



Research article

The interaction of genes controlling root traits is required for the developmental acquisition of deep and thick root traits and improving root architecture in response to low water or nitrogen content in rice (*Oryza sativa* L.) cultivars



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ABSTRACT

Most of the hot spots about rice research are related to roots; increasing rice yield is mainly associated with improving root traits. Understanding phenotype-gene regulation relationship in different rice cultivars can contribute to the genetic improvement of root system. The expression pattern of root genes in moroberekan (deep and thick roots and high root/shoot ratio “R/S”) was compared to that in Giza178 and PM12 (numerous but shallow roots) and IR64 (fewer but deeper roots than the latter ones). In contrast to the other genotypes, moroberekan did not cease developing deep and thick roots even after 60 days from sowing, perhaps because of not only the consistent upregulation but also the interaction of root genes. Xylem sap flow was significantly higher even under drought (low water content) in moroberekan. Auxin signaling-related *ARF12* and *PIN1* genes could play key roles in improving root traits in response to low water or nitrogen content. Their concurrent upregulation was coincided with developing 1) deeper roots in moroberekan under drought, 2) thicker and deeper roots in PM12 under low nitrogen content (LN) and 3) new roots with thicker and deeper characteristics in the four genotypes after root trimming. The upregulation of *PIN1* or *ARF12* in Giza178 at LN, PM12 at drought or in IR64 under drought or LN did not greatly change the root traits. Hierarchical analysis showed that *ARF12* and *PIN1* were distantly related, but overlapped with other genes controlling root traits. Overexpression of *ARF12* and *PIN1* could improve root traits in rice cultivars.

1. Introduction

Rice is one of the most important crops since it is the main diet for more than half of the world's population. The increasing population has been posing a successive pressure for increase in crop production (Zhang et al., 2007). To face this need of food for increasing population, raising the yield of rice remains the priority task for rice breeders (Ladha and Reddy, 2003; Gutierrez, 2012). Root system is an important organ for the uptake of the nutrients and water from the soil. The morphological and physiological root traits affect the growth and productivity of the plant. Well-developed rice roots increase the biomass and yield in response to different conditions in different cultivars (Arai-Sanoh et al., 2014 and ref. therein). Root growth and architecture are genetically controlled and also influenced by different environmental factors such as soil temperature, soil water content, soil physical,

chemical, and biological properties and solar radiation (Fageria et al., 2014).

Many studies have been focused on identifying genes related to the morphological characters and physiological functions of rice roots and with the aid of loss or gain of function, the roles of many of these genes were defined, which opened an opportunity for further research and thereby improving rice root traits (Uga et al., 2013). However, many of these studies have been concentrated on identifying the role of gene/s by quantitative trait locus (QTL) analyses, the mutation or by over/under-expression of a gene of interest in one rice genotype, which limits our systemic understanding of the regulatory mechanisms of root development in different rice cultivars (Meng et al., 2019). For example: *DEEPER ROOTING 1* (*DRO1*) was isolated as a functional allele that controls the gravitropic curvature of the roots. This gene was identified in the deep-rooting cultivar Kinandang Patong (a traditional tropical

Abbreviations: HN, High Nitrogen; LN, Low Nitrogen; RA, Root Angle; RD, Root Diameter; RDW, Root Dry Weight; RFW, Root Fresh Weight; RL, Root Length; RSA, Root Surface Area; RV, Root Volume

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Table 1
Abbreviation, name and function of the genes examined in the present study in rice roots.

Gene abbr.	Gene name	Function in rice roots	Reference
ARF12	AUXIN RESPONSE FACTOR12	Transcription activator that can facilitate auxin transport and it is implicated in regulating root elongation.	Qi et al. (2012)
ARL1	ADVENTITIOUS ROOTLESS 1	<i>CRL1</i> and <i>ARL1</i> are key regulators for the initiation of crown roots and lateral roots	Liu et al. (2005)
CaMK	CALMODULIN-dependent protein Kinase	Expressed in roots grown in paddy field/its expression required for mycorrhization.	Bao et al. (2014)
CRL1	CROWN ROOTLESS 1	They are essential for crown root formation.	Inukai et al. (2005)
CRL2	CROWN ROOTLESS 2		Inukai et al. (2005)
CRL4	CROWN ROOTLESS 3		Kitomi et al. (2011)
DRO1	DEEP ROOTING 1	Functions in downstream of the auxin signaling pathway and controls in gravitropic curvature of the roots.	Arai-Sanoh et al. (2014)
EXPA2	α EXPANSIN 2	They are multigene family in higher plants, play a critical role in regulating cell wall extension during development and are involved in regulating root system architecture	Zou et al. (2015)
EXPA5	α EXPANSIN 5		
EXPB5	β EXPANSIN 5		
FON1	Floral Organ Number	<i>FON1</i> and <i>FON4</i> function as regulators for meristem development	Chu and Zhang (2007)
GLR3.1	GLUTAMATE Receptor Like 3.1	It is essential for the maintenance of cell division and individual cell survival of root apical meristem at the early seedling stage.	Li et al. (2006)
NAC5	NAM, ATAF, and CUC	NAM, ATAF, and CUC domains-containing proteins constitute one large plant-specific family in rice and Arabidopsis. <i>NAC5</i> overexpression enlarges root diameter in rice.	Jeong et al. (2013)
SCR1	SCARECROW	Responsible for asymmetric cell division	Kamiya et al. (2003)
SHB	SHOEBOX	An AP2/ERF transcription factor. It directly binds to and activates gibberellin biosynthesis gene in the root meristem, leading to local production of GA that promotes elongation of meristem following germination and confer the phenotypic plasticity during the early developmental stages. At later stages, <i>SHB</i> and gene of GA biosynthesis participate in the modulation of cell proliferation in the root meristem.	Li et al. (2015)
(PIN1) REH	PIN FORMED	Involved in auxin-dependent adventitious root emergence and tillering.	Xu et al. (2005)
RH2	ROOTHAIRLESS2	Responsible for root hair formation.	Suzuki et al. (2003)
TUB6	TUBULIN 6	Tubulins are important component of microtubules that are involved in cell division, cell motility, and cell morphogenesis. <i>TUB6</i> is expressed in root.	Goddard et al. (1994)
WOX3A	WUSCHL-related homebox protein 3 and 9, respectively.	Transcriptional regulators control various developmental process including root and shoot apical meristem maintenance. Overexpression of <i>WOX3A</i> increased the number of lateral roots	Yoshikawa et al. (2003)
WOX9			Cheng et al. (2014)
			Cho et al. (2016)

japonica upland rice cultivar from Philippines) and originated in the genetic background of the shallow rooting parent IR64, a lowland indica rice cultivar, which is widely grown in south and south-east Asia (Uga et al., 2013). The effect of *DRO1* functional allele on rice growth was examined by comparing IR64 with *DRO1*-NIL Kinandang Patong under droughted upland and flooded lowland conditions (Uga et al., 2013; Arai-Sanoh et al., 2014). The results showed that the functional *DRO1* had effective role in acquisition of resources, leading to higher yield. Moreover, the expression of functional *DRO1* positively affected the growth of rice roots under different levels of soil compaction (Ramalingam et al., 2017).

Downregulation of *PIN-FORMED* gene (*PIN1*) has reduced crown root numbers (Wang et al., 2009). Crown root formation was impaired in *crown rootless 4/osgnom1* mutant (Liu et al., 2009). *GNOM* is membrane-associated guanine nucleotide exchange factor for the G-protein and plays a key role in polar auxin transport by mediating coordinated polar localization of *PIN1* in Arabidopsis (Steinmann et al., 1999; Geldner et al., 2003). Distortion of polar auxin transport and altered expression of *OspINs* were observed in *crown rootless 4/osgnom1* mutant, implying that polar auxin transport is essential for crown root formation in rice (Kitomi et al., 2018). Auxin signal is transmitted by pathway regulated by Aux/IAA and auxin response factor (*ARF*) (Liscum and Reed, 2002). Transgenic plants that produced Aux/IAA constitutively have reduced crown root numbers (Nakamura et al., 2006). *Crl1/ar1* mutant developed few crown roots. Meanwhile *CRL1/ARL1* encodes plant specific ASL (asymmetric leaves2-like), which acts as downstream of Aux/IAA and *ARF*-mediated auxin signaling pathway and whose expression is directly regulated by *OsARF* (Liu et al., 2005). The mutant *crl2* was also devoid of the ability of gravitropism and crown root initiation, suggesting that *CRL2* may play role in auxin signaling (Yamamoto et al., 2010). Most of crown root mutants showed defects in lateral root formation and root hair development, suggesting that auxin signaling is important in overall root morphogenesis (Kitomi et al.,

2018).

