RESEARCH ARTICLE

Evolution of Deeper Rooting 1-like homoeologs in wheat entails the C-terminus mutations as well as gain and loss of auxin response elements

Almas Ashraf1*, Obaid Ur Rehman2*, Shumaila Muzammil3, Jens Léon3, Ali Ahmed Naz3*, Fatima Rasool2, Ghulam Muhammed Ali1,4, Yusuf Zafar5, Muhammad Ramzan Khan1,2,4*

1 PARC Institute for Advanced Studies in Agriculture, National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC, Islamabad, Pakistan, 2 National Centre for Bioinformatics (NCB), Quaid-i-Azam University, Islamabad, Pakistan, 3 Institute of Crop Science and Resource Conservation (INRES), Department of Crop Genetics and Biotechnology, Rheinische Friedrich-Wilhelms University of Bonn, Bonn, Germany, 4 National Institute for Genomic and Advanced Biotechnology, National Agricultural Research Centre, Park Road, Islamabad, Pakistan, 5 Pakistan Agricultural Research Council, Islamabad, Pakistan

☯ These authors contributed equally to this work.

* mrkhan@qau.edu.pk (MRK); a.naz@uni-bonn.de (AAN)

Abstract

Root growth angle (RGA) in response to gravity controlled by auxin is a pertinent target trait for obtaining of higher yield in cereals. But molecular basis of this root architecture trait remain obscure in wheat and barley. We selected four cultivars two each for wheat and barley to unveil the molecular genetic mechanism of Deeper Rooting 1-like gene which controls RGA in rice leading to higher yield under drought imposition. Morphological analyses revealed a deeper and vertically oriented root growth in “NARC 2009” variety of wheat than “Galaxy” and two other barley cultivars “Scarlet” and “ISR42-8”. Three new homoeologs designated as TaANDRO1-like, TaBNDRO1-like and TaDNDRO1-like corresponding to A, B and D genomes of wheat could be isolated from “NARC 2009”. Due to frameshift and intronization/exonization events the gene structures of these paralogs exhibit variations in size. DRO1-like genes with five distinct domains prevail in diverse plant phyla from mosses to angiosperms but in lower plants their differentiation from LAZY, NGR and TAC1 (root and shoot angle genes) is enigmatic. Instead of IGT as denominator motif of this family, a new C-terminus motif WxxTD in the V-domain is proposed as family specific motif. The EAR-like motif IVLEM at the C-terminus of the TaADRO1-like and TaDDRO1-like that diverged to KLHTLIPNK in TaBDRO1-like and HvDRO1-like is the hallmark of these proteins. Split-YFP and yeast two hybrid assays complemented the interaction of TaDRO1-like with TOPLESS—a repressor of auxin regulated root promoting genes in plants—through IVLEM/KLHTLIPNK motif. Quantitative RT-PCR revealed abundance of DRO1-like RNA in root tips and spikelets while transcript signals were barely detectable in shoot and leaf tissues. Interestingly, wheat exhibited stronger expression of TaBDRO1-like than barley (HvDRO1-like), but TaBDRO1-like was the least expressing among three paralogs. The underlying cause of this expression divergence seems to be the presence of AuxRE motif TGTCTC and core TGTC with a coupling AuxRE-
like motif ATTTTCTT proximal to the transcriptional start site in TaBDRO1-like and HvDRO1-like promoters. This is evident from binding of ARF1 to TGTCTC and TGTC motifs of TaBDRO1-like as revealed by yeast one-hybrid assay. Thus, evolution of DRO1-like wheat homoeologs might incorporate the C-terminus mutations as well as gain and loss of AuxREs and other cis-regulatory elements during expression divergence. Since root architecture is an important target trait for wheat crop improvement, therefore DRO1-like genes have potential applications in plant breeding for enhancement of plant productivity by the use of modern genome editing approaches.

Introduction

Root architecture appears as the most relevant target trait for breeding against nutrient and water scarce conditions [1–4]. It pertains to spatial underground distribution of roots in the soil. It is demonstrated by growth rate, extent of branching, biomass, texture, length, width, steepness, shallowness, orientation and root angle in response to gravity. Unlike tap root system in dicots which comprise primary and lateral roots, the fibrous root system of monocots contains wide mass of equally sized roots contributed by seed borne seminal roots or shoot borne crown roots [5–7]. Root depth is an important feature for plant growth as it allows better access to nutrients such as nitrogen and stored water present in deeper layers of soil thus, leading to higher yield [8–10]. It is generally acknowledged that a deeper, thicker and more branched root system with a high root to shoot ratio can enhance the tolerance of rice to water deficit [11, 12]. In cereal crops, deeper rooting is manifested by the combination of a large root growth angle (RGA) which is the angle between the soil surface and the shallowest primary root, and long seminal and nodal roots [13]. Because of direction control, RGA determines whether a plant develops shallow or deep rooting system. Thus, requirement of an efficient, steeper and vertically oriented root system to exploit water expeditiously is a desirable strategy in major food safety crops such as wheat [14]. But this organ i.e., root remained orphan not only in wheat but also in other crops mainly due to lack of investment in the form of projects, underground nature that makes it inaccessible to direct observations or efficient phenotyping and scarcity of genetic data pertinent to root growth and architecture.

Root development is a complex phenomenon that involves constitutive and adaptive mechanisms of genes as well as close cis-regulatory correlations with the shoot parts of the plant [15]. However, due to its extreme importance in breeding programs especially under adverse climate, unveiling of molecular mechanism of root development is inevitable. Genetic determinants of root development in rice, maize, Arabidopsis and prunes were identified using complementary direct and reverse genetic approaches such as QTL detection, mutant analysis, transcriptomics and functional genomics. Research on root architecture has brought into light the implication of various factors including key genes, hormones, gene networks and nutrients and hitherto, little progress has been made in connecting RGA, steepness and orientation with yield [7, 16–18]. The first report on influence of auxin on root growth angle of lateral growth was published in Arabidopsis [19]. Later on in rice, in addition to DRO1 involved in RGA, 3 other major QTLs including DRO2, DRO3 and qSOR1 (quantitative trait locus for SOIL SURFACE ROOTING 1) were also mapped that might control the root architecture system under water deficient conditions for greater rice production [20–24]. Negatively regulated by auxin, DRO1 enforces downward bending of roots leading to asymmetric growth. The RGA seems to be controlled by elongation of cells in root tips which may facilitate root in bending downward
direction. Due to greater expression of DRO1 gene, RGA increases towards gravity. DRO1 gene enabled the scientists to reduce drought problem by allowing the root to penetrate deeper thereby help in increasing yield even under shortage of water. Guseman et al. [25] found that DRO1-related genes are present across diverse plant phyla, and fall within the IGT gene family. The IGT family also includes NGR (NEGATIVE GRAVITROPIC RESPONSE OF ROOTS), TAC1 (TILLER ANGLE CONTROL 1) and LAZY1. Previously it was reported that LAZY1 and its orthologs affect the shoot and root gravitropism in rice, Arabidopsis, maize and Medicago [26–32]. In Arabidopsis, there are 6 LAZY genes which influence tropism, auxin gradient and plant architecture in response to gravity [33]. The IGT family manifests 5 domains (I-V) with IGT as conserved motif in the region II. The AtTAC1 and OsTAC1 lack V region. In line with a potential role in root development, AtDRO1 and PpeDRO1 expression is restricted to root [25]. Transgenic Arabidopsis and peach exhibited deeper root angles and phenotypes under ectopic expression of DRO1-like while mutation of DRO1-like resulted in the growth of lateral roots in horizontal orientation. An EAR-like motif IVLEI at the C-terminus seems to be involved in generating overexpression phenotypes. This EAR-like is present in many other proteins as well as in IGT family including TAC1 and LAZY1 [25]. The DRO1-like is an important gene with potential to improve yield in wheat under drought regimes. However, its cloning, expression analysis and interaction with other proteins involved in root architecture needs to be unraveled before using it as a marker in the new breeding method i.e. genome editing. Moreover, the contribution of cis-regulatory elements in the expression and evolution of DRO1-like genes in wheat and barley has not yet been explored.

Wheat and barley are the most important food safety crops cereal crops adapted to diverse climatic conditions. Modern wheat (Triticum aestivum L.) is hexaploid with complex genetics comprising AA, BB and DD genomes contributed by wild relatives. In contrast the diploid genome of barley is closely related to one of the wheat genomes. It will be interesting to study the expression patterns of DRO1-like paralogs in wheat and role of cis-regulatory elements underlying expression divergence since paralogs evolve under similar evolutionary constraints post duplication/triplication as compared to the orthologs [34]. Therefore the major objective of this study was to unveil expression divergence of DRO1-like genes driven by cis-regulatory elements during the course of evolution in tritici (here refers to Triticum aestivum, Triticum urartu, Aegilops speltoids and Aegilops tauschii only).

This study reiterates that 3 DRO1-like homoeologs exist in wheat corresponding to AA, BB and DD genomes. Due to deletions, insertions, frameshifts or intronization/exonization events the genomic locus of TaBDRO1-like exhibit different exon/intron structures than other paralogs. All the three wheat homoeologs can interact with TPL with different intensities, probably through IVLEM/KLHTLIPNK motif at the C-terminus. Absence of an AuxRE binding motif TGTCTC and core TGTC as well as a coupling AuxRE-like ATTTTCTT motif proximal to the transcriptional start site (TSS) in TaADRO1-like, might be responsible for enhanced expression of this gene in root tissues. Conversely, weaker expression of TaBDRO1-like in root tips is attributed to stronger binding of these elements by ARF1 as evident form yeast one hybrid assay.

Results

Wheat and barley exhibit variations in root length and angle

One week old plantlets of two varieties of wheat "NARC 2009 & Galaxy", and barely cultivars "ISR42-8 & Scarlet" grown in pots were evaluated for root growth architecture (Fig 1A and 1B). Both the species developed fibrous hairy rootlets. NARC 2009 exhibited longer and/or deeper roots than the Galaxy. The average root length was 21cm in NARC 2009 in comparison
Expression divergence of DRO1-like genes in wheat and barley

A

Scarlet  ISR42-8  NARC 2009  Galaxy

B

Root length (cm)

Root Dry Weight (g)

C

Days after Sowing

D

NARC2009  Galaxy  Scarlet  ISR42-8

E

Root Angle (°)
with 14cm in Galaxy. Barley cultivar Scarlet developed steeper roots with length of 14.9 cm than the wild barley ISR42-8 which produced only 11.4 cm long roots. Interestingly, wild barley generated shortest or the shallowest roots among the selected wheat and barley cultivars while NARC 2009 developed the longest or deepest roots. However, NARC 2009 demonstrated a significance difference at \( P = 0.05 \). In other varieties, the \( P \) values remained non-significant. Overall, wheat plants of these varieties demonstrated deeper roots than barley. Moreover, drought tolerant wheat plants were taller with higher survival rate.

