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An improved agar-plate method for studying root growth and response of *Arabidopsis thaliana*

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Arabidopsis thaliana **is a widely used model plant for plant biology research. Under traditional agar-plate culture system (TPG, traditional plant-growing), both plant shoots and roots are exposed to illumination, and roots are grown in sucrose-added medium. This is not a natural environment for the roots and may cause artifact responses. We have developed an improved agar-plate culture system (IPG, improved plant-growing) where shoots are illuminated but roots are grown in darkness without sucrose addition. Compared to TPG, IPG produced plants with significantly less total root length, lateral root length and root hair density, although their primary roots were longer. Root gravitropism, PIN2 (an auxin efflux carrier) abundance, H^+ efflux or Ca^{2+} influx in root apexes, were weaker in IPG-grown roots than those in TPG-grown roots. We conclude that IPG offers a more natural way to study the root growth and response of** ***Arabidopsis thaliana.***

Plasticity in root growth and response allows plants to survive dramatic changes in their environments, despite their sessility. Plants also make use of environmental signals to guide root growth and response so that they can adapt to the environment[1–3]. For instance, roots can direct their growth and response in relation to gravity, light, gradients of temperature, humidity, ions, chemicals and oxygen. Importantly, the information provided by these environmental signals is integrated by specific signal transduction pathways, and interpreted into specific regulators that dictate the patterns of root growth and other responses[4,5].

Arabidopsis thaliana has been established as the model plant for a wide range of basic and applied research in plant biology[6]. Its short and self-pollinated life cycle, simple genome and the easiness to generate various transgenic or mutant plants have led to many plant scientists use it to study the physiological and molecular processes in higher plant in response to environmental signals. A problem that has been overlooked so far, however, is that the Arabidopsis plants are raised largely in unnatural conditions for the study of their responses to natural changes.

In the natural growth environment of higher plants, only shoots are illuminated, while roots are grown in darkness. However, under traditional agar-plate culture system (TPG, traditional plant-growing) for Arabidopsis plants, roots and shoots are both illuminated. Recently, some researchers proposed that light affects the root growth and responses, and urge plant researchers to keep living roots in darkness and bring them into the light only when necessary[3,7–10]. Sucrose also affects root growth and responses. Under TPG, about 1% – 3% sucrose is added into the agar medium. For example, an Arabidopsis mutant, *hps1* (*hypersensitive to Pi starvation 1*), shows enhanced sensitivity in almost all the aspects of plant responses to phosphorus starvation under TPG. However, when grown on MS (Murashige and Skoog) sucrose-free medium or soil, there was no obvious phenotypic difference between WT and *hps1* mutant[11]. This paper also proposed that Arabidopsis plants (shoot or root) can take up sucrose directly from the culture medium of TPG[11]. These results suggest that under TPG, root lighting or sucrose addition may cause artifact root responses. Although hydroponic-cultured or soil- cultured Arabidopsis root are grown in darkness without sucrose addition, it is inconvenient to investigate root growth and response *in situ*, which can be facilitated in agar-plate system (TPG). There is a need to improve the current agar-plate culture system and help plant researchers to overcome these pitfalls.

In the present study, we developed an improved agar-plate culture system (IPG, improved plant-growing) for studying the root growth and response of *Arabidopsis thaliana*. Under the IPG, Arabidopsis shoots are illuminated, while roots are grown in darkness without sucrose addition. Some Arabidopsis natural accessions, relevant Arabidopsis mutants and transgenic lines were used when we investigated the root growth responses (total root length, lateral root length, primary root length, root hair density), PIN2 abundance, gravitropic response, H^+ or Ca^{2+} flux in the root tip under TPG or IPG. Our results suggest that compared to TPG, IPG allows us to study the root growth and responses of Arabidopsis plants under the most approximate natural growing conditions.

Results

Root growth. Roots growth of Arabidopsis plants under TPG and IPG were investigated. Compared to TPG, Arabidopsis shoots were illuminated, but roots were grown in darkness without sucrose addition in the IPG situation (Fig. 1). Using Arabidopsis ecotype Col-0, we have found that although no significant difference was found in the shoot dry weight under TPG or IPG in Arabidopsis ecotype Col-0, the root dry weight in TPG was significantly higher than that in IPG (Fig. 2). Furthermore, the total root length, lateral root length or root hair density were significantly higher in TPG than those in IPG roots; and the primary root lengths in TPG were significantly lower than that in IPG (Fig. 3). Finally, as shown in Fig.S3, the root growth changes observed between TPG and IPG for the ecotype Ws were similar with those in Col-0.

