

Ectopic Expression of *WUSCHEL* (*AtWUS*) Gene Alters Plant Growth and Development in Rice

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Abstract: Developmental genes (DG)/ morphogenic genes are involved in enhancing the transformation and regeneration of plants. One such DG is the *WUSCHEL* (*WUS*) gene, a homeodomain transcription factor, and is involved in the stem cell maintenance of shoot apical meristem (SAM). In dicots, ectopic expression of *WUS* induced the embryogenic calli formation and organogenesis. On the other, *WUS* overexpression resulted in pleiotropic effects in most of the monocots. Also, very few dicots failed to regenerate due to the overexpression of *WUS*. In our study, the 35S driven *WUS* (*AtWUS*) gene expressing transgenic rice plants were generated. All the transgenic plants with the *WUS* (W) gene along with the vector (V) and untransformed (U) lines were confirmed by detailed Southern analyses. The single-copy W and V plants with complete T-DNA were taken for detailed analyses. The W plants exhibited few phenotypic changes such as thick stem, reduction in the internode length, enclosed panicle, unopen flower, pale yellow colour of the anther, and loss of viable pollens compared to the U and V plants. Interestingly, crown root formation and small vein formation in the leaves were detected in the W plants. The expression of the *WUS* gene was confirmed by RT-PCR analysis in the W plants. The seeds from the hemizygous plants showed enhanced embryogenic calli formation and attained early regeneration compared to U and V plants thereby confirming the role of the *WUS* gene in embryogenesis and regeneration.

Keywords: *WUSCHEL*, Embryogenic Calli, Regeneration, Pleiotropic Effects, Organogenesis

1. Introduction

Developmental genes (DGs) also known as morphogenic genes are involved in plant transformation and regeneration through a variety of mechanisms such as embryogenesis and organogenesis [1]. Somatic embryogenesis and plant regeneration can be increased by the exogenous supply of hormones which would reprogramme the fate of the cell [2]. Previous reports revealed that many morphogenic/related genes/transcription factors (TF) reprogramme the cell fate to increase the transformation [3] and regeneration efficiency [4, 5] in plants. Many TFs are involved in each stage of embryogenesis such as callus induction, embryogenic calli formation [6, 7]. Similarly, many TFs play a major role in plant regeneration and determine the fate of the cell under epigenetic [8-11], hormone-induced [12], or stress-induced conditions [13, 14]. Among the different categories of the

TFs, the TFs encoded by homeobox genes play a major role in regulating plant development and determining the cell fate. These homeobox TFs have at least 2 domains: one for recognition and binding, and the other for organizing additional proteins involved in transcription. The *WUSCHEL*- related homeobox (*WOX*) gene family belongs to the homeobox TF superfamily. All the members in this family possess homeodomain (HD) with 60-66 amino acid residues and bind DNA through a helix-turn-helix (HTH) structure [15, 16]. The HTH domain of the *WUS* gene possesses a nuclear localization signal (NLS) that has a strong binding affinity towards DNA and regulates the gene expression precisely. The *WUS* gene that encodes an HD TF is required for shoot apical meristem (SAM) and contributes to plant development that includes embryo patterning, stem cell maintenance, and organ formation. In *Arabidopsis*, the embryonic cell identity was maintained by the *WUS* gene

[17, 18]. Upon mutation of the *wus* gene, *Arabidopsis* failed to maintain shoot and floral meristem (FM) [19, 20] while there is an increase in meristematic cells rather than differentiation. *WUS* plays a role in the maintenance of stem cells not only in SAM but also in FM [21]. In rice, the *WUS* orthologue is called *OsWUS* which plays a major role in the tillering and fertility of rice [22]. The *indica* rice variety has 12 *WOX* genes and *japonica* has 17 *WOX* genes. Other orthologues of *WUS* are *MONOCULM 3 (MOC3)* [23] and *TILLERS ABSENT1 (TAB1)* [24-26]. These orthologues differ in their functions like tillering, fertility, tiller bud formation, and axillary meristem formation. The undifferentiated state of SAM in *Oryza sativa*, *Arabidopsis thaliana*, *Antirrhinum majus*, and *Petunia* was maintained by the homeobox gene, *OsWOX4* [27], *WUS (WUSCHEL)* [20], *ROA (ROSULATA)* [28] and *TER (TERMINATOR)* [29], respectively.

