Article

A VIN3-like Protein OsVIL1 Is Involved in Grain Yield and Biomass in Rice

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Abstract: In chromatin remodeling, the post-translational modification of histone proteins is mediated by multimeric protein complexes. VERNALIZATION INSENSITIVE3 (VIN3) forms a complex with Polycomb Repressive Complex 2 (PRC2), which mediates the trimethylation of H3K27 to repress target gene expression. In rice, four genes (OsVIL1–OsVIL4) encoding the VIN3-like proteins are expressed ubiquitously in various tissues. Null mutants of *osvil2* display pleiotropic phenotypes such as altered flowering time, floral organ defects, and reduced tiller size. In contrast, *osvil1* mutants did not show significant phenotypes except in fertilization compared with the wild type. However, transgenic plants overexpressing *OsVIL1* showed phenotypes of increased biomass and grain yield. Cross-sections of the basal region of elongating stems revealed that the increased biomass was mediated by inducing cell proliferation in the meristem. Chromatin immunoprecipitation assay indicated that OsVIL1 repressed expression of cytokinin oxidase/dehydrogenase gene (*OsCKX2*) by binding to the promoter and genic regions of *OsCKX2*. We also observed that OsVIL1 modified the levels of H3K27me3 in the *OsCKX2* chromatin. Because *OsCKX2* encodes an enzyme that degrades active cytokinin, we conclude that OsVIL1 functions in the regulation of endogenous active cytokinin levels, thereby increasing plant height and productivity.

Keywords: rice; grain yield; biomass; histone modification; *OsVIL1*; *OsCKX2*

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1. Introduction

Rice is a staple food, and increasing productivity of this crop is an important agricultural goal. To this end, much research has been carried out on increasing rice grain yield. In rice, plant architecture is an important factor that determines productivity, which is well known to depend on meristem activity [1,2]. Cytokinins are important in regulating meristem activity and maintenance by increasing cell division [1–5]. *LONELY GUY* (*LOG*) encodes a cytokinin riboside 5'-monophosphate phosphoribohydrolase that functions during the final step in bioactive cytokinin synthesis [3]. *LOG* mutants exhibit defective maintenance of shoot meristems and a severe reduction in panicle size [3]. The most effective QTL for increasing grain yield, *GRAIN NUMBER 1a* (*Gn1a*), encodes cytokinin oxidase/dehydrogenase (*OsCKX2*), which degrades active cytokinin [6]. Decreased expression of *OsCKX2* causes cytokinin to accumulate in inflorescence meristems and leads to a large number of tillers and spikelets per plant [6,7]. Several factors that influence grain productivity and biomass are associated with *OsCKX2* regulation [6–8]. The zinc-finger transcription factor DROUGHT AND SALT TOLERANCE (*DST*) directly activates *OsCKX2* expression in the meristem [9]. The mutant allele *DST*^{reg1} causes down-regulation of *OsCKX2*, thus increasing cytokinin accumulation in the inflorescence meristem and increasing

the number of branches and grains [9]. Also, *LARGER PANICLE (LP)*, which encodes a Kelch repeat-containing F-box protein, improves the panicle architecture and grain yield in rice [10]. LP is an ER-localized protein and interacts with rice SKP-like proteins, suggesting that LP might be involved in ER-associated protein degradation. Mutation of the *LP* gene reduces the expression of *OsCKX2* in young panicles, causing cytokinin accumulation. Also, *OsVIL2* represses the expression of *OsCKX2* by direct interaction with the promoter region of *OsCKX2* [7]. In addition, a chromatin interacting factor *OsVIL2* increases panicle development and biomass by increasing active cytokinin levels.

Polycomb repressive complexes contain two major protein complexes, PRC1 and PRC2, in plants [11,12]. PRC2s are methyltransferases that introduce trimethylation of lysine 27 on histone H3 (H3K27me3) to repress down-stream target genes [13,14]. In Arabidopsis, the VERNALIZATION 2 (VRN2)-PRC2 complex has several components: VRN2, SWINGER (an E(Z) Histone Methyltransferase homolog), FERTILIZATION-INDEPENDENT ENDOSPERM (ESC homolog), and Musashi-1 (p55 homolog), which form the canonical part, while three plant-specific PHD (plant homeodomain) finger proteins (VRN5, VERNALIZATION INSENSITIVE3, and VEL1) create the accessory part [13,15,16]. Previous reports have indicated that VRN2-PRC2 plays important roles in the vernalization pathway by mediating the accumulation of H3K27me3 at the *FLOWERING LOCUS C (FLC)* locus after vernalization [13,17,18]. The VRN2 complex functions in the vernalization pathway associated with VIN3, VIL1/VRN5, and VIL2 /VERNALIZATION-LIKE 1 [15,19]. VIN3 and VIN3-like proteins consist of conserved domains of the PHD finger, the fibronectin type III domain (FNIII), and the VIN3 interacting domain (VID). The PHD finger recognizes and binds to histone proteins, the FNIII domain is involved in protein-protein interactions, and VID domain is responsible for interactions between other VIL proteins [7,20,21].

