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HvWOX3A Gene Controls Plant Height and Leaf Size in Barley

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Abstract: Plant height and leaf morphology are considered complex agronomy traits; both are significantly related to nutrient utilization, lodging resistance, and photosynthetic efficiency, which ultimately affect biomass and yield. However, the molecular mechanism of leaf morphogenesis is still unclear. WOX is a group of plant-specific transcription factor families that regulate growth and development, cell division, and differentiation in plants. In the present study, we identified and characterized the *m-876* mutant in barley (*Hordeum vulgare* L.), which exhibited an extreme reduction in leaf width and plant height. Using a map-based cloning strategy, the *m-876* mutant was narrowed down to an 11.4 Mb genomic interval on the long arm of chromosome 5. By analyzing the gene annotation information and nucleotide sequences, we found that *HvWOX3A* (*HORVU.MOREX.r3.5HG0467090*) had a G-to-A substitution at the second exon in the *m-876* mutant, resulting in a change of the coding amino acid from Tryptophan to a premature stop codon at the 200th amino acid position. Remarkably, the mutation of the *HvWOX3A* gene leads to changes in gene expression in the *m-876* mutant. Collectively, our results indicate that the loss function of the *HORVU.MOREX.r3.5HG0467090* gene might be responsible for the phenotypic variation in barley mutants.

Keywords: barley; EMS; WUSCHEL-RELATED HOMEOBOX; plant height; leaf width

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1. Introduction

The application of high-yielding semi-dwarf varieties plays an important role in food production and the grain yield of cereal crops. In barley, decreasing plant height was the main strategy for increasing the grain yield and the harvest index through reduced crop lodging [1]. To date, more than 30 types of dwarfs or semi-dwarfs have been extensively explored in barley, such as *breviaristatum-e* (*ari-e*), *semi-brachytic 1* (*uzu1*), and *semi-dwarf 1* (*sdw1*), which have been widely used for modern barley cultivar breeding programs [2]. The *ari-e* mutant from Golden Promise is located on chromosome 5HL, encoding the γ-subunit of the heterotrimeric G-protein (*HvDep1*), which has mainly been used in several European cultivars [3,4]. The *uzu* (*HvBRI1*) gene is located on the long arm of chromosome 3HL. It encodes a protein kinase involved in the reception of brassinosteroids, the main dwarfing gene used for East Asia barley breeding programs [4,5]. The dwarfism controlled by *uzu* is caused by a missense mutation of a single-nucleotide substitution that results in an amino acid change at a highly conserved residue in the kinase domain of the BR-receptor protein HvBRI1, which is displayed similarly to the BR-insensitive mutant [5]. The barley *sdw1* locus is located on 3H and encodes a gibberellin 20-oxidase enzyme (*HvGA20ox2*). *sdw1* alleles have been widely used to develop modern varieties in Europe, East Asia, North America, South America, and Australia. At the same time, these dwarfing genes in barley have a negative impact on spike agronomical traits such as spike length, grain density, delay in heading, and day-length sensitivity [6–8].

In addition to plant height, leaf size plays a fundamental role in light absorption and photosynthetic efficiency and ultimately impacts biomass, plant performance, and crop yield [9,10]. Mutant screens in barley have been identified as narrow, wide, long, or short leaves [11]. The recessive *narrow-leafed dwarf1* (*nld1*) mutant is identified by a reduced plant height and leaf width due to a reduced cell number across the plants. Map-based cloning revealed that the *Nld1* gene encodes a WUSCHEL-RELATED HOMEOBOX (WOX) transcription factor, an ortholog gene of maize factors *NARROW SHEATH1/2* (*NS1/2*), and the rice-redundant factors *Narrow Leaf*2/3 (*NAL2/3*) [12–14]. *BIP* (*HvWOX3*) is another barley *WOX3* gene; the leaf-blade length and width of the *bifurcated palea* (*bip*) mutant is decreased as compared to the wild type, which exhibited a smaller reduction in the leaf size than that of the *nld1* mutant [14]. The *bipnld1* double mutants showed more severe phenotypes than those of either single mutant, indicating that *HvWOX3* and *NLD1* exert a conserved function to regulate leaf size [15]. Barely *broad leaf1* (*blf1*) was identified with wider leaf blades due to increased longitudinal cell numbers and cells along the leaf length; positional cloning showed that *BLF1* encodes a deductive *INDETERMINATE DOMAIN* family transcriptional regulator [16]. Genome-wide association studies have been employed to further analyze the natural genetic variation in plant leaf size. Digel et al. [17] detected *PHOTOPERIOD-H1* (*Ppd-H1*) as a candidate gene regulating the major QTL for leaf size and flowering time in the barley population; further analysis revealed that differences in the duration of leaf growth and consequent variation in leaf cell number may contribute to the leaf size difference among the *Ppd-H1* variants. Although recent studies have revealed that leaf founder cells greatly affect the final leaf shape, the genetic basis of leaf size is not well characterized in barley.

