Tillering or branching is an important agronomic trait in plants, especially cereal crops. Previously, in barley (*Hordeum vulgare*), 'Vlamingh', we identified the high number of tillers1 (*hnt1*) mutant from a $\gamma$-ray-treated segregating population. *hnt1* exhibited more tillers per plant, narrower leaves, and reduced plant height compared with the wild-type parent. In this study, we show that the *hnt1*-increased tiller number per plant is caused by accelerated outgrowth of tiller buds and that *hnt1* narrower leaves are caused by a reduction in vascular tissue and cell number. Genetic analysis revealed that a 2-hp deletion in the gene *HORVU2Hr1G098820* (*HvHNT1*), encoding a trypsin family protein, was responsible for the *hnt1* mutant phenotype. Gene function was further confirmed by transgenic complementation with *HvHNT1* and RNA interference experiments. *HvHNT1* was expressed in vascular tissue, leaf axils, and adventitious root primordia and shown to negatively regulate tiller development. Mutation of *HvHNT1* led to the accumulation of a putative cyclophilin-typt peptide cis/trans-isomerase (*HvPPIase*), which physically interacts with the *HvHNT1* protein in the nucleus of plant cells. Our data suggest that *HvHNT1* controls tiller development and leaf width through *HvPPIase*, thus contributing to understanding of the molecular players that control tillering in barley.

There are two stages in tiller development, bud formation and outgrowth. In recent years, analyses of mutants from various plant species have revealed that many genes are involved in the regulation of each stage. Mutants such as *barren stalk1* (*ba1*; Gallavotti et al., 2004) and *barren inflorescence2* (*bif2*; McSteen et al., 2007) in maize (*Zea mays*) and *monoculm1* (*moc1*; Li et al., 2003) in rice lost their ability to form tiller buds due to intrinsic defects in axillary meristem initiation, indicating that bud formation is genetically controlled. In contrast, axillary bud outgrowth is regulated by genes, phytohormones, environmental factors, and their interactions. Several genes that inhibit the outgrowth of tiller buds have been identified in some mutants, including *Teosinte branched1* (*Tb1*; Doebley et al., 2006) and *Grassy tillers1* (*gt1*; Whipple et al., 2011) in maize and *Teosinte Branched1* (*OsTB1*; Takeda et al., 2003), *Ideal Plant Architecture1* (*ipa1*; Jiao et al., 2010; Miura et al., 2010), *Dwarf27* (*D27*; Lin et al., 2009), *D14/HDTD2* (Arite et al., 2018), and *Dwarf20* (*D20*; Steubing et al., 2015).
and D53 (Jiang et al., 2013; Zhou et al., 2013) in rice. The TBL1-like genes inhibit bud outgrowth in response to several dormancy-inducing hormonal and environmental signals, indicating their role as an integrator of signals controlling dormancy versus the outgrowth fate of an axillary bud (Takeda et al., 2003). Rice dwarf (d14, d27, and d53) mutants had significantly more tillers than their wild-type counterparts, which was closely associated with phytohormone metabolism, including strigolactone (Jiang et al., 2013; Zhou et al., 2013). Moreover, the wheat tiller inhibition (till) mutant was characterized by precocious internode elongation that diverts Suc from the developing tillers (Kebrom et al., 2012). The till gene has no direct effect on the initiation of axillary meristems or bud outgrowth but instead regulates tillering indirectly by controlling the timing of internode elongation. In short, tiller development is regulated by many factors, and continued identification of mutants affected in tillering may reveal further genes controlling tiller development.

Barley is the fourth largest cereal crop in terms of planting area in the world. It has been used as a Triticeae model plant because of its diploid genome. Compared with other Triticeae species, such as wheat and rye (Secale cereale), barley has a low tillering capacity and fewer spikes per plant. Although several tiller mutants have been identified, only a few relevant genes, such as UNICULME4 (Cul4) that reduces tiller number per plant (Tavakol et al., 2015) and ELIGULUM-A (ELI-A) that regulates leaf and tiller development (Okagaki et al., 2018), have been identified. In addition, the candidate gene JucBel2 was identified in the low number of tillers1.a mutant, which encodes a BELL-like homeodomain transcription factor (Dabbert et al., 2010). Moreover, the homologous gene of TBL1 in barley (named INT-C) has a dramatic influence on lateral spikelet fertility but a minor effect on tiller development (Ramsay et al., 2011). Therefore, more tiller-related mutants and gene isolation in barley will enrich the understanding of barley tiller development.