Although the vernalization response is not involved in controlling flowering time in rice, it is associated with four VIN3-like proteins: *Oryza sativa* VIN3-like 1–4 (*OsVIL1–4*) [7,22]. *OsVIL2* binds to *O. sativa* EMBRYONIC FLOWER 2b (*OsEMF2b*), a component of PRC2, and the PHD-PRC2 complex induces flowering by repressing *O. sativa* *LEAFY COTYLEDON 2 and FUSCA 3-LIKE 1 (OsLFL1)* [23]. Moreover, *OsVIL1* binds to *OsEMF2b* and induces flowering by repressing *OsLF* under short-day conditions but delays flowering by inducing *Grain number, plant height, and heading date 7 (Ghd7)* expression under long-day conditions [24,25]. *OsVIL2* also plays essential roles in regulating meristem activity and affects biomass, grain yield, tiller outgrowth, and spikelet development [7,26,27]. Although both *OsVIL1* and *OsVIL2* are understood to control flowering time, how *OsVIL1* affects other developmental processes has not been studied to date. Here, we focused on how *OsVIL1* functions differ from those of *OsVIL2*, which have already been studied. We showed that *OsVIL1* increases grain yield and biomass through the direct regulation of *OsCKX2* expression, similarly to *OsVIL2*.

2. Results

2.1. Mutation of *OsVIL1* Produces No Obvious Phenotypes in Spikelet Development and Tiller Outgrowth

The rice genome contains four VIN3-like genes: *OsVIL1*, *OsVIL2*, *OsVIL3*, and *OsVIL4* (Figure 1A). Interestingly, *OsVIL* genes extensively existed in higher plants, including lycophytes, gymnosperms, amborellales, eudicots, and monocots, which indicates their functional importance in these plants (Figure 1A). Similar to Arabidopsis VIN3-like proteins, *OsVIL1–4* proteins contain PHD, FNIII, and VID domains (Figure 1B). The expression of the four *OsVIL* genes was broadly detected in various tissues of rice (Figure 1C–F and Supplementary Figure S1).

Figure 1. Comparative analysis of OsVIL genes. (A) Phylogenetic analysis of proteins OsVIL1–4 in rice, Arabidopsis, maize, *Sorghum bicolor*, and *Glycine max*. (B) Protein structures of OsVIL1–4. (C–F) Expression levels of *OsVIL1* (C), *OsVIL2* (D), *OsVIL3* (E), and *OsVIL4* (F) in various tissues. Rice *Actin1* was used as an internal control. The error bars indicate the standard deviations, $n = 4$.

To study the functions of *OsVIL1*, we generated *osvil1* null mutants using the CRISPR/Cas9 method. In the T₂ generation, we found three independent lines containing 1-bp insertion that causes early termination of translation (Figure 2A). Compared with the wild type (WT), plant height, and tiller outgrowth were not different in these *osvil1* mutants (Figure 2B). The development of panicles and spikelets was also normal in the mutants (Figure 2C–F). Although there were no significant defects in floral organ development, fertility was slightly decreased in the mutants compared with the WT (Figure 2G). These results indicated that *OsVIL1* functions redundantly with *OsVIL2* in most developmental processes except for a reduction in fertilization.

Figure 2. Phenotypes of the wild-type (WT) and *osvil1* null mutants. (A) Schematic structure of *OsVIL1* and mutation events obtained using the CRISPR/Cas9 method. In the gene structure, the black boxes indicate the four exons and the gray boxes indicate the 5'UTR and 3'UTR regions. The arrow indicates the mutation sites revealed using the CRISPR/Cas9 method. The target sequence is underlined with the protospacer adjacent motif shown in bold. Altered DNA sequences are indicated in red. (B) Phenotype of plant growth in WT and *osvil1* mutants at the seed-ripening stage under paddy field conditions. (C) Panicle phenotypes. Bar = 2 cm. (D) Seed phenotypes. Bar = 1 mm. (E) Total number of spikelets on one panicle. (F) Number of primary branches on the main panicle. (G) Seed fertility. The error bars indicate the standard deviations, n = Statistical significance is indicated by *** ($p < 0.001$).

2.2. Overexpression of *OsVIL1* Induces Increased Grain Yield and Biomass

To determine the function of the other rice VIN3-like proteins, we generated *OsVIL1* OX transgenic plants (Figure 3A). Under natural paddy field conditions, the *OsVIL1* OX plants showed a >20% increase in height compared with the segregated WT (Figure 3B). When each internode length of the WT and *OsVIL1* OX plants was measured, we found that the length of each internode was increased in the OX plants compared with the WT (Figure 3C). In addition, two more elongated internodes were observed in the OX plants, indicating that increased plant height of the transgenics was due to increased internode number and length (Figure 3C). Cross-section of the stems showed that the diameter of the main culms was increased in *OsVIL1* OX plants when compared with the WT (Figure 3D–F).

Figure 3. Internode development of wild-type (WT) and *OsVIL1* overexpression (OX) transgenic plants. (A) Phenotypes at about 152 days after sowing (DAS) in the paddy field. Bar = 10 cm. (B) Plants height at about 152 DAS. (C) Comparison of each internode length at about 152 DAS. (D,E) Cross sections of the main culm from the first internode of the WT and *OsVIL1* OX #1 transgenic line at about 112 DAS. Bars = 1 mm. (F) Comparison of the internode diameters at about 112 DAS. Error bars indicate the standard deviations, $n = 4$ or Statistical significance is indicated by *** ($p < 0.001$).

