Biochemical Genetics (2019) 57:403–420
https://doi.org/10.1007/s10528-018-9896-z

ORIGINAL ARTICLE

Identification and Characterization of a Novel Strigolactone-Insensitive Mutant, *Dwarfism with High Tillering Ability 34* (*dhta-34*) in Rice (*Oryza sativa* L.)

Rong Liang, et al. ***[full author details at the end of the article]***

Received: 26 May 2017 / Accepted: 4 December 2018 / Published online: 1 January 2019

Abstract

Rice tillering ability and plant height are two of the important traits determining the grain yield. A novel rice (*Oryza sativa* L.) mutant *dhta-34* from an *Indica* cultivar Zhenong 34 treated by ethyl methy1 sulfonate (EMS) was investigated in this study. The *dhta-34* mutant significantly revealed thrifty tillers with reduced plant height, smaller panicles and lighter grains. It also exhibited late-maturing (19.80 days later than the wild type) and withered leaf tip during the mature stage. The length of each internode was reduced compared to the wild type, belonging to the dn type (each internode of the plant stem decreased in the same ratio). The longitudinal section of *dhta-34* internodes showed that the length of cells was reduced leading to the dwarfism of the mutant. The F_2 population derived from a cross between *dhta-34* and an *Japonica* cultivar Zhenongda 104 were used for gene mapping by using the map-based cloning strategy. The gene *DHTA-34* was fine mapped in 183.8kb region flanked by markers 3R-7 and 3R-10. The cloning and sequencing of the target region from the mutant revealed that there was a substitution of G to A in the second exon of *LOC_Os03g10620*, which resulted in an amino acid substitution arginine to histidine. *DHTA-34* encoded a protein of the α/β-fold hydrolase superfamily, which could suppress the tillering ability of rice. *DHTA-34* was a strong loss-of-function allele of the *Arabidopsis thaliana D14* gene, which was involved in part of strigolactones (SLs) perception and signaling. Moreover, the relative expression of *DHTA-34* gene in leaf was higher than that in bud, internode, root or sheath. This study revealed that *DHTA-34* played an important role in inhabiting tiller development in rice and further identifying the function of *D14*.

Keywords *DHTA-34* (*dwarfism with high tillering ability 34*) · Strigolactones (SLs) · Fine mapping · Dwarfism and high tillering ability · Rice

---

Rong Liang and Ran Qin contributed equally to this study and share the first authorship.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10528-018-9896-z) contains supplementary material, which is available to authorized users.

Abbreviations

dhta-34	*Dwarfism with high tillering ability 34*
EMS	Ethyl methyl sulfonate
FAA	Formalin-acetic acid-alcohol
SLs	Strigolactones

Introduction

Rice tillering ability is an important agronomic trait, as tiller number per plant determines the panicle number, which is a key component of rice grain yield (Liang et al. 2014). Tillers development includes the initiation from axillary buds at the phase of vegetative growth and branching of fluorescence that determines the number of spikelets born on each tiller (Wang and Li 2008). Remarkable progress has been made in understanding the molecular basis of the control for shoot branching through studies of a set of highly branched mutants isolated from sorghum (*Sorghum Bicolor*), pea (*Pisum sativum*), *Arabidopsis* (*Arabidopsis thaliana*) and *petunia hybrida* (Kim 2010; Braun 2012; Ongaro and Leyser 2008; McSteen 2009; Beveridge and Kyozuka 2010). Previous study showed that more tillers in rice (Booker et al. 2005) could lead to dwarfism (Wang and Li 2008). According to the ratio of internode length to plant height, these dwarf mutants are classified into six groups including dn, dm, d6, nl, sh, and dm types. The length of different internodes for dn type could be proportionally reduced, the dm-type mutants showed a specific shortening in the second internode from the top (internodes mentioned below all were counted from the top), the second and third internodes were shortened at the same time for d6 type, while nl type had the characters of the second internode shortened and the forth internode elongation, and the first internode of sh type was almost not be found in the stem (Takeda 1997).

The gene identified as a key regulator controlling rice tiller and branching was *MOC1* (*MONOCULM 1*) (Li et al. 2003), an ortholog of lateral suppressor (*LS*/*LAS*) gene from tomato (*Solanum lycopersicum*) (Schumacher et al. 1999) and *Arabidopsis thaliana* (Greb et al. 2003). *MOC1* encoded a putative GRAS family nuclear protein which was mainly expressed in the axillary buds and functioned to initiate axillary buds and to promote their outgrowth at both vegetative and reproductive stages. The rice mutant *moc1* almost completely lost the tillering ability, and had a monoculm characteristic, which showed that *MOC1* positively regulating rice tillers. Rice *OsTB1* was first identified based on maize (*Zea mays*) homolog *TB1* (*TEOSINTE BRANCHED1*), which was involved in lateral branching in maize. Expression of *TB1* homolog *BRC1*/*TBL1* in *Arabidopsis* hormone signaling mutants suggested that *TB1* acted downstream of the auxin and *MAX* (*MORE AUXILIARY GROWTH*) pathways (Takeda et al. 2003; Doebley et al. 1995). According to the phenotypes of *OsTB1*, loss-of-function mutation of *OsTB1* exhibited enhanced lateral branching and finally it negatively regulated lateral branching in rice (Kulkarni et al. 2014).

Among the four *Arabidopsis MAX* genes, *MAX1*, *MAX3* and *MAX4* acted in hormone synthesis, and *MAX2* acted in perception (Booker et al. 2005). Mutations at the *MAX1-MAX4*, *RMS1-RMS5*, and *DAD1-DAD3* loci of *Arabidopsis*, pea and petunia, respectively, increased shoot branching phenotypes (Stirnberg et al. 2002; Turnbull et al. 2002; Sorefan et al. 2003; Booker et al. 2004; Beveridge 2000; Beveridge et al. 2003; Snowden and Napoli 2003). In addition, *D3*, *HTD1* and *D10* in rice were orthologous to *Arabidopsis MAX2*, *MAX3* and *MAX4* genes, respectively (Ishikawa et al. 2005; Zou et al. 2006; Arite et al. 2007). The mutations of these rice and *Arabidopsis* genes displayed similar phenotypes of high tillering and dwarfism. In fact, this hormone was belonging to strigolactone or the downstream product of strigolactone. *D14* was reported to work in the strigolactones (SLs) signaling pathways, which could inhibit rice tillering ability and propose to act as SLs receptor (Florian et al. 2014). From a series of rice dwarf mutants conferred with increased number of tillers, the corresponding genes were identified: *D3* (Ishikawa et al. 2005), *D17* (Booker et al. 2004), *D10* (Arite et al. 2007), *D14* (Arite et al. 2009), *D27* (Lin et al. 2009), and *D53* (Zhou et al. 2013). These genes had been suggested to be involved in the regulation of axillary bud outgrowth for tillering in rice. In fact, some dwarf genes including *D10*, *D27*, and *D17* functioned in SLs biosynthesis, and others including *D3*, *D53*, and *D14* were involved in SLs perception and signaling (Jiang et al. 2013). The results in recent researches showed that *D53*, which encoded a substrate of the SCF^{D3} ubiquitination complex and functions, worked as a repressor of SLs signaling (Zhou et al. 2013). It showed that *D14* was a receptor of SLs which also probably had other roles in growth and development including seed germination, root growth, flower development, and leaf senescence (Bradow et al. 1988, 1990; Snowden et al. 2005; Woo et al. 2001; Yan et al. 2007). Their gene products could play important roles in germination and early development of *Arabidopsis* (Mashiguchi et al. 2009). The mutants with the recessive traits in rice were defective in these genes which had lead to reduced statures and increased numbers of tillers (Ishikawa et al. 2005).

