The auxin efflux carrier PIN1a regulates vascular patterning in cereal roots

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Summary

- Barley (Hordeum vulgare) is an important global cereal crop and a model in genetic studies. Despite advances in characterising barley genomic resources, few mutant studies have identified genes controlling root architecture and anatomy, which plays a critical role in capturing soil resources.
- Our phenotypic screening of a TILLING mutant collection identified line TM5992 exhibiting a short-root phenotype compared with wild-type (WT) Morex background. Outcrossing TM5992 with barley variety Proctor and subsequent SNP array-based bulk segregant analysis, fine mapped the mutation to a cM scale. Exome sequencing pinpointed a mutation in the candidate gene HvPIN1a, further confirming this by analysing independent mutant alleles.
- Detailed analysis of root growth and anatomy in HvPIN1a mutant alleles exhibited a slower growth rate, shorter apical meristem and striking vascular patterning defects compared to WT. Expression and mutant analyses of PIN1 members in the closely related cereal brachypodium (Brachypodium distachyon) revealed that BdPIN1a and BdPIN1b were redundantly expressed in root vascular tissues but only Bdpin1a mutant allele displayed root vascular defects similar to HvPIN1a.
- We conclude that barley PIN1 genes have sub-functionised in cereals, compared to Arabidopsis (Arabidopsis thaliana), where PIN1a sequences control root vascular patterning.

Introduction

Barley is the fourth most significant cereal crop in the world, following wheat, maize and rice. This crop is increasingly utilised in genetic studies due to its diploid nature, low chromosome number, ease of cross-breeding and cultivation in different climatic conditions. In recent years, considerable progress has been made in generating novel genetic resources in barley, including a high-quality genome, gene knockout collections for reverse genetics, marker rich genetic diversity panels and synthetic recombinant populations for association genetics (Nice et al., 2016; Mascher et al., 2017; Schreiber et al., 2019).

To effectively utilise these resources in genetic improvement or crop breeding approaches, it is necessary to efficiently link genes to important agronomic traits. For instance, root traits play critical roles in water and nutrient foraging in soil. Thus, pinpointing genes underlying root function would enable the development of crop varieties with improved root systems. Although several barley mutants related to root hair, cytokinin homeostasis and root angle have been characterised (Gahoonia et al., 2001; Chmielewska et al., 2014; Gasparis et al., 2019; Kirschner et al., 2021, 2024; Fusi et al., 2022), genes that regulate key root architectural and anatomical traits remain to be identified.
The plant hormone auxin plays a crucial role in plant morphogenesis, including root growth and development (Bennett et al., 1996; Benková et al., 2003; Reinhardt et al., 2003; Petrášek et al., 2006; Wiśniewska et al., 2006; Swarup & Péret, 2012; Adamowski & Friml, 2015). Auxin is unique amongst plant hormones in having a specialised transport system that mobilises this signal in a polarised manner between cells and tissues (Bennett et al., 1996; Wiśniewska et al., 2006; Swarup & Péret, 2012). AUXI/LAX auxin influx carriers transport auxin into plant cells, while PIN efflux carriers pump auxin out of plant cells (Swarup & Bhosale, 2019). The PIN class of auxin efflux carriers are often asymmetrically localised in plant cells plasma membranes, which contributes to the polarised movement of auxin and formation of hormone gradients that regulate plant growth, positioning of organs and patterning of tissues (Petrášek et al., 2006; Wiśniewska et al., 2006; Adamowski & Friml, 2015). In the plant model Arabidopsis (Arabidopsis thaliana), PIN1 is a major auxin transporter whose functions have been described during leaf initiation, leaf margin definition and shoot vascular patterning, embryo development, root gravitropism (Benková et al., 2003; Reinhardt et al., 2003; Leyser, 2005; Hay et al., 2006; Scarpella et al., 2006; Bilsborough et al., 2011; Xi et al., 2016). Recent research has revealed functional diversity of PIN1 in grasses compared to Arabidopsis and the Brassicaceae. Brachypodium (Brachypodium distachyon) has two PIN1 clade members PIN1a and PIN1b and a phylogenetic sister to the PIN1 clade SoPIN1, which collectively recapitulate expression domains and polarisation behaviours observed for ArPIN1 in Arabidopsis (O’Connor et al., 2014, 2017). Similarly, barley has PIN1a, PIN1b and SoPIN1 genes that are one-to-one orthologues of brachypodium PIN1 members (Supporting Information Fig. S1).