Similar to the *OsVIL2* OX plants that exhibit increased grain yield, *OsVIL1* OX plants showed increased grain yield (Figure 4). *OsVIL1* OX lines had larger panicles (Figure 4A,B) and an increased number of primary (Figure 4C) and secondary branches (Figure 4D) compared with the WT. The increased number of primary and secondary branches caused an increase in the total number of spikelets per main panicle (Figure 4E).

Figure 4. Panicle development in wild-type (WT) and *OsVIL1* overexpression (OX) plants. (A) Panicle phenotype of WT and *OsVIL1* OX transgenic lines. Bar = 5 cm. (B) Main panicle lengths. (C) Number of primary branches on the main panicle. (D) Number of secondary branches on the main panicle. (E) Total number of spikelets on the main panicle. The error bars indicate the standard deviations, $n =$ Statistical significance is indicated by *** ($p < 0.001$), ** ($p < 0.05$).

2.3. Overexpression of *OsVIL1* Affects Cell Numbers in Meristem Regions

To investigate the differences between the WT and *OsVIL1* OX plants at the cellular level, we analyzed the size and number of the cells in the first internode at about 149 DAS. First, we checked the elongation region of the internode. The longitudinal section of the region showed that the cell number and size in the *OsVIL1* OX plants were not significantly increased compared with those of the WT (Figure 5A and Supplementary Figure S2). This observation suggested that increased plant height was not due to cell size. To evaluate whether the increase in plant height observed in the *OsVIL1* OX transgenic lines was due to increases in the cell number, the meristem region at the basal parts of the first internode were examined at the about 149 days after sowing (DAS) in the paddy field (Figure 5B). The cell size and number were analyzed in a 0.2-mm² sample area in the division zone after longitudinal sections. The analysis indicated that cell size from the *OsVIL1* OX plants was decreased to approximately 52% and 30% in length and width, respectively (Figure 5C). In contrast, the cell number was significantly increased to about 180% in *OsVIL1* OX plants compared with the segregated WT (Figure 5D). These results suggested that *OsVIL1* promoted cell division in the meristem region of the stem.

Figure 5. Longitudinal section of the first internode from wild-type (WT) and *OsVIL1* overexpression (OX) plants. (A) Longitudinal section analyses of the elongation zone from the first internodes in the WT and *OsVIL1* OX #1 line at about 149 DAS. Bars = 200 μm. (B) Longitudinal section analyses of the meristem zone from the first internodes at about 149 DAS. Bars = 200 μm. (C) Comparison of cell length and width in the upper 0.5 cm of the cell division region in the first internode of the WT and *OsVIL1* OX #1 line, $n = 30$ cells from three individual plants. (D) Comparison of cell numbers. Cells were counted in a sample 0.2-mm² area in the meristem region of the internode. The error bars indicate the standard deviations, $n =$ Statistical significance is indicated by *** ($p < 0.001$), ** ($p < 0.05$).

2.4. *OsVIL1* Represses *OsCKX2* Expression

Previously, we showed that gene expression associated with cell division/cell cycle, cell organization, DNA synthesis, and protein synthesis/amino acid activation was increased in *OsVIL2* OX plants [7]. We also reported that *OsVIL2* suppresses the expression of *OsCKX2*, which encodes a cytokinin-degrading enzyme [7]. The down-regulation of *OsCKX2* causes accumulation of active cytokinin that promotes cell division in *OsVIL2* OX plants [7]. Here, we investigated whether *OsVIL1* also affected *OsCKX2* expression. Analyses of the samples from the cell division zones showed that *OsCKX2* expression was significantly reduced in *OsVIL1* OX plants compared to WT (Figure 6B). However, the transcript levels of *GA 2-oxidase 1 (GA2ox1)* that controls GA homeostasis by inactivating bioactive GA through 2β-hydroxylation [28] were not significantly altered (Figure 6C). Similarly, the expression levels of *LP*, *DST*, and *OsSPL14*, which regulate panicle size and grain yield [29,30], did not differ between the WT and *OsVIL1* OX plants (Figure 6D–F). Our results suggest that *OsVIL1* increases active cytokinin levels through the control of *OsCKX2* expression.

Figure 6. Expression levels of several genes regulating plant height and grain yield in *OsVIL1* overexpression (OX) plants. Samples were prepared from the cell division zones (0.5 cm) of the first internodes (4 cm) under paddy field conditions. The expression levels of *OsVIL1* (A), *OsCKX2* (B), *OsGA2ox1* (C), *OsLP1* (D), *OsDST* (E), and *OsSPL14* (F) in the first internode. Rice *Ubiquitin1* was used as an internal control. The error bars indicate the standard deviations, $n = 3$. Statistical significance is indicated by *** ($p < 0.001$).

2.5. *OsVIL1* Directly Suppresses *OsCKX2* Expression by Regulating H3K27 Chromatin States

OsVIL2 represses *OsCKX2* expression by regulating the chromatin states of H3K27me3 [7]. To investigate whether *OsVIL1* also enhances H3K27me3 in *OsCKX2* chromatin, we generated transgenic plants that expressed Myc-tagged *OsVIL1* as well as Myc alone as a negative control. To perform chromatin immunoprecipitation (ChIP) assay, the basal parts of the second internode were collected before the heading stage. When we observed the levels of H3K27me3 in *OsCKX2* chromatin using H3K27me3 antibodies (Figure 7A), the *OsCKX2* chromatin was significantly enriched in the *OsVIL1*-Myc transgenic plants compared with the transgenic plants expressing the Myc-tag alone (Figure 7C). Moreover, when we observed the direct binding of *OsVIL1* in the *OsCKX2* chromatin region using anti-Myc antibodies, *OsVIL1*-Myc proteins were enriched at the *OsCKX2* chromatin sites P4 and P7–P9 (Figure 7E). As a negative control, we used *LP*, which was not significantly enriched in the *OsVIL1*-Myc transgenic plants (Figure 7D,F).