Kamiya et al. [30] isolated the *QHB* gene, a *WUS* type homeobox gene to study the stem cell maintenance in root apical meristem (RAM). As there is no homology for the *AtWUS* gene in rice, they used rice actin driven *AtWUS* gene for comparison studies. The overexpression of *QHB* and *WUS* gene in rice revealed defects in crown root formation. The seedlings showed severe dwarfism with malformed leaves. The morphology of leaves and the roots were interrelated. The defects in the crown root formation tend to result in the formation of malformed leaves. These results confirmed that the *WUS*-type homeobox gene is involved in the maintenance of the stem cells in RAM, which is similar to the mechanism in SAM. In the case of tobacco, the overexpression of the *AtWUS* gene affected the seedling stage, and later ectopic outgrowth was found in laminae, stem, and leaf axils. At the flowering stage, no outgrowth was seen and resulted in shorter stigma, style, and filament compared to the wild type. Yet the ectopic organogenesis was obtained by *AtWUS* in tobacco transformants and played a role in leaf development. They provided the evidence that *WUS* gene played a role in organ formation apart from stem cell maintenance. The ectopic expression of inducible (estradiol) promoter-driven *AtWUS* gene in *Capsicum chinense* [31], and *Picea glauca* [32] resulted in embryogenic calli formation and increased seedling growth. Similar to an inducible promoter, constitutive (35S) expression of the *AtWUS* gene also increased the somatic embryo formation in *Coffea canephora* [33]. Many of the cotton cultivars were difficult to regenerate due to the failure of embryogenic calli formation [34]. The 35S driven *AtWUS* expression in *Gossypium hirsutum* enhanced the embryogenic calli formation. Of the three different stages of *in vitro* regeneration, the *WUS* gene expression was found to be in the first stage only. The calli size increased in stages 2 and 3. The increased *WUS* expression turned the calli greenish and the plants failed to regenerate as a result of *WUS* overexpression [35]. In recalcitrant cotton variety CRI12, the embryogenic calli differentiation was obtained by overexpression of the 35S promoter-driven *AtWUS* gene. Also, the *WUS* gene enhanced the expression of *LEC1 (LEAFY COTYLEDON)*, *LEC2*, and *FUS3 (FUSCA3)* during somatic embryogenesis [36]. *LEC1*, *LEC2*, and *FUS3* play a major role in embryogenic calli

formation [37, 38]. The soybean TF *GmWOX18* expression increased the clustered buds in genetic transformation and increased regeneration [39]. Overexpression of the *Chrysanthemum WUS* gene in *A. thaliana* led to the formation of polysymmetric flowers rather than monosymmetric flowers.

Apart from single gene expression, a combination of DGs would help in enhancing the transformation and regeneration in plants [1]. Combined expression of maize *WUS* and *BABY BOOM (BBM)* genes increased the monocot transformation under weak and strong promoter, respectively [3]. Upon constitutive expression in maize, the transformation efficiency increased and had some pleiotropic effects. The expression pattern of *BBM* and *WUS* genes were found to be enhanced at different developmental stages and also at specific tissues. When maize immature embryos were used as explants more somatic embryos were formed and the regeneration increased rapidly. Under auxin-induced promoter, the plants were healthier compared to non-inducible promoter expression [40, 41]. Meanwhile, Mookan et al. [42] also described that the combined expression of maize *BBM* and *WUS2* genes using cre-lox technology increased the monocot transformation in recalcitrant sorghum and maize inbred lines and obtained fertile plants. Coexpression of *WOX* genes (*WOX2* with *WOX8/WOX9*) also induced the genes involved in the regeneration of tobacco and increased the shoot like structures in tissue culture without the external supply of cytokinin [43].

Until now all these previous studies involved the controlled expression of DGs either by transient, inducible, or tissue-specific expression, and excision of DGs using cre-lox mechanism [5]. Upon constitutive expression of the *AtWUS* gene, the transgenic plants displayed pleiotropic effects. The rice actin promoter-driven *AtWUS* gene affected the rice seedlings and resulted in dwarfism along with malformed leaves and the absence of crown root formation. In this study, more emphasis was given to explore the role of the *WUS* type homeobox gene, *QHB* in RAM [30]. The expression of the *AtWUS* gene under the 35S promoter in *Arabidopsis* induced aberrant cell division at the calli stage but failed to regenerate [36]. In other plants, the 35S promoter-driven *WUS* gene enhanced the embryogenic calli formation. However, the role of *AtWUS* gene expression towards embryogenic calli formation and regeneration needs to be studied in detail to improve the transformation of recalcitrant cultivars. Towards that, we overexpressed 35S driven *AtWUS* gene in rice and generated transgenic plants. These transgenic plants were confirmed by detailed Southern analyses. The transgenic plants exhibited some morphological differences compared to control plants. The seeds from hemizygous single copy plants were evaluated for the role of embryogenic calli formation and regeneration.

2. Materials and Methods

2.1. Binary Vector and Rice Transformation

The *WUSCHEL (AtWUS)* gene was taken as a *SpeI/XhoI*

fragment (1.1 kb) from pER10 (purchased from Dr. Nam-Hai Chua, Rockefeller University, USA) and cloned into *XbaI/XhoI* site in between 35S promoter and polyA in pRT100. The resultant clone was designated as pMG1. The whole cassette was taken as a *SphI* fragment and treated with the Klenow enzyme. The Klenow treated fragment was cloned into the *SmaI* site of pCAMBIA1301 and the resultant clone was designated as pMG2 (Figure 1). The binary vector, pMG2, and pCAMBIA1301 were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (pSB1) by triparental mating individually and confirmed by Southern analyses. Rice seeds (*Oryza sativa* cv IET10364) were surface sterilized [44] and placed in the callus induction medium. The 21-day-old scutellum derived embryogenic rice

calli were used for transformation and pre-incubated for 3 days. Rice transformation was performed using the *Agrobacterium* strain LBA4404 (pSB1, pMG2) and LBA4404 (pSB1, pCAMBIA1301), with appropriate controls. After 3 days of co-cultivation, the infected calli were placed on the selection medium harbouring 50 mg/l hygromycin and 250 mg/l cefotaxime for 14 days. Subsequently, after two rounds of selection (21 days for each selection), the calli were placed on to the regeneration medium containing 40 mg/l hygromycin and 250 mg/l cefotaxime [45]. GUS staining [46] was performed with the calli and leaves to identify the putative transformants. The regenerated plantlets were acclimatized to soil and maintained in the transgenic greenhouse till maturity.