PIN2 change. Auxin efflux carrier PIN2 plays an important role in root signaling and behavior[3]. Therefore, we have compared the PIN2 expression and distribution in both TPG and IPG situations. In Arabidopsis root, PIN2 undergos constitutive cycling between the plasma-membrane (PM) and endosomal compartments via endocytic trafficking. Using laser confocal scanning microscopy, we have found that in the TPG root, PIN2 was predominantly PM-localized (Fig. 4A). However, in the IPG root, the PM localization of PIN2 was largely reduced, and some PIN2 signal was detected in the endosomal-like compartments (Fig. 4B). In addition, the abundance of *PIN2* mRNA transcript or PIN2: GFP fluorescence in IPG was significantly lower than those observed in TPG (Fig. 4C).

Root gravitropic response. PIN2 is required for root gravitropism[12], and our above results suggest that PIN2 is involved in the differences recorded between the TPG roots and IPG roots (Fig. 4). Using the wild-type Arabidopsis (Ws) and PIN2 partial mutant (*agr1-2*), we have explored the gravitropic responses of Arabidopsis roots grown in the TPG or IPG. During gravi-stimulation for 24 h, no significant difference was found in primary root elongation of Ws and *agr1–2* in TPG or IPG. However, root-tip cure-angle of Ws in TPG was significantly higher than that of Ws in IPG, *agr1–2* in TPG or *agr1–2* in IPG. Further, no significant difference was found in root-tip cure-angle of Ws in IPG or *agr1–2* in TPG, and the root-tip cure-angle of *agr1–2* in TPG was significantly higher than that of *agr1–2* in IPG (Fig. 5). Additionally, under gravi-stimulation for 1.5 h, in root cap, H^+ efflux or Ca^{2+} influx of Ws in TPG was significantly higher than those of Ws in IPG, *agr1–2* in TPG or *agr1–2* in IPG. Moreover, no significant differences were found in H^+ efflux or Ca^{2+} influx patterns of Ws in IPG or *agr1–2* in TPG, and the H^+ efflux or Ca^{2+} influx of *agr1–2* in TPG situation was significantly higher than that of *agr1–2* in IPG (Fig. 6).

