Review

DEVELOPMENT OF *IN PLANTA* TRANSFORMATION METHOD USING *Agrobacterium tumefaciens* THAT IS SIMPLE AND EFFICIENT AS WELL AS APPLICABLE TO VARIOUS PLANTS [1)]

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[1)] This is a revised paper with addition of some new data of: Kojima, M., Suparthana. P., and Nogawa, M.; Journal of Japanese Science of Bioengineering (Seibutukogakukaishi) 85, 57-62 (2007)

ABSTRACT

A simple and efficient *in planta* transformation method was developed. In the method, meristems of either apical or axillary buds of immature plants or apical buds of embryos in imbibed seeds, depending on plants, were inoculated by *Agrobacterium tumefaciens* after being pricked with a needle. The inoculated plants were grown to maturation in pots under non-sterile conditions. Transformation was demonstrated by several lines of evidence obtained with mostly the progenies of T1 generation; phenotypic inheritance from T0 plants to plants of the following generation, resistance of seed germination to antibiotics, detection of β-glucuronidase activity in transformants of T1 generation, detection of transgene by Southern blot and PCR analyses in T1 generation transformants and rescue from T1 generation transformants of the plasmids composed of T-DNA of binary vector and flanking plant genomic DNA. The diverse species of plants such as buckwheat (*Fagopyrum esculentum*), mulberry (*Morus alba* L.), kenaf (*Hibiscus cannabinus*), rice (*Oryza sativa*), wheat (*Triticum aestibvum* L.), maize (*Zea mays*), and soybean (*Glycine max*) were shown to be efficiently transformed by our *in planta* method.

Keywords: *in planta* transformation, *Agrobacterium tumefaciens*

Conventional transformation methods and their disadvantages

In the conventional transformation methods being currently used, transgene is first introduced into either calli or tissues *in vitro* culture, using either *Agrobacterium*-mediated method or microparticle bombardment method and then plants are regenerated on medium. The *Agrobacteium*-mediated method has several advantages over the microparticle bombardment method: the former allows for a more stable integration of a defined segment of DNA into plant genome and generally results in a lower copy number and fewer rearrangements than the latter.

Although the conventional transformation methods are commonly used, they have some disadvantages. First, they require sterile conditions. Second, they take rather long periods of time. Third, somatic mutation or somaclonal variation occurs frequently in plant cells during *in vitro* culture. Finally, some plants are recalcitrant to regeneration. These disadvantages of the conventional methods are mainly caused by *in vitro* culture of plant cells. Therefore, some *in planta* transformation methods, which require no *in vitro* culture of plant cells, have been reported to overcome the disadvantages of the conventional methods (Chee *et al*., 1989; Touraev *et al*.1997; Clough *et al*., 1998; Hu *et al*., 1999; Qing *et al*., 2000). However, none of the reported *in planta* transformation methods except one for *Arabidopsis* have been widely used because of the problem of efficiency and reproducibility (Bent, 2000). On the other hand, the *in planta* transformation method, or

floral dip method, for *Arabidopsis* is reliable and thereby widely used. Unfortunately, the method is, however, applicable to only *Arabidopsis*. Thus, we attempted to develop the general *in planta* transformation method that can be applied to various plants.

The hint for development of new method

In our method, a meristem was selected as a target tissue for inoculation of *Agrobacterium.* The hint for this idea came from the experiment as follows. *Kalanchoe pinnata* has many adventitious buds on the edge of its leaf (Fig. 1A). Each bud contains a meristem with totipotency and thereby is capable to produce a whole plant. When a leaf is detached from stem and kept on soil or wet vermiculite, a young plantlet emerges from each bud in two or three weeks. The leaves from one plant are a clone, having an identical genome.

The adventitious buds of one leaf were inoculated, after being pricked with a needle, by *A.tumefaciens* containing a 2.5 kb of buckwheat cDNA with unknown function in a sense orientation, while ones of another leaf from the same plant were inoculated by *A.tumefaciens* containing the same cDNA but in an anti-sense orientation. The inoculated leaves were incubated on wet vermiculite and then transferred to separate pots to grow to mature plants. As shown in Fig.1B, the plants emerged from the respective leaf exhibited phenotypes opposite to each other; the plants from the leaf inoculated by *A.tumefaciens* containing the cDNA in a sense orientation were tall and aged earlier, while the plants from the leaf inoculated by *A.tumefaciens* containing the cDNA in an anti-sense orientation were short and late in aging. From the result of the experiment, we were convinced that it is possible to transform plant by inoculating *A.tumefaciens* onto meristem of a plant, but not plant cell or tissue *in vitro* culture.

