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Bioactive Beads-Mediated Transformation of Rice with Large DNA Fragments Containing *Aegilops tauschii* Genes, with Special Reference to Bead-Production Methodology

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1. Introduction

Plant transformation is a technique that allows us to transfer genes from one species to another in order to introduce new characteristics into the recipient. The plant transformation technique has become widely adopted as a method both to understand plant physiology and to improve plant characteristics. There are now many established gene-transfer methods, both direct and indirect, for the stable introduction of novel genes into plant species. Examples include *Agrobacterium*-mediated transformation, particle bombardment (biolistic), and protoplast electroporation (Klein and Fitzpatrick-McElligott, 1993; Tzfira and Citovsky, 2006). Recently, a direct gene-transfer method called bioactive beads (BABs)-mediated transformation has been developed (Sone et al., 2002; Wada et al., 2011a, 2011b). This method involves immobilization of DNA molecules on alginate beads and their transfer to plant cells with the assistance of a polyethylene glycol (PEG) solution. Alginate, a hydrophilic polysaccharide that solidifies in the presence of Ca^{2+} ions, is utilized as a barrier membrane to produce calcium-alginate beads for immobilizing high-density DNA molecules. The original procedure for bead production was described in Sone et al. (2002) (Fig. 1). Firstly, a plasmid DNA solution was mixed with a $CaCl_2$ solution. Isoamyl alcohol was then added to a 1.5 ml micro-tube containing an aqueous phase 1% sodium alginate solution to form a water/oil mixture that was emulsified by sonication using an ultrasonic disrupter (UR-20P; Tomy Seiko, Tokyo, Japan) for 10 s. Bioactive beads (BABs) encapsulating DNA molecules were generated after immediately adding a $CaCl_2$ solution containing plasmid DNA into the emulsified solution. The DNA-immobilizing BABs were utilized in combination with PEG solution for protoplast transformation.

This method was successfully used for transformation of tobacco BY-2 protoplasts with a ten-fold higher transformation efficiency than the conventional PEG transformation method that is using naked plasmid DNA (Sone et al., 2002). The BABs transformation method has a wide applicability beyond tobacco BY-2 protoplasts to many organisms, both in plant cells, e.g., eggplant (*Solanum integrifolium*, Liu et al., 2004), tobacco (*Nicotiana tabaccum* SR-1, Liu et

al., 2004) (Fig. 2), carrot (*Daucus carota*, Liu et al., 2004), rice (*Oryza sativa*, Wada et al., 2009) and in human cells, e.g., lymphocyte cell line K562 cells and human carcinoma HeLa cells (Higashi et al., 2004).

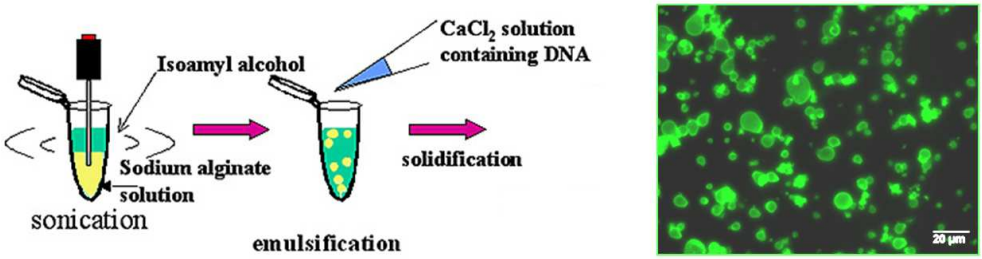

Fig. 1. Schematic diagram shows the preparation of bioactive beads (BABs) immobilizing DNA molecules

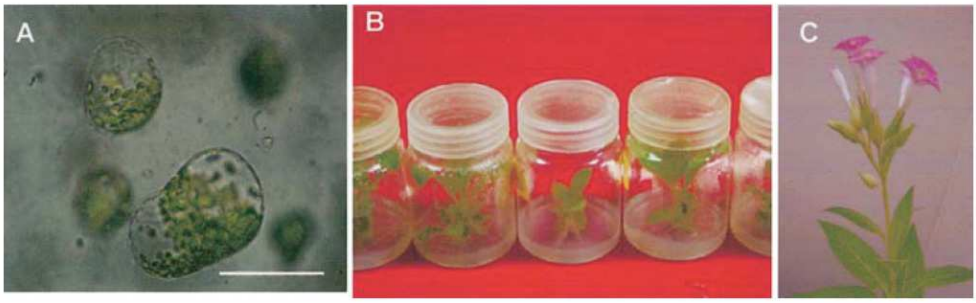

Fig. 2. Transgenic tobacco SR-1. A Cell division of protoplast 5 days after co-transformation. B Transgenic tobacco SR-1 regenerated plants 4 months after being transformed. C Flowering transgenic tobacco SR-1, 5.5 months after transformation. (With kind permission from Springer Science+Business Media: Journal of Plant Research, Obtaining transgenic plants using the bio-active beads method, 117, 2004b, 95-99, Liu, H.; Kawabe, A.; Matsunaga, S.; Murakawa, T.; Mizukami, A.; Yanagisawa, M.; Nagamori, E.; Harashima, S.; Kobayashi, A & Fukui, K., Figure 2)

Mizukami and associates (2003) have introduced yeast artificial chromosomes (YACs) into yeast spheroplasts using this method, and revealed the higher physical stability of 468 kb of YAC DNA embedded in and/or on the bioactive beads in solution compared to naked chromosome DNA molecules. The authors also checked whether YAC DNA molecules immobilized on bioactive beads would be intact even after vortex treatment. The result showed that naked YAC DNA molecules was degraded in the solution, resulting in no visible band after electrophoresis, but the band was clearly observed in the case of the YAC DNA molecules immobilized on the beads. This shows that BABs can stabilize large DNA fragments in solution. Moreover, yeast chromosomal DNA with YAC DNAs (128 kb, 256 kb and 468 kb) was embedded in and/or on the beads and transferred into recipient yeast cells lacking those YAC DNAs. Pulse field gel electrophoresis clearly showed that YAC DNAs up to 468 kb in size were successfully introduced into recipient

cells by PEG treatment with bioactive beads. This utility, coupled with the fact that the method does not require any sophisticated equipment and is easy to practice, clearly suggests the bioactive beads method as an alternative transformation method, especially for large DNA molecules.