Furthermore, we calculated the dry weight of the cultivars from 6 to 21 days at the intervals of 3 days (Fig 1C). A positive correlation was observed between increase in number of days and root biomass in the form of dry weight for all the cultivars. Nevertheless, NARC 2009 revealed the maximum dry weight of 20 g after 21 days, while ISR42-8 showed minimum weight of 9 g. Hence, root dry weight seems to be directly related to root length. An important parameter evaluated was the shape or quantification of root tip angles of 6-day-old plants grown on vertically oriented culture plates (Fig 1D and 1E). In case of wheat, NARC 2009 exhibited narrower root angle and featured more vertically oriented root growth. The average root angle of NARC 2009 was 30˚ in comparison with 32˚ in Galaxy. The ISR42-8 showed greater root angle of 48˚ and promoted less vertically oriented root growth than NARC 2009 and Galaxy.

As \( DRO1 \) gene is involved in RGA in response to gravity under drought conditions in rice, therefore we focused on the isolation and analysis of \( DRO1 \)-like orthologs from wheat and barley.

**\( DRO1 \)-like orthologs belong to the IGT family of genes in plants.** In order to isolate coding sequences of \( DRO1 \)-like homoeologs from selected wheat varieties, genes specific primers corresponding to A, B and D genomes of wheat were employed using root tip cDNA of NARC 2009 as template (S1 Table). PCR amplification following by sequencing and BLAST search in “Ensembl Plants” revealed three \( DRO1 \)-like gene products designated as \( TaAN-DRO1 \)-like, \( TaBN-DRO1 \)-like and \( TaDN-DRO1 \)-like (Fig 2A). These newly isolated paralogs clustered with their respective clades comprising \( TuDRO1 \)-like (\( Triticum urartu \)), \( AsDRO1 \)-like (\( Aegilops speltoids \)) and \( AtaDRO1 \)-like (\( Aegilops tauschii \)) in the phylogeny of tritici (Fig 2B). Coding sequences analysis revealed that the size of the newly isolated \( DRO1 \)-like paralogs remained conserved corresponding to their homologs already present in IWGSC database. This size conservation reflects the absence of any deletion, insertion or nonsense mutations in the CDS (S1A–S1C Fig). Pairwise alignment of newly isolated genes showed that the TaAN-DRO1-like with amino acid length of 251 shared 98.4% identity with TaADRO1-like from database harboring only 4 amino acids substitutions. Similarly, TaBN-DRO1-like and TaDN-DRO1-like shared 97.8% and 98.6% identity with their counterparts, respectively (S2–S4 Figs).

In order to discern the different structural domains of the \( DRO1 \)-like proteins, we generated a multiple alignments of the CDS collected from diverse plants including \( Triticum \), \( Aegilops \), \( Hordeum \), cereals, monocots, dicots, \( Arabidopsis \), \( Amborella \), \( Selaginella \) and \( Physcomitrella \). Fig 2C features 5 distinct domains (I–V) with different types of amino acids for \( DRO1 \)-like proteins (S5 and S6 Figs). In \( Triticum \), \( Aegilops \) and \( Hordeum \) all the 5 conserved
Fig 2. Gene isolation, phylogenetic reconstruction and conservation of DRO1-like homoeologs and orthologs in tritici and close relatives. (A) PCR amplification of TaADRO1-like, TaBDRO1-like and TaDDRO1-like from NARC 2009 which is a drought tolerant variety of wheat. M stands for 1kb ladder. (B) Phylogenetic tree of DRO1-like CDS of Tritici, Aegilops and Hordeum generated in neighbor-joining algorithm by taking Arabidopsis as an outgroup. Bootstrap values for 10000 replicates were calculated for reliability of tree. Scale is given at the base. (C) Multiple alignments were generated using ClustalW program in Bio-Edit software. Domains are
domains of variable length are observed. However, 3 insertions of total 4 amino acids are prominent in *Hordeum* in the region I, III and IV.

The common denominator of this gene family is an IGT motif [GΦL(A/T)GT] which is present downstream of the N-terminus in the region II. This motif is also conserved in other DRO1 related proteins such as LAZY and TAC1 [25]. A very prominent feature of this alignment is the conservation of 3 amino acid residues WxxTD at the C-terminus in the 5th domain. This motif seems to be more highly conserved in all the DRO1-like proteins than IGT. In *Selaginella*, the IGT is located 28 aa residues further downstream of the conserved region in the 2nd domain. But WxxTD remained intact in 5th domain in all the DRO1-like proteins. Another hallmark of DRO1-like proteins is the occurrence of EAR-like motif (LxLxL) “IVLEM” in the region V at the extreme C-terminus. Ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif-mediated transcriptional repression is one of the principal mechanisms of plant gene regulation [35]. In DRO1-like genes of dicots, this motif diverged into IVLEI. In triticci however, IVLEM is present in TaADRO1-like and TaDDRO1-like but it is completely altered in TaBDRO1-like and HvDRO1-like with a consensus sequence of LHTLIPNK marked with an addition of IPNK amino acids. The hypothetical progenitor of triticci, *Brachypodium* (in this study) and a primitive plant *Amborella*, kept this IVLEM sequence conserved. Nevertheless, in *Oropetium* grass and other lower plants such as *Physcomitrella* this C-terminal motif is variable in length and sequence composition but it is difficult to distinguish whether these are more DROI-like or LAZY1-like proteins.

Comparison of gene structures of the 17 DRO1-like orthologs from various plant phyla indicated the conservation of 5 exons and 4 introns in most of the plants (S7 Fig). But sizes of their genomic loci were variable. Lower plants exhibited larger size of the DRO1-like gene due to larger exon and smaller introns. However, a reduction in gene size took place during the course of evolution. For example, *Physcomitrella* encode a protein of 431aa residues followed by *Amborella* of 357 amino acids but rice contains only 221 aa which is even shorter than wheat with 251 residues. It is mainly due to deletions in the coding regions. But in some cases such as *TaBDRO1-like* and *HvDRO1-like* genes of wheat and barley respectively, there might occurred intronization or a merger of exon 4 and 5. Nevertheless, the other two paralogs *TaADRO1-like*, *TaDDRO1-like* and their progenitors (*TuDRO1-like* (Triticum urartu) and (*Ata-DRO1-like*) *Aegilops tauschii* kept their exon-intron structures conserved. All the protein coding regions started with ATGAAG exon but *Oropetium* is an exception to this. The first exon of DRO1-like gene in *Oropetium* is 576 bp in size containing only two very short introns. The longest 2nd exon of 962bp was detected in *Physcomitrella*. Remarkably, the shortest introns were found in *Oropetium* and *Arabidopsis*. The length variations in gene structures occurred probably due to deletion, insertions and frameshifts or intronization/exonization events.

In spite of length and sequence variations in gene structures, nevertheless, all the DRO1-like proteins kept the IGT signature, EAR-like and WxxTD motifs conserved.

**DRO1-like interacts with TOPLESS protein.** Previously it was reported that TPL gene involved in lateral branches growth interacts with EAR-like proteins [36]. This EAR-like motif IVLEI at the extreme C-terminus of the DRO1-like proteins in *Arabidopsis* is implicated in narrowing lateral root branching by reducing angle and shortening root lengths as well as in shoot upward curling at leaf margin and alterations in shoot branch angles. With amino acid
sequence variations this motif is present in all the DRO1-like and LAZY1-like proteins selected for study (Fig 3A). Interestingly, in TaBDRO1-like and HvDRO1-like proteins of wheat and barley, respectively, this motif is altered and extended as KLHTLLIPNK. It will be very interesting to find whether TaTPL (S8A and S8B Fig) interacts with wheat and barley homoeologs or not. For this purpose, we adopted transient bimolecular fluorescence complementation (BiFC) approach in Nicotiana benthamiana. Split-YFP analyses in Fig 3Babc indicate that Nicotiana leaves infiltrated with TaADRO1-like, TaBDRO1-like and TaDDRO1-like exhibit YFP signals in cytoplasm, cell membrane and nucleoli, though at different intensities. TaBDRO1-like-TaTPL complementation showed stronger YFP signals than the other two paralogs. As TaBDRO1-like is very similar to HvDRO1-like, therefore these interactions might hold true for HvDRO1-like barley ortholog as well.

In order to test the function of the EAR-like motif we deleted it from all the three wheat DRO1-like proteins. Interestingly, the truncated DRO1-like proteins i.e., TaADRO1-likeΔ246, TaBDRO1-likeΔ245 and TaDDRO1-likeΔ246) were unable to interact with TPL as revealed by complete absence of YFP signals (Fig 3Bdef). Snapshots for the authenticity of YFP signals in black, white and chlorophyll backgrounds using LSCM are given in Fig 3C and 3D. Furthermore, in vitro binding assay using Y2H system was also performed to validate the interaction of TaDRO1-like with TPL proteins (S9 Fig).

These results allow us to infer that TaDRO1-like proteins interact with TPL through EAR-like motifs. How this protein i.e. TPL is involved in DRO1-like pathway needs to be empirically validated.

**DRO1-like orthologs exist in diverse phyla of plant kingdom.** Since DRO1-like is an important developmental protein recruited in root architecture [22] therefore it may exist in entire plant kingdom. We compiled a dataset of 82 DRO1-like orthologs from sequenced genomes of plants representing different phyla. In order to probe the evolutionary relationship among different plants phylogenetic reconstruction was performed in Neighbor Joining algorithm.

A circular tree (S10 Fig) demonstrates the occurrence of DRO1-like orthologs in diverse plants that can be differentiated into 9 to 10 different clades. At the base a small clade of moss Physcomitrella and lycophyte Selaginella is present. This lineage splits into cereals mostly tritici and grasses such as Oropetium which harbors a fibrous root system. In the tritici, all the progenitors or genome contributors of modern wheat (Triticum aestivum) including Triticum urartu, Aegilops speltoids and Aegilops tauschii are clustered together. Remarkably, barley HvDRO1-like is more closely related to TaBDRO1-like and AsDRO1-like. Brachypodium seems to be basal to the tritici. Cereals monocots are next neighbor to palms and banana. Remarkably, Solanaceous plants occupy the position as a sister clade of very diverse plants including Coffea, Populus and Jatropha which in turn are close relatives of lotus and beans instead of Arabidopsis and other Brassica family members. Cotton is clustered with melons and citrus. Prunes, leguminous crops and other trees show close relationship to each other probably due to their root system architecture similarities. Surprisingly, Amborella trichopoda is sister to Beta vulgaris though these two have quite different root morphologies.