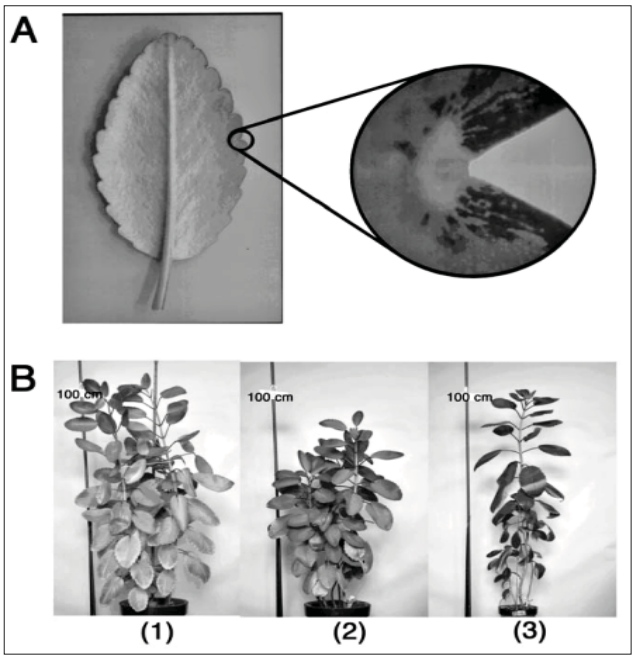

Fig. 1. Adventitious buds on leaf blade of *Kalanchoe pinnata* Lam (A) and appearance of the plants emerged from the adventitious buds inoculated by *A.tumefaciens* (B). The plants of B- (1) are ones emerged from the buds inoculated by *A. tumefaciens* (LBA4404) strain containing a 2.5 kb of buckwheat cDNA with unknown function in a sense orientation. The plants of B- (2) are ones emerged from the buds inoculated by *A.tumefaciens* (LBA4404 strain) containing the same cDNA in an anti-sense orientation. The plant of B- (3) is one emerged from the bud treated with water.

Seek for a model plant and transformation procedure

Kalanchoe plant seemed to have great advantages as a model plant for the *in planta* transformation; it has many big adventitious buds containing meristems on its leaf which are easy to access. Moreover, they are a clone. However, *Kalanchoe* plant has a fatal disadvantage; it is very difficult to obtain seeds. Thus, we sought for other plant as a model plant. Besides adventitious buds, meristem is included in various tissues or organs of any higher plant; it is included in a shoot, axillary bud, root tip and so on. We selected buckwheat (*Fagopyrum esculentum* var. Shinano No.1) as a model plant because it grows rapidly and produces many seeds within three months. Using buckwheat, we made up the *in planta* transformation procedure as follows (Kojima *et al*., 2000[a]; Kojima *et al*., 2000[b]). Buckwheat seeds sterilized with sodium hypochlorite were sown on soil in a pot and grown for 4 to 5 days to a stage of 7 to 8 cm height with two expanded leaves. Two to three points in top (0 to 3 mm part from top) of hypocotyl that included an apical meristem were pricked with a needle (ϕ 0.71

mm) and then inoculated with a cotton swab drenched with *A.tumefaciens* water suspension (ca 1.0×10^8 cells/ml). The inoculated seedlings were kept under the unfavorable conditions, at 22^0C in the dark, for 3 days to suppress defense action of plant so as to facilitate infection by *A.tumefaciens.* Subsequently, the seedlings were grown to maturation to set seeds (T_1 generation) under non-sterilized and ambient conditions. Buckwheat plants develop two types of flowers with different pistil lengths, or hetrostylism. Only pollination between different types of flowers can result in fertilization. The flowers of plants in a pot were pollinated randomly by using a plumage.

Various plants such as mulberry (*Morus alba* L.), kenaf (*Hibiscus cannabinus*), rice (*Oryza sativa*), wheat (*Triticum aestibvum* L.), maize (*Zea mays*), and soybean (G*lycine max*) were successfully transformed by essentially the same method as one for buckwheat described above (Ping *et al*., 2003; Kojima *et al*., 2004; Suparthana *et al*., 2005; Suparthana *et al*., 2006; Kojima *et al*., 2006), except that *A.tumefaciens* was inoculated onto meristem of a different part of a plant among those plants. *A. tumefaciens* was inoculated onto meristems of axillary buds of young plants for mulberry, either apical or axillary buds of young plants for kenaf, apical buds of embryos in imbibed seeds for rice, wheat, and maize and axillary buds of decapitated seedlings for soybean.

