TaMOR is essential for root initiation and improvement of root system architecture in wheat

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Summary
Optimal root system architecture is beneficial for water-fertilizer use efficiency, stress tolerance and yield improvement of crops. However, because of the complexity of root traits and difficulty in phenotyping deep roots, the study on mechanisms of root development is rarely reported in wheat (Triticum aestivum L.). In this study, we identified that the LBD (LATERAL ORGAN BOUNDARIES DOMAIN) gene TaMOR (MORE ROOT in wheat) determines wheat crown root initiation. The mor mutants exhibited less or even no crown root, dwarfism, less grain number and lodging caused by few roots. The observation of cross sections showed that crown root initiation is inhibited in the mor mutants. Molecular assays revealed that TaMOR interacts with the auxin response factor ARF5 to directly induce the expression of the auxin transporter gene PIN2 (PIN-FORMED 2) in the root base to regulate crown root initiation. In addition, a 159-bp MITE (miniature inverted-repeat transposable element) insertion causing DNA methylation and lower expression of TaMOR-B was identified in TaMOR-B promoter, which is associated with lower root dry weight and shorter plant height. The results bring new light into regulation mechanisms of crown root initiation and offer a new target for the improvement of root system architecture in wheat.

Introduction
Wheat (Triticum aestivum L.) is a staple food crop with deep root system. Root system is vital for plants to absorb water and nutrients from the soil, to perceive environmental stresses and anchor the plant in the soil. For monocots, the fibrous root system is composed of seed-borne seminal roots and stem-borne crown roots. Seminal roots (primary seminal root and lateral seminal roots) grow deeply and absorb water in the deep soil (Chen, 1989; Golan et al., 2018). Crown roots are the majority determining root surface area that is crucial for water and nutrients uptake (Comas et al., 2013; Li et al., 2021). Thus, uncovering the mechanisms of root initiation and identifying natural variation associated with root traits will contribute to improvement of root system architecture, abiotic stress tolerance and yield of wheat.

Crown roots are the main components of root system in monocots. In the model monocot Oryza sativa, a number of genes controlling crown root formation have been reported. CROWN ROOTLESS 1 (CRL1) is the first identified gene positively regulating crown root formation in rice. It encodes a plant-specific ASYMMETRIC LEAVES 2 (AS2)/LATERAL ORGAN BOUNDARIES (LOB) domain protein acting as a direct target of AUXIN RESPONSE FACTOR 1 (OsARF1) in the auxin signalling pathway (Inukai et al., 2005). The mutant of CRL1/ OsGNO1M1 exhibited few crown roots because of the altered expression of PIN-FORMEDs (OsPINs) and impaired polar auxin transport (Kitomi et al., 2008; Liu et al., 2009). The auxin-induced APETALA 2 (AP2)/ EHYLENE RESPONSIVE FACTOR (ERF) transcription factor gene CRL5 is required for crown root initiation by repression of cytokinin signalling (Kitomi et al., 2011). The WUSCHEL-related homeobox gene WOX11 activates crown root emergence through interaction with a transcription factor ERF3 and the ADA2-GCN5 histone acetyltransferase complex and enhances drought tolerance by modulating root system architecture (Zhao et al., 2009, 2015; Zhou et al., 2017). Rice CULLIN-ASSOCIATED AND NEDDYLYATION-DISSOCIATED 1 (OsCAND1) is involved in auxin signalling pathway that maintains the G2/M transition in the crown root primordia after fundamental organization formation (Wang et al., 2011). The chromodomain, helicase/ATPase and DNA-binding domain (CHD) protein CRL6 promotes crown roots formation by regulating expression of the above reported genes and OsIAA genes (Wang et al., 2016). Rice SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (OsSPL3/OsSPL12-OsMADS50 module represses crown root formation by regulating auxin signalling and transport (Shao et al., 2019). The NAM, ATAF1/2 and CUC2 (NAC) protein OsNAC2 acts as an integrator of auxin and cytokinin pathways to negatively control primary root length and crown root number (Mao et al., 2020).

Natural sequence variations are widely distributed among genomes of varieties. The sequence variations contributing to phenotypic differences are considered to be the driving force of crop evolution and breeding process. Transposable elements (TEs) are present in more than 80% of wheat genome (Clavijo et al., 2017). The miniature inverted-repeat transposable elements (MITEs) are short non-autonomous DNA transposons active in plants. They are found in promoters and other regulatory regions to influence gene expression. For instance, an 82-bp MITE in the promoter of ZmNAC111 is associated with drought tolerance by the suppression of ZmNAC111 expression through RNA-directed
DNA methylation (RdDM) (Mao et al., 2015). A stowaway-like MITE in the 3'-untranslated region of Ghd2 represses Ghd2 translation to modulate rice heading date (Shen et al., 2017). A miniature Jing (mjing) insertion in rice HIGH-TILLERING DWARF1 (OsHDT1) causes a premature stop codon and high-tillering dwarf phenotype (Tang et al., 2019). The tillering regulation factors OsMIR156d and OsMIR156j are repressed by MITEs in their promoters, and DWARF14 (D14) is activated by MITE in its downstream sequence (Xu et al., 2020). Therefore, the identification of MITE insertion related to target traits and its effects on gene expression will offer new strategies to crop breeding.