Figure 7. Chromatin immunoprecipitation assay of OsVIL1 in *OsCKX2* chromatin. Samples were prepared from the upper 0.5 cm of the cell division region in the second internode before the heading stage, $n = 10$. (A,B) Genomic structure of *OsCKX2* and *OsLP*. OsVIL1-Myc transgenic plants were used for the chromatin immunoprecipitation assay. As a control, we used Myc-empty transgenic plants. (C,D) Analysis of H3K27me3 chromatin states in *OsCKX2* chromatin (C) and *OsLP* (D). (E,F) Enrichment of *OsCKX2* (E) and *OsLP* (F) chromatin in OsVIL1-Myc transgenic plants. The entire experiment was conducted three times and the graph shown are from one of the three experiments. The error bars indicate the standard deviations. Statistical significance is indicated by *** ($p < 0.001$), * ($p < 0.01$).

3. Discussion

3.1. OsVIL1 Is Involved in Chromatin Remodeling in Rice

The rice genome contains four *VIN3*-like genes (*OsVIL1*–*OsVIL4*) that are homologous to *Arabidopsis* *VIN3* [31]. They are composed of a PHD finger that is a conserved motif for histone binding, an FNIII domain, and a VID domain [20,21,25]. The PHD finger of *OsVIL2* binds to the native histone H3 in vitro and induces H3K27me3 histone modification in the chromatin of target genes [7,23]. In this study, we showed that *OsVIL1* also modifies chromatin states by regulating H3K27me3 to repress the expression of down-stream genes. H3K27me3 is an epigenetic modification of histone H3 and affects the stability and plasticity of gene regulation during various developmental processes. The chromatin interacting factor of *OsVIL2* binds to *OsEMF2b*, which is a core protein in the PRC2 complex, and

induces flowering by repressing *OsLFL1* in rice [23]. The *osvil2* null mutants display late flowering phenotypes, as do *OsEMF2b* mutants. Although *OsVIL2* OX plants display increased biomass and grain yield, the overexpression of *OsEMF2b* does not (unpublished data). Similar to *OsVIL2*, *OsVIL1* represses the expression of target genes by inducing H3K27me3. Moreover, *OsVIL1* binds to *OsEMF2b* through the FNIII domain [25]. However, further studies are needed to elucidate the molecular mechanisms that underlie how the interactions between the *OsVIL* proteins and *OsEMF2b* control biomass and grain yield in rice.

3.2. VIN3-like Proteins Regulate Cell Division of Meristem in Rice

Vernalization is an acceleration of floral transition from vegetative growth to reproductive growth after exposure to a prolonged period of low temperature [31–33]. In Arabidopsis, VIN3 that is a repressive chromatin-remodeling component is induced only after perceiving a sufficient duration of cold [34,35]. The VIN3 protein induces the trimethylation of H3K27 of the target loci to regulate gene expression. For example, VIN3 increases the levels of H3K27me3 in the *FLC* chromatin region and other *FLC* clade members [18]. Rice does not require vernalization to control flowering time, and no *FLC*-homologous gene is present in rice [23]. Although the detailed mechanisms for controlling flowering time differ between rice and Arabidopsis, the *OsVIL1* and *OsVIL2* proteins in rice play a role in promoting flowering by reducing the expression of *OsLFL1*, a flowering repressor [23,25].

In rice, *osvil2* null mutants display pleiotropic phenotypes, including altered flowering times, reduced tiller numbers, alterations in the leaf angles, and defects in floral organ development [23,26,27]. In addition, *OsVIL2* OX plants exhibit increased biomass and grain yields caused by an accumulation of active cytokinin [7]. In this study, we showed that overexpression of *OsVIL1* led to similar phenotypes as those seen in *OsVIL2* OX plants, including increased biomass and grain yield. When we examined the histone modification states of *OsCKX2* chromatin, a gene responsible for cytokinin degradation, *OsVIL1* affected trimethylation of H3K27 in *OsCKX2* chromatin, similarly to *OsVIL2*. Cytokinin plays an essential role in cell proliferation and regulating the size of meristem [4]. In rice, the VIN3-like proteins *OsVIL1* and *OsVIL2* repressed *OsCKX2* expression to create a balance between cytokinin synthesis and catabolism to control meristem activity and cell proliferation. In light of these results, it will be interesting to study how VIN3-like proteins in other plant species differ from those in rice and Arabidopsis.

