CHAPTER IV
DISCUSSION

1. Transformation of green fluorescent protein into embryogenic calli without selection pressure

In this study, embryogenic calli were co-cultivated with *Agrobacterium* carrying the *hpt* deleted pCAMBIA5305 (having only GFP in T-DNA). Total of GFP –positive calli were then cut into small pieces and transferred to regenerated medium. We found that GFP positive calli were obtained. However, the regeneration of transformed calli were not yet successful. It was difficult to maintain the preferential growth of green fluorescent cell in culture. This observation suggested that the prevented overgrowth of nontransformed calli by cut into small pieces caused them injure. Furthermore, calli were cut into pieces not less than 5 mm. whereas smaller calli may reduce callus regeneration. This experiment was similar to the report of Ahlandsberg et al. (1999). But both barley cultivars (Baroness and Golden Promise) were transformed by bombardment. A transformation vector , pN1473GFP, contained GFP as a reporter system. Transformation efficiences were approximate 1.3-1.8%.

2. Detection of stable GFP expression and inheritance in T_1 progeny under hygromycin selection.

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In this study, we transformed green fluorescent protein into embryogenic calli by *A. tumefaciens* EHA105. Hygromycin (50 mg/l) was used as a selectable agent. The infected conditions were varied from 10 min infection / 2 days co-cultivation to 15 min infection / 3 days cocultivation. Transformation efficiency was close between 2 conditions. However, 3 days co-cultivation gave approximately 2 fold higher GFP positive calli than 2 days co-cultivation. In contrast, transgenic calli at 3 days co-cultivation reduced regeneration than 2 days co-cultivation. This study suggested that the excessive growth of bacteria on the calli caused them to brown and resulted in reduced callus initiation. The majority of plants produced by this study were free of

morphological aberration, probably as a result of the fact that the cells were maintained *in vitro* for a short time.

Hiei et al. (1994) reported *Agrobacterium* mediated transformation of *japonica* rice; Tsukinohikari , Asanohikari and Koshihikary using scutellum-derived calli as starting materials. *A. tumefaciens* strain LBA4404 (pTOK233), habouring genes for kanamycin resistance(nptII), hygromycin resistance (*hpt*) as selectable marker and GUS(uidA) as a reporter gene was used in transformation. Transformation efficiency determined by hygromycin-resistance plants stably expressed GUS activity relative to the number of pieces of scutellum-derived calli. Then the calli were co-cultivated with bacterial cells and showed transformation cfficiency in range between 10-30%.

Zhang et al.(1997) reported the transformation of calli induced from embryos of mature seeds with *A. tumefaciens* strain LBA4404 carrying the plasmid pTOK233. The *indica* rice cultivar, Pusa Basmati, and the *japonica* cultivars, E-yi 105, E-wan5, and Zhong-shu-wan-geng(ZSWG), were used as targets for *Agrobacterium*-mediated transformation. Transformation efficiency was 13.5%, 13.0%, 9.1% and 9.3%, respectively.

Rashid et al. (1996) reported *Agrobacterium*-mediated transformation of *indica* rice, Basmati 370, Basmati 385 and Basmati 6129. The scutellum-derived calli were co-cultivated with *A. tumefaciens* strain EHA101 (pIG121Hm), carrying *hpt* as a selectable marker and GUS as a reporter gene. Basmati 370 showed a transformation efficiency of 22%, Basmati 385 showed a transformation efficiency of 4.8% whereas *Basmati* 6129 fell to form plantlet and no regenerant was obtained.

These indicated that the efficiency of transformation varies with both plant genotypes and the transformation system used. In general, *japonica* cultivars of rice tissue culture-responsive gave higher efficiency of transformation than *indica* cultivars. Transformation efficiency in this work determined by plants stably expressed GFP fluorescence relative to the number of pieces of scutellum derived calli that had been co-cultivated with bacterial cells was 4.0-5.5%.

In this study, healthy transgenic plantlets were transferred in pot in December. During 2-3 months, all of plants grew to flower, Of the 9 plant lines, only 3 lines were able to set seed. This observation suggested that rice (KDML105) was photoperiod sensitive. In general, rice seedlings were grown to flower for 3 months. Mature rice set seed in appropriate photoperiod. In this study, transgenic plantlets were lately grown. The flower of immature plants were unable to set seed. Moreover, ear of rice had low yield.

After harvesting, the germinated seedling was tested for the expression of GFP and its inheritance by molecular analysis. In this study, we found that GFP fluorescence and PCR analysis were obtained in total of seedling. These results suggested that two copies of *gfp* gene is inserted on homologous chromosome of such a plant. The transgene is able to pass to the progeny though both male and female gametes. The result of segregation is all dominant expression of GFP in all progeny. If a single copy *gfp* gene is inserted on heterozygous chomosome of such a plant. The Mendelian segregation for dominant GFP expression to recessive no GFP expression will be 3 : 1 ratio. But the number of seeds in each line, which were investigated, were low number. Perhaps, the recessive was not obtained in this experiment.

Mohanty et al., 1999 reported *Agrobacterium*- mediated transformation of *indica* variety Pusa Basmati 1. Molecular and genetic analysis of transgenic plants revealed the integration , expression and inheritance of transgenes in the progeny of these plants. Twenty seven percent plants contained single copy gene insertion and copy number of transgenes had been found to vary from 1-4 in transgenic plants. Mendelian as well as non- Medelian inheritance patterns of introduced genes had been obtained in the R_1 progeny.

