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Comparative analysis reveals gravity is involved in the MIZ1-regulated root hydrotropism

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Abstract

Hydrotropism is the directed growth of roots toward the water found in the soil. However, mechanisms governing interactions between hydrotropism and gravitropism remain largely unclear. In this study, we found that an air system and an agar-sorbitol system induced only oblique water-potential gradients; an agar-glycerol system induced only vertical water-potential gradients; and a sand system established both oblique and vertical water-potential gradients. We employed obliquely oriented and vertically oriented experimental systems to study hydrotropism in Arabidopsis and tomato plants. Comparative analyses using different hydrotropic systems showed that gravity hindered the ability of roots to search for obliquely oriented water, whilst facilitating roots' search for vertically oriented water. We found that the gravitropism-deficient mutant *aux1* showed enhanced hydrotropism in the oblique orientation but impaired root elongation towards water in the vertical orientation. The *miz1* mutant exhibited deficient hydrotropism in the oblique orientation but normal root elongation towards water in the vertical orientation. Importantly, in contrast to *miz1*, the *miz1/aux1* double mutant exhibited hydrotropic bending in the oblique orientation and attenuated root elongation towards water in the vertical orientation, providing further insight into the role of gravity in root hydrotropism.

Keywords: Arabidopsis, gravitropism, hydrotropism, MIZ1, root, water potential.

Abbreviations: OHES, obliquely oriented hydrotropic experimental system; SSO, sand system for oblique orientation hydrotropism; SSV, sand system for vertical orientation hydrotropism; VHES, vertically oriented hydrotropic experimental system.

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Introduction

Because plants are sessile organisms, plant growth and development display exceptional plasticity, allowing for rapid acclimation to a changing environment (Zhu, 2016; Dietrich et al., 2017; Su et al., 2017; Shkolnik et al., 2018). One such acclimation response is tropism, which is the directed growth of plant tissues in response to various stimuli, including gravity, light, temperature, oxygen, touch, and water (Silva-Navas et al., 2016; Evsholdt-Derzsó and Sauter, 2017; Dietrich, 2018; Vandenbrink and Kiss, 2019; Muthert et al., 2020). Among these is hydrotropism, the directed growth of roots in response to water potential gradients, which can help plants overcome the detrimental effects of drought (Eapen et al., 2003, 2017; Kobayashi et al., 2007; Miyazawa et al., 2011; Lynch, 2013). However, there have been few studies on hydrotropism in the past century because of the difficulty in designing experimental systems that can reproducibly simulate the water potential gradients found in soils and the simultaneous effect of gravity on directing root growth (Takahashi and Scott, 1991; Stinemetz et al., 1996; Kiss, 2007; Takahashi et al., 2009).

A classic system to examine the hydrotropic response of roots is the one used by Sachs (1872), which involved placing a few plant seeds in a hanging cylinder of wet sawdust held together by a mesh screen, resulting in root growth toward the wet substratum, exhibiting true hydrotropism. Using Julius Sachs's experimental system, Darwin found that water potential gradients influenced directional root growth in Phaseolus, Vicia, Avena, and Triticum (Darwin and Darwin, 1880; Hooker, 1915). More modern systems to examine hydrotropism in Arabidopsis have been developed by two groups who used the systems to isolate hydrotropism mutants (Takahashi et al., 2002; Eapen et al., 2005). Takahashi et al. (2002) developed obliquely oriented hydrotropic experimental systems (OHESs), including an air system and an agar-sorbitol system, and identified the non-hydrotropic mutant miz1 (Kobayashi et al., 2007). These screening systems also led to the isolation of the miz2 mutant, which is deficient in the GNOM gene (Miyazawa et al., 2009). In another approach, Saucedo et al. (2012) developed vertically oriented hydrotropic experimental systems (VHESs), including an agar-glycerol system, and isolated a mutant with altered hydrotropic responses (ahr1). To date, however, the genetic locus of AHR1 has not been identified (Saucedo et al., 2012). In addition, although Cole and Mahall (2006) developed a sand-based system for analyses of root hydrotropism in dune shrubs, there was no compelling evidence for hydrotropic root growth. Iwata et al. (2013) studied hydrotropism in Arabidopsis under natural conditions using an image scanner. However, the investigation of root hydrotropism using a natural drought system is challenging, and the operating procedures may be impractical and especially cumbersome. Thus, the development of an improved system that accurately simulates natural conditions is essential for the elucidation of the mechanisms underlying hydrotropic responses.

Previous studies have reported that gravitropism might interact with hydrotropism to influence the direction of the growth of roots (Jaffe *et al.*, 1985; Takahashi *et al.*, 2003; Kaneyasu *et al.*, 2007; Takahashi *et al.*, 2009). Consequently, agravitropic mutants, clinorotation, and space experiments with plants (microgravity in space) have been used to differentiate hydrotropic from gravitropic responses (Takahashi et al., 1999, 2009; Takahashi et al., 2003; Morohashi et al., 2017). Arabidopsis roots of the gravitropism-deficient mutant or starchless mutant tend to display a greater hydrotropic response (Chang et al., 2019), and anti-auxin reagents enhance the hydrotropic response in Arabidopsis roots (Takahashi et al., 2009; Krieger et al., 2016; Shkolnik et al., 2016). Furthermore, the hydrotropism-deficient mutant miz1 shows reduced phototropism and altered wavy growth responses, suggesting that hydrotropism may interfere with other tropisms in Arabidopsis (Kobayashi et al., 2007). However, it has not yet been elucidated how MIZ1-modulated root hydrotropism is influenced by gravitropism, and thus it remains necessary to disentangle the relevance of the two major root tropisms.

In this study, we performed a quantitative survey of the scientific literature on root hydrotropism and assessed to what extent the imperfections in each of the experimental systems used present challenges to our interpretations of root-tropic responses. Furthermore, we designed an improved sand system to study hydrotropism, in which a realistic water potential gradient is established, not only in the vertical orientation but also in the oblique orientation under natural conditions. Moreover, based on previous systems and our sand system, we used RNA sequencing, cell biology, and genetic crossing and found that gravitropism is important for root hydrotropism. Notably, we found that gravity hinders the ability of roots to search for obliquely oriented water, while it helps their search for vertically oriented water; MIZ1 is involved in these two processes.