In present study, a new dwarf mutant *dhta-34* with high tillering ability was identified, which showed reduced sensitivity to strigolactones and accumulated tillering number. The genetic and molecular analyses indicated that the loss of function of the *LOC_Os03g10620* gene by a single base substitution was responsible for the mutant phenotype. Positional cloning revealed that *DHTA-34* was found to be allelic to *D14*, encoding a protein of the α/β-hydrolase superfamily (Chevalier et al. 2014). We proposed that *dhta-34* was a novel mutant to help us understand in the strigolactone pathway and functions at a late step of active hormone synthesis or in its signaling pathway further.

Materials and Methods

Plant Materials and Growth Conditions

The rice (*Oryza sativa* L.) mutant with dwarfism and high tillering ability, *dhta-34*, was isolated from *Indica* cultivar Zhenong 34 treated with ethyl methy1 sulfonate (EMS), which Zhenong 34 showed normal plant height and tillering number. F_2 segregating population for gene mapping were derived from *dhta-34* crossed with a flat leaf *Japonica* cultivar Zhenongda 104, which is from different background from

Zhenong 34 but had normal height and tillering number. Rice plants were grown in the paddy field in Hangzhou of Zhejiang Province and Lingshui in Hainan Province, China, from March of 2015 to July of 2015, where the average temperature and the average relative humidity were 25 °C and 75%, respectively. We planted the rice plants from December of 2015 to April of 2016 in Lingshui of Hainan Province, China, where the average temperature and the average relative humidity were 20 °C and 80%, respectively.

Agronomic Trait Statistics

The tillering number was counted, and plant heights were measured from the base of the shoot to the tip of the longest leaf before heading and from the base of the shoot to the panicle tip in the longest stem at mature stage. Ten rice plants of the wild type (WT) and mutant *dhta-34* were sampled to calculate the number of empty grains and filled grains per plant, respectively. For getting the seed setting rates per plant, the number of filled grains was divided by the total grains including the number of empty grains and filled grains. The length of different internodes, length of panicles and grains, and 1000 seeds weight were also measured in laboratory.

Observation of Cytological Structure

The second internode from the top of the wild type and mutant plants as well as the flag leaves at the heading stage were put into FAA fixation (70% (*v*/*v*) ethanol:40% (*v*/*v*) formaldehyde:acetic acid =18:1:1 (*v*/*v*/*v*)) for at least 24 h at 4 °C, dehydrated in a graded ethanol series, completely transparentized with xylene and then embedded in paraffin. Slices of 4 µm thick were cut out from the embedded materials using a microtome (Type: LEICA RM2016), and stained with 0.5% toluidine blue solution. Finally, the sections were fixed in neutral resins and observed under a microscope (Type: NIKON DS-U3). By moving the slides, cytological structure was recorded.

Map-Based Cloning of *DHTA-34* Gene

For map-based cloning of the *DHTA-34* gene, the mapping population consisted of 534 F_2 individuals with the mutant phenotypes generated from a cross between mutant *dhta-34* and *Japonica* Zhenongda 104. The rice genomic DNA extraction was performed as described (Kang et al. 2004). To fine map the target gene, more InDel markers were developed by online tool primerblast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) or Primer 5.0 software. The primer sequences of these InDel markers used were listed in Table 1. The genetic linkage between the *DHTA-34* locus and molecular markers was determined by using Mapmaker 3.0. The entire genomic region spanning target region was amplified for the mutants and their corresponding wild-type plants by PCR, which was performed as follows: denaturation at 94 °C for 5 min, followed by 31 cycles of

Table 1 Primer sequences of quantitative real-time PCR analysis

Markers	Forward primer	Reverse primer
HTD2	GGTTCTTGAACGACAGCGACTA	CGTCGAACACCTGCTGTATCTC
HTD1	ACCTCGTCCAGAAGCGTGAGT	AGGCCCAGTCGTGGATCA
D10	GGCTTCCGGCACCTGTTCG	CGCCTTGTACGCCTCCGACTC
D3	AAGCCGGTTTATCCAATTCC	GCACCAAGAATCGTCTGGAT
D53	GAGGAGGATAGGAAACCTGTGCC	GTCTCCTTTCACTGCTGGTAC
D27	TCTGGGCTAAAGAATGAAAAGGA	AGAGCTTGGGTCACAATCTCG
Actin	CTTCATAGGAATGGAAGCTGCGGGTA	CGACCACCTTGATCTTCATGCTGCTA

94 °C for 30 s, annealing for 30 s (annealing temperature determined by primer pair sequences), 72 °C for 30 s, and with a final extension step at 72 °C for 10 min. The PCR products were separated on 6% polyacrylamide gels. After electrophoresis, the amplified DNA bands were detected using the silver staining.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from leaves, internodes, sheaths, roots and buds of wild-type Zhenong 34 and *dhta-34* mutant using a Trizol RNA mini-kit following the manufacturer's protocol (Roche, Mannheim, Germany). For RT-PCR, the first strand cDNA was transcribed reversibly from total RNA with random primers as the primer using SuperScript II (Invitrogen) and used as the template for real-time PCR.

Primers for *HTD*2 (gene ID: *LOC_Os03g10620*), *HTD1* (*LOC_Os04g46470*), *D10* (*LOC_Os01g54270*), *D3* (*LOC_Os06g06050*), *D53* (*LOC_Os11g01330*), *D27* (*Os11g0587000*) and Actin (*Os03g0718150*), were listed in the Table 1. The real-time PCR was conducted in a volume of 20 µl with 0.8 µl of each primers (10 µM), 10 µl 2 × SYBR *Premix Ex Taq* II (TAKARA), 1.6 µl cDNA (12.5 ng/µl), and 6.8 µl dH_2O. Every volume of 20 µl repeated three times in three different wells of the 96-well plates. Procedure for the real-time PCR amplification was 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s with 40 cycles, followed by the generation of a dissociation curve to check for specificity of amplification. For each sample, real-time PCR was repeated three times for independent biological replication. Quantification cycles were determined by LightCycler 480 II (Roche, Sweden).

The expression levels of genes *HTD*2, *HTD1*, *D10*, *D3*, *D53*, and *D27* were calculated relative to the reference gene (Actin) following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical significance of differences between means was determined by Student *t*-test.

Hormone Treatments

Strigolactones (SLs) treatments were performed between two groups of Zhenong 34 and mutant *dhta-34*. Rice seeds were sterilized in a 10% (*v*/*v*) sodium hypochlorite solution for 45 min. After washed with deionized water for three times, sterilized rice seeds were put on a piece of gauze soaked in water at 28 °C in the dark for 2 d. GR24 (dissolved in 95% acetone) was added to the rice Yoshida nutrient solution for preparing the solutions, whose concentrations were 0 μmol/L, 10^{-2} μmol/L, 10^{-1} μmol/L and 1 μmol/L finally. The germinated seeds of wild type Zhenong 34 and mutant *dhta-34* were placed in these four treatments, respectively. The shoot branching was observed and photographed after two weeks. One group of rice with shooting branching was transferred to Yoshida nutrient solution (Yoshida et al., 1976) with 0.5 mM GR24 (CX23880, Chiralix) and another was treated without GR24. And both of these two groups were grown in the growth chamber under a 16-h light (28 °C)/8-h dark (25 °C) cycle for 3 weeks and the Yoshida nutrient solution was replaced every two days.

Homology Analysis and Phylogenetic Tree of *DHTA-34* Homologs

For homology analysis, alignment of DHTA-34 homologs was carried out by ClustalW. The gene sequences of *Arabidopsis thaliana* (Gene ID: *AT3G03990*), *Sorghum bicolor* (*Sb01g043630*), *Vitis vinifera* (*VIT_18s0001g09140*), *Zea mays* (*Zm00001d028094*), *Brachypodium distachyon* (*BRADI1G70930*) and *Theobroma cacao* (*TCM_034711*) were downloaded from Rice Genome Annotation Project (https://rice.plantbiology.msu.edu/).