An important feature that can be observed from this tree is the occurrence of gene/genome duplication of DRO1-like orthologs in different plants. In addition to occurrence of genome triplication in modern wheat, this phylogeny reveals genome duplication in Glycine max, Phaseolus vulgaris, Brassica napus, banana, oil palm, grapes and Nicotiana etc. The duplicates in certain cases are so diverged that these are not even sister to each other as in the case of Phaseolus vulgaris.

This phylogenetic reconstruction reveals wide spread occurrence of DRO1-like genes in plants. Their clustering in different clades alludes towards existence of evolutionary
Fig 3. Recruitment of EAR-like motif in interaction of DRO1-like proteins with TOPLESS. (A) Amino acid sequence alignment of the 5th domain of the DRO1-like proteins of Triticci, Aegilops, Hordeum, Brachypodium, monocot cereals, Arabidopsis, Prunes, Oropetium thomaeum, Amborella, Selaginella, Physcomitrella and LAZY1 of Prunus is shown. The EAR-like motif is highlighted in red letter while blue residues depict an EAR-like motif in AsDRO1-like, TaBDRO1-like and HvDRO1-like with addition of IPNK residues. (B) Bimolecular fluorescence complementation analysis. Protein-protein interactions studies of DRO1-like homoeologs with TOPLESS (TPL)
relationship in plants with respect to root architecture. Hence expression patterns of these genes are predicted to be divergent even in triticci.

Barley and wheat DRO1-like paralogs exhibit divergent and stronger expression in root tissues. Quantitative and semi-quantitative RT-PCR was carried out to compare the expression patterns of TaBDRO1-like and HvDRO1-like in wheat and barley root tips, respectively. Interestingly, root tips of NARC 2009 and Galaxy showed stronger transcript expression over both the barley cultivars (Figs 4A and S11). Wild barley ISR42-8 displayed the weakest gene expression while NARC 2009 demonstrated the strongest signals. We further compared the expression patterns of DRO1-like paralogs of wheat (TaADRO1-like, TaBDRO1-like and TaDDRO1-like) in root tissues of NARC 2009. Surprisingly, the expression of TaBDRO1-like was the lowest among three paralogs. On the contrary, TaADRO1-like showed the strongest transcript signals in root tips whereas TaDDRO1-like featured moderate level of gene expression. Remarkably, the gene expression of TaBDRO1-like was even weaker than its close ortholog HvDRO1-like in barley.

In order to compare the expression of three paralogs in different tissues includes roots, shoot, leaf and spikelets of NARC 2009 and Galaxy, semi-quantitative and quantitative real time RT-PCR was performed. Fig 4C–4F demonstrates that NARC 2009 surpasses Galaxy in the accumulation of RNA transcripts for the three DRO1-like wheat paralogs. All the three genes show stronger expression in root tissues for both the varieties but transcripts are barely detectable in shoot tissues. Root tips exhibit the strongest transcript signals for all three homo-eloges in both the varieties. Interestingly, expression of DRO1-like wheat paralogs also culminates in spikelets. Nevertheless, all the three paralogs are expressed in root tissue albeit to different level.

These data allow us to infer that besides, showing structural differences, the DRO1-like triplicates of wheat also feature divergence in expression. Thus, there is a need to probe into their cis-regulatory regions specifically promoters.

**Gain and loss of cis-regulatory elements in the promoters of DRO1-like wheat paralogs might influence the expression divergence.** The underlying cause of expression divergence is generally the incremental contribution of cis-regulatory elements residing in the promoter and introns [37]. For cis-regulatory comparison—using the PipMaker tool—we generated dot plots of -2kb upstream promoter regions of wheat and barley DRO1-like orthologs as well as their progenitors including TuDRO1-like, AsDRO1-like and AtaDRO1-like (S12 Fig). All the DRO1-like wheat paralogs showed divergence from each other and only up to -600bp region near the TSS could be aligned. However, these paralogs exhibited strong conservation with their progenitors, particularly TaADRO1-like with TuDRO1-like and TaDDRO1-like with AtaDRO1-like. Interestingly, TaBDRO1-like seemed to be quite divergent from its progenitor AsDRO1-like. It also shows very less homology with HvDRO1-like in comparison with other wheat paralogs although the CDSs of TaBDRO1-like and HvDRO1-like are quite similar. Interestingly, HvDRO1-like promoter is more closely related to TaDDRO1-like than TaBDRO1-like. This is surprising because their CDS and genes structures are similar, and expression patterns also do overlap. Their expression patterns might be governed by the same cis-regulatory motifs. This is one of the speculations that can be put forward for this anomaly.
Previously it was reported that an auxin response factor element (AuxRE) TGTCTC near the TSS in the DRO1 promoter bound by auxin response factor (ARF1) influences the expression of this gene in rice roots [22]. In this study we mapped the position of TGTCTC and core ARF motif TGTC in the promoter and intronic regions of DRO1-like of tritici and other plants (Table 1). The core motif TGTC was located in the intronic and promoter regions more frequently with almost equal rate for all the orthologs. But AuxRE with complete TGTCTC sequence was found only in Sorghum, rice, Amborella and tritici. Interestingly, among tritici only the AsDRO1-like, TaBDRO1-like and HvDRO1-like, which are sister to each other phylogenetically, contain this motif in the promoter proximal region.

We further probed into the promoters dataset and narrowed down to tritici only. Partial multiple alignment of conserved regions of tritici in Fig 5A (S13 Fig) features a 25bp insertion immediately upstream of the TSS in hypothetical diploid progenitor Brachypodium. Interestingly, this insertion encloses an AuxRE core motif TGTC in all the tritici except TaADRO1-like and TuDRO1-like promoter where it is mutated to GGTC. As DRO1 is negatively regulated by auxin, therefore presence of TGTCTC upstream and core TGTC in this insertion might be responsible for weaker expression of TaBDRO1-like and HvDRO1-like genes. On the other hand, their absence in other paralogs TaADRO1-like and TaDDRO1-like might underlie stronger and moderate expression in root tissues, respectively. Remarkably, further upstream of this AuxRE, a composite AuxRE-like motif is present in all the tritici except TaADRO1-like and TuDRO1-like. The combination of both these motifs in close vicinity is reported to enhance the chances of ARF1 binding. Thus, expression divergence of TaDRO1-like paralogs may also be attributed to the possibility of combinatorial interactions of the AuxRE-AuxRE-like motifs. This is very interesting findings because this might hint the relative functional contribution of DRO1-like paralogs in roots architecture in wheat. This facilitates in selecting the paralogs that might be used for further functional characterization through genome editing approach.

The binding activity of ARF1 with AuxREs was tested by the Y1H assay using three 200 bp DNA fragments from different regions of TaDRO1-like promoters. Fig 5B (upper panel) shows that only yeast clones harboring pGAD-ARF1 plasmid in the presence of TaBDRO1\textsubscript{P-TGTC}, grow on the medium indicating that ARF1 is indeed able to recognize and interact with AuxREs. In contrast, in the presence of TaADRO1\textsubscript{P-GGTC} which contains no AuxRE, yeast growth is undetectable suggesting that this sequence no longer serves as a binding site for ARF1. Remarkably, yeast growth is visible for interaction of TaBDRO1\textsubscript{P-TGTC} with pGAD-D-ARF1 indicating that true AuxRE has strong binding affinity for ARF1. Non-lethal β-galactosidase assay confirmed the above mentioned interactions (Fig 5C lower panel). The unit activity of β-galactosidase was calculated for the interaction affinity. The results show that there is maximum number of miller units (more than 81) for TaBDRO1\textsubscript{P-TGTC} with pGAD-ARF1. However, no units of activity were observed for TaADRO1\textsubscript{P-GGTC} as this region is devoid of any AuxRE. Moreover, the values obtained were also statistically significant.

Importantly, the TGTC element also occurs at various other positions in the promoter and intronic regions (Table 1). There are 6 TGTCs in the promoter and 7 in the introns (6+7) of
TaBDRO1-like, and 4+7 in TaDDRO1-like. Though HvDRO1-like contains 5+5 but it is present singly in highly expressing TaADRO1-like and its progenitors TuDRO1-like. In Selaginella, this motif is present but not existing in Physcomitrella (Fig 5A). Its position is a little bit downstream in Oropetium. In other cereals and monocots, TGTC core motif is mutated and even absent in Brachypodium.

Taken together, during the evolution of DRO1-like from TuDRO1-like to TaADRO1-like and AsDRO1-like to TaBDRO1-like there was a gain of one TGTC in each but in contrast, TaD-DRO1-like lost one TGTC after hybridization (Fig 6). Thus, cis-regulatory regions including promoters and introns diverged, and gain and loss of TGTC might have occurred during genome triplication or hybridization.