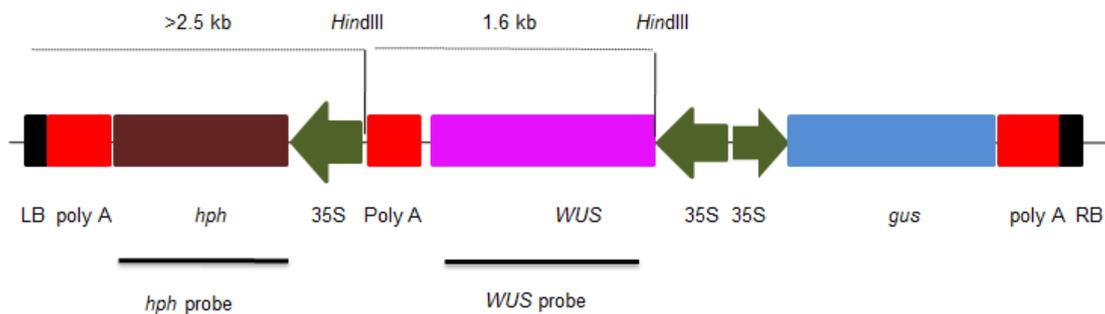


Figure 1. Linear map of pMG2. The T-DNA portion of the binary plasmid pMG2 harbours p35S-hph gene in the HindIII site of pCAMBIA1301. Left border junction fragments (>2.5 kb) generated by HindIII digestion are indicated by a line. 35S – CaMV35S promoter, LB – Left border, hph – hygromycin phosphotransferase, WUS – wuschel, gus – β -glucuronidase gene, RB – right border.

2.2. PCR Analyses

Genomic DNA from untransformed (U), vector (V), and WUS (W) transformed plants were extracted using the CTAB method [47] and quantified by fluorimeter [48]. Genomic DNA (100 ng) was used as a template and PCR analysis was performed using *gus*, *hph*, and *WUS* gene-specific primers. The *gus* fragment (0.5 kb) was amplified using the primers: (5' TATCAGCGCGAAGTCTTTATACC 3') and (5' CAGTTGCAACCACCTGTTGAT 3') with the following condition [94°C for 2 min followed by 30 cycles (94°C for 30 sec, 58°C for 40 sec, 72°C for 45 sec) and final extension with 72°C for 6 min]. The *hph* fragment (1.1 kb) was amplified using the primers: (5' AAAGCCTGAACTCACCGC 3') and (5' GGTTTCCACTATCGGCGAG 3') with the following conditions [94°C for 1 min followed by 30 cycles (94°C for 1 min, 56°C for 1 min, 72°C for 1 min) and final extension with 72°C for 6 min]. The *WUS* fragment (0.83 kb) was amplified with the primers: (5' CATCAAGCCGACCAAGAAAGC 3') and (5' TAGCTCAAGAGAAGCGCAAGG 3') with the following conditions [94°C for 1 min followed by 30 cycles (94°C for 40 sec, 60°C for 30 sec, 72°C for 1 min) and final extension with 72°C for 5 min].

2.3. Southern Blot Analyses

Five micrograms of DNA from U, V, and W plants were digested with the enzyme *HindIII* and electrophoresed. The *hph* and *WUS* probes were labeled with the digoxigenin and non-radioactive Southern [49] was performed following the

manufacturer's instruction (Roche, Germany).

2.4. Segregation Analyses

The T₀ seeds were surface sterilized and placed in half MS medium [50] for 3 days. After three days the germinated seedlings were transferred to MS medium harbouring 50 mg/l hygromycin and scored for hygromycin resistance. A Chi-square test was performed to validate statistically hygromycin sensitive and resistant plantlets. Further GUS staining was used to identify the zygosity in T₁ and T₂ lines.

2.5. Leaf Development and Pollen Staining

Leaf development was studied at regular intervals in the U, V, and W lines. Mature pollen from untransformed (U), null (N), vector (V) and WUS (W) plants were taken for iodine staining (I₂-KI) [51] and the stained images were visualized under Nikon light microscopy [Nikon (C-DSS230) microscope, Tokyo, Japan].

2.6. RT-PCR Analysis

The total RNA from U, V, and W plants were extracted, estimated, and subjected to DNase treatment [52]. Two-step RT-PCR kit (TaKaRa, Kyoto, Japan) was used to synthesize cDNA from DNase treated RNA samples. In the first step, 1 µg of RNA was taken along with oligo dT and dNTPs and kept at 65°C for 5 min for the first-strand synthesis. Further RNase inhibitor, RTase, and 5X RT buffer were added and kept at

42°C for 30 min and 95°C for 5 min for cDNA synthesis. The cDNA (100 ng) was used for *OsACTIN* and *AtWUS* PCR. The actin fragment was amplified using forward primer (5'CTTGCTGGCGTGATCTCA 3') and reverse primer (5' CAGGGCGATGTAGGAAAGCT 3'). The following condition was used to amplify the actin fragment: [94°C for 2 min followed by 35 cycles (94°C for 40 sec, 55°C for 40 sec, 72°C for 1 min) and final extension with 72°C for 6 min]. The *WUS* cDNA was amplified by the forward primer 5'TATATGGCGGCTAACGA3' and reverse primer 5' GATGATAGAGATGATGGTCTTGG 3'. The following condition was used to amplify the *WUS* fragment: [94°C for 1 min followed by 30 cycles (94°C for 40 sec, 60°C for 30 sec, 72°C for 1 min) and final extension with 72°C for 5 min].