In order to conduct the phylogenetic analysis of *DHTA-34* gene family, predicted full-length amino acid sequences were used to generate the tree by the maximum likelihood method in MEGA V6.0 in bootstrap mode with 100 replications.

Results

Characterization of *dhta-34* Mutant

The rice mutant *dhta-34* was isolated from EMS treated mutagenized population of Zhenong 34. Compared to the wild type, mutant *dhta-34* exhibited significantly increased tillering number, reduced plant height and panicle length (Fig. 1; Table 2). Morphological analysis showed that all internodes of mutant *dhta*-34 were decreased. In addition, compared to the wild type, plant height, seed setting rate and panicle length of mutant *dhta-34* decreased, but the tillering number increased dramatically.

Genetic Analysis

The experimental design for genetic analysis of mutant used to test F_2 population in paddy field was conducted in Hangzhou of Zhejiang Province in 2016. There were

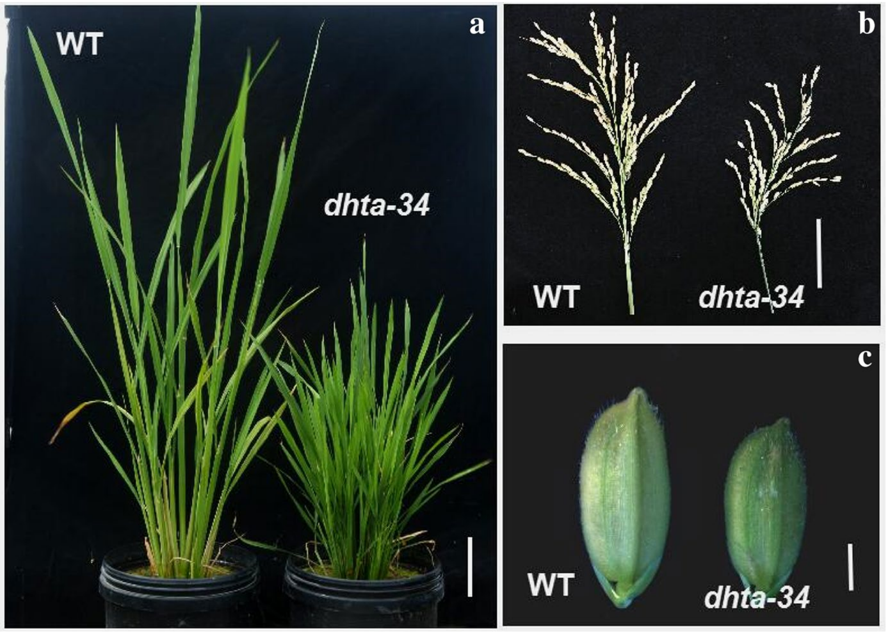


Fig. 1 Phenotypes comparison of WT and mutant *dhta-34*. **a** plants in tillering stage, bar = 10 cm; **b** panicles in mature stage, bar = 5 cm; **c** grains in later stage, bar = 2 mm

Table 2 Comparison of agronomic traits between wild type and *dhta-34*

Trait	WT	*dhta-34*
Plant height (cm)	92.88 ± 3.35	62.1 ± 1.95**
Panicle length (cm)	18.8 ± 0.94	14.1 ± 1.42**
First internode length from the top (cm)	32.83 ± 3.48	24.02 ± 2.39**
Second internode length from the top (cm)	20.85 ± 1.24	11.88 ± 1.14**
Third internode length from the top (cm)	8.59 ± 2.45	4.35 ± 1.98**
Fourth internode length from the top (cm)	2.98 ± 1.98	1.93 ± 0.59
Tiller number (no.)	8.25 ± 3.30	53.35 ± 6.76**
Heading time (day)	103.01 ± 2.11	122.99 ± 1.20**
1000 seeds weight (g)	25.29 ± 0.20	21.89 ± 0.20**
Seed-setting rate (%)	91.04 ± 0.82	84.11 ± 9.83*

* and ** indicate significant difference at the 0.05 and 0.01 probability level, respectively

425 rice plants with normal traits and 143 rice plants with dwarfism and high tillers in F_2 and the segregation ratio of F_2 population was 3:1 ($\chi^2 = 0.094 < \chi^2_{0.05} = 3.84$).

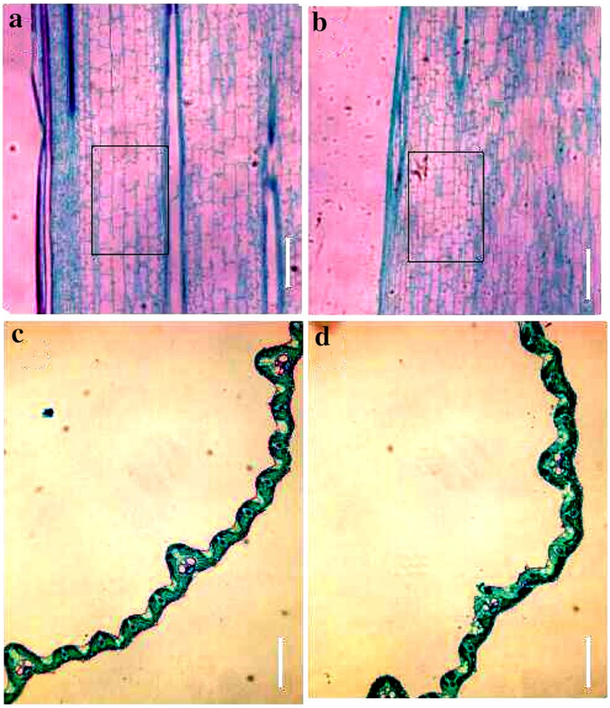


Fig. 2 Microstructure of the second internode and flag leaf of wild type and *dhta-34*. **a**, **b**, longitudinal section of second internode, bar = 50 μm; **c**, **d** transversal sections of flag leaf; **a** and **c** stand for wild type Zhenong 34, **b** and **d** stand for *dhta-34*; bar = 0.5 mm

Histological Observation

Wild type and *dhta-34* longitudinal sections of the second internode were observed through light microscopy. The cells (marked out by black blocked box) in the wild type (Fig. 2a) were longitudinally elongated, whereas those in the mutant were shorter (Fig. 2b). The number of small vascular bundles between two large vascular bundles in *dhta-34* (Fig. 2c) was less than that in the transverse sections of wild type (Fig. 2d), which were related to the width of blades.