Agrobacterium is eliminated by defense action of plant

In our transformation method, the inoculated immature plants did not receive any treatment to eliminate *Agrobacteria.* As for the inoculated imbibed seeds, they were treated once with antibiotics (*Cefotaxime*). It seemed unlikely, however, that *Agrobacterium* could be eliminated completely by such only one time antibiotic treatment. We, therefore, examined the presence of the remaining *Agrobacteria* used for transformation in the shoot apices of mature transformants in T_0 and T_1 generations by the two experiments described below. No *Agrobacteria* was detected in the shoot apices, which produce germ cells for the next generation.

In the conventional transformation methods, in which *Agrobacteria* are inoculated onto plant cells *in vitro* culture, it is very difficult to eliminate *Agrobacteria* completely. This may be because the conditions of *in vitro* culture are suitable not only for plant cells but also for *Agrobacteria*. On the other hand, under natural conditions, *Agrobacterium* is a rather week pathogenic bacterium; when *Agrobacteria* were inoculated onto leaves or stems of *Kalanchoe* plants grown in pots, galls emerged only on the inoculated sites, never on other parts of plants. From this result, the plants grown under such natural conditions appear to be resistant enough to confine *Agrobacteria* to the inoculated sites and eliminate them finally. Just like in the case above, the inoculated plants were also grown in pots under natural conditions in our *in planta* transformation method. Consequently, it is the most likely that *Agrobacteria* are eliminated by defense action of plant in our method.

In order to examine the presence of remaining *Agrobacteria* in the inoculated plants, we carried out two experiments. In the first experiment, shoot apices of mature plants in T_0 and T_1 generations were aseptically homogenized with autoclaved water using a mortar and pestle. The homogenates were inoculated onto LB media containing various antibiotics, on which *Agrobacteria* used for transformation could grow. The inoculated media were incubated at 28^oC for 3 days. No colonies appeared for any homogenates, indicating the absence of *Agrobacteria* used for transformation. In the second experiment, genomic DNAs were isolated from shoot apices of mature plants in T_0 and T_1 generations and subjected to PCR analysis using them as templates. When the primer sets for genes in T-DNA region of binary vector were used, PCR products were obtained, while no PCR products were obtained when the primer sets for genes outside T-DNA region of binary vector were used. This result also indicated the absence of *Agrobacteria* used for transformation.

Plants of T_0 generation are chimeras, while ones of T_1 generation are true transformants

In our method, A*grobacteria* were inoculated onto meristems of either apical or axillary buds of young plants or apical buds of embryos in imbibed seeds, depending on plants, in which various organs and primodia had already formed. Therefore, it is likely that the plants of T_0 generation are chimeras. However, it is reasoned that transformants of T_1 and subsequent generations produced by our method are not chimeric plants but truly transformed plants made from all transformed cells on the basis of the following consideration. *Agrobacteria* would likely transfer transgene not only to genomes of cells of meristems but also to genomes of already differentiated cells in buds. The transgenes integrated into genomes of these already differentiated cells could determine only phenotype of lower parts of mature T_0 plants. In contrast, transgenes integrated into the genomes of as yet undifferentiated cells in meristems, which are destined to become apical meristems of mature plants, could contribute to production of shoot apices and hence to phenotype of mature T_0 plants. On maturation, germ cells (egg) and pollen (sperm) are produced by an apical meristem. An individual germ cell contains a single haploid copy of genomic DNA. Two germ cells, an egg and a sperm, produce a single fertilized cell, from which the entire body of cells of each transformant in the next generation is derived. Thus, transformants of T_1 and subsequent generations should not be chimeric plants but true transformants. A considerable portion of T_0 generation plants transformed by our method exhibited the phenotype different from that of nontransformed plants, being an indication that transgenes had been integrated efficiently in genomes of apical meristems of those mature T_0 generation plants.

Based on the above consideration, we examined the transformation and integration of transgene in genomes of transformants using plants of T_1 generation but not T_0 generation in most of the experiments.