Despite the advantages of BAB transformation mentioned above, there is insufficient information regarding the ideal production conditions, such as the shape and size of BABs, or the concentration of DNA that is suitable for the most efficient BABs transformation. Thus, the production conditions for BABs should be optimized. Here, improvement of the BAB production system enabling uniform size and shape will be reported. Using this system, various sizes of beads immobilizing the pUC18-sGFP construct could be produced. Its applicability to plant transformation has already been examined by using tobacco BY-2 protoplasts.

As described above, the BAB method has the ability to transfer large DNA fragments into yeast spheroplasts and into the protoplasts derived from tobacco BY-2 cells suspension. Moreover, recently our group has successfully produced transgenic rice with large DNA inserts containing *Aegilops tauschii* hardness genes by using this method. The detailed transformation procedures and characterization of transgenic plants will be described in this chapter.

2. Improvement of efficacy of a BAB transformation method

The capturing of DNA fragments by BABs can prevent their physical damage during transformation processes. As a result, the BAB transformation method provides a feasible large DNA fragment transfer method into many organisms (Mizukami et al., 2003; Wada et al., 2009). However, the mechanism for DNA transfer is still obscure and experimental conditions are not yet optimized, resulting in low DNA transfer efficiency (Liu et al., 2004). During preparation of BABs by the original sonication method, $CaCl_2$-containing DNA molecules were added into an alginate emulsion solution, and the beads were then collected by centrifugation and used for transformation (Sone et al. 2002). It was, however, found that large amounts of DNA remained in the $CaCl_2$ solution, resulting in a poor efficiency, both in DNA immobilization and transformation. Therefore, a more efficient BAB production system with higher DNA immobilization and DNA transformation efficiencies is desired.

A new system for bead production was developed using an in-house device called a bead-maker. The bead-maker has 2 major components; an automated micro-syringe, and a vibrator (Fig. 3). The automated micro-syringe was assembled by placing a syringe (100 μl gastight syringe, Hamilton, Nevada, USA) on a micro-syringe pump (MSP-RT, AS ONE) so that the flow rate of the solution could be accurately controlled. The vibrator consists of a loudspeaker (FR-8, 4Ω, Visaton, Germany), attached to a moveable rod fixed to a wooden board and connected to a sine wave sound generator (AG-203D Kenwood, Tokyo, Japan). A capillary tube (30 μm ϕ, GL Sciences, Tokyo, Japan) from the syringe was connected to the moveable rod so it could be vibrated simultaneously when the rod was vibrated. The frequency and amplitude of the sine wave sound generator are selectable. As a result, the

vibration of the moveable rod as well as the capillary tube linking the micro-syringe and the vibrator are controllable.

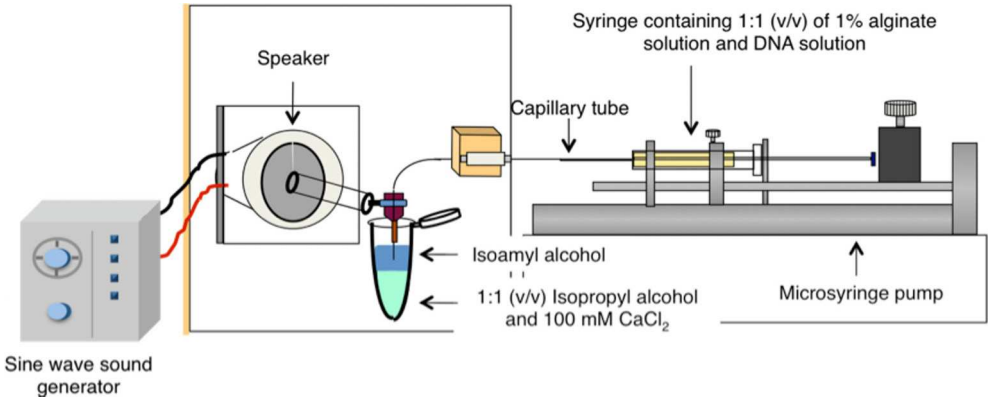

Fig. 3. Schematic diagram of a bead-maker

To test the effectiveness of this new system for plasmid DNA immobilization, bead production using 1% alginate containing 0.5 μg/μl of pUC18-sGFP was carried out. First, a DNA-containing alginate solution was prepared by mixing 100 μl of sodium alginate solution (1% w/v) with 100 μl of pUC18-sGFP (0.5 μg/μl), and then the freshly-prepared solution was slowly loaded into the micro-syringe. The syringe was connected to the capillary tube and placed on the micro-syringe pump. The solidifying solution was then prepared by firstly adding 750 μl of a mixture of 0.1 M $CaCl_2$ and isopropyl alcohol (1:1) in a 1.5 ml micro-centrifuge tube, followed by adding 750 μl of isoamyl alcohol and placing the tube in a plastic rack as shown in Fig. 3. As the bead-maker was working, the DNA-containing alginate solution was pumped out at a steady flow rate. Simultaneously, the sine wave sound generator produced sound waves at the speaker resulting in the vibration of the moveable rod connecting to the speaker. Consequently, vibration of the capillary tube linked to the moveable rod dropped alginate-DNA solution into the solidifying solution. The isoamyl alcohol kept the droplets spherical and the mixture of $CaCl_2$ and the isopropyl alcohol solidified the alginate-DNA droplets. The spherical beads were collected and washed at least twice in 0.1 M $CaCl_2$ solution by centrifugation (5000 *g* for 5 min) and re-suspended in a sufficient volume of 100 mM $CaCl_2$ solution. To verify the efficiency of DNA immobilization, the beads were stained by a DNA-staining dye, YOYO-1, and were observed under a fluorescence microscope (Zeiss). Images were captured using a CCD (charge-coupled device) camera (Fig. 4a-d). Observation of bead shape under the microscope revealed them to be spherical. Contrastingly, the shapes of beads made using the original system, with normal sonication, were irregular; many were not spherical. Qualitatively, the green fluorescence intensity emitted from DNA-immobilized beads, which correlates with the amount of DNA immobilized by the BABs, was measured using image analysis software, Image J (http://rsbweb.nih.gov/ij/), to further compare the efficiency of DNA immobilization of these two systems. The intensity of green fluorescence emitted from beads made using the new system was obviously

higher than that of the beads made by sonication. To quantify this result, the green fluorescence intensities from more than 50 beads made using both the new system and the sonication system were measured (Fig. 4e). The mean intensity from the improved beads was much higher than that from beads made by sonication, indicating that the amount of DNA immobilized by the improved beads was higher than that by beads made by the original sonication system.

