNA mutant line, and compared to wild-type (FIG. 7D). While the transgene was confirmed to be overexpressed via qRT-PCR compared to wild-type (FIG. 7A), we hypothesized that gene silencing was occurring. To test this, we evaluated native AtDRO1 gene expression using

primers targeting the 3' UTR that was not present in the OE transgene construct. Consistent with this hypothesis, OE Line 1 showed substantially reduced native *AtDRO1* expression compared with the wild-type (FIG. 7B). See also FIG. 7C for quantitative data on lower root branch angles compared to wild-type. These results are consistent with the phenotype observed in *atdro1* T-DNA line.

[0064] Shoots of AtDRO1 OE plants exhibited pronounced phenotypes. Both the rosette and cauline leaves displayed a distinct upward curling at the leaf margins (FIG. 8B) compared to wild-type Arabidopsis plants (WT) (FIG. 8A). With cauline leaves, this often led to the leaf curling completely around the lateral shoot above it. This may be due to differential cell expansion or division on upper and lower leaf surface. Siliques of AtDRO1 OE plants were also typically shorter than in WT, and the outlines of the seeds were more defined (FIGs. 8A and 8B). In addition, shoot branch angles were narrower by ~12 or ~10 degrees in Arabidopsis AtDRO1 OE Lines 3 and 8 (respectively) compared to WT (FIG. 8D).

[0065] In rice, Uga *et al.* found that the shallow rooting IR64 varieties contain a C-terminal truncated DRO1 allele. Within the C-terminal truncated sequence are two conserved motifs – a KWVKTDS (SEQ ID NO: 4) motif of unknown function, and an IVLEI (SEQ ID NO: 5) motif that is very similar to the EAR motif present in LAZY1 (FIGs. 9A-9C). To test whether this EAR-like motif is required for *AtDRO1* function, we deleted the last 5 residues (IVLEI) from AtDRO1 (AtDRO1ΔEAR) and overexpressed this truncated version in Arabidopsis. When grown on vertically oriented plates, we observed that, for AtDRO1ΔEAR Lines 6 and 7, the roots showed neither significant change in lateral root angle compared with wild-type Arabidopsis (WT) (FIGs. 10A and 10B) nor any shoot phenotype (FIG. 8C). This suggests that the EAR-like motif is required for AtDRO1 overexpression plants to have the desired altered root phenotypes.

EXAMPLE 4 Plum Transformation

[0066] The functionality of PpeDRO1 was tested by generating overexpression lines in plum. Peach transformation remains highly intractable, therefore the closely related and readily transformed plum is often used for *in planta* assays (Petri *et al.* 2012. *Methods Mol. Biol.*

847:191-199). *PpeDRO1* overexpression (OE) constructs were made by amplifying the coding sequence of PpeDRO1 (Ppa021925) from peach cDNA, and cloning downstream of the 35S promoter in our in-house pBIN-AFRS overexpression vector, using SalI and BamHI restriction sites. The OE construct was subsequently transformed into *Agrobacterium tumefaciens*. European plums (*Prunus domestica L.*) were transformed with the PpeDRO1 OE strain and a control (pSUC2::GUS) strain using a previously established protocol (Petri *et al.*, supra). Briefly, cold (4°C) stored seeds of the 'Stanley' plum variety were used for transformation. Seeds were surface sterilized with 15% commercial bleach for 15 min and washed three times with sterile water. Hypocotyls were excised from the zygotic embryos under sterile conditions using a stereomicroscope, and then sliced into 2-3 segments. After slices were immersed in an *Agrobacterium* suspension for 20 min, the transformed hypocotyl segments were cultured for 3 days in co-cultivation medium. Hypocotyl segments were then plated on antibiotic (80mg/l kanamycin) selection medium to regenerate transgenic shoots. Recovered kanamycin resistant transgenic shoots were multiplied in multiplication medium before being transferred to rooting medium. Finally, plants were transferred to soil and acclimatized in a growth chamber for several weeks before being moved to greenhouse conditions.

[0067] PpeDRO1 overexpression in plum results in root growth and leaf curling phenotypes. Consistent with Arabidopsis, expression of PpeDRO1 was highest in peach roots, about 7-14 fold higher than in leaf, node and internode samples (FIG. 11A). Shoot meristems also showed expression, about half as much as the root samples (FIG. 11A). Shoots of select lines regenerated under antibiotic selection in tissue culture displayed a similar upward leaf-curling phenotype to Arabidopsis AtDRO1 OE plants (compare FIG. 11B and FIG. 8B). Unlike Arabidopsis, the curling phenotype disappeared within 2-3 weeks after the plants were transplanted to soil. During the plum regeneration process, abnormal rooting phenotypes were also observed. The process of plum transformation involves generating plant shoots from callus, then transferring regenerated shoots first to shoot multiplication medium (FIGs. 11C and 11E), followed by transfer to rooting medium (FIGs. 11D and 11F), which stimulates root production. Shoot multiplication media contains the cytokinin BA that normally inhibits root growth. Before the transfer to rooting medium, wild-type plum regenerated shoots do not typically form roots (FIGs. 11C and 11E). Unlike control plum plants (wild-type), regenerated transgenic plum

shoots overexpressing PpeDRO1 frequently formed thin roots while growing on shoot multiplication medium (FIGs. 11D and 11F).

EXAMPLE 5 Plum Root Assays

[0068] Transformed plums (genetically altered plums) that had been recovered from tissue culture and placed in soil (Metromix 360) in 3-inch pots and then placed in a growth chamber for 2-3 weeks were tested to determine whether PpeDRO1 overexpression has an effect on rooting depth. Once root systems were established in these pots, PpeDRO1 expression levels in multiple lines of recovered seedlings were confirmed. (FIG. 12) using qPCR. Then, seedlings were transplanted to 9 inch pots containing two tiers of wire mesh. Mesh grids were placed at a soil depth of 2 and 5 inches below the surface. The wire mesh grids capture the number of roots growing to each depth. Seedlings were allowed to grow in the mesh-containing pots until shoot heights reached approximately 8-12cm. Plants were then carefully removed from soil, and the number of roots and diameter of root system growing through each mesh grid were recorded. Root and shoot lengths were recorded, and plants were subsequently dissected and dried in a drying oven overnight to measure root and shoot dry weight. For tissue culture rooting assays, shoots were generated on multiplication medium over 3-4 weeks, then transplanted to jars containing either the same shoot multiplication medium or rooting medium and allowed to grow for another 3-4 weeks.

[0069] Individual seedlings reached this shoot height at different times, however this was not a genotype-specific effect. For the fixed shoot height window, the roots of genetically altered PpeDRO1 OE plums were found to be significantly longer than roots of wild-type plum (control) (FIGs. 13A and 13D). Both root dry weight (as a population) and shoot dry weight of genetically altered PpeDRO1 OE plums were greater than controls (FIG. 13B). In addition, genetically altered PpeDRO1 OE plums lines had more roots (when considered as a population) growing through the lower mesh grids than the number of roots growing through the lower mess grids of control plums (FIG. 13C). Asterisks indicate Student t-test values of $p < .05$.

EXAMPLE 6 *AtDRO1* and *PpeDRO1* Expression

[0070] Expression profiles of *AtDRO1* and *AtDRO1* were assessed via qualitative real-time PCR (qPCR) analysis on Arabidopsis seedlings. Arabidopsis seedlings were grown on vertical plates for 14 days, and then hand dissected to separate the shoot and the root. Each biological replicate consisted of a plate of 12 seedlings. Three biological replicates were used. Arabidopsis RNA was extracted using a Directzol RNA Extraction Kit (Zymogenetics).

[0071] The expression profiles of *PpeDRO1* was assessed on plum tissue. Plum tissue containing the PpeDRO1 OE construct was collected from apical meristems of 2-month old plants growing in soil. Peach roots were collected for use as a standard. Peach tissue was collected and flash frozen, lyophilized for ~1 week and ground. 20-30mg of tissue was used in the RNeasy Plant Mini Kit (Qiagen), then treated with the Ambion Turbo DNA-free kit (Ambion).

[0072] QPCR was performed as previously described by Dardick *et al.* (2013. *Plant J.* 75:618-630). Briefly, each reaction was run in triplicate using 50 ng of RNA in a 12 µl reaction volume, using the Superscript III Platinum SYBR Green qRT-PCR Kit (Invitrogen). The reactions were performed on a 7900 DNA sequence detector (Applied Biosystems). Quantification for Arabidopsis samples was performed using a relative curve derived from a serially diluted standard RNA run in parallel. Quantification for peach and plum samples was performed using the delta Ct method, and normalized to actin.

[0073] AtDRO1 expression was evaluated in dissected roots and shoots, and *AtDRO1* expression was shown to be largely root specific (FIG. 4A). qPCR data comparing PpeDRO1 expression in different peach organs indicates highest expression in roots (FIG. 11A).

[0074] All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

[0075] The foregoing description and certain representative embodiments and details of the invention have been presented for purposes of illustration and description of the invention. It is

not intended to be exhaustive or to limit the invention to the precise forms disclosed. It will be apparent to practitioners skilled in this art that modifications and variations may be made therein without departing from the scope of the invention.

Claims

We claim:

Claim 1. An expression vector comprising a heterologous promoter operably linked to a polynucleotide encoding PpeDRO1, wherein said PpeDRO1 comprises an amino acid sequence of SEQ ID NO: 2.

Claim 2. The expression vector of Claim 1, wherein said polynucleotide comprises SEQ ID NO: 1.

Claim 3. A transgenic cell comprising the expression vector of Claim 1.

Claim 4. The transgenic cell of Claim 3, wherein said transgenic cell is a transgenic *Prunus* cell.

Claim 5. The transgenic *Prunus* cell of Claim 4, wherein said transgenic *Prunus* cell is a transgenic cell from the group consisting of transgenic *Prunus persica*, transgenic *Prunus domestica*, transgenic *Prunus avium*, transgenic *Prunus salicina* and transgenic *Prunus armeniaca*.