During shoot development in Arabidopsis, PIN1 is expressed in the epidermis and vasculature (Guenot et al., 2012). During spikelet development in brachypodium, SoPIN1 is expressed in the epidermis, PIN1a in inner vasculature and PIN1b in parenchymatic tissues between epidermis and vasculature, indicating a potential role in supporting vasculature formation in the new developing organ (O’Connor et al., 2014). In Arabidopsis roots, the PIN1 protein is asymmetrically localised rootwards in central stele and cortical cells, whereas it is localised shootwards in epidermal cells (Blilou et al., 2005). This pattern of localisation, when combined with other PIN family members (PIN2, 3, 4, and 7), plays a crucial role maintaining the rootward/shootward auxin polarity which sustain the regular cell division and organ patterning in roots (Gälweiler et al., 1998; Reinhardt et al., 2003; Blilou et al., 2005; Zhang et al., 2019). Similarly, to Arabidopsis PIN1, HvPIN1a is localised in barley root meristem cells in a rootward direction towards the QC in the inner tissues and shootwards in outer cortex epidermis lateral root cap cells, suggesting a similar pattern of auxin fluxes in this species (Kirschner et al., 2018). However, the roles of the individual cereal PIN1 clade members during root growth and development remains unclear.

Through a combination of forward genetic and genomic approaches in barley, we identified mutations in the PIN1a gene that cause defects in root developmental and vascular patterning. These defects were mimicked in wild-type (WT) roots by blocking PIN1-mediated polar auxin transport using the inhibitor NPA. Furthermore, expression domain and root growth analysis of reporters and mutants of PIN1 members in the cereal model brachypodium suggested that BdPIN1a and BdPIN1b act redundantly during root vascular patterning with BdPIN1a functioning as a central regulator. We conclude by discussing the functional diversity of PIN1 in cereals and conserved role of PIN1a controlling root vascular patterning.

Materials and Methods

Plant material

Barley (Hordeum vulgare L.) HvPIN1a mutant alleles (TM5992, TM5141 in cultivar cv Morex and TB13081 in cultivar cv Barke background), brachypodium BdPIN1 member mutants (BdPIN1a and BdPIN1b in Bd21.3 background) (O’Connor et al., 2017) and respective WT (Morex, Barke and TB_WT and Bd21.3, respectively) were used for root length and cross-section imaging analyses. All barley Hopin1a mutant alleles and their respective WT lines were used for root tip staining and confocal imaging. Morex was used in NPA treatment assay. Morex, TM5141 and TM5992 were used in NAA treatment assay. The brachypodium auxin response reporter DR5 and auxin efflux carrier reporters PIN1a, PIN1b, and SoPIN1 (O’Connor et al., 2017) were employed for expression analysis in root tip and vasculature tissues through confocal microscopy.

Growth conditions

For all root growth and cross-section phenotyping analyses of barley, seeds were surface sterilised with 20% Sodium hypochlorite solution for 5 mins followed by five washes with sterile water. Sterilised seeds were germinated on sterile Whatman filter paper placed in a Petri dish for 1 d at 21°C in dark. Germinated barley seedlings were transferred to 1% agar plates (without MS) at pH 5.7 in growth room (21°C, 16 h : 8 h, day : night cycle; 125–150 µmol m $^{-2}$ s $^{-1}$) and grown vertically up to 7 d. Brachypodium seeds were surface sterilised using the same method above and then grown in ½-strength Murashige & Skoog medium, 1% agar plates at pH 5.7 at the same environmental conditions as above for 7 d.

Identification of barley short-root mutant lines TM5992, TM5141 and TB13081

Barley short-root mutant (TM5992) was identified as described in Bovina et al. (under preparation). Briefly a chemically (NaN$_3$) mutagenised population (TILLMore population) (Talamè et al., 2008) of c. 4000 M6 lines in Morex cv background was screened for root traits at the seedling (2-wk-old) stage. TM5992 showed a short-root phenotype when grown using semi-hydroponics method adapted from (Talamè et al., 2008), when compared to Morex WT. From the same mutagenised population, a second short-root mutant line (TM5141) was
identified. A third *HvPIN1a* mutant allele (*TB13081*) was further identified by TILLING (McCallum *et al.*, 2000) by screening an EMS (Ethyl methanesulfonate) treated population of cv Barke consisting of 10 279 individuals, for which a multidimensional pool of individual DNA samples is available (Gottwald *et al.*, 2009). All mutations found using a capillary electrophoresis system were further validated by re-sequencing using Sanger sequencing and following primers: 5141_F1 GGACTTCTACACGTCATGAC, 5992_F2 GGACTTCTACACGTCATGAC. *TB13081* was initially identified as heterozygous segregating family. One heterozygous plant was selfed and homozygous progenies (both WT or mutant) were identified by Sanger sequencing and further selfed to produce seed for phenotypic evaluation.