Demonstration of transformation

To allow transformation to be verified by various means, we used four different strains of *A.tumefaciens,* an avirulent M-21 mutant strain (Majumder *et al.*, 2000), LBA4404 strain harboring a pBI 121 binary vector (Toyobo Co.), LBA4404 strain harboring a pIG121-Hm binary vector (Ohta *et al.*, 1990) and LBA4404 strain harboring a binary vector (pBI-res2) modified for rescue of plasmid (Suparthana *et al.*, 2006). M-21 strain is an avirulent mutant of which auxin synthesizing gene (*iaaM*) is destructed by Tn*5*-insertion, but all other genes including cytokinin synthesizing enzyme gene are intact. As a result, the transformants by M-21 strain were expected to synthesize a high level of cytokinin, resulting in a dramatically altered phenotype due to hormone imbalance, which is a good visible indication of transformation. Some altered phenotype also would be expected for transformants produced using other strains of *A.tumefaciens* since endogenous gene of plant might be destructed or modified by insertion of transgene into its genome. The second *A.tumefaciens* strain, LBA4404 strain with a pBI121 binary vector, is a standard strain for transformation of plants and contains a neomycin phosphotransferaseII (nptII) gene and a β-glucuronidase (GUS) gene. The third *A.tumefaciens* strain, LBA4404 strain with a pIG121-Hm binary vector, was used to indicate transformation by histochemical detection of β-glucuronidase as well as resistance to hygromycin B, since the pIB121-Hm binary vector contained a GUS gene with an intron and a hygromycin B resistance gene in its T-DNA. The fourth *A.tumefacien*s strain, LBA4404 strain with a pBI-res2 binary vector, was used to demonstrate definitively integration of transgene (T-DNA) in genome of transformant by rescuing the plasmid that contained integrated T-DNA and flanking plant chromosomal DNA in a contiguous segment. The plasmids were rescued from transformants of T_1 generation as follows; genomic DNAs were extracted from transformants and digested with BamHI, which did not split the segment of

ampicilin resistance gene and replication origin of pBuescript plasmid in T-DNA region of the binary vector. The digested DNAs were self-ligated and then transformed into *E.coli*. The transformed *E.coli* were screened on LB medium containing ampicilin and plasmids were isolated from *E.coli* grown on the medium. The structure of the isolated plasmid was determined by sequencing.

Many species of plants were transformed by using four strains of *A.tumefaciens* mentioned above and transformation was examined by various means. First, there appeared plants in T_0 generation, which showed appearance different from that of parent plants, and their phenotype (appearance) was inherited by the progenies in the subsequent generations (Fig.2). Second, the seeds (T_1 generation) of wheat transformants by LBA4404 strain harboring a pIG121-Hm binary vector showed hygromycin B resistance in their germination (Fig.3). Third, the wheat transformants (T_1 generation) by *A.tumefaciens* LBA4404 strain harboring a pIG121-Hm binary vector showed positive β-glucuronidase activity (GUS) staining (Fig.4). Fourth, transgene integration into genome was demonstrated by Southern blot analysis with the wheat transformants (T_1 generation) by *A.tumefaciens* LBA4404 strain harboring a pBI-res2 (Fig. 5). Fifth, PCR analysis detected transgene (GUS gene) in the genomes of buckwheat transformants (T_1 generation) by *A.tumefaciens* LBA4404 strain harboring a pBI121 binary vector (Fig.6). Finally, the plasmids containing the integrated T-DNA and flanking genomic DNA in a contiguous segment were rescued from the T_1 generation wheat transformants by *A.tumefaciens* LBA4404 strain harboring a pBI-res2 binary vector (Fig.7).

Transformation efficiency was calculated based on the results of the analyses described above. The transformation efficiency was from 40% to 60% with most of the plants tested such as buckwheat, rice and wheat. The transformation efficiency values of 40% to 60% seemed to be extremely high as compared with those by the conventional methods reported previously.