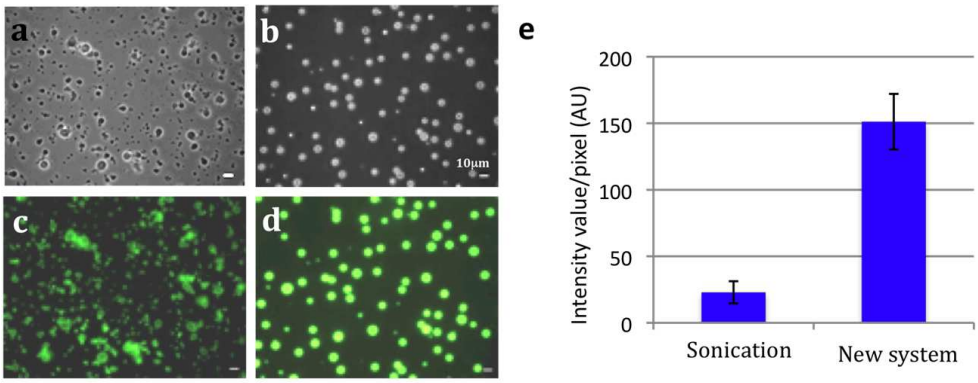

Fig. 4. Improvement of BAB production was achieved using the new BABs production system. BABs made by the new system were spherical, size-controllable and more highly efficient at DNA immobilization compared to BABs made by the original sonication system. Phase contrast (a-b) and fluorescent images (c-d) of plasmid DNA-immobilized beads made using the sonication system (a and c), and using the new system (b-d). Bars = 10 μm. Mean intensity of green fluorescent intensity from plasmid-DNA immobilized beads stained by YOYO-1 (e). Error bars: ±1 s.d.

We further investigated whether the bead size influenced transformation efficiency. As described above, solution flow rate and the frequency and amplitude of the sine wave were adjustable. Various combinations of these parameters were tested to obtain uniformly sized BABs. Investigation of the effect of solution flow rate, vibration frequency, and vibration amplitude on the size of beads indicated that a solution flow rate at 0.4 μl/min was the most suitable for producing beads of a uniform size, compared to other flow rates tested at 0.2, 0.8, 2 and 5 μl/min. However, the bead size was controllable by changing the vibration frequency and amplitude. The vibration amplitude had a direct effect on bead sizes: smaller beads are produced with a higher amplitude. The frequency of the sine wave affected the bead size through the strength of the capillary tube vibration; frequencies causing strong vibration produced smaller beads while weak vibration produces larger beads.

Three different sizes of BABs immobilizing the same amounts of pUC18-sGFP were selected (Fig. 5) and used for transformation into tobacco BY-2 protoplasts in combination with PEG treatment (Fig. 6). Transient assays of GFP-expressing protoplasts were carried out 24 h and 48 h after transformation (Fig. 6c). The results showed a negative correlation between bead size and transformation efficiency, that is, as the size of beads decreased, the transformation

efficiency increased. Moreover, with the new system, a *ca.* 10% transformation efficiency was achieved (Fig. 6 (ⓒ)). One to six µm diameter beads provided higher transformation efficiencies than beads made by sonication method. Thus it is likely that immobilization of DNA molecules on beads made by the new system is more efficient than that made by sonication system. Furthermore, it was also found that transformation efficiency obtained from one to six µm beads was higher than that obtained from seven to twelve µm beads. Two explanations are possible to reveal out why smaller beads have better transformation efficiency. One is that DNA is introduced into plant cells through physical uptake of bioactive beads. Smaller beads should be more easily incorporated into plant protoplasts. The other is because a higher number of smaller beads means a larger total surface area for the same volume of alginate solution being used. This means that a higher number or a larger total surface area of smaller beads might adhere to the protoplast membrane than when using larger beads, consequently enhancing the interaction between DNA-immobilized beads and protoplasts, and ultimately increasing DNA transfer between beads and protoplasts. From these results, it is concluded that, aside from DNA concentration, bead size optimization is also an important factor in achieving high transformation efficiency. This new system developed for BABs production with higher transformation efficiency should facilitate more applicability of BABs transformation and even enable multiple gene delivery into plant cells.

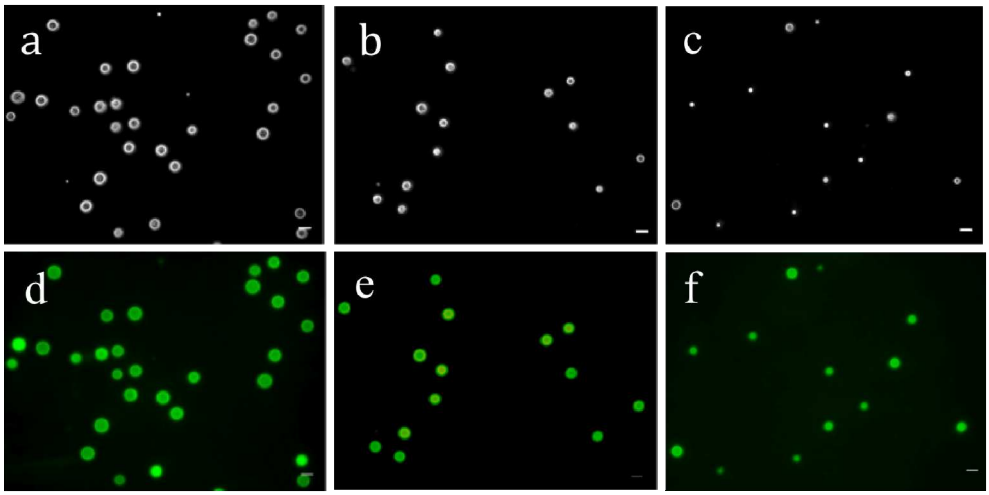

Fig. 5. Phase contrast (a-c) and fluorescence image (d-f) of BABs made under optimized conditions. BABs of 7-12 µm diameter made with 0.4 µl/min solution flow rate, frequency of 250 Hz, and amplitude set to 7 (a,d). BABs of 5-8 µm diameter made with 0.4 µl/min solution flow rate, frequency of 270 Hz, and amplitude set to 10 (b,e). BABs of 1-6 µm diameter made with 0.4 µl/min solution flow rate, frequency of 250 Hz, and amplitude set at 10 (c,f). Bars = 10 µm