Materials and methods

Plant materials and growth conditions

The Col-0 ecotype of Arabidopsis (Arabidopsis thaliana (L.) Heynh) was used as the wild-type (WT) unless otherwise indicated. Other Arabidopsis plants used in the study were previously described: miz1 and ahr1 (Miao et al., 2018), DR5::GFP (Xu et al., 2013a), DII-VENUS (Band et al., 2012), and AUX1pro::AUX1-YFP (Li et al., 2011) lines. The Arabidopsis mutant pgm1-1 (CS210) was obtained from the Arabidopsis Biological Resource Center. The following gravitropic mutants were used: auxininflux transporter mutant aux1-7 (Bennett et al., 1996), gravitropismdeficient auxin response factor double-mutant arf10/arf16 (Wang et al., 2005), and starch-deficient mutant pgm1-1 (Kiss et al., 1989), which displays a substantial impairment in gravitropic responses. Seeds were surface sterilized with 1.5% (w/v) NaClO for approximately 15 min, washed three to five times with autoclaved water, and stratified at 4 °C in the dark for 2 d to break seed dormancy and synchronize germination. Subsequently, the seeds were placed on half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich) supplemented with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.8). Five-day-old uniform seedlings were subsequently transferred and planted vertically in a growth chamber at 22–23 °C under 100–150 μ mol photons m⁻² s⁻¹ illumination with a 16 h-8 h light-dark cycle and 60% relative humidity. Tomato (Solanum lycopersicum LA0534) seeds were germinated on filter paper at 28 °C in the dark.

Root hydrotropism systems

The air system shown in Figs 1A-C, 4D, and 5E was established as previously described (Takahashi *et al.*, 2002). To determine the root



Fig. 1. Hydrotropic responses in mutants and wild-type Arabidopsis in the oblique orientation using an air or agar–sorbitol system. (A) Diagram illustrating the air system for testing hydrotropism. The system is described in detail in 'Materials and methods' and in previous studies. (B) Representative micrographs of mutant and wild-type Arabidopsis seedlings hydrostimulated for 4 h in the air system. All roots were photographed using a stereomicroscope 4 h after the start of hydrostimulation. Scale bar: 1 mm. The following mutants were analysed: non-hydrotropic mutant *miz1*, altered hydrotropic response mutant *ahr1*, and three gravitropism-deficient mutants (the auxin response factor double-mutant *arf10/arf16* displaying loss of columella cell identity and a disorganized root cap, the *aux1–7* mutant carrying a mutation in the auxin transport gene, and the starchless *pgm1–1* mutant). (C) Hydrotropic bending after 4 or 8 h incubation in the air system. (D) Diagram illustrating the agar–sorbitol system for testing hydrotropism. The system is described in detail in 'Materials and methods' and in previous studies. (E) Representative images of mutant and wild-type Arabidopsis roots hydrostimulated for 6 h in the agar–sorbitol system. Scale bar: 2 mm. (F) Hydrotropic bending of mutant and wild-type roots after 6 h or 12 h of growth under control (half-strength MS medium) or hydrostimulation (half-strength MS medium + 400 mM sorbitol) conditions. Data in (C, F) are presented as means ±SE of three independent biological replicates; different letters denote significant differences (*P*<0.05, Duncan's test). (This figure is available in colour at *JXB* online.)

hydrotropic bending, we carefully cut off each Arabidopsis root grown on agar together with the agar block; the cut agar block with one seedling was photographed using a stereomicroscope and analysed with ImageJ software. The agar–sorbitol system shown in Figs 1D–F and 4A–C, E was established as previously described (Takahashi *et al.*, 2002). To determine the root hydrotropic bending, we photographed the square plates using a digital camera (Nikon D7100) and analysed the images using ImageJ. The direction of gravity was considered 0°, and the angle of deviation from the direction of gravity was determined as the hydrotropic angles of roots in both systems (Fig. 1). The agar–glycerol system shown in Fig. 2 was established as previously described by Eapen *et al.* (2003). Roots were photographed using a digital camera (Nikon D7100), and images were analysed using ImageJ to measure the length of roots.

The sand system for oblique orientation hydrotropism (SSO) shown in Figs 3A–D and 5F consisted of a square plate $(10\times10\times1.5 \text{ cm})$ filled with sand (approximately 210 g). The sand had previously been passed through a sieve with a 0.8 mm mesh, sifted, and washed three to five times with water, followed by autoclaving and drying for 2 d at 65 °C. Six-day-old seedlings grown under normal conditions (half-strength MS medium) were transplanted onto square plates equipped with square holes $(1.5\times1.5 \text{ mm}, L\timesW)$ in the medium plate, and covered with sand taking care not to damage the roots; the sand was immediately saturated with approximately 55 ml distilled water (pH 6.3). To maintain a high water content, we fully covered the plate with a lid. To establish experimental water potential gradients, we covered the left half of the plate with sterile gauze; the right half of the plate was sealed with plastic film, which prevented evaporation. To maintain the water content gradient in the hydrostimulated chamber for a relatively long period of time, we supplied 10 ml water only to the right side of the plate through an injection port 5 and 7 d after transplantation. The chambers were held at an angle of 25 degrees from the vertical axis to promote root growth along the transparent bottom side of the plate. Then the device was placed in a growth chamber with a relative humidity of 60% at 22 $^{\circ}$ C.

The sand system for vertical orientation hydrotropism (SSV) shown in Figs 3E-H and 6 consisted of a polystyrene cup (5 cm lower diameter, 6.5 cm upper diameter, 8 cm high for Arabidopsis, or 6.5 cm lower diameter, 8 cm upper diameter, 12 cm high for tomato), filled with sand and distilled water (pH 6.3). The sand used in the SSV was the same as that used in the SSO described above and was sieved and sterilized. All cups were saturated with distilled water from the bottom of the container. Arabidopsis seeds were surface sterilized and sown onto the surface sand layer using a transfer pipette. For all experiments presented here, treatments started 5 or 7 d after sowing (DAS). For each chamber, one of the halves was randomly selected to serve as the 'control' and the other as 'hydrostimulated'. To maintain the sand at a high water content, we immersed the bottom of the cups in distilled water throughout the experiments. To establish experimental water potential gradients along the gravity vector, we withheld water (simulating natural drought) after DAS 7 or 9, so that the water potential of the hydrostimulated chamber in the sand surface gradually decreased due to drainage and evaporation over time, resulting in relatively higher water content in the deeper sand layers. During hydrotropic growth, the main roots elongated towards the wet sand (sites of higher water potential) at the bottom of the cups following the gravitropic vector. Then the device was placed in a 22 °C growth chamber, with a relative humidity of 60%.



Fig. 2. Hydrotropic responses in mutants and wild-type Arabidopsis in vertical orientation using the agar–glycerol system. (A) Diagram illustrating the agar–glycerol system for testing hydrotropism. The system is described in detail in 'Materials and methods'. (B) DR5::GFP, DII-VENUS, and AUX1pro::AUX1-YFP signals after 4 d of growth under control (1/2 MS) or hydrostimulation. Scale bar: 20 μ m. (C) Quantification of fluorescence intensities in the roots of plants described in (B). (D, E) Representative micrographs (D) and quantification (E) of amyloplasts in wild-type (Col-0) plants grown in the agar–glycerol system. Scale bar: 20 μ m. (F) Representative images of mutant and wild-type seedlings under hydrostimulation. Scale bar: 5 mm. (G) Primary root length of mutant and wild-type seedlings. Primary root length was defined as the growth of roots after transferring into a new medium (Control or AGS). Data in (C, G) are presented as means ±SE of three independent biological replicates; asterisks denote significant differences (*P*<0.05, Student's *t*-test). (This figure is available in colour at *JXB* online.)