3. Results

3.1. Generation of T_0 Transgenic Plants

Agrobacterium strain LBA4404 (pSB1) harbouring the binary vector, pMG2 (Figure 1), and vector (pCAMBIA1301) were used for rice transformation. After two rounds of selection, a portion of the calli was used for GUS staining (Data not shown). The putative *gus* positive transgenic calli were transferred to the regeneration medium. Sixteen *WUS* (*W*) plants were obtained from two batches of transformation. A portion of the leaf was subjected to GUS staining (Data not shown). The GUS positive putative transformants were transferred to the transgenic greenhouse and maintained till maturity. The PCR performed in the six *WUS* (*W*) and five

vector (*V*) plants confirmed the presence of 1.1 kb *hph* amplicon, and 0.5 kb *gus* gene (data not shown).

3.1.1. Transgene Integration and Copy Number Determination

Six *W* plants and five *V* plants obtained from the first batch of transformation were taken for detailed analyses. All the six putative transgenic plants (*V* and *W*) were digested with *Hind*III and probed with *hph* (LB probe) to determine the junction fragments and the copy number. All the *V* and *W* plants hybridized fragments >2.5 kb except in plants (*W*3 and *V*10). Five vector plants (*V*10, *V*12, *V*13, *V*14, and *V*16) and 6 *WUS* transgenic plants (*W*4, *W*5, *W*6, *W*7, *W*17, and *W*18) possessed single-copy T-DNA integration (Figure 2A). The T_0 plants, *W*5 and *W*6 might be siblings as they possessed similar junction fragments for the LB probe. The *W* transgenic plants were subjected to Southern analyses using the *WUS* gene (1.6 kb) as a probe. Upon *Hind*III digestion, a 1.6 kb internal fragment of the *WUS* gene is expected to be hybridized for the *WUS* probe. Among the *W* plants, 5 plants (*W*3, *W*4, *W*7, *W*17, and *W*18) hybridized to *WUS* fragment (1.6 kb) (Figure 2B).

In the case of *W*5 and *W*6, the T-DNA might be truncated at the RB lacking the *WUS* gene and possess the LB with the *hph* gene. This is further evident from the PCR analyses using *WUS* primers. Both the plants revealed a higher sized amplicon apart from the expected 0.83 kb (Data not shown). This may be due to the presence of truncated T-DNA at the LB. Henceforth, further analyses were carried out only with the single copy plants (*W*4, *W*7, and *V*13) harbouring complete T-DNA.

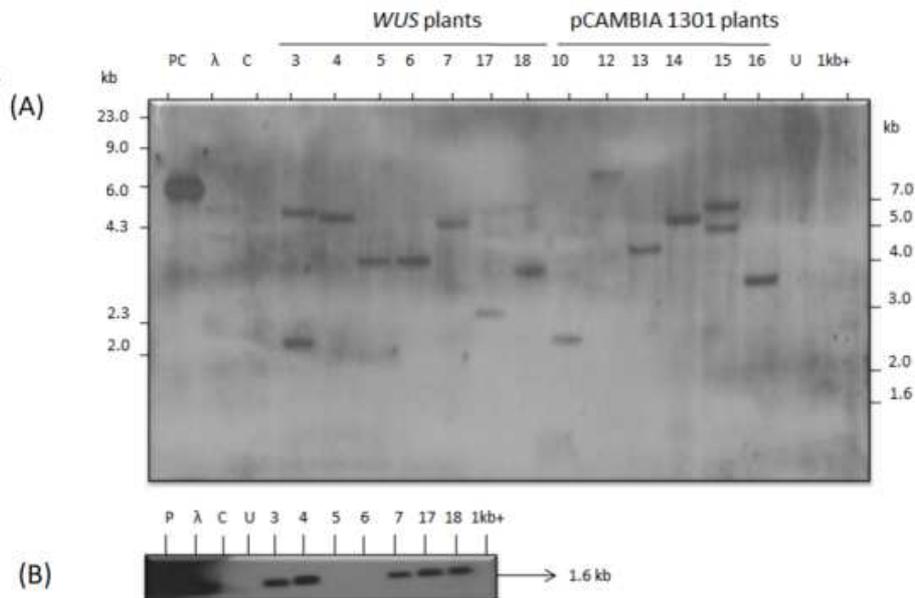


Figure 2. Southern analysis of T_0 rice plants transformed with *A. tumefaciens* LBA4404 (pSBI, pMG2) and LBA4404 (pSBI, pCAMBIA1301) using (A) *hph* as the probe and (B) *WUS* as the probe. Numbers on the top refers to T_0 lines. Five μ g of plant DNA was digested with *Hind*III and electrophoresed. C – DNA from untransformed control plant digested with *Hind*III, U – Undigested DNA from T_0 plant, 1 kb+ – 1 kb+ ladder, PC – Positive control, P – Probe.

3.1.2. Morphology of T_0 Plants

The control (*U*) plants and putative transgenic lines, *V* and *W* were acclimatized in the transgenic greenhouse. The *U*

and *V* plants started flowering after 60-70 days while the *W* transgenic plants were still in the vegetative stage with faded leaf color (Figure 3A). The *W* transgenic plants started flowering only after 90-120 days. They also exhibited a

panicle enclosed phenotype. As the T₀ plants were hemizygous, few of the panicles were found to be closed (Figure 3B). The heading date was found to be increased in

W plants compared to U and V plants (Figure 3C). The total number of seeds obtained from the T₀ transgenic plants of W was less (Figure 3C) compared to U and V plants.