Genetic mapping of short-root mutant *TM5992*

For genetic mapping of short-root mutant *TM5992*, seeds were immersed in a 0.5% sodium hypochlorite solution for 5 min, rinsed with demineralised water and incubated in the dark at 28°C for 24 h. Germinated seeds were placed between two sheets of 50 × 25 cm of filter paper (Carta filtro Labor, Gruppo Corendons SpA, Milan, Italy) soaked in demineralised water, rolled, positioned vertically in a 5 l plastic beaker with 1 l of demineralised water and grown for 10 d at 24°C with a 16 h photoperiod. Bulked segregant analysis (BSA) (Michelmore *et al.*, 1991) was carried out using 7 d old F2 plants derived from the cross *-TM5992 × Barke* (a 2-row barley cv). For bulks preparation, 15 WT s (bulk +/+ ) and 15 short-root (bulk −/− ) individuals were chosen. DNA from a leaf of each selected plant was extracted with a commercial kit (Macheray-Nagel Nucleospin Plant II) and bulked together in equal amounts in two (+/+ and −/− ) bulks, reaching a final concentration of 50 ng µl⁻¹. The two DNA bulks along with eight short-root single-plant DNA samples and eight WT single-plant DNA samples were genotyped using the 9 k Illumina Infinium iSelect barley SNP array (Comadran *et al.*, 2012). SNP’s signal was analysed with the GENOMESTUDIO software (Illumina, San Diego, CA, USA) calculating the delta theta score as the squared difference between the theta values (Hyten *et al.*, 2008) of the two bulks.

Exome capture and sequencing

DNA samples from *TM5992* were subjected to exome capture (Mascher *et al.*, 2013b) and Next Generation Shogun sequencing with Illumina HiSeq2000 PE100, at IPK, Gatersleben, Germany. Mutation mapping was carried out using previously described pipeline (Mascher *et al.*, 2014). Reads were aligned to the reference genome sequence of barley cultivar Morex (Mascher *et al.*, 2013a) to retrieve the POPSEQ positions of the sequenced BACs. Called mutations were intersected with the annotation of the barley cv Morex reference (Mascher *et al.*, 2017) and SNP effects were predicted with SNPEff (Cingolani *et al.*, 2012).

Nondestructive X-ray microCT root phenotyping of barley *PIN1a* mutants

Morex and *HvPIN1a* mutant alleles *TM5141* and *TM5992* (*n* = 6 independent replicates) were pregerminated in Petri dishes for 1 d at 21°C in dark. Equally germinated seedlings were transferred to PVC columns (8 cm diameter, 15 cm height) filled with well water sandy loam soil and grown until 9 d after germination. Each column was scanned using a Phoenix vtomex M® CT scanner (GE Measurement & Control Solutions, Wunstorf, Germany) at the Hounsfield Facility (University of Nottingham, Sutton Bonington Campus, UK), using a voltage and current of 180 kV and 180 µA, respectively, also at a voxel size resolution of 55 µm, with the specimen stage rotating through 360 degrees at a rotation step increment of 0.166 degrees over a period of 7.5 min, so a total of 2160 projection images were obtained by averaging 4 frames with an exposure of 250 ms each, at every rotation step. A 0.1 mm Copper filters was applied for the scans in front of the exit window of the X-ray tube. Scans were reconstructed into 16-bit greyscale 3D volumes using DATOSRec software (GE Sensing and Inspection Technologies, Wunstorf, Germany). Reconstructed image stacks were segmented and root traits where quantified using RootTrak (Mairhofer *et al.*, 2012).

Barley root growth kinetics and root tip imaging

Root length data of barley cv Morex, *TM5992, TM5141*, Barke, TB_WT and *TB13081* were tracked daily for 3–5 d to assess the root growth kinetics. Only roots touching the agar surface were considered for the analysis. From the same roots, root tip samples were sectioned at 1.5 cm from the root mature zone (mid root region) and an adapted protocol from (Kurihara *et al.*, 2015; Ursache *et al.*, 2018) was used to prepare root tips for confocal imaging. Samples were fixed in paraformaldehyde for 1.5 h in...
vacuum and then were left on ClearSee solution for 7 d before staining with Yellow Direct 96 dye in vacuum for 1.5 h. Cell counting from epidermal, cortical and central metaxylem tissues were performed using image stacks which have been imported in Fiji (ImageJ) and then aligned in order to have an in-focus image of the whole root apical meristem (RAM) which includes cells from columella to the first visible root hair.