The ability of genotype to alter its phenotype in response to the environment is defined as plasticity (O'Toole and Bland, 1987). Water shortage (drought) negatively affects crop growth and productivity. About one third of the areas cultivating rice is rain-field lowland ones and prone to drought (Maclean et al., 2002). In response to drought stress, plants modified root architecture (Asch et al., 2005) by developing deeper and thicker roots. Since nitrogen is a major constituent of amino acids, ATP, nucleic acid, coenzymes, plant hormones, and secondary metabolites, N deficiency negatively affects all aspects of plant function from metabolism, resources allocation, growth and development (Xu et al., 2012; Giehl and von Wirén, 2014). To cope with N deficiency, plants have evolved complicated physiological, biochemical, and morphological adaptations. For example, plants will increase the capacity to acquire N by stimulating root growth at the expense of shoot growth (Giehl and von Wirén, 2014). There have been some attempts toward phenotypic characterization of various traits in response to nitrogen and nitrogen use efficiency in rice such as root length and density (e.g. Peng et al., 2015; Steffens and Rasmussen, 2016). The ability of rice roots to accumulate biomass (root sink size) is independent from the ability of shoot base to initiate new roots, but rather dependent on the ability of new roots to compete for resources with the shoot system (Chang and Zhu, 2017), determining the plasticity degree of the root system to cope with the environmental constraints.

It is expected that one root trait could be controlled by more than one gene or by the interaction among different genes. Uga et al. (2015) have suggested that *DRO1* alone cannot completely explain the difference in root growth angle between Kinandang Patong and IR64. However, the interaction of different genes controlling root traits in different rice cultivars is not fully examined. Recently, some studies have been examined the effect of possible cross-talk among genes related to one family on regulating root traits in rice cultivars. Cheng

et al. (2014) have studied the role of WUSCHEL-related homeobox genes in rice development, hormone signaling, and abiotic stress response in different rice organs.

Out of 62 traits in 3024 rice varieties available in the database in the International Rice Research Institute (IRRI), none of root traits is included. This suggests that root traits are not widely included in rice breeding program at least because of the lack of data on the root traits on the available resources (Nada and Abogadallah, 2018). Roots are difficult to study because they are underground and are naturally complex (Shrestha et al., 2014). However, the molecular examination of rice root traits could add to the field of rice improvement programs. In the present study, we examined the expression pattern of twenty genes (Table 1) controlling different root traits during the developmental stages and in response to different conditions (water or nitrogen) in four rice genotypes possessing different root phenotypes.

We chose four rice genotypes with different root phenotypes: Giza178 is Egyptian cultivar with a range of drought resistance and it is characterized by high number of tillers (Abd-Allah et al., 2010) and thin and shallow roots. PM12 was isolated from a population of Giza178 as it was characterized by greater shoot and root compared to Giza178 (Nada and Abogadallah, 2018). Roots of PM12 are greater than those of Giza178 but they are still shallow and fine. IR64 is a known rice cultivar with few, shallow roots (Arai-Sanoh et al., 2014); but deeper than those of Giza178 and PM12. Moroberekan is African cultivar with limited number of tillers and deeper roots (Champoux et al., 1995) and it possesses thicker and deeper roots (Lilley et al., 1996; Ganapathy et al., 2010) in comparison with the previous three genotypes.

Based on the previous data, we hypothesized that 1) the developmental acquisition of deep and thick roots requires an interaction of consistently upregulated genes controlling root traits through all the developmental stages, 2) the regulation of root genes and the plasticity of roots in response to different conditions are dependent on rice genotype and 3) the interaction of root genes may improve the root performance in response to low water or nitrogen content. To test these hypotheses, the objectives of this study were: 1) to characterize the morphological traits of the four genotypes at different developmental stages and at different growth conditions, 2) to investigate the relationship between the genes expression pattern and the root traits in each rice genotype at different developmental stages and in response to different growth conditions and 3) to verify whether the expression pattern will be varied in the new roots after root trimming from that in the old roots and it will be genotype-dependent.

2. Materials and methods

2.1. Plant materials

Seeds of Giza178 were provided from Agricultural Research Centre, Egypt. PM12 is a F4 progeny of an off-type plant isolated from a population of Giza178 in 2012 as taller plant that was hard to pull from soil. PM12 was propagated by self-pollination over the next 4 years. IR64 and Moroberekan seeds were kindly supplied by IRRI, Manila, Philippines.

2.2. Root traits and genes expression at different developmental stages

The seeds of the four genotypes were sowed in black plastic bags (20 cm diameter x 45 cm height) containing a mixture of clay and peat moss soil (1:1). The seeds were covered with thin layer of soil and irrigated by water every day. The experiment was held in the green house of Botany and Microbiology Department, Faculty of Science, Damietta University under the appropriate field conditions for rice growth (26–28/22–24 °C day/night temperature, 49–52% relative humidity, and $2150 \mu\text{mol m}^{-2} \text{s}^{-1}$ maximum light intensity). Samples of rice plants were harvested after 7, 15, 30, 45 and 60 days after sowing (DAS). For root morphological and biomass measurements, bags were

cut and soil particles were gently removed to keep roots intact. Some seeds were germinated as previously mentioned in bags containing stainless steel mesh to detect the angles of the growing roots at each developmental stage (Tomita et al., 2017). Root and shoot biomass, root length, root volume, root angles, and number of roots per plant were measured. Measurements for each parameter at each developmental stage were made from ten replicates. For the molecular analyses, new roots close to the shoot bases were cut and frozen immediately in liquid nitrogen and then stored at -80°C .

2.3. Root traits and genes expression in response to water or nitrogen content

The seeds of the four genotypes were grown in soil mixture of clay and peat moss in plastic bags as previously mentioned. After fifteen days of sowing, seedlings were divided into four groups. The first group was irrigated with water every day (the control) and the second group was irrigated with 70% of the field capacity (the drought, Nada and Abogadallah, 2014; Nada et al., 2018) in order to evaluate the effect of soil water content on the root traits and the expression of the related genes. The third and fourth groups were used to evaluate the effect of soil nitrogen content on the root traits and the expression of the related genes where the third group was irrigated with water and 1.5 g of urea fertilizer per 1 Kg soil was added as nitrogen source every 5 days until the end of the experiment (high nitrogen group, HN) and the fourth group was irrigated with water only without nitrogen source (low nitrogen group, LN).

Before starting this experiment, soils were washed thoroughly to remove any nitrogenous residues. The nitrogen content was evaluated in the used soils and HN and LN treated-plants were compared to the control ones that were grown under proper nitrogen content. The seedlings were harvested as previously mentioned at 35 DAS. Ten replicates were used for each parameter at each treatment. For the molecular analyses, new roots close to the shoot bases were cut and frozen immediately in liquid nitrogen and then stored at -80°C .

2.4. Characterization of root traits and genes expression of the new roots after root trimming

The seeds of the four genotypes were germinated in 15 cm x 20 cm trays containing perlite under field conditions as previously mentioned. Trays were irrigated with full strength Ruakura nutrient solution (Smith et al., 1983). When seedlings were 25 days old, roots were clipped at 1 cm below the shoot base and then they were transplanted separately into 10 cm x 20 cm x 35 cm pots containing perlite. These transplanted seedlings were divided into three groups; the first one was irrigated with full strength Ruakura nutrient solution (HNT), the second one was irrigated with full strength Ruakura nutrient solution devoid of any nitrogen source (LNT) and the third group was irrigated with 70% of the field capacity with full strength Ruakura nutrient solution (drought, DT). These three groups were compared to seedlings with intact roots (no trimming roots, NT) irrigated with full strength Ruakura nutrient solution. After 7 days from root trimming, root length was measured and new roots close to the shoot base were cut and frozen immediately in liquid nitrogen and then stored at -80°C . Some of these seedlings were transplanted into pots containing stainless steel mesh fixed at 10 cm from the pot top and the clipped roots were directly placed on this mesh. The new roots, re-grown throughout this mesh, were used in root angle measurements. Three to ten replicates were used for each parameter at each treatment.