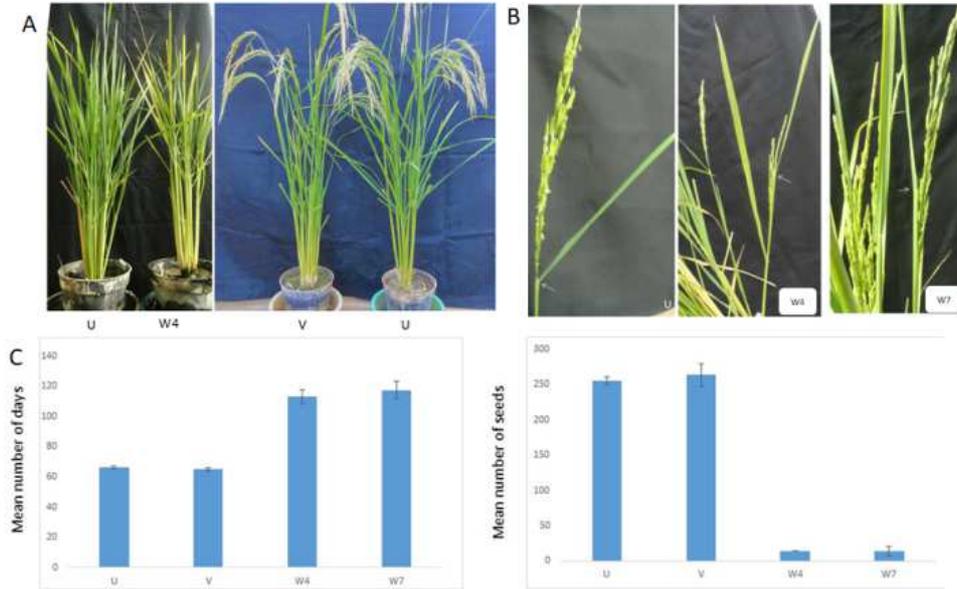


Figure 3. A. Morphology of T₀ WUS line 4 (W4) compared to untransformed (U) and vector (V), B. Panicle enclosure type in U and two W lines (W4 & W7) (arrow indicates the collar), C. Analyses of heading date and seed formation in U, V and two W lines (W4 & W7).

3.1.3. Inheritance of Transgene

The seeds from two T₀ plants, W4 and W7 along with V13 were scored for hygromycin resistance and the data were statistically validated by the χ^2 test. All the T₁ lines (V13, W4, and W7) revealed a 3:1 segregation ratio thus confirming the inheritance of single-copy T-DNA at a single locus (Table 1). Ten T₁ transgenic plants from W4 and eight transgenic plants from W7 were taken for further homozygosity analysis. Of these, only 3 plants (W4-1, W4-10, and W7-2) had more than 20 seeds (Table 2). For further validation, 12 T₁ plants from line W4-10 along with 3 null (N), and U plants were analyzed (data not shown). There was no difference between U, V, N, and W plants during the vegetative stage until 60 days. After 60 days the stem was hollow in U and V plants (data not shown). On the other, the stem was still thick (vegetative) in the W plants (Figure 4).

The vegetative stage of W plants was found to be increased than the U, and V plants (Data not shown), and there is the inheritance of the panicle enclosed phenotype in W lines (Data not shown). Such a panicle enclosed phenotype might have been due to the defective internode elongation (Data not shown). The loss of panicle exertion affected the panicle morphology (Figure 5A) and it subsequently affected the grain numbers also to a greater extent (Figure 3C). Seed setting (Figure 6B) was highly affected in W plants due to the presence of an unopen flower (Figure 6A) and defective pollen. The WUS expressed T₁ plants, W4-6, and W4-9 were screened for anther color and pollen viability (Figure 6D). The anther color and its morphology under a light microscope revealed pale yellow compared to yellow color in control (Figure 6B). The difference in the anther color intensity was also confirmed using ImageJ software (Figure 5B).

Table 1. Segregation analysis of T₀ lines.

| T ₀ plant line no. | Total no. of seeds | GUS ⁺ ve | GUS ⁻ ve | χ^2 (3:1) | P-value |
|-------------------------------|--------------------|---------------------|---------------------|----------------|---------|
| W4 | 20 | 16 | 4 | 0.27 | >0.60 |
| W7 | 40 | 34 | 6 | 2.13 | >0.14 |
| V13 | 40 | 31 | 9 | 0.13 | >0.72 |

Table 2. Segregation analysis of T₁ plant seeds harbouring WUS gene.

| T ₁ WUS plant no. | Total no. of seeds | GUS ⁺ ve | GUS ⁻ ve | χ^2 (3:1) | P-value |
|------------------------------|--------------------|---------------------|---------------------|----------------|---------|
| W4-1 | 23 | 18 | 5 | 0.13 | >0.72 |
| W4-10 | 33 | 24 | 9 | 0.09 | >0.76 |
| W7-2 | 20 | 12 | 8 | 2.40 | >0.12 |
| W7-9 | 16 | 11 | 5 | 0.02 | >0.89 |

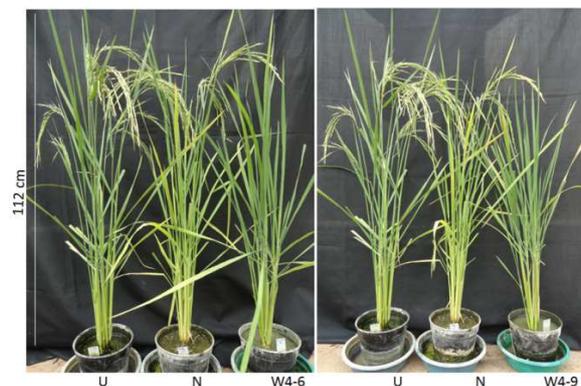


Figure 4. Morphology of untransformed (U), vector (V), and WUS (W) lines.