Map-Based Cloning of the *DHTA-34* Gene

SSR and InDel markers distributed on rice 12 chromosomes (supplementary 1) were used to identify the target genes in *dhta-34* mutant. *DHTA-34* gene was primarily mapped between the InDel markers 3R-4 and R3M10 on the short arm of chromosome 3 using 32 recessive plants derived from the F_2 population (Fig. 3b). Subsequently several InDel markers including 3R-1, 3R-8, 3R-12, 3R-7, 3R-11, and 3R-10 were developed (Table 3). Finally, the locus was further narrowed to a

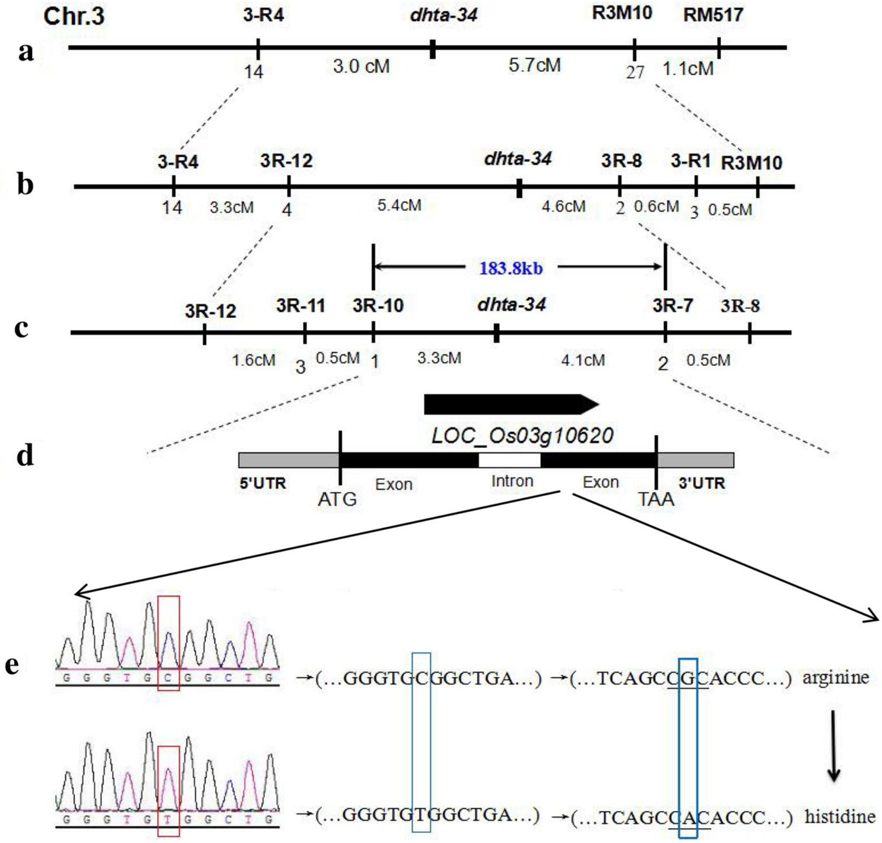


Fig. 3 Fine mapping and isolation of *DHTA-34* gene. **a** the *dhta-34* gene was primarily mapped on chromosome 3 between markers 3-R4 and R3M10. **b** the numerals indicated the number of recombinants identified from 268 recessive F_2 plants were showed above the vertical lines and the genetic distances (cM) between adjacent markers were listed **c** the *DHTA-34* gene was fine mapped to an interval of 183.8 kb using 560 F_2 mutant individuals. **d** there was a cloned gene *D14* (*LOC_Os03g10620*) with high tillering and dwarf mutant traits in the 183.8 kb region, which was related to a kind of α/β fold family domain containing protein, suspected to be the *DHTA-34* gene. Black and gray boxes indicated the ORF region and untranslated region (UTR) of *dhta-34* gene, respectively. **e** there was a C to T nucleotide change in the reverse complement sequence (light blue boxed), which meant a G to A substitution (dark blue boxed) in the second exon of the *dhta-34* mutant, encoding amino acid change from arginine to histidine (Color figure online)

183.8 kb interval between markers 3R-7 and 3R-10, where existed a cloned high tillers and dwarf gene *D14*, *LOC_Os03g10620* (Fig. 3d). We speculated that this gene mutation leaded to *dhta-34*'s dwarfism and high tillers. Cloning and sequencing of the target region from the mutant revealed that there was a substitution G to A (CGC–CAC) in the second exon of *LOC_Os03g10620*, which resulted in an amino acid substitution from arginine to histidine in 227th amino acid (Fig. 3e). Therefore, *dhta-34* was a new allelic mutant of *D14*, and was named as *dhta-34* hereafter.

Table 3 Primer sequences for fine mapping of *dhta-34* gene

Markers	Forward primer	Reverse primer
R3M10	CCGAGTACCATTGCTTTC	CTGCCATAGTTACTGCTCTGTT
3R-4	TCCTTTGCATGCCATCTGAACTAC	TGGCCCAACAAAACACATAAGC
3R-12	TCTTGCCTCCGACCCTCTGT	TTTTGAATTCATGGCTTTGTGC
3R-8	TCAAGGAGGCCGATAGTTA	CAGGGGCATGTTCAAAGA
3R-1	GTGCGGGTCGTCGATGTTCT	GTCATCGCCATTGCCATCG
3R-11	CATTATAGTTGCTTGTGGTTTT	AAAAATTGGATAGGCAGTAAG
3R-10	TGACGGATGCAGAGACAC	TCGGCATTAGGGACATC
3R-7	TTGGGAGTTGGGAGGTAGA	CTAAGAAAAGAAAAGAAAAGAAAACT

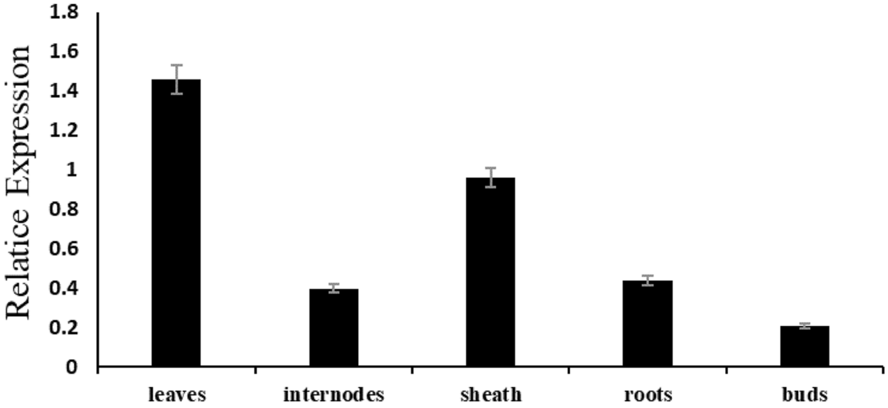


Fig. 4 Real-time PCR analysis of *DHTA-34* transcript levels in different plant organs. Values are the means ±SD of three replicates

Expression Pattern of *DHTA-34*

The expression pattern of *DHTA-34* in different tissues of leaves, internodes, sheaths, roots, and buds from heading stage was evaluated by RT-PCR analysis (Fig. 4). The highest level of *dhta-34* mRNA expression was detected in leaves, which was significantly higher than those in internodes, sheaths, roots, and buds which were 0.40, 0.96, 0.44, and 0.21, respectively, compared with those of the wild type. For determining whether *DHTA-34* acted in the strigolactone signal pathways or strigolactone synthesis, some high tillers and dwarfism-related genes, such as *HTD1*, *D10*, *D3*, *D53*, and *D27* were analyzed for transcript level in wild type and mutant *dhta-34*.