Barley and brachypodium mutants and WT root cross-sectioning

An adapted protocol from (Atkinson & Wells, 2017) was used for root cross-sectioning and confocal imaging of barley pin1a mutant alleles (TM5992, TM5141 and TB13081) and brachypodium pin1a and pin1b mutants. Root sections of c. 1.5 cm were taken from the root mature zone (mid root region) and fixed in 4.5% agarose blocks. Blocks where then sliced (Slice height = 100 μm, Frequency = 50 Hz, Amplitude = 1 mm) and for barley, the samples were stained either using calcofluor white for 30 s–1 min before confocal imaging or a combination of Calcofluor White (Sigma 18909-100ML-F) and Basic Fuchsin to stain lignin (Ursache et al., 2018). For this latter method, the agarose slices obtained from the vibratome were then stained with a 50% mixture of Calcofluor White and Basic Fuchsin (0.2%) for 5 min, washed 2 times in ClearSee every 30 min and then left clearing for 2 d in a fresh ClearSee solution. Images were collected using confocal microscope Leica-SP5 by exciting root cross-sections with UV laser at 405 nm for calcofluor white and 543 nm for Basic Fuchsin. For brachypodium cross-sectioning, plants were dipped in water for 30 min–1 h before collecting the samples and fixing them in agarose blocks. This avoids roots getting hyper oxidated which causes cell wall to degrade and turn brown in colour, compromising the staining and the imaging. Samples were then stained as above using Calcofluor White for 30 s–1 min and imaged using confocal microscope Leica-SP5.

Protein structure and binding site prediction

AlphaFold2 (Jumper et al., 2021; Mirdita et al., 2022) was used to predict the structures of the full-length HvPIN1A protein separately using the WT sequence and the two-point mutations (TM5141 and TM5992). Since these de novo structure predictions did not reveal any significant structural changes in the regions surrounding the sites of the point mutations compared to the WT, the WT sequence was further analysed via PHYRE2.0 (Kelley et al., 2015) and SUSPECT (Yates et al., 2014) webserver to study the effects of mutations on the protein structure, stability, and possible function. This analysis revealed a strong sequence conservation and mutational sensitivity at position 490 compared to position 75 in the sequence, indicating a possible effect of the mutations at these positions on protein structure stability and function. To probe this further, binding sites were predicted on the WT structure using the DoGSiteScorer (Volkamer et al., 2010) algorithm and auxin was computationally docked into the predicted sites using JAMDA (Flachsenberg et al., 2024).

NPA treatment

For barley, germinated seedlings of WT Morex were transferred to paper rolls supplemented with DMSO and DMSO with 1, 5, 25 and 50 μM NPA and grown for 5 d. The paper rolls were initially soaked with 30 ml of treatment solution before germinated seeds were placed on them. Once prepared, the paper rolls were placed in 500 ml cylinders containing 100 ml of solution. To maintain adequate moisture, 5 ml of solution was added to each cylinder daily. Seedlings were then grown for 5 d in a growth room under conditions of 21°C temperature, 16 h : 8 h, day : night cycle, and 125–150 μmol m⁻² s⁻¹ light intensity. Root growth images were taken and then sections of c. 1.5 cm were harvested from the mature root zone (mid root region) and cross-sectioning was performed as described in the method section: Barley and brachypodium mutants and WT root cross-sectioning.

NAA treatment

Seedlings of barley WT (Morex) and HvPIN1a mutants (TM5141 and TM5992) were pregerminated in darkness for 1 d before being transferred to either 1% agar mock media with DMSO or 1% agar media supplemented with 100 nM NAA and were grown for 3 d under a 16 h : 8 h, day : night cycle at 21°C. Root growth images were taken and then root sections of 1.5 cm were harvested from the mature root zone (mid root region) and cross-sectioning was performed as described in the section above. Cross-sections were stained using Basic Fuchsin and Calcofluor White using the protocol described above (see ‘Barley and brachypodium mutants and WT root cross-sectioning’ in the Materials and Methods section).

Results

A novel barley short-root mutant shows defects in root growth rate and meristem size

The barley mutant line TM5992, identified in the TILLMore TILLING population, exhibited a short-root phenotype (Fig. 1a). This phenotype was further characterised using 2D and 3D root phenotyping methods. A growth pouch-based method (Atkinson et al., 2015) validated the short-root phenotype, while the noninvasive X-ray microCT imaging (Mairhofer et al., 2015) directly confirmed it in soil (Figs 1b, S2).

To elucidate the cellular basis of the TM5992 root length defect, we initially monitored the root growth rates of mutant TM5992 and WT Morex lines grown vertically on 1% agar plates. Root growth analysis during the first 5 d postgermination revealed a significantly slower growth in TM5992 during the initial 2 d, compared to the accelerated growth observed in the Morex. Subsequently, over the next 3 d, the growth rate decelerated in both genotypes compared to the initial 2 d. Nevertheless, TM5992 exhibited more than twice the reduction in growth rate compared to Morex (Fig. 1c).

Next, we investigated whether these growth differences were due to altered sizes of root apical meristem and/or elongation...
zones, where cells divide and expand, respectively. Root zone measurements were performed by generating maximum surface projection images of 7-d-old root tips stained with propidium iodide, using a confocal microscope. This approach revealed that roots of mutant line TM5992 had shorter apical meristem and elongation zones compared to the Morex, causing the root hair differentiation zone in the mutant to appear closer to the root apex (Fig. 1d).

