Evaluating the efficacy of *Agrobacterium tumefaciens* mediated transformation in recalcitrant crops

Synopsis submitted to Madurai Kamaraj University

for the degree of

DOCTOR OF PHILOSOPHY

By

T. VICTORATHISAYAM

Reg. No. F9440

Department of Plant Biotechnology

School of Biotechnology

Madurai Kamaraj University

Madurai- 625 021

INDIA

November 2020

Evaluating the efficacy of *Agrobacterium tumefaciens* mediated transformation in recalcitrant crops

SYNOPSIS

Introduction

The Rice genome has been completely sequenced in 2005. The size of the rice genome is 430 Mb. There are three rice varieties available such as *japonica*, *indica*, and *javanica*. Among the varieties, *japonica* and *indica* were the most commonly used rice varieties for the crop improvement program. The crop improvement was successfully achieved with the *Agrobacterium tumefaciens* mediated transformation. The introduction of a foreign gene by *A. tumefaciens* is a routine method in dicots. Monocot plants lack the wounding signal to attract *Agrobacterium*, hence it lacks the T-DNA transfer mechanism. However, with the aid of acetosyringone, the monocot transformation was successful in a few *japonica* and *indica* varieties. The transformation efficiency was found to be less in *indica* varieties compared to the *japonica* varieties due to its recalcitrance nature. With the identification of *rat* and *hat*, mutant studies scientists found the plant proteins play a role right from *Agrobacterium* attachment till the T-DNA integration. Even the plant defense pathway was also elicited upon *Agrobacterium* attachment.

Later Tie et al. (2012) with the advent of microarray technology characterized the genes from *japonica* and *indica* varieties and its expression from 0 h to 48 h during infection. Upon attachment initially, the plant defense was activated in *japonica* variety but immediately within 6 h the defense pathway was reduced. In the case of *indica* variety, the plant defense was constitutively active from the 0 h to 48 h. This is one of the major reasons for the increased transient transformation efficiency in many *indica* varieties than the *japonica* varieties. Other limiting factors for transformation were the type of tissue, strain, binary vector, and other tissue culture factors. To increase the transformation efficiency either we have to defeat the

limiting factors by optimizing the protocols for transformation or reduce the plant defense proteins or plant defense activation by overexpressing plant proteins. Such overexpressed plant proteins have been reported and discussed in detail. Previously, Sardesai et al. (2007) found that the *UGT* gene activation by T-DNA tagging of mutating cellulose synthase gene might increase the *Agrobacterium* transformation. Other reports revealed that glycosylation of small lipophilic molecules reduces the plant defense activation signaling. Hence the *UGT* gene was explored in detail for increasing the transformation efficiency.

Another important barrier in the *Agrobacterium* transformation was the less regeneration of plants. The overexpression of plant genes renders the plant tissues susceptible to the plant transformation by altering the texture of the explant. The texture of the explant was changed by producing more somatic embryos with regeneration potential. Somatic embryogenesis (SE) is important for inducing the embryogenic calli formation. Friable embryogenic calli are the prerequisite for the regeneration of lateral aerial parts. To induce the embryogenic calli many plant genes play a major role. The regeneration of lateral organ development requires proper SAM/RAM development. The maintenance of proper SAM/RAM requires several homeotic genes and transcription factors. The overexpression of homeotic genes or transcription factors increases the plant regeneration ectopically. The misregulation of homeotic or transcription factors in the SAM/RAM affected the plant regeneration and also the proper development.

Homeotic genes are otherwise called morphogenic genes or developmental genes. These developmental genes have the ability to change the fate of the cell. The well-known developmental genes are *WUSCHEL, LEAFY COTYLEDON, BABY BOOM,* and *MYOBLASTOSIS*. All the above genes are the master regulator, which controls the other genes either positively or negatively. The controlled expression of these developmental genes is essential for the maintenance of the SAM and organogenesis. Sometimes these developmental genes were controlled by the transcription factors. Hence transcription factors are also involved in plant growth and development.

The regeneration was increased by many master regulators, homeotic genes, and transcription factors (TFs). The master regulators, *WUS, LEC, BBM,* and *MYB* genes are involved in regulating plant growth and development. The plant regeneration fate was determined by either epigenetic, hormone, or stress-related factors. To modify the plant cell fate, overexpression of many heterologous genes is reported to increase the regeneration efficiency. Some seed maturation genes also play a role in morphogenesis.