Because wheat roots grow deeply in the soil and identification of underground phenotypes is difficult, understanding of molecular mechanisms and natural variations related to crown root initiation are still limited. To investigate the initiation of crown root in wheat, we previously identified that the LBD gene from wheat, TaMOR, is induced by auxin treatment and ectopic expression in rice showed more crown root and higher yield (Li et al., 2016). In this work, we generated transgenic overexpression lines and mutants of TaMOR in a bread wheat cultivar Kenong 199 background. Phenotypic analyses showed that TaMOR is necessary for crown root initiation. We found that TaMOR interacts with auxin-responsive factor ARF5 to activate the expression of direct target PIN2. A 159-bp MITE insertion in the promoter of TaMOR-B causes DNA methylation and reduced expression of TaMOR-B, which leads to lower root dry weight and shorter plant height. These findings not only reveal the molecular mechanism of TaMOR regulating root initiation but also provide key insights into the genetic basis of natural variation associated with root traits and improvement of root system architecture in wheat breeding.

**Results**

**TaMOR is essential for wheat crown root initiation**

To examine the role of TaMOR in wheat root development, we generated mutants by CRISPR-Cas9 editing and overexpression lines in bread wheat cultivar Kenong 199 background. The expression level of TaMOR in two independent homozygous overexpression lines OE-1 and OE-2 was shown in Figure 1a. Because of the high similarity of TaMOR-A, TaMOR-B and TaMOR-D coding sequences (Li et al., 2016), we designed guide RNA spacer sequences editing TaMOR in three genomes simultaneously by CRISPR/Cas9 genome-editing technology. However, the mutants with TaMOR edited in all three genomes A, B and D only had a small primary seminal root and could not grow normally (Figure S1a). Thus, two mutants mor-5 and mor-6 with editing on TaMOR-A and TaMOR-D were selected for detailed analysis (Figure 1b and 1c). At the 2-week-old seeding stage, the total numbers of roots including seminal roots and crown roots in wild type (WT) and transgenic plants were recorded. There are often 5-6 seminal roots and 1-3 crown roots in the WT plants, which was similar with TaMOR overexpression lines, while mutants had only one or two seminal roots without crown roots. There was no significant difference in maximum root length among different wheat lines (Figure 1d and 1e).

We also investigated the root traits at juvenile stage in pipes and field. The phenotypes in the pipes showed that root number and root dry weight of OE-1 were higher than WT, whereas mutants only had one or two roots that much less than WT. In addition, root depth, root dry weight and shoot dry weight of mutants were lower than WT (Figure 2a, 2b, and 2d). Minirhizotron images in the field displayed that the root number of mutants distributed in soil was less than that of WT and overexpression lines (Figure 2c and Table S1). Because of the weak root systems, the mutants were prone to lodging at the later development stage (Figure 2b). In addition, plant height, spike length, tiller number and grain number per spike were decreased significantly in the mutants (Figure 2c). These results suggested that TaMOR plays crucial roles in the formation of lateral seminal roots and crown roots.

The development of crown root was proposed divided into seven stages from initial cells establishment to crown root emergence (Itoh et al., 2005). To test which stage TaMOR is involved in, we made cross sections of the stem base where crown roots occur. In the overexpression and WT plants, the primordia of crown roots formed near the peripheral vascular cylinder, but there was no crown root initiation in mor-6 (Figure 1f). The results suggested that TaMOR is essential for wheat crown root initiation.

**TaMOR interacts with an auxin response factor ARF5**

To study the function of TaMOR in the control of crown root initiation, we performed yeast two-hybrid screening assay. Previously, we found that full length of TaMOR has transactivation activity (Li et al., 2016). Truncated sequences of TaMOR were constructed into vector pGBK7 to detect their transactivation activity. The TaMOR210 (1-210 aa) fragment fused with binding domain (BD) did not show transactivation activity (Figure S2) and was used to screen the cDNA library prepared from wheat root and root base. As a result, an auxin response factor ARF5 (TraesCS6D02G100900) was identified. ARF5 is a protein with 102 aa only containing an AUX/IAA domain. To confirm their interaction in yeast, coding sequence of ARF5 was isolated to construct pGADT7-ARF5. The results showed that TaMOR interacts with ARF5 in yeast (Figure 3a).

To further confirm their interaction in vivo, we conducted luciferase complementation imaging (LCI), biomolecular fluorescence complementation (BIFC) and co-immunoprecipitation (Co-IP) assays in Nicotiana benthamiana leaves. In LCI assays, TaMOR was fused with N-terminal part of LUC (TaMOR-nLUC) and ARF5 was fused with C-terminal part of LUC (cLUC-ARF5). As shown in Figure 3b, strong luciferase activity was detected when TaMOR-nLUC and cLUC-ARF5 were co-expressed, whereas there was no signal in the control samples. In BIFC assays, yellow fluorescence signal in nucleus was observed when TaMOR fused with N-terminal element of YFP (TaMOR-YNE) and ARF5 fused with C-terminal element of YFP (ARF5-YCE) were co-expressed (Figure 3c). Finally, Co-IP assays showed that ARF5-Flag was co-immunoprecipitated by TaMOR-GFP, but not free GFP (Figure 3d). Together, the results demonstrated that TaMOR interacts with ARF5 in vitro and in vivo.

The expression pattern of ARF5 was analysed by qRT-PCR. At 2-week-old seedling stage, ARF5 was highly expressed in root base (Figure S3a). At juvenile stage, ARF5 was constitutive expressed with high levels in root, root base and leaf (Figure S3b). From germination to crown root formation, the expression of ARF5 increased gradually in root base (Figure S3c). To investigate the role of ARF5 in crown root formation, we generated ARF5 overexpression rice lines in the Kitaake background. Three T3 homologous overexpression lines were selected for phenotype
Expression levels of ARF5 were markedly increased in overexpression lines OE-10, OE-11 and OE-53 (Figure S3d). The root number of the overexpression lines was significantly more than wild type at seedling stage (Figure S3e) and booting stage (Figure S3f). The results suggested that ARF5 is involved in the crown root formation.