To determine whether cell division or elongation was affected in root tissues, we cleared 7-d-old root tips using ClearSee and stained them with Yellow Direct 96 stain (Fig. 1e). This enabled us to visualise and measure barley root cell numbers and lengths in cortical, epidermal and central metaxylem tissues from the stem cell initials at the root tip to the first differentiated root hair (as shown in images in e). Morex and TM5992 were used. *Indicates significance (P-value < 0.01, t-test). Dots and horizontal lines inside the box indicates mean and median, while dots outside the box indicate outlier data points.

The short-root phenotype is caused by a recessive mutation in the HvPIN1a gene

To pinpoint the underlying genetic basis of the short-root phenotype in TM5992, we tested its inheritance by crossing TM5992 with the 2-row barley cv Proctor. F1 plants exhibited a WT phenotype, whereas evaluation of 91 F2 plants for root length revealed segregation for either short or WT roots (example given in Fig. 2a). Numbers and rate of phenotypic classes fitted a Mendelian pattern (72 : 19 WT : short-root plants, P = ns. χ² test 3 : 1). These results revealed that the root phenotype segregating in the cross TM5992 × Proctor was controlled by a single locus, with a WT dominant allele from cv Proctor and a mutated recessive allele from TM5992.
A SNP-based BSA was performed to genetically map TM5992 using the F2 population. BSA results indicated that the mutated locus mapped on chromosome 7 (Fig. 2b), in a large pericentromeric region spanning c. 200 Mb between markers SCRI_RS_150016 and BOPA1_4850–969 (38.8–68.4 cM based on the barley POPSEQ reference map) (Beier et al., 2017). To pinpoint the mutation, we conducted exome sequencing of TM5992 and mapped the reads to the Morex reference genome. A total 27 missense variants were identified to be in the coding region spanning 20 Mb between markers BOPA1_4850–969 and BOPA1_6462–11372 (38.8–969 and 64.36–11372 cM respectively). While the mutation in TM5992 failed to reveal results highly similar to those observed in TM5141, other genetic background was not harboured by other candidate genes. The mutation within the first exon and showed an even stronger short-root phenotype compared to TM5992 (Fig. 2c.e). Analyses of root growth rate and root development in TM5141 and TB13081 revealed results highly similar to those observed in TM5992 (Figs S3, S4).

While the mutation in TB13081 (Y231*) introduces a premature stop codon, those in TM5992 (G490E) and TM5141 (D75N) lead to missense substitutions. To elucidate how these substitutions disrupt gene function, we mapped them onto the predicted HvPIN1a protein structure using AlphaFold2 (Jumper et al., 2021). Overall, the predicted WT and mutant protein structures displayed remarkable similarity in regions containing the mutations (Fig. S5a,d). However, G490 is a highly conserved residue sensitive to mutation (Fig. S5c) and resides close to a predicted binding pocket on the WT protein structure (Fig. S5d). Therefore, in TM5992, the G490E substitution, replacing a neutral glycine with a negatively charged glutamic acid, is highly likely to alter the electrostatic properties of this pocket and consequently impact PIN1A function in this region. Residue D75 has low sequence conservation but moderate sensitivity to mutation (Fig. S5b). Thus, in TM5141, the D75N substitution, changing a negatively charged aspartate to positively charged asparagine, is also likely to affect the local structural stability of PIN1A. To probe this further, we predicted potential binding sites on the WT structure and

![Image of short root F2 plants](image-url)
computationally docked auxin into the predicted sites. Two high-ranking binding sites with favourable auxin docking scores were predicted close to the sites of both these mutations (Fig. S5d), indicating that either of the G490E or D75N substitutions are likely to have a strong negative effect on auxin binding in these regions and hinder overall protein function.

Taken together, the results of root growth and the analysis of mutations on PIN1a protein structure function provide conclusive evidence that mutated HvPin1a is likely responsible for the short-root phenotype observed in TM5992, TM5141 and TB13081.

Longitudinal sections revealed striking irregularities in the root vasculature of all three Hupin1a mutant alleles compared to their respective WT backgrounds (Figs 1e, S4a,b). To further investigate these irregularities, we examined cross-sections of 7-d-old mutant alleles and WT root samples grown in 1% agar plates (Figs 3a–c, S6). Remarkably, all mutant alleles showed enlarged cells in the Inner Parenchymatic Cell Layer (IPSL) adjacent to the central metaxylem, with a correspondingly smaller number of cells in that cell layer compared to their WTs (Figs S7). This cell layer enlargement was associated with a significantly larger stele diameter in all mutant alleles compared to their WTs (mean increase > 13% in TM5992 and TM5141 compared to Morex and > 20% bigger in TB13081 compared to Barke and TB_WT) (Fig. S8). Consequently, the root tips of all mutant alleles were slightly thicker than their WT controls (Fig. S8c,d).