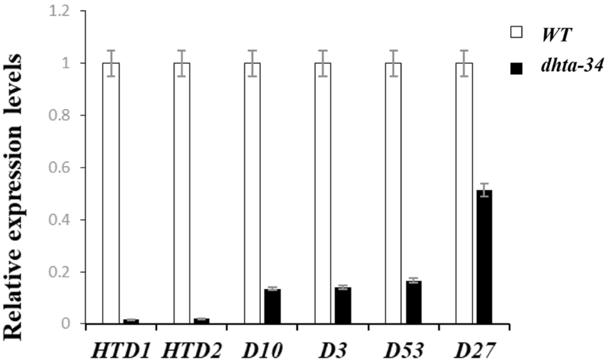


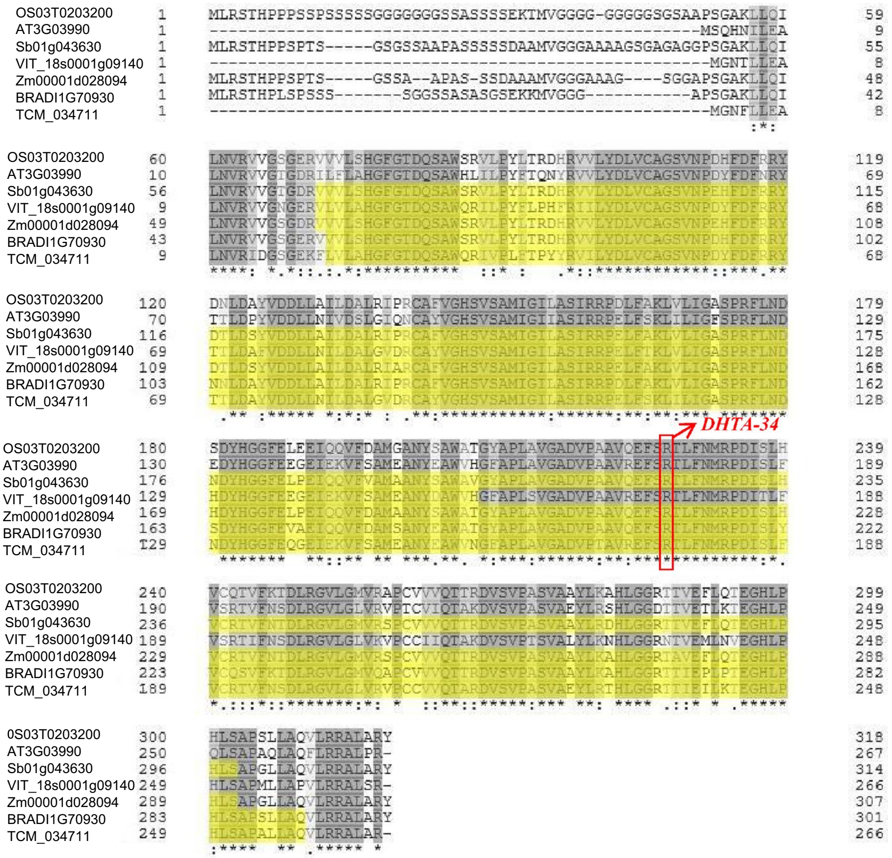
Fig. 5 Expression analysis of genes related to dwarf and high tillering by qRT-PCR. Total RNA was isolated from WT and *dhta-34* mutant and the expression levels of *HTD1*, *HTD2*, *D10*, *D3*, *D53*, and *D27* were measured by qRT-PCR

The Expression of Tillering Ability-Related Genes in Buds

For examining whether the gene(s) associated with the expression of tiller formation were affected by the *dhta-34* mutation, the expression of *HTD1*, *HTD2*, *D3*, *D10*, *D53*, and *D27* in buds were compared between the *dhta-34* mutant and the Zhenong 34 wild-type at the buds of tillering stage. The results showed that the expression of *HTD1*, *HTD2*, *D3*, *D10*, *D53*, and *D27* of buds decreased significantly in the *dhta-34* mutant compared to that in the wild-type (Fig. 5). These genes negatively regulated the rice tillering ability and their expression levels were suppressed because of the synthesis of hormone SLs which inhibiting the outgrowth of tillers. From the results, expressions of *HTD1* and *HTD2* regulated the SLs signal pathway were mostly lower than those of other related genes, leading to result which *DHTA-34*, allele of HTD2/*D14*, might be the candidate gene.

Homology Analysis and Phylogenetic Tree of *DHTA-34* Homologs

BLAST search website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed the existence of six homologs of DHTA-34 in *Arabidopsis thaliana* (Gene ID: *AT3G03990*), *Sorghum bicolor* (*Sb01g043630*), *Vitis vinifera* (*VIT_18s0001g09140*), *Zea mays* (*Zm00001d028094*), *Brachypodium distachyon* (*BRADI1G70930*), and *Theobroma cacao* (*TCM_034711*), respectively. DHTA-34 protein shared 61.32%, 83.33%, 62.89%, 82.08%, 82.70%, and 65.09% homology with these six amino acid proteins (Fig. 6). All of the homologues had the relative conserved domain sequences, which constituted the main structure of proteins. The amino acid site with the mutagenesis in rice was similar to those in *Arabidopsis thaliana* and *Zea mays*. Mutagenesis site of DHTA-34 protein was similar to those in *Arabidopsis thaliana* and *Zea mays* marked out with red bract, which further revealed that the mutations occurred in conserved domain could bring about functional modification. The phylogenetic analysis of *DHTA-34* homologs was made by MEGA V6.0 software (Fig. 7).



```
OS03T0203200       1   MLRSTHPPPSSPSSSSSGGGGGGGSSASSSSEKTMVGGGG-GGGGGSGSAAPSGAKLLQI   59
AT3G03990          1   ------------------------------------------------MSQHNILEA    9
Sb01g043630        1   MLRSTHPPSPTS-----GSGSSAAPASSSSSDAAMVGGGAAAAGSGAGAGGPSGAKLLQI  55
VIT_18s0001g09140  1   -------------------------------------------------MGNTLLEA    8
Zm00001d028094     1   MLRSTHPPSPTS-----GSSA--APAS-SSDAAAMVGGGAAAG----SGGAPSGAKLLQI  48
BRADI1G70930       1   MLRSTHPLSPSSS-------SGGSSASASGSEKKMVGGG-----------APSGAKLLQI  42
TCM_034711         1   -------------------------------------------------MGNFLLEA    8
                                                                            :*:

OS03T0203200      60   LNVRVVGSGERVVVLSHGFGTDQSAWSRVLPYLTRDHRVVLYDLVCAGSVNPDHFDFRRY  119
AT3G03990         10   LNVRVVGTGDRILFLAHGFGTDQSAWHLILPYFTQNYRVVLYDLVCAGSVNPDYFDFNRY   69
Sb01g043630       56   LNVRVVGTGDRVVVLSHGFGTDQSAWSRVLPYLTRDHRVVLYDLVCAGSVNPEHFDFRRY  115
VIT_18s0001g09140  9   LNVRVVGNGERVLVLAHGFGTDQSAWQRILPYFLPHFRIILYDLVCAGSVNPDYFDFRRY   68
Zm00001d028094    49   LNVRVVGSGDRVVVLSHGFGTDQSAWSRVLPYLTRDHRVVLYDLVCAGSVNPEHFDFRRY  108
BRADI1G70930      43   LNVRVVGSGERVVVLSHGFGTDQSAWSRVLPYLTRDHRVVLYDLVCAGSVNPDHFDFRRY  102
TCM_034711         9   LNVRIDGSGEKFLVLAHGFGTDQSAWQRIVPLFTPYYRVILYDLVCAGSVNPDYFDFRRY   68
                       ****: *.*::.:.*:**********   ::* :   .*::************::***.**

OS03T0203200     120   DNLDAYVDDLLAILDALRIPRCAFVGHSVSAMIGILASIRRPDLFAKLVLIGASPRFLND  179
AT3G03990         70   TTLDPYVDDLLNIVDSLGIQNCAYVGHSVSAMIGIIASIRRPELFSKLILIGFSPRFLND  129
Sb01g043630      116   DTLDSYVDDLLAILDALRIPRCAFVGHSVSAMIGILASIRRPELFAKLVLIGASPRFLND  175
VIT_18s0001g09140 69   TTLDAFVDDLLNILDALGVDRCAYVGHSVSAMIGILASIRRPELFTKLVLIGASPRFLND  128
Zm00001d028094   109   DTLDSYVDDLLAILDALRIARCAFVGHSVSAMIGILASIRRPELFAKLVLIGASPRFLND  168
BRADI1G70930     103   NNLDAYVDDLLAILDALRIPRCAFVGHSVSAMIGILASIRRPDLFAKLVLIGASPRFLND  162
TCM_034711        69   TTLDAYVDDLLNILDALGVDRCAYVGHSVSAMIGILASIRRPELFSKLILIGASPRFLND  128
                        .** :***** *:*:* : .**:***********:******:**:**:*** *******

OS03T0203200     180   SDYHGGFELEEIQQVFDAMGANYSAWATGYAPLAVGADVPAAVQEFSRTLFNMRPDISLH  239
AT3G03990        130   EDYHGGFEEGEIEKVFSAMEANYEAWVHGFAPLAVGADVPAAVREFSRTLFNMRPDISLF  189
Sb01g043630      176   NDYHGGFELPEIQQVFVAMAANYSAWAVGYAPLAVGADVPAAVQEFSRTLFNMRPDISLH  235
VIT_18s0001g09140 129  HDYHGGFEEGEIEKVFSAMEANYDAWVHGFAPLSVGADVPAAVREFSRTLFNMRPDITLF  188
Zm00001d028094   169   HDYHGGFELPEIQQVFDAMAANYSAWATGYAPLAVGADVPAAVQEFSRTLFNMRPDISLH  228
BRADI1G70930     163   SDYHGGFEVAEIQQVFDAMSANYEAWATGYAPLAVGADVPAAVQEFSRTLFNMRPDISLY  222
TCM_034711       129   NDYHGGFEQGEIEKVFSAMEANYEAWVNGFAPLAVGADVPTAVREFSRTLFNMRPDISLF  188
                        *******   **::** ** ***.**. *:***:******:**:************:*.

OS03T0203200     240   VCQTVFKTDLRGVLGMVRAPCVVVQTTRDVSVPASVAAYLKAHLGGRTTVEFLQTEGHLP  299
AT3G03990        190   VSRTVFNSDLRGVLGLVRVPTCVIQTAKDVSVPASVAEYLRSHLGGDTTVETLKTEGHLP  249
Sb01g043630      236   VCRTVFNTDLRGVLGMVRSPCVVVQTTRDVSVPASVAAYLRDHLGGRTTVEFLQTEGHLP  295
VIT_18s0001g09140 189  VSRTIFNSDLRGVLGLVKVPCCIIQTAKDVSVPTSVALYLKNHLGGRNTVEMLNVEGHLP  248
Zm00001d028094   229   VCRTVFNTDLRGVLGMVRSPCVVVQTTRDVSVPASVAAYLKAHLGGRTAVEFLQTEGHLP  288
BRADI1G70930     223   VCQSVFKTDLRGVLGMVQAPCVVVQTTRDVSVPASVAAYLKAHLGGRTTIEPLPTEGHLP  282
TCM_034711       189   VCRTVFNSDLRGVLGLVRVPCCVVQTARDVSVPASVAEYLRTHLGGRTTIEILKTEGHLP  248
                       *.:::*::*******:*: *   ::**::*****:*** **: **** .::* * .*****

0S03T0203200     300   HLSAPSLLAQVLRRALARY   318
AT3G03990        250   QLSAPAQLAQFLRRALPR-   267
Sb01g043630      296   HLSAPGLLAQVLRRALARY   314
VIT_18s0001g09140 249  HLSAPMLLAPVLRRALSR-   266
Zm00001d028094   289   HLSAPGLLAQVLRRALARY   307
BRADI1G70930     283   HLSAPSLLAQVLRRALARY   301
TCM_034711       249   HLSAPALLAQVLRRALAR-   266
                       :****  ** .***** *
```