The validation and cloning of plant-type-related genes are the foundation for improving crop yield. However, only a few genes have been cloned in barley, which limits the application of the barley molecular design in breeding. The present study was aimed at (1) identifying and cloning the gene controlling plant height and narrow leaves and (2) exploring the changes in the transcriptomics between the wild type and the mutants and investigating the role of differentially expressed genes in the regulation of barley plant development.

2. Materials and Methods

2.1. Plant Materials

Yangnongpi 5 (wild type, WT) is a variety of two-row malting barley. In the autumn of 2017, Yangnongpi 5 and 1200 M_3 families derived from its ethylmethanesulfonate (EMS) mutagenesis were planted in the experimental field at Yangzhou University. The barley *m-876* mutant with narrow-leaf and dwarf phenotypes was obtained from the M_3 families, which were inherited stably after continuous cultivation. Two mapping populations of 50 F_2 and 1114 F_3 mutant plants were generated from a cross between *m-876* and the cultivar Morex with two-row malting barley. The parents and genetic populations were planted in Yangzhou City (32°15′ N, 119°01′ E); the annual average temperature is approximately 15 °C and the average sunshine duration throughout the year is 2140 h. All barley materials were sown in October, and phenotypic investigations were carried out in May of the next year before harvest. The F_2 and F_3 individuals with narrow-leaf and dwarf phenotypes were collected and used for gene mapping. The wild-type and mutant-type lines were recorded in the segregated F_2 generations, and the segregation ratio was calculated using the chi-square test.

2.2. Phenotypic Analysis

The leaf length and width were measured at the heading stage. The plant height, internode number, and the length, spike length, seed set rate, and grain number per spike were determined at maturity using the data from three independent replications with 10 randomly selected individuals in each replication. To evaluate the thousand-grain weight, grain length, and grain width, 10 independent barley plants were collected for

phenotypic analysis via an automatic seed size analysis system (SC-G, Wanshen, Hangzhou, China). In total, three replicates were evaluated for statistical analysis.

2.3. Molecular Marker Development

The reference genome sequence for Morex was downloaded from the IPK database (https://galaxy-web.ipk-gatersleben.de/, accessed on 13 October 2020). The genetic polymorphism between Morex and Yangnongpi 5 was obtained using the SnpHub database model (http://wheat.cau.edu.cn/Wheat_SnpHub_Portal/collaboration_GBJ_210711/, accessed on 13 October 2020). Primers were designed to scan the InDels, with a flanking region length of 200 bp. The amplified fragment size ranged from 100 to 350 bp. InDel markers on the barley genome between Yangnongpi 5 and Morex were employed for mapping (Table S1).

2.4. Fine Mapping and Cloning of the Candidate Gene

A total of 50 F_2 and 1114 F_3 individuals with narrow-leaf and dwarf phenotypes were used for gene mapping. The genomic DNA of the parents and of each F_2 and $F_{2:3}$ plant was extracted using the CTAB method [18]. PCR was performed in a final reaction volume of 20 μL containing 50 ng of DNA template, 2.0 μL of primer pairs (10 μM), and 10 μL of 2×Taq Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China). The PCR reaction was carried out with an initial cycle of 3 min at 94 °C, 34 cycles of 15 s at 94 °C, 15 s at 55–58 °C, 60 s at 72 °C, and a final 5 min cycle at 72 °C. Finally, the PCR products were separated on 8% polyacrylamide gel.