In a previous study, we identified a high-tillering mutant of barley ‘Vlamingh’ isolated from a segregating population treated with γ-rays. In addition to more tillers per plant, this mutant, designated high number of tillers1 (hnt1), also had narrower leaves and shorter plants than the wild-type parent. In this study, we investigated the genetics behind the hnt1 mutant phenotype. We first identified the candidate gene of HvHNT1 on chromosome 2HL by molecular mapping, then confirmed that HvHNT1 encodes a trypsin family protein using transgenic analysis. The expression pattern of HvHNT1 was highly associated with tiller formation and leaf development. We identified a putative cyclophilin-type peptidyl-prolyl cis-trans-isomerase (HvPPIase), which is considered the substrate of the HvHNT1 protein. Accordingly, we assume that HvHNT1 controls tiller development and leaf width in barley through regulating HvPPIase.

RESULTS

Phenotyping of the hnt1 Mutant

The hnt1 mutant exhibited more tillers per plant, narrower leaves, and shorter plants than the wild type (Fig. 1; Table 1). The hnt1 mutant had several fold more tillers per plant than the wild type at all sampling times (Fig. 1B). Leaf length showed no significant difference between the hnt1 mutant and the wild type; however, leaf width of the mutant was approximately half that of the wild type (Fig. 1D). The hnt1 mutant plants had shorter internode lengths than the wild type, hence the shorter plants (Fig. 1G; Table 1). Moreover, the hnt1 mutant had lower grain weights than the wild type (Fig. 1F; Table 1).

To elucidate the mechanisms behind high tillering in the hnt1 mutant, the base tissues including tiller buds were examined histologically. In 15-d-old seedlings, the hnt1 mutant had two or more tillers per plant, with one tiller bud in each leaf axil, whereas the wild type had only one small tiller bud (Fig. 2A). Similarly, the hnt1 mutant had more tiller bud primordia than the wild type when examined with a scanning electron microscope (Fig. 2B). A quantitative analysis of bud and tiller development showed that the hnt1 mutant took significantly fewer days to produce the same number of tillers than the wild type (Figs. 1B and 2C), indicating that tiller development in the hnt1 mutant is significantly faster than in the wild-type plants. Since both the hnt1 mutant and the wild type had only one tiller bud in each leaf axil, more tillers per plant in the hnt1 mutant can be attributed to more rapid outgrowth of tiller buds (Figs. 1B and 2).

Anatomical observations found significantly fewer veins per leaf on average for the hnt1 mutant (14.8) than for the wild type (17.8; P < 0.01; Fig. 3A). Furthermore, the mutant had 11.2 epidermal cells between two
adjacent veins, significantly fewer than those of the wild type (14.4; \( P < 0.01 \); Fig. 3B). However, no significant difference between \( hnt1 \) and the wild type was observed regarding epidermal cell size. Thus, the narrower leaves of the \( hnt1 \) mutant are attributed to fewer veins and cells, indicating that the mutant is affected in cell division rather than cell elongation.

**Mapping and Identification of \( HvHNT1 \)**

To map the \( hnt1 \) locus, a double haploid (DH) mapping population was developed from a cross of the \( hnt1 \) mutant and cv Boluke. The segregation ratio of mutant and normal plants, specifically 148 of the 269 DH lines exhibiting the mutant phenotype, suggested that \( hnt1 \) is controlled genetically by a single locus. In addition, the plants with more tillers were consistently associated with narrower leaves and shorter stature.

Linkage analysis using 360 molecular markers showed that the \( hnt1 \) locus was primarily located in an interval between microsatellite marker Ebmac0039 and insertion/deletion marker M458438 on the long arm of chromosome 2H. To narrow the locus region, 25 new markers were developed, and the \( hnt1 \) locus was located in a region...
between insertion/deletion markers M39589 and M54556 (Supplemental Table S1). Within this region, there are 32 high-confidence genes (Supplemental Table S2) according to the results reported by Mayer et al. (2012) and (Mascher et al., 2017). A 2-bp deletion in a putative gene, \textit{HORVU2Hr1G098820} (MLOC_67307), was detected after cloning and sequencing the candidate genes in the \textit{hnt1} mutant and the wild type (Fig. 4A). The 2-bp deletion at the fourth exon caused a frameshift and generated a premature translation termination product in the \textit{hnt1} mutant allele. A cleaved-amplified polymorphic sequence (CAPS) molecular marker was developed to screen the genotypes with the 2-bp deletion. As a result, the PCR product of the wild type was 267 bp and could be digested into 153-bp and 114-bp fragments by endonuclease \textit{AluI}. In contrast, the \textit{AluI}-digested product of the \textit{hnt1} mutant was 265 bp, as the 2-bp deletion disrupted the \textit{AluI} restriction enzyme cutting site (Fig. 4B). All lines of the DH population were screened with the CAPS marker, which cosegregated with the phenotype. Therefore, \textit{HORVU2Hr1G098820} was considered the candidate gene responsible for the \textit{hnt1} mutant phenotype, herein denoted \textit{HvHNT1}.