**Figure 1** Root phenotypes of TaMOR overexpression lines and mutants at seedling stage. (a) Expression of TaMOR in WT, overexpression lines OE-1 and OE-2. Values are means ± SD (n = 3). ***, P < 0.01. (b) Schematic diagram of TaMOR-A, -B and -D and the positions of two guide RNA targeting sequences S1 and S2. The gray boxes represent coding sequences. (c) Identification of TaMOR mutants mor-5 and mor-6 generated by CRISPR/Cas9 technology. The sequences of target and protospacer adjacent motif (PAM) are indicated. The dots represent deletion of corresponding sequences. (d) and (e) Root phenotypes of OE-1, OE-2, WT, mor-5 and mor-6 at 2-week-old seedling stage. Bars, 5 cm. Values are means ± SD (n = 30). ***, P < 0.01. (f) Cross sections of root base in 10-day-old OE-1, WT and mor-6 seedlings. Root base is the stem base where crown roots occur. The arrows indicate crown root primordia. Bars, 250 μm.

analysis. Expression levels of ARFS were markedly increased in overexpression lines OE-10, OE-11 and OE-53 (Figure S3d). The root number of the overexpression lines was significantly more than wild type at seedling stage (Figure S3e) and booting stage (Figure S3f). The results suggested that ARFS is involved in the crown root formation.
TaMOR regulates genes involved in root development

To explore genes regulated by TaMOR, we compared the transcriptomes of root bases from 2-week-old WT and mor-6. A total of 6976 genes were up-regulated and 8386 genes were down-regulated to more than twofold in mor-6 compared with WT (Figure 4a). Gene ontology analysis showed that the differential expressed genes were mainly enriched in photosynthesis, defense response, cell wall biogenesis and organization and metabolic process (Figure 4b), implying the role of TaMOR in these biological pathways. Auxin is the major phytohormone controlling root development. The differential expressed genes involved in the auxin signal pathway, auxin metabolism and transport and known genes regulating root development such as PLETHORA gene PLT1 (Aida et al., 2004) and EXPANSIN gene EXPB6 (Ramakrishna et al., 2019) were verified to be regulated by TaMOR (Figure 4c), indicating that TaMOR regulates root development through these genes. The expression levels of PLT1, EXPB6 and PIN2 were decreased severely in the mutant compared with WT, suggesting that they might be direct targets of TaMOR.

TaMOR recruits ARF5 to directly induce PIN2 expression

To confirm whether PLT1, EXPB6 and PIN2 were direct targets of TaMOR, we conducted yeast one-hybrid assays. TaMOR was fused into vector pB42AD to form AD-TaMOR. The promoter sequences of PLT1, EXPB6 and PIN2 were constructed into vector pLacZi (Figure 5a and Figure S4a. As shown in Figure 5b and Figure S4b, TaMOR bound to P1 of PIN2 promoter, but not promoters of PLT1 and EXPB6, suggesting that PIN2 is the direct target of TaMOR. To further investigate the binding region, P1 was divided into three fragments for yeast one-hybrid assays. TaMOR bound to P5, but not P3 and P4 (Figure 5b). We next designed biotin-labelled probes according to P5 sequence to perform electrophoretic mobility shift assay (EMSA). TaMOR bound to Probe 3 and the binding was suppressed by the competitor probe (Figure 5c), indicating that TaMOR specifically binds to the Probe 3 sequence in the promoter of PIN2.

To confirm that PIN2 acts downstream of TaMOR, we detect PIN2 expression in WT and mor-6 using RNA in situ hybridization. PIN2 was strongly expressed in the root primordium of WT, but

Figure 2 Root phenotypes of TaMOR overexpression lines and mutants at jointing stage. (a) and (b) Root phenotypes of OE-1, OE-2, WT, mor-5 and mor-6 grown in pipes at jointing stage. Bars, 20 cm. (c) Minirhizotron images of OE-1, OE-2, WT, mor-5 and mor-6 grown in field at jointing stage. a-h, soil layers of different depths. a, 0-14.1 cm; b, 14.1-28.3 cm; c, 28.3-42.4 cm; d, 42.4-56.6 cm; e, 56.6-70.7 cm; f, 70.7-84.8 cm; g, 84.8-99.0 cm; h, 99.0-113.2 cm. (d) Comparisons of root depth, root number, root dry weight and shoot dry weight. Values are means ± SD (n = 9). *, P < 0.05. **, P < 0.01.
the signal in mor-6 was very weak (Figure 5e), showing that PIN2 expression is dependent on TaMOR in the root base.

As the interaction protein of TaMOR, ARF5 could not bind to PIN2 promoter directly (Figure 5b). So we speculated that ARF5 was recruited by TaMOR and regulates PIN2 expression. To confirm the regulation of PIN2 expression by TaMOR and ARF5, we performed dual-luciferase reporter assays in N. benthamiana leaves. PIN2 promoter was fused with LUC gene as a reporter. TaMOR-GFP and ARF5-Flag were constructed as effectors. The LUC activity was significantly induced when TaMOR and ARF5 were co-expressed, whereas there was no difference of LUC activity compared with control when TaMOR or ARF5 was expressed alone (Figure 5d). These results suggested that TaMOR directly binds to PIN2 promoter and recruits ARF5 to induce PIN2 expression.