3.3. Loss-of-Function of *OsVIL1* Causes a Reduction in Fertility

Although similar phenotypes were seen when *OsVIL1* or *OsVIL2* were overexpressed, the *osvil1* null mutants did not show remarkable developmental phenotypes in contrast with *osvil2* null mutants, which exhibit pleiotropic phenotypes. Because both *OsVIL1* and *OsVIL2* induced the trimethylation of H3K27 in the *OsCKX2* chromatin region, we expected that their PHD domains would share similar conserved target loci. In addition, *OsVIL1* and *OsVIL2* bind to *OsEMF2b*, the core protein of the PRC2 complex, through the FNIII domain. *OsEMF2b* null mutants display abnormal floral organ identity, reduced tiller size, and pleiotropic phenotypes as in *osvil2* null mutants [23]. For these reasons, we suggested that *OsVIL2* functions with *OsEMF2b* in the PRC2 complex to regulate cell division and meristem identity. The null mutants in *OsVIL1* did not cause observable developmental defects except in fertilization, suggesting that *OsVIL2* can rescue most of *OsVIL1* functions. The cause of the reduced fertility of the *osvil1* mutant has not yet been clearly identified. The *osvil2* has no research results on pollen or the anther development because the floral organ is completely broken in this mutant. The *OsEMF2b*, interaction partner of *OsVIL1* and *OsVIL2*, functions in anther and pollen development in rice [36]. Both *OsVIL1* and *OsVIL2* are expected to play a role in anther and pollen development, but further studies are needed to identify more detailed functions.

However, biomass and grain yield increased when *OsVIL1* was overexpressed, indicating that *OsVIL1* serves to assist the functions of *OsVIL2* in regulating cell division

and meristem activity. Further study, such as generation of double, triple, or quadruple mutants of OsVIL gene mutants, is required to reveal detailed molecular mechanisms of interaction among the four OsVIL proteins to determine how they regulate various developmental processes.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Oryza sativa var. *japonica* cultivar Nipponbare was used to generate transgenic plants. Target regions for the CRISPR/Cas9-induced *osvil1* KO mutants were genotyped through sequencing (Table S1). For DNA sequencing of the target regions, PCR products were gel purified using *Pfu* polymerase and subjected to subcloning. More than 10 transformed *E. coli* colonies for each line were then randomly picked up and sequenced. DNA mutations were subsequently identified through sequence alignment between sequenced alleles and WT alleles using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov> (accessed on 1 November 2021). Seeds were germinated either on an MS medium or in soil. We selected two T_0 lines that highly expressed the introduced *OsVIL1* and renamed the recombinant gene OX #1 and #2. The T_1 progeny were grown on an MS medium containing $40 \mu\text{g mL}^{-1}$ hygromycin. Finally, plants were grown either in the paddy field or a controlled growth room under a light/dark cycle (14.5-h light at 28°C /10-h dark at 23°C ; 50% humidity) as previously reported [37].

4.2. Vector Construction and Rice Transformation

To generate *OsVIL1* OX plants and Myc-tagged transgenic plants, *OsVIL1* full-length complementary DNA (cDNA) was amplified and digested with restriction enzymes *SpeI* and *SacI*, and inserted into binary vector pGA3426 that contains the maize *Ubiquitin 1* (*Ubi1*) promoter, or pGA3438 that contains the maize *Ubiquitin1* (*Ubi1*) promoter and Myc epitope [38]. To construct the CRISPR/Cas9 plasmid, the target sequence was cloned into the entry vector pOs-sgRNA; then, the target sequence and sgRNA were further cloned into a destination vector, pH-Ubi-cas9-7, using the Gateway^{TM} system as previously reported [39,40]. The constructs were introduced into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method [41]. Rice transformation via *Agrobacterium*-mediated co-cultivation was performed as previously described [42]. All primers used for cloning are listed in Table S1.

4.3. RNA Extraction and Real-Time PCR Analysis

For RNA extraction, 0.5-cm samples were collected from the leaf blades or basal parts of the first and second internodes. Total RNA was isolated with RNAiso Plus (Takara, Kyoto, Japan) and $2 \mu\text{g}$ of total RNA were annealed with 10 ng of oligo (dT). First-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, Fitchburg, WI, USA), RNasin (Promega, Fitchburg, WI, USA), and 2.5 mM deoxyribonucleotide triphosphate. Transcript levels were analyzed by quantitative RT-PCR (qRT-PCR) with SYBR Green I Prime Q-Master mix (GENETBIO, Daejeon, Korea) in a Rotor-Gene Q system (Qiagen, Hilden, Germany) [39]. For normalization, we used rice gene *Ubiquitin1* (*OsUbi1*) or *Actin1* (*OsActin1*) as an internal control. The relative expression level was calculated using the $\Delta\Delta\text{Ct}$ method. All primers used for qRT-PCR are listed in Table S1.

4.4. Histochemical Analysis

To observe internode development, we used paraffin to obtain sections from the samples of the first internode. The samples were collected from the basal and middle parts of the first internode, and fixed with FAA solution as previously reported [43]. Then, samples were dehydrated and infiltrated with paraffin before being embedded in the embedding ring. Samples were cut to a thickness of $10 \mu\text{m}$, rehydrated, and stained with toluidine blue to observe cell differentiation and elongation. Samples were visualized using a BX61 microscope (Olympus, Tokyo, Japan).

4.5. Chromatin Immunoprecipitation (ChIP) Analysis

Transgenic plants expressing OsVIL1-Myc were used for ChIP analysis as previously reported [44]. Briefly, about 2 g of the basal parts from the second internodes were collected and incubated in 3% formaldehyde; then, the nuclei were isolated. Chromatins were fragmented to approximately 500 to 1000 bp lengths by sonication. Before preclearing, 1% of the sample was gathered as an input. For immunoprecipitation, we used anti-Myc monoclonal antibodies (#2276; Cell Signaling, Bethesda, MD, USA) as described previously [23,39]. Data were normalized according to the percentage input method as reported previously [45]. The primer sequences used for the ChIP assays are listed in Table S1. All experiments were conducted at least three times, each involving three biological replicates.