Basic Fuchsin staining of Hvpin1a mutant allele root cross-sections showed increased staining intensity in the central metaxylem, its adjacent inner parenchymatic cell layer, and the

Fig. 3 Barley (Hordeum vulgare) pin1a mutant alleles show root vascular defects that can be mimicked by blocking PIN activity in Morex by NPA treatment. (a–c) Representative images showing root cross-section of Morex (a) and Hupin1a mutant alleles TM5992 (b) and TM5141 (c). Seedlings were grown vertically for 4 d on 1% agar and mid regions (c. 3–4 cm from the root apex) of the seminal root were cross-sectioned using vibratome, stained with a 50% mixture of Calcofluor White and Basic Fuchsin (see Materials and Methods section) and imaged using confocal microscope. Blue, green and white arrows indicate irregularities in protoxylem, peripheral metaxylem vessels and first inner parenchymatic cell layer around the central metaxylem. Bar, 100 µm. n > 3 independent biological replicates and n > 6 seedlings per replicate. (d–g) Representative images showing Yellow Direct 96 stained longitudinal sections (d,e) and 50% mixture of Calcofluor White and Basic Fuchsin stained root cross-sections (f,g) of 5-d-old seedlings of Morex, grown on 1% agar supplemented with DMSO (control) and 50 µM NPA dissolved in DMSO. Bar, 100 µm (d,e) and 50 µm (f,g). Red arrowhead (d,e) indicates the first visible root hair and white arrowhead shows visible defects in vascular patterning. Blue, green and white arrows in (g) indicate irregularities in protoxylem, peripheral metaxylem vessels and first inner parenchymatic cell layer around the central metaxylem. n = 3 independent biological replicates, and seminal roots from n > 6 seedlings were analysed per replicate.

HvPIN1a is essential for vascular patterning in barley roots
neighbouring cells of peripheral metaxylem and protoxylem, compared to the WT. These signals suggest enhanced lignification of secondary cell wall and possibly ectopic divisions in these tissue types (Fig. 3a–c). Further image quantification confirmed that among HvPin1a mutants in Morex background, TM5141 displayed the greatest deposition of lignin in the stele and more pronounced short-root phenotype compared to the WT (Fig. 2e). Overall, the increased numbers of lignified cells indicated that HvPIN1a could be important for patterning or differentiation of root vascular tissues.

To directly validate the role of polar auxin transport (PAT) in controlling the root vascular patterning in barley, we employed the PAT inhibitor NPA. We investigated its impact on root growth and anatomy by treating Morex seedlings on agar containing increasing NPA concentrations (1, 5, 25 and 50 μM). At 5–50 μM NPA, roots phenocopied the short-root phenotype of Hvpin1a mutant alleles (Figs 3d,e, S9). Consistent with this, longitudinal and cross-section analysis revealed similar developmental and anatomical defects in NPA-treated WT Morex roots compared to the mutants (Figs 3f,g, S10). These findings demonstrate that blocking PIN activity with NPA mimics the short-root and vascular developmental defects observed in Hvpin1a mutants, strongly supporting the crucial role of HvPIN1a in PAT and root vascular patterning in barley. Additionally, the exogenous application of 100 nM NAA could not restore the WT phenotypes in HvPin1a mutants (Fig. S11), suggesting that elevating auxin levels alone is not sufficient, rather, functional PIN1a is required to distribute the auxin correctly in relevant tissues.

**PIN1a plays a conserved role regulating root vascular patterning in other cereals**

HvPIN1a belongs to a clade of three PIN1-related genes in the barley genome (Fig. S1). Phylogenetic analysis revealed that barley PIN1 members exhibit a high degree of conservation with brachypodium PIN1 family members (Fig. S1). We initially investigated whether the function of HvPIN1a in root growth and root vascular patterning was conserved in brachypodium by characterising the root length and root anatomy of a recently described BdPIN1a mutant line (O’Connor et al., 2014, 2017). BdPIN1a mutant showed shortened primary root compared to the WT (Brachypodium distachyon accession 21.3) Bd21-3 roots (Fig. S12a,b), but not as striking as observed for Hvpin1a mutants. Hence, BdPIN1a and HvPIN1a appear to play some orthologous developmental functions.