### Gene Validation of \textit{HvHNT1}

To confirm that the 2-bp deletion in \textit{HvHNT1} was responsible for the \textit{hnt1} mutant phenotype, a genetic complementation analysis was performed. A 6.75-kb genomic DNA fragment containing a promoter (3,078-bp upstream sequence), coding region (3,348 bp), and 3' untranslated region (324-bp downstream sequence) of the \textit{HvHNT1} gene was introduced into the \textit{hnt1} mutant through \textit{Agrobacterium tumefaciens}-mediated transformation. The sequence information of the constructed genetic complementation vector was used to develop a PCR-based marker, Re_F/R (Supplemental Table S1), for determining the genetic background in the complementation test. Of the 22 transgenic plants, 20 lines

| Trait                        | \textit{hnt1}       | Wild Type      |
|------------------------------|---------------------|----------------|
| Plant height (cm)            | 49.24 ± 2.46        | 64.76 ± 4.32** |
| Leaf width (cm)              | 0.52 ± 0.03         | 0.93 ± 0.08**  |
| Leaf length (cm)             | 11.66 ± 1.11        | 12.61 ± 1.02   |
| Internode I length (cm)      | 15.04 ± 0.63        | 22.53 ± 0.9**  |
| Internode II length (cm)     | 12.07 ± 0.39        | 14.33 ± 0.46** |
| Internode III length (cm)    | 6.84 ± 0.68         | 10.37 ± 0.71** |
| Internode IV length (cm)     | 5.68 ± 0.74         | 7.25 ± 0.51**  |
| Internode V length (cm)      | 2.84 ± 0.25         | 3.09 ± 0.13    |
| No. of fertile tillers       | 9.40 ± 1.03         | 5.12 ± 0.89**  |
| Total tiller number          | 40.35 ± 4.61        | 15.68 ± 3.43** |
| 1,000 kernel weight (g)      | 34.43 ± 1.13        | 38.18 ± 1.49** |

Figure 2. Tiller development in the \textit{hnt1} mutant and its wild-type parent. A, Morphology of \textit{hnt1} and wild-type (WT) stem bases containing tiller buds. B, Scanning electron microscopy images of the stem base. M, Main stem; SAM, shoot apical meristem; T1, first tiller; T2, second tiller; T3, third tiller. C, Number of days to reach five and 10 tillers.
were confirmed by genomic PCR as positive transgenic seedlings (Fig. 5A). The identified 20 primary lines (T0) exhibited phenotypes similar to the wild type, thus demonstrating complementation of \( hnt1 \). The CAPS marker was also used to verify the transformed lines; the positive T0 seedlings were the heterozygote of the mutant and wild-type alleles of \( HvHNT1 \) (Fig. 5B). Five of these lines were selected to generate T2 seedlings for further characterization. Reverse transcription quantitative PCR (RT-qPCR) analysis of the \( HvHNT1 \) gene showed that the complemented lines had much higher expression levels than the mutant and no significant difference from the wild type (Fig. 5C). In addition, the complemented phenotype was inheritable in the T1 plants (Fig. 5D), thus supporting that the \( hnt1 \) mutant phenotype is caused by mutation of the \( HvHNT1 \) gene.

To further examine the role of \( HvHNT1 \), we used an RNA interference (RNAi) approach to inhibit \( HvHNT1 \) expression. Several \( HvHNT1 \)-RNAi lines were obtained, and the corresponding wild type (cv Golden Promise) was used as the control for each one. Four independent and positive T2 lines were selected for further characterization. The expression level of \( HvHNT1 \) in these four RNAi lines ranged from 24% to 34.9% of that in the wild type (Fig. 5F), and tillers per plant ranged from 19.5 to 22, being significantly higher than in the wild type (11 tillers per plant; Fig. 5G). Meanwhile, leaf width and plant height did not significantly differ between the RNAi lines and the wild type (Fig. 5E). These findings indicate that the phenotypic change in \( HvHNT1 \) knockdown lines is in partial consensus with the \( HvHNT1 \) knockout line (\( hnt1 \) mutant) and that \( HvHNT1 \) negatively regulates tiller development in barley.

\( HvHNT1 \) Encodes a Trypsin Family Protein

The sequence comparison between genomic DNA and cDNA revealed that \( HvHNT1 \) comprised five exons and four introns (Fig. 4A). The gene was predicted to encode a trypsin family protein with 648 amino acids, and bioinformatic analysis showed that the amino acid sequence 210 to 425 was the functional domain region using the InterPro online search tool (https://www.ebi.ac.uk/interpro/).