Claim 6. A method of producing a genetically altered *Prunus* plant having an altered root architecture of narrower lateral root branch angles and longer root system compared to lateral root branch angles and root system depth of a wild-type *Prunus* plant, said method comprising:

(a) transforming at least one wild-type *Prunus* cell with an expression vector comprising a heterologous promoter operably linked to a polynucleotide encoding PpeDRO1, wherein said PpeDRO1 comprises an amino acid sequence of SEQ ID NO: 2 to produce at least one transformed *Prunus* cell; and

(b) selecting at least one transformed *Prunus* cell that produces an increased amount of said PpeDRO1 compared to amount of PpeDRO1 produced by said wild-type *Prunus* cell to produce a transgenic *Prunus* cell that produces an increased amount of said PpeDRO1;

(c) inducing said transgenic *Prunus* cell that produces said increased amount of said PpeDRO1 to grown into a genetically altered *Prunus* plant that produces said increased amount of said PpeDRO1 compared to said amount of PpeDRO1 produced by said wild-type *Prunus* plant, wherein said increased amount of PpeDRO1 causes said genetically altered *Prunus* plant to have said altered root architecture of narrower lateral root branch angles and longer root system compared to said lateral root branch angles and root system depth of a wild-type *Prunus* plant.

Claim 7. The method of Claim 6, wherein said polynucleotide comprises SEQ ID NO: 1.

Claim 8. A genetically altered *Prunus* plant produced by the method of Claim 6 or the progeny thereof, wherein said genetically altered *Prunus* plant and said progeny produce increased amount of PpeDRO1 compared to amount of PpeDRO1 produced by a wild-type *Prunus* plant, and wherein said increased amount of PpeDRO1 causes said genetically altered *Prunus* plant and said progeny thereof to have said altered root architecture of narrower lateral root branch angles and longer root system compared to said lateral root branch angles and root system depth of a wild-type *Prunus* plant.

Claim 9. A genetically altered *Prunus* seed of the transgenic plant of Claim 8, wherein said genetically altered *Prunus* seed comprises an expression vector comprising a heterologous promoter operably linked to a polynucleotide encoding PpeDRO1, wherein said PpeDRO1 comprises an amino acid sequence of SEQ ID NO: 2.

Claim 10. A genetically altered *Prunus* cell comprising an expression vector comprising a heterologous promoter operably linked to a polynucleotide encoding PpeDRO1, wherein said PpeDRO1 comprises SEQ ID NO: 2, wherein a genetically altered *Prunus* plant generated from said genetically altered *Prunus* cell produces increased amount of PpeDRO1 compared to amount of PpeDRO1 produced by a wild-type *Prunus* plant, and wherein said increased amount of PpeDRO1 causes said genetically altered *Prunus* plant to have altered root architecture of narrower lateral root branch angles and longer root system compared to said lateral root branch angles and root system depth of a wild-type *Prunus* plant.

Claim 11. The genetically altered *Prunus* cell of Claim 10, wherein said polynucleotide comprises SEQ ID NO: 1.

Claim 12. A germplasm of said genetically altered *Prunus* cell of Claim 10.

Claim 13. A genetically altered *Prunus* plant having an altered root architecture of narrower lateral root branch angles and longer root system compared to lateral root branch angles and root system depth of a wild-type *Prunus* plant, said genetically altered *Prunus* plant comprising a heterologous promoter operably linked to a polynucleotide encoding PpeDRO1, wherein said PpeDRO1 comprises SEQ ID NO: 2, wherein said genetically altered *Prunus* plant produces increased amount of PpeDRO1 compared to amount of PpeDRO1 produced by a wild-type *Prunus* plant, and wherein said increased amount of PpeDRO1 causes said genetically altered *Prunus* plant to have said altered root architecture of narrower lateral root branch angles and longer root system compared to said lateral root branch angles and root system depth of a wild-type *Prunus* plant.

Claim 14. The genetically altered *Prunus* plant of Claim 13, wherein said polynucleotide comprises SEQ ID NO: 1.

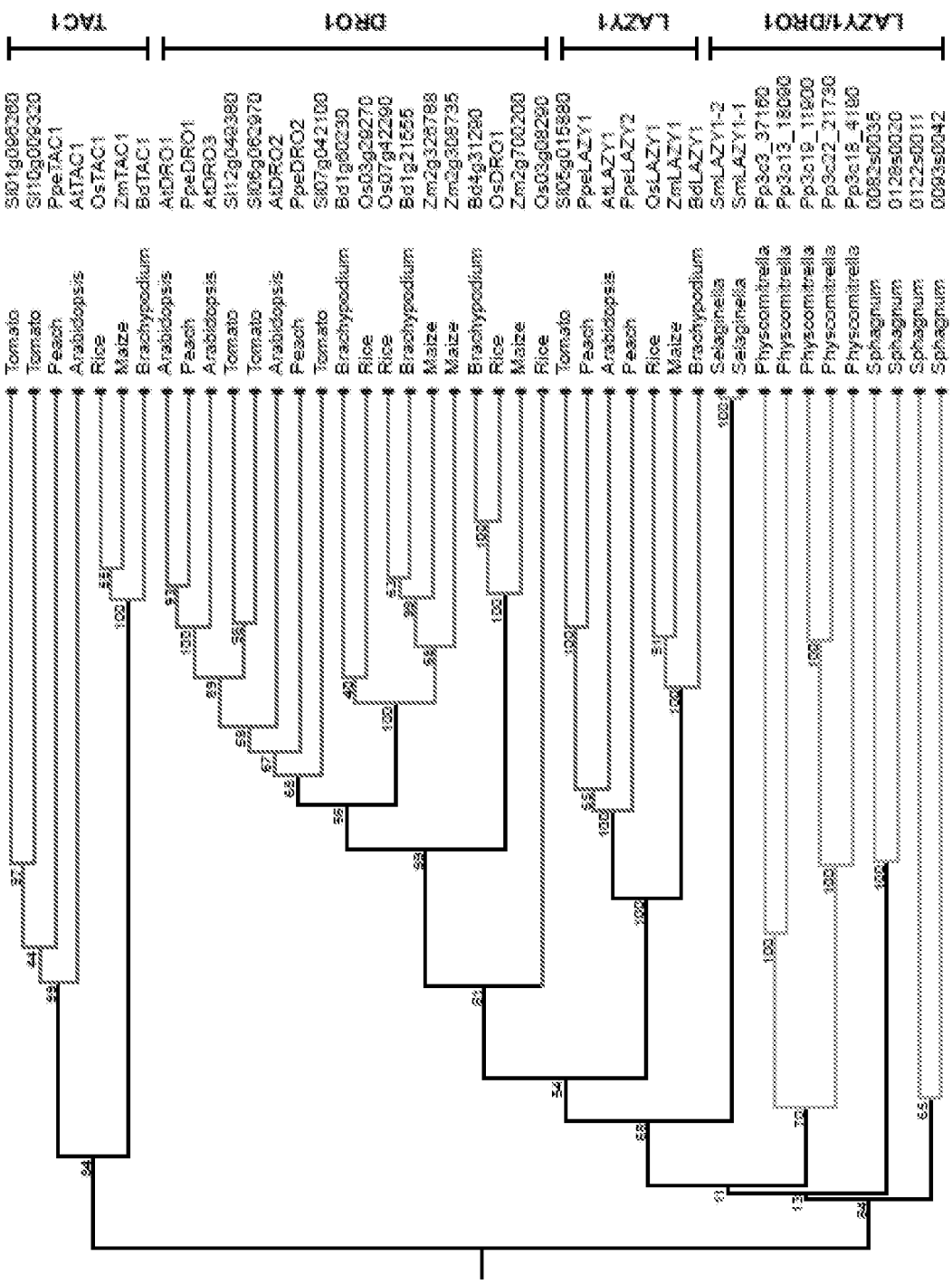

TAC1
DRO1
LAZY1
LAZY1/DRO1
Solyc01g067360
Solyc01g096320
PpeTAC1
AtTAC1
OsTAC1
ZmTAC1
BdTAC1
AtDRO1
PpeDRO1
AtDRO3
Solyc12g044380
Solyc06g062970
AtDRO2
PpeDRO2
Solyc07g042100
Bd1g60230
Os03g39270
Os07g42290
Bd1g21555
Zm2g328788
Zm2g308735
Bd4g31280
OsDRO1
Zm2g700200
Os03g68290
Solyc05g015880
PpeLAZY1
AtLAZY1
PpeLAZY2
OsLAZY1
ZmLAZY1
BdLAZY1
SmLAZY1-2
SmLAZY1-1
Pp3c3_37160
Pp3c13_18060
Pp3c19_11800
Pp3c22_21730
Pp3c18_41180
0083s0035
0122s0020
0122s0011
0083s0042
Tomato
Tomato
Peach
Arabidopsis
Rice
Maize
Brachypodium
Arabidopsis
Peach
Arabidopsis
Tomato
Tomato
Arabidopsis
Peach
Tomato
Brachypodium
Rice
Rice
Brachypodium
Maize
Maize
Brachypodium
Rice
Maize
Rice
Tomato
Peach
Arabidopsis
Peach
Rice
Maize
Brachypodium
Selaginella
Selaginella
Physcomitrella
Physcomitrella
Physcomitrella
Physcomitrella
Physcomitrella
Sphagnum
Sphagnum
Sphagnum
Sphagnum
0.600