A schematic diagram showing the evolution of DRO1-like genes in Tritici including Aegilops and Hordeum. The empty upward arrows at the left side indicate the occurrence of DRO1-like evolutionary events from hypothetical diploid progenitor to gene expression stage in the roots of wheat. Dotted arrows symbolize the hypothetical progenitor and originators whereas solid upward arrows mark the changes in gene structures and cis-regulatory elements along with variations in gene expression from TuDRO1-like, AsDRO1-like and AtaDRO1-like to TaADRO1-like, TaBDRO1-like and TaDDRO1-like, respectively in wheat paralogs. Exons in gene structures are symbolized by filled boxes while promoters by empty boxes. Upwards arrows between the exon boxes in the gene structures, show the introns, and numbers above indicate the length of exons and promoters in base pairs. Blue boxes depict the reduction in exon number in AsDRO1-like, HvDRO1-like and TaBDRO1-like as well as extension of an EAR-like motif (from IVLEM to KLHTLINNK) at the C-terminus. Numbers above the nucleotides on gene structures represent the times occurrence of TGTCTC (marked red) and TGTC (marked black) in the promoter regions. An asterisk in the promoter boxes of HvDRO1-like, AsDRO1-like and TaBDRO1-like symbolizes the presence of TGTCTC (red) and TGTC

Table 1. Identification of AuxREs in DRO1-like promoters and introns of Tritici and other plants.

| Sr. No. | Gene   | TGTCTC Position in Promoter | TGTCTC Position in Introns | TGTC Position in Promoter | TGTC Position in Introns |
|--------|--------|----------------------------|----------------------------|---------------------------|--------------------------|
| 1      | AtDRO1 | -1287                      | -                          | -36, -634, -1196, -1851   | +233                     |
| 2      | SiDRO1 | -                          | -                          | -167, -1414, -1427, -1677 | -                        |
| 3      | SiDRO1 | -1486                      | +77                        | -769, -823, -1099         | +221, +609, +632, +804, +1037, +2164 |
| 4      | OsDRO1 | -375                       | -                          | -91                       | +631, +653, +731, +755, +777, +852, +2618 |
| 5      | ZmDRO1 | -                          | -                          | -309, -501, -607, -770, -994 | +308, +1080, +1382, +1586, +2052, +2857, +2888 |
| 6      | TaDRO1 | -                          | -                          | -772                      | +72, +452, +838, +1044, +1059, +1147, +1397 |
| 7      | TaADRO1 | -                         | -                          | -772, -853                | +72, +453, +1046, +1061, +1149, +1406 |
| 8      | AtaDRO1 | -                         | -                          | -372, -1708, -1726, -1827 | +510, +676, +852, +886, +901, +977, +1245 |
| 9      | TaDDRO1 | -                         | +72                        | +72, +453, +1046, +1061, +1149, +1406 |
| 10     | AsDRO1 | -1802                      | -                          | -367, -753, -809, -938, -1785 | +513, +827, +848, +882, +897, +1002, +1015 |
| 11     | TaBDRO1 | -1251                     | -                          | -742, -798, -926, -1738, -1749, -1841 | +509, +824, +845, +879, +894, +999 |
| 12     | HvDRO1 | -1967, -1977               | -                          | -364, -1330, -1747       | +392, +757, +791, +902 |
| 13     | BdDRO1 | -                          | -                          | -825                      | +254, +382, +677, +684, +862, +1018, +1268 |
| 14     | AtDRO1 | -1333                      | -                          | -818                      | +1071, +1155, +4031, +4055, +4574 |
| 15     | OtDRO1 | -                          | -                          | -205, -350, -929, -1281, -1843 | -                        |
| 16     | SmDRO1 | -                          | -                          | -107, -388, -587, -1412, -1669 | -                        |
| 17     | PpDRO1 | -                          | -                          | -851, -875, -1482, -1591 | +36, +355, +1669 |

The position of TGTCTC and TGTC are shown in the promoters with “-” sign while in the introns are indicated with “+” sign. The only “-” sign indicates the absence of TFBSs in the respective regulatory sequence. The position of nucleotide upstream of ATG is taken as -1 while that of downstream as +1.

https://doi.org/10.1371/journal.pone.0214145.t001
Fig 5. ARF1 binds more strongly with the AuxREs in the promoter region of TaBDRO1-like. (A) Multiple alignment of partial promoter sequence of DRO1-like orthologs from 17 different plants is shown (right panel). The conservation of AuxRE core motif (right panel) and upstream N10-like CArG-boxes (left panel) is depicted. Nucleotides marked as yellow indicate the standard TGTCTC motif and TGTC as core near the TSS while nucleotides underlined as yellow represent the N10-like CArG-box which is a binding site for MADS-box transcription factors. B) Yeast 1-hybrid analysis was performed for the detection of binding of ARF1 with cis-DNA fragments of TaDRO1-like (upper panel). The lower panel indicates non-lethal β-galactosidase assay. (C) Calculations of β-galactosidase activity of binding of ARF1 with AuxREs (TGTCTC and TGTC) of TaDRO1-like. * Significant (P<5%).

https://doi.org/10.1371/journal.pone.0214145.g005
(black) motifs. The TGTC asterisk is also there in AtaDRO1-like and TaDDRO1-like but not in TaADRO1-like, and even in hypothetical progenitor that may be BdDRO1-like which is
symbolized by a question mark. Gain and loss of TGTCTC and TGTC is indicated by +1 and -1 in the boxes whereas qualitative expression in wheat root tips (RT) is shown as +, ++ and +++ for weaker, moderate and stronger, respectively.

In addition to TGTC, there is loss of MADS-box cis-regulatory elements (CARG-boxes) at various positions in the promoters of tritici (S2 Table). These CARG-boxes (CCAAATATGG or CCWWWWWWGG, an N10-like box) might be binding sites for the AGL17 clade member such as AGL21 reported to play a crucial role in lateral roots growth induced by auxin [38]. Different variants of CARG-box were detected in tritici. But interestingly, this element occurred with greater frequency in TaBDRO1-like while but remained absent in TaADRO1-like. Hence, expression of TaBDRO1-like was decreased while that of TaADRO1-like increased in the root tips.

We further determined the number and positions of all the plant transcription factor binding sites along the entire length of tritici and barley promoters by taking BdDRO1-like as hypothetical progenitor (S3 Table). A total of 597 TFBSs for 35 different types TFs were detected in all the promoters. Remarkably, AsDRO1-like, TaBDRO1-like and HvDRO1-like gained the maximum TFBSs sites while TaDDRO1-like the least, when compared with Brachypodium. These sites include some of the very important TFs such as MYB84, ABF1, ANT (as highlighted in S12 Fig) ERF2 and NAC69 etc. that play important role in root and shoot development through hormones and stress responsive pathways.

Thus, it is speculated that gain and loss of TFBSs might be responsible for alterations in expression of DRO1-like triplicates during root tip development in wheat.

**Materials and methods**

**Plant material and growth conditions**

Seeds of two varieties of hexaploid wheat (Triticum aestivum L.), "NARC 2009" (drought tolerant) and “Galaxy” (drought susceptible) were obtained from Bio-Resources Conservation Institute (BCI) and Crop Sciences Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan. The Institute of Resource Conservation (INRES) at Bonn University provided the seeds of barley (Hordeum vulgare L.) cultivars ISR42-8 (wild barley) and Scarlet (cultivated barley). Seeds were sown in the pots in glasshouses of INRES and National Institute for Genomic and Advanced Biotechnology (NIGAB), NARC, Islamabad. Nicotiana benthamiana plants were also grown under standard conditions for split-YFP analysis. The work related to plant morphology and protein-protein interactions was performed at NIGAB but rest of the research work regarding gene isolation, cloning, expression and bioinformatics analyses was executed at INRES as well as in the Genome Editing and Sequencing Lab at National Centre for Bioinformatics, Quaid-i-Azam University, Islamabad, Pakistan.

**Root length, angle and dry weight determination**

For determination of root length, 6-days-old germinated seedlings of NARC 2009, Galaxy, ISR42-8 and Scarlet were selected. Plants were shifted from the pots, separated and roots were gently washed with tap water until cleaned. The root length of wheat and barley varieties were measured with a scale and data were recorded as means of replicates.

In order to measure the root angle of selected wheat and barley varieties, surface sterilization of seeds were done with 15% Clorox solution for 12 min. Seeds were rinsed three to four times with sterile distilled water and germinated for 2 d on vertical orientated 0.5 X MS media containing 0.7% agar and 1% sucrose. Seedlings were imaged and root angles were measured using ImageJ software. Furthermore dry weight of the roots was also compared. Seven to 21-days-old plantlets of NARC 2009, Galaxy, ISR42-8 and Scarlet were uprooted at intervals of 3 days. Roots were kept in oven at 37.8°C for 24 hours. They were sealed inside Ziploc bags to
avoid regaining of the moisture and weighed on balance after cooling. The procedure was adopted for every single plant separately. Data were recorded as means of replicates. A Student’s T-test was applied to calculate the statistical significance of the bar chart data.

Genes isolation
Coding sequences of DRO1-like genes were retrieved from IPK Barley BLAST Server (http://webblast.ipk-gatersleben.de/barley_ibsc/) and International Wheat Genome Sequencing Consortium (IWGSC; https://www.wheatgenome.org/Tools-and-Resources/Sequences) databases. Primers were designed from the CDS of TaADRO1-like, TaBDRO1-like, TaDDRO1-like and HvDRO1-like for gene isolation, expression pattern analysis and protein-protein interaction experiments (S1 Table). Total RNA was extracted from root tips of the NARC 2009, Galaxy, ISR42-8 and Scarlet using TRIZole reagent kit (Thermo Fisher Scientific). The cDNA template was generated by RevertAid kit (Thermo Fisher Scientific). In order to amplify full length coding sequences of DRO1-like genes by PCR, rTaq polymerase (TaKaRa, Tokyo, Japan; Code R001) was used. The PCR profile was set at 95˚C for 5 minutes, followed by 94˚C for 30 seconds, 58˚C for 30 seconds and 68˚C for 1 minute for 37 cycles. PCR products were resolved and photographed on 1% agarose gel containing ethidium bromide. The purified amplicons were cloned in pTZ57R/T vector by using InstAclone PCR Cloning Kit (Thermo Fisher Scientific) following the manufacturer’s guidelines, and then sequenced commercially. Similarly, TPL ortholog from wheat was also amplified, cloned and sequenced. Newly isolated TaADRO1-Like-N, TaBDRO1-Like-N and TaDDRO1-Like-N genes have been deposited in NCBI database under accession numbers MK639010, MK639011 and MK639012, respectively.

Genomic loci of 17 DRO1-like genes form different plants including tritic, Aegilops, Hordeum and other higher and lower plants were retrieved using Ensembl genome browser. These sequences were annotated for determination of CDS, UTRs, start-stop codons and intron-exon structures. All the information was documented in the form of a flowchart with nucleotide numbers and positions in base pair unit.

Bimolecular fluorescence complementation (BiFC)
The TPL protein interacts with other proteins through EAR motif regulated by auxin. As DRO1 is also regulated by auxin, therefore we did the BiFC experiments in planta to validate the interaction of TPL with TaADRO1-like, TaBDRO1-like and TaDDRO1-like, and their truncated versions (TaADRO1-likeΔ246, TaBDRO1-likeΔ245 and TaDDRO1-likeΔ246) by removing IVLEM/KLHTLIPNK motif. For this purpose split-YFP system elaborated by Bracha-Drori (2004) and Walter (2004) was employed [39, 40]. The full-length ORFs of these three proteins were first cloned into the pENTR/D-TOPO-201 vector to generate entry clone (Invitrogen) and then Gateway technology (Invitrogen) was used for introducing it into the pBaTL-YFPc and pBaTL-YFPn vectors. The Agrobacterium tumefaciens strain GV3101RK was electroporated with the above mentioned 6 constructs. The constructs were co-infiltrated into the 2- to 3-week-old leaves of Nicotiana benthamiana from abaxial surface after pinching with a yellow tip. The addition of the p19 viral silencing suppressor was must in the infiltration mixture to avoid co-suppression [41]. The plants with infiltrated leaves were kept under glasshouse conditions for 2 to 3 days. A normal fluorescence microscope Nikon Digital SIGHT DS- i2, ECLIPSE Ni-U was used for scanning YFP signals in leaves after three days of growth. YFP signals were also scanned with CLSM (Leica) and snapshots were taken.