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**Fig. 4** The role of PIN1a in root vascular patterning is conserved in cereal model brachypodium (Brachypodium distachyon). (a–c) Representative confocal images of root cross-sections of wild-type brachypodium accession Bd21-3 (a), Bdpin1a (b) and Bdpin1-b (c) mutants. Seedlings were vertically grown for 7 d on 1% agar and mid regions (c. 2–3 cm from the root apex) of the main root were cross-sectioned, stained with 50% mixture of calcofluor white and Basic Fuchsin, and imaged using confocal microscope. n > 3 independent biological replicates and n > 5 seedlings per replicate were analysed. Bar, 100um. Blue, green and white arrows in (b) indicate irregularities in protoxylem, peripheral metaxylem vessels and first parenchymatic cell layer around the central metaxylem. (d–k) Representative confocal images showing brachypodium roots expressing (d) auxin response reporter DR5, (e, f) auxin efflux carrier reporters PIN1a, PIN1b and SoPIN1 reporters (O’Connor et al., 2017). Bar, 100 μm. (h–k) represents orthogonal views of (d–g, respectively) showing YZ axis (cross-sections) in root meristem at position marked by the white dotted line. Bar, 100 μm.
As the PIN1a and PIN1b duplication in grasses is conserved and SoPIN1 and PIN1 clades appear to mediate distinct functions (O’Connor et al., 2014, 2017), we wondered whether any functional redundancy exists between PIN1a and PIN1b in controlling root growth and root vascular patterning. As no material was available to investigate the role of HvPIN1b in root vascular patterning, we were able to characterise anatomy in a recently described Bdpin1b mutant line (O’Connor et al., 2014, 2017). However, we did not observe any patterning defects in Bdpin1b mutants (Figs 4c, S12c–f). Although we cannot conclude that PIN1b necessarily behaves the same way in brachypodium and barley, our results indicated that, while PIN1b may function redundantly with PIN1a to control vascular patterning, PIN1a plays a master regulatory role, and this is likely to be conserved across cereal species.

We noted that the Bdpin1a and Hvip1a patterning defects are mainly associated with shape of central metaxylem and surrounding parenchymatic cell layer. To explore whether PIN1 clade members are specifically expressed in these root vascular cell types, we examined available GFP-based reporters of brachypodium PIN1a and PIN1b (and SoPIN1) for differences in their expression domains. Analysis of PIN1 reporters revealed both Bdpin1a and Bdpin1b are expressed redundantly in root vascular tissues specifically in protoxylem and metaxylem (central and peripheral), while SoPIN1 is mainly expressed in the peripheral metaxylem vessels (Fig. 4e–g). The overlapping spatial expression patterns revealed for PIN1a and PIN1b suggest they could function redundantly to regulate vascular patterning. Nevertheless, genetic evidence demonstrates that PIN1a may play a more critical regulatory role patterning vascular tissues in cereal roots.

**Discussion**

Studies across different monocot species, including the cereal model brachypodium (O’Connor et al., 2014, 2017) and maize (Forestan et al., 2012), have highlighted the impact of genome duplication events in monocots on PIN family members. This duplication has led to an expansion of the PIN family compared to the PIN1 clade, such as PIN1a, play a key role regulating root growth vascular patterning in roots. However, the (Forestan et al., 2012), whereas in brachypodium, BdPIN1a and BdPIN1b are expressed primarily in the inner tissues of the apical shoot primordia (O’Connor et al., 2014). This suggests further levels of sub-functionalisation of PIN1 members within cereals. AtPIN1 is expressed in roots and its mutants display a low penetrance phenotype with either ectopic or loss of protoxylem differentiation (Bishopp et al., 2011). While previous studies in maize (Forestan et al., 2012) and rice (Li et al., 2019, 2022) showed expression of PIN1 members in root apex and their role in root growth regulation, it remained unclear whether these cereal PIN1 members exhibit any root vascular phenotypes.

This paper reports the characterisation a barley mutation in the HvPIN1a auxin efflux carrier encoding gene which causes a short-root phenotype with slower root growth rate and reduced meristem size (Figs 1, 2d,e). Closer examination of root anatomy in Hvip1a mutant alleles revealed a striking vascular patterning defect. These defects included irregular central metaxylem, larger and thicker parenchymatic cells around central metaxylem vessels, higher lignification of cell walls in stele tissues including protoxylem and metaxylem (central as well as peripheral) vessels. Characterisation of a mutant line disrupting the orthologous Bdpin1a gene in brachypodium revealed a similar root vascular patterning defect. These results for the first time show that PIN1a plays a key role regulating root vascular patterning in cereals. Moreover, we show that when treated with high concentrations of NPA, barley WT seedlings display root anatomical defects resembling those of Hvip1a mutant alleles.