A BLAST analysis was performed on the HNT1 protein sequence of different plant species. Based on the predicted protein sequences, a phylogenetic tree was constructed to reveal the evolutionary relationship of the gene family members (Fig. 6). Another homologous gene became apparent in the barley genome,
namely MLOC_62326.2, which shared 69.4% similarity to HvHNT1 in the amino acid sequence. Moreover, the HvHNT1 gene in barley shared high sequence similarity with that in other species of Gramineae, including wheat, maize, rice, sorghum (Sorghum bicolor), and Brachypodium distachyon. In wheat, each subgenome has two homologous genes, where the genes from chromosome 2 in each subgenome share the closest genetic relationship. In addition, homologous proteins of HvHNT1 were also found in other plants, including Arabidopsis (Arabidopsis thaliana) and tomato (Solanum lycopersicum). The rice narrow leaf1 (nal1) mutant with narrow-leaf and dwarf phenotype (Qi et al., 2008) showed a degree of phenotypic similarity to the hnt1 mutant. The existence of HNT1-like genes in other plant species and the partial phenotypic similarity of the nal1 rice mutant and hnt1 barley mutant indicate partial conservation of biological functions for the HvHNT1 protein family.

Expression Pattern of HvHNT1

To examine the expression pattern of the HvHNT1 gene, total RNA was isolated from the basal stem, leaf, leaf sheath, root, stem, and spike of wild-type plants at different growth stages, including seedling, tillering, stem elongation, booting, and heading. Tills grow from the nodes of unelongated internodes in the base stem near the shoot apical meristem, axillary...
meristem, and tiller buds. The results of RT-qPCR analysis showed that HvHNT1 was mainly expressed in shoots, with low expression in roots at the seedling and tillering stages (Fig. 7A). At the heading stage, there was no obvious difference in the gene expression levels between the base stem and other tissues. In short, the HvHNT1 gene was mainly expressed in the base stem at the tillering stage. As tillers are formed on the basal nodes, the high expression level of HvHNT1 in seedling bases indicates that the gene is involved in tillering.

Based on the RT-qPCR results, cellular localization of HvHNT1 was further determined using different tissues of barley ‘Vlamingh’, including leaf, stem, and basal stem, by in situ PCR analysis (Fig. 7, B–M; Supplemental Fig. S1). The presence of the transcripts was visualized by the color reaction of the alkaline phosphates bound to the antibody, which is specific to the DIG group incorporated during the amplification of the PCR product from specific cDNA produced by the specific reverse primers (Q-HNT1-F2/R2) that recognize HvHNT1. Cross sections of leaves and stem revealed that HvHNT1 was mainly expressed in the vascular system. A longitudinal section of the basal stem at the seedling stage showed that HvHNT1 was mainly expressed in the leaf axil and adventitious root primordia and thus highly related to vascular, tiller, and adventitious root formation. Therefore, HvHNT1 expression is highly associated with tiller development and leaf width.

HvHNT1 Might Regulate Barley Tiller Development through HvPPIase

To understand the possible pathway involved in the high-tillering hnt1 mutant phenotype, proteomic analysis was conducted using the basal stems of four-leaf-aged wild-type and hnt1 mutant plants. The results showed that the wild type and the hnt1 mutant had similar protein profiles except for an 18-kD protein (Fig. 8A), which was more abundant in the hnt1 mutant. The protein spot was excised and digested by trypsin for further analysis using liquid chromatography-mass spectrometer (LC-MS). The accumulated protein in the mutant was identified as a putative cyclophilin-type HvPPIase. However, the RT-qPCR analysis showed that the transcriptional level of HvPPIase did not differ significantly between hnt1 and the wild type. It may be assumed that HvPPIase is negatively regulated by HvHNT1 at the posttranscriptional level. The homologous gene of HvPPIase is DIAGEOTROPICA (DGT) in tomato. Mutation of the DGT gene resulted in pleiotropic phenotypes, including apical dominance reduction and vascular development alteration (Balbi and Lomax, 2019).
Therefore, it is logical that the accumulation of HvPPiase protein could increase tiller number and alter the leaf pattern in the hnt1 mutant.

We further determined the subcellular localization pattern of HvHNT1 and HvPPiase by examining transient coexpression of HvHNT1-YFP and HvPPiase-RFP in barley leaf protoplasts and observed their localization using confocal microscopy. The fluorescence signal of HvHNT1-YFP was detected in the nucleus, whereas the fluorescence signal of HvPPiase-RFP was detected in the cytomembrane, cytoplasm, and nucleus (Fig. 8B). The overlay of HvHNT1-YFP and HvPPiase-RFP fluorescence signals in the cell nuclei suggests that the HvHNT1 and HvPPiase proteins are colocalized and may physically interact with each other in the nucleus (Fig. 8B).