According to the reference genome of the Barley V3.0 (Morex) [19], the genomic DNA sequences of the candidate gene were amplified from the WT and *m-876* mutants using the primer pairs shown in Table S1. The PCR products were separated via 1.0% agarose gel electrophoresis. The DNA fragments were cut from the gel and purified with the GeneJET Gel Extraction kit (Thermo Scientific, Waltham, MA, USA). The fragments were connected to the pEASY-T1 cloning vector and sequenced. Sequence analysis was performed using DNAMAN software version 10 (https://www.lynnon.com/dnaman.html, accessed on 13 October 2020).

2.5. Transcriptome Sequencing and Data Analysis

The seedlings of WT and *m-876* mutants aged about two weeks old were collected for RNA extraction and library construction. Three replicates were set up for each experiment. The sequencing library construction was carried out using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEW ENGLAND BioLabs inc., Massachusetts, USA) according to the kit's instruction manual. The library quality was detected using the Qubit 2.0 and the Agilent Bioanalyzer 2100 systems (Agilent Technologies, Inc., Santa Clara, CA, USA). The quality control library was collected based on pre-designed target data volume and then deep-sequenced with the Illumina Solexa sequencing platform. Clean data with high quality were obtained by filtering raw data, then removing the adapter oligonucleotides and low-coverage population sequence reads. These clean data were then aligned to the pre-defined reference version Barley v3.0 (Morex), generating mapped data. Read count statistics for each gene were calculated using Feature Counts v1.5.0-p3. The fragments per kilobase of the transcript per million fragments mapped (FPKM) was carried out to measure the relative expression level of each gene by StringTie v2.2.3 software using the maximum-flow algorithm. The differentially expressed genes (DEGs) were identified via edgeR, and the cutoff value for filtering was established as $|\log2(\text{Fold change})| \geq 1$ and FDR < 0.05 for a normalized expression level.

3. Results

3.1. Phenotypic Characterization of the Barley Mutant m-876

The EMS-mutagenized strategy provides novel, valuable genetic resources for gene clones and for creating breeding materials with high yield, high quality, and high resistance.

In the present study, a *m-876* mutant was obtained from a mutant library of cultivar Yangnongpi 5 via EMS mutagenesis, which displayed a severe dwarf phenotype with a significantly reduced plant height (Figure 1A,E). The statistical analysis confirmed that the reduction in plant height of the *m-876* mutant was due to the decreasing length of all internodes (Figure 1B,F). However, no significant differences were observed in the internode numbers and spike lengths, while the spike and the first and second internode below the spike exhibited bending phenotypes (Figure 1B,G,H). It is worth noting that the seed setting rate decreased slightly (about 13.3%), and the grain number per spike reduced by about 6.3 (Figure 1I,J). In addition to the dwarf phenotype, the *m-876* mutation affected the length and width of the leaf blade (Figure 1C). Each leaf length of the *m-876* was about 56.9% to 77.5% that of the WT for each leaf blade, while each leaf width of the *m-876* mutant plants was significantly reduced to a range of 39.5% to 60.1% that of the WT plants (Figure 1K,L). We also tested the grain shape of WT and *m-876*, and *m-876* produced a lower weight due to reduced grain length and width (Figure 1D,M–O). These results indicate that the *m-876* mutant caused pleiotropic effects in the development of vegetative and reproductive organs.