FIG. 1

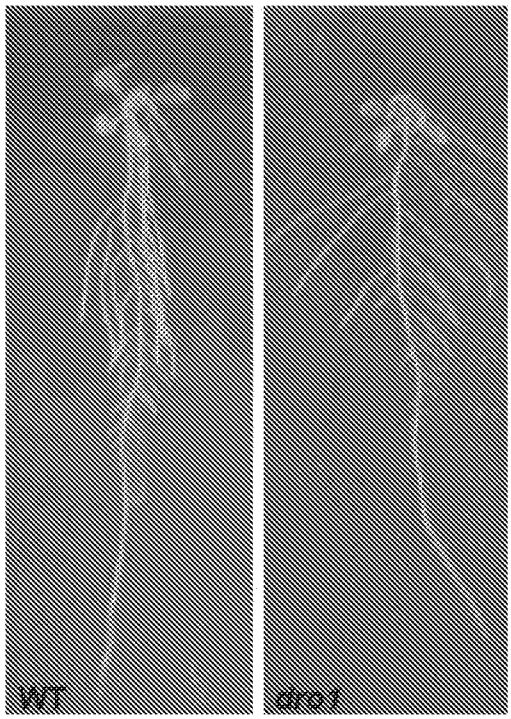


FIG. 2A

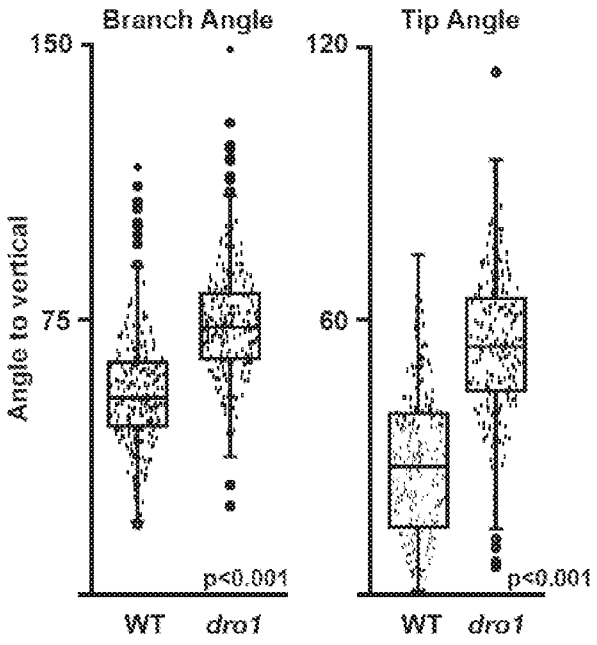


FIG. 2B

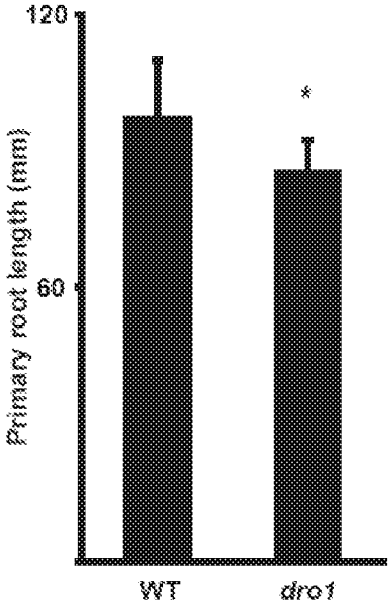


FIG. 2C

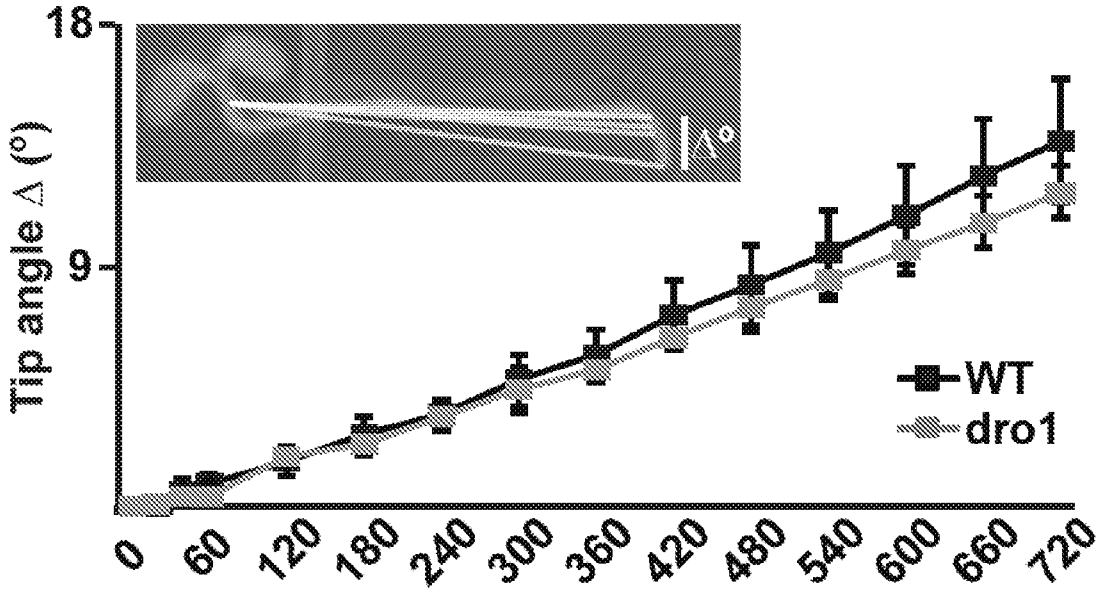

18
9
Tip angle Δ (°)
Δ°
WT
dro1
0
60
120
180
240
300
360
420
480
540
600
660
720

FIG. 3A

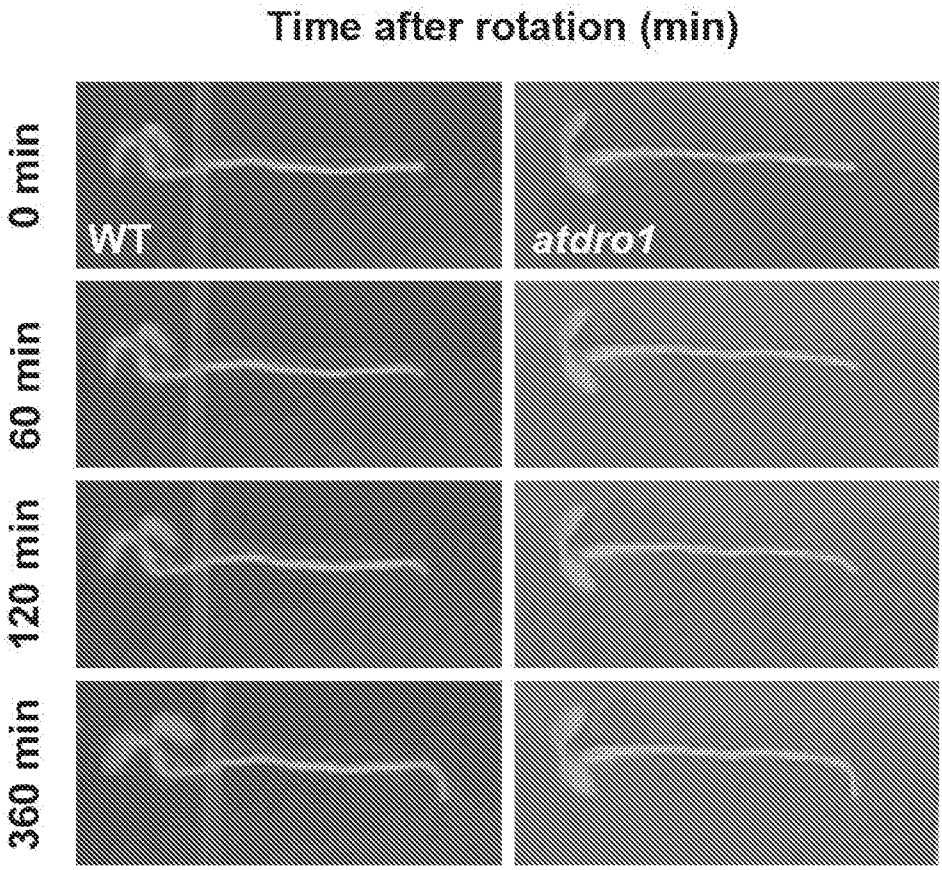


FIG. 3B

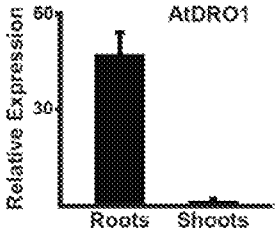


FIG. 4A

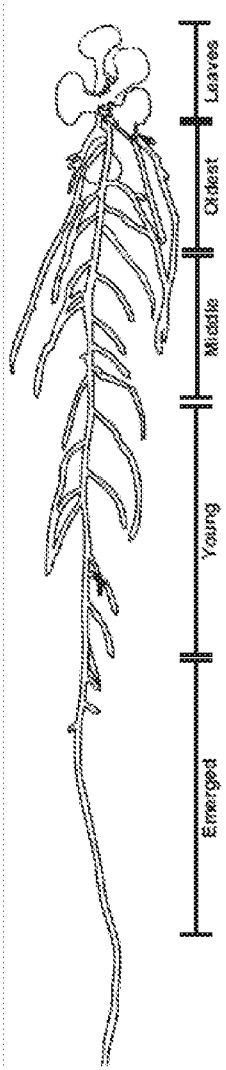


FIG. 4H

FIG.4B

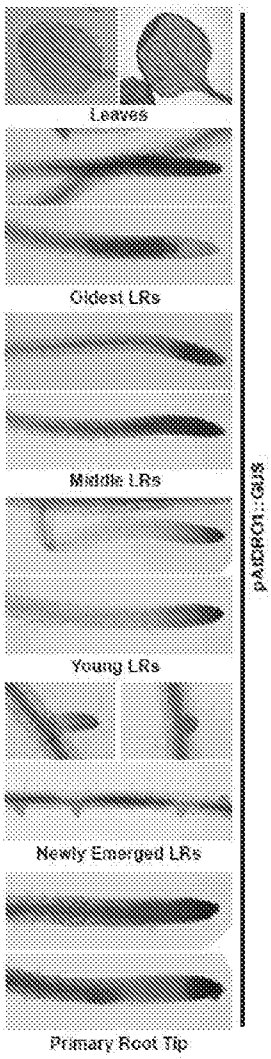
FIG. 4C

FIG. 4D

FIG. 4E