To visualize the physical interaction of HvHNT1 and HvPPiase, a bimolecular fluorescence complementation (BiFC) assay was performed. For the BiFC assay, nonfluorescent N-terminal YFP fragment (nYFP) was fused to the C terminus of HvPPiase to create the nYFP-HvPPiase fusion, and the N terminus of HNT1 was fused to nonfluorescent C-terminal YFP fragment (cYFP) to create the HvHNT1-cYFP fusion. Interactions between these two fusion proteins were then transiently coexpressed in barley protoplasts. As a result, a strong YFP signal was detected using confocal microscopy (Fig. 8C). Coexpression between nYFP-HvPPiase and cYFP and between nYFP and HvHNT1-cYFP was used as a negative control. Therefore, the results of the BiFC assay suggest that HvHNT1 and HvPPiase physically interact in the nucleus of plant cells. Moreover, the
in situ PCR analysis showed that the gene expression pattern of HvPPIase was partly similar to HvHNT1, with expression mainly in the vascular system and small tillers (Supplemental Fig. S2).

The HNT1 protein belongs to the trypsin protein family. The trypsin-like protease superfamily is defined by its cleavage of substrates with Arg or Lys as the site with broad-spectrum cleavage activity (Lin et al., 1999). The HvPPIase protein has 171 amino acids, including 14 Lys and six Arg. These amino acid sites are the potential cleavage sites for HNT1 protein. As HvHNT1 and HvPPIase interact physically in the nucleus and the HvPPIase protein accumulated in the hnt1 mutant, we propose that HvPPIase is a substrate that may be digested by HvHNT1, thus underlying the mechanism through which HvHNT1 regulates barley tiller development and leaf width.

DISCUSSION

Several genes controlling tiller development in barley have been identified using low-tillering or even uniculum mutants (Tavakol et al., 2015; Okagaki et al., 2018). In this study, the HvHNT1 gene was identified that appears to be closely related to high-tillering ability in barley. Compared with the wild type, the hnt1 mutant has more tillers per plant and displays narrower leaves and shorter plant stature. Genetic analysis showed that the mutant phenotype is controlled by HORVU2Hr1G098820, which was denoted HvHNT1 and encodes a trypsin family protein consisting of 648 amino acids. Transgenic analysis demonstrated that a 2-bp deletion in HvHNT1 is responsible for the hnt1 mutant phenotype. Moreover, the HvHNT1 gene is highly expressed in the shoot base at the tillering stage and preferentially expressed in vascular tissues, leaf axils, and adventitious root primordia. Proteomic analysis, as well as BiFC and bioinformatics analyses, showed that HvPPIase physically interacts with and may be digested by HvHNT1. This was further validated by in situ PCR analysis of HvPPIase expression. Therefore, HvPPIase accumulated specifically in the hnt1 mutant. In short, we propose that HvHNT1 affects barley tiller development through its regulation of HvPPIase.

In addition to controlling tiller formation, the HvHNT1 gene in barley is also related to leaf width and plant height. The homologous genes in rice were NAL1 and PLANT ARCHITECTURE AND YIELD1 (PAY1). The NAL1 gene encodes an unknown protein related to polar auxin transport. A loss-of-function mutation in the NAL1 gene leads to a narrow-leaved and dwarf phenotype (Qi et al., 2008). PAY1 improves plant architecture by regulating polar auxin transport and altering endogenous indole-3-acetic acid distribution (Zhao et al., 2015). Consequently, the mechanisms by
which *HvHNT1* influences leaf width and plant height could be similar to those associated with the *NAL1* gene. In this study, the narrower leaves of the *hnt1* mutant resulted from a significant reduction in the number of vascular veins per leaf (Fig. 3A), indicating that *hnt1* is related to vascular formation. In situ PCR analysis of the *HvHNT1* gene in leaves and stems also shows that the gene is preferentially expressed in vascular tissues (Fig. 7, C, D, F, and G). Therefore, *HvHNT1* may be involved in vascular development. During leaf differentiation, veins develop from leaf primordia in a specific pattern. The commonly accepted molecular model for vein development is the canalization-of-auxin-flow hypothesis, where MONOPTEROS (MP), PIN-FORMED1 (PIN1), and auxin are involved in the formation of leaf veins (Friml et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). *NAL1* affects vascular development as well as polar auxin transport in rice (Qi et al., 2008). Our preliminary results demonstrated that expression of the *PIN1* homologous gene was not impacted in the *hnt1* mutant, which differs from the rice *nal1* mutant (Qi et al., 2008). Furthermore, RNA interference of the *HvHNT1* gene increased tiller number but did not affect leaf shape in this study (Fig. 5E). Thus, the function of *HvHNT1* in barley may be similar to, but not the same as, *NAL1* in rice.