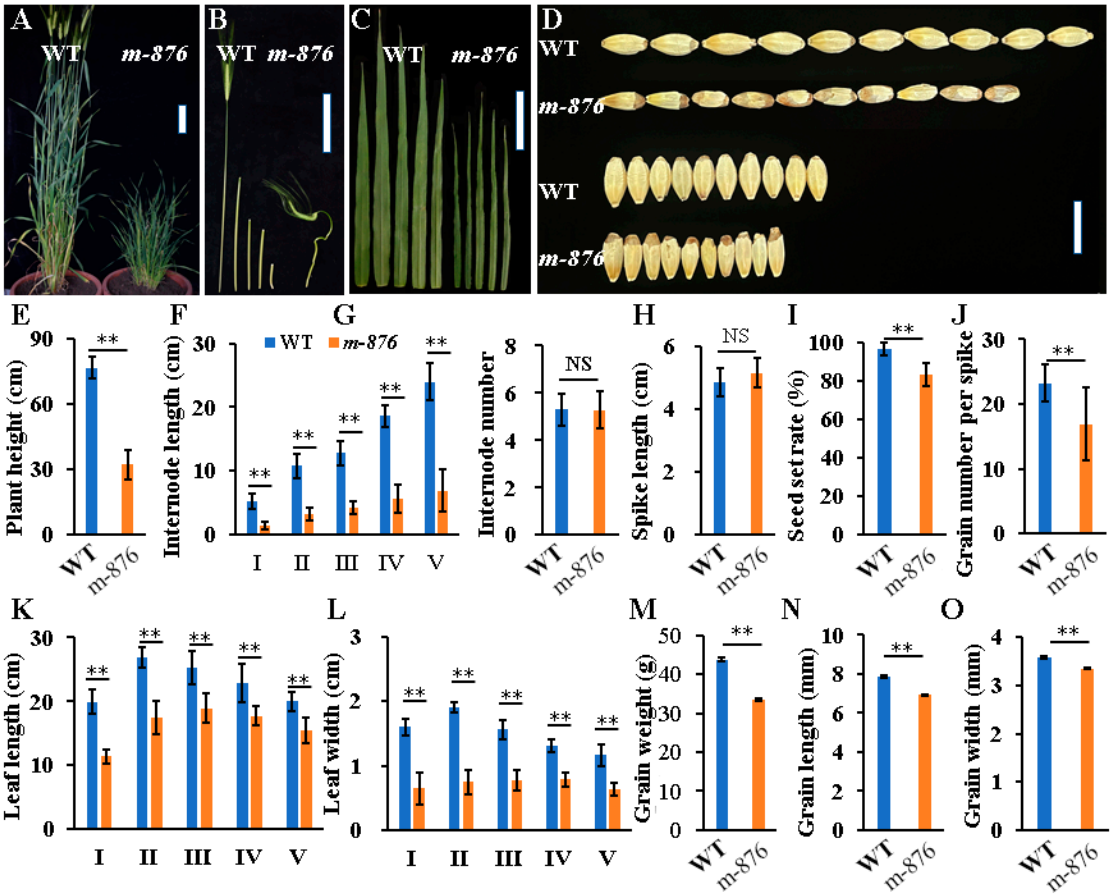

Figure 1. The morphology of Yangnongpi 5 (WT) and the *m-876* mutant. (**A**) Plant morphology of the WT and *m-876* at the heading stage. Scale bar = 10 cm. (**B**) The 1st–5th internodes of the WT and the *m-876* mutant at the heading stage. Bars = 10 cm. (**C**) The 1st–5th leaves of the WT and the *m-876* mutant at the heading stage. Scale bar = 5 cm. (**D**) The morphology of mature grains from WT and *m-876* plants. Scale bar = 1 cm. (**E**) Plant height. (**F**) Internode length. (**G**) Internode number. (**H**) Spike length. (**I**) Seed set rate. (**J**) Grain number per spike. (**K**) Leaf length. (**L**) Leaf width. (**M**) Thousand-grain weight. (**N**) Grain length. (**O**) Grain width. ** Significant difference at $p < 0.01$ as compared to WT using Student's *t*-test, NS indicates no significant difference.

3.2. Genetic Analysis and Map-Based Cloning of *HvWOX3A*

To analyze the inheritance of the *m-876* mutant, the *m-876* mutant in the genetic background of Yangnongpi5 was crossed with Morex. The F_1 plants showed a similar phenotype to Morex. The phenotypic observation of the F_2 population (162 normal phenotype plants and 50 mutant plants; $\chi^2 = 0.05 < \chi^2(0.05, 1) = 3.84$) revealed that the abnormal phenotype of the *m-876* mutant was controlled by a single recessive gene. Using 50 F_2-mutant phenotype individuals, we mapped the *m-876* gene between the markers 5H-79 and 5H-94 on chromosome 3 (Figure 2A). We then developed 10 molecular markers, distributed on the primary mapping region, to test polymorphisms between Morex and the *m-876* mutant. Five of them showed that polymorphism was used for genotyping the F_3 population (to 1114 mutant phenotype plants), which allowed us to further narrow down the mapping location of the candidate gene to the 11.4 Mb physical region between markers 5H-81 and 5H-86 (Figure 2B).