Generation of yeast 2-hybrid constructs and protein-protein interaction studies. For detecting TaDRO1-like interactions with TPL protein, AH109 strain with two yeast vectors pGBKT7 (bait) and pGADT7 (prey) were employed (Clontech). Y2H in vitro assay was done
according to the methods described in the yeast protocols handbook (Clontech). A total of 6 yeast constructs, two each for TaADRO1-like, TaBDRO1-like and TaDDRO1-like, and their truncated versions (TaADRO1-like^{Δ246}, TaBDRO1-like^{Δ245} and TaDDRO1-like^{Δ246}) were tested. Autoactivation and autobinding were tested by co-transformation TaDRO1-like-TPL proteins with empty bait and prey vectors. Finally, all the constructs were co-transformed into yeast strain AH109 to study their interactions with each other. High-stringency (SD/-Trp-Leu-His-Ade) yeast media were used for plating. The Y2H analysis and interaction matrix was generated as mentioned in Ihsan et al. [42].

Gene expression
Gene expression patterns of DRO-like in wheat and barley were discerned by semi-quantitative and quantitative real-time RT-PCR using real-time PCR machine 7500 Fast (ABI). Total RNA was extracted from different tissues of wheat and barley cultivars using TriZole reagent (Invitrogen). These tissues included crown root, root tip, leaf, middle root, basal root, basal shoot and spikelet. A reaction volume of 25μl was used for each sample. The elf and tubulin served as endogenous controls. Primers specific to TaADRO1-like, TaBDRO1-like, TaDDRO1-like and HvDRO1-like were used on cDNA templates, and RT-PCR was performed as described by Khan et al. [43]. The relative expression of DRO1-like transcripts was compared in wheat and barley cultivars. The transcript abundance was also compared for three wheat homoeologs in root tips of NARC 2009. Three biological as well as technical replicates were undertaken. Semi-quantitative RT-PCR products were resolved on 1.5% agarose gel whereas normalized fold expression of real-time PCR products was attained using 7500 Fast Software v2.3.

Sequence analysis and phylogenetic reconstruction
Newly isolated sequences of TaANDRO1-like, TaBNDRO1-like and TaDNDRO1-like were analyzed using Bio-Edit tool. Sequences were BLAST-searched and only sequences with maximum number of hits with quality score were selected. Complete coding sequences were assembled. In addition to newly isolated sequences, the retrieval of both the coding and promoter sequences of DRO1-like orthologs from the different phyla of plant kingdom with sequenced genomes (82 around) including mosses, lycophytes, Amborella, Oropetium grass, tritici, monocots, dicots, rosids, astrids etc. was carried out using NCBI, Ensembl genome browser [44], IWGSC databases and IPK barley blast server. For sequence analysis, different datasets were generated for titici, monocots, dicots and entire plant phyla. Multiple and pairwise alignments were generated using ClustawW program in the Bio-Edit Sequence Alignment Editor (http://www.mbio.ncsu.edu). Conserved domains and important motifs were highlighted. In order to infer the evolutionary relationship of DRO1-like orthologs, a circular neighbor-joining tree with bootstrap replications of 10000 was reconstructed by MEGA6 [45, 46].

Promoter analysis and identification of cis-regulatory elements
Without a special note we retrieved -2kb upstream sequence as a promoter for all the DRO1-like orthologs. Among these, most of the promoter sequences consisted of tritici but also included mosses, liverworts, tuft grass, monocots, dicots and rosids etc. We also selected entire genomic loci of the above mentioned plants comprising all the exon-introns for the identification of cis-regulatory elements. Dot plots analyses in PipMaker program (http://pipmaker.bx.psu.edu/pipmaker/) were done [47] for comparison of promoter sequences and identification of regions of close similarity. For this purpose, promoter sequence -2kb upstream of ATG for each gene was inserted in the input box and a dot plot was generated through pairwise
alignment. For identification of cis-regulatory motifs including auxin response elements (AuxREs), we used Bio-Edit and different online programs such as Mulan (Multiple-sequence local alignment) (http://mulan.dcode.org) with TBA and MultiTF (http://rvista.dcode.org/cgi-bin/mTTF.cgi), and also did it manually [48]. The number and position of TGTC and TGTC core motifs was documented for each promoter and intronic region individually. The positions of conserved regions containing AuxREs and AuxRE-like were located and TGTC motif was highlighted.

In order to find all the plant specific TFBSs in the promoter of all the tritici, Mulan program was used. Putative cis-acting elements in the individual promoters and downstream regions were predicted using the plant specific cis-acting DNA elements in the TRANSFAC (http://www.gene-regulation.com/pub/databases.html) database. The type, number and times occurrence of TFBSs with position was recorded separately for each gene promoter. Presence and absence of TFBSs was compared and gain and loss of TFBBs was inferred from the tables generated.

**Yeast 1-hybrid assay.** In order to confirm the in vitro binding of TaDRO1-like by trans-regulators ARF1 to AuxREs (TGTC and TGTC) in the promoters, we performed Y1H analysis. The Y1H assay was carried out using the MATCHMAKER one-hybrid system (Clontech). Three types of TaDRO1-like DNA fragments each around 200bp in length were selected. The type 1 fragment contains core AuxRE TGTC of TaBDRO1-like while sequence of type 2 fragment was GGTC in case of TaADRO1 near the TSS. The region towards more proximal promoter from -1100 to -1300 bp containing complete TGTC motif was included in the 3rd type of fragment. These three fragments were directly inserted into the multiple cloning sites of reporter plasmids of pLacZi and pHISi-1, respectively. After linearization these three bait constructs were integrated into the genome of yeast strain YM4271. The dual reporter strain was selected and maintained on synthetic dextrose (SD)/-His/-Ura medium. For construction of the pGAD-ARF1 fusion, the cDNA of ARF1 was ligated with GAL4 activation domain in pGAD424 plasmid. Finally constructs were introduced into yeast strain with dual reporter genes, with a blank pGAD424 plasmid as control. After co-transformation, the yeast transformants were tested on SD/-Leu/-His/-Ura medium containing 3.0 mM 3-amino-1, 2, 4-triazole (3-AT) and 80 mg l⁻¹ of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 1x BU salt.

Non-lethal β-galactosidase assay was performed as described in Clontech manual. The ORF of ARF1 was fused in-frame with the GAL4 activation domain of the one-hybrid vector pGAD424, and then transferred into yeast cells containing pLacZi-AuxREs plasmids, respectively; the blank pGAD424 plasmid was used as control. The unit of β-galactosidase activity was calculated by the equation of U = 1000×[OD₄₂₀]/(time (in min)×volume (in ml)×[OD₆₀₀]. A Student’s T-test was applied to calculate the statistical significance of the bar chart data.

**Discussion**

“The Power of Movement in Plants” is famous book in which Charles and Francis Darwin [49] long ago documented the capability of plants for orienting their root growth towards gravity through root tip bending. Gravitropism is necessary for roots to grow into the soil not only for water and nutrient acquisition but to anchor plant as well [50]. Presence of a profuse and deeper root system may have a direct impact on yield under adverse climatic conditions [51]. Therefore, in this study we focused on root system architecture of wheat and barley. When morphologically compared, both the wheat varieties NARC 2009 (drought tolerant) and Galaxy (drought susceptible) showed longer roots in comparison with wild ISR42-8 (wild barley) and Scarlet (cultivated variety). Among all cultivars NARC 2009 demonstrated a deeper root network, more vertical RGA, gain of dry weight, healthier and taller plants.
Development of drought resistant varieties with improved root architecture is the most desirable strategy to increase the crop production [52]. Molecular basis of this orphan trait revealed that DRO1 gene which is negatively regulated by auxin, controls RGA in response to gravity in rice leading to increase in yield under drought imposition [22]. From NARC 2009, we were able to isolate 3 DRO1-like paralogs designated as TaANDRO1-like, TaBNDRO1-like and TaDNDRO1-like. Phylogenetically, HvDRO1-like of barley is sister to TaBDRO1-like of wheat. Comparison of gene structures of the 17 DRO1-like orthologs from various plant phyla indicated the conservation of 5 exons and 4 introns in most of the plants. But size of the gene structures remained variable. The length variations in gene structures occurred probably due to deletion, insertions and frameshifts or intronization/exonization events.

Here we found that DRO1-like proteins are present across diverse plant phyla and fall within the IGT gene family [26]. LAZY, NGR and TAC1 are also reported as member of this family. These genes control orientations of above and underground plant parts. Six LAZY genes (LZY1-6) with differential expression in various tissues were identified in Arabidopsis by Taniguchi et al. [32]. Arabidopsis LAZY1 family plays a key role in gravity signaling within statocytes and in branch angle control of roots and shoots. Yoshihara and Spalding [33] identified the role of LAZY family in mediating the effects of gravity on auxin gradients and plant architecture. The previous name of LAY3 was AtDRO1 or AtNGR2. LAZY and TAC1 contain IGT motif but their function is to control lateral shoot development rather than RGA in monocots and dicots [53–56, 27, 57–59]. In lower plants such as Physcomitrella and Selaginella, the differentiation between LAZY, TAC1 and DRO1-like proteins remains enigmatic.

Though IGT motif is reported as the signature for this family but our study revealed that another motif WxxTD in the 5th domain can also act as the prominent feature of this family because its position is more conserved across the entire plant phyla than the IGT of 2nd domain. Another common feature of this family is an EAR-like motif at the extreme C-terminus. This motif is present in numerous co-repressors that show co-repression through interaction with TPL protein [60]. Best known for interaction with auxin-regulated proteins such as BODENLOS (BDL) involved in root development, TPL interacts with other transcription factor complexes involved in various other pathways in plants [61–66] thereby converting them to transcriptional repressors [63]. These protein-protein interactions are mediated by a small conserved motif known as the ethylene response factor (ERF)-associated amphiphilic repression (EAR) domain [46] with a consensus sequence (L/F)DLN(L/F)xP, which has also been identified in several other transcription factors with repressive activity [46, 67, 68]. Around 219 candidate proteins belonging to 21 transcription regulator families harbor this EAR-like motif and show protein repression [45].