Fig. 6 ClustalW alignment of *DHTA-34* homologs. Multiple sequence alignment of *DHTA-34* revealed that DHTA-34 protein shared 61.32%, 83.33%, 62.89%, 82.08%, 82.70%, and 65.09% homology with its homologs in *Arabidopsis thaliana (AT3G03990)*, *Sorghum bicolor (Sb01g043630)*, *Vitis vinifera (VIT_18s0001g09140)*, *Zea mays (Zm00001d028094)*, *Brachypodium distachyon (BRADI1G70930)* and *Theobroma cacao (TCM_034711)*, respectively (marked with black and gray bracts). The yellow bracts highlighted for conserved domains and red bracts for mutagenesis in mutant *dhta-34* (Color figure online)

Phylogenetic analysis indicated that homology proteins could be divided into to two groups and both of bootstraps were 66 but further exploration exhibited that DHTA-34 protein was related monocot and high developing plant.

Hormone Treatment

For identifying the *dhta-34* functioning in the same way as *D14* and understanding the role of *D14*, hormone of GR24, a synthetic strigolactone analog treatment had been applied to WT and the mutant *dhta-34*. GR24 (dissolved in 95% acetone)

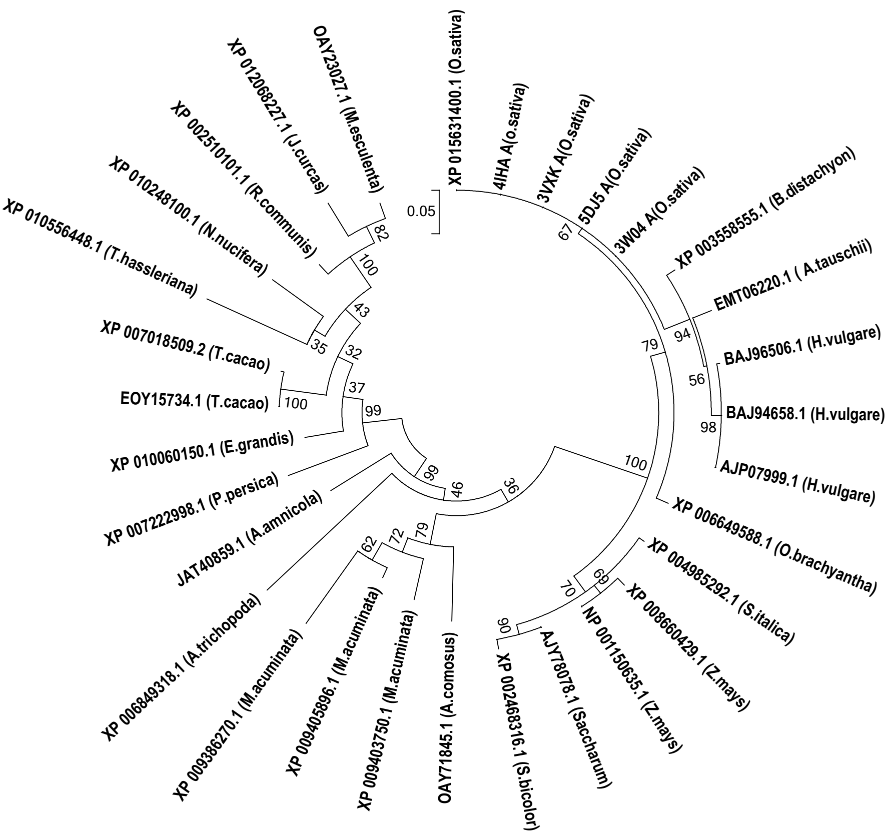


Fig. 7 Phylogenetic tree of DHTA-34 homology protein identified in this study. The tree was constructed by Neighbor-Joining method with bootstrap of 100 in MEGA V6.0 software. The number at each node represented the bootstrap support (percentage)

was added to the rice nutrient solution to prepare the solution with concentration of 0 μmol/L, 10^{-2} μmol/L, 10^{-1}μmol/L, and 1 μmol/L, finally. The germinated seeds of wild-type Zhenong 34 and mutant *dhta-34* were placed in these four treatments, respectively. The results showed that lower (0 μmol/L and 10^{-2} μmol/L in this experiment) hormone strigolactone concentration might not inhibit the tillering capacity of strigolactone-insensitive mutant *dhta-34*, but higher concentrations (10^{-1} μmol/L and 1 μmol/L) would stop tillering both in Zhenong 34 and *dhta-34* (Fig. 8 a&b). As previous reported, the application of 0.5 μM GR24 could not rescue the strigolactone-insensitive mutant *dhta-34* phenotype, and its first and second leaf buds of *dhta-34* were not suppressed by exogenous GR24 (Fig. 8 c&d).