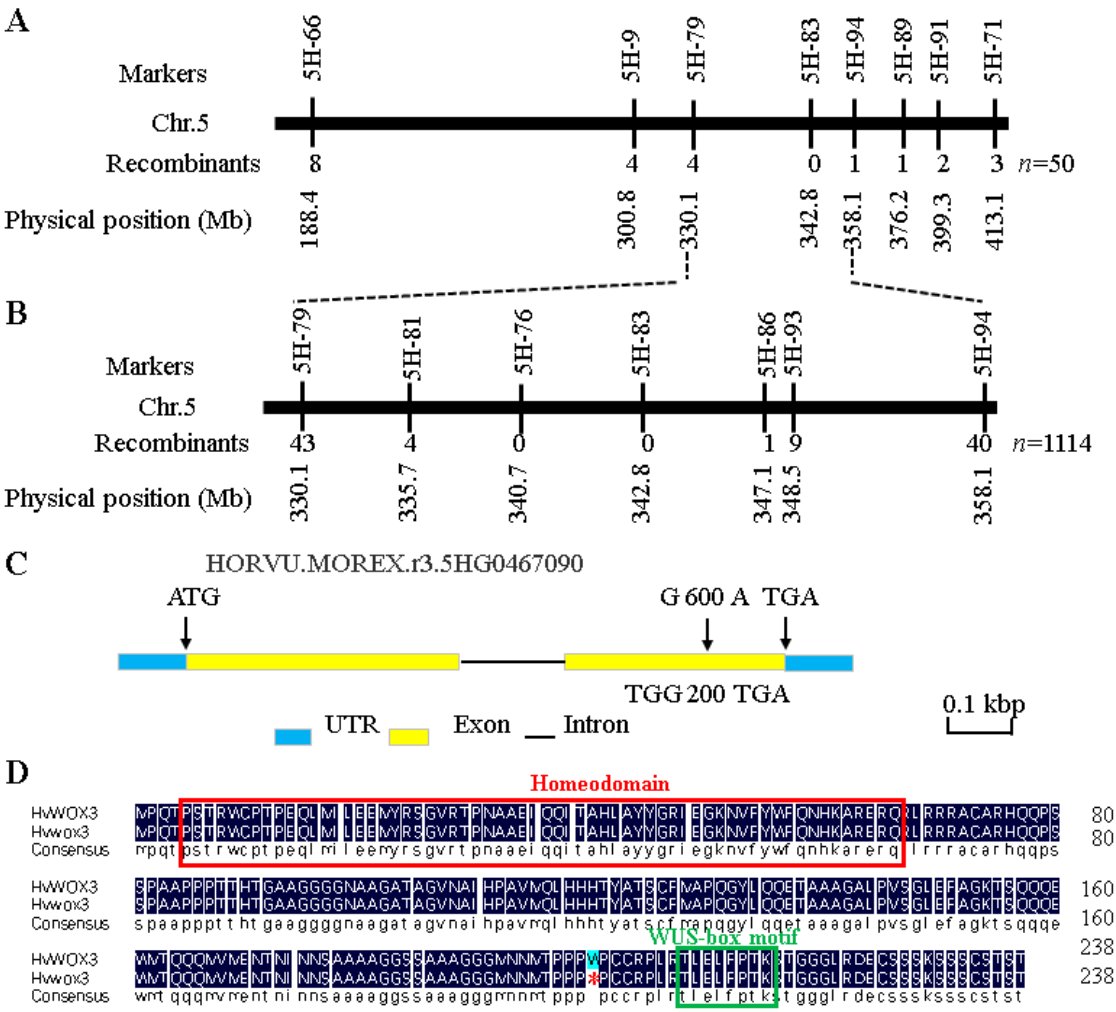

Figure 2. The map-based cloning and mutation-site analysis of the *m-876* mutant. (**A**,**B**) The physical positions of the DNA markers used for the primary mapping of the mutant using 50 F_2 and 11,114 F_3 plants homozygous for the *m-876* mutant phenotype. (**C**) The schematic of the candidate gene. The yellow and blue boxes represent the coding sequence and untranslated region, respectively, and the black line represents the intron sequence. (**D**) The amino acid sequence alignment of the candidate between the WT and the *dnl* mutant. The red boxes represent the homeodomain, the green box represents the WUS-box motif, and the red star indicates the mutant positions.