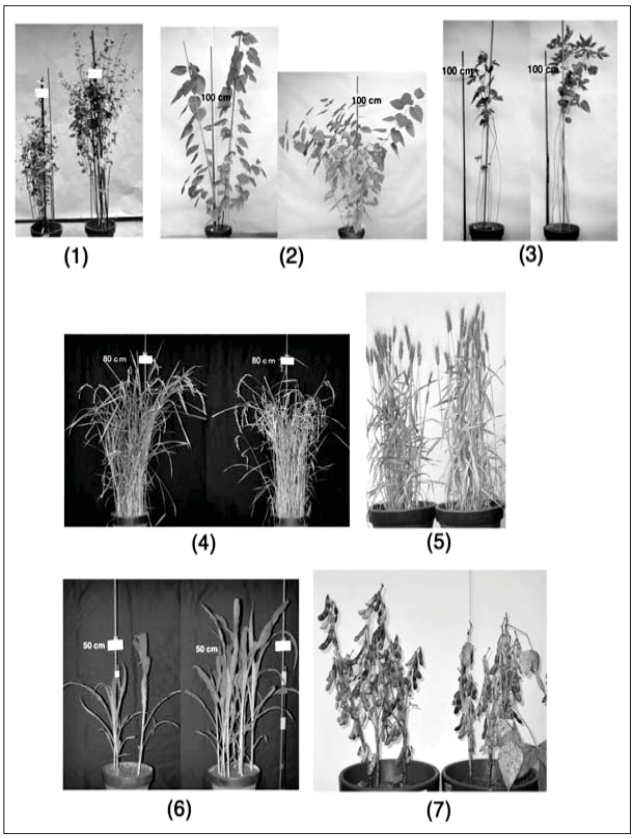

Fig. 2. Phenotypes of various species of transformants generated by the *in planta* transformation method. In the respective panel, the left side plants are nontransformants and the right side plants are transformants. (1) Buckwheat (*Fagopyrum esculentum*) transformants (T_4 generation) by *A.tumefaciens* (LBA4404 strain) containing a rice MADS box gene (Acc. No. AB00325), (2) Mulberry (*Morus alba* L.) transformants (offspring produced from T_0 transformants by stem cuttage) by *A.tumefaciens* (M-21 strain), (3) Kenaf (*Hibiscus cannabinus*) transformants (T_1 generation) by *A.tumefaciens* (M-21 strain), (4) Rice (*Oryza sativa*) transformants (T_1 generation) by *A.tumefaciens* (LBA4404 strain, pIG121-Hm), (5) Wheat (*Triticum aestivum*) transformants (T_0 generation) by *A.tumefaciens* (M-21 strain), (6) Maize (*Zea mays*) transformants (T_1 generation) by *A.tumefaciens* (LBA4404 strain, pIG121-Hm), (7) Soybean (*Glycine max*) transformants (T_1 generation) by *A.tumefaciens* (M-21 strain)

However, such a high transformation efficiency obtained with our *in planta* method is reasonable in light of the experimental evidence and the reasons given below.

Reason for high transformation efficiency of our method

Kalanchoe plants (*Kalanchoe daigremontiana*) grown in a pot were inoculated on their stems or leaves with a virulent strain of *A.tumefaciens*