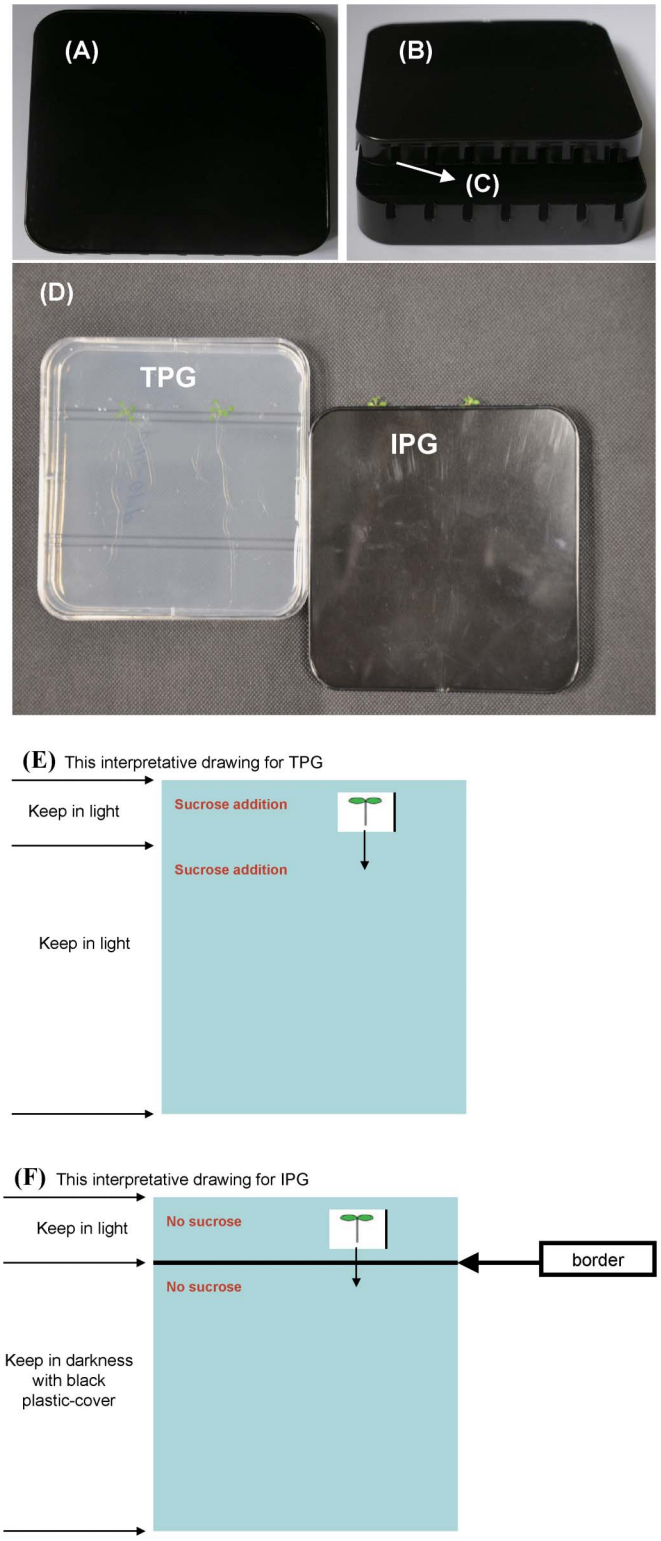

Figure 1 | The improved agar-plate culture system (IPG) for studying the root growth of *Arabidopsis thaliana*. Note: (A) Upper view of IPG; (B) Side view of IPG (C) Oblong holes (6 × 3.5 mm, L × W) in the IPG; (D) Wild-type Arabidopsis plants (Col-0) grown in the traditional agar-plate system (TPG) or IPG. (E) and (F) this interpretative drawing for TPG (E) or IPG (F).

Discussion

Root growth is modulated by the convergence of multiple environmental and endogenous signals[13,14]. *Arabidopsis thaliana* has been used as the model plant for the study of many such processes that integrate the signaling pathways and regulate the physiological responses. In nature, roots are plant organs that typically lie below the soil and grow in darkness without sucrose addition. Arabidopsis roots can be easily grown in non soil agar-plate, which facilitates *in vitro* study and analysis. It is often that the plant is raised in agar

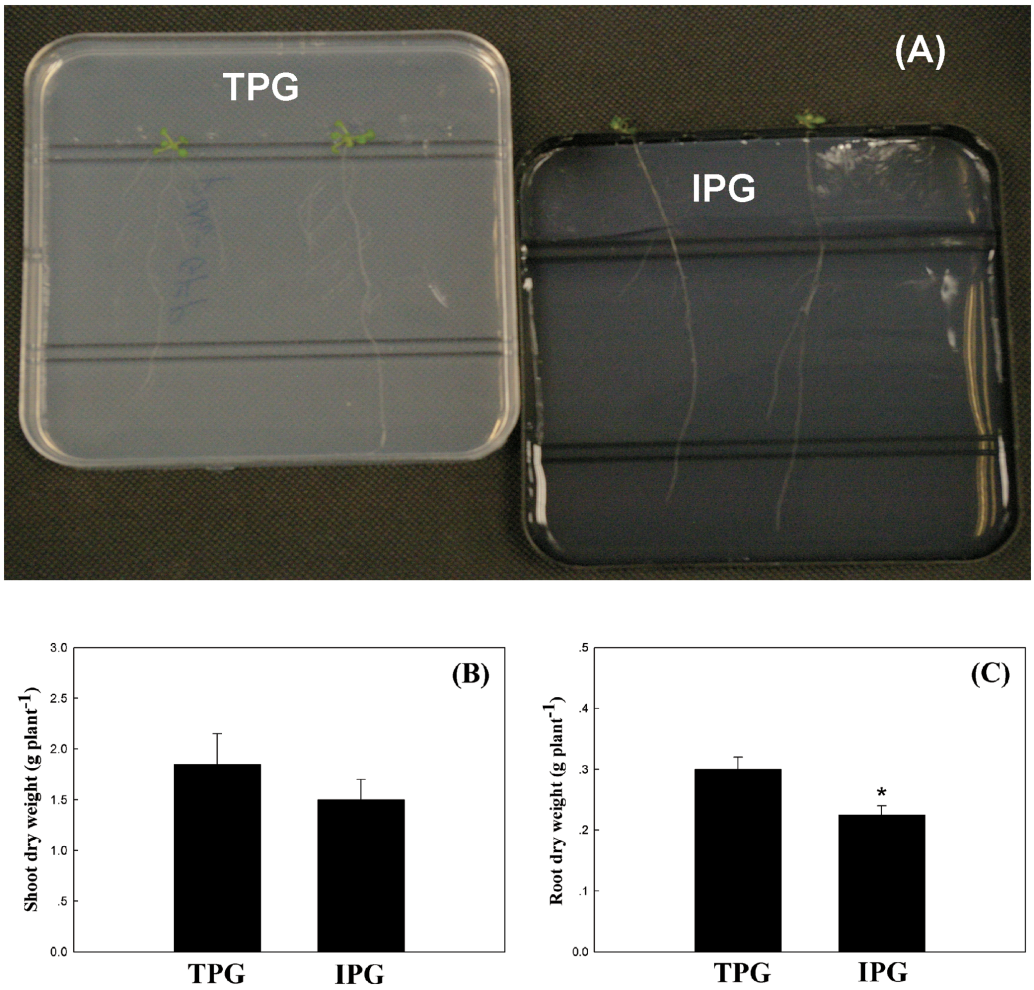

Figure 2 | The phenotype or dry weight of wild-type Arabidopsis plant (Col-0) grown in the traditional agar-plate culture system (TPG) or improved agar-plate system (IPG). 15-d-old Arabidopsis plants were grown in TPG or IPG. Note: (A) Phenotype; (B) Shoot dry weight; (C) Root dry weight.