Measurement of water content in the sand system

For sand system-based analyses of obliquely oriented water (Fig. 3A, B), the control and hydrostimulated chambers were divided into four zones (I–IV) from left to right (2.5 cm width for each zone). For vertically oriented water (Fig. 3E, F), the control and hydrostimulated chambers were divided into four zones (I–IV) from top to bottom (2 cm height for each zone). Each zone was collected carefully with a spoon, and its weight determined (fresh weight). The dry weights of the sand were also determined after a 48 h incubation at 65 °C. The water content was calculated using the following formula:

$$W = \frac{F - D}{F} \times 100\%$$

where W represents the water content, F is the fresh weight, and D is the dry weight (Munné-Bosch and Alegre, 2002).

Raw and pseudo-coloured sand images

For the SSO, the lid covering the control and plastic film/sterile gauze covering the hydrostimulated chamber were removed after hydrostimulation. For the SSV, the cups were carefully cut lengthwise with scissors after hydrostimulation. Raw sand images were taken using a digital camera (Nikon D7100). To enhance the visualization of moisture distribution in the raw sand images, we enhanced the differences in grey-scale intensity values using ImageJ and a 16-colour look-up table (LUT) (Rellan-Alvarez *et al.*, 2015). Pseudo-coloured images (Fig. 3B, F) were generated from the raw images (Fig. 3A, E), and water contents were colour-coded using an appropriate LUT. The differences in grey-scale intensity values indicated moisture content gradients. Water contents in the sand were mapped and visualized (Fig. 3B, F).



Fig. 3. Oblique orientation hydrotropism (A–D) and vertical orientation hydrotropism (E–H) in mutant and wild-type Arabidopsis grown in the sand system. (A) Representative raw sand images showing the sand system for oblique orientation hydrotropism (SSO). After hydrostimulation, the lid covering the control (top) and plastic film/sterile gauze covering the hydrostimulated chamber (bottom) were removed, and raw sand images were taken using a digital camera (Nikon D7100). Scale bars: 1.0 cm. Water content was calculated as (fresh weight–dry weight)/fresh weight. The system is described in detail in 'Materials and methods'. (B) Water distribution in the control and SSO. Pseudo-coloured images (B) were generated from the raw images (A) to show water distribution in the sand. Differences in grey-scale intensity values were enhanced using a 16-colour LUT. Scale bars: 1.0 cm. (C) Diagram illustrating the control and SSO. For a detailed description of the assays see 'Materials and methods'. (D) Hydrotropism (SSV). After hydrostimulation, cups were carefully cut lengthwise, and raw sand images were taken using a digital camera (Nikon D7100). Scale bars: 0.85 cm. Water content was calculated as (fresh weight–dry weight)/fresh weight). The system is described in detail in 'Materials and methods'. (F) Water distribution in the sand. Differences in grey-scale intensity values were enhanced using a digital camera (Nikon D7100). Scale bars: 0.85 cm. Water content was calculated as (fresh weight–dry weight)/fresh weight. The system is described in detail in 'Materials and methods'. (F) Water distribution in the control and SSO. (E) Representative raw sand images were taken using a digital camera (Nikon D7100). Scale bars: 0.85 cm. Water content was calculated as (fresh weight–dry weight)/fresh weight. The system is described in detail in 'Materials and methods'. (F) Water distribution in the control and sand system for SSV. Pseudo-coloured images (F) were generated from the raw images (E) to show water distribution in the sa

Root harvest and phenotypic analyses in the sand system

The gauze and plastic film in the SSO system were removed using scissors, and the sand was removed from the plates by rinsing carefully with water. After all of the sand was removed, wet roots along the bottom side of the plate were covered with a dry filter paper taking care to leave the root structure undisturbed; the filter paper and all of the roots attached to it were carefully transferred onto the bench with tweezers and photographed with a digital camera (Nikon D7100). For each root sampling, we extracted the root systems by hand. The cups in the SSV were cut along both sides with scissors, followed by rinsing with water. Seedlings were clamped with tweezers, and sand was removed from roots by shaking carefully. Seedlings were photographed using a digital camera (Nikon D7100).

Primary root length analyses

The height of the cup in the SSV system was 8 cm; the top of the cup was considered 0 cm deep. To determine the length of the primary root, we cut the cup lengthwise with scissors; after rinsing with water, the sand was removed from the roots by shaking carefully. To minimize the influence of inherently shorter roots (e.g. some gravitropism mutants), we used the relative primary root length. The primary root length of control plants was considered 100%, and the relative primary root length was calculated based on the primary root length relative to the displacement of the primary root apex for the duration of hydrostimulation, as described previously (Xu *et al.*, 2013*b*).

RNA sequencing and data analyses

Because the hydrotropic angles of WT roots in oblique agar-sorbitol systems are approximately 30° 12 h after the start of hydrostimulation. we obtained root samples 10 h after the start of hydrostimulation. Approximately 0.2 g whole root tissues was harvested. The harvested roots were immediately frozen in liquid nitrogen and stored at -80 °C. Three biological replicate samples were collected. For RNA sequencing, total RNA was extracted from roots according to the method described by Miao et al. (2018). The quality and quantity of the total RNA were assessed using the Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Sequencing was performed using the BGISEQ-500 sequencer (BGI, Shenzhen, China). RNA-Seq experiment raw data in the fastq format were first processed by SOAPnuke (version 1.5.2). Clean data were obtained by removing reads containing adapter, poly-N, and low-quality reads from raw data. The remaining high-quality reads were mapped against the Arabidopsis Information Resource reference genome (TAIR10) using HISAT2 (version 2.1.0). To determine transcript abundance, we calculated the values of fragments per kilobase of transcript per million mapped fragments (FPKM) using Bowtie2 (Version 2.2.5). Differential gene expression analyses were carried out using DEGseq. For each pairwise comparison, genes with log2(fold change) >1.5 or <-0.5 and false discovery rate <0.05 were considered differentially expressed genes (DEGs). Gene annotations were obtained from TAIR. Colour-coded gene expression was visualized using the Heatmapper (http://www2.heatmapper.ca/expression/).

Confocal microscopy

The fluorescence of DR5::GFP, DII-VENUS, and AUX1pro::AUX1-YFP in Arabidopsis root tips was observed with a Zeiss LSM 780 laser spectral scanning confocal microscope as described by Xu *et al.* (2013*b*). For DR5::GFP, excitation at 488 nm and emission at 510–540 nm, for VENUS, excitation at 488 nm and emission at 520–570 nm, and forYFP, excitation at 514 nm and emission at 530–550 nm were used. Roots were stained with a solution of 25 μ M propidium iodide in water for 2 min and rinsed prior to imaging. Propidium iodide was excited at 552 nm and detected at 600–700 nm. Signal intensity was quantified as the mean grey value using the open-source software ImageJ. Approximately 10 seedlings were imaged per group, and at least two independent experiments were performed. All images were taken under identical conditions.