2.5. Root angle measurements

As previously mentioned, plastic bags were cut and roots grown through the stainless steel mesh were put in trays filled with water and soil particles were gently removed from the roots. Root angles of the

crown roots in water were measured using a protractor on eight-score scales [20° (10°–20°), 30° (20°–30°), 40° (30°–40°), 50° (40°–50°), 60° (50°–60°), 70° (60°–70°), 80° (70°–80°), and 90° (80°–90°)]. Root angles were measured at each developmental stage and for the new roots after root trimming in the four genotypes. The number of roots at each scale was counted and calculated as a percentage of the total number of the roots for each plant from each genotype. Ten to twenty-five plants were used as replicates for each genotype at each developmental stage.

2.6. Quantification of the expression of genes controlling root traits

Total RNA was extracted from 50 mg frozen roots using IQeasy plus plant RNA extraction kit (Cat. No. 17491, iNtRON biotechnology, Korea) according to the manufacturer's protocol. cDNA was synthesized as previously described in Nada and Abogadallah (2014). Semi-quantitative RT-PCR was performed using primer sets (Table S1). PCR conditions were adjusted as follows: initial denaturation at 95 °C for 3 min followed by 36–45 cycles of denaturation at 95 °C for 30 s, annealing at 51–55 °C for 30 s and extension at 72 °C for 50–60 s and then final extension at 72 °C for 3 min. For each gene, the PCR conditions were adjusted to show the maximum differences among samples within the linear phase of the amplification. The conditions and the number of cycles for each gene (Table S1) were adjusted to avoid DNA saturation. The amplified DNA was resolved on 1% agarose gel stained with EtBr in 1X TAE buffer using BioRad equipment and visualized by a gel documentation system (UVi, Tec, Cambridge, UK). The volume of bands was measured by using Image Studio v3.1.4 software. The measurements were normalized for equal 18S rRNA. For each gene, three replicates were used.

2.7. Statistical analysis

All measurements were replicated as mentioned under each section. To compare among data, one-way and two-way ANOVA analyses with Fisher's Least Significant difference (LSD) post hoc test were performed by using Sigma Plot v11.0 at significant level $P \leq 0.05$.

3. Results

3.1. Root traits during the developmental stages

Root length (RL) was significantly higher in PM12 by about 28, 37 and 18% than that in Giza178, IR64 and moroberekan, respectively at 15 DAS. Thereafter, moroberekan possessed the highest RL at the following stages compared to the other genotypes. RL of moroberekan was 42, 25 and 17% higher than that of Giza178, PM12 and IR64, respectively at 60 DAS (Fig. 1G).

There were no significant differences in root fresh weight (RFW) and root dry weight (RDW) among the four genotypes either at 15 or 30 DAS. However, RFW increased in moroberekan by about 34% of that in PM12 at 60 DAS and RDW of moroberekan was higher by about 22 and 27% than that of PM12 at 45 and 60 DAS, respectively (Fig. 1H and I).

Root/shoot (R/S) ratio was significantly higher in Giza178 and PM12 by about 25–28% than that in IR64 and moroberekan at 15 DAS. This ratio became significantly similar in all genotypes at 30 DAS. Thereafter, R/S ratio was significantly higher in moroberekan by 18 and 23% than that in PM12 at 45 and 60 DAS, respectively and by about 32 and 38% than that in Giza178 and IR64 (had similar R/S ratio) at 45 and 60 DAS, respectively (Fig. 1J).

MANOVA results for RL ($F = 73.6$, $P < 0.001$), RFW ($F = 45.8$, $P < 0.01$), RDW ($F = 61.1$, $P < 0.01$) and R/S ($F = 32.3$, $P < 0.001$) showed that there were significant differences among the four genotypes at different developmental stages.

3.2. Characterization of root diameter and root angles in the four genotypes

Root diameter (RD, $F = 56.2$, $P < 0.01$) was measured at different distances from the root tip for the four genotypes (Fig. 1K). Moroberekan possessed the greatest RD (Fig. 3A and B for magnification) compared to the other genotypes at all distances except at 2 cm, at which RD of moroberekan was significantly similar to that of PM12 and higher than that of IR64 and Giza178. PM12 and IR64 had significantly similar RD at distance of 10, 14 and 18 cm from the root tip. Giza178 possessed the smallest RD compared to the other genotypes.

Root angle (RA, $F = 35.9$, $P < 0.01$) was measured at each scale and calculated as a percentage of the total number of roots per each replicate for each genotype. In Giza178, PM12 and IR64, the range of root angles was between 20° to 90°. The highest percentage of roots was detected at angles of 60°, 50° and 70° in Giza178, PM12 and IR64, respectively. Meanwhile, the range of moroberekan root angles was between 40° to 90° and the highest percentage of roots was detected at angles of 80°, 70° and 90°, respectively (Fig. 1L).

3.3. Root traits in response to soil water or nitrogen content

RL was significantly stimulated by drought in PM12, IR64 and moroberekan (by about 16, 22 and 24% increase of the corresponding control, respectively) (Fig. 2E). HN treatment significantly increased RL in moroberekan by about 13% of the control but it significantly reduced RL of Giza178 and PM12 compared to the corresponding control. RL was enhanced by LN treatment in PM12 and IR64 (14 and 21% increase of the corresponding control, respectively) but no significant change was detected in Giza178 or moroberekan compared to the corresponding control.

RFW was significantly reduced by drought in the four genotypes. However, moroberekan RFW was significantly similar to that of PM12 and they were 54 and 51% higher than those of Giza178 and IR64, respectively. RFW decreased at HN condition in Giza178 and PM12 by about 49 and 63% of the corresponding control and there was no significant change in RFW of IR64. Meanwhile, RFW of moroberekan was significantly enhanced (to 113%) compared to the corresponding control. At LN condition, RFW did not significantly change in PM12 but it increased significantly (to 116%) in IR64 compared to the corresponding control. However, RFW decreased significantly down to 72 and 59% of the control in Giza178 and moroberekan, respectively (Fig. 2F).

Root surface area (RSA) was significantly declined by drought in the four genotypes compared to the corresponding control (down to 63, 77, 78 and 69% of the control in Giza178, PM12, IR64 and moroberekan, respectively). At HN condition, RSA did not significantly change in IR64 and it showed 11% increase in moroberekan. Meanwhile, RSA decreased significantly in Giza178 and PM12 (down to about 63 and 62%, respectively) compared to the corresponding control. LN condition significantly reduced RSA of Giza178 and moroberekan by about 12 and 16% of the corresponding control, respectively whereas RSA increased significantly by 11% of the control in IR64 and no significant change was recorded for RSV of PM12 compared to the control (Fig. 2H).

Root volume (RV) was significantly lowered by drought in the four genotypes (down to 56, 86, 84 and 90% in Giza178, PM12, IR64 and moroberekan, respectively) compared to the corresponding control. Under HN condition, RV of moroberekan was significantly enhanced (up to 118%) but that of Giza178 and PM12 decreased significantly (down to 65 and 56%, respectively) of the corresponding control. Under LN condition, RV decreased significantly in Giza178 and moroberekan by 11 and 15% of the corresponding control, respectively but RV of PM12 and IR64 was higher by 11 and 27% than the corresponding control, respectively (Fig. 2I).

R/S ratio was significantly unchanged by drought in the four genotypes compared to the corresponding control. HN treatment did not