In Gramineae species, such as rice, wheat, and barley, tillers develop from the axillary meristem. Axillary bud development consists of two stages: (1) axillary bud formation when the bud is dormant, and (2) axillary bud outgrowth when bud dormancy is broken (Schmitz and Theres, 2005; Kebrom et al., 2012). Therefore, tillering depends not only on the initiation of axillary buds but also on the regulation of bud outgrowth. In this study, the *hnt1* mutant had almost four times more tillers than the wild type (Fig. 1B). The histological study showed no difference between the *hnt1* mutant and the wild type regarding the initiation of axillary buds, with one axillary bud in each leaf axil. However, bud outgrowth differed dramatically between the *hnt1* mutant and the wild type, with *hnt1* plants displaying significantly faster bud outgrowth than wild-type plants (Fig. 2). In other words, the *hnt1* mutant has a shorter dormancy duration of axillary buds than the wild type. In general, bud outgrowth is inhibited by the primary shoot apex, referred to as apical dominance. Therefore, we assume that more tillers per plant in the *hnt1* mutant is attributed to weakened or even lost apical dominance.

Our results indicate that *hnt1* is a pleiotropic mutant. Compared with the wild type, the *hnt1* mutant exhibited an abnormal phenotype. Both genetic analysis and transgenic validation demonstrated that all abnormal phenotypes were caused by a mutation in the *HvHNT1* gene, suggesting that *HvHNT1* is a crucial gene for tiller development regulation.

The results of the 2D proteomic analysis showed an accumulation of HvPPIase protein in *hnt1* that was not accompanied by changes at the transcriptional level. Together with the results obtained from the bioinformatic analysis and BiFC assay, we conclude that HvPPIase might be the substrate of the HvHNT1 protein. The PP1ase superfamily in eukaryotes includes cyclophilins, FK506-binding proteins, parvulins, and PP2A phosphatase activators (Jing et al., 2015). Several plant CYCLOPHILIN (CYP)-like genes have been functionally characterized and are involved in a variety of physiological and developmental processes, including flowering, phytohormone signaling, and stress responses, and play important roles in regulating auxin signaling in plants (Trupkin et al., 2012; Ma et al., 2013; Yoon et al., 2016). Rice plants over-expressing OsCYP19-4 showed cold-resistance phenotypes with significantly more tillers and spikes per plant (Yoon et al., 2016). The increase in tiller number in OsCYP19-4-overexpressing plants is consistent with the *hnt1* mutant phenotype. Mutations in the tomato cyclophilin *DGT* gene causes pleiotropic phenotypes, including a slow gravitropic response, reduced apical dominance, and altered vascular development (Balbi and Lomax, 2003; Lavy et al., 2012), and results in a similar phenotype to the *hnt1* mutant. Interestingly, the *dgt* mutant does not exhibit altered levels of auxin (Fujino et al., 1988) and appears to be affected in the expression of a subset of auxin-regulated genes in a tissue- and developmental stage-specific manner (Nebenführ et al., 2000; Balbi and Lomax, 2003), which is similar to that found in the *hnt1* mutant. There was no significant difference in endogenous auxin (indole-3-acetic acid) between *hnt1* and the wild type, but the transcriptional levels of many auxin-regulated genes, including some members of the *HoloAA* and *HvARF* families, changed in the mutant (Supplemental Fig. S3). It is well documented that auxin controls many aspects of plant physiology and morphology, including cell division, vascular tissue formation, adventitious root initiation, apical dominance, and tiller development. Therefore, more tillers per plant and narrower leaves in the *hnt1* mutant may be attributed to accumulation of the HvPPIase protein and changes in the expression of auxin-related genes (Supplemental Fig. S3). However, it is worth noting that the results of the BiFC assay on the physical interaction between HvHNT1 and HvPPIase have not been validated by an independent assay and that HvPPIase digestion by HvHNT1 has not been tested in vitro. Hence, further research is required to confirm if HvPPIase is a direct substrate of the HvHNT1 trypsin protein.

The identification of molecular players in plant development is important for genetic studies and plant breeding. In this case, the *HvHNT1* gene could be useful in barley breeding, as it affects tiller development, plant height, and leaf size. Moreover, this report describes the involvement of trypsin in controlling tiller development, plant height, and leaf size in a cereal crop.

**MATERIALS AND METHODS**

**Plant Material and Tiller Observation**

The *hnt1* mutant was isolated from the wild-type barley (*Hordeum vulgare*) ‘Vlamingh’ treated with γ-rays as follows. M1 seeds were sown in a field to
select the mutant with more tillers per plant in M2 and then was progressed to M5 via self-pollination. The M5 seedlings were genotypically and phenotypically stable and named the hnt1 mutant. In addition, a DH population consisting of 269 lines derived from a cross between hnt1 and cv Boluke was developed for use in this study. All DH lines and the two parents were grown in an experimental field at the Department of Agriculture and Food in Western Australia. Tillers per plant and leaf width were recorded at the heading stage, and plant height and 1,000 kernel weight were measured at maturity. Both hnt1 and the wild-type cv Vlamingham were grown in a growth chamber with a 14-h/10-h day/night cycle at 23°C/18°C. The dynamic change in tillers per plant was recorded weekly by counting tillers on each of 12 hnt1 and wild-type plants.