Starch staining and light microscopy

The amyloplast is a starch-containing non-coloured plastid. Measurement and observation of amyloplasts in the columella cells of the root caps were performed by staining with iodine/potassium iodide solution, as previously described by Takahashi *et al.* (2003).

Statistical analyses

All statistical tests and error bars are described in the figure legends. All statistical analyses were carried out using SPSS, version 17.0. For comparison between two groups, a two-tailed Student's *t*-test was used. *P*-values <0.05 were considered statistically significant. For comparisons between more than two groups, Duncan's test with an α -level of 0.05 was used.

Results

Single-orientation hydrotropism systems are commonly used to investigate root hydrotropism in Arabidopsis

To assess how many and which hydrotropism systems have been used in previous studies, PubMed Central open-access full-text articles were screened for keywords related to hydrotropism in Arabidopsis (Claeys et al., 2014). As shown in Supplementary Fig. S1 at JXB online, two types of system have been commonly used to study root hydrotropism in Arabidopsis: OHESs, including air systems (Takahashi et al., 2002; Noriega-Calixto et al., 2019), agar-sorbitol systems (Takahashi et al., 2002), and natural drought systems (Iwata et al., 2013); and VHESs, commonly using agar-glycerol systems (Miao et al., 2018). Air systems were used in 68% of the studies, while agar-sorbitol, agar-glycerol, and natural drought systems were used in 36%, 24%, and 4% of studies, respectively (see Supplementary Fig. S1). The vast majority of studies employed single-orientation hydrotropism systems (88%), whereas agar-glycerol systems and OHESs were used in only 12% of the studies (Supplementary Fig. S1).

The hydrotropic response induces oblique root orientation in air systems and agar–sorbitol systems

Air and agar–sorbitol systems were the first systems used to study root hydrotropism in Arabidopsis (Takahashi *et al.*, 2002). In this study, we employed both to evaluate the hydrotropic response of Arabidopsis roots (Fig. 1A, D). Arabidopsis (WT, Col-0) roots bent towards the wet agar and deviated from the gravity vector in the hydrostimulated chamber (Fig. 1B, E), while WT root growth followed the gravity vector and did not display bending toward the agar in the control chamber (Fig. 1C, F). As expected, *miz1* root growth followed the gravity vector and the root did not bend toward water-rich agar (Fig. 1B, E), confirming the previously described performance of the two hydrotropic experimental systems (Kobayashi *et al.*, 2007). Next, we compared the hydrotropic responses of gravitropic mutants (see Supplementary Fig. S2A) using these two systems. Roots of the auxin response factor double-mutant arf10/arf16 displayed loss of columella cell identity and a disorganized root cap (Wang et al., 2005), while the aux1-7 mutant, which carries a mutation in the auxin transport gene (Bennett et al., 1996), and the starchless pgm1-1 mutant, showed a substantial reduction in gravitropic responses (Kiss et al., 1989). When hydrostimulated for 4 h in the air system, gravitropism-deficient mutants (aux1-7, arf10/arf16, and pgm1-1) exhibited significantly higher hydrotropism than WT roots (Fig. 1C). The same was true of mutants stimulated for 6 h in the agar-sorbitol system (Fig. 1F). However, the hydrotropism of WT roots was similar to that of the mutants when hydrostimulated for 8 h (Fig. 1C). The WT (Col-0) and arf10/arf16 double-mutant showed a similar hydrotropism when hydrostimulated for 12 h, whereas the aux1-7and pgm1-1 mutants exhibited significantly higher hydrotropism (Fig. 1F). These results suggest that gravitropismdeficient mutants have increased hydrotropism early during hydrostimulation in OHESs. In both systems, the root angles in the ahr1 mutants (isolated in VHES systems) were identical to those in WT plants (Fig. 1C, F). Primary root elongation of these mutants was similar to that in WT plants (Supplementary Fig. S2B, C). Collectively, these results suggest that the oblique orientation hydrotropic response of Arabidopsis roots in air systems is similar to that in agarsorbitol systems and that gravitropism-deficient mutants exhibit enhanced hydrotropism in both systems.

Agar–glycerol systems induce vertical orientation hydrotropism in roots

The agar-glycerol system consists of two different media: the top half contains a water stress medium (WSM), and the lower half contains a normal medium (NM) (Saucedo et al., 2012; Miao et al., 2018). The WSM contains 0.5% (v/v) glycerol and 0.1% (w/v) alginic acid (Fig. 2A). First, we employed transgenic plants expressing auxin response reporters (DR5::GFP), an auxin sensor (DII-VENUS) and the principal auxin-influx carrier (AUX1pro::AUX1-YFP), and found that the DR5 promoter activity, DII-VENUS signal intensity, and AUX1 expression levels were significantly reduced in the root tips in the presence of the water potential gradient compared with controls (Fig. 2B, C). Second, we found that the amount of amyloplast in WT roots was substantially decreased under hydrostimulation (Fig. 2D, E). Finally, we analysed the response of the primary root to water potential gradients in hydrotropic and gravitropic mutants. Ahr1 mutants showed remarkable growth toward water-rich substrates compared with WT roots, while miz1 showed reduced growth (Fig. 2F, G), in line with previous reports (Saucedo et al., 2012; Miao et al., 2018). In the agar-glycerol system, Arabidopsis root growth was inhibited under hydrostimulation (Fig. 2G), and WT roots were significantly longer than those of gravitropic mutants under hydrostimulation (Fig. 2F, G). These results suggest that the agar-glycerol system can induce vertical orientation hydrotropism and that gravitropism-deficient mutants have decreased hydrotropism under this system.

Sand systems can be used to investigate root hydrotropism in the oblique or vertical orientation

To investigate root hydrotropism under natural conditions, we designed an improved hydrotropism system wherein root system development occurs in the sand in the presence of realistic water potential gradients (Fig. 3A-C). To enhance visualization of distribution of moisture in the raw image of sand in the sand system for oblique orientation hydrotropism (SSO) (Fig. 3A), we imaged the sand and enhanced the differences in grey-scale intensity values (Fig. 3B), as previously described (Rellan-Alvarez et al., 2015). Thus, differences in grey-scale intensity values corresponded to moisture content gradients (e.g. the water levels in the hydrostimulated chamber of SSO from left to right were 4.1±0.13%, 7.79±1.50%, 10.10±1.64%, and 11.77±1.77%). Using this method, we mapped and visualized water levels in the sand (Fig. 3A, B). Sand maintained a constantly high water content (approximately 14%) in all zones for 9 d under control conditions; there were no detectable gradients in the water level in the control chamber (see Supplementary Table S1). Oblique water content gradients ranging from 4.1% to 11.8% were formed in the hydrostimulated chamber (Supplementary Table S1).