When the genome annotation derived from the Morex V3 database was analyzed, 66 annotated genes with high confidence were found in the mapping interval

(https://apex.ipk-gatersleben.de/apex/f?p=284:10:::NO:::, accessed on 13 October 2020). When analyzing the gene annotation information and nucleotide sequences in the WT and the *m-876* mutant, we found that *HvWOX3A* (*HORVU.MOREX.r3.5HG0467090*) had a G-to-A (at position 600 from translation start site) substitution at the second exon in the *m-876* (Figure 2C), resulting in a change of the coding amino acid from Tryptophan to a premature stop codon at the 200th amino acid position (Figure 2D). The sequence analysis revealed that *HvWOX3A* contains a 717-bp coding sequence and encodes a 238-amino acid (aa) protein containing a WUS homeodomain at the N-terminus (5-66 aa) and a WUS-box motif at the C-terminus (208-215 aa). A blast analysis showed that the HvWOX3A protein was an orthologous gene of rice NAL2/3 and maize NS1/2. We further analyzed the *HvWOX3A* coding sequences of the pan-genome, comprising landraces, cultivars, and a wild barley. We found that all 20 varieties had identical nucleotide sequences as compared to the Yangnongpi 5 variety at the mutation site (Table S2), implying that the *HvWOX3A* gene may be a candidate gene for the *m-876* mutant.

3.3. Transcriptome Analysis of m-876 Mutant

According to the transcriptome profiling analysis, 434 (346 upregulated and 88 downregulated) differentially expressed genes were detected between the WT and the *m-876* mutant (Figure 3A). GO and KEGG enrichment analyses were performed on the DEGs, which showed that the GO was mainly enriched in the integral component of the membrane, the ATP binding, the plasma membrane, the protein kinase activity, etc. Moreover, KEGG was enriched in the plant hormone signal transduction, the starch and sucrose metabolism, the phenylpropanoid biosynthesis pathway, the MAPK signaling pathway, etc. (Figure 3B,C).