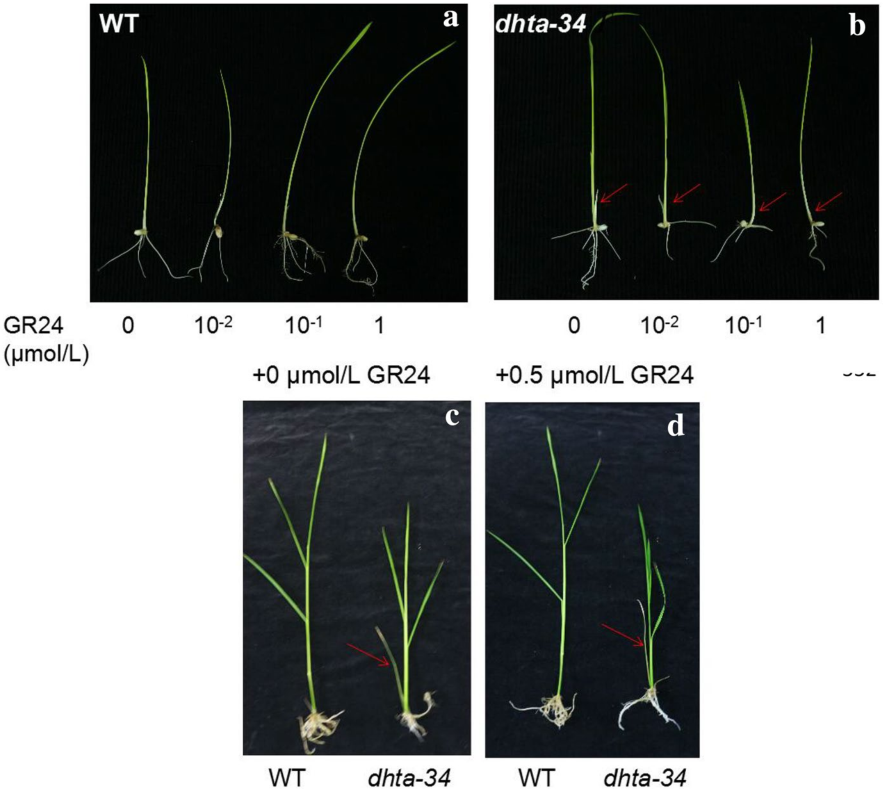


Fig. 8 Hormone treatment of mutant *dhta-34* and wild type Zhenong 34. **a** and **b** adding GR24 of 0 μmol/L, 10^{-2} μmol/L, 10^{-1} μmol/L and 1 μmol/L into nutrient solution for WT and *dhta-34*; **c** without GR24, mutant *dh-34* demonstrated the first tiller (marked out by red arrow in (**a**)) at 3 weeks after germination, while wild type was not. **d**, with 0.5 μmol/L GR24 treatment, mutant *dhta-34* also demonstrated the first tiller (marked out by red arrow in (**b**)) at 3 weeks after germination (Color figure online)

Discussion

Rice tillering ability is strongly affected by plant hormones auxin, cytokinin and strigolactone. Strigolactones (SLs) are plant hormones that inhibiting tillering ability. The rice gene *FINE CULM 1* (*FC1*) (Leyser 2011; Takeda et al. 2003; Hubbard et al. 2002), which was orthologous to *TEOSINTE BRANCHED 1* (*TB1*) in corn (Doebley et al. 1997), worked downstream of SLs. Loss-of-function mutants of *TB1* exhibited the excess branching phenotype which resembled the five *d* mutants. From EMS-treated Zhenong 34, we identified the mutant *dhta-34*, which exhibited a dramatic increase in tiller number and a reduction in height, similar to the mutants involved in SLs biosynthesis or signal transduction (Ishikawa et al. 2005; Zou et al. 2006). The average tillering number of mutant *dhta-34* was 53, which was almost similar to *d14* and *d88* who had 56 and 64 tillers, but *d14* and *d88* (plant height 32.5 cm and 32.1 cm, respectively) were more dwarf when compared to *dhta-34* (62.1 cm). So different mutageneses leaded to phenotypic discrepancy. The dwarf and high tillering ability phenotype of *dhta-34* could not be rescued by exogenous GR24 which was a synthetic strigolactone analog, demonstrating that *DHTA-34* was involved in SLs signaling pathway. And moreover, the synthesis genes *HTD1*, *HTD*, *D10* and the signal transduction genes *D3*, *D53*, and *D27* were all down-regulated in *dhta-34*, especially the expression levels of *HTD1* and *HTD2* were extremely lower than those in others genes, which indicated that the synthesis and signal pathways were much affected due to the defect in mutant. In addition, these genes negatively regulated the rice tillering ability, while their expression levels were suppressed within synthesis of hormone SLs which inhibiting the outgrowth of tillers. From the results, expression level of *HTD1*, one of the important enzymes involved in the biosynthesis of SLs, was mostly lower than those of other related genes, leading to the result that *DHTA-34*, allele of *HTD2*/*D14*, might be the candidate gene. It also could support that *DHTA-34* gene was related to SLs signaling but not SLs biosynthesis. The result showed that *dhta-34* is a novel allelic mutant of *D14*/*HTD2*/*d88* genes and there was a substitution G to A in the second exon of *LOC_Os03g10620*, which resulted in an amino acid substitution arginine to histidine (Fig. 3). The new allele will facilitate further functional characterization of this gene, which may lead to unfolding of newer signaling pathways involving plant development and architecture.

These three allelic mutants *dhta-34*, *d14*, and *d88* belong to the dn-type of dwarfism as the *htd-1* mutant (Zou et al. 2005; Ishikawa et al. 2005). In rice, cell division in the meristem and cell elongation in the cell elongation zone cause stem elongation. Usually, dwarfing could be the result of a defect in one or both of the two processes. According to the observation, dwarfism in the *dhta-34* mutant could be attributed to a defect in elongation of parenchyma cells (Fig. 2).

In the previous study, *D3*, one of the tillering dwarf genes, responsible for SLs signal capturing and transmitting, encoded an F-box leucine-trich repeat (LRR) protein orthologous to *Arabidopsis MAX2*/*ORE9*, implying a conservation of mechanisms controlling axillary bud activity between monocots and eudicots. SKP, Cullin and F-box E3 ubiquitin protein ligase comprised a complex $\mathrm{SCF}^{\mathrm{D3}}$ (Han et al. 2004; Zhou et al. 2013), and it inhibited tillering ability of rice with *D14* jointly (Zhao et al. 2013). Recently, a repressor in SLs signaling, i.e., *D53* (Zhou et al. 2013), was identified, which was belonging to the double Clp-N motif-containing P-loop nucleoside triphosphate hydrolase superfamily and acted as a substrate of the $\mathrm{SCF}^{\mathrm{D3}}$ complex. *D53* could act as an adaptor protein linking D14 and D3 to form a D14-D3-*D53* complex.

Six homologs of DHTA-34 in *Arabidopsis thaliana*, *Sorghum bicolor*, *Vitis vinifera*, *Zea mays*, *Brachypodium distachyon* and *Theobroma cacao*, respectively, were DHTA-34 protein shared 61.32%~83.33% identity with directly orthologous proteins of six orthologous proteins (Fig. 6). To go insight into the phylogenetic relationship and functional associations of DHTA-34, a phylogenetic tree based on neighbor-joining (NJ) methods was constructed for phylogenetic tree of DHTA-34 homology proteins. Phylogenetic analysis indicated that homology proteins could be divided into to two groups and both of bootstraps were 66 but further exploration exhibited that DHTA-34 protein was related monocot and high developing plant.