A MITE in the promoter of TaMOR-B is associated with root dry weight

To investigate the natural variations of TaMOR, we study the sequence polymorphisms of TaMOR from A, B and D genomes using a high polymorphic population containing 32 wheat accessions. The coding sequences of TaMORs were highly conservative (Li et al., 2016). Sequencing of over 1900 bp upstream from the start codon in the high polymorphic population revealed that a 159-bp insertion exists in the promoter of TaMOR-B, while no polymorphism was detected in TaMOR-A and TaMOR-D. The BLAST result showed that the insertion sequence is unique to wheat and distributed in multiple chromosomes. According to the 9 bp target site duplications (TSDs) in the insertion sequence, we inferred that it belongs to a Mutator-like MITE (Figure 6a). Primers MOR-BlfF and MOR-BlfR were designed to distinguish the natural variation in TaMOR-B promoter (MITE' or MITE) in a natural population of 323 wheat accessions. We next analysed the relationship between the MITE insertion and root traits of the natural population. There was a significant correlation between the insertion and root dry weight (RDW) at grain filling stage. The RDW of MITE' accessions was lower than MITE accessions (Figure 6b). In addition, the MITE insertion was associated with plant height (PH) under multiple conditions. The PH of MITE' accessions were lower than MITE accessions (Figure 6c). Thus, the MITE insertion in the promoter of TaMOR-B has effect on the development of under- and above-ground parts in wheat.

The MITE insertion leads to DNA methylation and lower TaMOR-B expression

To determine whether the MITE insertion in the promoter alters the expression of TaMOR-B, we detected the activities of TaMOR-B promoter with and without MITE insertion using the dual-luciferase reporter system. We found that the MITE insertion represses the promoter activity of TaMOR-B (Figure S5a). To examine the negative effect of the MITE insertion on TaMOR-B expression, transcription levels of TaMOR-B in different genotypes were analysed. We selected fifteen MITE' and fifteen MITE' accessions randomly from the wheat natural population. The TaMOR-B expression in MITE' accessions was significantly lower than that in MITE accessions (Figure 6d).

In view of the essential role of TaMOR in root initiation, to further confirm the repression of TaMOR expression and root number by the MITE insertion, we constructed a BC3F3 backcross population derived from a wheat cultivar with high root dry weight Yumai 18 (MITE' genotype) and a cultivar with low root dry weight Jingxuan 25 (MITE genotype). There were 5 MITE' genotypes, 152 MITE genotypes and 3 heterozygotes in the population (Figure 5b). TaMOR-B expression and root number at early tillering were lower in the MITE' lines and the parent Jingxuan 25 (Figure 6e and 6f).
DNA methylation is one of the reasons for the repressed expression of adjacent gene caused by MITE insertion (Castelletti et al., 2014; Mao et al., 2015). To determine whether the 159-bp MITE insertion is correlated with DNA methylation, we checked the DNA methylation status near the MITE of TaMOR-B promoter in the MITE+ and MITE- genotypes using bisulfite conversion method. The results showed that MITE and its nearby region were hypermethylated in the MITE+ genotype compared with the MITE- genotype (Figure 6g and 6h). Taken together, the MITE insertion in the TaMOR-B promoter causes DNA methylation and lower TaMOR-B expression.

The distribution of the natural variation of TaMOR-B in China

To explore breeding potential of the natural variation of TaMOR-B, we investigated the distribution of the natural variation in Chinese 10 major agro-ecological production zones of wheat using Population 1 with 157 landraces and Population 2 with 348 modern cultivars. In Population 1, MITE+ and MITE- genotypes account for 11.46% and 88.54%, respectively. In Population 2, MITE+ and MITE- genotypes account for 9.2% and 90.8%, respectively. Compared with Population 1, the distribution of MITE+ in Population 2 increased in seven wheat zones except zones VI, VIII and IX (Figure 7a and 7b). We also detected the frequency of the two genotypes released in different decades using the modern cultivars in Population 2 and the natural population. In Population 2, the frequency of MITE+ genotype increased from pre-1950 to 1990s (Figure 7c). In the natural population, from pre-1950 to 2010s, the frequency of MITE+ genotype also increased while the PH and RDW at filling stage decreased (Figure 7d and 7e). The results showed that the MITE+ genotype with short plant height has been positively selected during the modern breeding process. However, the frequency of MITE- genotype with high root dry weight decreased gradually.
Our findings suggest that the MITE genotype should be considered for the future improvement of wheat root system architecture.

Discussion

Root system architecture improvement is one of the wheat breeding targets because of its unique roles in water and nutrients uptake, stress signal perception and plant anchorage in the soil. However, due to lack of high-throughput method to investigate root phenotypes in the field, the molecular mechanisms and the natural variations associated with wheat root development are largely unknown. Here, we identified that TaMOR is essential for wheat seminal and crown root initiation. It interacts with the auxin response factor ARF5 and directly binds to PIN2 promoter to induce PIN2 expression in the root primordium. Furthermore, we found that a 159-bp MITE insertion in TaMOR-B promoter is associated with root dry weight and plant height. The MITE insertion results in the decrease of TaMOR-B expression and root number. Therefore, our findings not only reveal the molecular mechanism of TaMOR regulating root initiation but also provide new genetic basis for wheat root system architecture improvement in the future.

TaMOR controls crown root initiation by recruiting ARF5 to induce PIN2 expression directly

LBD proteins have been reported to be essential for the formation of lateral roots and crown roots in Arabidopsis, maize and rice. In Arabidopsis, LBD16, LBD18 and LBD29 are required for the lateral root formation downstream of ARF7 and ARF19 (Feng et al., 2012b; Lee et al., 2009; Okushima et al., 2007). Their mutants exhibited impaired pericycle cell division and decreased