FIG. 4F

FIG. 4G



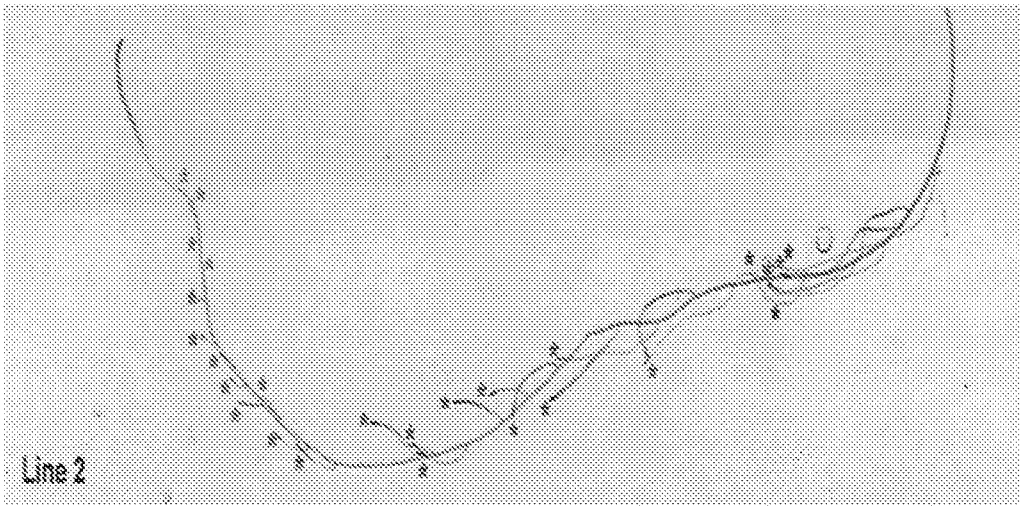


FIG. 5A

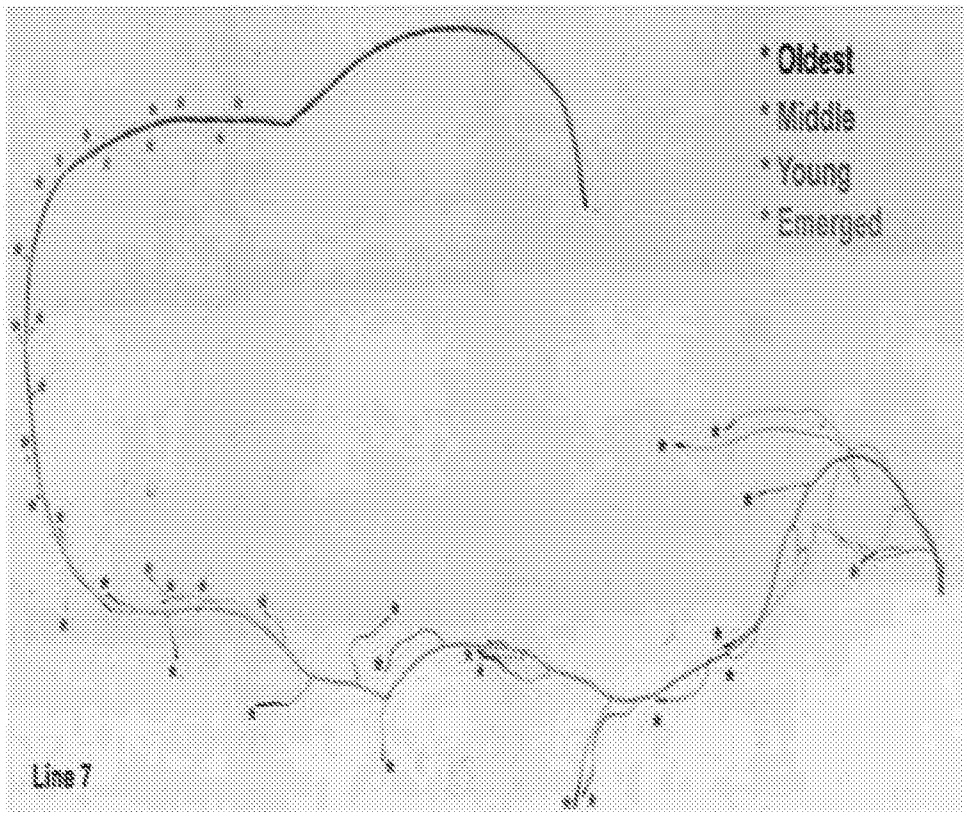


FIG. 5B

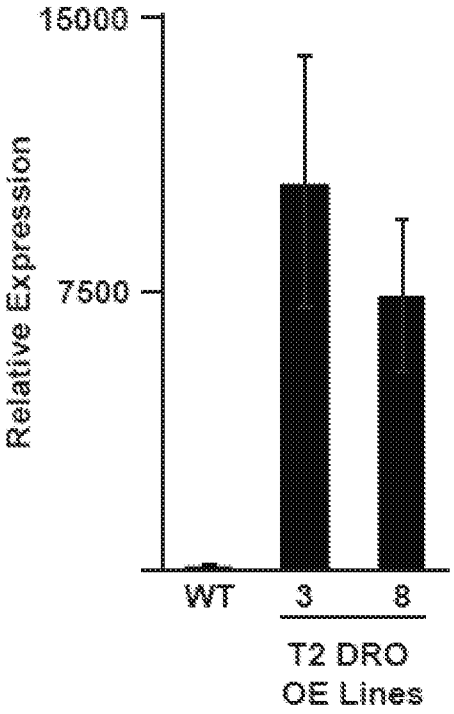


FIG. 6A

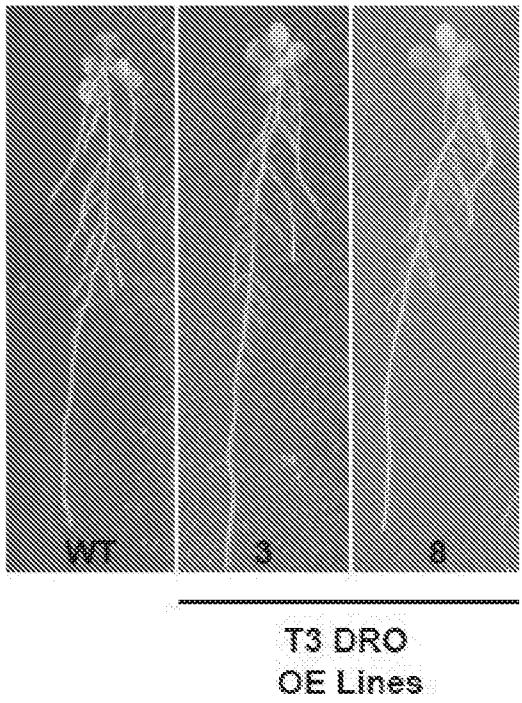


FIG. 6B

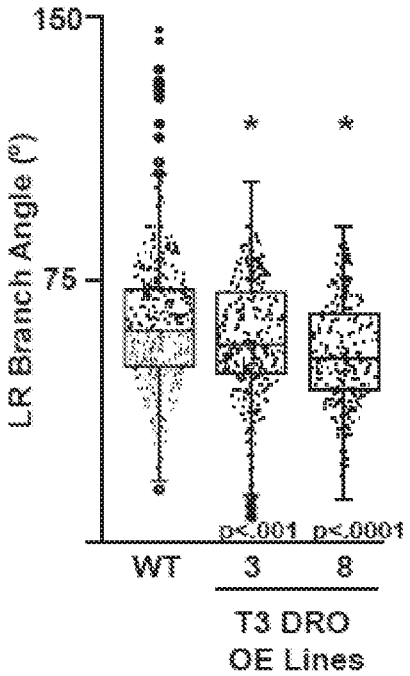


FIG. 6C

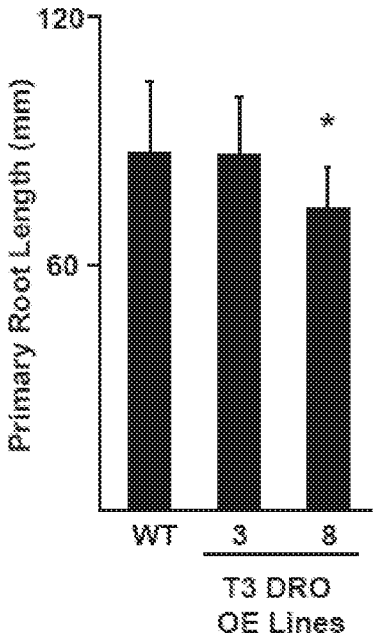


FIG. 6D

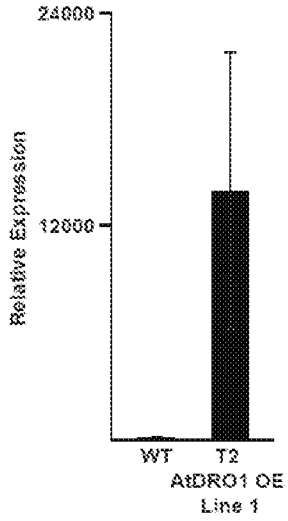


FIG. 7A

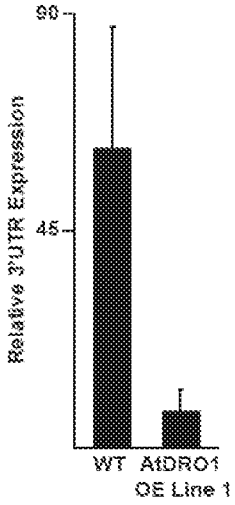


FIG. 7B

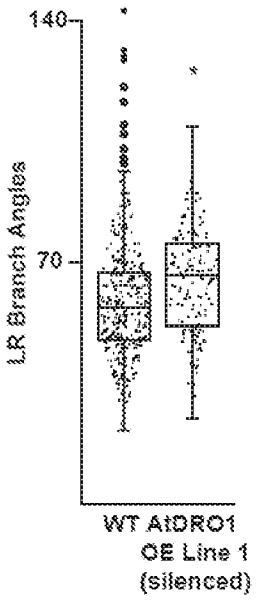


FIG. 7C

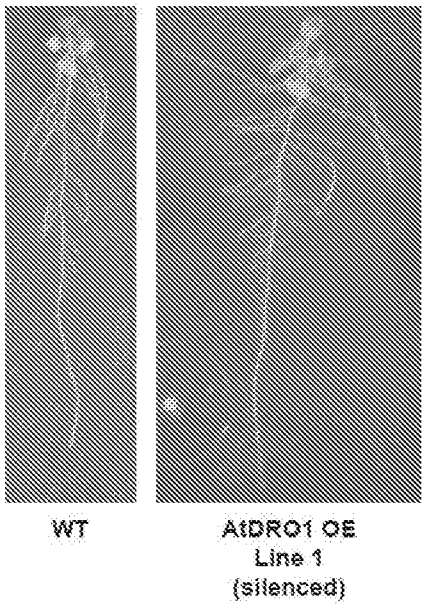


FIG. 7D