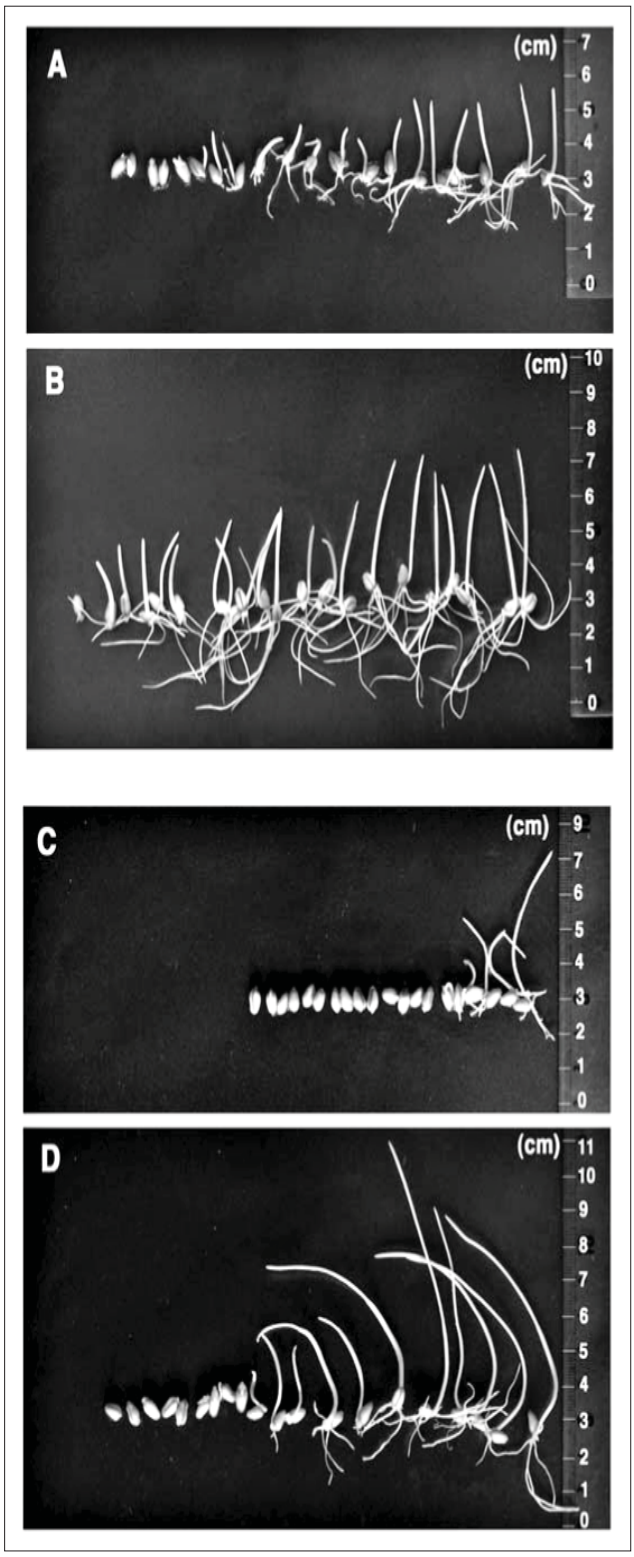

Fig. 3. Germination of seeds of the wheat transformants (T_1 generation) by *A.tumefaciens* (LBA4404) strain harboring a pIG121-Hm binary vector in presence of hygromycin B (30 ppm). (A) germination of the transformants in presence of hygromycin B; (B) germination of the transformants in water; (C) germination of the nontransformants in presence of hygromycin B; (D) germination of the nontransformants in water (Suparthana *et al*., 2006).

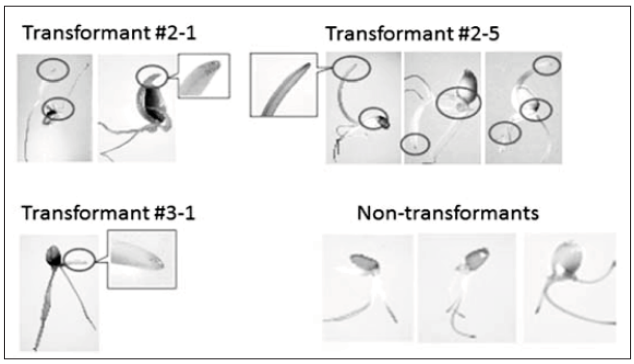

Fig. 4. Histochemical assay of β-glucuronidase (GUS) in the wheat transformants (T_1 generation) by *A.tumefaciens* LBA4404 strain harboring a pIG121-Hm

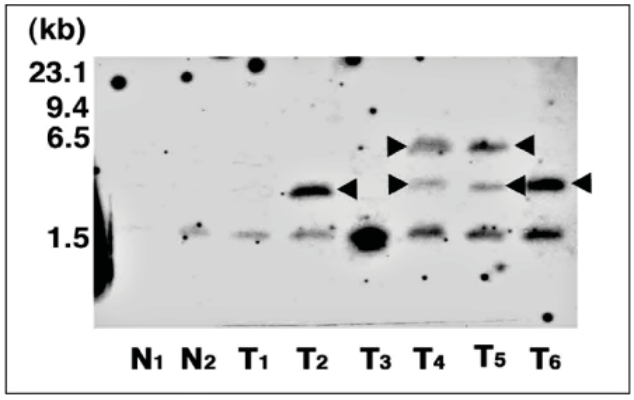

Fig. 5. Southern hybridization analysis of the wheat transformants (T_1 generation) by *A.tumefaciens* LBA4404 strain harboring a pBI-res2 binary vector. Genomic DNAs of the transformants were digested with BamHI and SphI, which did not split the *ori* gene and ampicilin resistance gene of pBluscript plasmid segment in T-DNA region of the binary vector. The digested DNAs were fractionated on 0.8 % agarose gel and subsequently transferred to the membrane, which was probed with a Dig-labeled probe containing an *ori* gene. Lanes N1 and N2, different nontransformants; lane T_1-T_6, different transformants. The bottom band at 1.5 kb is not a band due to the integrated transgene but a band due to some segment of endogenous DNA of buckwheat genome (Suparthana *et al*., 2006).