Figure 5 TaMOR recruits ARF5 to induce PIN2 expression. (a) Schematic diagram of PIN2 and its promoter fragments used in yeast one-hybrid assays. (b) TaMOR binds to P5 fragment of PIN2 promoter in yeast. The fragments were constructed into plac2i vector. AD-TaMOR, TaMOR fused with pB42AD. AD-ARF5, ARF5 fused with pB42AD. AD, pB42AD used as negative control. Control, the empty plac2i vector. (c) EMSA showed TaMOR directly binds to Probe 3 of PIN2 promoter. Probe, biotin-labelled probes. Cold probe, unlabelled probe. +, absence of proteins or probes; +, presence of proteins or probes. 200×, the concentration of cold probe is 200 times of probe. (d) Induction of PIN2 expression by TaMOR-ARF5 verified by dual-luciferase reporter assays. 35S: REN-ProPIN2: LUC was reporter. 35S: ARF5-Flag and 35S: TaMOR-GFP were effectors. Empty vectors with free GFP and Flag were used as negative controls. Values are means ± SD (n = 3). *, P < 0.05. **, P < 0.01. (e) RNA in situ hybridization showed that PIN2 is expressed in root primordium of WT, but not expressed in mor-6. The sense probe of PIN2 was used as the negative control. Bars, 250 μm.
expression of PINs, PLTs and CYCDs which are instrumental for lateral root initiation (Feng et al., 2012a; Goh et al., 2012). LBD18 directly activate expression of E2Fa and EXP14 to regulate lateral root initiation and emergence (Berckmans et al., 2011; Lee et al., 2013). A pathogen-responsive protein PRH1 was found to be the target of ARFs and LBDs in the auxin-induced lateral root formation (Zhang et al., 2020). In maize, mutation of the LBD gene RTCS caused lack of seminal roots, crown roots and lodging resistance (Taramino et al., 2007). The homolog of LBD29 in rice, CRL1, acts downstream of ARF proteins to regulate crown root and lateral root formation (Inukai et al., 2005). The gene regulatory network dependent on CRL1 in crown root formation demonstrated that genes involved in root meristem specification and maintenance, cell proliferation, hormone homeostasis and transport are regulated by CRL1, which showed the conservation and specification between lateral root formation in Arabidopsis and crown root formation in rice (Coudert et al., 2011, 2015; Lavarenne et al., 2019).

However, in wheat, the knowledge regarding functions and molecular mechanisms of LBD proteins in root formation is still limited. Our study showed that mutation of TaMOR inhibits the formation of embryonical lateral seminal roots and post-embryonal crown roots (Figure 1 and 2). The TaMOR overexpression line OE-1 exhibited a slightly increased root number phenotype at jointing stage, and OE-2 has no significant difference with the wild type (Figure 2). It is consistent with the expression level of TaMOR in the overexpression lines (Figure 1a). The observation of cross sections illustrated that TaMOR is required for the initiation of crown roots (Figure 1f). In the previous studies, root phenotypes were often evaluated at jointing stage.
seedling stages under controlled conditions because it is difficult to investigate root phenotypes at the later stage of development in the field. Here, we used PVC pipes and minirhizotron images to investigate root phenotypes at different stages and confirmed the crucial function of TaMOR in the crown root initiation.

ARFs are key factors in auxin signal pathway and reported to be upstream of LBDs in the lateral root formation (Inukai et al., 2005; Lee et al., 2009; Okushima et al., 2007). In wheat, like Arabidopsis LBDs, TaMOR promoter is directly bound by ARF (Figure S6). Here, the cDNA library prepared from wheat roots and root bases was used for the yeast two-hybrid screening assay to explore the interacting proteins of TaMOR and their function in the crown root initiation. We found that TaMOR interacts with ARF in vivo and in vitro (Figure 3). ARF5 encodes a novel ARF protein that lack DNA-binding domain. Similar with TaMOR, ARF5 has positive effects on crown root formation (Figure S3). PINs are responsible for the polarized auxin transport and cell division during the initiation of lateral roots and crown roots (Benkova et al., 2003; Biliou et al., 2005; Xu et al., 2005). PLTs are key factors determining root stem cell specification and root development (Aida et al., 2004). Expansin proteins controlling cell expansion are involved in the initiation of lateral roots and crown roots (Kong et al., 2019; Ma et al., 2013; Ramakrishna et al., 2019). We found that the expression levels of PIN2, PLT1 and EXPB6 are significantly decreased when TaMOR is mutated, which indicates that TaMOR is essential for cell proliferation and auxin transport in the initiation of crown roots (Figure 4). PIN2 is the direct target of TaMOR, but TaMOR could not induce PIN2 expression in the absence of ARF5 demonstrated that TaMOR recruits ARF5 to induce PIN2 expression (Figure 5).

In the previous study, TaMOR was found to interact with a pentatricopeptide repeat (PPR) protein TaMRRP (Li et al., 2016). PPR proteins have been reported to participate in RNA metabolism and root growth. For example, the pentatricopeptide repeat proteins ABA overly-sensitive 5 (ABO5) and ABO8 are required for splicing of NAD2 intron 3 and NAD4 intron 3 in mitochondrial complex I, respectively. Their mutants exhibited more reactive oxygen species (ROS) accumulation in root tips and sensitive to ABA of root growth (Liu et al., 2010; Yang et al., 2014). The auxin transport, signalling, and genes PLT1 and PLT2 were repressed in the abo8 mutant (Yang et al., 2014). Therefore, in addition to the transcriptional regulation in the auxin signalling pathway, TaMOR may be involved in the posttranscriptional regulation and the crosstalk of phytohormone signals.

The MITE insertion in TaMOR-B promoter has negative effects on TaMOR-B expression and root development. MITEs are widely dispersed in eukaryotic genomes and essential for genome evolution and gene expression. Numerous MITEs are found within or close to gene coding regions in wheat (Keidar-
The knowledge about their impacts on neighbouring genes and contributes to important traits will be helpful to reveal the genetic basis and improve the related traits. A MITE in the Vrn-A1 promoter induces Vrn-A1 expression and promotes flowering without vernalization (Gorafi et al., 2016). A stowaway-like MITE, TaMITE81, in the 5’ UTR of a chalcone synthase gene TaCHS7BL negatively regulates TaCHS7BL expression (Xi et al., 2016). A 125-bp MITE inserted in the promoter of TaVSR1-B reduced TaVSR1-B expression and root depth (Wang et al., 2021).