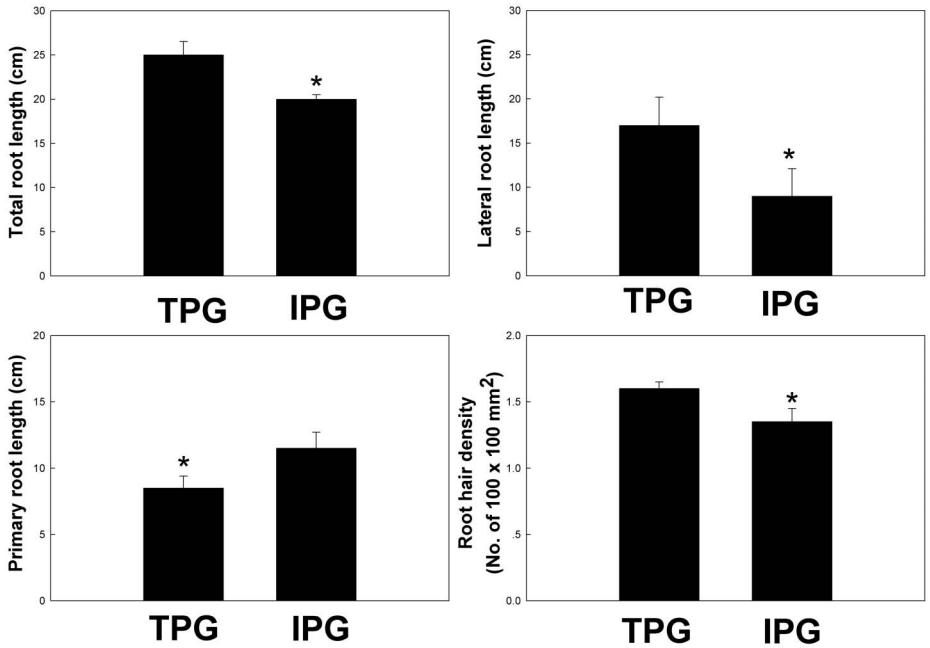

Figure 3 | The root growth of wild-type Arabidopsis plant (Col-0) grown in the traditional agar-plate culture system (TPG) or improved agar-plate system (IPG). 15-d-old Arabidopsis plants were grown in TPG or IPG. Note: (A) Total root length; (B) Lateral root length; (C) Primary root length; (D) Root hair density.