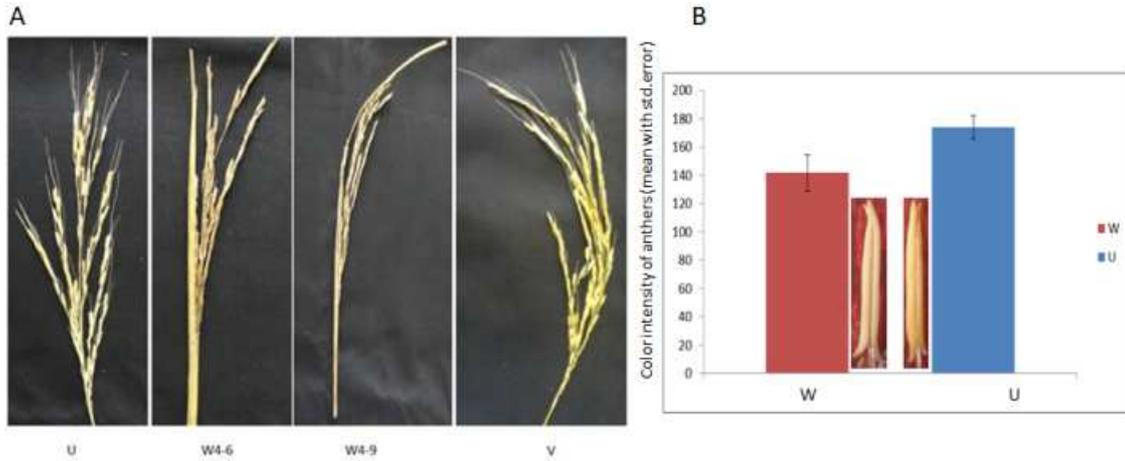


Figure 5. A. Disorganized panicle structure in T1 *WUS* lines (W4-6 and W4-9), B. Measurement of colour intensity of anthers using Image J software in W and U plants.

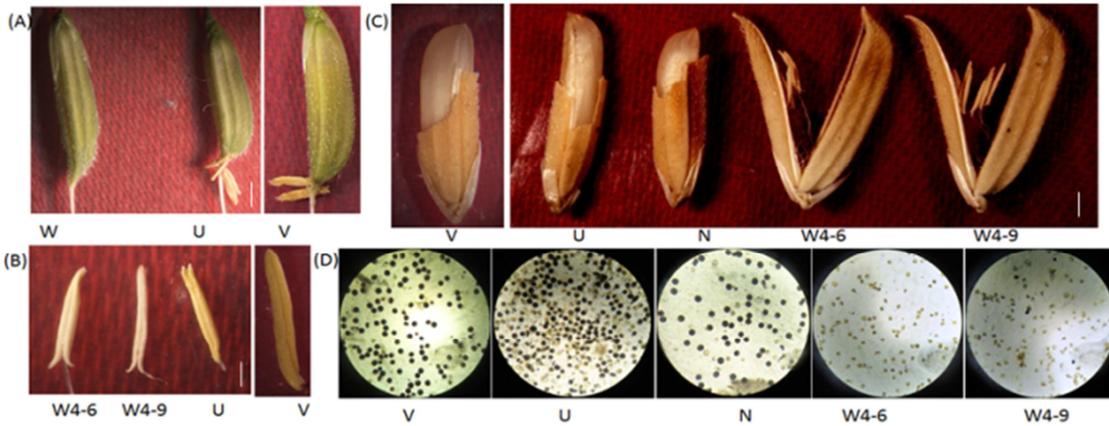


Figure 6. Phenotypic characteristics of (A) individual spikelet (unopen), (B) pale yellow color anther, (C) Seed setting and (D) pollen viability of untransformed (U), null (N), vector (V) and *WUS* (W) Lines (W4-6, W4-9) (Bars – A – 10 mm, B – 2 mm, C - 100 mm, D – 10X magnification).

Among the T₁ plants of W4, only three plants (W4-6, W4-7, and W4-9) lost pollen viability and there were no seeds. These 3 plants were completely abnormal in phenotype and those might be homozygous plants due to the expression of *WUS* at both locus. The homozygosity was further confirmed by the RT-PCR by analyzing two homozygous plants along with the hemizygous and U plants (Figure 7C). The cross-section of 20 days old leaf sheath revealed mature vascular bundles that might have been arisen earlier than the U plants (data not shown). Besides, the leaves of W plants possess one smaller vein which was absent in the U and V plants (Data not shown). The RT-PCR analysis revealed that the expression of *WUS* in the leaves (Figure 7A).

3.2. Embryogenic Calli Formation and Regeneration

The *WUS* expression in homozygous plants affected the panicle phenotype and pollination. The RT-PCR result confirmed the *WUS* gene expression in young panicles of W4-6 compared to the U and V plants (Figure 7B). Hence, there was no seed formation in homozygous plants. Due to the failure in generating homozygous plants, the hemizygous plants were subjected to callus induction and regeneration.