These findings suggest that our sand system accurately simulated oblique water content gradients (Fig. 3C). Therefore, we used it to examine the hydrotropic response of Arabidopsis roots in the oblique orientation. The number of lateral roots that emerged on the left side of the primary root was similar to the number that emerged on the right side of the primary root under control conditions (Fig. 3D; Supplementary Fig. S3A). However, the number that emerged on the right (wet) side of the primary root was significantly higher in the presence of water content gradients compared with the number that emerged on the left (dry) side (Fig. 3D). The roots of the Col-0, Ws, and Ler Arabidopsis ecotypes showed a similar response (Fig. 3D), indicating that the capacity of the root growth response to oblique water potential gradients is a general phenomenon across genetic backgrounds in Arabidopsis. Additionally, the root growth of tomato also exhibited a similar oblique hydrotropic response (see Supplementary Fig. S4), while we found no differences in the number of lateral roots on the wet and dry sides of the primary roots under control or hydrostimulated conditions in *miz1* plants (Fig. 3D; Supplementary Fig. S3A). Furthermore, the ratios between the lateral root length on the wet and dry sides of the chamber were significantly higher in the presence of water content gradients compared with control conditions (Supplementary Fig. S3B), in accordance with previous studies that have used natural drought systems (Iwata et al., 2013; Bao et al., 2014; Robbins and Dinneny, 2018). miz1 plants did not show asymmetry in the length of lateral root on the wet and dry sides of the primary roots under control or hydrostimulated conditions (Supplementary Fig. S3B). These results indicated that in addition to OHESs (air system and agar-sorbitol system), the sand system for oblique orientation hydrotropism (SSO) could be used to investigate oblique orientation hydrotropism.

In nature, roots grow in response to water gradients, and most of the gradients are vertical (Russell, 1977). We developed

an improved sand system for vertical orientation hydrotropism (SSV), with water content gradients (ranging from 0.6% to 3.4%) established parallel to the direction of gravity after 9 d of hydrostimulation (Fig. 3E-G). Furthermore, in time-course experiments, we found that the water content gradients were established as early as 5 d after the start of hydrostimulation (see Supplementary Table S2). The results further confirmed that this experimental system accurately simulated vertical water content gradients. Therefore, we used this growth system to investigate the hydrotropic response of Arabidopsis roots in the vertical orientation. We found that the 5 d hydrostimulation significantly enhanced primary root elongation (Supplementary Fig. S5), and this was closely associated with a steeper water potential gradient (Supplementary Table S2). To investigate if the positive hydrotropic response of roots occurred only in the sand or also in soil microcosms, we quantified the growth of WT primary roots in a vertical soil culture system. In this set-up, the soil maintained a consistently high water content (~36%) in all zones for 9 d (Supplementary Table S3). Compared with control roots, the primary root length of WT seedlings was significantly higher in the soil (Supplementary Fig. S6). Because the sand system induced a steeper water potential gradient than real soil (5.7fold versus 1.3-fold gradient; Supplementary Tables S2, S3), and the sand system was easily adjustable, we used it in subsequent experiments.

Under hydrostimulation, the roots of the Ws and Ler Arabidopsis ecotypes exhibited a significantly higher hydrotropic elongation than the respective controls, indicating that the positive hydrotropic response is a general phenomenon across various genetic backgrounds (Fig. 3H; Supplementary Fig. S7A). Moreover, tomato roots showed a similar vertical hydrotropic response under hydrostimulation (see Supplementary Fig. S7B, C). In the agar-glycerol system, the ahr1 mutant showed significantly higher primary root elongation than WT plants (Fig. 2G); in contrast, in the sand system, primary root elongation in the *ahr1* mutant was similar to that in WT (Fig. 3H). Hydrostimulation also inhibited the growth of WT roots in the agar-glycerol system (Fig. 2G), suggesting that the osmolyte glycerol contained in the agar-glycerol system caused severe osmotic stress. Taken together, these results suggest that the increased primary root elongation is the consequence of an enhanced hydrotropic response because roots continued to grow towards the high-water zone; the sand system may offer a more natural system to study hydrotropism in a vertical orientation.

Hydrostimulation regulates the expression of auxinand amyloplast-related genes in miz1 plants

To investigate the molecular expression profile associated with root hydrotropism, we performed RNA sequencing analyses using WT (Col-0) and *miz1* roots grown under control (C) and hydrostimulation (H) conditions. We found that hydrostimulation significantly up-regulated (>1.5-fold) 365 and 1468 genes in WT and *miz1* roots, respectively (Fig. 4A), while 469 and 701 genes were significantly downregulated (<0.5-fold; Fig. 4B). There were six DEGs in WT roots and 21 DEGs in miz1 roots that were related to auxin signalling pathway or auxin biosynthesis. For example, NIT2 (AT3G44300, encoding a nitrilase), WAG1 (AT1G53700, involved in wavy root growth), and YUC3 (AT1G04610, a member of the YUCCA family) were up-regulated under hydrostimulation in WT roots, while the expression levels of IAA1 (AT4G14560, indole-3-acetic acid-inducible 1), SAUR61 (AT1G29420, small auxin up-regulated RNA), and SAUR78 (AT1G72430, small auxin up-regulated RNA) were downregulated (Fig. 4C; Supplementary Table S4). Similarly, eight auxin-related genes, including NIT2, were up-regulated under hydrostimulation in *miz1* roots, while 13 auxin-related genes, such as IAA14 and SAUR78, were down-regulated (Fig. 4C; Supplementary Table S4). DEGs related to starch (amyloplast) metabolism were also enriched under hydrostimulation (three DEGs in WT roots and six DEGs in miz1 roots). Notably, AMY3 (AT1G69830, a plastid-localized α -amylase) and LUP1 (AT1G78970, a lupeol synthase 1) were up-regulated under hydrostimulation in WT roots, while BAM9 (AT5G18670, a β-amylase) was down-regulated (Fig. 4C; Supplementary Table S5). SEX4 (AT3G52180, a plant-specific glucan phosphatase), SGR5 (AT2G01940, involved in shoot gravitropism), and SS3 (AT1G11720, a starch synthase) were up-regulated under hydrostimulation in miz1 roots, while AMY2 (AT1G76130, an α -amylase), BAM3 (AT4G17090, a β -amylase), and SGR9 (AT5G02750, involved in shoot gravitropism) were downregulated (Fig. 4C; Supplementary Table S5). These results suggest that hydrostimulation influences the expression of auxin-related and amyloplast-related genes.

Further investigation of changes in the expression of auxin-regulated genes was performed using transgenic plants expressing auxin response reporters (DR5::GFP) in WT and *miz1* roots (Fig. 4D; Supplementary Fig. S8A). Under hydrostimulation, the DR5::GFP signal intensity was significantly lower in the root tips of *miz1* plants than in WT plants (Fig. 4D; Supplementary Fig. S8A), consistent with the RNA sequencing data (Fig. 4C; Supplementary Table S4). In addition, the amounts of amyloplasts were substantially decreased under hydrostimulation, in both WT and *miz1* plants (Fig. 4E; Supplementary Fig. S8B).