Apart from the growth and development *UGT* and *MYB* genes are involved in biotic and abiotic stresses. The other classes of *UGT* genes involved in the bacterial and fungal resistance were reported in wheat and other monocots. The *UGT* gene is involved in the secondary metabolic pathway. Since it is involved in phenylpropanoid metabolism and its steps, the *UGT* gene was validated in disease resistance against the fungi, *Rhizoctonia solani*. In the case of MYB, many R2R3 genes were reported in fungal resistance. The reason is *MYB* involved in the glucosinolate pathway. Glucosinolate imparts broad fungicidal activity. Also, *MYB115* and its close relative *MYB118* gene evolved a new pathway benzyloxy glucosinolate pathway. As a result, the role in contributing resistance was evaluated for sheath blight resistance causing fungi, *R. solani*.

Summary of findings reported in the thesis

TRANSFORMATION EFFICIENCY

1. Overexpression of *Arabidopsis thaliana UGT* gene in rice

The initial findings of Sardesai et al. (2007) prompted us to propose a hypothesis. They found eight *hat* mutants and 5 of them were isolated based on the T-DNA/junction fragments. One among the five mutants, *hat1* contains T-DNA insertion in the 7^{th} exon of a gene encoding cellulose synthase-like protein (CSL). The downregulation of this gene highly up-regulated the nearby *UGT* (UDP-glucosyl transferase) gene. Upon overexpression of this *UGT* gene, the root transformation was increased in *Arabidopsis.* The *UGT* gene was amplified from the *Arabidopsis* and confirmed by sequencing. Previously in our lab, the *UGT* gene was cloned into a

binary vector, pCAMBIA1301, and confirmed with multiple restriction analyses. The binary clone was also mobilized into the *Agrobacterium* strain LBA4404 (pSB1) and confirmed by Southern analysis. Earlier in our lab pCAMBIA1301 was mobilized into the *Agrobacterium* strain LBA4404 (pSB1) and confirmed by Southern analysis for vector transformation.

The *Agrobacterium* strain LBA4404 (pSB1, pVENKAT3) and LBA4404 (pSB1, pCAMBIA1301) were used for rice transformation (two batches independently). GUS staining was performed to identify the putative transgenic plants in *UGT* and vector plants. Out of 28 *UGT* plants only 13 plants were taken for analysis and 12 plants showed *hph, gus,* and *UGT* PCR amplification. In the case of vector, among 13 plants only the first 8 plants were taken for analysis and all the plants were showed amplification for *hph* and *gus* gene. The *UGT* transformation efficiency was 3% higher than the vector. All the 12 UGT and 8 vector plants were subjected to Southern analysis. All the *UGT* plants possessed a single copy except plant no. 15 (double copy). In the case of vector, 5 plants possessed a single copy and the remaining were two copy plants. Based on the banding pattern, the *UGT* and vector plants have 10 and 4 independent transgenic events. Of this, only the single copy UGT1 and V4 were taken for T_1 and T_2 analyses. The semi-quantitative Southern analysis and GUS staining results showed that UGT1-1 and V4-10 were homozygous. Supertransformation was performed with the *Agrobacterium* strain, LBA4404 (pSB1, pCAMBIA3300) in the homozygous lines (UGT1-1 and V4-10) along with control. The transformation efficiency increased 4-fold in the UGT line compared to V and control. The bioassay was performed against *R. solani* and validated for disease resistance. However, the *UGT* gene did not play a role in fungal resistance.

REGENERATION EFFICIENCY

1. Overexpression of *Arabidopsis thaliana WUS* gene in rice

The *Agrobacterium* strain LBA4404 (pSB1, pMG2) and LBA4404 (pSB1, pCAMBIA1301) were used for rice transformation (two batches independently). GUS staining was performed to identify the putative transgenic plants in *WUS* and vector plants. Out of 16 *WUS* plants, only 7 plants were taken for analysis. All 7

plants showed *gus* amplification, 5 plants showed *hph* amplification and 4 plants showed *WUS* PCR amplification. In the case of vector among 10 plants, all the plants were showed amplification for *gus* and 6 plants showed amplification for the *hph* gene. We took 5 *WUS* and 6 vector plants for Southern analysis. Only 4 *WUS* plants possessed a single copy and except plant no 3 (double copy). In the case of vector 5 plants possessed a single copy and the remaining was a double copy. Based on the banding pattern *WUS* and vector plants have 3 and 5 independent transgenic events. The T_0 plants showed pleiotropic effects under a constitutive promoter. Of this only the single copy W4, W7 and V13 were taken for further analysis. The pleiotropic effects include, delayed heading date, enclosed panicle, and less seed setting. Hence we obtained a fewer number of seeds in W4 and W7. The 12 T_1 plants were taken for analyses for identifying a homozygous plant. Among 12 plants 3 plants were completely affected by delayed heading date, enclosed panicle, unopen flower, pale yellow colour anther, defective pollen, and seed setting. The other 9 plants have less number of seeds like T_0 plants. Hence, the three plants (W4-10-6, W4-10-7, and W4-10-9) might be homozygous and the remaining were hemizygous. Due to a defective seed setting, the regeneration efficiency was not validated in homozygous *WUS* plants. The regeneration efficiency was validated by W4-10-5 and W4-10-11 hemizygous seeds. The regeneration efficiency was found to be increased under the hemizygous condition than the control.