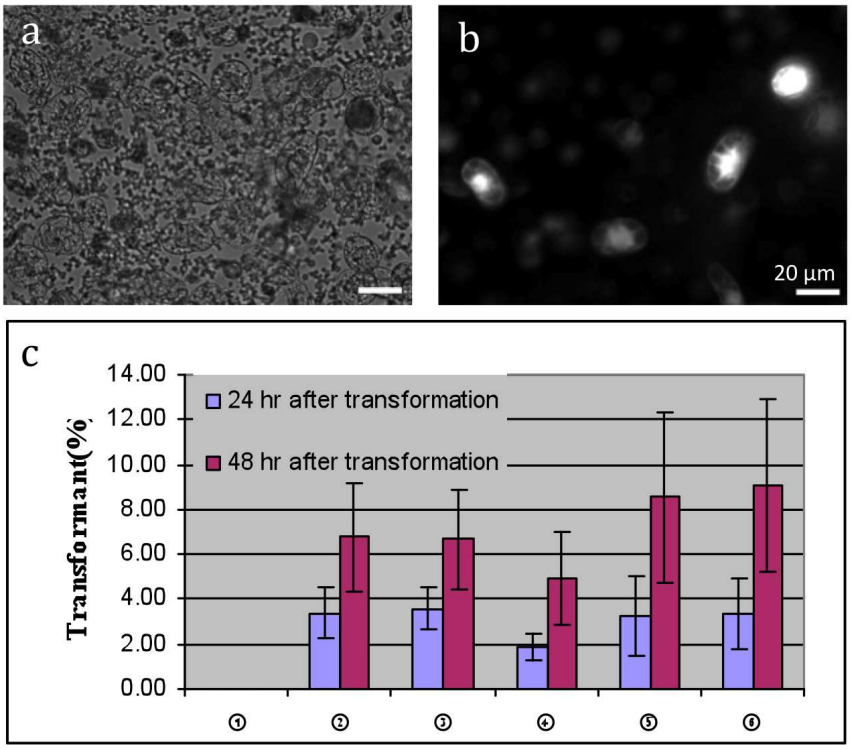

Fig. 6. Transformation of tobacco BY-2 protoplasts using BABs immobilizing pUC18-sGFP. Phase contrast image of tobacco BY-2 protoplast transformed with the improved BABs (a), tobacco BY-2 protoplasts expressing GFP protein (b). Bars = 20 µm. Transformation efficiency after 24 h and 48 h of transformation: ①, negative control (distilled water); ②, 50µg naked pUC18-sGFP; ③, sonicated beads; ④, beads made using the new system, size: 7-12 µm (68%); ⑤, beads made using the new system, size: 5-8 µm (62%), 2-3 µm (16%); ⑥, beads made using new system, size: 1-6 µm (70%). Error bars: ±1 s.d.

3. Large DNA transfer into plants using the bioactive beads method

3.1 Transformation of rice with large DNA molecules using BAB method

Transformation of large DNA fragments is a promising approach to extend the reach of plant genetic engineering. Until now, plant genetic engineering has been performed using single or small numbers of genes, resulting in successful production of genetically engineered plants such as herbicide- and insect-resistant plants (Gonsalves, 1998; Khan et al., 2009; Song et al., 2003b; Tai et al., 1999; Wang et al., 2005). To produce transgenic plants with more variety of phenotypes, however, multiple gene transfer will be required (Dafny-Yellin et al., 2007; Daniell et al., 2002; Halpin, 2005; Naqvi et al., 2010). Even single traits are often the result of expression of multiple genes. For example, a single metabolic pathway may be related to several genes. Thus if we want to manipulate the metabolic pathway, it will be best achieved by manipulating several genes simultaneously. Introducing these

genes at same time is preferable because: (1) introduction of multiple genes through crossing of different transgenic plants is time-consuming and laborious, and (2) co-transformation of different kinds of plasmid DNAs needs preparation of many constructs, which also takes time and needs different kinds of marker genes depending on the number of plasmids to be introduced. Therefore, transformation with large DNAs including multiple genes is promising to enhance the efficiency of transformation. In addition, large DNA transfer will enable the regulatory regions of transgenes to be transferred with the genes of interest. This will allow introduced genes to be expressed at the physiological level.

Although this approach is expected to have such advantages, it is still difficult to transfer large DNA fragments into plants. This difficulty is due to the lack of a reliable method of introducing large DNA fragments into plants. For general plant transformation, *Agrobacterium*-mediated transformation and particle bombardment are the methods normally used (Bhalla, 2006; Rakoczy-Trojanowska, 2002). However, these methods are difficult to apply to large DNA transfer into plants because of the instability of large DNAs in *Agrobacterium* cells and during the bombardment process. Song and associates (2003a) and Nakano and co-workers (2005) have recently reported that DNA fragments larger than 100 kb are unstable in *Agrobacterium* cells, resulting in deletion and/or rearrangements in *Agrobacterium* cells before their transformation into plants. Their data suggest that *Agrobacterium*-mediated transformation is not suitable for large DNA transfer. In particle bombardment, DNAs coated on metal particles generally suffer from physical damage during bombardment, resulting in fragmentation of large DNAs. However, some laboratories, have reported success in introducing DNA fragments over 100 kb in size by particle bombardment (Van Eck et al., 1995; Mullen et al., 1998; Phan et al., 2007). This is a promising improvement, but successful reports of large DNA transfers using this method are still limited. In addition, particle bombardment needs the equipment. Thus, an easy and inexpensive method needs to be developed for large DNA transfer. As described above, our group has recently developed such a new transformation method, namely the BAB method (Higashi et al., 2004; Liu et al., 2004a; Liu et al., 2004b; Mizukami et al., 2003; Sone et al., 2002; Wada et al., 2011a, 2011b). This method is easy and inexpensive. The method has been applied to yeast, mammalian cells (HeLa, K562 cells), and plant cells (tobacco BY-II, tobacco SR-I, carrot, egg plant, rice). Transformation efficiency is about 10 times higher than PEG treatment without bioactive beads. An important characteristic of the BAB method is its transformation capability with large DNA fragments. As mentioned above, 468 kb of YAC DNA was effectively transferred into yeast cells. This result clearly suggests that the BAB method is a suitable for large DNA transfer.