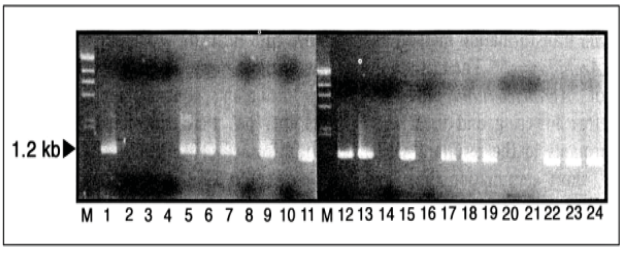

Fig. 6. PCR detection of β-glucronidase gene in the buckwheat transformants (T_1 generation) by *A.tumefaciens* LBA4404 strain harboring a pBI121 binary vector. The 1.2 kb DNA is a β-glucronidase gene segment amplified. M, size marker of DNA; lane 1, pBI121 binary vector; lane 2-4, different nontransformants; lane 5-24, different transformants (Kojima *et al*., 2000[a]).

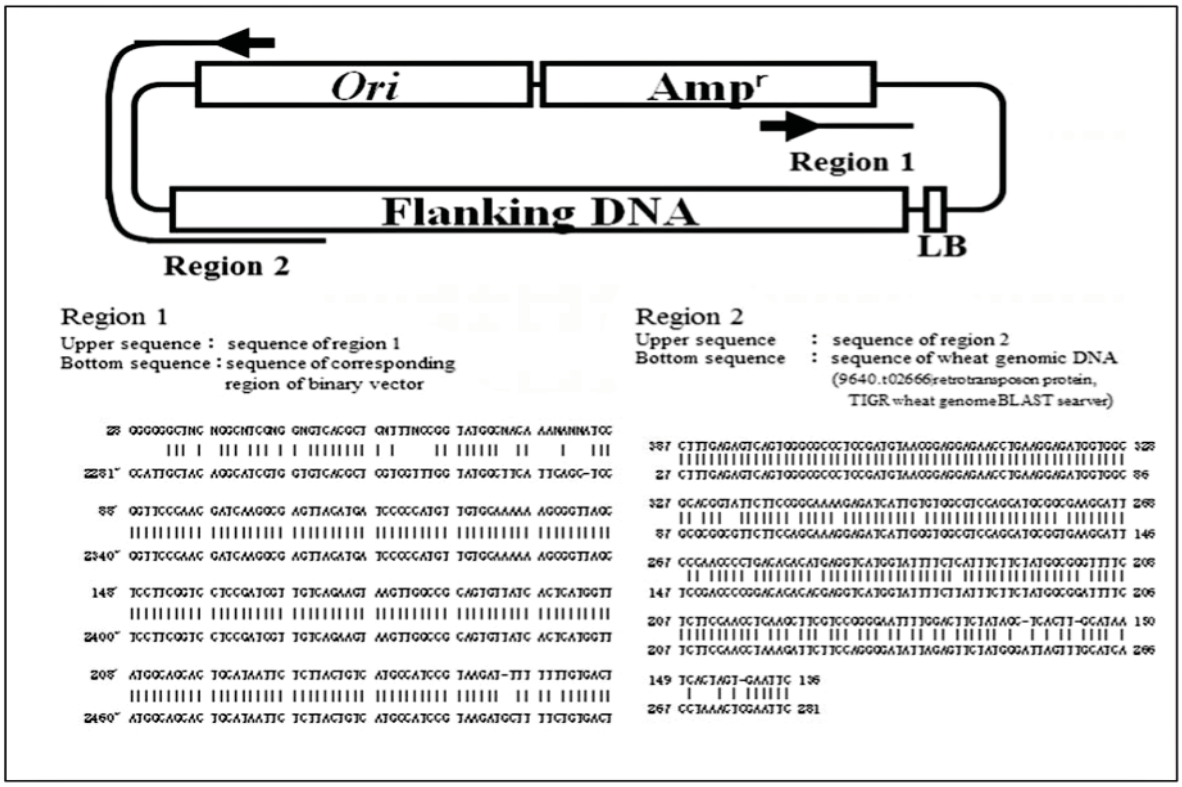

Fig. 7. Assumed structure of a plasmid rescued from a wheat transformant (T_1 generation) by *A.tumefaciens* LBA4404 strain harboring a pBI-res2 binary vector, and alignment of the determined sequence of the plasmid and the corresponding sequence of the assigned gene. The arrows indicate the positions and directions of the primers used for sequencing. The sequence of region 1 in the rescued plasmid was homologous with that of the corresponding region of the binary vector used for transformation, while the sequence of region 2 was homologous with that of a wheat genomic DNA (Acc. No. 9640 t02666, retrotransposon protein) (Suparthana *et al*., 2006).