4.6. Statistical Analyses

Using R software, the *p* values were obtained by using one-way analysis of variance (ANOVA; Tukey's HSD test) for the test groups [46].

4.7. Phylogenetic Analysis

The orthologous and/or closely paralogous *OsVIL1* genes among the 44 representative plants were identified, using an OrthoFinder pipeline (version 2.5.1) [47]. Afterward, phylogenetic relationships among these VIL genes were retrieved as previously reported [48]. Briefly, multiple alignment of the coding sequences from identified VIL genes were aligned using MAFFT (version 7.243) [49]. Next, the Neighbor-Joining (NJ) phylogenetic tree was inferred through MEGA6 [50] with bootstraps = 1000.

4.8. Meta-Expression Analysis

To analyze tissue specific expression patterns of *OsVIL* genes, we used a CAFRI-Rice database site (<http://cafri-rice.khu.ac.kr/inspector> (accessed on 20 December 2021) [51] based on public available rice Affymetrix microarray data set from the NCBI Gene Expression Omnibus (GEO). The expression values are normalized by R language and then turned into log2 values. With downloaded log2 expression values, we constructed the heatmap with TB tools program [52].

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/plants11010083/s1>, Figure S1. Expression pattern heatmaps of *OsVIL* genes; Figure S2. Longitudinal section of the first internode; Table S1. Primers used for this study.