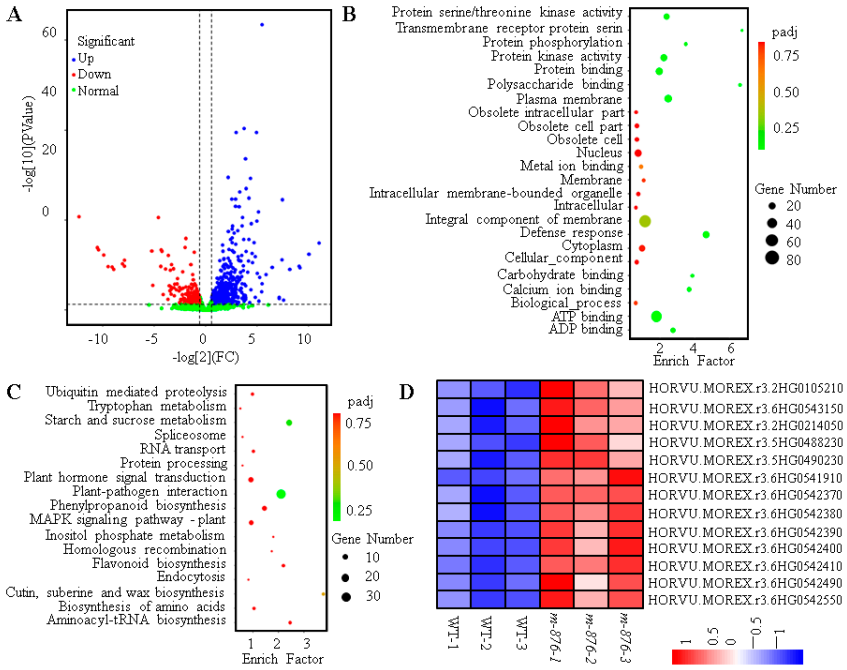

Figure 3. The transcriptome analysis revealed multiple signaling pathways changed by the *HvWOX3A* gene. (**A**) Volcano plot representing the differentially expressed genes in the *m-876* mutant. The x-axis exhibits the significance scores, and the y-axis shows the expression fold-changes of the DGEs. The blue and red dots represent the up- and downregulated genes, respectively. (**B**) The gene ontology (GO) categories of the DGEs. (**C**) Kyoto Encyclopedia of Genes and Genomes (KEGG) categories of the DGEs. (**D**) A heatmap showing the expression levels of the KAO and WAK gene members in barley. The colors represent high (red) and low (blue) expression levels in terms of fragments per kilobase of exon per million mapped fragments (FPKM) values for each gene.

Currently, *OsWOX3A* is a GA-responsive gene and directly interacts with the ent-kaurenoic acid oxidase (KAO) promoter involved in the negative feedback regulation of the GA biosynthetic pathway. In the present study, the *HORVU.MOREX.r3.2HG0105210* (*HvKAO*) gene encoded a cytochrome P450 family protein, which was the homologous gene of *OsKAO*. *HvKAO* was upregulated in the *m-876* mutant. Similarly, the expression levels of genes involved in protein kinase activity, HORVU.MOREX.r3.6HG0543150, HORVU.MOREX.r3.2HG0214050, HORVU.MOREX.r3.5HG0488230, HORVU.MOREX.r3.5HG0490230, HORVU.MOREX.r3.6HG0541910, HORVU.MOREX.r3.6HG0542370, HORVU.MOREX.r3.6HG0542380, HORVU.MOREX.r3.6HG0542390, HORVU.MOREX.r3.6HG0542400, HORVU.MOREX.r3.6HG0542410, HORVU.MOREX.r3.6HG0542490, HORVU.MOREX.r3.6HG0542550, and HORVU.MOREX.r3.6HG0543150, encoding wall-associated kinases (WAKs), were also upregulated in the *m-876* mutant (Figure 3D).

4. Discussion

WOX has been identified as a family of transcription factors that regulates various development processes of plants, including embryogenesis; somatic embryogenesis; flower, leaf, and root development; stem cell maintenance, etc. [20]. In plants, the *WUSCHEL* gene and *WOX1-14* genes of *Arabidopsis* have been identified; meanwhile, 13 WOX family members have been extensively elucidated in rice [21]. In plants, WOX proteins are divided into three clades, including the WUS, ancient, and intermediate clades [22]. Rice Os*WOX3A* and *OsWOX4* belong to the WUS clade. Os*WOX3A* is a typical gene and controls plant development, such as leaves, lemma, and palea morphogenesis, even tiller and lateral root, although *OsWOX4* has been employed in maintaining the shoot apical meristem [13,22,23]. The *OsWOX9A* and OsWOX11 genes belonging to the intermediate clade lead to narrow adaxially rolled leaves and shoot development, respectively [24]. In the present study, phylogenetic analysis revealed that *HvWOX3A* belongs to the WUS clade [14,22]. It is worth noting that duplicated *NS* and *NAL* genes in maize and rice are clearly paralogs, *NS1* and *NS2* are products of ancient allopolyploidization in maize, and the overexpression of *NAL* in rice results in a wide-leaf phenotype, suggesting that dosages of *NAL* and *NS* control leaf width in rice and maize, respectively [25]. However, only one copy of the *HvWOX3A* gene was identified based on the barley genome assembly, indicating that the function of *WOX3A* is conserved in maize, rice, and barley genomes undergoing different evolutionary histories after their divergence.