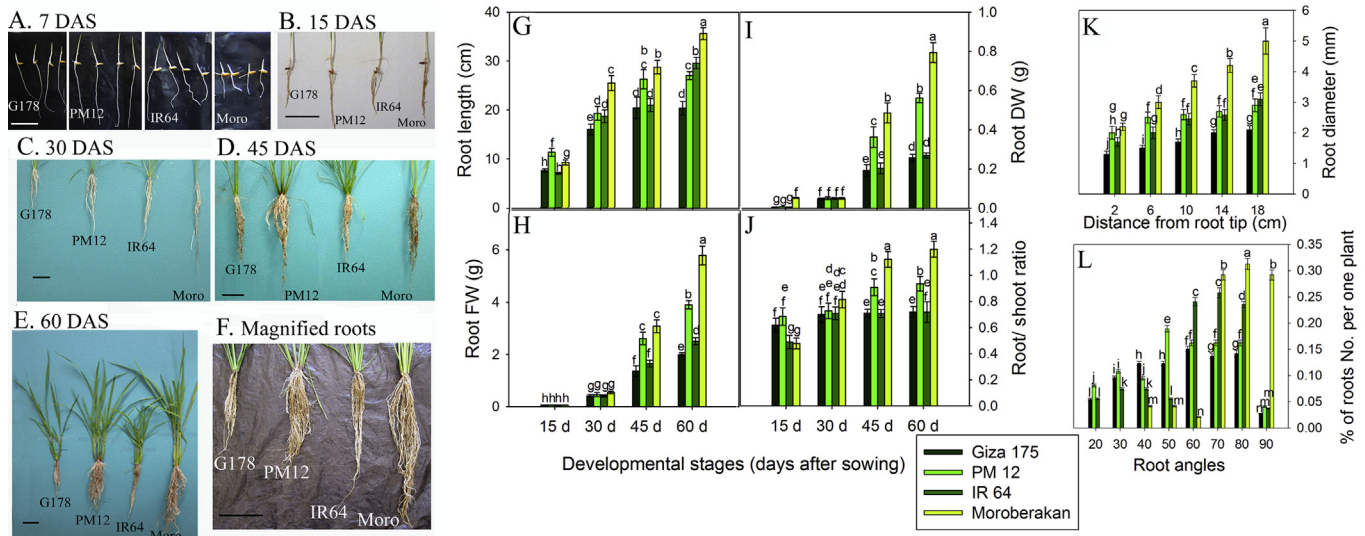


Fig. 1. The four rice genotypes at different developmental stages (days after sowing, DAS). A, B, C, D and E : Roots morphology at 7, 15, 30, 45 and 60 DAS, respectively. F: Magnified roots for the four genotypes at 60 DAS. G178: Giza178, PM12: PM12, IR64: IR64 and Moro: Moroberakan. G-L are root morphological characteristics for the four rice genotypes at different developmental stages. G: Root length (RL, cm), H: Root fresh weight (FW, g), I: Root dry weight (DW, g) and J: Root/Shoot ratio (R/S), K: Root diameter (RD, mm) from the root tip and L: Root angles (RA) as percentage of the total root number per plant. Data are means ($n = 10-25$ according to the measurement) $\pm$ SE. Data with different labels are significantly different at $P \leq 0.05$.

significantly affect this ratio in moroberakan compared to the control but R/S of Giza178, PM12 and IR64 was significantly lowered by 30, 31 and 25% of the corresponding control. At LN condition, R/S ratio did not significantly change in Giza178 but it increased significantly in PM12, IR64 and moroberakan to 113, 121 and 116% of the corresponding control, respectively (Fig. 2G).

Xylem sap flow under control condition was higher in moroberakan, PM12, IR64 and Giza178, respectively. Drought stress significantly

decreased xylem sap flow in the four genotypes with the maintenance of the same order among the four genotypes. At HN condition, xylem sap flow decreased significantly in Giza178 and PM12 (by about 19 and 27%, respectively of the corresponding control) whereas it increased significantly in moroberakan and IR64 (to 114 and 110% of the corresponding control). LN condition significantly enhanced the xylem sap flow in PM12 and IR64 (to 117 and 124% of the corresponding control, respectively) but it significantly reduced that in moroberakan (by about

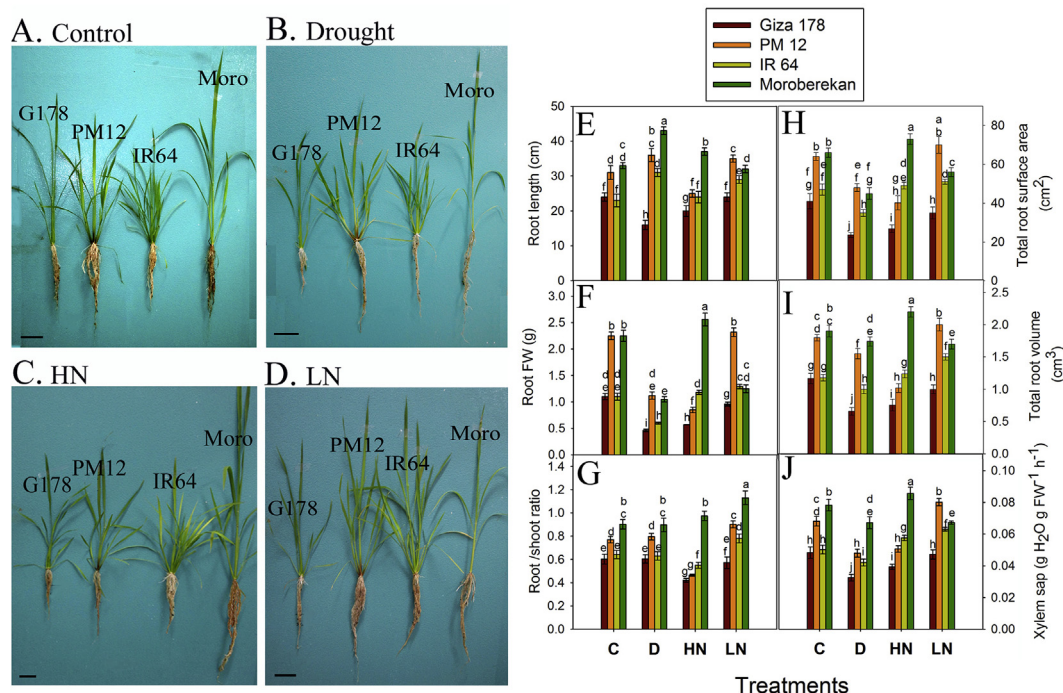


Fig. 2. The four rice genotypes at different growth conditions. Morphology of the four genotypes grown under A: control conditions (adequate water and nitrogen), B: 70% of the control field capacity (drought condition), C: high nitrogen condition (HN) and D: low nitrogen condition (LN). G178: Giza178, PM12: PM12, IR64: IR64 and Moro: Moroberakan. E – J are root traits of the four rice genotypes at different growth conditions. E: Root length (RL, cm), F: Root fresh weight (FW, g), G: Root/shoot ratio (R/S), H: Root total surface area (RSA, cm²), I: Root total volume (RV, cm³) and J: Xylem sap flow (g H₂O per g FW per hour). Data are means ($n = 10$) $\pm$ SE. Data with different labels are significantly different at $P \leq 0.05$.

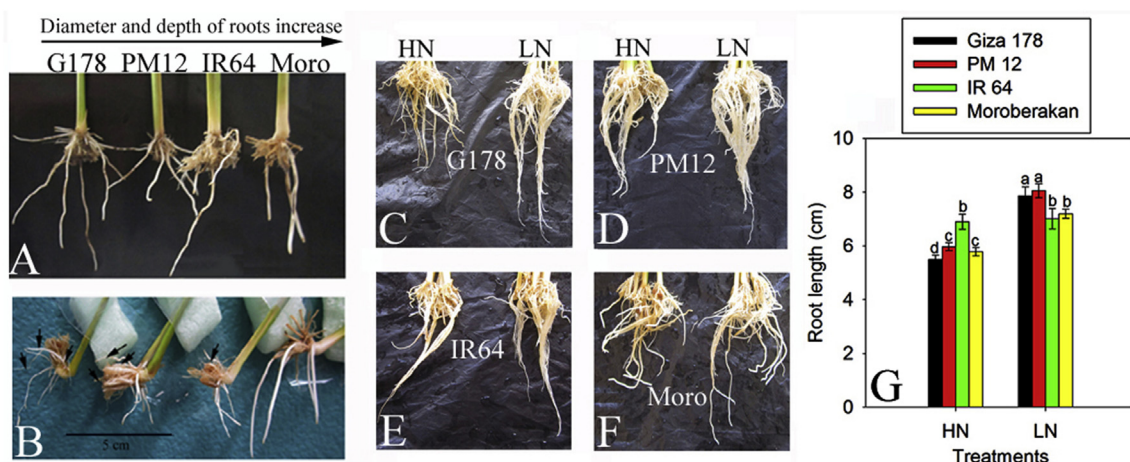


Fig. 3. A and B: Root traits of the four genotypes after root trimming. C–F: The regrowth of the new roots after root trimming in the four genotypes at high nitrogen (HN) and low nitrogen (LN) conditions. G: Root length (RL) of the new roots after root trimming in the four genotypes at HN and LN conditions. Data are means ($n = 10$) $\pm$ SE. Data with different labels are significantly different at $P \leq 0.05$. G178: Giza178, PM12: PM12, IR64: IR64 and Moro: Moroberakan.

15%) compared to the control (Fig. 2J).