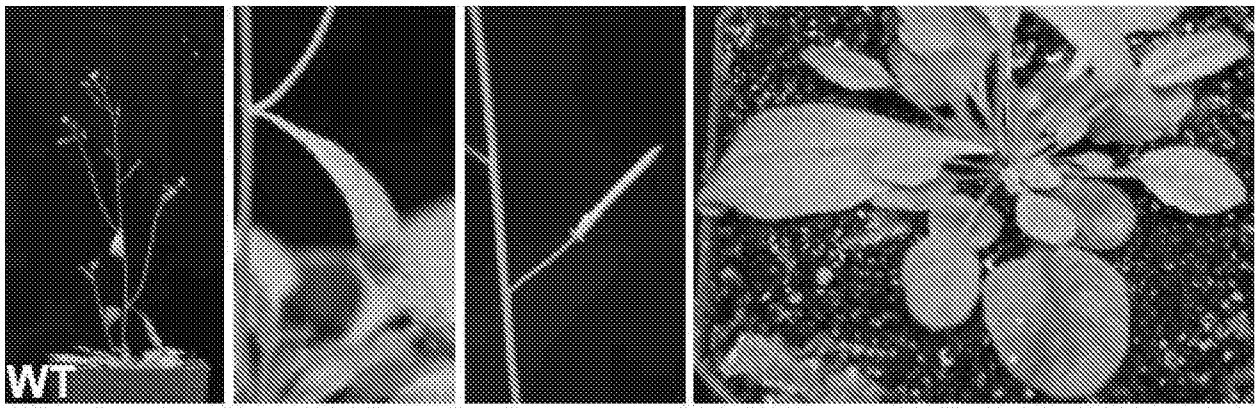


FIG. 8A

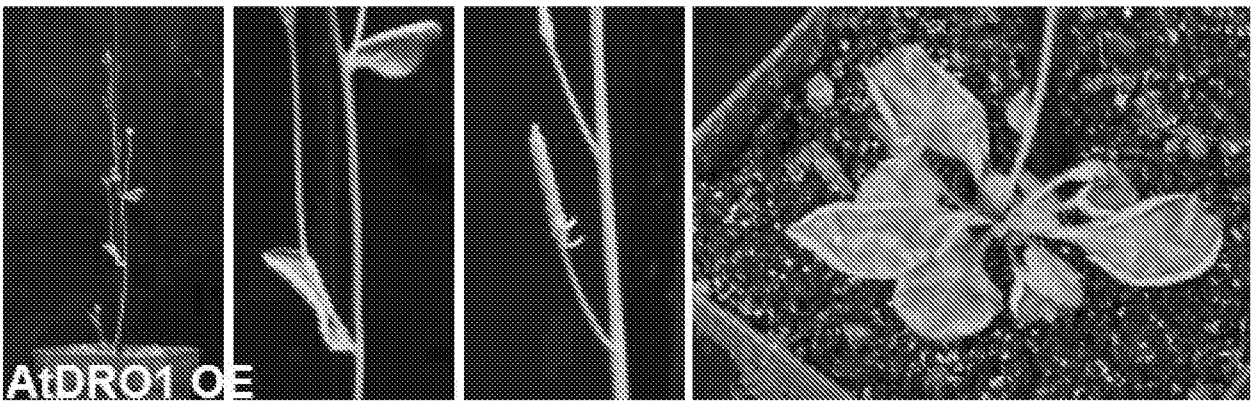


FIG. 8B

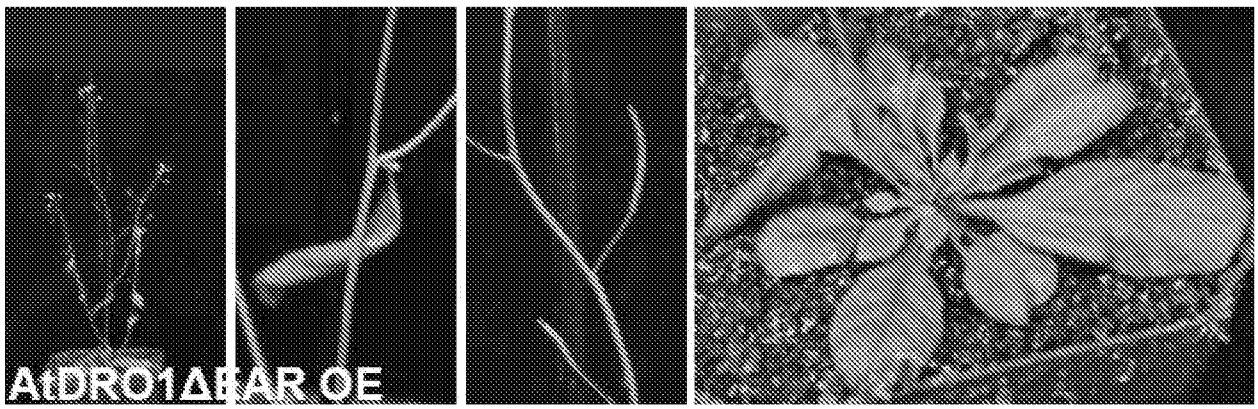


FIG. 8C

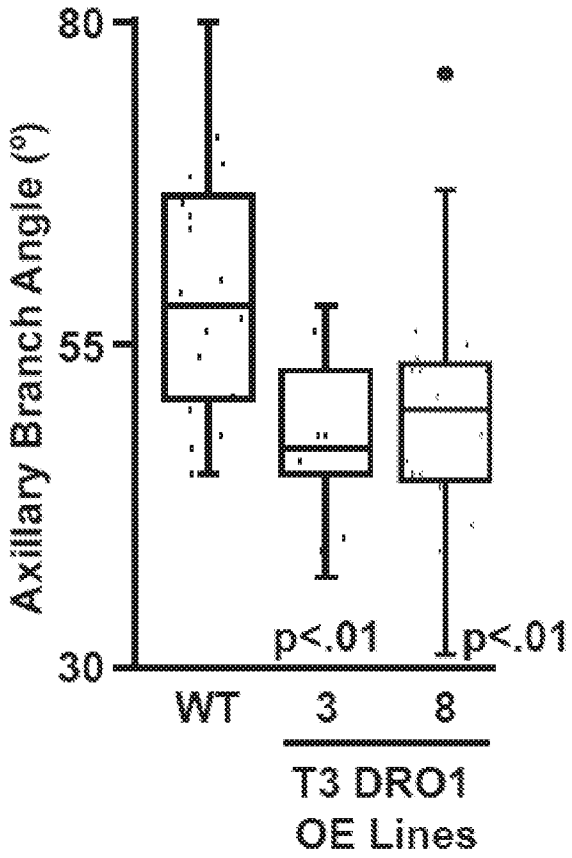


FIG. 8D

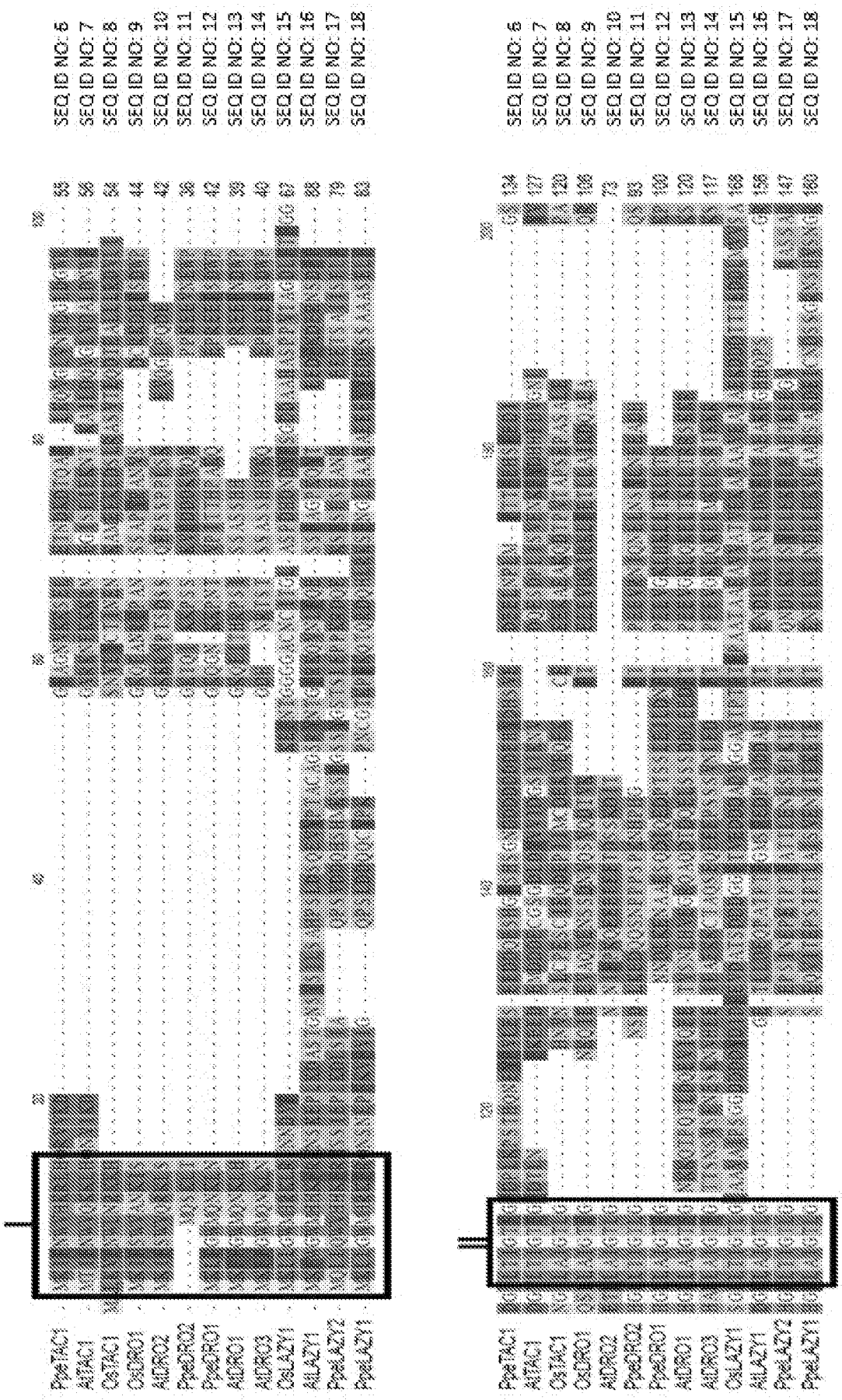

PpeTAC1
AtTAC1
OsTAC1
OsDRO1
AtDRO2
PpeDRO2
PpeDRO1
AtDRO1
AtDRO3
OsLAZY1
AtLAZY1
PpeLAZY2
PpeLAZY1
SEQ ID NO: 6
SEQ ID NO: 7
SEQ ID NO: 8
SEQ ID NO: 9
SEQ ID NO: 10
SEQ ID NO: 11
SEQ ID NO: 12
SEQ ID NO: 13
SEQ ID NO: 14
SEQ ID NO: 15
SEQ ID NO: 16
SEQ ID NO: 17
SEQ ID NO: 18