(A-208 strain, a parent strain of M-21 avirulent strain used in this paper) by piercing with a needle previously smeared with *A.tumefaciens* and then the inoculated plants were continued to grow in a pot. After six weeks, galls were formed on every (100 %) inoculated site (Majumder *et al*., 2001). It has been revealed that gall formation results from integration of T-DNA region of Ti-plasmid indwelling in *A.tumefaciens* cell into plant genome, that is, "natural transformation".

Some reasons are conceivable for that such an efficient natural transformation, gall formation, is accomplished under the conditions mentioned above. First, the factors necessary for gall formation such as auxin, cytokinin and other as yet unidentified factors might be provided at the best time, at the optimal concentrations and in the best combinations. Second, the meristems in the plants grown in a pot should have a stronger resistance to pathogen, *A.tumefaciens* in this case, and stress and a higher capacity for differentiation and regeneration than cells or tissues *in vitro* culture. Finally, there might be significant roles in wounding of plant tissues that occurs on inoculation of *A.tumefaciens.* When wounded, plant should activate cell division to heal the wounded tissue. The activation of cell division must accompany activation of chromosome replication. Some special conditions allowing T-DNA to integrate effectively into plant chromosome might happen during the activated chromosome replication. Furthermore, the wounded plant also synthesizes many kinds of phenolic compounds and accumulates them in the wounded tissue and the surrounding tissue. The accumulated phenolic compounds have antimicrobial activity and so act as a chemical defense against pathogens. In addition, some of the phenolic compounds produced in response to wounding are reported to show the

same activity as acetosyringone; they induce the genes in *vir* region of Ti-plasmid or helper plasmid (Bolton *et al.*, 1986). Consequently, the phenolic compounds in the wounded tissue might promote integration of T-DNA into plant genome.

There is close similarity between gall induction on *Kalanchoe* plants by *A.tumefaciens* mentioned above, where "natural transformation" occurred on every (100 %) inoculated site, and our *in planta* transformation method; in the former case, the stems or leaves of *Kalanchoe* plants grown in pots were inoculated, accompanied by wounding, by *A.tumefaciens* and continued to grow in pots. In the later case, the meristems of immature plants were inoculated, accompanied by wounding, by *A.tumefaciens* and grown to maturation to set seeds in pots. The similarity between the two cases might account for the high transformation efficiency of our method.

Advantages of our *in planta* transformation method

Generally, the transformant generated by the *in planta* transformation method is superior to one by the conventional transformation method in that the former is the least likely accompanied with mutation since *in vitro* culture of plant sells, being prone to induce somatic mutation, is not involved in the process of the former.

In addition, our *in planta* method has four other advantages. First, it is simple as well as efficient. Third, we do not need to use a special *A.tumefaciens* strain; instead a standard and popular strain of *A.tumefaciens*, LBA4404 strain, is enough for our method. Finally, our method is applicable to wide species of plants. As shown in Fig.2, diverse species of crop plants have been successfully transformed by our method; they include dicotyledonous plants (buckwheat, kenaf and soybean), woody plant (mulberry) and monocotyledonous plants (rice, wheat and maize). It has been recognized that monocotyledonous crops, especially wheat and maize, are rather difficult to be transformed by the conventional transformation method using *A.tumefaciens* (Hiei *et al.*, 1994; Cheng *et al.*, 1997). This possibly has to do with the phenomenon that crown gall disease is never seen with these monocotyledonous crops on the field. It is, therefore, noteworthy that monocot crops such as rice, wheat and maize were transformed with high efficiency by our *in planta* transformation method.

The remaining subject with plant transformation method

There remains an essentially important but unsolved problem with the method of plant transformation; that is the problem that we can neither designate location on the chromosome where the transgene should be integrated nor regulate the number of transgene to be integrated into chromosome. Such a present situation of plant transformation method is as if we were launching randomly a rocket without a control device toward plant cells. This situation is the case for both the conventional transformation methods and the *in planta* transformation methods. In order to solve this problem, we should develop a practical gene targeting method for plants as early as possible.

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