Puzzlingly, Bdpin1a and its closely related homologue Bdpin1b exhibit overlapping expression patterns in brachypodium root vascular cell types. Using DR3 and Bdpin1a, Bdpin1b and SoPIN1 reporters revealed that such gene expression accumulates surrounding the areas where auxin accumulates in the vasculature tissues. Specifically, Bdpin1a and Bdpin1b expression is localised in the central metaxylem and peripheral metaxylems (as well as the endodermis), while SoPIN1 expression is localised in only the peripheral metaxylem region. Interestingly, we only observed a vasculature patterning defect in Bdpin1a but not in Bdpin1b mutant roots. This observation contrasts results in Bdpin1b leaves which reveals Bdpin1b plays a more important vascular patterning role in this organ (Leyser, 2005; Xi et al., 2016). Accordingly, a moderate root length reduction was observed in Bdpin1b compared Bdpin1a mutants where a more evident reduction was observed in the shoot length in these mutants when compared to WT (Fig. S12a). Our mutant studies suggest that members of the PIN1 clade in cereals have undergone a process of sub-functionalisation, resembling the process seen in brachypodium during shoot organogenesis. However, in contrast to what happens in the shoot (O’Connor et al., 2014, 2017), we hypothesise that Bdpin1a and Bdpin1b synergically regulate auxin flux in the root vasculature, rather than being in between vasculature and epidermal tissues, where Bdpin1a plays a prevalent role. In addition, our study clearly demonstrates that also in other cereals such as barley, conserved members of the PIN1 clade, such as PIN1a, play a key role regulating root growth vascular patterning in roots. However, the
crosstalk between different PIN1 members process still remains unclear due to the current unavailability of PIN1b and SoPIN1 mutants/reporters and the current challenges in developing stable DII/DR5 auxin reporters in cereals (Kirschner et al., 2018). Further research is necessary to address these challenges and generate genetic resources for other barley PIN1 members. The availability of these resources will not only enable us to unravel the functional diversification of PIN1 members but also shed light on potential diversification of their interacting proteins or downstream effectors.

What is the physiological relevance of PIN1a mediated root vascular patterning in crops? Root vasculature is required for long-distance transport of water, mineral salts and molecular signalling from root to shoot. Especially metaxylem vessel organisation (number and area) is considered important for drought tolerance as it regulates hydraulic conductivity and conservative water usage (Comas et al., 2013). Our results suggest that PIN1a is crucial for auxin mediated normal development of central metaxylem and its surrounding parenchymatic layer and overall root growth. Thus, PIN1a function could be important for root system architecture and conferring tolerance to drought and other abiotic stresses. Consistently, a recent study in maize demonstrated that overexpression of ZmPIN1a increases auxin levels in root and produces deeper root architecture system, resulting in more stress resilient plants with an increased yield under drought and phosphate starvation (Li et al., 2018). Further work will be required to investigate physiological impact of observed vasculature defect in pin1a mutants in barley and brachypodium for water uptake in order to provide more insight for developing stress resilient crops.

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Competing interests

None declared.

Author contributions

R Bhosale, MJB and SS designed research; RF, SGM, SR, R Bovina, CDJVT, HL, BSA, ANB, LMY, DHJ, CJS, DO’C and R Bhosale performed experiments and analysed data. NS, MM, RT and AB provided material and resources. R Bhosale, MJB and SS wrote the manuscript. All authors read and edited manuscript.

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Data availability

All study data are included in the main text and Supporting Information.

References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Exome sequencing and mapping of TM5992 reads to Morex reference identified 16 mutations within genes on Chromosome 7H. Red highlighted rows indicate three mutations within region of interest identified by Bulk Segregant Analysis.

Fig. S1 Phylogenetic analysis of PIN Protein sequences in Arabidopsis, barley, wheat, rice, maize and brachypodium.

Fig. S2 Measured root growth parameters of TM5992 and Morex.

Fig. S3 HvPIN1a allelic mutant lines TM5141 and TB13081 exhibit similar root growth rate as line TM5992.

Fig. S4 HvPIN1a allelic mutant lines TM5141 and TB13081 exhibit similar root developmental defects as line TM5992.

Fig. S5 Structure prediction of HvPIN1a and mutation mapping.

Fig. S6 HvPIN1a allelic mutant line TB13081 exhibit similar root anatomical defects as line TM5992 and TM5141.

Fig. S7 HvPIN1a allelic mutants show enlarged parenchymatic cells in the inner stele layer surrounding central metaxylem.

Fig. S8 HvPIN1a allelic mutants show enlarged stele and root diameter.

Fig. S9 NPA treatment induces short-root phenotype and vascular patterning defects in the wild-type (WT) Morex roots similar to those observed in HvPIN1a mutant alleles.

Fig. S10 NPA treatment increases root and stele diameter in wild-type (WT) Morex similar to those observed in HvPIN1a mutant alleles.

Fig. S11 NAA treatment does not rescue wild-type (WT) phenotypes in HvPIN1a mutant alleles.

Fig. S12 Root growth and anatomy comparison between brachypodium wild-type (WT) (Bd21-3) and Bdpin1 mutants (Bdpin1a, Bdpin1b).

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