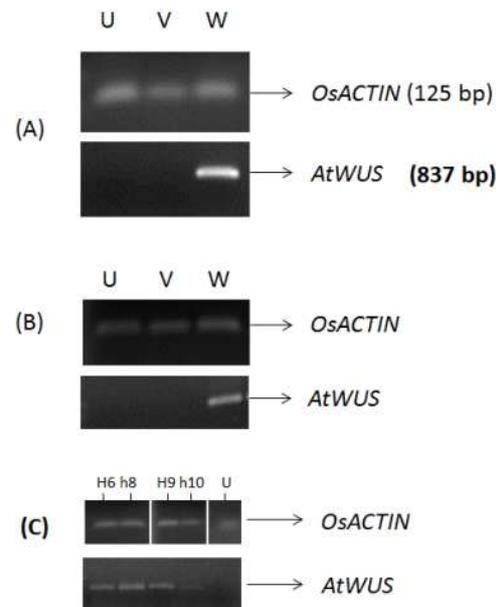


Figure 7. RT-PCR expression of the leaf (A) and panicle (B) (less than 1.5 cm) from U, V, and W plants (C) RT-PCR expression in homozygous (H), hemizygous (h) and U plants.

The seeds from two hemizygous lines (W4-10-5 and W4-10-11) along with U and V lines were evaluated for the embryogenic calli formation and regeneration. The T₁ hemizygous seeds were surface sterilized, kept in dark for 21 days, and observed at regular intervals for embryogenic structures. The number of seeds from W lines in generating embryogenic calli were not statistically different from the control seeds (data not shown). The only difference was that

the calli from W lines were whitish with more embryo-like structures compared to U plants. The calli from W lines turned greenish (Figure 8A) earlier than the U and V lines. Further, the transition to greenish like structures from the embryogenic calli was found to be higher in W lines compared to U and V plants. The shoots obtained from the W callus were numerous compared to the U callus (Figure 8B).



Figure 8. Embryogenic calli regeneration under the microscope (A) and multiple shoot formation in W plants compared to U plants.

4. Discussion

One of the major hurdles in transgenic technology includes the regeneration potential is difficult in recalcitrant crops. Efficient plant regeneration can be achieved via somatic embryogenesis and organogenesis. Nowadays, basic research on the above well-defined areas is expanding. The role of the DGs and their network involved in morphogenesis are explored in detail. But the detailed characterization of such DGs includes either ectopic overexpression or downregulation in plants. The overexpression of DGs had resulted in deleterious pleiotropic effects, it's of utmost importance to understand the negative impact of overexpression of DGs [53]. One such DG is *AtWUS*, a master regulator in plant growth signaling. The overexpression of *AtWUS* led to embryogenic calli formation, vegetative to embryonic transition, delay in heading date and flowering, and panicle enclosure phenotype.

4.1. *AtWUS* Gene Promotes Embryogenesis and Regeneration

Somatic embryogenesis is an important tool for crop improvement and depends on calli induction and

regeneration. The embryogenic calli and the subsequent regeneration depends upon the genotype, preculturing, hormonal conditions, and methods of transformation [54]. Three types of calli were developed in callus induction, embryogenic, non-embryogenic, and rhizogenic. Only embryogenic or friable embryogenic calli has the potential to regenerate through somatic embryogenesis [55]. Such embryogenic calli formation requires plant growth regulators, carbon sources, and basal media. Apart from the external sources, several genes need to be activated at the seed level to promote somatic embryogenesis [56-58] as evident in *Arabidopsis*. The *WUS* gene expression increased the embryogenic calli formation in *G. hirsutum* [36], embryo-like structures in *Arabidopsis* [17], and ectopic changes in the calli of *C. chinense* [31]. In our results, the calli obtained from T₁ seeds (hemizygous) of W plants tend to be embryogenic compared to U and V plants (data not shown). Ectopic organogenesis was also increased in *G. hirsutum* [35]. The regeneration ability of the calli was also increased by more embryogenic calli formation [40]. In our study, the regeneration of the embryogenic calli was enhanced compared to U and V plants. Also, the calli turned greenish and generated multiple shoots compared to U and V plants. Our study also confirmed the ectopic organogenesis (Figure

8B) in rice, as evident in other plants upon *WUS* overexpression.

4.2. Overexpression of *AtWUS* Increased the Vegetative Development

The *WUS* overexpression in rice under the actin promoter resulted in the small and twisted leaf [30]. Apart from *WUS*, *WOX* genes also play a role in somatic embryogenesis, regeneration from leaf segments, early and lateral leaf development. Among the *WOX* genes, overexpression of the *WOX3* gene induced the expression of *KNOX* genes at embryo development and inflorescence meristem. The *WOX3* overexpression altered the leaf morphology while *WOX4* failed to develop vegetative meristem revealed by RNAi studies [27]. *WOX3* is highly conserved in gymnosperms, basal angiosperms, eudicots, and monocots. In rice, *WOX3* plays a role in leaf development [59]. *WOX4* is also highly conserved in *Arabidopsis* and rice and strongly expressed in leaf primordia while the lateral organs showed reduced expression [27]. Previously in tobacco, the *WUS* expression altered the leaf structure, conjoined leaves, and vein pattern [60]. In our study, overexpression of the *AtWUS* gene increased the leaf primordia in the leaf sheath and increased the matured vascular bundle in leaf. Interestingly, one smaller vein was additionally evident in the leaves of W plants while it was absent in U and V plants. From our studies, the role of the *WUS* gene plays a role in vein formation apart from leaf development (Data not shown). Apart from leaf, the tiller was cross-sectioned at 60 days to check any alteration in the vegetative development. The results showed the W plants were not yet bolted compared to U and V plants (Figure 4).