Morphological Analysis

The shoot apices in 15-d-old seedlings of wild-type cv Vlamingham and the hnt1 mutant were obtained for anatomical observation with a stereomicroscope. A minimum of eight shoot apices were dissected for each genotype for analysis by scanning electron microscopy.

The rolled and mature leaves from wild-type and hnt1 seedlings were embedded in 5% (w/v) agarose and sliced into 80-μm sections for leaf vein, cell number, and size counting using a microscope with a camera. Cell size was calculated using ImageJ software with 20 biological repetitions.

Mapping of HvHNT1

The genomic DNA of all DH lines and the two parents was extracted using the cetyl-trimethyl-ammonium bromide method. In total, 360 molecular markers scattered across all chromosomes were selected to examine the parents for polymorphism. The genotypes of all lines were then determined using 114 polymorphic markers. Tillers per plant and leaf width of each DH line and the two parents were recorded at the heading stage. Because multtiller plants are consistently associated with narrow leaves, the phenotype of the DH population was scored as A for wild type and B for hnt1 type. In this study, we used the trait score as a marker. Using polymorphic markers, a genetic linkage map was constructed using Joinmap 4.0 software (https://www.kyazma.nl/index.php/JoinMap/). The molecular markers located on both sides of the trait marker were regarded as primary segregation markers. The region between the primary segregation markers was considered the primary candidate region, where 24 primary recombination lines were identified.

New molecular markers, including simple sequence repeats and small insertions and deletions, were developed based on sequence differences between cv Morex and cv Barke. The physical map and genomic sequence data were obtained from the MIPS barley genome database (http://mips.helmholtz-zuerchen.de/plant/barley). The new markers and recombination lines were simultaneously used to narrow the candidate region. High-confidence genes in the candidate region were confirmed based on Mayer et al. (2012). The identified polymorphisms were used to develop new markers (CAPS_F/R). The sequence alignment and primer design were performed using GENEIOUS software (https://www.geneious.com/).

Genetic Complementation Analysis

A 6.75-kb genomic DNA fragment containing a promoter (3,070-bp upstream sequence), coding region (3,348 bp), and 3' untranslated region (324-bp downstream sequence) of the HvHNT1 gene was amplified from wild-type genomic DNA using primers HNT1-ProHindIII-F and HNT1-ORF-Sac-R (Supplemental Table S1). The fragment was cloned into the HindIII and SacI sites of the binary vector pCAMBIA1300 without the 35S promoter to create the transformation plasmid for the complementation test. The constructed complementation vector was introduced into Agrobacterium tumefaciens strain AGL1 using electroporation and transferred into the hnt1 mutant using the callus induction method (Hatwood, 2014). Based on the sequence information of the constructed genetic complementation vector, a PCR-based marker was designed to verify the transformed lines. The forward primer (RE_F) was designed based on the initial vector fragment, and the reverse primer (RE_R) was designed based on the 6.75-kb genomic DNA. The CAPS marker was also used to verify the transformed lines. In addition, RT-qPCR was conducted to determine the HvHNT1 gene expression level in the five independent transgenic lines (T2), the wild type, and the mutant. Total RNA from leaf tissue of transgenic lines (T2), wild-type, and mutant seedlings at the tillering stage was extracted using a centrifugal filter kit according to the user manual (Takara). After RT, qPCR was performed using gene-specific primers Q-HNT1_F and Q-HNT1_R. The relative expression level of each transcript was obtained by normalization to the HvACTIN gene.

Generation and Analysis of the HvHNT1 Knockdown Lines

To generate the hairpin RNAi construct, we cloned a 300-bp fragment (37-263 bp from ATG) of HvHNT1 cDNA as inverted repeats into the pANDA vector (Miki and Shimamoto, 2004) under the control of the maize (Zea mays) ubiquitin1 promoter and subsequently transformed the vector into A. tumefaciens strain AG1L. The primer (RNAi-clon_F/R) sequences used for amplifying a 300-bp fragment of HvHNT1 cDNA are shown in Supplemental Table S1. Immature embryos of barley ‘Golden Promise’ were used for A. tumefaciens-mediated transformation. The positive transgenic lines were confirmed by primers RNAi_F and RNAi-clon_R (Supplemental Table S1). The expression levels of HvHNT1 were determined in the leaves of the T2 generation RNAi lines and cv Golden Promise (wild type) using RT-qPCR with primers Q-HNT1_F and Q-HNT1_R and HvACTIN gene normalization. The phenotype, including tiller number, leaf width, and plant height, was calculated at the heading stage.