Plant type is one of the key factors that determine crop yield. Plant height and leaf size are important aspects of plant type, which are closely related to the lodging resistance and photosynthetic efficiency of crops. Through map-based cloning, we identified a single G-to-A point mutant in *HvWOX3A* that causes a truncated protein in the deduced HvWOX3A protein and results in dwarf and narrow-leaf phenotypes (Figure 1A,C). The truncated protein with 199 amino acids only contains a homeodomain. Still, it lacks a WUS-box motif, which was defined in a strict sense with T-L-[DEQP]-L-F-P-[GITVL]-[GSKNTCV] and belongs to the highly conserved WUSCHEL-related homeobox protein family [22]. Studies of maize and rice also confirmed that mutations in *WOX3A* genes may potentially cause plant development defects. For example, the maize genes *NS1* and *NS2* are redundant; duplicated *WOX3* genes and *ns1ns2* double mutants display a narrow-leaf sheath and a margin-deleted phenotype in the lower portion of the leaf blades [12]. *NAL2* and *NAL3* genes are orthologous to maize *NS1/2*, which is consistent with the narrow-leaf phenotype in rice [13,25]. Remarkably, the barley *nld1* and *bip* mutants exhibit narrow leaves as compared to those of the wild types and the dwarf phenotype, with only two internodes from the *nld1* of the top showing a decrease in length. Map-based cloning revealed that both *NLD1* and *BIP* genes encode a WOX3 [14,15]. In the present study, *m-876* exhibited an extremely severe defective phenotype as compared to the *nld1* mutant in the leaf width and plant height, which may contribute to varied genetic backgrounds. These results

are consistent with our finding that mutation in *HvWOX3A* triggers the developmental deficiency of *m-876* via a dominant negative effect.

The *WOX3* gene belongs to the *WUSCHEL-RELATED HOMEOBOX3* gene family and regulates lateral organ development differentially in plants. In rice, *OsWOX3A* acts as a transcriptional repressor and specifically recognizes the TTAATCG motif in the promoter of *OsKAO*. *OsKAO* encodes ent-kaurenoic acid oxidase, a GA biosynthetic enzyme. OsWOX3A is a blocker of GA biosynthesis, whose expression is drastically and temporarily upregulated by GA3 and downregulated by paclobutrazol [26]. *NAL21* is another gene regulating the leaf width and plant height and encodes a ribosomal small subunit protein RPS3A in rice; transformation with modified OsWOX3A genomic DNA with lacking uORFs can better rescue the narrow-leaf phenotype of the *nal21* mutant than the native genomic DNA, implying that the protein expression level of OsWOX3A is subject to the translational regulation of RPS3A [27]. It is worth noting that *HvKAO* expression is upregulated in mutant lines, indicating that the *WOX3* gene may be involved in a conserved regulatory network in plants. In addition, WAK members demonstrated that they are involved in pathogen resistance, heavy metal tolerance, and plant development [28]. *OsWAK11* is defined as a cell-length suppressor and determines rice plant morphology and grain traits that are critical for yield; overexpressing *OsWAK11* exhibited a semi-dwarf phenotype, displayed erect leaves with smaller inclination angles, and produced smaller seeds than the wild-type plants [29]. In the present study, 11 WAK members were upregulated in the mutant lines as compared to the WT. The elucidation of the relationship between WOX3A and WAKs is required for further genetic analysis in the future.

5. Conclusions

In summary, we identified a mutant, *m-876,* with dwarf and narrow-leaf phenotypes. Our genetic analysis revealed that the *m-876* mutant was controlled by a single recessive gene. The candidate gene was narrowed down to an 11.4 Mb genomic interval on the long arm of chromosome 5 using map-based cloning. By analyzing the gene annotation information and nucleotide sequences, we found that *HvWOX3A* had a G-to-A single nucleotide substitution at the second exon in the *m-876* mutant, resulting in a change of the coding amino acid from Tryptophan to a premature stop codon at the 200th amino acid position. Remarkably, the mutation of the *HvWOX3A* gene leads to changes in gene expression in the *m-876* mutant. This research improves our understanding of the mechanisms of *HvWOX3A* in plant development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14081846/s1. Table S1: Primers pairs used in this study; Table S2: Comparison of the CDS of *HvWOX3A* gene among landraces, cultivars and a wild barley variety.

Author Contributions: Conceptualization, B.G. and R.X.; methodology, X.H. (Xinyao Hong), H.D., Y.Z., J.Q. and X.H. (Xinyu Huang); formal analysis, C.L. and F.W.; investigation, X.H. (Xinyao Hong) and H.D.; writing—original draft preparation, B.G. and X.H. (Xinyao Hong); writing—review and editing, B.G. and R.X.; project administration, B.G. and J.Z.; funding acquisition, B.G. and R.X. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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