MANOVA results for RL ($F = 27.9$, $P < 0.04$), RFW ($F = 44.2$, $P < 0.03$), RSA ($F = 37.9$, $P < 0.01$), RV ($F = 41.8$, $P < 0.03$), R/S ($F = 28.9$, $P < 0.04$) and xylem sap flow ($F = 26.9$, $P < 0.03$) showed that there were significant differences among the four genotypes in response to different treatments.

3.4. Characterization of root traits after root trimming

Roots of the four genotypes were clipped as mentioned in the Material and Methods section. The plants with clipped roots were transplanted under HN or LN condition (Fig. 3C–F). RL ($F = 69.1$, $P < 0.03$) was significantly higher at LN condition than at HN one (by about 27, 25 and 14% for Giza178, PM12 and moroberakan, respectively). RL of IR64 was significantly similar at HN and LN conditions and it was significantly higher than that of the other genotypes at HN condition (Fig. 3G).

3.5. Expression pattern of genes controlling root traits during the developmental stages

The transcript level of root genes at different developmental stages (15, 30, 45 and 60 DAS) was compared to that at 7 DAS for the four genotypes (Figs. 4 and 5, Table S2).

In Giza178: *CaMK*, *CRL1*, *CRL4*, *EXPA2*, *EXPB5*, *TUB6* and *WOX3* were upregulated at most stages. Meanwhile, *DRO1*, *FON1*, *GLR3.1*, *NAC5*, *PIN1* and *SHB* were downregulated at most stages.

In PM12: *CaMK*, *CRL1*, *CRL4*, *PIN1*, *SCR1*, *SHB*, *TUB6* and *WOX3* were upregulated at most stages. Meanwhile, *GRL3.1*, *FON1*, *NAC5* and *RH2* were downregulated at most stages.

In IR64: *ARF12*, *PIN1*, *SCR1* and *WOX3* were upregulated at most stages. Meanwhile, *ARL1*, *CRL1*, *EXPA2*, *FON1*, *GLR3.1*, *NAC5* and *RH2* were downregulated at most stages. For these three cultivars, the other studied genes showed no consistent pattern of regulation during the developmental stages. In moroberakan: all genes were upregulated at all stages except *NAC5*, which was downregulated at all stages and *GLR3.1* that had no consistent pattern of regulation.

3.6. Regulation pattern of genes controlling root traits in response to water or nitrogen content

The regulation pattern in the treated roots was compared to that in the control roots (Fig. 6, Table S3). The expression levels of *ARF12*, *CaMK*, *CRL1*, *DRO1*, *EXPA2*, *FON1*, *GLR3.1*, *NAC5*, *SCR1*, *SHB*, *TUB6*,

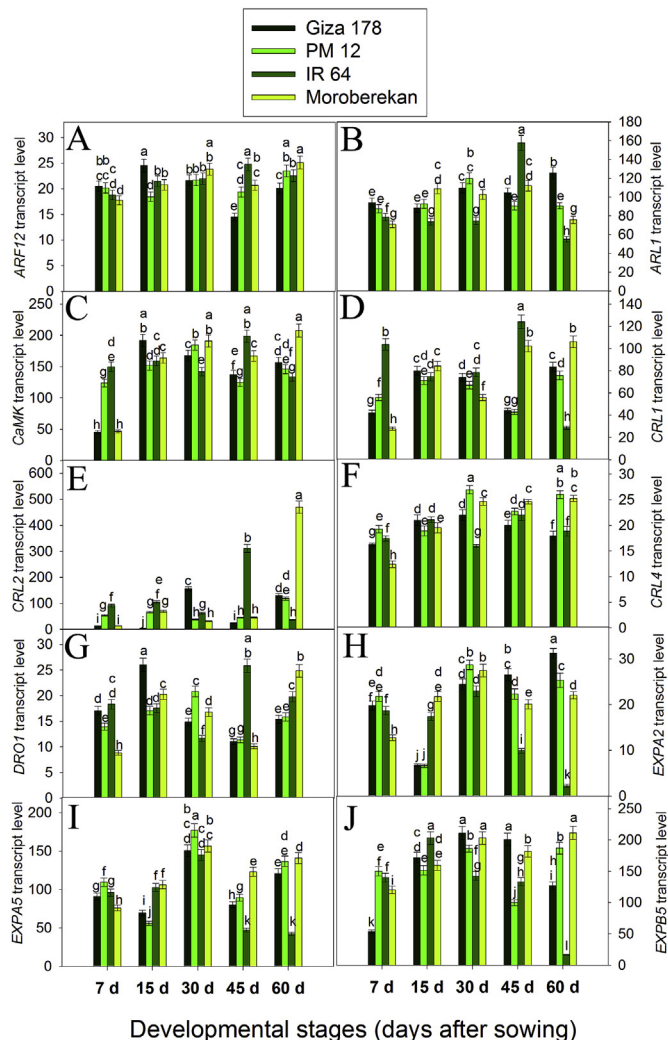


Fig. 4. The expression of genes controlling root traits at different developmental stages (DAS). A: *ARF12*, B: *ARL1*, C: *CaMK*, D: *CRL1*, E: *CRL2*, F: *CRL4*, G: *DRO1*, H: *EXPA2*, I: *EXPA5* and J: *EXPB5*. Semi-quantitative analysis was used and the bands detected are the PCR products at the non-saturated linear phase. Data are the product of the normalized bands with *18S rRNA*. Data are means $\pm$ SE ($n = 3$). Data labeled with different letters are significantly different at $P \leq 0.05$.

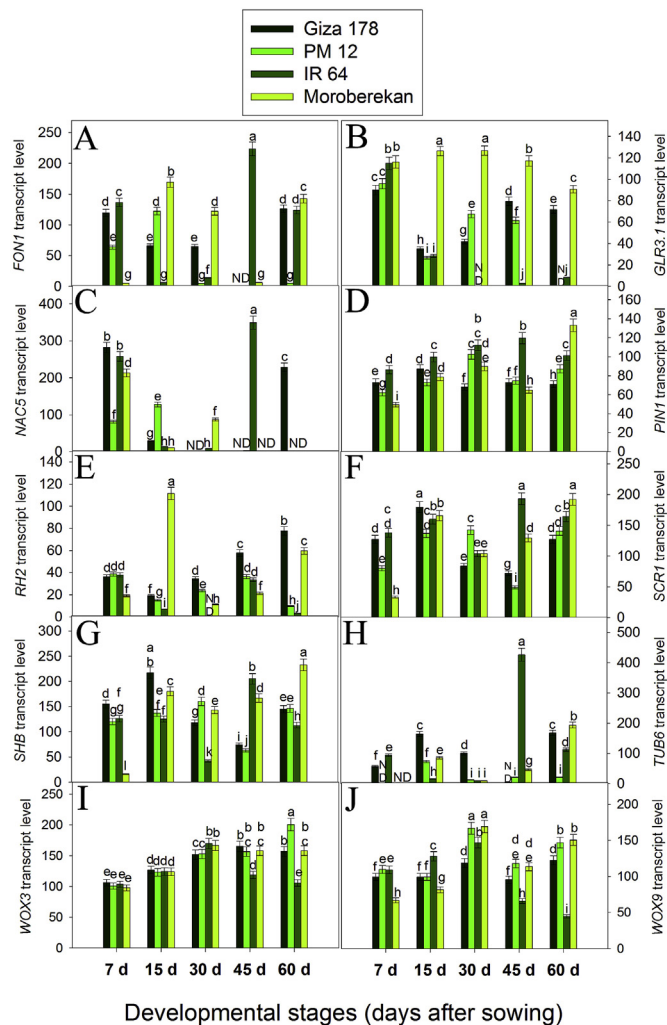


Fig. 5. The expression of genes controlling root traits at different developmental stages (DAS). A: *FON1*, B: *GRL3.1*, C: *NAC5*, D: *PIN1*, E: *RH2*, F: *SCR1*, G: *SHB*, H: *TUB6*, I: *WOX3* and J: *WOX9*. Semi-quantitative analysis was used and the bands detected are the PCR products at the non-saturated linear phase. Data are the product of the normalized bands with *18S rRNA*. Data are means $\pm$ SE ($n = 3$). Data labeled with different letters are significantly different at $P \leq 0.05$. ND: Non Detectable band.

WOX3, *WOX9* and *PIN1* were examined in this experiment for the four genotypes.