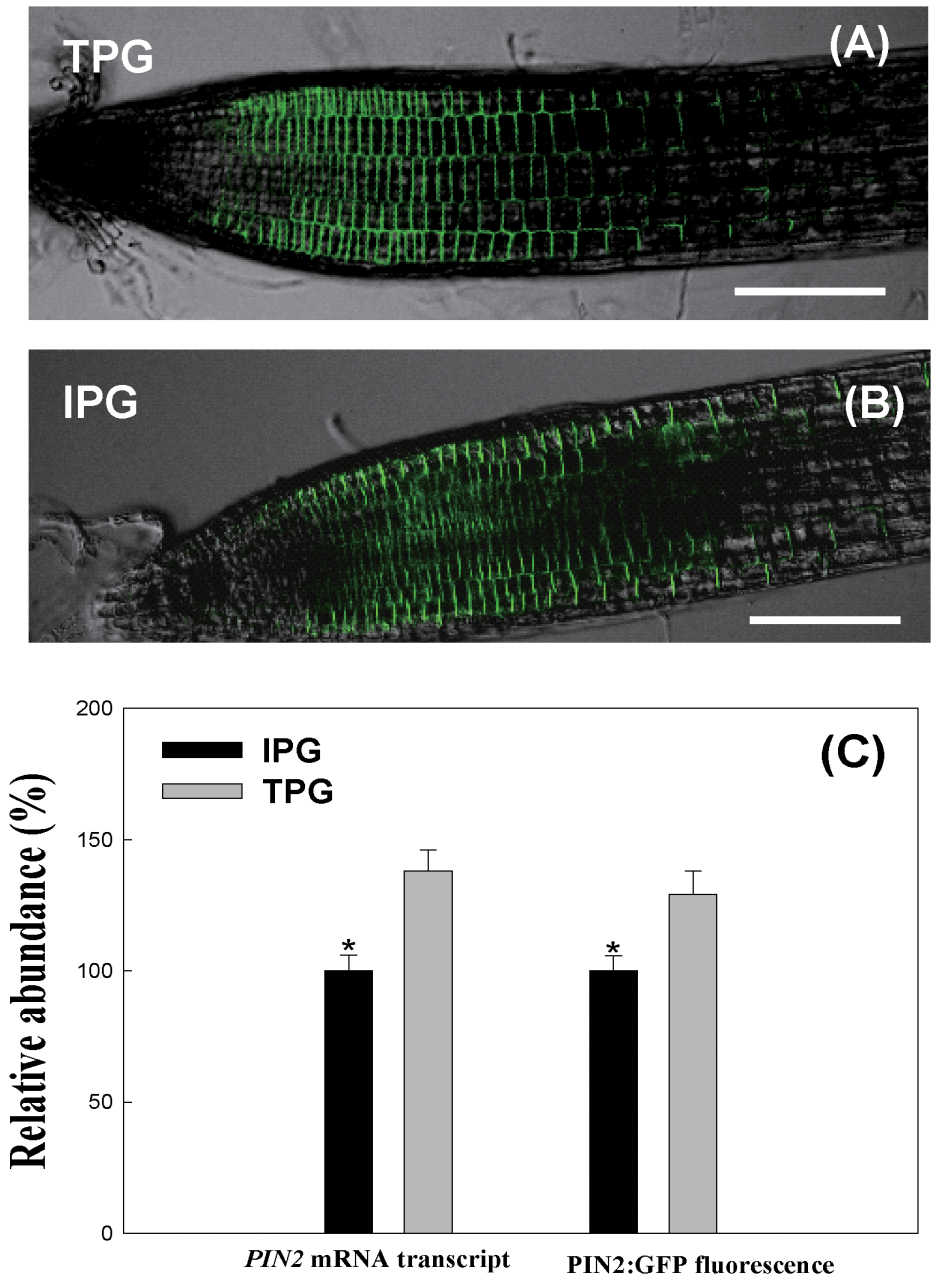

Figure 4 | PIN2 distribution and abundance in the root tip of wild-type Arabidopsis plants (Col-0) (DR5rev:GFP) grown in the traditional agar-plate culture system (TPG) or improved agar-plate system (IPG). 15-d-old Arabidopsis plants were grown in TPG or IPG. (A) PIN2 distribution in TPG; (B) PIN2 distribution in IPG; (C) Transcript abundance of *PIN2* or PIN2:GFP abundance. Bar = 100 μm (white line). "PIN2 abundance in IPG" is plotted as "100%".

culture with its roots illuminated and exposed to sucrose in the medium. However, light and sucrose are known affecting root growth and response[7–11]. Our results confirm that such unnatural treatments to roots can cause substantial differences in terms of root growth and responses to environmental signals.

Using Arabidopsis ecotype Col-0 or Ws, we have found that the total root length, lateral root length or root hair density in IPG (roots are grown in darkness without sucrose addition) was significantly lower than that in TPG (roots are illuminated and grown in the sucrose-added environment), while the primary root length in IPG was significantly higher than that in TPG (Fig. 2, Fig. 3 and Fig.S3). There are several potential reasons for this root growth difference between TPG and IPG. Firstly, app:addword:differencevia activation of PHOT1, a blue light photoreceptor, light stimulates Arabidopsis root growth[7,10,15,16]. Illuminated roots display the light-escape tropism via faster root growth[8,9]. Secondly, elongation rate of Arabidopsis primary root in darkness is higher than that in light under long photoperiods (16 h : 8 h or 12 h : 12 h, light : dark)[17]. Thirdly, in the presence of exogenous sucrose, Arabidopsis root can grow both in light and darkness. However, in sucrose-free medium, the growth rate of Arabidopsis root is decreased whether in light or in darkness[18]. Further, light and sucrose promote root hair formation[19].

Root gravitropism is regulated by auxin transport, and auxin efflux carrier PIN2 exhibits asymmetric plasma-membrane location, determining the polarity of auxin transport[12]. Compared with the TPG roots, plasma-membrane location of PIN2 was affected, and some PIN2 accumulated within endosomal-like compartments in the IPG roots (Fig. 4). Root gravitropic response in IPG root of wild-type Arabidopsis (Ws) mimic the TPG root gravitropism of *agr1–2* (PIN2 partial mutant of Arabidopsis) with altered response to gravity