2. *MYB115*

The *Agrobacterium* strain LBA4404 (pSB1, pKS4) and LBA4404 (pSB1, pCAMBIA1301) were used for rice transformation (three batches independently). GUS staining was performed to identify the putative transgenic plants in *MYB115* and V plants. The T_0 *MYB115* plants were dwarf in phenotype. Out of 10 *MYB115* plants, only 3 plants were taken for analysis. All the 3 plants showed *gus*, *hph*, and *MYB115* PCR amplification. All the *MYB115* plants were subjected to Southern analysis. Plant no. 8 (MYB115-8) is the single copy plant and the others were double copy. Based on the segregation analysis of T_1 plant seeds we found the homozygous line MYB115-8-9. Similar to T_0 plants T_2 plants were also dwarf in phenotype under a constitutive promoter. The *MYB115* gene is involved in seed maturation pathways.

Hence the nutritional composition of the *MYB115* homozygous transgenic rice seeds was analyzed. There is an increased fat content in the *MYB115* seeds compared to the controls. The bioassay was performed against *R. solani* and validated for disease resistance. However, the *MYB115* gene did not involve in fungal resistance. The *MYB115* gene might be an active player in reducing the transformation efficiency. Due to the dwarf phenotype, adventitious root formation, and lower transformation efficiency, the regeneration in T_2 seeds was not validated.

Outcome of the thesis

- The *AtUGT* gene enhanced the *Agrobacterium*-mediated transformation at the T_0 level in rice. At T_0 state the transformation efficiency was increased by 3%.
- The supertransformation in the homozygous rice transgenic plants was increased fourfold than the vector.
- The *WUS* overexpression in rice altered the plant phenotypes. It caused the enclosed panicle phenotype, delayed heading date, and less number of seed setting. Despite all these pleiotrophic effects, the two T_0 lines were validated.
- The T_1 and T_2 plants also inherited the pleiotropic effects. Hence we obtained a fewer number of seeds. Few plants in T_2 generation lost seed setting and remaining inherited the pleiotropic effects.
- The regeneration efficiency was evaluated in two hemizygous lines. These lines revealed enhanced embryogenic calli formation and regeneration.
- The *AtMYB115* gene was overexpressed in rice, to validate the regeneration efficiency.
- The rice transgenic plants harbouring the *MYB115* gene were generated. Single copy plants were identified and the plants showed dwarf phenotype.
- The T_1 and T_2 plants also inherited the same dwarf phenotype along with adventitious root formation in rice.

- The gene *MYB115* might play a role in transformation instead of regeneration. A very fewer number of plants were obtained in the T_0 generation.

- As the less transformation efficiency was less, the regeneration efficiency was not evaluated. The *MYB115* gene could not be a potential candidate for overcoming the bottlenecks in transformation.

Poster presentations

1. Presented poster entitled "Overexpression of *Arabidopsis UDP-glucosyl-transferase (UGT)* gene in rice to enhance plant transformation" in National seminar on Challenges and Innovative approaches in Crop Improvement. December 16, 17 (248) 2014, AC and RI, TNAU, Madurai, India

Oral presentations

2. Oral presentation paper entitled "Overexpression of *Arabidopsis thaliana WUS (WUSCHEL)* gene affects the lateral organ development in rice" in 3rd National Conference on "Frontiers in Ecobiological Sciences and its Applications" Theme: Water-Food-Energy Nexus (FESA 2018) which held at Periyar University, Salem from February 7-9, 2018.

3. Oral presentation paper entitled "Overexpression of *Arabidopsis MYB* genes enhance the regeneration efficiency in rice" in International Conference on Impact of Innovations in Science and Technology for Societal Development (IISTSD– 2019) which held at Kongunadu Arts and Science College, Coimbatore from 19-21 September 2019.

Publications

Thiveyarajan Victorathisayam, Ganapathy Sridevi (2020) Ectopic expression of *WUSCHEL (AtWUS)* gene alters plant growth and development in rice. Plant 8(3): 45-53

T. Victorathisayam, G. Sridevi (2020) Ectopic expression of *AtUGT* gene increases the transformation efficiency in recalcitrant rice" communicated in Frontiers in Plant Science, section Plant Biotechnology.

Manuscript in Preparation

T. Victorathisayam, A. Muthuganesan, G. Sridevi. The *AtMYB115* gene altered the plant transformation and revealed dwarf phenotype plants.