Under drought condition, the expression levels of *CRL1*, *SHB* and *TUB6* were significantly enhanced in Giza178. The transcript levels of *ARF12*, *CaMK*, *DRO1*, *GLR3.1* and *WOX9* were upregulated in PM12. The transcript levels of *ARF12*, *EXPA2* and *NAC5* were upregulated in IR64. *ARF12*, *FON1* and *PIN1* transcript levels increased significantly in moroberekan (Fig. 6A-N).

Under HN condition, the expression level of *CRL1* only was upregulated in Giza178. The transcript levels of *CaMK*, *DRO1* and *FON1* were significantly enhanced in PM12. The expression levels of *ARF12*, *GLR3.1*, *EXPA2*, *WOX3* and *NAC5* were upregulated in IR64. The transcript levels of *ARF12*, *FON1*, *EXPA2* and *PIN1* were upregulated in moroberekan (Fig. 6A-N).

Under LN condition, the transcript levels of *CRL1*, *GLR3.1*, *TUB6* and *PIN1* increased significantly in Giza178. The transcript levels of *ARF12*, *CaMK*, *DRO1*, *FON1*, *SCR1*, *NAC5* and *PIN1* were upregulated in PM12. The expression levels of *ARF12*, *GLR3.1* and *WOX3* were upregulated in IR64 and the transcript levels of *ARF12*, *FON1* and *PIN1* were significantly enhanced in moroberekan (Fig. 6A-N).

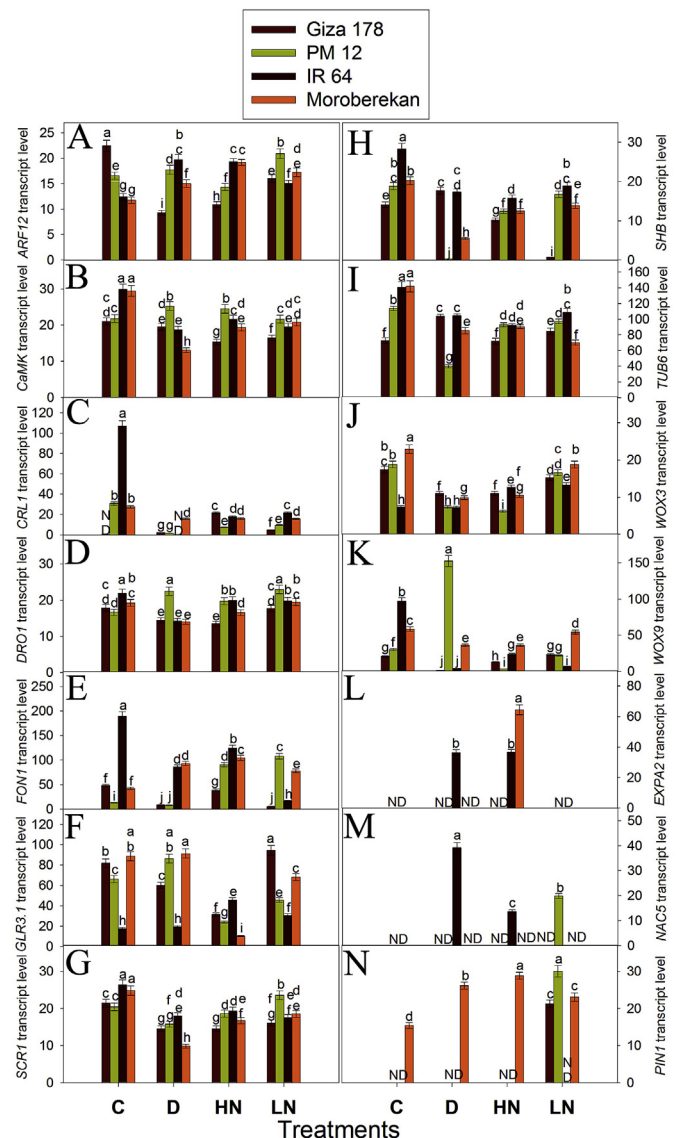


Fig. 6. The expression of genes controlling root traits at different growth conditions (control, drought and high and low nitrogen HN, LN, respectively). A: *ARF12*, B: *CaMK*, C: *CRL1*, D: *DRO1*, E: *FON1*, F: *GLR3.1*, G: *SCR1*, H: *SHB*, I: *TUB6*, J: *WOX3*, K: *WOX9*, L: *EXPA2*, M: *NAC5* and N: *PIN1*. Semi-quantitative analysis was used and the bands detected are the PCR products at the non-saturated linear phase. Data are the product of the normalized bands with *18S rRNA*. Data are means $\pm$ SE ($n = 3$). Data labeled with different letters are significantly different at $P \leq 0.05$. ND: Non Detectable band.

3.7. Expression pattern of genes controlling root traits in the new roots after root trimming

The expression levels of *ARF12*, *CaMK*, *CRL1*, *DRO1*, *FON1*, *GLR3.1*, *SCR1*, *SHB*, *TUB6*, *WOX3*, *WOX9*, *EXPA2*, *EXPB5* and *PIN1* were examined in this experiment for the four genotypes. The transcript levels in the new roots were compared to those in the control roots (no-trimming, NT, Fig. 7, Table S4).

Under drought condition, all genes were upregulated in Giza178. The transcript levels of these genes were significantly enhanced in the other three genotypes except *SCR1*, *TUB6* and *EXPA2* in PM12; *CRL1*, *SCR1*, *WOX9* and *EXPA2* in IR64 and *TUB6*, *WOX9* and *EXPA2* in moroberekan (Fig. 7A-N).

Under HN condition, the expression levels of all genes in the four genotypes were upregulated except *CaMK* and *EXPB5* in Giza178; *FON1*, *GLR3.1*, *SCR1* and *EXPB5* in PM12; *CaMK* in IR64 and *FON1*,

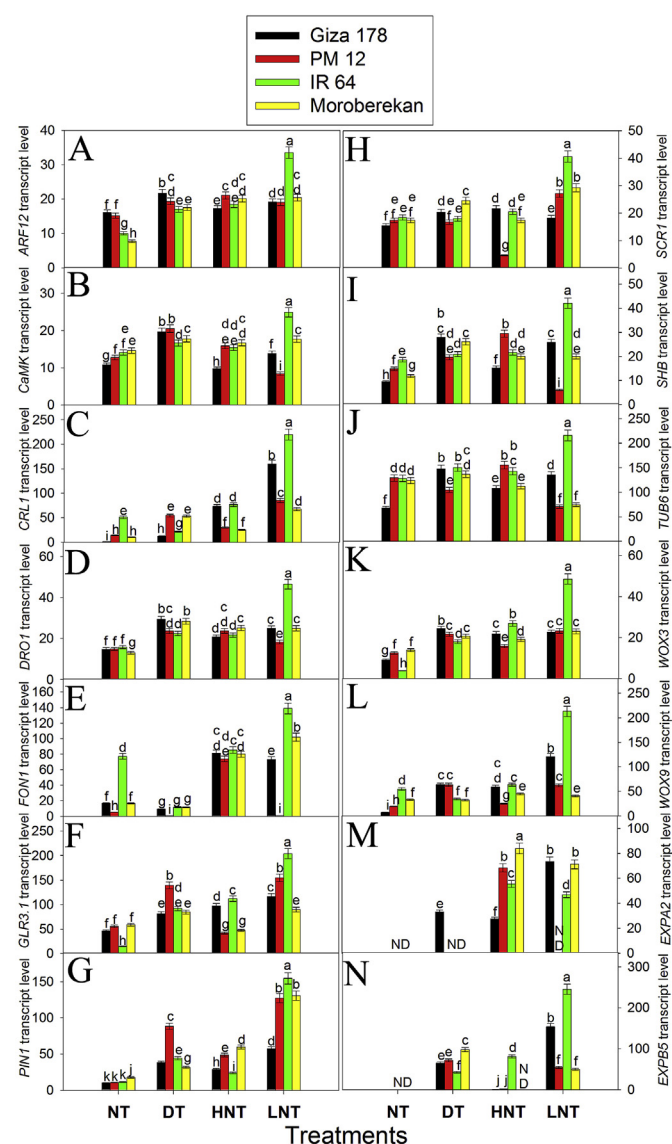


Fig. 7. The expression of genes controlling root traits at the new roots after root trimming at different growth conditions (no trimming, drought, HN and LN). A: *ARF12*, B: *CaMK*, C: *CRL1*, D: *DRO1*, E: *FON1*, F: *GLR3.1*, G: *PIN1*, H: *SCR1*, I: *SHB*, J: *TUB6*, K: *WOX3*, L: *WOX9*, M: *EXPA2* and N: *EXPB5*. Semi-quantitative analysis was used and the bands detected are the PCR products at the non-saturated linear phase. Data are the product of the normalized bands with *18S rRNA*. Data are means $\pm$ SE ($n = 3$). Data labeled with different letters are significantly different at $P \leq 0.05$. ND: Non Detectable band.