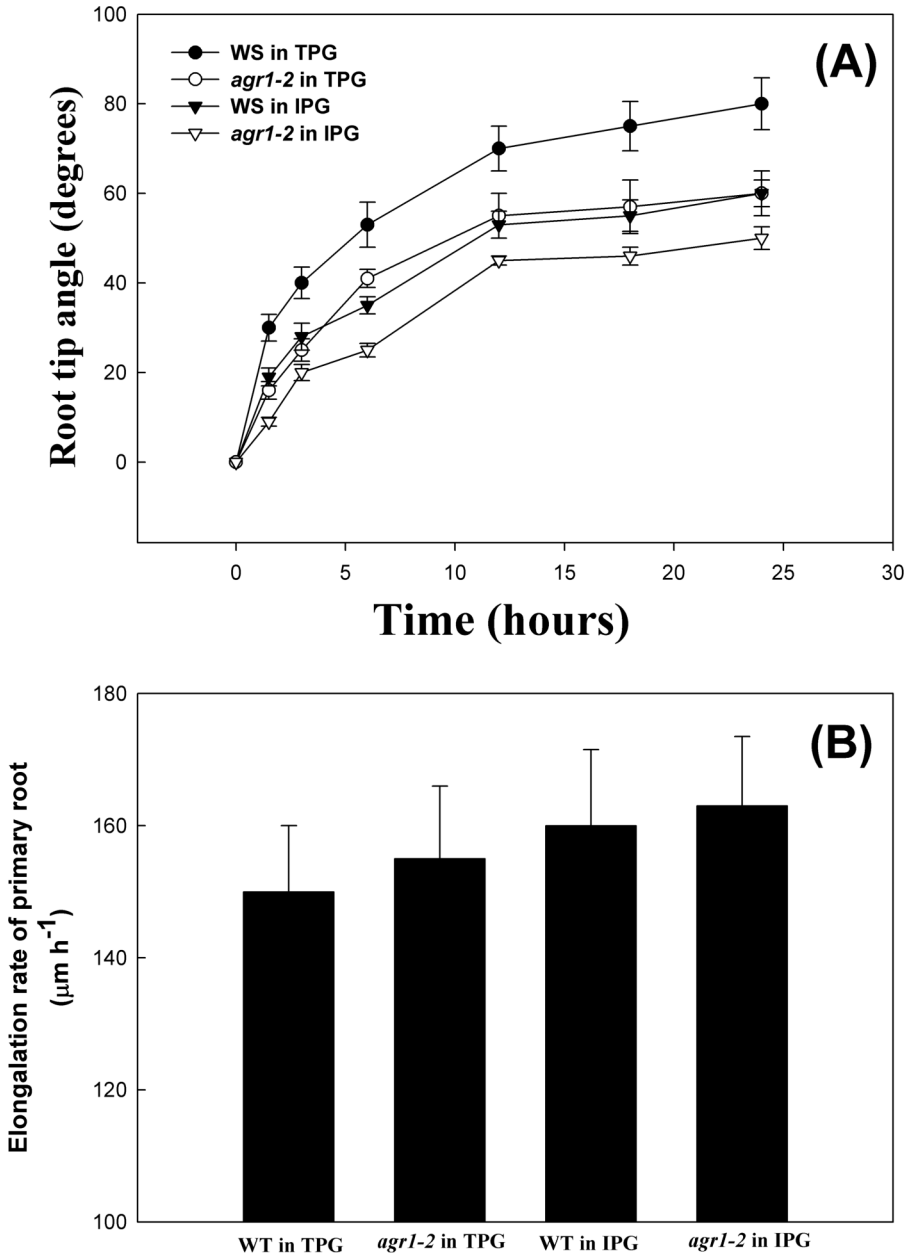

Figure 5 | Gravitropic response or primary root elongation of wild-type (Wassilewskija: Ws) or mutant (*arg1–2*) Arabidopsis plants grown in the traditional agar-plate culture system (TPG) or improved agar-plate system (IPG). 15-d-old Arabidopsis plants were grown in TPG or IPG. Note: (A) Root tip angle during reorientation of root; (B) Elongation rate of primary root under gravistimulation for 24 h.

(Fig. 5). This finding suggests that changes in root tip PIN2 alters the gravitropism in roots grown in the IPG mode. Moreover, H^+ or Ca^{2+} flux modulation in root cap is important for the gravity response of Arabidopsis root[2,20]. H^+ efflux or Ca^{2+} influx was largely reduced in IPG root cap of Ws or *agr1–2* compared to TPG root, which suggests that changes in the two parameters may also contribute to the altered gravitropism in IPG roots.

In conclusion, Arabidopsis root growth and responses to environmental signals in an improved agar-plate system (IPG) are different from those under the traditional agar culture system (TPG). This is due to the fact the roots are exposed to lighting and sucrose (TPG) during culture or not (IPG). Since lighting and sucrose can directly regulate root growth and its responses to other environmental signals, studies using the *in vitro* cultured Arabidopsis plants should adopt the most approximate natural-growth-environment such as that in IPG.