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References

- Wu, Y.; Wang, Y.; Mi, X.F.; Shan, J.X.; Li, X.M.; Xu, J.L.; Lin, H.X. The QTP *GNP1* encodes GA20ox1, which increases grain number and yield by increasing cytokinin activity in rice panicle meristem. *PLoS Genet.* **2016**, *12*, e1006386. [\[CrossRef\]](#)
- Wang, C.; Yang, X.; Li, G. Molecular insights into inflorescence meristem specification for yield potential in cereal crops. *Int. J. Mol. Sci.* **2021**, *22*, 3508. [\[CrossRef\]](#)
- Kurakawa, T.; Ueda, N.; Maekawa, M.; Kobayashi, K.; Kojima, M.; Nagato, Y.; Sakakibara, H.; Kyozuka, J. Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* **2007**, *445*, 652–655. [\[CrossRef\]](#)
- Jameson, P.E.; Song, J. Cytokinin: A key driver of seed yield. *J. Exp. Bot.* **2015**, *67*, 593–606. [\[CrossRef\]](#)
- Bartrina, I.; Otto, E.; Strnad, M.; Werner, T.; Schmulling, T. Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. *Plant Cell* **2011**, *23*, 69–80. [\[CrossRef\]](#)
- Ashikari, M.; Sakakibara, H.; Lin, S.; Yamamoto, T.; Takashi, T.; Nishimura, A.; Angeles, E.R.; Qian, Q.; Kitano, H.; Matsuoka, M. Cytokinin oxidase regulates rice grain productivity. *Science* **2005**, *309*, 741–745. [\[CrossRef\]](#)
- Yang, J.; Cho, L.H.; Yoon, J.; Yoon, H.; Wai, A.H.; Hong, W.J.; Han, M.; Sakakibara, H.; Liang, W.; Jung, K.H.; et al. Chromatin interacting factor OsVIL2 increases biomass and rice grain yield. *Plant Biotechnol. J.* **2019**, *17*, 178–187. [\[CrossRef\]](#)
- Yeh, S.Y.; Chen, H.W.; Ng, C.Y.; Lin, C.Y.; Tseng, T.H.; Li, W.H.; Ku, M.S.B. Down-regulation of Cytokinin Oxidase 2 expression increases tiller number and improves rice yield. *Rice* **2015**, *8*, 36. [\[CrossRef\]](#)
- Li, S.; Zhao, B.; Yuan, D.; Duan, M.; Qian, Q.; Tang, L.; Wang, B.; Liu, X.; Zhang, J.; Wang, J.; et al. Rice zinc finger protein DST enhances grain production through controlling *Gn1a/OsCKX2* expression. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 3167–3172. [\[CrossRef\]](#)
- Li, M.; Tang, D.; Wang, K.; Wu, X.; Lu, L.; Yu, H.; Gu, M.; Yan, C.; Cheng, Z. Mutations in the F-box gene LARGER PANICLE improve the panicle architecture and enhance the grain yield in rice. *Plant Biotechnol. J.* **2011**, *9*, 1002–1013. [\[CrossRef\]](#)
- Molitor, A.; Shen, W.H. The polycomb complex PRC1: Composition and functions in plants. *J. Genet. Genom.* **2013**, *40*, 231–238. [\[CrossRef\]](#)
- Kim, D.H.; Sung, S. Polycomb-mediated gene silencing in *Arabidopsis thaliana*. *Mol. Cells* **2014**, *37*, 841–850. [\[CrossRef\]](#)
- Mozgova, I.; Henning, L. The polycomb group protein regulatory network. *Annu. Rev. Plant Biol.* **2015**, *66*, 269–296. [\[CrossRef\]](#)
- Yan, B.; Lv, Y.; Zhao, C.; Wang, X. Knowing when to silence: Roles of polycomb-group proteins in SAM maintenance, root development, and developmental phase transition. *Int. J. Mol. Sci.* **2020**, *21*, 5871. [\[CrossRef\]](#)
- De Lucia, F.; Crevillen, P.; Jones, A.M.; Greb, T.; Dean, C. A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of *FLC* during vernalization. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 16831–16836. [\[CrossRef\]](#)
- Qiu, Y.; Liu, S.L.; Adams, K.L. Concerted divergence after gene duplication in polycomb repressive complexes. *Plant Physiol.* **2017**, *174*, 1192–1204. [\[CrossRef\]](#)
- Sheldon, C.C.; Finnegan, E.J.; Peacock, W.J.; Dennis, E.S. Mechanisms of gene repression by vernalization in *Arabidopsis*. *Plant J.* **2009**, *59*, 488–498. [\[CrossRef\]](#)
- Sung, S.; Amasino, R.M. Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* **2004**, *427*, 159–164. [\[CrossRef\]](#)
- Wood, C.C.; Robertson, M.; Tanner, G.; Peacock, W.J.; Dennis, E.S.; Helliwell, C.A. The *Arabidopsis thaliana* vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 14631–14636. [\[CrossRef\]](#)
- Sung, S.; Schmitz, R.J.; Amasino, R.M. A PHD finger protein involved in both the vernalization and photoperiod pathways in *Arabidopsis*. *Genes Dev.* **2006**, *20*, 3244–3248. [\[CrossRef\]](#)
- Greb, T.; Mylne, J.S.; Crevillen, P.; Geraldo, N.; An, H.; Gendall, A.R.; Dean, C. The PHD finger protein VRN5 functions in the epigenetic silencing of *Arabidopsis* *FLC*. *Curr. Biol.* **2007**, *17*, 73–78. [\[CrossRef\]](#)
- Zhao, S.Q.; Hu, J.; Guo, L.B.; Qian, Q.; Xue, H.W. Rice leaf inclination2, a VIN3-like protein, regulates leaf angle through modulating cell division of the collar. *Cell Res.* **2010**, *20*, 935–947. [\[CrossRef\]](#) [\[PubMed\]](#)
- Yang, J.; Lee, S.; Hang, R.; Kim, S.R.; Lee, Y.S.; Cao, X.; Amasino, R.; An, G. OsVIL2 functions with PRC2 to induce flowering by repressing *OsLFL1* in rice. *Plant J.* **2013**, *73*, 566–578. [\[CrossRef\]](#)
- Wang, J.; Hu, J.; Qian, Q.; Xue, H.W. LC2 and OsVIL2 promote rice flowering by photoperiod-induced epigenetic silencing of *OsLF*. *Mol. Plant.* **2013**, *6*, 514–527. [\[CrossRef\]](#)
- Jeong, H.J.; Yang, J.; Cho, L.H.; An, G. OsVIL1 controls flowering time in rice by suppressing *OsLF* under short days and by inducing *Ghd7* under long days. *Plant Cell Rep.* **2016**, *35*, 905–920. [\[CrossRef\]](#)
- Yoon, H.; Yang, J.; Liang, W.; Zhang, D.; An, G. OsVIL2 regulates spikelet development by controlling regulatory genes in *Oryza sativa*. *Front. Plant Sci.* **2018**, *9*, 102. [\[CrossRef\]](#)
- Yoon, J.; Cho, L.H.; Lee, S.; Pasriga, R.; Tun, W.; Yang, J.; Yoon, H.; Jeong, H.J.; Jeon, J.S.; An, G. Chromatin interacting factor OsVIL2 is required for outgrowth of axillary buds in rice. *Mol. Cells* **2019**, *42*, 858–868. [\[PubMed\]](#)
- Hsieh, K.T.; Chen, Y.T.; Hu, T.J.; Lin, S.M.; Hsieh, C.H.; Liu, S.H.; Shiue, S.Y.; Lo, S.F.; Wang, I.W.; Tseng, C.S.; et al. Comparisons within the Rice GA 2-Oxidase gene family revealed three dominant paralogs and a functional attenuated gene that led to the identification of four amino acid variants associated with GA deactivation capability. *Rice* **2021**, *14*, 70. [\[CrossRef\]](#)
- Miura, K.; Ikeda, M.; Matsubara, A.; Song, X.J.; Ito, M.; Asano, K.; Matsuoka, M.; Kitano, H.; Ashikari, M. OsSPL14 promotes panicle branching and higher grain productivity in rice. *Nat. Genet.* **2010**, *42*, 545–549. [\[CrossRef\]](#)