Phylogenetic Analysis

Homologous protein sequences of HvHNT1 were obtained from the Arabidopsis (Arabidopsis thaliana), tomato (Solanum lycopersicum), maize, sorghum (Sorghum bicolor), wheat (Triticum aestivum), Brachypodium distachyon, and rice (Oryza sativa) genome databases using an HvHNT1 amino acid sequence with ViroBLAST (Deng et al., 2007). A neighbor-joining tree was built using amino acid sequences of predicted proteins using GENEIOUS via the neighbor-joining method.

Gene Expression Analysis (RNA Isolation and RT-qPCR Analysis)

Total RNAs were isolated from the basal stem, leaf, sheath, root, stem, and spike of wild-type plants at different growth stages, including seedling, tillering, stem elongation, booting, and heading, using a centrifugal filter kit according to the manufacturer’s instructions (Takara). First-strand cDNA synthesis was performed with 1 μg of total RNA using the PrimeScrip RT reagent kit with gDNA Eraser (Takara), according to the user manual. cDNA samples were diluted 3-fold, with 1 μl used for further analysis. qPCR analyses were performed using gene-specific primers Q-HNT1_F and Q-HNT1_R (Supplemental Table S1) in the reaction system of SYBR Green Supermix (Bio-Rad) on a Roche 480 real-time PCR machine (Roche). The basal stem of hnt1 and wild-type seedlings in the tillering stage was also used to analyze the gene expression of auxin-related genes. The barley ACTIN gene was used as an internal control. RT-qPCR was carried out with three biological and technical replications. The 2^-ΔΔCq relative quantification method was used to evaluate quantitative variation. All primers are listed in Supplemental Table S1.

In Situ PCR

The in situ PCR analysis of HvHNT1 and HvPPLase was performed using a described method (Athman et al., 2014) with several modifications. The leaf and basem samples from the seedling and booting stages were immersed in ice-cold FAA (containing 63% [v/v] ethanol, 5% [v/v] acetic acid, and 2% [v/v] formaldehyde) for 3 h. The samples were then embedded in 5% (w/v) agarose and sectioned to 80 μm. Ten sections were collected in one tube to perform the DNase treatment, and RT-qPCR was performed using the primers Q-HNT1-F2/R2 and Q-PPLase-F2/R2 listed in Supplemental Table S1. Sections were then treated by blocking the treatment, anti-DIG-AP binding, and staining with BM Purple AP substrate (Roche). After staining, the sections were washed and mounted in 40% (v/v) glycerol and observed with a Leica microscope. The negative controls followed the preparation of the test samples, but with the reverse transcriptase enzyme omitted. The positive control was carried out with the ribosomal 18S transcript; the primers are listed in Supplemental Table S1.

Proteomic Analysis

Total proteins from the base stem tissues of the wild type and hnt1 mutant at the four-leaf stage were subjected to 2D electrophoretic proteomic analysis.

A Gene Controlling Barley Tillering
Three biological and two technical replicates of 2D gel images were scanned using a GS-800 2DE scanner (Bio-Rad), and protein spots were analyzed with ImageMaster 2D (Amersham Biosciences). To compare spot quantities between gels, the spot volumes were normalized as a percentage volume of three biological replicates. The significantly differentially expressed protein spots were excised and digested by trypsin for LC-MS/MS analysis. The procedure was performed as described by Wu et al. (2014) with some small modifications.

Subcellular Localization and BiFC in Barley Protoplasts

For the constructs transiently expressing in barley leaf protoplasts, the coding regions of HvHNT1 were amplified and cloned into pCAMBIA1300-YFP and BiFC vectors pSAT1-cEYFP-C1-B, and the coding regions of HvPPIase were amplified (primers are listed in Supplemental Table S1) and cloned into pCAMBIA1300-RFP and BiFC vectors pSAT1-cEYFP-C1, using the homologous recombination technology (Vazyme ClonExpress II One Step Cloning kit) to generate HvHNT1-YFP, HvHNT1-cEYFP, HvPPIase-RFP, and nYFP-HvPPIase, respectively.

Barley protoplast isolation and polyethylene glycol-mediated transformation were performed according to Bai et al. (2014) with some minor modifications. In brief, the primary leaves of barley cv Golden Promise was cut into 2-mm segments and incubated at 25°C in the dark for 12 to 18 h, and protoplasts were detected. Confocal microscopy images were taken using an LSM780 confocal laser scanning microscope (Zeiss).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. HvHNT1 expression in various tissues at different growth stages (supplemental information for Fig. 7).

Supplemental Figure S2. In situ PCR analysis of the HvPPIase gene.

Supplemental Figure S3. Gene expression levels of auxin-related genes between hnt1 mutant and wild-type seedlings.

Supplemental Table S1. Information on the primers used in this study.

Supplemental Table S2. The 32 high-confidence genes in the mapping region.

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