GLR3.1, *SCR1* and *EXPB5* in moroberekan (Fig. 7A–N).

Under LN condition, the transcript levels of all examined genes increased significantly in Giza178 and IR64. For PM12 and moroberekan, the transcript levels of all genes were upregulated except *CaMK*, *SHB*, *TUB6* and *EXPA2* in PM12 and *TUB6* in moroberekan (Fig. 7A–N).

4. Discussion

4.1. Effect of genes expression pattern on the root traits during the developmental stages

Although moroberekan RL was shorter at 15 DAS, it was significantly higher than the other genotypes at all the following stages. Roots of moroberekan extended to more than 30 cm depth at 60 DAS (Fig. 1G). Rice roots more than 30 cm depth were considered deep roots and were assumed to be effective for water and nutrient uptake under

drought conditions (e.g. Azhiri-Sigari et al., 2000; Yue et al., 2006). Moreover, moroberekan had higher percentage of deep roots (in the range of 70–90%) followed by IR64, PM12 and Giza178, respectively. RFW and RDW were also higher in moroberekan and then in PM12 than those of Giza178 and IR64 (Fig. 1H and I). Moroberekan was also characterized by high R/S ratio followed by PM12, Giza178 and IR64, respectively especially at 45 and 60 DAS (Fig. 1J). Nada and Abogadallah (2018) suggested that the high R/S ratio in moroberekan was not simply because of the root sink strength/activity but rather due to the restricted shoot sink size (few number of tillers). This is consistent with the present study where R/S ratio was lower at 15 and 30 DAS, when the growing shoot system was not restricted by sink size, than that at 45 and 60 DAS, when the shoot system reached just about the maximum growth potential. Increasing R/S ratio may improve plant growth and yield under conditions of water or nutrients shortage (Chang and Zhu, 2017). RD of moroberekan recorded the superior value over the other genotypes except at 2 cm (Fig. 1K). Increasing root diameter could improve the tolerance of plants to water shortage and consequently, higher yield (Jeong et al., 2013).

All the examined genes (except *NAC5* and *GLR3.1*, Table S2) were upregulated at all stages (compared to 7 DAS) in moroberekan. The consistent upregulation and perhaps the interaction of these genes particularly at 45 and 60 DAS could be responsible for moroberekan root traits. The overexpression of *NAC5* led to increasing root diameter in rice cultivar (Jeong et al., 2013). Although moroberekan roots were thicker than the other genotypes, *NAC5* expression was downregulated in moroberekan, suggesting that different gene/s other than *NAC5* is/are controlling root diameter trait in moroberekan.

PM12 is representing average traits between moroberekan in one hand and Giza178 and IR64 on the other hand. PM12 is an off type variant from Giza178 population with greater roots. The regulation of genes (Figs. 4 and 5, Table S2) at different developmental stages was quite similar in these two genotypes except for *PIN1*, which was upregulated at all stages in PM12. *PIN1* has been suggested to play an important role in auxin dependent adventitious roots and tillering (Xu et al., 2005). Moreover, *ARF12*, *SCR1* and *SHB* were upregulated at 60 DAS in PM12 contrasting that in Giza178. *ARF12* is contributed to the regulation of root elongation in rice (Qi et al., 2012). *OsSCR1* and *OsSHR1* (SHORT ROOT) are the key regulators of the quiescent cells maintenance and root radial patterning (Sabatini et al., 2003). *OsSCR1* and *OsSHR1* control the division of epidermis-endodermis initial cells in rice (Kitomi et al., 2018). Therefore, the difference of the root traits in PM12 over Giza178 (Fig. 1) could be attributed to the regulation of these genes.

Although there was inconsistent pattern for genes expression in IR64 during the developmental stages (Figs. 4 and 5, Table S2), *ARF12* and *PIN1* were upregulated at all stages, explaining that RL of IR64 was significantly higher than that of PM12 at 60 DAS. It seems that not the interaction among genes only is controlling the root traits but also the pattern of genes expression/regulation may be involved. Although *ARF12* and *PIN1* were simultaneously upregulated at 60 DAS, PM12 maintained similar RL at 45 and 60 DAS (Fig. 1), that perhaps due to the inconsistent regulation of *DRO1*, *ARF12* and *CRL2* (*CRL2* may play role in auxin signaling, Yamamoto et al., 2010) during all developmental stages. Furthermore, the increased RSA of PM12 compared to IR64 at 60 DAS may be attributed to the inconsistent regulation of *ARL1*, *CRL1* and *CRL4*, which play roles in crown root formation (Inukai et al., 2005; Kitomi et al., 2011) during the developmental stages of IR64. Samejima and Tsunematsu (2016) identified the deep-rooting rice accessions by those continued to develop deep roots even at 60 DAS and from our study, the acquisition of deep and thick roots (as in moroberekan) could require not only an interaction of different genes but also a consistent pattern of upregulated genes during the development of root system.

4.2. Root architecture and genes regulation in response to water or nitrogen content

Root architecture is genetically controlled as well as influenced by different environmental factors (Russell, 1977; Klepper, 1992; Merrill et al., 1996; Fageria, 2009). The question arises here how different soil resources (water or nitrogen content) may affect the regulation of genes controlling root traits in each genotype.

Rice is more sensitive to drought than other crops and that is due to inefficient roots and the shoots of rice can be as resilient to water deficit as those of other crops if deficiencies from roots are neutralized (Parent et al., 2010). Droughted Giza178 initiated the emergence of new roots via the upregulation of *CRL1*, *SHB* and *TUB6* (Table 1 for references) genes (Table S3 and Fig. 6), but drought did not stimulate genes controlling RL, RSA and RV (*ARF12* or *PIN1*), which led to that large decrease in RFW and xylem sap flow (Fig. 2). In PM12, drought stress induced the expression of *ARF12*, which controls the root elongation (Qi et al., 2012), *DRO1*, which plays a vital role in gravitropic curvature of roots (Arai-Sanoh et al., 2014), *GLR3.1* (Li et al., 2016) and *WOX9* (Cho et al., 2016), which both are involved in the maintenance of cell division of apical meristem of roots, resulting in increasing RL compared to the control (Fig. 2). IR64 responded to drought stress by up-regulating *ARF12* (Qi et al., 2012) and *NAC5* (Jeong et al., 2013) and *EXPA2* (Zou et al., 2015), which both can be contributed to increasing root diameter. Therefore, RL (Fig. 2) and RD (data not shown) of IR64 were significantly increased by drought compared to the control. Moreover, RL, RSA, RV, RFW and xylem sap flow were significantly increased in droughted IR64 compared to Giza178 despite possessing similar root traits under control condition (Fig. 2). Meanwhile, moroberekan under drought induced the transcript level of *ARF12* and *PIN1*; both are involved in the regulation of auxin transport and root elongation (Table 1 for references) and also *FON1* that is contributed to the regulation of the meristem development (Chu and Zhang, 2007). Therefore, moroberekan had higher RL and RV than PM12, although they possessed quite similar RSA and RFW (Fig. 2), resulting in the significant increase in xylem sap flow (Fig. 2J) compared to the other genotypes. Rice genotypes with larger root length and root surface area can explore lower soil surfaces for higher nutrient uptake and thereby improving the yield (Sadana et al., 2002, 2005). From these results, we can conclude that the plasticity of root system to cope with soil water shortage is dependent on rice genotype and *ARF12* and *PIN1* could play vital roles in improving root traits in response to drought.

Nitrogen is one of the most important elements that affect crop growth and production (Hakeem et al., 2011; Sutton and Bleeker, 2013). Moroberekan invested the available nitrogen mainly in the root system, emphasizing by the upregulation of genes (Table S3, Fig. 6) contributed to increasing RL, RSA, RV and RFW (Fig. 2) in response to HN condition compared to the control. However, LN condition enhanced the transcript level of *ARF12*, *FON1* and *PIN1*, maintaining RL similar to the control. In contrast to Nada and Abogadallah (2018), R/S of moroberekan at HN condition was lower than that at LN one, suggesting that moroberekan was not operating at the maximal shoot growth potential (moroberekan was younger than that in the previous study). Compared to the other genotypes at HN and LN, moroberekan had higher R/S, suggesting higher root-favored biomass allocation.