FIG. 9A

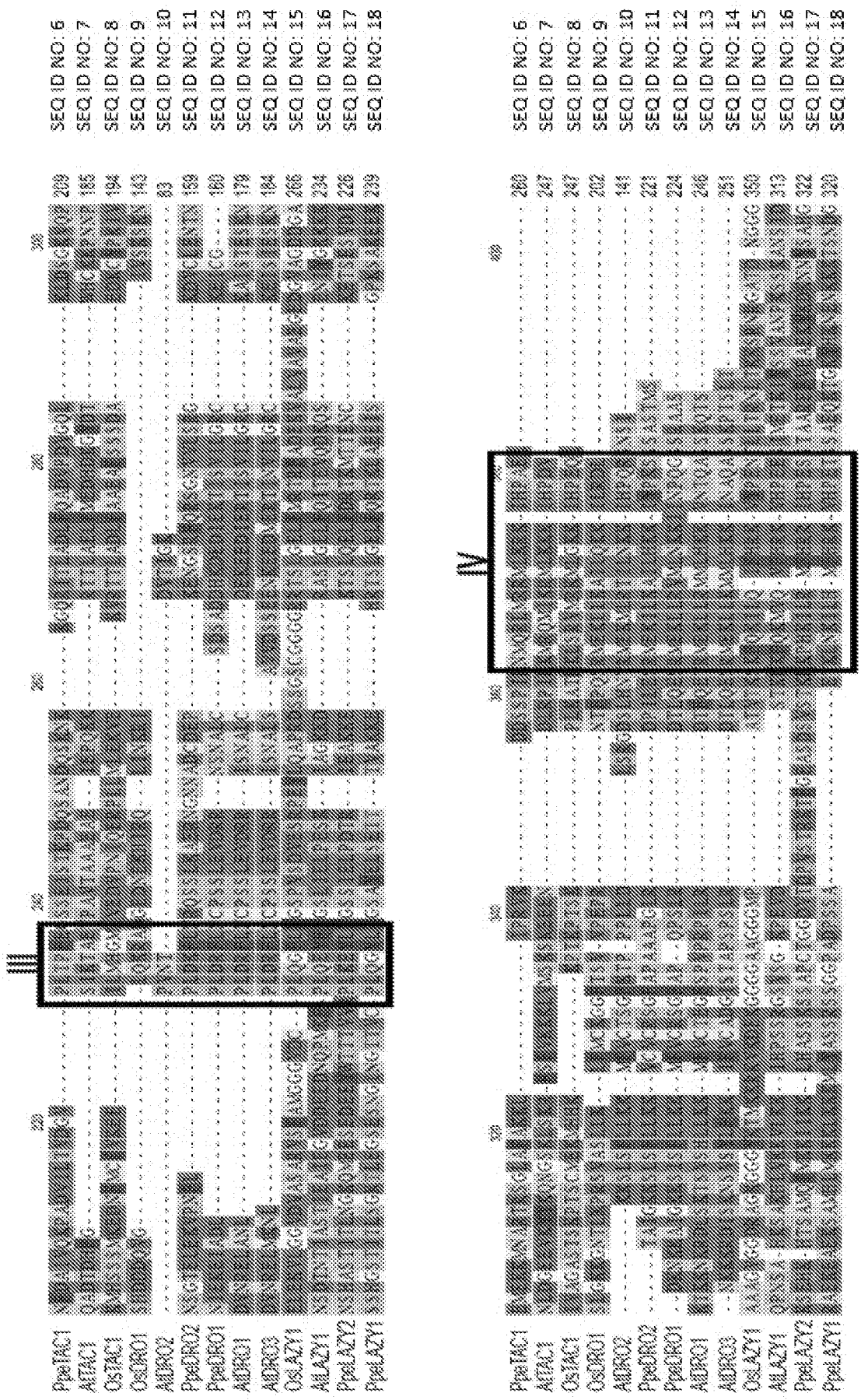

PpeTAC1
AtTAC1
OsTAC1
OsDRO1
AtDRO2
PpeDRO2
PpeDRO1
AtDRO1
AtDRO3
OsLAZY1
AtLAZY1
PpeLAZY2
PpeLAZY1
SEQ ID NO: 6
SEQ ID NO: 7
SEQ ID NO: 8
SEQ ID NO: 9
SEQ ID NO: 10
SEQ ID NO: 11
SEQ ID NO: 12
SEQ ID NO: 13
SEQ ID NO: 14
SEQ ID NO: 15
SEQ ID NO: 16
SEQ ID NO: 17
SEQ ID NO: 18

FIG. 9B

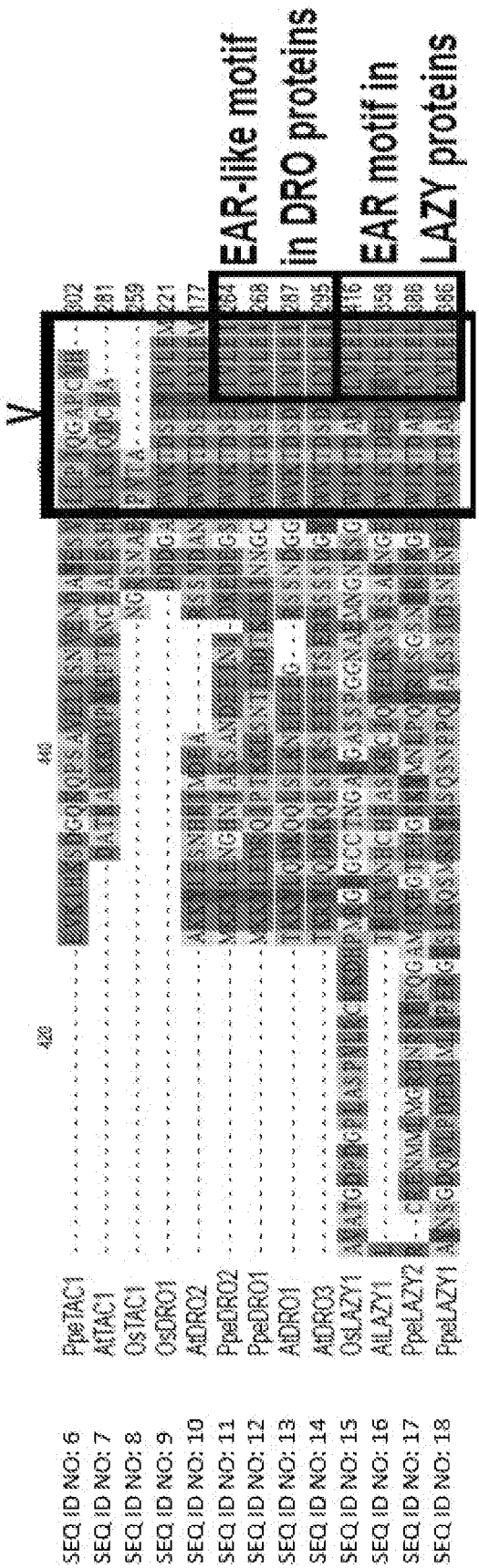

EAR-like motif
in DRO proteins
EAR motif in
LAZY proteins
SEQ ID NO: 6 PpeTAC1
SEQ ID NO: 7 AtTAC1
SEQ ID NO: 8 OsTAC1
SEQ ID NO: 9 OsDRO1
SEQ ID NO: 10 AtDRO2
SEQ ID NO: 11 PpeDRO2
SEQ ID NO: 12 PpeDRO1
SEQ ID NO: 13 AtDRO1
SEQ ID NO: 14 AtDRO3
SEQ ID NO: 15 OsLAZY1
SEQ ID NO: 16 AtLAZY1
SEQ ID NO: 17 PpeLAZY2
SEQ ID NO: 18 PpeLAZY1