Here, we found a novel Mutator-like MITE insertion in TaMOR-B promoter (Figure 6a). Mutator is a MITE superfamily with a small proportion in wheat genome. It is characterized by 100–700 bp sequence, 7–11 bp TSD and varying or undetectable TIR (Keidar-Friedman et al., 2018; Wicker et al., 2007). The MITE insertion in TaMOR-B promoter leads to lower root dry weight and plant height and reduced TaMOR-B expression in wheat (Figure 6b-f). It is reported that RdDM pathway is involved in the regulation of neighbouring gene expression by MITEs (Wei et al., 2014). Our study revealed that the MITE insertion in TaMOR-B promoter correlates with DNA methylation (Figure 6g and h). Therefore, the MITE insertion in TaMOR-B promoter represses TaMOR-B expression through DNA methylation, and then has negative effects on underground root development and aboveground plant development. A working model for the effect of MITE insertion in TaMOR-B and regulation of crown root initiation by TaMOR is proposed in Figure S7.

The natural variation of TaMOR-B provides insights into root system architecture improvement

Since the middle of the 20th century, the ‘Green Revolution’ has resulted in reduced plant height and increased yield (Khush, 1999). The goal of wheat breeding has always been to focus on the population performance, such as aboveground shoot architecture and yield, but the group selection for high yield under the suitable water and fertile soil conditions ignored root traits, leading to the smaller root systems with fewer root number (Fradgley et al., 2020; Zhu et al., 2019). Because of the indispensable role of roots in absorbing soil water and nutrients, cultivars bred under sufficient water and fertilizer conditions will have the risk of significant yield reduction due to their weak root system under the condition of limited resources. Meanwhile, the second ‘Green Revolution’ has been proposed to increase yield with less fertilization, in which the root traits should be the main target of improvement (Lynch, 2007). However, although root traits are important for wheat breeding, the study on root system architecture improvement is still limited.

Natural variations in germplasm resources are the genetic basis of crop breeding. In the present, we found the MITE’ genotype of TaMOR-B with lower PH and RDW has been positively selected in the most of Chinese major wheat zones, which is consistent with the changing trends of plant height and root number during wheat breeding (Figure 7). However, in the Northwestern Spring Wheat Zone (VIII) and Qinghai-Tibetan Plateau Spring-Winter Wheat Zone (IX) where are dry and water-scarce, the MITE’ genotype has been positively selected, implying that the natural variation of TaMOR-B may have effects on drought tolerance.

Overall, our findings demonstrated that TaMOR is an essential gene for root initiation, and two variants of TaMOR-B, MITE’ and MITE’, are selection targets for the improvement of wheat root system architecture and plant height, respectively. It provides new insights into wheat breeding for simultaneous improvement of aboveground and underground traits.

Methods

Plant materials and growth conditions

The wheat cultivar Kenong 199 was used for transgene of TaMOR. For root phenotypic observation of two-week old seedlings, plants were hydroponically cultured in light incubator under controlled long-day conditions (16 h light at 23 °C and 8 h darkness at 20 °C). For root phenotypic observation at jointing stage, plants were grown in polyvinyl chloride (PVC) pipes and field where translucent pipes were embedded under natural long-day conditions (Beijing) (Li et al., 2019b). The planting and scanning methods of minirhizotrons referred to Li et al., 2019b. Eight images were scanned in each minirhizotron pipe for analysis at jointing stage. Total root number in every image was measured using WinRHIZO Tron MF image analysis software. For transcriptomic analysis, mor-6 and Kenong 199 were hydroponically cultured for two weeks and root bases (the stem bases where crown roots occur) were sampled. The backcross population was grown in a container under natural long-day conditions to early tillering and roots were washed to count root number.

The japonica rice cultivar Kitaake was used for transgene of ARFS. For root phenotypic observation of two-week old rice seedlings, plants were hydroponically cultured in light incubator under controlled long-day conditions (16 h light at 30 °C and 8 h darkness at 25 °C). For root phenotypic observation at booting stage, plants were grown under natural long-day conditions (Beijing) in the container.

A panel of 32 highly polymorphic wheat accessions screened using SSR markers (list in Table S2) was used for nucleotide polymorphism analysis. The natural population with 323 wheat accessions mainly collected from the Northern Winter Wheat Zone and Yellow and Huai River Valleys Facultative Wheat Zone of China was used for association analysis and the frequency of two genotypes released in different years. Population 1 with 157 landraces and Population 2 with 348 modern cultivars collected from ten major wheat zones of China were used to analyse the distribution of the genotypes. The accessions in the three populations refer to Wang et al. (Wang et al., 2019).

The natural population was planted for three years (2015–2017) at Shunyi (40°23′N, 116°56′E) and Changping (40°13′N, 116°13′E) in Beijing to record agronomic traits. The drought stress (DS) environment was not irrigated during the three whole growing seasons but had rainfalls of 173 mm, 143 mm and 116 mm, respectively. The well-watered (WW) environment was irrigated with 750 m3/ha water at pre-overwintering, jointing, flowering and grain filling when the amounts of rainfall were insufficient. The heat stress (HS) environment was covered by a polythene film at the one-week post-anthesis stage. The methods and effects of the stress treatments refer to Li et al. (Li et al., 2019a). For analysis of root dry weight, the natural population was grown in PVC pipes, and roots were washed, sampled, dried and weighed at grain filling stage.