Gruiseman et al. [25] demonstrated that C-terminus motif IVLEM in DRO1-like is required for lateral root phenotypes in Arabidopsis. It also narrows the lateral root growth angles. In the present study, through split-YFP and Y2H analyses we were able to confirm that all the 3 wheat paralogs could interact with TPL protein with different intensities. Remarkably, the difference of IVLEM in TaADRO1-like and TaDDRO1-like, and KLHTLIPNK in TaBDRO1-like did not affect the interactions spectrum of TaDRO1-like with TPL. But TaDRO1-like proteins with removed IVLEM/ KLHTLIPNK abolished the interactions. Thus, IVLEM is recruited in interaction with TPL. Root architecture seems to be trait controlled by a network of genes and this is what our protein-protein interaction studies reveal. How this interaction can change the function in any pathway with repressing activity remained to be unveiled. We may speculate that it might have a role in auxin flux that negatively regulate the expression of TaDRO1-like or block its expression in shoots. Hence, this interaction may serve as a link between roots and shoot development.
The role of cis-regulatory elements after duplication/triplication of genes during speciation in expression divergence can hardly be exaggerated [69]. Different models have been proposed and genome duplications seems to be the best option to study the gain and loss of cis-regulatory elements as genomic paralogs i.e., homoeologs are provided with the same environment than orthologs [34]. DRO1-like triplicates of wheat in comparison with barley seems to be ideal for studying the contribution of cis-regulatory elements in expression divergence is proposed by this study. The analysis of promoter and intronic regions in wheat indicated that gain and loss of cis-regulatory elements might have contributed in the expression divergence of DRO1-like paralogs. Presence of an ARF1 binding motif TGTCTC in TaBDRO1-like and HvDRO1-like upstream of the TSS at positions -1251bp and -1967, -1977, respectively, might be responsible for stronger gene expression in wheat root tips than other two paralogs. Uga et al. [22] observed this TGTCTC motif near the TSS in rice. Through electrophoretic mobility shift assay (EMSA), they validated it as binding site for OsARF1/OsARF23. These auxin-responsive elements (AuxREs) occur in upstream regions of some early-auxin-response genes, and ARFs bind AuxREs to regulate the transcription of these genes [70]. Within AuxREs, the TGTCTC motif makes the greatest contribution to ARF binding strength [71]. In promoter analysis, we detected a conserved region near the TSS where TGTC was conserved in TaBDRO1-like and TaDDRO1-like but mutated to GGTC in TaDDRO1-like. This is speculated to be an important element, which by binding with ARF suppresses the expression of TaBDRO1-like and HvDRO1-like. Mironova et al. [72] suggested that AuxRE with its coupling sequence AuxRE-like (ATTTTCTT) may form a composite element for stronger binding of ARF. Interestingly, this coupling motif is detected in TaBDRO1-like but not in TaADRO1-like promoters. Remarkably, through Y1H we are able to empirically validate the binding of TaBDRO1-like TGTC and TGTCTC while no binding of GTGC with ARF1 protein was detectable.

Another important motif that can bind MADS-box TFs i.e. CARG-box (CCWWWWWWGGG, other variants also do occur) was detected in TaBDRO1-like promoter with greater frequency. Root specific AGL 17 clade MADS-box TF such as AGL21 [44] may bind it. These TFs play a crucial role in lateral root development. In Arabidopsis, AGL21 was found positively regulating auxin accumulation in lateral root primordia and lateral roots by enhancing local auxin biosynthesis, thus stimulating lateral root initiation and growth. It is speculated that binding of AGL21 to CARG-motif of TaBDRO1-like decreases the auxin level and thus decreasing the expression of these genes in roots. Other cis-element binding TFs including NAC69, MYB84, ER, and DOF might also play an important role. Most of these sites were lost in the TaA-DDRO1-like and TaDDRO1-like but gained in HvDRO1-like, AsDRO1-like and TaBDRO1-like. Thus, gain and loss of these cis-regulatory elements might have played a major role in expression divergence among TaDRO1-like homoeologs during evolution.

Speculations about the evolution of DRO1-like in tritici may be many (Fig 6). Earlier in the history, the hypothetical diploid progenitor, for example Brachypodium contains a single DRO1-like gene harboring an EAR-like IVLEM C-terminus motif but lacking TGTCTC/TGTC core motif in the promoter region. It went through genome triplication/duplication or hybridization event. TuDRO1-like of Triticum urartu, AsDRO1-like of Aegilops speltoides and Ata-DRO1-like of Aegilops tauschii originated through genome triplication from BdDRO1-like of Brachypodium. It is denoted by dotted line. HvDRO1-like of Hordeum vulgare seems to be a progenitor of AsDRO1-like after Brachypodium. During speciation procedure, changes in gene structures such as reduction in number of exons through reading frameshifts in HvDRO1-like and AsDRO1-like, and other promoter mutations where gain and loss of cis-regulatory motifs occurred. In the lineage leading to TuDRO1-like, the CDS remained unchanged but there was a gain of one core TGTC (AuxRE) motif upstream of the TSS. During the course of evolution from TuDRO1-like to TaADRO1-like there was gain of one more TGTC in the proximal...
promoter though it lacked standard TGTCTC which is a complete sequence motif. Probably due to the absence of TGTCTC and TGTC core motif near the TSS this gene transcript exhibited stronger expression in the root tips that make it a good candidate for the functional control of root architecture in wheat. On the other hand, in the lineages leading to HvDRO1-like and AsDRO1-like, in addition to 4 bp extension in IVLEM motif there was a gain of 2 TGTCTC motifs in the promoter proximal region, and 3 to 5 TGTCs including one core motif in the conserved region near the TSS. However, AsDRO1-like lost one TGTCTC but gained two TGTC. The TaBDRO1-like re-gained one more TGTC and maintained standard TGTCTC as well. Gain of this TGTCTC/TGTC might be responsible for the weakest expression of TaBDRO1-like and of HvDRO1-like. The evolution of TaDDRO-like is similar to TaADRO1-like except it gained 3 core sequences that include TGTC conserved near the TSS. Hence, this gene exhibited moderate expression in root tips. This gain and loss of cis-regulatory elements during the evolution might have played a role in the expression divergence of these DRO1-like orthologs in tritici.

In nutshell, the variations at the C-terminus as well as gain and loss of AuxREs and other cis-regulatory elements might have played a role in the evolution of DRO1-like wheat paralogs involved in deeper rooting. Since root architecture is an important target trait for wheat crop improvement therefore, isolation, expression and interaction studies of DRO1-like genes in wheat have potential applications in plant breeding for enhancement of plant productivity. It is imperative to study the regulation of stress responses at cellular level in roots, allele replacement for QTL validation and the epigenetic regulation of roots. The unveiling of gene networks and complete pathway and most importantly expression repertoires in wheat/crops in root growth and drought avoidance by using genomic/transcriptomic data is inevitable. In this regard, RNA-seq based high throughput sequencing can be a suitable approach. Drought avoidance strategies by exploiting root growth genes through genome editing technique of CRISPR-Cas9 may be very advantageous for designing annual and perennial crops that are productive in moisture-poor soils. CRISPR-Cas9 can be used to disrupt the DRO1-like genes for producing shallower and more profuse wider root systems in some crops for productively enhancement. Ideally, the expression of DRO1-like can be enhanced using CRISPRa manipulation.

Supporting information
S1 Fig. Isolation of DRO1-like genes from NARC 2009 cultivar of wheat. Pairwise amino acid sequence alignments of the newly isolated DRO1-like paralogs in comparison with those existing in “Ensembl Plants” browser are shown. (A) TaADRO1-like-N with TaADRO1-like. (B) TaBDRO1-like-N with TaBDRO1-like. (C) TaDDRO1-like-N with TaADRO1-like. Conserved nucleotides are shown as asterisks. (TIF)

S2 Fig. Isolation of TaADRO1-like gene from NARC 2009 cultivar of wheat. Pairwise nucleotide sequence alignment of newly isolated TaADRO1-like-N with TaADRO1-like is shown. Conserved nucleotides are shown as asterisks. (TIF)

S3 Fig. Isolation of TaBDRO1-like gene from NARC 2009 cultivar of wheat. Pairwise nucleotide sequence alignment of newly isolated TaBDRO1-like-N with TaBDRO1-like is shown. Conserved nucleotides are shown as asterisks. (TIF)
S4 Fig. Isolation of TaDDRO1-like gene from NARC 2009 cultivar of wheat. Pairwise nucleotide sequence alignment of newly isolated TaDDRO1-like-N with TaADRO1-like is shown. Conserved nucleotides are shown as asterisks. (TIF)

S5 Fig. Multiple partial protein alignment of DRO1-like paralogs and orthologs in tritici and close relatives. Multiple alignments for first 3 domains (I-III) were generated using ClustalW program in Bio-Edit software. Domains are enclosed in a solid line box and domain number is given under the alignment in each row. One and two aa insertions in *Hordeum vulgare* HvDRO1-like are indicated in grey color. Amino acids highlighted in yellow in domain II indicate the IGT motif which acts as the signature of this gene family. Amino acids highlighted in yellow in domain II indicate the IGT motif which is the denominator of this family of genes. (TIF)

S6 Fig. Multiple partial protein alignment of DRO1-like paralogs and orthologs in tritici and close relatives. Multiple partial alignments for last 2 domains (IV, V) were generated using ClustalW program in Bio-Edit software. Domains are enclosed in a solid line box and domain number is given under the alignment in each row. The bold amino acids in yellow (WxxTD) in the 5th domain at the C-terminus are the newly reported conserved amino acids for DRO1-like and related proteins. An EAR-like motif of 5 amino acids (IVLEM) is shown in red color but this motif is diverged and extended to 9 aa KLHTLIPNK in a few proteins by the addition of IPNK highlighted in blue. This extended motif is specific for AsDRO1-like, TaB-DRO1-like, TaBNDRO1-like and HvDRO1-like proteins. (TIF)

S7 Fig. Comparison of gene structures of DRO1-like orthologs. Gene structures of 17 DRO1-like orthologs from Tritici and other plants were analyzed using Ensembl genome browser. The gene structures comprise CDS from ATG to stop codon including introns. Exons in gene structures are symbolized by filled grey boxes while introns by upward lines between the exonic boxes. The numbers above indicate the length of exons in base pairs. The 5’- and 3’- UTRs are shown as empty boxes at the ends. The first two codons of the CDS are shown as ATGAAG in most of the structures except *Oropetium thomaeum*. (TIF)

S8 Fig. Isolation of TOPLESS (TPL) gene form wheat. Pairwise sequence alignments of the newly isolated TaTPL-N with existing sequence of TaTPL in Ensembl Plants are shown. (A) Nucleotide alignment. (B) Amino acid alignment. Conserved nucleotides/amino acids are shown as asterisks. (TIF)

S9 Fig. In vitro interaction assay of TaDRO1-like proteins with TPL. (A) Protein-protein interactions studies of DRO1-like homoeologs with TOPLESS (TPL) proteins were carried out using yeast two hybrid analysis. The binding domain full-length CDS of TaADRO1-like, TaBDRO1-like and TaDDRO1-like constructs as well as with truncated versions (TaADRO1-likeΔ246, TaBDRO1-likeΔ245 and TaDDRO1-likeΔ246) were co-transformed in yeast and grown on stringent dropout media. Note; no growth of yeast colonies in case of truncated DRO1-like proteins. (B) Non-lethal β-galactosidase assay. (TIF)

S10 Fig. Phylogenetic reconstruction of DRO1-like genes. A circular neighbor-joining tree of 82 DRO1-like orthologs from different phyla of plant kingdom was generated in MEGA6. Bootstrap values of 10000 pseudo replicates are indicated at the nodes of the tree. Each clade of
the tree is marked with different plant family name and related species.