FIG.9C

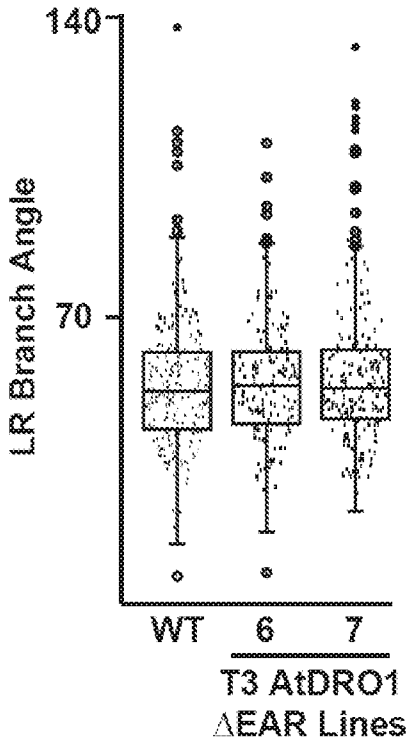


FIG. 10A

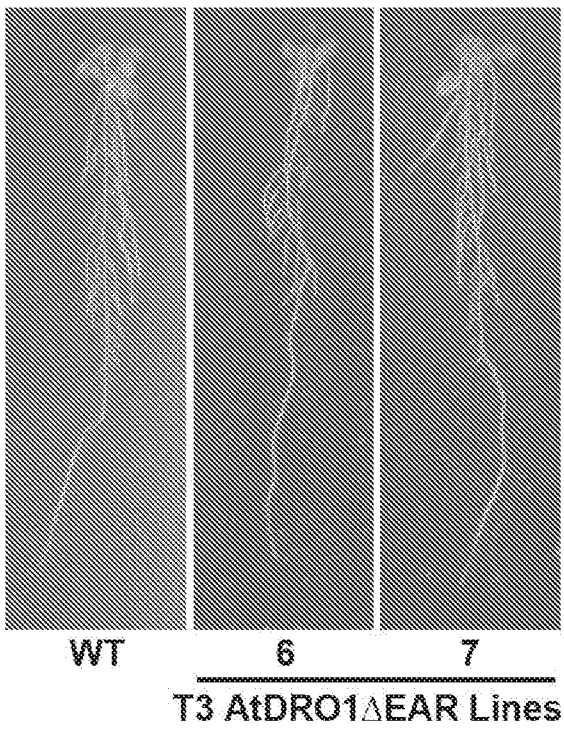


FIG. 10B

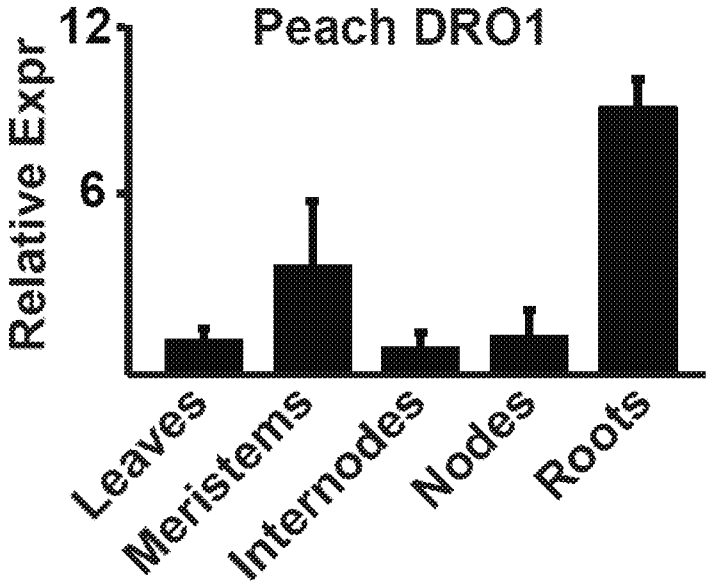


FIG. 11A

FIG. 11B

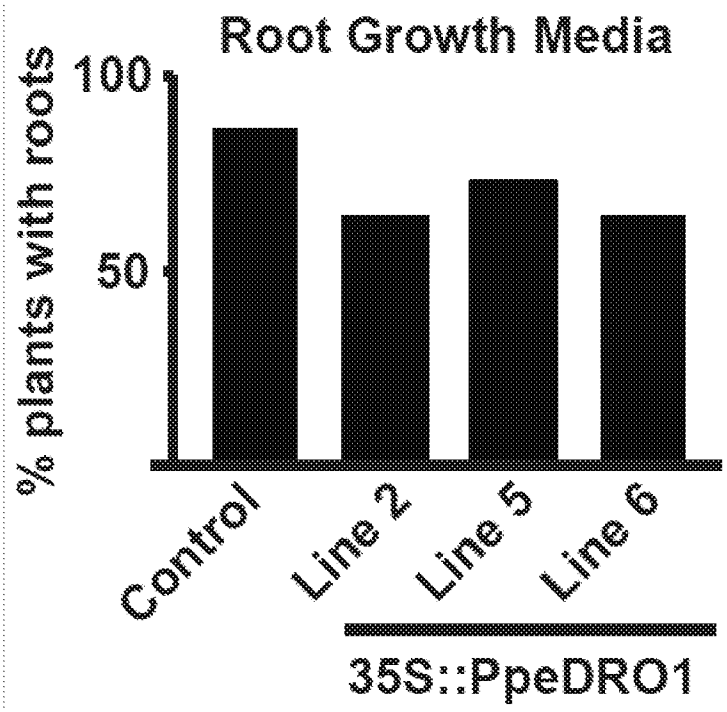


FIG. 11C

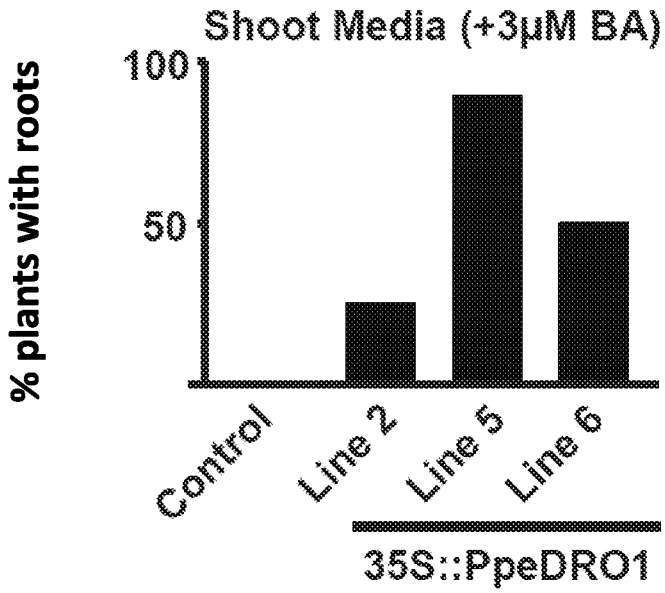


FIG. 11D

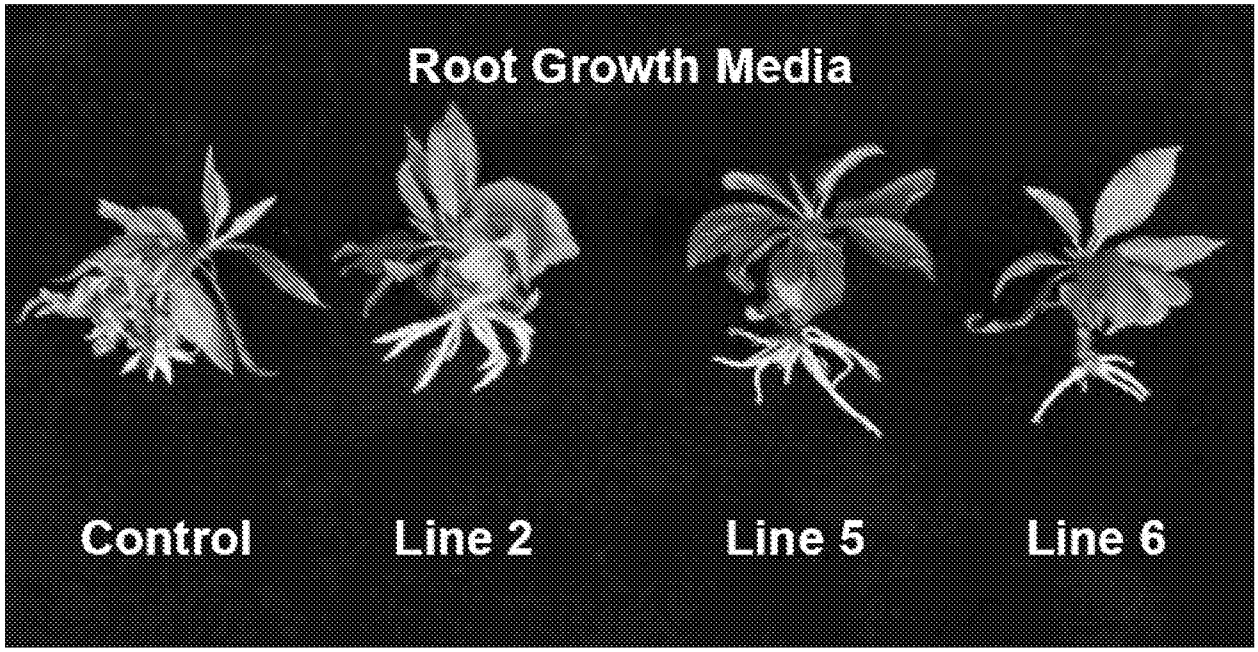


FIG. 11E

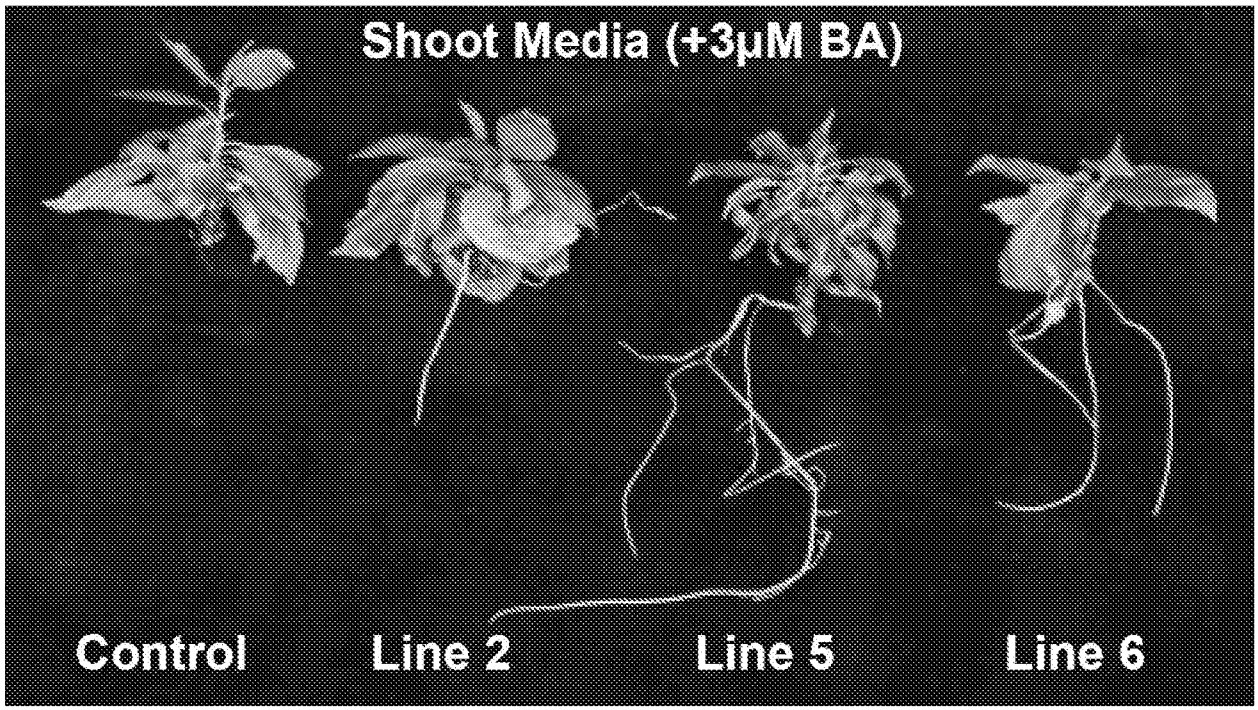


FIG. 11F

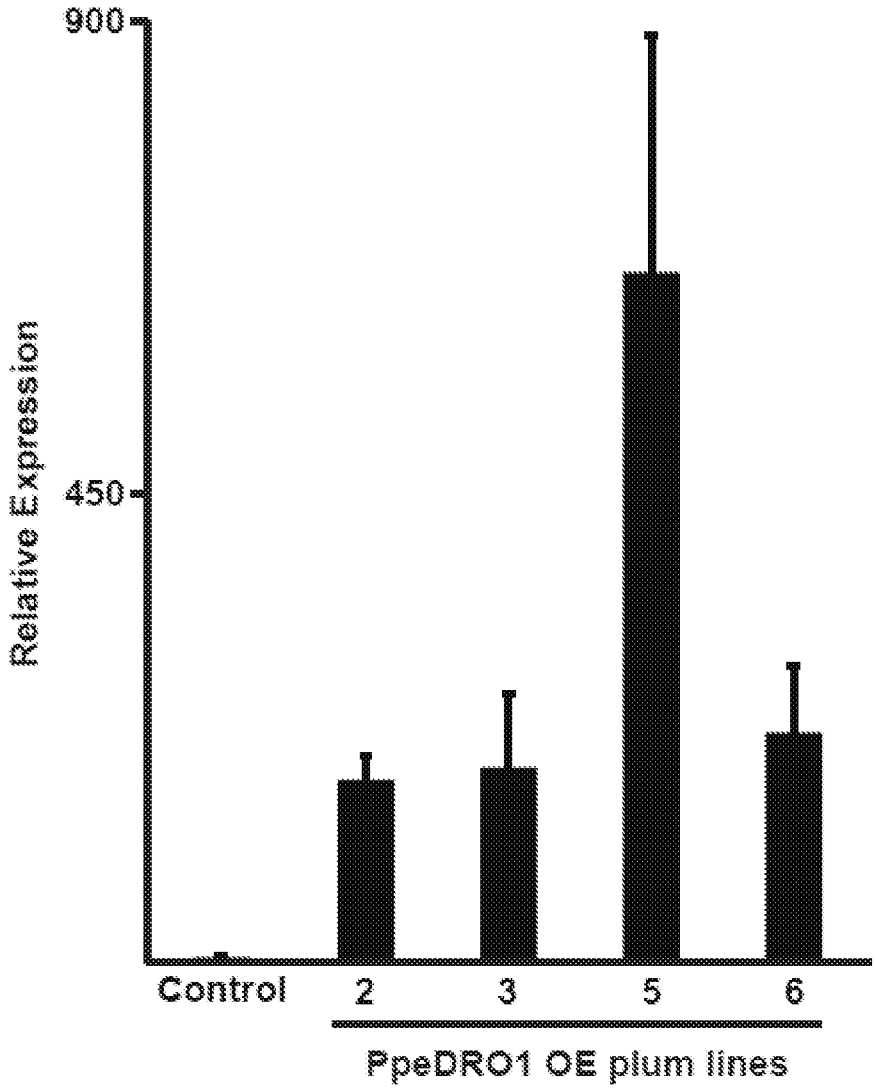

900
450
Relative Expression
Control
2
3
5
6
PpeDRO1 OE plum lines

FIG. 12

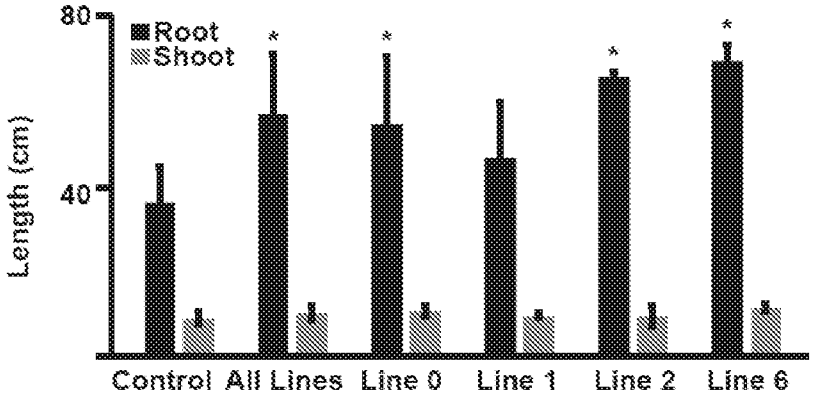


FIG. 13A

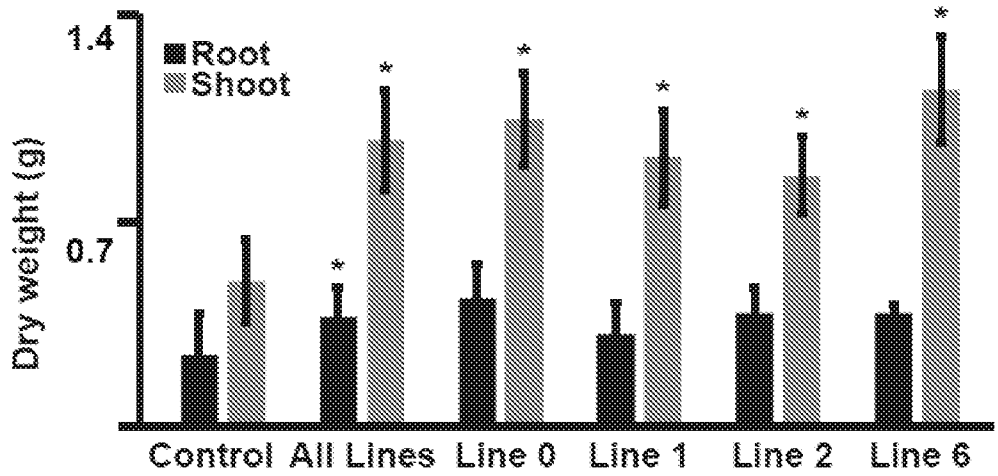


FIG. 13B

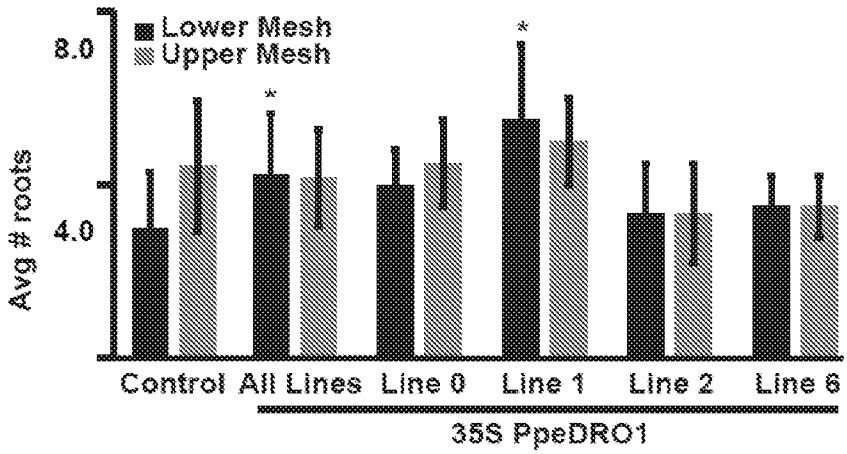


FIG. 13C

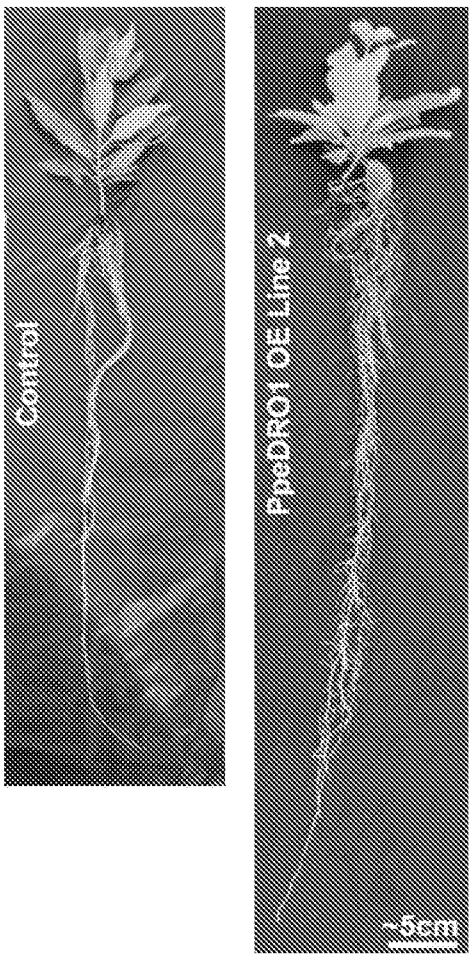


FIG. 13D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/053487

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/82(2006.01)i, C07K 14/415(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/82; A01H 5/00; A01H 11/00; C07K 14/415

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:PpeDRO1, Prunus, vector, transformation, root angle, root length

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUSEMAN, JESSICA M et al., "Deeper rooting 1 controls root orientation and depth in Arabidopsis and Prunus species", In: 33rd Annual Mid-Atlantic Plant Molecular Biology Society, 15-16 August 2016, National Wildlife Visitor Center, US See page 18.	1-14
A	NCBI Reference Sequence: XP_008228169.1 (11 May 2016) See the whole document.	1-14
A	US 2006-0130185 A1 (CHANG, SHUJUN et al.) 15 June 2006 See the whole document.	1-14
A	UGA, YUSAKU et al., "Control of root system architecture by DEEPER ROOTING 1 increases rice yield under drought conditions", Nature Genetics, 2013, Vol. 45, No. 9, pp. 1097-1102 See the whole document.	1-14