A backcross population (BC3F3) with 160 lines was constructed to confirm the effects of TaMOR-B alleles. The population was generated from a recurrent parent Yumai 18 and a donor parent Jingxuan 25, through three randomly backcross and three self-cross. The genotypes of TaMOR-B in the backcross lines were analysed using primers MOR-BIFn and MOR-BIRn. The MITE’ and MITE’ genotypes were grown in the soil in plastic container. Root
number was counted at tillering stage after root washing, and gene expression in two genotypes was detected using sampled root bases.

Generation of transgenic plants

To obtain TaMOR overexpression lines, coding sequence of TaMOR was amplified and cloned into the binary vector pCAMBIA1390 to generate the pUbi: TaMOR construct. The construct was transformed into wheat cultivar Kenong 199 by physical biolistic particle approach. Homozygous lines were selected and used for phenotypic observation.

To generate TaMOR mutants, four primers containing two 19-bp gene-specific sequences of TaMOR-A, -B and -D were designed to clone the fragment of pCBC-MT1T2 to the CRISPR/Cas9 vector pBUE414 with two guide RNAs targeting TaMOR-A and -B. The construct was introduced into Agrobacterium tumefaciens strain EHA105 and transformed into wheat cultivar Kenong 199. Transgenic lines were analysed by PCR and sequencing. Homozygous transgenic lines were selected and used for phenotypic observation.

To overexpress ARF5 in rice, coding sequence of ARF5 was amplified and cloned into the binary vector pCAMBIA1390 to generate the pUbi: ARF5 construct. The construct was introduced into Agrobacterium tumefaciens strain EHA105 and transformed into rice cultivar Kitaake. Homozygous lines were selected and used for phenotypic observation. Primers are listed in Table S3.

Transactivation activity assay

Full-length and fragments encoding N-terminal 108, 144, 200, 210 and 220 amino acids of TaMOR were cloned into pGBK7 to construct TaMOR-BD, TaMOR108-BD, TaMOR144-BD, TaMOR200-BD, TaMOR210-BD and TaMOR220-BD. They were co-transformed with pGADT7 into the yeast strain Y2HGold, respectively. The yeast transformed with empty pGBK7 and pGADT7 vector was used as negative control. The transformed yeasts were incubated on SD/-Leu-Trp (SD/-LW) and SD/-Leu-Trp-His-Ade (SD/-LWHA) medium at 30 °C. Primers are listed in Table S3.

Yeast two-hybrid assay

For yeast two-hybrid screening assay, TaMOR210-BD was used as bait. The cDNA library from root and root base of Kenong 199 at jointing stage fused with pGADT7 was used as prey. Mating procedure was performed with the yeast strain Y2HGold transformed with the bait TaMOR210-BD and the yeast strain Y187 transformed with the prey library. The positive clones were identified by PCR with primers T7 and 3' AD and sequencing.

For yeast two-hybrid assay, CDS of ARF5 was cloned into pGADT7 to construct ARF5-BD and co-transformed with TaMOR210-BD into the yeast strain Y2HGold. Empty vectors pGBK7 and pGADT7 were used as negative controls. Primers are listed in Table S3.

Luciferase complementation imaging assay

The coding sequence of TaMOR was fused with the N-terminal fragment of luciferase (nLUC) to form TaMOR-nLUC. The coding sequence of ARF5 was fused with the C-terminal fragment of luciferase (cLUC) to form cLUC-ARF5. The constructs were introduced into the Agrobacterium tumefaciens strain EHA105. Transformed cells were resuspended in induction buffer (10 mM MES [pH 5.7], 10 mM MgCl2 and 0.2 mM AS) and incubated for 2 h. The about 28-day-old tobacco (Nicotiana benthamiana) leaves were infiltrated with mixed suspension of nLUC and cLUC, TaMOR-nLUC and cLUC, nLUC and cLUC-ARF5, TaMOR-nLUC and cLUC-ARF5, respectively. After 48 h, transfected leaves were sprayed with the substrate (10 mM luciferin), and then incubated in darkness for 5 min and photographed using CCD imaging apparatus (NightShade LB 985, Berthold).

Bimolecular fluorescence complementation assay

The coding sequence of TaMOR was fused with the N-terminal fragment of YFP (YNE). The coding sequence of ARF5 was fused with the C-terminal fragment of YFP (YCE). The constructs were introduced into Agrobacterium tumefaciens strain EHA105 and infiltrated into N. benthamiana leaves. pCAMBIA2300-H2B-mCherry was co-infiltrated as a nuclear marker. Transfected leaves were detected by laser confocal microscopy (LSM880, Zeiss) after 48 h.

Co-immunoprecipitation assay (Co-IP)

The coding sequence of TaMOR was cloned into vector pCAMBIA1300-GFP to form TaMOR-GFP. The coding sequence of ARF5 was cloned into vector to pCAMBIA1300-Flag to form ARF5-Flag. They were introduced into Agrobacterium tumefaciens strain EHA105 and infiltrated into N. benthamiana leaves. After 48 h, the infiltrated leaves were ground to powder in liquid nitrogen and resuspended in 500 μl protein extraction buffer. The Co-IP assay was performed as previously described (Cai et al., 2021). Anti-GFP mAb magnetic beads (Medical Biological Laboratories, D153-11), Anti-GFP (Medical Biological Laboratories, 598-7) and anti-Flag (Medical Biological Laboratories, M185-7) antibodies were used in the assay.

Transcriptomic analysis

For RNA-seq analysis, root bases were sampled from two-week-old seedlings of WT and mor-6. Total RNA was isolated using RNA Extraction Kit (Tianmo Biotech, TR205). The RNA quantification, qualification, library preparation and 150-bp paired-end sequencing were performed at Novogene (Beijing). Differential expression analysis of genes in WT and mor-6 was performed using the DESeq2R package (1.20.0). Genes with P value < 0.05 were assigned as differentially expressed. Gene ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package. GO terms with P value < 0.05 were considered significantly enriched by differential expressed genes.