30. Jiao, Y.; Wang, Y.; Xue, D.; Wang, J.; Yan, M.; Liu, G.; Dong, G.; Zeng, D.; Lu, Z.; Zhu, X.; et al. Regulation of *OsSPL14* by *OsmiR156* defines ideal plant architecture in rice. *Nat. Genet.* **2010**, *42*, 541–544. [\[CrossRef\]](#)
31. Fu, D.; Dunbar, M.; Dubcovsky, J. Wheat *VIN3*-like PHD finger genes are up-regulated by vernalization. *Mol. Genet. Genom.* **2007**, *277*, 301–313. [\[CrossRef\]](#)
32. Sung, S.; Amasino, R.M. Vernalization and epigenetics: how plants remember winter. *Curr. Opin. Plant Biol.* **2004**, *7*, 4–10. [\[CrossRef\]](#)
33. Amasino, R.M. Vernalization and flowering time. *Curr. Opin. Biotechnol.* **2005**, *16*, 154–158. [\[CrossRef\]](#)
34. Kim, D.H.; Zografos, B.R.; Sung, S. Vernalization-mediated *VIN3* induction overcomes the LIKE-HETEROCHROMATIN PROTEIN1/POLYCOMB REPRESSION COMPLEX2-mediated epigenetic repression. *Plant Physiol.* **2010**, *154*, 949–957. [\[CrossRef\]](#)
35. Kim, D.H.; Sung, S. Coordination of the vernalization response through a *VIN3* and *FLC* gene family regulatory network in *Arabidopsis*. *Plant Cell* **2013**, *25*, 454–469. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Deng, L.; Zhang, S.; Wang, G.; Fan, S.; Li, M.; Chen, W.; Tu, B.; Tan, J.; Wang, Y.; Ma, B.; et al. Down-regulation of *OsEMF2b* caused semi-sterility due to anther and pollen development defects in rice. *Front. Plant Sci.* **2017**, *8*, 1998. [\[CrossRef\]](#)
37. Cho, L.H.; Yoon, J.; Pasriga, R.; An, G. Homodimerization of *Ehd1* is required to induce flowering in rice. *Plant Physiol.* **2016**, *170*, 2159–2171. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Kim, S.R.; Lee, D.Y.; Yang, J.I.; Moon, S.; An, G. Cloning vectors for rice. *J. Plant Biol.* **2009**, *52*, 73–79. [\[CrossRef\]](#)
39. Cho, L.H.; Yoon, J.; Wai, A.H.; An, G. *Histone deacetylase 701 (HDT701)* induces flowering in rice by modulating expression of *OsIDS*. *Mol. Cells* **2018**, *41*, 665–675. [\[PubMed\]](#)
40. Yoon, J.; Cho, L.H.; Kim, S.R.; Tun, W.; Peng, X.; Pasriga, R.; Moon, S.; Hong, W.J.; Ji, H.; Jung, K.H.; et al. CTP synthase is essential for early endosperm development by regulating nuclei spacing. *Plant Biotechnol. J.* **2021**, *19*, 2177–2191. [\[CrossRef\]](#)
41. An, G.; Ebert, P.R.; Mitra, A.; Ha, S.B. Binary vectors. In *Plant Molecular Biology Manual*; Kluwer Academic Publisher A3: Dordrecht, The Netherlands, 1988; pp. 1–19.
42. Jeon, J.S.; Lee, S.; Jung, K.H.; Jun, S.H.; Jeong, D.H.; Lee, J.; Kim, C.; Jang, S.; Lee, S.; Yang, K. T-DNA insertional mutagenesis for functional genomics in rice. *Plant J.* **2000**, *22*, 561–570. [\[CrossRef\]](#)
43. Yoon, J.; Cho, L.H.; Antt, H.W.; Koh, H.J.; An, G. *KNOX* protein *OSH15* induces grain shattering by repressing lignin biosynthesis genes. *Plant Physiol.* **2017**, *174*, 312–325. [\[CrossRef\]](#)
44. Yoon, J.; Cho, L.H.; Yang, W.; Pasriga, R.; Wu, Y.; Hong, W.J.; Bureau, C.; Wi, S.J.; Zhang, T.; Wang, R.; et al. Homeobox transcription factor *OsZHD2* promotes root meristem activity in rice by inducing ethylene biosynthesis. *J. Exp. Bot.* **2020**, *71*, 5348–5364. [\[CrossRef\]](#)
45. Haring, M.; Offermann, S.; Danker, T.; Horst, I.; Peterhansel, C.; Stam, M. Chromatin immunoprecipitation: Optimization, quantitative analysis and data normalization. *Plant Methods* **2007**, *3*, 1–16. [\[CrossRef\]](#)
46. Cohen, Y.; Cohen, J.Y. *Analysis of Variance, in Statistics and Data with R: An Applied Approach through Examples*; John Wiley & Sons Ltd.: Chichester, UK, 2008; pp. 417–461.
47. Emms, D.M.; Kelly, S. OrthoFinder: Phylogenetic orthology inference for comparative genomics. *Genome Biol.* **2019**, *20*, 1–14. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Peng, X.; Tun, W.; Dai, S.; Li, J.; Zhang, Q.; Yin, G.; Yoon, J.; Cho, L.H.; An, G.; Gao, L. Genome-wide identify genes contributing to photoperiodic flowering in *Oryza rufipogon*. *Front. Plant Sci.* **2021**, *12*, 736419. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [\[CrossRef\]](#)
51. Hong, W.J.; Kim, Y.J.; Kim, E.J.; Chandran, A.K.N.; Moon, S.; Gho, Y.; Yoou, M.H.; Kim, S.T.; Jung, K.H. CAFRI-rice: CRISPR applicable functional redundancy inspector to accelerate functional genomics in rice. *Plant J.* **2020**, *104*, 532–545. [\[CrossRef\]](#)
52. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant* **2020**, *13*, 1194–1202. [\[CrossRef\]](#)