Cheng et al., 1997 reported *Agrobacterium*-mediated transformation of wheat *Triticum aestivum* cv. Bobwhite. More than 100 transgenic events had been produced. Stable integration , expression and inheritance of the transgenes were confirmed by molecular and genetic analysis. One to five of the transgene were integrated into the wheat genome without rearrangement. Approximately 35% of the transgenic plants received a single copy of the transgenes based on Southern analysis

of 26 events. Transgenes in T_1 progeny segregated in a Mendelian fashion in most of the transgenic plants.

The expression of transgenic plantlets was observed under fluorescence microscope. Both leaves and roots showed GFP expression. Then they were cut by cross-section. In young root section, the expression of GFP was found in vascular bundle cylinder , cortex and epidermal cell. While expression in leaves of older plants was limited in vascular bundle tissue. Jefferson et al. (1987) previously reported that the CaMV 35S promoter was more strongly expressed in cells of the vascular bundle.

3. Transformation of the barley *hva*1 gene into rice (KDML105) by co-cultivation with *Agrobacterium* EHA105.

The pCAMBIA5305*hva*1 was constructed by replacing the hygromycin resistance gene with two cassettes DNA of *hva*1 gene and *bar* gene. The direction of these cassettes DNA were reversed to the direction of 35S promoter which controlled *gfp* gene. In this study, we found that three genes within the left and right border of T-DNA showed expression. Moreover the rice actin 1 gene showed that an efficient promoter for regulation the *hva*1 gene in rice KDML 105.

Cao et al., 1992 transformed plasmids carrying *bar* gene ,under control of either the 5' region of the rice actin 1 (*Act* 1) or the 35S promoter, into suspension cells of *Oryza sativa* cv. Taipei 309 by microprojectile bombardment. The selection efficiencies, when the *bar* gene expression was driven by two different promoters, gave no apparent difference. However, in evaluating the effectiveness of inactivation of phosphinothricin in pDM302 and pDM307 transformed plants in terms of the ammonia levels, before and 26 hours after spraying the plants with herbicide BASTA™, the ammonia content of pDM302 transformed plants carrying *Act* 1 –bar gene increased one to three fold after spraying. But the ammonia content in pDM307 transformed plants carrying 35S-*bar* gene increased two to fifteen fold with average increase by seven fold.

The use of constitutive promoters in transgenic cereals had generally been restricted to the expression of genes encoding resistance to antibiotics or herbicides. In transient assays of reporter gene, the constitutive promoter commonly used in cereal transformation showed differences in their relative activity in monocot cells (McElroy and Brettel, 1994). Plant actin promoter was likely to be active in all tissues because actin was a fundamental component of the plant cell cytoskeleton. In rice, there was at least eight actin like sequences per haploid genome; four of these had been isolated and shown to differ from each other in the tissue and stage-specific abundance of their respective transcripts (McElroy et al., 1990). Therefore, the actin 1 promoter form rice may be a choice of appropriate promoter to drive expression of the transgenes in rice KDML105 replacing the Cauliflower Mosaic Virus 35S promoter in further study.

Maintenance of the embryogenesis status of calli on the selection medium was important for the efficient recovery of regenerants. To have effective phosphinothricin selection, it was important to omit not only glutamine from the selective medium, but also several other amino acids. Addition of 10 mM glutamic acid, 25 mM proline, 10 mM arginine, or 25 mM proline in combination with 10 mM arginine to medium with 20 mg/l phosphinothricin restored the growth rate from 9% to 31,49,65 or 80%, respectively compared to the growth on medium without selection(Dekeyser, et al. 1989). For this reason, we excluded both proline, casein hydrolysate and glutamine from the first selection medium during the transformation experiments.

In this study, we transformed the barley *hva*1 gene into embryogenic calli by co-cultivation with *A. tumefaciens* EHA105 carrying pCAMBIA5305*hva*1. After 3 days on 8 mg/l glufosinate selection, the transformation efficiency was 20.5%. When the embryogenic calli were selected for 2 months, the transformation efficiency was 2.7%. Moreover, the transformation efficiency was low when also selected on 6 and 10 mg/l glufosinate. The transformation efficiency were 1.8 and 2.2%, respectively. Of the 12 callus lines produced from three concentrations selection, 1 line from each 8, 10 mg/l glufosinate selection gave rise to plants. This observation suggested that the loss of transformed calli , regeneration capacity and increase of albinism were usually associated with prolonged periods of culture and the length of time in culture

on the selection medium. This results were similar to the study of Witrzens et al. (1998).

Witrzens et al.,1998 reported the transformation of immatured embryos by microprojectile bombardment. The pEmuPAT and pDM803 plasmids were used for transformation. Of the plantlets surviving (glufosinate) selection, 95% were escaped, compared to 50% escaped through geneticin selection. The transformation efficiency was 0.25% of embryo bombardment.

In this study, we reported on the successful *Agrobacterium*- mediated transformation and expression of *hva*1 gene in rice KDML105. Expression of the synthetic *hva*1 gene was shown by RT-PCR of rice mRNA. In further study, accumulation of HVA1 protein will be investigated under stress conditions. It will improve to understand the role of this protein confer increase tolerance to water deficit and salt stress.