We suggest that dwarf and tillering mutants are suitable for the study of bud activity control in rice and believe that future molecular and genetic studies using them may enable significant progress in understanding the control of dwarfism and tillering capacity.

Acknowledgments This work was supported by the Science and Technology Office of Zhejiang Province (2016C32085 and 2012C12901-2), Open Foundation of Key Laboratory for Crop Germplasm of Zhejiang Province and the Program for Innovative Research Team in University (IRT1185).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest to this work and have no financial and personal relationships with other people or organizations that can inappropriately influence their work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

References

Arite T, Iwata H, Ohshima K, Maekawa M, Nakajima M, Kojima M et al (2007) *DWARF10*, an *RMS*1/*MAX4*/DAD1 ortholog, controls lateral bud outgrowth in rice. Plant J 51:1019–1029

Arite T, Umehara M, Ishikawa S, Hanada A, Maekawa M, Yamaguchi S et al (2009) *D14*, a strigolactone-insensitive mutant of rice, shows an accelerated outgrowth of tillers. Plant Cell Physiol 50(8):1416–1424

Beveridge CA (2000) Long–distance signaling and a mutational analysis of branching in pea. Plant Growth Regul 32:193–203

Beveridge CA, Kyozuka J (2010) New genes in the strigolactone–related shoot branching pathway. Curr Opin Plant Biol 13:34–39

Beveridge CA, Weller JL, Singer SR, Hofer JMI (2003) Axillary meristem development, budding relationships between networks controlling flowering, branching and photoperiod respon siveness. Plant Physiol 131:927–934

Booker J, Auldridge M, Wills S, McCarty D, Klee H, Leyser O (2004) *MAX3*/*CCD*7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. Curr Biol 14:1232–1238

Booker J, Sieberer T, Wright W, Williamson L, Willett B, Stirnberg P et al (2005) *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3*/*4* to produce a carotenoid–derived branch–inhibiting hormone. Dev Cell 8:443–449

Braun N, De Saint A, Germain JP, Pillot SBoutet-Mercey, M. Dalmais, I. Antoniadi, C. Rameau, et al (2012) The Pea TCP Transcription Factor PsBRC1 Acts Downstream of Strigolactones to Control Shoot Branching. Plant Physiol 158:225–238

Chevalier F, Nieminen K, Sánchez-Ferrero JC, Rodríguez ML, Chagoyen M, Hardtke CS, Cubas P (2014) Strigolactone promotes degradation of *DWARF14*, an α/β hydrolase essential for strigolactone signaling in *Arabidopsis*. Plant Cell 26:1134–1150

Doebley J, Stec A, Gustus C (1995) Teosinte branched 1 and the origin of maize, evidence for epistasis and the evolution of dominance. Genetics 141:333–346

Doebley J, Stec A, Hubbard L (1997) The evolution of apical dominance in maize. Nature 386:485–488

Han L, Mason M, Risseeuw EP, Crosby WL, Somers DE (2004) Formation of an scf ztl complex is required for proper regulation of circadian timing. Plant J 40(2):11

Hubbard L, McSteen P, Doebley J, Hake S (2002) Expression patterns and mutant phenotype of teosinte branched1 correlate with growth suppression in maize and teosinte. Genetics 162:1927–1935

Ishikawa S, Maekawa M, Arite T, Onish K, Takamure I, Kyozuka J (2005) Suppression of tiller bud activity in tillering dwarf mutants of rice. Plant Cell Physiol 46:79–86

Jiang L, Liu X, Xiong G, Liu H, Chen F, Wang L et al (2013) *DWARF 53* acts as a repressor of strigolactone signalling in rice. Nature 504:401–405

Kang TJ, Yang MS (2004) Rapid and reliable extraction of genomic DNA from various wild–type and transgenic plants. BMC Biotech 4:20–20

Kim HK, Luquet D, Van Oosterom E, Dingkuhn M, Hammer G (2010) Regulation of tillering in sorghum, genotypic effects. Ann Bot 106:69–78

Kulkarni KP, Vishwakarma C, Sahoo SP, Lima JM, Nath M, Dokku P et al (2014) A substitution mutation in *OsCCD7* cosegregates with dwarf and increased tillering phenotype in rice. J Genet 93:389–401

Leyser O (2011) Auxin, self–organisation, and the colonial nature of plants. Curr Biol 21:R331–R337

Li X, Qian Q, Fu Z, Wang Y, Xiong G, Zeng D et al (2003) Control of tillering in rice. Nature 422:618–621

Liang WH, Shang F, Lin QT, Lou C, Zhang J (2014) Tillering and panicle branching genes in rice. Gene 537:1–5

Lin H, Wang R, Qian Q, Yan M, Meng X, Fu Z et al (2009) *DWARF27*, an iron–containing protein required for the biosynthesis of strigolactones, regulates rice tiller bud outgrowth. Plant Cell 21:1512–1525

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. Methods 25:402–408

McSteen P (2009) Hormonal regulation of branching in grasses. Plant Physiol 149:46–55

Ongaro V, Leyser O (2008) Hormonal control of shoot branching. J Exp Bot 59:67–74

Snowden KC, Napoli C (2003) A quantitative study and lateral branching in petunia. Funct Plant Biol 30:987–994

Sorefan K, Booker J, Haurogne K, Goussot M, Bainbridge K, Foo E et al (2003) *MAX4* and *RMS1* are orthologous dioxygenaselike genes that regulate shoot branching in *Arabidopsis* and pea. Genes Dev 17:1469–1474

Stirnberg P, Van De Sande K, Leyser HMO (2002) *MAX1* and *MAX2* control shoot lateral branching in Arabidopsis. Dev 129:1131–1141

Takeda K (1997) Internode elongation and dwarfism in some gramineous plants. Gamma Field Sym 16:l–18

Takeda T, Suwa Y, Suzuki M, Kitano H, Ueguchi-Tanaka M, Ashikari M et al (2003) The *OsTB1* gene negatively regulates lateral branching in rice. Plant J 33:513–520

Turnbull CGN, Booker JP, Leyser HMO (2002) Micrografting techniques for testing long–distance signalling in *Arabidopsis*. Plant J 32:255–262

Wang Y, Li J (2008) Molecular basis of plant architecture. Annu Rev Plant Biol 59:253–279

Zhao JF, Wang T, Wang MX, Liu YY, Yuan SJ, Gao YN et al (2013) *DWARF3* participates in an SCF complex and associates with *DWARF14* to suppress rice shoot branching. Plant Cell Physiol 55:1096–1109

Zhou F, Lin Q, Zhu L, Ren Y, Zhou K, Shabek N et al (2013) *D14*–SCF^{D3}–dependent degradation of *D53* regulates strigolactone signalling. Nature 504:406

Zou J, Chen Z, Shang Z, Zhang W, Jiang G, Zhao X et al (2005) Characterizations and fine mapping of a mutantgene for high tillering and dwarf in rice (*Oryza sativa* L.). Planta 222:604–612

Zou J, Zhang S, Zhang W, Li G, Chen Z, Zhai W et al (2006) The rice *HIGH–TILLERING DWARF1* encoding anortholog of *Arabidopsis MAX3* is required for negative regulation of the outgrowth of axillary buds. Plant J 48:687–696

Affiliations

Rong Liang[1] · Ran Qin[1] · Chengcong Yang[1] · Dongdong Zeng[1] · Xiaoli Jin[1] · Chunhai Shi[1]

✉ Chunhai Shi
chhshi@zju.edu.cn

Rong Liang
15925663413@163.com

Ran Qin
369346842@qq.com

[1] Department of Agronomy, Zhejiang University, Hangzhou 310058, China