A	US 2012-0317678 A1 (UGA, YUSAKU) 13 December 2012 See the whole document.	1-14

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 December 2017 (28.12.2017)	**28 December 2017 (28.12.2017)**

Name and mailing address of the ISA/KR
International Application Division
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/053487

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	GUSEMAN, JESSICA M. et al., "DRO1 influences root system architecture in Arabidopsis and Prunus species", The Plant Journal, 2017 (published online: 28 December 2016), Vol. 89, pp. 1093-1105 See pages 1093, 1098, 1100, 1104; and Supplementary Figure 1.	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2017/053487

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2006-0130185 A1	15/06/2006	AU 2004-246018 A1	16/12/2004
		AU 2004-246018 B2	21/01/2010
		AU 2004-246018 B8	28/01/2010
		BR PI0411030 A	19/09/2006
		CA 2528408 A1	16/12/2004
		CL 2004001427 A1	04/03/2005
		EP 1639082 A2	29/03/2006
		EP 1639082 A4	13/08/2008
		JP 2007-525954 A	13/09/2007
		JP 2012-115266 A	21/06/2012
		JP 4964588 B2	04/07/2012
		NZ 544391 A	26/06/2009
		US 7960614 B2	14/06/2011
		WO 2004-108903 A2	16/12/2004
		WO 2004-108903 A3	07/06/2007
US 2012-0317678 A1	13/12/2012	AU 2010-336253 A1	14/06/2012
		AU 2010-336253 B2	18/09/2014
		BR 112012017055 A2	01/09/2015
		CA 2782300 A1	30/06/2011
		CN 102918154 A	06/02/2013
		CN 102918154 B	17/02/2016
		EP 2518148 A1	31/10/2012
		EP 2518148 A4	23/10/2013
		EP 2518148 B1	26/07/2017
		JP 5791049 B2	07/10/2015
		KR 10-1806342 B1	01/12/2017
		KR 10-2012-0101542 A	13/09/2012
		WO 2011-078308 A1	30/06/2011