To examine the applicability of the BAB method to plant transformation with large DNA fragment, 124 kb of YAC DNA was introduced into cultured tobacco BY-2 cells (Liu et al., 2004b). The transient expression of introduced genes was detected in the transgenic suspension cells. To investigate this in more detail, the bioactive beads method was applied to the transformation of rice with *ca*. 100 kb BAC DNA (Wada et al., 2009). Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) was used as the plant material and BAC DNA including hardness genes from *Agilops tauschii* was introduced using the bioactive beads method. The hardness genes consist of three genes (*puroindoline a*, *puroindoline b, GSP-1* gene) and are scattered in a *ca*. 100 kb region on the short arm of chromosome 5D in *Aegilops tauschi* (Turnbull et al., 2003). The BAC DNA encompasses the hardness locus and also contains the regulatory regions of each gene (Fig. 7). Because of this, it was expected that the expression of each gene would be shown at the regular physiological level.

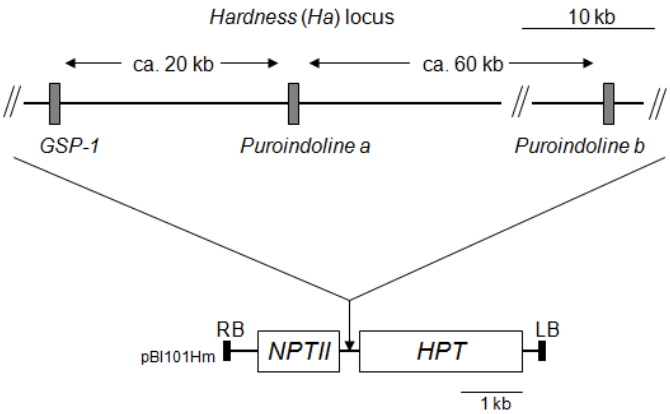

Fig. 7. Schematic diagram of the construct, used in this transformation, pBI BAC10-60. (With kind permission from Springer Science+Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750-68, Wada, N.; Kajiyama, S.; Akiyama, Y.; Kawakami, S.; No, D.; Uchiyama, S.; Otani, M.; Shimada, T.; Nose, N.; Suzuki, G.; Mukai, Y.; Fukui, K.; Figure 1)

As a result, nine transgenic plants were obtained and analyzed. The PCR analyses showed that each gene was integrated into the rice genome (Table 1). Some transgenic plants contained most transgenes, but transgenic plants with all the transgenes could not be obtained. This indicates that rearrangement of introduced DNA molecules occurred during transformation.

	Lines	*NPTII* gene	*Pinb* gene	*Pina* gene	*GSP-1* gene	*HPT* gene
Non-transgenic plant		–	–	–	–	–
Transgenic plants with pBI BAC10-60	9-1-1	–	+	–	+	+
	9-1-2	–	+	–	+	+
	9-1-3	+	+	–	+	+
	9-1-4	+	+	–	+	+
	9-1-6	–	+	–	+	+
	9-1-7	+	+	–	+	+
	9-1-8	+	+	–	+	+
	9-1-9	+	+	–	+	+
	9-1-10	–	–	–	+	–

Table 1. Profiles of T_0 transgenic plants as determined by PCR analysis. +: gene detected, – : gene not detected. (With kind permission from Springer Science+Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750-68, Wada, N., Kajiyama, S., Akiyama, Y., Kawakami, S., No, D., Uchiyama, S., Otani, M., Shimada, T., Nose, N., Suzuki, G., Mukai, Y., Fukui, K.; Table 3)

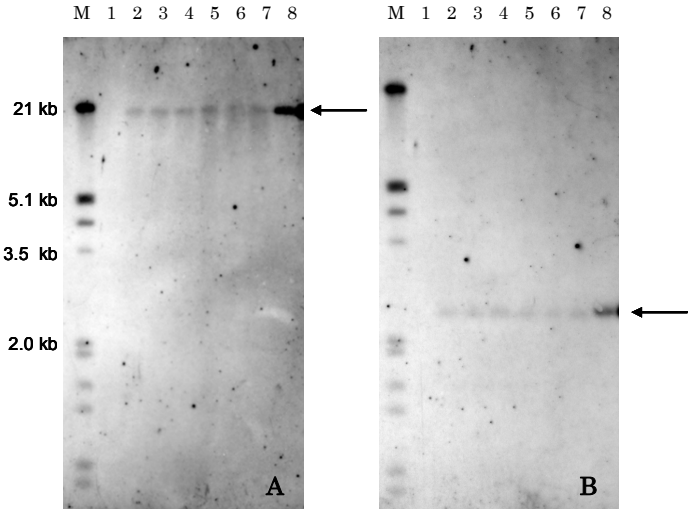

Fig. 8. Genomic Southern blot analysis of T_0 transgenic plants. Total DNA of rice plants was digested with *Hin*dIII and probed with (A) the *HPT* gene and (B) *Pinb* gene. Lane 1 : control plant, Lane 2 : transgenic plant 9-1-3, Lanes 3 to 7 : transgenic plants 9-1-6 to 9-1-10, Lane 8 : pBI BAC DNA digested with *Hin*dIII. The amount of BAC DNA corresponds to two copies insertion of the transgene in rice genome. Arrows indicate the locations of the observed bands (These figures, from Wada et al. (2011), are reproduced with permission from John Wiley and Sons, Inc.)