Methods

The improved growth plate. Traditional agar-plate culture system (TPG, traditional plant-growing) can raise the *in vitro* Arabidopsis plants but cannot eliminate the effects of light and sucrose on the physiology of roots (Fig.S1 and Fig.S2). Thus, we developed the improved agar-plate system (IPG, improved plant-growing) for controlling the effect of light and sucrose on root growth. Firstly, IPG has the same size with the traditional growth plate (12.5 × 12.5 × 1.5 cm, L × W × H), but IPG is black and light-tight (Fig. 1A, Fig. 1D, Fig. 1E and Fig. 1F). Secondly, compared with TPG, there are seven oblong holes (6 × 3.5 mm, L × W) in the medium container or upper cover of IPG (Fig. 1B and Fig. 1C). Thirdly, there is no sucrose included in the medium container of IPG compared to TPG (Fig. 1E and Fig. 1F). Further, when Arabidopsis plants are grown in IPG, shoots are illuminated without sucrose addition, while roots are grown in darkness environment without sucrose addition which is very different from the TPG mode (Fig. 1D, Fig. 1E and Fig. 1F).

Plant materials and growth conditions. The wild-type Arabidopsis plants (WT) were ecotype Col-0 or Wassilewskija (Ws), and the *arg1-2* mutant with Ws background which was obtained from Rujin Chen (Samuel Roberts Noble Foundation, Ardmore, USA). Before planting, Arabidopsis seeds were surface

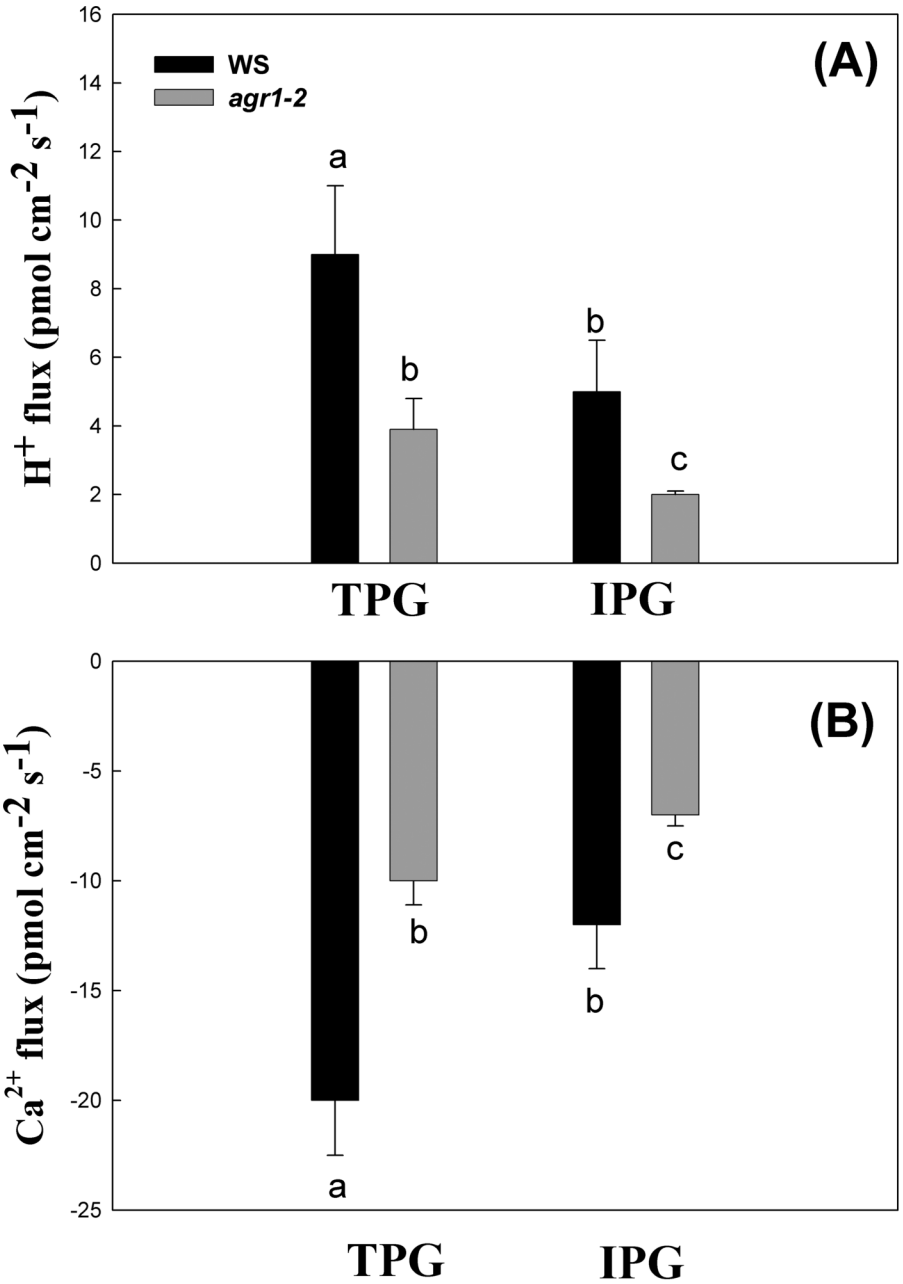

Figure 6 | H^+ (A) or Ca^{2+} (B) flux in the root cap of wild-type (Wassilewskija: Ws) or mutant (*arg1–2*) Arabidopsis plants grown in the traditional agar-plate culture system (TPG) or improved agar-plate system (IPG). 15-d-old Arabidopsis plants were grown in TPG or IPG, and then gravistimulation was given for 1.5 h.