Giza178, PM12 and IR64 invested mainly the available nitrogen at HN condition in increasing the shoot biomass at the expense of root growth (Fig. 2) where their R/S ratio was higher at LN condition than that at HN one. The regulation of genes (Table S3, Fig. 6) and root traits (Fig. 2) of IR64 may indicate that some of the available resources (Nitrogen) could be invested in the root system as well as in the shoot one at HN condition but to lower extent if compared to moroberekan.

In response to HN treatment, Giza178, PM12 or IR64 enhanced the regulation of genes related to initiate new roots (*CRL1*, Table 1 for references), auxin transport and initiating meristem development (*ARF12*, *DRO1* and *FON1*, Table 1 for references) or related to auxin

transport, maintenance of cell division and increasing lateral roots and root diameter (*ARF12*, *GLR3.1*, *WOX3*, *EXPA2* and *NAC5*, Table 1 for references), respectively, but not those genes related to increase RL or RV, perhaps because the availability of nitrogen and water stimulated the investment in the shoot system rather than in the root one (low R/S ratio). LN condition enhanced the transcript levels of genes related to increasing RL, RSA, RV and RFW and thereby increasing R/S ratio. Plants increase their capacity to acquire nitrogen by stimulating root growth in relative to shoot one i.e. increasing R/S ratio (Giehl and von Wirén, 2014). Compared to the control condition and to the other genotypes, the concurrent upregulation of *ARF12* and *PIN1* has resulted in improving root morphological traits (Fig. 2) in PM12 in response to LN condition, suggesting their vital roles not only in response to drought but also to soil nitrogen deficiency.

4.3. Effect of root trimming on resources translocation and root genes regulation

From the previous results, the regulation of genes controlling root traits in response to drought or nitrogen content was genotype-dependent. The ability of roots to accumulate biomass was independent from the ability of shoot base to initiate new roots but rather was dependent on the ability of new roots to compete for resources with shoot system (Chang and Zhu, 2017). This translocation of resources from the shoot system to the root one could be affected by the available resources (water or nitrogen) and thereby operating the functional genes. Therefore, we found that RL was greater under LN condition than that under HN one (Fig. 3) after root trimming.

The expression profile of the genes was significantly changed in Giza178, PM12 and IR64 after root clipping in response to different conditions (Table S4, Fig. 7), which has resulted in new roots with different traits from the not-clipped roots (the previous experiment). Moroberekan maintained its expression profile and hence the root traits were maintained. The upregulation of all genes and the fast regrowth of new roots in Giza178 suggest that the growth of normal roots (Experiment 1 and 2) was restricted by root sink activity/strength in contrast to moroberekan that favored root resources allocation because of the limited sink size of the shoot system (few tillers, Champoux et al., 1995). The transcript level (Fig. 7) in the four genotypes after root trimming was dependent on water or nitrogen content in the growth medium, explaining the longer RL at LN condition (Fig. 3C–G). The transcript levels of all genes in PM12 at LN condition in the new roots were higher than those in the other genotypes (Fig. 7), suggesting that LN condition enhanced the resources translocation from the shoot to produce numerous and long roots (Fig. 3D) to cope with LN content and thereby maintaining the growth. Therefore, producing new roots was not dependent only on the ability of new roots to compete for resources with shoot system (dependent on genotype) but also on the availability of the surrounding nutrients (nitrogen in our experiment).

The simultaneous upregulation of *ARF12* and *PIN1* along with the traits of the new roots in Giza178 under different conditions (Fig. 7, Table S4) added more highlights on our suggestion that these two genes can play vital roles in regulating root traits during the developmental stages and in improving these traits in response to low water or nitrogen content. There is evidence that rice root traits are under complex or probably redundant gene networks and genetic loci (e.g. Li et al., 2015; Cheng et al., 2016). *ARF12* and *PIN1* regulate the transmitted auxin signal (Liscum and Reed, 2002) and polar auxin transport (Meng et al., 2019), respectively. Auxin signaling is important in overall root morphogenesis as suggested by Kitomi et al. (2018). It seems that these genes are involved in different routes in the molecular regulatory pathway of rice root development (Kitomi et al., 2018; Meng et al., 2019). Hierarchical analysis of the expression profile (Fig. 8A,B,C) shows that *PIN1* and *ARF12* can be found in two different, distinct clades and each of them is closely related to different genes controlling different root traits (for example, *ARF12* and *DRO1* - *PIN1* and *CRL1*).

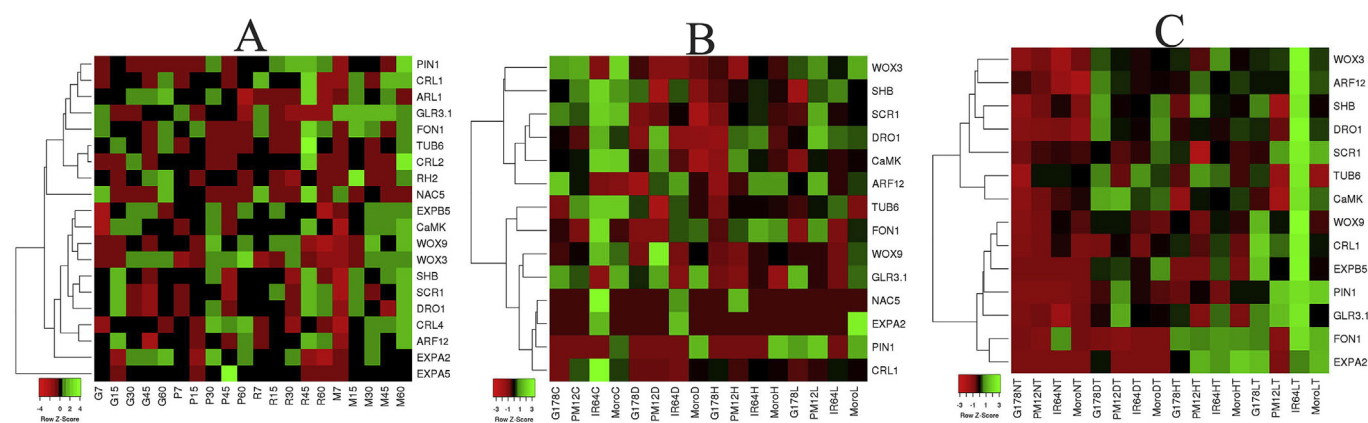


Fig. 8. Hierarchical analysis of expression profiles of genes controlling root traits at A: different developmental stages, B: different growth conditions and C: different growth conditions after root trimming. The signal value is log transformed and subjected to complete linkage hierarchical clustering with Heatmapper software: <http://www.heatmapper.ca/expression/>. G178: Giza178, PM12: PM12, IR64: IR64 and Moro: Moroberekan. Genotype name + 7, 15, 30, 45 or 60 numbers refer to DAS, respectively. Genotype name + C, D, H or L refers to rice genotype at control, drought, HN or LN condition, respectively. Genotype name + NT, DT, HT or LT refers to plants with no-trimmed roots, with trimmed roots at drought, with trimmed roots at HN or with trimmed roots at LN condition, respectively.

Accordingly, the concurrent and constitutive overexpression of *ARF12* and *PIN1* may improve root traits and hence the productivity and yield could be enhanced under different soil conditions.

In conclusion, the interaction of consistently upregulated root genes during all developmental stages in moroberekan but not in Giza178, PM12 or IR64 has resulted in deeper and thicker roots. Moreover, *ARF12* and *PIN1* could play key roles in improving root traits in response to different conditions (water or nitrogen content). Root plasticity was dependent on rice genotype (resources allocation/translocation and root sink activity and strength) and on the availability of water or nutrients in the growth medium. The concurrent and constitutive overexpression of *ARF12* and *PIN1* in other rice genotype (e.g. PM12) could improve root traits and thereby enhancing the growth and yield even under water or nitrogen shortage.

Author contributions

RM Nada and GA Abogadallah: designed the research and contributed to fieldwork.

RM Nada: supervised and contributed to lab work, collected the data, performed the data analysis and interpretation and wrote the manuscript.

EG Budran: contributed to the fieldwork and the research design.

SE Abo-Hegazy: performed the field and lab work and contributed to data collection and analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.05.018>.

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