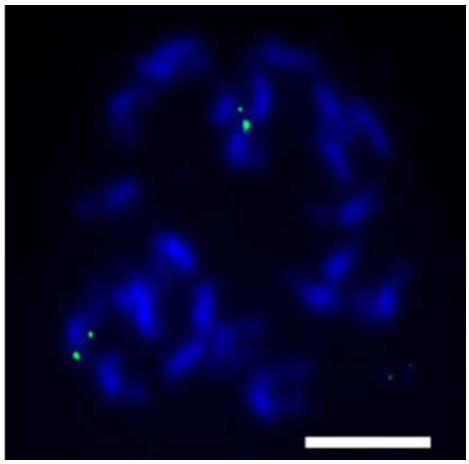

Fig. 9. FISH analysis of the kernels of the T_1 transgenic plant 9-1-6-3. The two paired green signals indicate the integration sites of pBI BAC 10-60. Bar = 10 µm. (With kind permission from Springer Science+Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750-68, Wada, N.; Kajiyama, S.; Akiyama, Y.; Kawakami, S.; No, D.; Uchiyama, S.; Otani, M.; Shimada, T.; Nose, N.; Suzuki, G.; Mukai, Y.; Fukui, K.; Figure 4)

The copy numbers of *puroindoline b* (*Pinb*) and *HPT* genes were checked by genomic Southern blot analysis. The results showed that these two regions were integrated into the rice genome as a single copy of intact fragment (Fig. 8). T_0 plants showed sterility: 7 out of 9 transgenic plants did not produce any seeds. Two transgenic plants were partially fertile and produced some seeds. They recovered their fertility in successive generations. Segregation tests of the T_1 generation showed that transgenes were inherited into the next generation in Mendelian mode. Homozygous plants were also obtained. FISH analysis revealed that the transgenes were integrated into the telomeric region of a pair of rice chromosomes in the homozygous plants (Fig. 9). The expression of the introduced gene, *Pinb*, was also confirmed at the mRNA and protein levels. Thus, the promoter region of *Pinb* was functional even in rice cells. These results indicate that the BAB method can introduce multiple genes into plants and produce stable transgenic plants that can pass introduced transgenes on to successive generations.

To examine if the introduced gene is functional in transgenic rice, a phenotypic analysis was performed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Wada et al., 2010). The results indicated that the endosperm structure changed in the transgenic rice to a more loosely packed structure (Fig. 10). The hardness genes are known to affect the softness of wheat endosperms by giving the loosely packed endosperm structure. Thus, the EM observations clearly indicated that the introduced hardness gene functioned in similar manner as in wheat endosperm. Analysis of physico-chemical properties of the rice flour also indicated that the transgenic rice endosperm had the phenotype of soft textured seed. The results suggest that the PINB protein localized at the surface of the starch compounds, resulting in preventing the adhesion of each starch compound and changing some physico-chemical properties, such as flour particle size, and pasting properties. These results indicate that the hardness locus introduced was functional in transgenic rice. This suggests that introduction of a genomic locus that controls a trait could be a good strategy for adding desirable traits to plants.

The results obtained indicated that the BAB method is a promising method for plant transformation with large DNA fragments. Co-transformation experiments with two or three kinds of BAC DNAs simultaneously were also successful (data not shown). Co-transformation can increase the number of genes that can be introduced simultaneously. However, some aspects of the BAB method could be improved. First, the intactness of introduced DNA fragments should be examined. In our experiments, the deletion of some transgenes was observed. The rearrangement of introduced DNA fragments might also occur in other regions that were not checked. How often and to what extent such rearrangements occur with the BAB compared to conventional methods needs to be investigated to fully establish that the BAB method has advantages over other conventional methods for transformation with large DNA fragments. Second, further improvement in the transformation efficiency of the BAB method should be achieved. We have succeeded in immobilizing proteins on BABs (data not shown). The immobilization of large DNAs with proteins, such as VirE2, might target the introduced DNA into the host genome more efficiently because VirE2 is known to target T-DNA into nuclei and protect the T-DNA during *Agrobacterium*-mediated transformation (Gelvin, 2003; Gopalakrishna et al., 2003). Despite these points that could be improved, our results clearly indicate that the bioactive beads method could be an alternative way of producing stable transgenic plants with multiple transgenes.