Role of gravity in oblique orientation hydrotropism in miz1 roots

MIZ1 is a positive regulator of root hydrotropism in the oblique orientation, and hydrostimulation significantly inhibited auxin responses in the root tips of *miz1* plants (Fig. 4D; Supplementary Fig. S8A). Therefore, we examined the genetic link between AUX1 and MIZ1. To generate double-mutant Arabidopsis plants, we crossed *miz1* mutant plants with *aux1* mutants. Subsequently, homozygous *miz1/aux1* double-mutant plants were selected on the basis of mutant-specific primers and DNA sequencing (Fig. 5A, B). Compared with WT plants, *miz1/aux1* double-mutants exhibited significantly impaired gravitropism (Fig. 5C, D). Next, we examined the hydrotropic response of the *miz1/aux1* double-mutant using different growth systems. In the air system, the *miz1/aux1* double-mutant exhibited a

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Fig. 4. The role of auxin- and amyloplast-related pathways in the roots of *miz1* mutant during the hydrotropic response. (A, B) Venn diagrams summarizing the changes in gene expression in *miz1* roots. RNA sequencing analyses were performed with wild-type and *miz1* roots, and the numbers of up-regulated (>1.5-fold, A) or down-regulated (<0.5-fold, B) genes in the *miz1* mutant under control (C) or hydrostimulated (H) conditions are shown. (C) Changes in the expression levels of auxin- and amyloplast-related genes in *miz1* roots. The means and SD were calculated using the RNA sequencing data from three biological replicates. (D) DR5::GFP signal in wild-type (Col-0) and *miz1* roots under control (C) or hydrostimulated (H) conditions. (E) The amount of amyloplasts in wild-type (Col-0) and *miz1* roots under control (C) or hydrostimulated (H) conditions (E) The assignificant differences in Col-0 or *miz1* mutant roots (two-tailed Student's *t*-test). Data in (D, E) are presented as means ±SE of three independent biological replicates; different letters denote significant differences (*P*<0.05, Duncan's test). (This figure is available in colour at *JXB* online.)

significantly higher hydrotropism than the miz1 single mutant (Fig. 5E), suggesting that the hydrotropism-deficient phenotype of miz1 roots could be partially rescued via loss of AUX1 function. Given that SSO also induces obliquely oriented water potential gradients, we further assessed the hydrotropic response of the agravitropic double-mutant *miz1/aux1* in SSO. Under hydrostimulation, we observed asymmetry in the lateral root number on wet and dry sides of the primary roots in WT plants and the *miz1/aux1* doublemutant; however, this asymmetry was not observed in *miz1* plants (Fig. 5F). Furthermore, the *miz1/aux1* doublemutant exhibited asymmetry in the lateral root length



Fig. 5. The role of gravity in oblique orientation hydrotropism in *miz1* roots. (A) The homozygous *miz1* mutant was identified using three mutant-specific primers (LBb1.3+LP+RP). (B) The DNA sequences of wild-type and *miz1/aux1* double-mutant. *Mutation at nucleotide 459. (C) Representative phenotypes of 6-day-old mutant and wild-type Arabidopsis seedlings. Scale bar: 0.5 cm. (D) Root gravitropic bending kinetics in mutant and wild-type plants. (E) Root hydrotropic bending in mutant and wild-type plants hydrostimulated for 8 h in the air system. (F) Hydrotropic response in mutant and wild-type roots grown in the sand system for SSO. Data in (D–F), are presented as means ±SE of three independent biological replicates; different letters denote significant differences (*P*<0.05, Duncan's test). (This figure is available in colour at *JXB* online.)

(Supplementary Fig. S9), suggesting that AUX1-regulated gravitropism is involved in the hydrotropic response in *miz1* plants.

Role of gravity in vertical orientation hydrotropism in miz1 roots

Next, we used the improved sand system for vertical orientation hydrotropism (SSV) and the transgenic plants DR5::GFP and AUX1pro::AUX1-YFP to further explore the mechanisms underlying the hydrotropic elongation of primary roots (Fig. 6A). Hydrostimulation enhanced auxin signalling, indicated by the increase in the levels of the auxin response marker DR5::GFP (Fig. 6A, B). Consistently, hydrostimulation treatment significantly increased the protein levels of the auxin importer AUX1pro::AUX1-YFP (Fig. 6A, B). In addition, we found that under hydrostimulation, WT roots had substantially higher amount of amyloplast compared with controls (Fig. 6C). These results indicate that auxin and amyloplast signalling are required for hydrotropic root elongation in a vertical orientation.



Fig. 6. Role of gravity in vertical orientation hydrotropism in *miz1* roots. (A) DR5::GFP and AUX1pro::AUX1-YFP fluorescence signals in root tips of plants grown in the sand system for vertical orientation hydrotropism (SSV) were detected by confocal microscopy. Scale bars: 20 μ m. (B) Quantification of fluorescence intensities in the root tip cells shown in A. (C) Representative micrographs and quantification of amyloplasts in wild-type (Col-0) columella cells in the sand system for SSV. (D) Relative primary root length in wild-type and mutant plants in the sand system for SSV. The primary root length of all plants under control conditions was considered 100%; the relative primary root length was calculated from the primary root length relative to the displacement of primary root apex for the duration of hydrostimulation. (E) Representative micrographs and quantification of amyloplasts in wild-type (Col-0) and mutant columella cells upon hydrostimulation in the sand system for SSV. (F) Primary root length in wild-type and mutant plants in the sand system for SSV. In (A–D) plants were grown under humidity-saturated conditions for 9 d and an additional 7 d under either humidity-saturated or hydrostimulated conditions. In E and F, plants were grown under humidity-saturated conditions for 5 d and an additional 7 d under either humidity-saturated or hydrostimulated conditions. Data in (B, C) are presented as means ±SE of three independent biological replicates; asterisks denote significant differences (*P*<0.05, Duncan's test). (This figure is available in colour at *JXB* online.)

Next, we analysed the root hydrotropism response in gravitropic and hydrotropic mutants (Fig. 6D). Hydrotropic stimulation markedly increased the primary root length (28.5% of control) in WT (Col-0) seedlings (Fig. 6D). However, primary root length only increased by 16.7%, 14.7%, and 9.8% under hydrostimulation in aux1-7, arf10/arf16, and pgm1-1 mutants, respectively (Fig. 6D). These results suggest an important role of gravitropism in hydrotropic root elongation. We also used the miz1/aux1 double-mutant to confirm the role of gravity in root hydrotropism in the SSV. The miz1 mutant exhibited similar amyloplast accumulation to WT plants. By contrast, the miz1/aux double-mutant showed less amyloplast accumulation than WT plants under hydrostimulation (Fig. 6E), suggesting that auxin is necessary for hydrostimulation-induced amyloplast accumulation in a vertical orientation. Furthermore, the primary root lengths of the miz1/aux1 double-mutant were shorter than those of WT plants (Fig. 6F). Taken together, these results suggest that gravity sensing steers root growth downward, facilitating access to water stored in deeper layers of the sand.