sterilized with 70% ethanol for 1 min, then with 1% sodium hypochlorite solution plus SDS for 9 min, and subsequently rinsed by sterile deionized water 6 times, and then kept in the dark for 3 days at 4° for stratification. Arabidopsis seeds were sown on half-strength Murashige and Skoog (MS) plates containing 0.8% agar and 1% sucrose. After that, Arabidopsis plants were grown at 22 ± 1°, with the light intensity of 120 μmol photons m^{-2} sec^{-1}, 16/8 h photoperiod, and relative humidity of 70%. After germination for 5 days, Arabidopsis seedlings were carefully transferred to TPG with full-strength Murashige and Skoog nutrients containing 0.8% agar and 3% sucrose or IPG with full-strength Murashige and Skoog nutrients containing only 0.8% agar (no sucrose). Additionally, in this transferred process for IPG, absorbent cotton was used in oblong hole to shut out light. After growth for 10 days, 15-d-old Arabidopsis plants were used to for experimental analyze.

Measurement of dry weight and root growth. Arabidopsis shoot or root was collected and dried at 70°C for 3 days, then weighed. The root growth (total root length, lateral root length, primary root length) was measured using a root analysis instrument (WinRHIZO; Regent Instruments Inc., Quebec, ON, Canada), as described previously[21]. The elongation rate of primary root (μm h^{-1}) was calculated from the primary root length with respect to the displacement of primary root apex for the duration of time. Root hairs were determined in the region 0–5000 μm from root cap junction in Arabidopsis plants using confocal laser scanning microscopy (Olympus FV-1000 spectral type SPD mar/G/R IX81 FLUOVIEW laser confocal system)[22]. Root hair densities were estimated from a 100 × 100 $μm^2$ section.

Confocal laser microscopy. The fluorescence of PIN2:GFP in Arabidopsis root tip was observed (with the same Olympus FV-1000 confocal microscope) as described previously[23]. To image GFP, the 488- and 514-nm lines of the argon laser were used for excitation, and emission was detected at 510 and 530 nm, respectively. Approximately 10 seedlings/images were examined, and at least two independent experiments were performed, giving the same statistically significant results. All images were taken under the same conditions.

Real-time RT-PCR. Total RNA was extracted from Arabidopsis plants, and then real-time RT-PCR was assayed as described previously[24]. Real-time RT-PCR was performed in 25 μL reaction mixture composed of cDNA by using the DNA Engine Opticon 2 system (MJ Research, USA) for continuous fluorescence detection. Gene-specific primers for real-time RT-PCR were designed using Primer 5 software (Table

S1). *At-ACT2* is a strongly and constitutively expressed "house-keeping" gene in Arabidopsis plants, so the quantification of mRNA levels was based on comparison with the level of mRNA for *At-ACT2*.

Gravitropic reorientation assay. Arabidopsis roots grown in TPG or IPG were gravistimulated by 90° rotation. Plates were photographed at specified time intervals after gravistimulation with a Nikon COOLPIX800 digital camera (Tokyo, Japan). ImageJ software was used for the measurement of root tip angles as described previously[25].

Assay of H^+ or Ca^{2+} flux in root cap. H^+ or Ca^{2+} fluxes were measured non-invasively using SIET (scanning ion-selective electrode technique, SIET system BIO-003A; Younger USA Science and Technology Corporation; Applicable Electronics Inc.; Science Wares Inc., Falmouth, MA, USA)[26,27]. Arabidopsis plants were equilibrated in measuring solution for 20–30 min, these equilibrated Arabidopsis plants were transferred to the measuring chamber, a small plastic dish (3-cm diameter) containing 2 to 3 mL of fresh measuring solution. When the root became immobilized at the bottom of the dish, the microelectrode was vibrated in the measuring solution between two positions, 5 μm and 35 μm from the root surface, along an axis perpendicular to the root. The background was recorded by vibrating the electrode in measuring solution not containing roots. Microelectrode were made and silanized by Xuyue Science and Technology Co., Ltd.

Statistical analyses. Data were subjected to analysis of variance and *post hoc* comparisons were done with Duncan's Multiple Range Test at $P < 0.05$ level. The statistical software program used was SPSS version 13.0. The values are the means and SD of six replicates from two independent experiments.

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Author contributions

W.X., J.Z., W.S., G.D., F.B. and K.Y. conceived the experiments. W.X., G.D., J.Z., W.S., F.B. and J.L. wrote the manuscript. W.X., G.D., K.Y., Q.L. and Y.L. performed the experiments.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

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