(TIF)

S11 Fig. Gene expression patterns of DRO1-like transcripts in wheat and barley. Semi-quantitative RT-PCR results of the HvDRO1-like and TaBDRO1-like transcripts in wheat and barley. The elf is an internal control. M stands for 1kb leader.

(TIF)

S12 Fig. Comparison of DRO1-like promoters of Tritici, Aegilops and Hordeum. Graph showing the results of DRO1-like promoter analysis of Tritici, Aegilops and Hordeum. The promoter sequences -2kb upstream of ATG were retrieved and comparison was performed using the PipMaker program (http://pipmaker.bx.psu.edu/pipmaker/).

(TIF)

S13 Fig. Promoter comparison and identification of transcription factor binding sites (TFBSs) in DRO1-like promoter sequences of Tritici, Aegilops and Brachypodium. Partial promoter sequences of DRO1-like of Tritici, Aegilops and Brachypodium were put in Bio-Edit program for generation of multiple alignment through ClustalW tool. Solid boxes enclose the TFBSs for MYB84, AuxRE-like, core Aux-RE in insertion, ABF1 and ANT marked as yellow. A backward arrow indicates the putative position of the transcriptional start sites (TSS). Note the mutation of AuxRE core i.e. TGTC to GGTC in TuDRO-like and TaADRO1-like, and also in the AuxRE-like motif (ATTTCTT to TTTTAGC) as a coupling motif upstream.

(TIF)

S1 Table. List of primer sequences used in the study. Primers used for different experiments including cDNA synthesis, gene isolation, expression and protein-protein interactions are shown. The sequence direction is 5’ to 3’.

(TIF)

S2 Table. Identification of different types of MADS-domain binding elements i.e. CArG-boxes in the DRO1-like promoters of tritici and closely related plants. Different types of CArG-boxes were detected using Mulan program and their positions were marked. The position of these elements was determined by taking first nucleotide upstream of ATG as -1bp. The core sequence motif is mentioned in capital letters.

(TIF)

S3 Table. Identification of all the plant specific regulatory elements in the DRO1-like promoters of tritici and closely related plants. For identification of TFBSs, -2kb sequence upstream of translational start site was selected as promoter and analyzed using Mulan program. The position and number of times occurrence of different TFBSs were recorded. The “-” sign indicates the absence of a particular site in that promoter sequence. The total number of TFBSs and their occurrence was also documented as highlighted in bold.

(TIF)

S1 File. Supporting Information Zip Archive. Raw data for measurement of root morphology (Fig 1B and 1C), root angle (Fig 1E) and real time gene expression (Fig 4A–4F) analysis.

(ZIP)

Acknowledgments

We are thankful to Bio-Resources Conservation Institute (BCI) and Crop Sciences Institute, National Agricultural Research Centre (NARC), Islamabad for providing the research.
Author Contributions
Conceptualization: Ali Ahmed Naz, Muhammad Ramzan Khan.
Data curation: Almas Ashraf, Obaid Ur Rehman, Shumaila Muzammil, Fatima Rasool.
Formal analysis: Almas Ashraf, Obaid Ur Rehman, Ali Ahmed Naz, Muhammad Ramzan Khan.
Investigation: Almas Ashraf, Obaid Ur Rehman, Muhammad Ramzan Khan.
Methodology: Fatima Rasool.
Project administration: Jens León, Ali Ahmed Naz, Muhammad Ramzan Khan.
Resources: Jens León, Ghulam Muhammad Ali, Muhammad Ramzan Khan.
Supervision: Jens León, Ali Ahmed Naz, Yusuf Zafar, Muhammad Ramzan Khan.
Validation: Muhammad Ramzan Khan.
Writing – original draft: Almas Ashraf, Obaid Ur Rehman, Muhammad Ramzan Khan.
Writing – review & editing: Yusuf Zafar, Muhammad Ramzan Khan.

References
1. Kong X, Zhang M, De Smet I, Ding Z. Designer crops: optimal root system architecture for nutrient acquisition. Trends in Biotechnology. 2014; 32:597–8. https://doi.org/10.1016/j.tibtech.2014.09.008 PMID: 25450041
2. Lopez-Arredondo D, González-Morales SI, Bello-Bello E, Alejo-Jacuinde G, Herrera Engineering food crops to grow in harsh environments. F1000Res. 2015; 4:651. https://doi.org/10.12688/f1000research.6538.1 PMID: 26380074
3. Sun YNing T, Liu Z, Panq J, Jiang D, Guo Z, et al. The OsSec18 complex interacts with P0(P1-P2)2 to regulate vacuolar morphology in rice endosperm cell. BMC Plant Biology. 2015; 15:55. https://doi.org/10.1186/s12870-014-0324-1 PMID: 25848690
4. Li X, Guo Z, Lv Y, Cen X, Ding X, Wu H, et al. Genetic control of the root system in rice under normal and drought stress conditions by genome-wide association study. PLoS Genetics. 2017; 17.1006889.
5. Hochholdinger F, Wen T-J, Zimmermann R, Chimot-Marolle P, da Costa e Silva O, Bruce W, et al. The maize (Zea mays L.) rootless1 gene encodes a putative GPI-anchored, monocot-specific, COBRA-like protein that significantly affects grain yield. The Plant Journal. 2008; 54(5):888–98. https://doi.org/10.1111/j.1365-313X.2008.03459.x PMID: 18298667
6. Mc Steen P. Auxin and Monocot development. Cold Spring Harbor Perspectives in Biology. 2010; pp. 001479.
7. De Smet I. Lateral root initiation: one step at a time. New Phytologist. 2012; 193:867–873. PMID: 22403823
8. Wasson A, Richards R, Chatrath R, Misra S, Prasad SS, Rebetzke GJ, et al. Traits and selection strategies to improve root systems and water uptake in water-limited wheat crops. Journal of Experimental Botany. 2012; 63(9):3485–98. https://doi.org/10.1093/jxb/ers111 PMID: 22653298
9. Lynch JP. Root phenes for enhanced soil exploration and phosphorus acquisition: Tools for future crops. Plant Physiology. 2011; 156(3):1041–9. https://doi.org/10.1104/pp.111.175414 PMID: 21610180
10. Lynch JP, Wojciechowski T. Opportunities and challenges in the subsoil: Pathways to deeper rooted crops. Journal of Experimental Botany. 2015; 66(8):2199–210. https://doi.org/10.1093/jxb/eur508 PMID: 25582451
11. Fukai S, Cooper M. Development of drought-resistant cultivars using physiomorphological traits in rice. Field Crops Research. 1995; 40:67–86.
12. Gowda VRP, Henry A, Yamauchi A, Shashidhar HE, Serraj R. Root biology and genetic improvement for drought avoidance in rice. Field Crops Research. 2011; 122:1–13.

13. Araki H, Morita S, Tatsumi J, Iijima M. Physiomeorongraphic analysis on axile root growth in upland rice. Plant Production Science. 2002; 5:286–93.

14. Rich SM, Watt M. Soil conditions and cereal root system architecture: review and considerations for linking Darwin and Weaver. Journal of Experimental Botany. 2013; 64:1193–208. https://doi.org/10.1093/jxb/ert043 PMID: 23505309

15. Puig J, Pauluzzi G, Guiderdoni E, Gantet P. Regulation of shoot and root development through mutual signaling. Molecular Plant. 2012; 5:974–83. https://doi.org/10.1093/mp/ssq047 PMID: 22628542

16. Satbhai SB, Ristova D, Busch W. Underground tuning: quantitative regulation of root growth. Journal of Experimental Botany. 2015; 66(4):1099–112. https://doi.org/10.1093/jxb/erq429 PMID: 21212298

17. Vermeer JEM, Geldner N. Lateral root initiation in Arabidopsis thaliana: a force awakens. F1000Prime Reports. 2015; 7:32. https://doi.org/10.12703/P7-32 PMID: 25926983

18. Wachsmann G, Sparks EE, Benfey PN. Genes and networks regulating root anatomy and architecture. New Phytologist. 2015; 208:26–38. https://doi.org/10.1111/nph.13469 PMID: 25989832

19. Rosquete MR, von Wangenheim D, Marhavy P, Barbez E, Stelzer EH, Benkova E, et al. An auxin transport mechanism restricts positive orthogravitropism in lateral roots. Molecular Plant. 2012; 5:974–83. https://doi.org/10.1093/mp/ssq047 PMID: 22628542

20. Uga Y, Okuno K, Yano M. Dro1, a major QTL involved in deep rooting of rice under upland field conditions. Journal of Experimental Botany. 2011; 62:2485–2494. https://doi.org/10.1093/jxb/erq429 PMID: 21212298

21. Uga Y, Hanzawa E, Nagai S, Sasaki K, Yano M, Sato T. Identification of qSOR1, a major rice QTL involved in soil-surface rooting in paddy fields. Theoretical and Applied Genetics. 2012; 124:857–86. https://doi.org/10.1007/s00122-011-1688-3 PMID: 21894467

22. Uga Y, Sugimoto K, Ogawa S, Rane J, Ishitani M, Hara N, et al. Control of root system architecture by DEEPER ROOTING 1 increases rice yield under drought conditions. Nature Genetics. 2013a; 45:1097–1102. https://doi.org/10.1038/ng.2725 PMID: 23913002

23. Uga Y, Yamamoto E, Kanno N, Kawai S, Mizubayashi T, Fukuoka S. A major QTL controlling deep rooting on rice chromosome 4. Scientific Reports. 2013b; 3:3040.