Discussion

Hydrotropic response in Arabidopsis under different experimental systems

Soil water in the plant root zone is heterogeneously distributed under natural conditions (Dietrich, 2018). Hydrotropism is the

mechanism by which plant roots sense the differences in water potentials in the soil and direct root growth toward water-rich areas (Jaffe *et al.*, 1985; Cassab *et al.*, 2013; Miao *et al.*, 2018). Several successful hydrotropic experimental systems have been established over the years (Takahashi *et al.*, 2002; Eapen *et al.*, 2003; Saucedo *et al.*, 2012). When the primary water source is the rainfall before the growing season, deeper soil layers have higher water content; by simulating this scenario, VHESs facilitate root growth straight down a vertically oriented water gradient column (Fig. 3G). On the other hand, when episodic/ occasional rainfall is the primary water source, upper or obliquely oriented soil layers have higher water content (Figs 1, 3C); hence, in OHESs, roots elongate preferentially within the upper soil or in obliquely oriented soil layers, leading to characteristic root bending patterns (Lynch, 2013; Feng *et al.*, 2016).

However, our results suggest that the same plant genotypes can show distinct root growth patterns in the two hydrotropism systems, owing to the fact that the different systems present different orientations of water potential gradients, with significant variation in space (Figs 1–3). Under most circumstances, single-orientation hydrotropism systems are used (see Supplementary Fig. S1). For instance, air and agar–sorbitol systems only induce oblique water potential gradients. Here, we found that gravitropic mutants exhibited hydrotropic root bending in both systems, highlighting the similarities between the two (Fig. 1C, F). Iwata *et al.* (2013) studied Arabidopsis hydrotropism under natural conditions and found that the water gradients were only oblique. When glycerol was used as an osmolyte in the agar–glycerol system (Fig. 2), auxin and amyloplast responses were dramatically decreased in the root tips in the presence of a water potential gradient (Fig. 2C, E), suggesting that osmotic stress inhibits amyloplast and auxin signalling, as previously reported (Takahashi *et al.*, 2003; Ponce *et al.*, 2008; Xu *et al.*, 2013*b*; Rowe *et al.*, 2016). Compared with the respective controls, hydrostimulation inhibited root growth in WT and auxin mutants (Fig. 2F, G). Taken together, these results suggest that the presence of the osmolyte glycerol in the growth system not only induces water potential gradients but also induces severe osmotic stress.

Taking these factors into consideration, we designed an improved hydrotropism system that is well suited for studying root hydrotropism in Arabidopsis (Fig. 3C, G); we also tested its utility in the tomato (see Supplementary Figs S4, S7). Importantly, this sand system can be used to investigate root hydrotropism in the oblique (Fig. 3C) or vertical orientation (Fig. 3G). We found that miz1 plants showed deficient hydrotropism in the oblique orientation under the sand system for oblique orientation hydrotropism (SSO) (Fig. 3D), suggesting that SSO is similar to the air system and agar-sorbitol system (Fig. 1). The sand system for vertical orientation hydrotropism (SSV) significantly promoted vertical root elongation in WT (Col-0), miz1, and ahr1 plants (Fig. 6D). By contrast, the agarglycerol system inhibited vertical root elongation in WT and miz1 plants (Fig. 2F, G), suggesting that the agar-glycerol systems might induce water stress due to the high concentration of glycerol. In addition, given that light influences root development (Xu et al., 2013a; Zheng et al., 2019), the fact that plant shoots and roots are exposed to illumination in most hydrotropism systems may cause artefacts. In the sand system (Fig. 3), although shoots are illuminated, roots are not exposed to light, simulating the natural environment. Thus, our sand system can reproducibly induce relatively stable and smooth water gradients, and offer a more natural system to study hydrotropism (Fig. 3).

Gravity plays an important role in the MIZ1-regulated root hydrotropism

Under natural conditions, hydrotropic stimulation in the roots occurs simultaneously with gravity sensing (Takahashi et al., 2002; Dietrich, 2018). Under these conditions, roots may first judge the location of water, followed by a change in the orientation of the root tip. When the water orientation is oblique to the gravity vector, roots must overcome gravitropism and ultimately initiate root bending toward the obliquely oriented water (Fig. 1). Consistently, previous studies that have used OHESs (air and agar-sorbitol systems) have reported that gravity inhibits root hydrotropism (Krieger et al., 2016). We also present evidence on the role of gravity in MIZ1-regulated root hydrotropism. Our transcriptomic data indicated dramatic changes in the expression of multiple genes involved in auxin and amyloplasts responses in miz1 plants during the hydrotropic response (Fig. 4C). Notably, auxin responses were reduced in *miz1* under hydrotropism conditions (Fig. 4D; Supplementary Fig. S8A). In addition, the degradation of

amyloplasts in columella cells occurred in both the WT and miz1 mutants upon hydrostimulation (Fig. 4E; Supplementary Fig. S8B). These results imply that auxin and amyloplast responses may be important for the hydrotropic mutant miz1. Because amyloplasts in root columella cells are required for gravity sensing, amyloplast degradation is thought to play an important role in negating gravisensing and might regulate hydrotropism-dependent root growth (Takahashi *et al.*, 2003). Furthermore, the genetically gravitropism-modified miz1/aux1 double-mutant exhibited hydrotropic bending due to gravitropism impairments (Fig. 5). Taken together, these results pinpoint an antagonistic relationship between hydrotropism and gravitropism, and indicate that in the absence of gravitropism (loss of AUX1 function), the response to lateral water potential gradients in miz1 plants is partially restored.

In our sand system, oblique orientation hydrotropism was similar to hydropatterning (Fig. 3C). Previous studies have indicated that primary roots exhibit obliquely oriented hydrotropism in both air and agar-sorbitol systems (Fig. 1), while during hydropatterning, lateral roots emerge toward water-rich areas (Orosa-Puente et al. 2018). The primary roots of the miz1 mutant did not show obliquely oriented hydrotropism in air or agar-sorbitol systems (Fig. 1), and the lateral roots of the miz1 mutant did not emerge toward water-rich areas in the soil (Iwata et al., 2013) or in our sand system (Fig. 3D); these findings suggest that oblique orientation hydrotropism was similar to hydropatterning. Importantly, the miz1 mutant not only exhibited oblique orientation hydrotropism deficiencies in primary roots, but also showed hydropatterning deficiencies in lateral roots (Figs 1, 3D). In addition, WT plants exhibited oblique orientation hydrotropism in primary roots and hydropatterning in lateral roots, further supporting that oblique orientation hydrotropism is similar to hydropatterning. Given that primary and lateral roots make up the majority of the plant root system (Malamy, 2005), obliquely oriented hydrotropism and hydropatterning in roots may be important for enhancing the growth of terrestrial plants when water availability is limited.