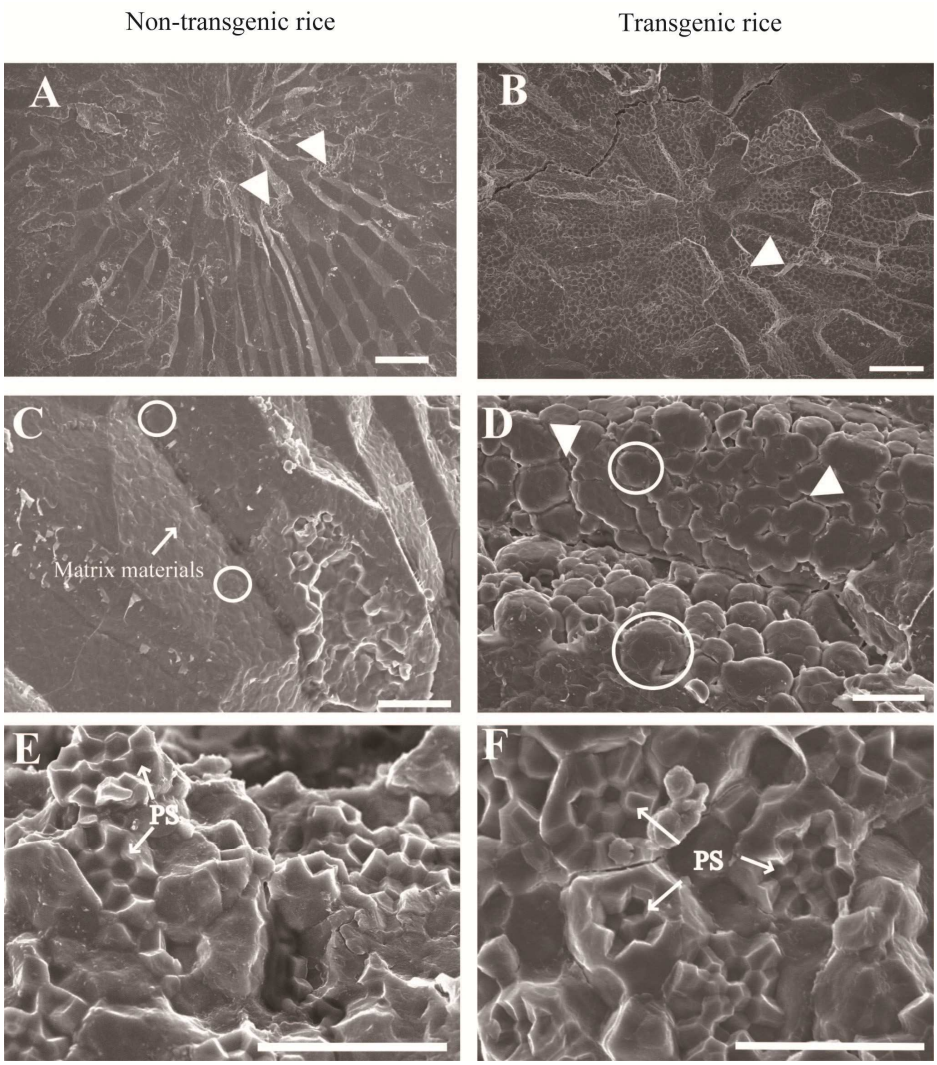

Fig. 10. SEM observation of rice endosperm cells. (A,B) Low magnification view of transversely fractured surface of milled rice of (A) non-transgenic rice and (B) transgenic rice. Arrowheads indicate intracellularly cleaved site. Bars: 100 µm. (C,D) Higher magnification view of intercellularly cleaved site of (C) non-transgenic rice and (D) transgenic rice. Bars: 20 µm. (C) Compound starch granules (circles) embedded within matrix material in non-transgenic rice. Intracellularly cleaved sites can also be seen. (D) Starch compound granules (circles) surrounded by airspaces (arrowheads). (E,F) Higher magnification view of intracellularly cleaved site of (E) non-transgenic rice and (F) transgenic rice. Partially split compound starch granules (PS) exposing individual starch granules with sharp angles and edges can be seen. Bars: 20 µm (Reproduced from Wada et al. (2010) with permission of Elsevier Science.)

In mammalian cells, chromosome engineering systems including artificial chromosomes, have been developed (Basu & Willard, 2005; Ikeno et al., 1998; Kazuki et al., 2011; Oshimura & Katoh, 2008). An artificial chromosome has a capacity to carry chromosomal fragments, with virtually no size limitation to the transgenes that can be transferred (Kuroiwa et al., 2000). In addition, microcell-mediated transfer (MMCT) has made it possible to introduce genes that cannot be transferred by conventional transfection. For example, a chromosomal region including the dystrophin gene (2.4 Mb) has been introduced into a mouse genome using human artificial chromosomes (HACs) and MMCT (Hoshiya et al., 2009). HACs can be engineered by recombination technology in cells that are the most suitable for each step (e.g. chicken DT40 cells for homologous recombination, hamster CHO cells for site-specific recombination, Kazuki et al. 2010). However, there is no such system for plants. Recently three reports have been published on the production of plant artificial chromosomes (Carlson et al., 2007; Yu et al., 2007; Ananiev et al., 2009). However, there is still no report of utilizing them for plant transformation with large DNA fragments. Thus, a plant transformation system using large DNA fragments has yet to be developed. The BAB method can be used to introduce large DNA fragments into a plant genome as a part of plant transformation system with large DNA fragments.

3.2 Further utility of bioactive beads

BABs can be not only applied to DNA transformation, but also to the immobilization of proteins (Zhou et al., 2009). BSA (Bovine serum albumin) protein was successfully entrapped by BABs and its interaction with FITC-labeled anti BSA was clearly observed. Moreover, the authors improved the efficacy of protein immobilization in BABs by treating the alginate solution with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHSS) to cross-link the desired protein (BSA) to the alginate carboxyl groups prior to solidification. It was found that cross-linking beads provided high protein-retention ability for up to 2 weeks after immobilization. Such improved protein-immobilizing beads with high retention capacity might have the potential to be an alternative choice for detecting antigen-antibody interactions.

BABs have also been successfully used in the immobilization of a single yeast cell displaying hydrolyzing enzymes to capture fluorescent molecules released after enzymatic reaction (Zhou et al., 2009). The retention of fluorescent products by yeast-encapsulating BABs enabled active and non-active cells to be differentiated by sorting in a flow cytometer. Using such a developed system, a library screening for novel enzymatic activities on the surface of yeast cells should be possible.

4. Conclusion

An alternative transformation method, BABs-mediated transformation, has been developed by applying a drug delivery system (DDS) in which highly concentrated DNA molecules are entrapped by small autonomously degradable alginate beads and transferred into plant cells in combination with polyethylene glycol (PEG) treatment. This transformation method is easy to perform, is applicable to a range of organisms, allows large-sized DNAs to be delivered, and facilitates the transportation of multiple genes of up to 468 kb size into yeast spheroplasts. Moreover, our latest results on transformation of BAC DNA containing *A. tauschii* hardness genes into rice protoplasts, along with an improvement in the efficiency of

DNA immobilization by BABs have verified that this method is capable of producing transgenic rice that carry large DNA fragments and can facilitate the production of useful transgenic plants by introduction of multiple genes simultaneously with high efficiency. Further development of the BAB method will contribute to the development of more flexible plant genetic engineering methodologies using large DNA fragments. This will open up new possibilities for plant genetic engineering and make it possible to produce a number of useful transgenic plants in the near future.

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6. References

Ananiev, E. V.; Wu, C.; Chamberlin, M. A.; Svitashev, S.; Schwartz, C.; Gordon-Kamm, W. & Tingey, S. (2009) Artificial chromosome formation in maize (Zea mays L.). *Chromosoma*, 118, 157–177

Basu, J. & Willard, H.F. (2005) Artificial and engineered chromosomes: non-integrating vectors for gene therapy. *Trends Mol Med*, 11, 251-258.

Bhalla, P. L. (2006) Genetic engineering of wheat--current challenges and opportunities. *Trends Biotechnol*, 24, 305-311.