4.3. *AtWUS* Overexpression Leads to Delayed Heading Date and Flowering

Heading date was genotype-dependent and it is controlled by basic vegetative growth and photoperiod sensitivity in *japonica* rice variety [61]. Many genes were involved in regulating the heading date in rice by complex interactions starting from the vegetative stage to the flowering stage. The gene *OsId1* (Indeterminate1) in rice is essential to activate either *Hd1* (Heading date 1) or the *Ehd1* (Early HD1), which will activate the expression of *Hd3a* and *RFT* genes [62]. The *ehd1* mutation affected the expression of *Hd3a* and also the floral transition. Hence it plays a significant role in floral meristem formation and its development [63]. The reduction in internode is associated with the reduced synthesis of IAA and GA resulted in panicle enclosure. The enclosed panicle affects the grain number due to the loss of pollen fertility. The delay in heading date affected the yield rate in different cultivars of rice in China [64]. In our results, both the T₀ plants (Figure 3A) and T₁ plants (Figure 4), the heading date (Figure 3C) was delayed. As a result, the numbers of seeds were decreased in W plants compared to U and V lines. Also, *WUS* expressed lines possessed a tightly packed stem rather than the normal one (data not shown). Overall our results revealed that the expression of the *WUS* gene (Figure 7B) is

associated with the delay in heading date, increased vegetative growth, and the severe reduction in internode elongation length (Data not shown).

4.4. Panicle Enclosure

Panicle enclosure refers to the enclosure of the panicles either partly or fully with the flag leaf sheath. This phenotype is commonly found in almost all CMS (cytoplasmic male sterile) lines. This phenotype seriously affected the hybrid rice production as the normal pollination is affected in rice. The level of the gibberellic acid A₁ (GA₁) played a major role in internode elongation in rice [65]. The GA1 deficiency in the uppermost internode (UI) leads to the decreased synthesis of the indole acetic acid (IAA) resulted in a panicle enclosure phenotype [66]. Panicle enclosure was also studied in mutants of rice *shortened uppermost internode 1 (sui1-1 and sui1-2)* plants which showed partly sheathed and fully sheathed panicle respectively. Gibberellin treatment also not increased the *SUI* due to the decreased longitudinal cell length in rice [67]. Panicle enclosure affected the panicle morphology. Previously the allele, *PANICLE PHYTOMER 1 (PPI)* upon mutation affected the panicle structure with increased primary rachis in two different rice cultivars in Japan [68]. In our study, the number of altered branches emerging out of the sheath was noted in *WUS* transgenic plants (W4-6 and W4-9) not in U and V plants (Figure 5A). Also, the *WUS* gene expression (Figure 7) affected the internode length that leads to the panicle enclosure phenotype (data not shown). Hence the further developmental stages were affected.

Panicle enclosed phenotype affected the pollination severely [67]. Our results also confirmed that severity in the phenotype affected the pollination and resulted in unopen flower morphology (Figure 6A). The anthers were not able to fertilize due to the pale yellow color of the anther (Figure 6B). Pale yellow color anther lacks mature pollen [67]. The pale yellow color anther was measured and confirmed by ImageJ software (Figure 5B). The defect in the pollination led to a reduction in the seed formation in T₀ plants. In our study, the T₁ plant seeds were screened to identify the homozygous lines. No homozygous plant was identified during the inheritance of *WUS* until T₂ generation. The expression of the *WUS* gene was confirmed in the hemizygous and homozygous plants (Figure 7C). The hemizygous plants also showed a delay in the heading. The N plants were checked whether the integration of T-DNA or the expression of *WUS* affected the plant development. However, the N plants were normal like the U plants. Among the 12 *WUS* plants, 3 plants did not set seeds due to the panicle enclosed phenotype and defective flowers. The T₁ hemizygous plants only produced seeds and were less in number compared to U, V, and N plants. Thus, we conclude that those 3 plants with defective seeds might be homozygous due to the expression of *WUS* at both the loci. The higher level of *WUS* expression affected the homozygous rice plants at all vegetative (Figure 7A), inflorescence (Figure 7B) and flower development stages, and lacked the seed formation.

5. Conclusion

The efficient transformation relies on embryogenic calli formation and regeneration through somatic embryogenesis. Many morphogenic genes were involved in somatic embryogenesis. Identification of such morphogenic genes would be a useful tool in improving transgene technology. Many reports revealed that timely expression of DGs would be useful in improving transformation in recalcitrant crops. Our study provides evidence that the *AtWUS* gene under constitutive promoter in rice tends to turn the calli embryogenic and enhanced the transition towards regeneration. However, the ectopic expression of the *AtWUS* gene in rice led to the pleiotropic effects such as tightly packed stem, panicle enclosure phenotype, delay in heading date, pale yellow color anther, defective in seed setting and pollen viability. Interestingly, the overexpression did not affect the crown root formation (Data not shown). The *AtWUS* overexpression increased the regeneration at the embryogenic level in cotton, *Capsicum*, and *Arabidopsis*. Our results also revealed that the ectopic expression led to increased somatic embryogenesis and attained early regenerative phase irrespective of all the pleiotropic effects.

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