In response to drought, plants increase the root-to-shoot ratio, by growing longer roots and inhibiting shoot growth (van der Weele et al., 2000; Sharp and LeNoble, 2002; Sharp et al., 2004; Uga et al., 2013). Polyethylene glycol is commonly added to the medium to reduce water potential (Rowe et al., 2016), and there is probably less of a gradient in the system. In addition, soil drying experiments (pot-grown plants and plants grown in the field) are typically employed to measure certain aspects of plant growth, survival, and water status (Verslues et al., 2006; Dodd et al., 2008a, 2008b). Water gradients in such experiments are affected by pot dimensions and irrigation placement (Dodd et al., 2011; Puértolas et al., 2013). Our sand system for vertical orientation hydrotropism (SSV) is similar to soil drying experiments in that vertical water content gradients were significantly established (Fig. 3G). For the measurement of vertical orientation hydrotropism, the agar-glycerol system can induce the vertical water-potential gradients from low to high (Fig. 2A). Then, the water content gradients in the sand system can be compared with the water-potential gradients of the agar-glycerol system: the lower water-content in the top of the sand system reflects the lower water-potential, and the higher water content in the bottom of the sand system reflects

A Gravity hinders roots to search for obliquely oriented water



Fig. 7. Proposed model illustrating the divergent roles of gravity in root hydrotropism. (A) When water orientation is oblique to the gravity vector, the gravity vector hinders root bending. In the presence of obliquely oriented water, wild-type and *miz1/aux1* double-mutant plants show hydrotropic root bending, whereas *miz1* mutant plants do not. (B) When water orientation is parallel to the gravity vector, gravity sensing promotes root growth in a straight downward direction allowing for the capture of vertically oriented water. Under these conditions, wild-type and *miz1* plants show root elongation towards the water in a vertical orientation, whereas *miz1/aux1* double-mutant plants do not. (This figure is available in colour at *JXB* online.)

the higher water-potential (Fig. 3G). Thus, based on establishing water contents in sand or soil, water content gradients in the sand system or soil drying experiments can also reflect the waterpotential gradients from low to high (Dodd et al., 2006, 2008b; Puértolas et al., 2013). In addition, compared with the agar-glycerol system, the sand system or soil drying experiments is more close to the natural conditions. Although amyloplast and auxin responses were enhanced upon hydrostimulation in the SSV system (Fig. 6A-C), these responses were reduced in the agar-glycerol system (Fig. 2B-E), further supporting that the sand system is superior to the agar-glycerol system in providing stable vertically oriented water gradients. Furthermore, hydrotropic stimulation in the SSV markedly increased the primary root length in WT seedlings, whereas agravitropic mutants were unable to reach the higher water potential present in deeper wet sand layers (Fig. 6D, F). This result is in agreement with previous studies showing that drought conditions induce steeper roots, aiding water uptake (Uga et al., 2013; Lynch, 2013; Rellan-Alvarez et al., 2015). Moreover, the miz1/aux1 double-mutant showed attenuated root elongation towards the water in a vertical orientation (Fig. 6F), suggesting that gravity is essential for the vertically oriented hydrotropism of roots. These findings also indicate that plants with an increased root growth angle in response to gravity stimulation will be better able to tap into deep water resources typically present in deeper soil layers, ensuring adequate water supply and thereby reducing the need for irrigation (Mingo *et al.*, 2004; Eapen *et al.*, 2017; Robbins and Dinneny, 2018).

In conclusion, we present a sand system that simulates natural conditions more accurately than traditional systems, with dynamic water potential gradients established not only in the oblique orientation but also in the vertical orientation. This system is, therefore, ideal for the study of root hydrotropic responses. We also provide evidence that gravity plays an essential role in the MIZ1-regulated root hydrotropism (Fig. 7). When the water orientation is oblique to the gravity vector, the gravity vector hinders root bending and the acquisition of obliquely oriented water. Wild-type and miz1/aux1 double-mutant plants show hydrotropic root bending, whereas miz1 mutants do not (Fig. 7A). On the other hand, when water orientation is parallel to the gravity vector, gravity sensing promotes root growth in a straight downward direction to allow for the capture of vertically oriented water. Wild-type and *miz1* plants show root elongation towards the water in a vertical orientation, whereas miz1/aux1 double-mutant plants do not (Fig. 7B). Overall, based on comparative analyses using various hydrotropic experimental systems, we propose that gravity impedes roots' search for obliquely oriented water while facilitating roots' search for vertically oriented water. Understanding the mechanisms of interaction between hydrotropism and gravitropism is pivotal to our understanding of the rooting patterns of plants under natural conditions, as efforts intensify to improve crop water uptake to meet the increasing demands for food under the rapidly changing climate conditions.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. The relative (percent) use of the three main research systems to study hydrotropism in Arabidopsis roots from data in PubMed Central (open-access articles).

Fig. S2. Primary root length and gravitropic bending in mutant and wild-type Arabidopsis.

Fig. S3. The hydrotropic response in mutant and wildtype Arabidopsis in sand system for oblique orientation hydrotropism (SSO).

Fig. S4. The hydrotropic response in tomato in sand system for oblique orientation hydrotropism (SSO).

Fig. S5. Changes in primary root length of Col-0 in the sand system for vertical orientation hydrotropism (SSV).

Fig. S6. Primary root length of Col-0 seedlings in the soil system or the sand system for vertical orientation hydrotropism (SSV) under control and hydrostimulation.

Fig. S7. Hydrotropic responses of Arabidopsis and tomato in the sand system for vertical orientation hydrotropism (SSV).

Fig. S8. Auxin and amyloplasts reponses in wild-type and *miz1* roots.

Fig. S9. The right/left-side lateral-root-length ratio in Col-0, *miz1* and *miz1/aux1* mutant in the sand system for SSO under hydrostimulation.

Table S1.Water content (%) of control and hydrostimulated containers in sand system for oblique orientation hydrotropism (SSO).

Table S2.Water content (%) of control and hydrostimulated containers in sand system for vertical orientation hydrotropism (SSV).

Table S3. Water content (%) in control and hydrostimulated chambers in soil.

Table S4. Fold changes in auxin-related gene expression in the roots under hydrostimulation conditions compared with under control in WT and *miz1* determined by RNA-seq.

Table S5. Fold changes in amyloplast-related gene expression in the roots under hydrostimulation conditions compared with under control in WT and *miz1* determined by RNA-seq.

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Author contributions

WX, YL, and WY planned and designed the research. LL, HD, and XD performed experiments and analysed the data.YL, WX, WY, JZ, RM, FB, HK, and CL discussed and wrote the manuscript. WX agrees to serve as the author responsible for scientific communication.

Data availability

The RNA sequencing data generated in this study have been deposited in NCBI (https://www.ncbi.nlm.nih.gov/sra/PRJNA641259) and SRA (Sequence Read Archive). The accession is PRJNA641259.

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