Quantitative real-time PCR analysis

Total RNA was extracted using an RNA Extraction Kit (Tianmo Biotech, TR205) and reverse transcribed using FastKing RT Kit (TIANGEN, KR116). qRT-PCR analyses were performed in an LightCycler 96 System (Roche) with the TB Green Premix Ex Taq (TaKaRa, RR420). The wheat TUBULIN (TUB) gene and rice ACTIN (ACT) gene were used as an internal control. Primers are listed in Table S3.

Yeast one-hybrid assay

To generate ARF5, TaMOR and ARF1, the coding sequences of TaMOR and ARF1 were cloned into the pB42AD vector. To generate ProPIN2::LacZ, ProPLT1::LacZ, ProEXPB6::LacZ and ProTaMOR::LacZ, fragments of PIN2, PLT1, EXPB6 and TaMOR promoter were cloned into the vector pLacZ. They were co-transformed into the yeast strain EGY48. Transformants were incubated on SD-Trp-Ura medium for 3 days and then
transferred onto SD/-Trp-Ura medium plus X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for β-galactosidase assays.

Electrophoretic mobility shift assay (EMSA)

The coding sequence of TaMOR was cloned into vector pMAL-c2x to construct MBP-TaMOR. MBP-TaMOR and the empty MBP vector pMAL-c2x were separately transformed into the E. coli expression strain Rosetta (DE3). The proteins were induced by 0.1 mM IPTG at 30 °C. The induced proteins were purified with amylose resin (New England Biolabs, E8022) and eluted by 10 mM maltose. The biotin-labeled probes were synthesized by BGI. The assay was performed using the LightShift Chemiluminescent EMSA Kit (Thermo, 20148). Primers and probes are listed in Table S3.

RNA in situ hybridization

Root bases of 10-day-old seedlings were sampled and fixed with FAA (Formalin-acetic acid–alcohol) solution at 4 °C overnight. Then they were dehydrated in 70%, 80%, 90% ethanol and 3 times in 100% ethanol and cleared in 25% xylene+75% ethanol, 50% xylene+50% ethanol, 75% xylene+25% ethanol and 3 times in 100% xylene. The samples were embedded in paraffin (Paraplast Plus, Sigma), and cross sectioned using automated microtome (RM2255, Leica). The slides were dried at 42 °C for 4 days.

For observation of cross section, the slides were dewaxed with 100% xylene for 2 times, 70% xylene+30% ethanol, 30% xylene+70% ethanol, 100% ethanol, 70% ethanol, 50% ethanol, 30% ethanol and water and then dyed with toluidine blue. For RNA in situ hybridization, the sections were prepared with RNase-free water. The assay was performed as described previously (Chen et al., 2015). Primers used for probes are listed in Table S3.

Dual luciferase reporter assay

TaMOR-GFP and ARF5-Flag were used as effectors. The promoter sequence of PIN2 was cloned into pGreenII 0800-LUC vector to construct the reporter 3SS: REN-ProPIN2: LUC. pcAMBIA1300-GPF and pCAMBIA1300-Flag were used as vector control. They were introduced into Agrobacterium tumefaciens strain GV3101 and infiltrated into N. benthamiana leaves. The activities of LUC and REN were detected using Dual-Glo Luciferase Assay System (Promega, E2920) 2 days after infiltration. The ratio of LUC activity/REN activity was analysed as relative luciferase activity.

Association analysis

The promoter sequences of TaMOR-A, -B and -D in the highly polymorphic population were sequenced by specific primers. Genotypes of the natural population were examined by primers MOR-BlnF and MOR-BlnR. The associaton analysis was performed using general linear model (GLM) in software TASSEL 5. Significant association was at the level of P < 0.05. The student’s-t test was used to determine the significance of phenotypic differences between two genotypes. Primers are listed in Table S3.

Bisulfite conversion assay

The DNA methylation level near MITE in TaMOR-B promoter was detected by bisulfite conversion method. Genomic DNA was extracted from the root bases of MITE⁺ and MITE⁻ genotypes in the backcross population. Bisulfite treatment was performed on 500 ng of genomic DNA using the EZ DNA Methylation-Gold kit (Zymo Research, D5005). The converted DNA was amplified by PCR with specific primers (Table S3). The PCR products were cloned into the pEASY-Blunt cloning vector (Transgen, CB101) for sequencing with primer M13FR. More than ten positive clones of each genotype were sequenced.

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Conflicts of interest statement

The authors declare no conflicts of interest.

Author contributions

R. J. and X. M. supervised the project. C. L. and R.J. designed the research and wrote the paper. C. L. performed most of the experiments. J. Y. W. and L. L. analysed the data. J. L., M. Z., B. L., Q. L., J. H., Y. D., J. P. W. and Z. F. helped with the preparation of plant materials and gene expression analysis.

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**Supporting information**

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

**Figure S1** The phenotypes of *TaMOR* overexpression lines and mutants.

**Figure S2** Transactivation activity of *TaMOR*.

**Figure S3** *ARF5* is involved in the crown root formation.

**Figure S4** *TaMOR* does not bind to *PLT1* and *EXPB6* promoter.

**Figure S5** The MITE insertion in *TaMOR* promoter.

**Figure S6** *TaARF1* directly binds to *TaMOR* promoter.

**Figure S7** A proposed model of the molecular mechanism of the root number reduction by MITE insertion in *TaMOR-B* promoter, namely a model of effect of MITE insertion on *TaMOR-B* expression and the regulation of crown root initiation by *TaMOR*.

**Table S1** Total number of roots in each minirhizotron image.

**Table S2** Accession names of the high polymorphic population.

**Table S3** Primers and probes in this study.