24. Uga Y, Kitomi Y, Ishikawa S, Yano M. Genetic improvement for root growth angle to enhance crop production. Breeding Science. 2015; 65; 111–119. https://doi.org/10.1270/jsbbs.65.111 PMID: 26069440

25. Guseman JM, Webb L, Srinivasan C, Dardick C. DRO1 influences root system architecture in Arabidopsis and Prunus species. The Plant Journal. 2017; 89:1093–105. https://doi.org/10.1111/tjp.13470 PMID: 28029738

26. Hollender CA, Dardick C. Molecular basis of angiosperm tree architecture. New Phytologist. 2015; 206:541–56. https://doi.org/10.1111/nph.13204 PMID: 25483962

27. Yoshihara T, Iino M. Identification of the gravitropism-related rice gene LAZY1 and elucidation of LAZY1-dependent and independent gravity signaling pathways. Plant Cell Physiology. 2007; 48: 678–688. https://doi.org/10.1093/pcp/pcm042 PMID: 17412736

28. Godbole R, Takahashi H, Hertel R. The latency mutation in rice affects a step between statoliths and gravity-induced lateral auxin transport. Plant Biology. 1999; 1: 379–381

29. Yoshihara T, Spalding EP, Iino M. LAZY1 is a signaling component required for gravitropism of the Arabidopsis thaliana inflorescence. The Plant Journal. 2013; 74: 267–279 https://doi.org/10.1111/tjp.12116 PMID: 23331961

30. Sasaki S, Yamamoto KT. Arabidopsis LAZY1 is a peripheral membrane protein of which the carboxyterminal fragment potentially interacts with microtubules. Plant Biotechnology. 2015; 32: 103–108

31. Ge L, Chen R. Negative gravitropism in plant roots. Nature Plants. 2016; 17; 2(11):16155 https://doi.org/10.1038/nplants.2016.155 PMID: 27748769

32. Taniguchi M, Furutani M, Nishimura T, Nakamura M, Fushita T, Iijima K, et al. The Arabidopsis LAZY1 Family Plays a Key Role in Gravity Signaling within Statocytes and in Branch Angle Control of Roots and Shoots. Plant Cell. 2017; 29(8):1984–1999. https://doi.org/10.1105/tpc.16.00575 PMID: 28765510

33. Yoshihara T, Spalding EP. LAZY Genes Mediate the Effects of Gravity on Auxin Gradients and Plant Architecture. Plant Physiology. 2017; 175(2):959–9693 https://doi.org/10.1104/pp.17.00942 PMID: 28821594

34. Li P, Wang Y, Qian Q, Fu Z, Wang M, Zeng D, et al. LAZY1 controls rice shoot gravitropism through regulating polar auxin transport. Cell Research. 2017; 17:402–10.
35. Kagale S, Links MG, Rozwadowski K. Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. Plant Physiology. 2010; 152(3): 1109–34. https://doi.org/10.1104/pp.109.151704 PMID: 20097792

36. Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M. Repression domains of Class II ERF transcriptional repressors share an essential motif for active repression. The Plant Cell. 2001; 13(8):1959–68. https://doi.org/10.1105/TPC.010127 PMID: 11487705

37. Wittkopp PJ, Kalay G. cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nature Reviews Genetics. 2012; 13:59–69.

38. Yu LH, Miao ZQ, Qi GF, Wu J, Cai XT, Mao JL, et al. MADS-Box transcription factor AGL21 regulates lateral root development and responds to multiple external and physiological signals. Molecular Plant. 2014; 7(11):1653–69. https://doi.org/10.1093/mp/ssu088 PMID: 25126697

39. Bracha-Dori K, Shichrrur K, Katz A, Oliva M, Angelovic R, Yalovsky S, et al. Detection of protein–protein interactions in plants using bimolecular fluorescence complementation. The Plant Journal. 2004; 40:419–27. https://doi.org/10.1111/j.1365-313X.2004.02206.x PMID: 15469499

40. Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Nake C, et al. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. The Plant Journal. 2004; 40:428–38. https://doi.org/10.1111/j.1365-313X.2004.02219.x PMID: 15469500

41. Voinnet O, Rivas S, Mestre P, Baulcombe D. Retracted: An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. The Plant Journal. 2003; 33:949–56. PMID: 12609035

42. Ihsan H, Khan MR, Ajmal W, Ali GM. WsMAGO2, a duplicated MAGO NASHI protein with fertility attributes interacts with MPF2-like MADS-box proteins. Pflanzen. 2015; 241:1173–87. https://doi.org/10.1007/s00425-015-2247-y PMID: 25630441

43. Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics. 2008; 9:299–306. https://doi.org/10.1093/bib/bbn017 PMID: 18417537

44. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 1987; 4:406–25. https://doi.org/10.1093/oxfordjournals.molbev.a040454 PMID: 3447015

45. Schwartz S, Zhang Z, Frazer KA, Smit A, Riemer C, Bouck J, et al. PipMaker-A web server for aligning two genomic DNA sequences. Genome Research. 2000; 10(4):577–86. PMID: 10779500

46. Comas LH, Becker SR, Cruz VMV, Byrne PF, Dierig DA. Root traits contributing to plant productivity under drought. Frontiers in Plant Science. 2013; 13:4–442.

47. Xiong L, Wang RG, Mao G, Koczan JM. Identification of drought tolerance determinants by genetic analysis of root response to drought stress and abscisic acid. Plant Physiology. 2006; 142(3):1065–74. https://doi.org/10.1104/pp.106.084632 PMID: 16963523

48. Roychoudhry S, Kepinski S. Shoot and root branch growth angle control—the wonderfulness of lateral-ness. Current Opinion in Plant Biology. 2015; 23:124–31. https://doi.org/10.1016/j.pbi.2014.12.004 PMID: 25597265

49. Yu B, Lin Z, Li H, Li X, Li J, Wang Y, et al. TAC1, a major quantitative trait locus controlling tiller angle in rice. The Plant Journal. 2007; 52:891–8. https://doi.org/10.1111/j.1365-313X.2007.03284.x PMID: 17908158

50. Ku L, Wei X, Zhang S, Zhang J, Guo S, Chen Y. Cloning and characterization of a putative tac1 ortholog associated with leaf angle in maize (Zea mays). PLoS ONE. 2011; 6:1–7.

51. Dardick C, Callahan A, Horn R, Ruiz KB, Zhebentyayeva T. PpeTAC1 promotes the horizontal growth of branches in peach trees and is a member of a functionally conserved gene family found in diverse plant species. The Plant Journal. 2013; 75:618–30. https://doi.org/10.1111/tjp.12234 PMID: 23663106
57. Yoshihara T, Iino M. Identification of the gravitropism-related rice gene LAZY1 and elucidation of LAZY1-dependent and–independent gravity signaling pathways. Plant Cell Physiology. 2007; 48:678–88. https://doi.org/10.1093/pcp/pcm042 PMID: 17412736

58. Dong Z, Jiang C, Chen X, Zhang T, Ding L, Song W, et al. Maize LAZY1 mediates shoot gravitropism and inflorescence development through regulating auxin transport, auxin signaling, and light response. Plant Physiology. 2013; 163:1306–22. https://doi.org/10.1104/pp.113.227314 PMID: 24089437

59. Yoshihara T, Spalding EP, Iino M. AtLAZY1 is a signaling component required for gravitropism of the Arabidopsis thaliana inflorescence. The Plant Journal. 2013; 74:267–79. https://doi.org/10.1111/tjp.12118 PMID: 23331961

60. Kagale S, Rozwadowski K. EAR motif-mediated transcriptional repression in plants: An underlying mechanism for epigenetic regulation of gene expression. Epigenetics. 2011; 6(2):141–6. https://doi.org/10.4161/epi.6.2.13627 PMID: 20935498

61. Kieffer M, Stern Y, Cook H, Clerici E, Maulbetsch C, Laux T, et al. Analysis of the transcription factor WUSCHEL and its functional homologue in arrthrinum reveals a potential mechanism for their roles in meristem maintenance. The Plant Cell. 2006; 18(3):560–73. https://doi.org/10.1105/tpc.105.039107 PMID: 16461579

62. Long JA, Ohn C, Smith ZR, Meyerowitz EM. TOPLESS regulates apical embryonic fate in Arabidopsis. Science. 2006; 312:1520–3. https://doi.org/10.1126/science.1123841 PMID: 16763149

63. Szemenyei H, Hannon M, Long JA. TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science. 2008; 319(5868):1384–6. https://doi.org/10.1126/science.1151461 PMID: 18258861

64. Gallavotti A, Long JA, Stanfield S, Yang X, Jackson D, Vollbrecht E, et al. The control of auxillary meristem fate in the maize ramosa pathway. Development. 2010; 137(17):2849–56. https://doi.org/10.1242/dev.051748 PMID: 20699296

65. Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Perez AC, et al. NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature. 2010; 464(7289):788–91. https://doi.org/10.1038/nature08854 PMID: 20360743

66. Zhu Y, Qian W, Hua J. Temperature Modulates Plant Defense Responses through NB-LRR Proteins. PLoS Pathogens. 2010; 6(4):e1000844. https://doi.org/10.1371/journal.ppat.1000844 PMID: 20368979

67. Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M. Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. The Plant Journal. 2004; 34:733–9.

68. Hill DP, Smith B, McAndrews-Hill MS, Blake JA. Gene Ontology annotations: what they mean and where they come from. BMC Bioinformatics. 2008; 9 Suppl 5:S2.

69. Innan H, Kondrashov F. The evolution of gene duplications: classifying and distinguishing between models. Nature Reviews Genetics. 2010; 11:97–108. https://doi.org/10.1038/nrg2689 PMID: 20051986

70. Hagen G, Guilfoyle T. Auxin-responsive gene expression: genes, promoters and cis-regulatory factors. Plant Molecular Biology. 2002; 49(3–4):373–85. PMID: 12036261

71. Ulmasov T, Hagen G, Guilfoyle TJ. ARF1, a transcription factor that binds to auxin response elements. Science. 1997; 276(5320):1865–8. PMID: 9188533

72. Mironova VV, Omelyanchuk NA, Wiebe DS, Levitsky VG. Computational analysis of auxin responsive elements in the Arabidopsis thaliana L. genome. BMC Genomics. 2014; 15.pp.S4.