Carlson, S. R.; Rudgers, G. W.; Zieler, H.; Mach, J. M.; Luo, S.; Grunden, E.; Krol, C.; Copenhaver, G. P. & Preuss, D. (2007) Meiotic transmission of an in vitro-assembled autonomous maize minichromosome. *PLoS Genet*, 3, 1965-1974.

Dafny-Yelin, M. & Tzfira, T. (2007) Delivery of multiple transgenes to plant cells. *Plant Physiology*, 145, 1118–1128.

Daniell, H. & Dhingra, A. (2002) Multigene engineering: dawn of an exciting new era in biotechnology. *Current Opinion in Biotechnology*, 13, 136–141.

Gelvin, B. (2003) Agrobacterium mediated plant transformation: The biology behind the "Gene-Jockeying" Tool. *Microbiology and Molecular Biology Reviews*, 67, 1, 16-37.

Gonsalves, D. (1998) Control of Papaya Ringspot Virus in Papaya: A Case Study. *Ann Rev Phytopath*, 36, 415-437.

Gopalakrishna, S.; Singh, P. & Singh, N. K. (2003) Enhanced transformation of plant cells following co-bombardment of VirE2 protein of agrobacterium tumefaciens with DNA substrate. *Current Science*, 85, 9, 1343-1347.

Halpin, C. (2005) Gene stacking in transgenic plants the challenge for 21st century plant biotechnology. *Plant Biotech. J.*, 3, 141-155.

Higashi, T.; Nagamori, E.; Sone, T.; Matsunaga, S. & Fukui, K. (2004) A novel transfection method for mammalian cells using calcium alginate microbeads. *J Biosci Bioeng*, 97, 191-195.

Hoshiya, H.; Kazuki, Y.; Abe, S.; Takiguchi, M.; Kajitani, N.; Watanabe, Y.; Yoshino, T.; Shirayoshi, Y.; Higaki, K.; Messina, G.; Cossu, G. & Oshimura, M. (2009) A highly stable and non-integrated human artificial chromosome (HAC) containing the 2.4Mb entire human dystrophin gene. *Mol Therapy*, 17, 309-17.

Ikeno, M.; Grimes, B.; Okazaki, T.; Nakano, M.; Saitoh, K.; Hoshino, H.; McGill, N.I.; Cooke, H. & Masumoto, H. (1998) Construction of YAC–based mammalian artificial chromosomes. *Nat Biotechnol*, 16, 431 – 439.

Kazuki, Y.; Hosoya, H.; Takiguchi, M.; Abe, S.; Iida, Y.; Osaki, M.; Katoh, M.; Hiratsuka, M.; Shirayoshi, Y.; Hiramatsu, H.; Ueno, E.; Kajitani, N.; Yoshino, T.; Kazuki, K.; Ishihara, C.; Takehara, S.; Tsuji, S.; Ejima, F.; Toyoda, A.; Sakaki, Y.; Larionov, V.; Kouprina, N. & Oshimura, M. (2010) Refined human artificial chromosome vectors for gene therapy and animal trasngenesis, *Gene Therapy,* 1-10.

Khan, E.U. & Liu, J.H.(2009) Plant biotechnological approaches for the production and commercialization of transgenic crops. *Biotechnol & Biotechnol Eq*, 23, 1281-1288.

Klein ,T. M. and Fitzpatrick-McElligott, S. (1993). Particle bombardment: A universal approach for gene transfer to cells and tissues. *Curr. Opin. Biotechnol.*, 4: 583-590.

Kuroiwa, Y.; Tomizuka, K.; Shinohara, T.; Kazuki, Y.; Yoshida, H.; Ohguma, A.; Yamamoto, T.; Tanaka, S.; Oshimura, M. & Ishida I.(2000) Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts. *Nat Biotechnol,* 18, 1086–1090.

Liu, H.; Kawabe, A.; Matsunaga, S.; Kim, Y.; Higashi, T.; Uchiyama, S.; Harashima, S.; Kobayashi, A. & Fukui, K. (2004a) An Arabidopsis thaliana gene on the yeast artificial chromosome can be transcribed in tobacco cells. *Cytologia*, 69, 235-240.

Liu, H.; Kawabe, A.; Matsunaga, S.; Murakawa, T.; Mizukami, A.; Yanagisawa, M.; Nagamori, E.; Harashima, S.; Kobayashi, A & Fukui, K. (2004b) Obtaining transgenic plants using the bio-active beads method. *J Plant Res*, 117, 95-99.

Mizukami, A.; Nagamori, E.; Takakura, Y.; Matsunaga, S.; Kaneko, Y.; Kajiyama, S.; Harashima, S.; Kobayashi, A. & Fukui K. (2003) Transformation of yeast using calcium alginate microbeads with surface-immobilized chromosomal DNA. *Biotechniques*, 35, 734-740.

Mullen, J.; Adam, G.; Blowers, A. & Farle, E. (1998) Biolistic transfer of large DNA fragments to tobacco cells using YACs retrofitted for plant transformation. *Mol Breeding,* 4, 449-457.

Nakano, A.; Suzuki, G.; Yamamoto, M.; Turnbull, K.; Rahman, S.; Mukai, Y. (2005) Rearrangements of large-insert T-DNAs in transgenic rice. *Mol Genet Genomics,* 273,123-129.

Naqvi, S.; Farré, G; Sanahuja, G.; Capell, T.; Zhu, C. & Christou, P.(2010) When more is better: multigene engineering in plants. *Trends in Plant Science*, 15, 48–56.

Oshimura, M. & Katoh, M.(2008) Transfer of human artificial chromosome vectors into stem cells. *Reprod Biomed Online*, 16, 57–69.

Phan, B.H.; Jin, W.W.; Topp, C.N.; Zhong, C.X.; Jiang, J.M.; Dawe, R.K.& Parrott, W.A. (2007) Transformation of rice with long DNA-segments consisting of random genomic DNA or centromere-specific DNA. *Transgenic Res*, 16, 341-351.

Rakoczy-Trojanowska, M. (2002) Alternative methods of plant transformation - A short review. *Cell Mol Biol Lett*, 7, 849-858.

Sone, T.; Nagamori, E.; Ikeuchi, T.; Mizukami, A.; Takakura, Y.; Kajiyama, S.; Fukusaki, E.; Harashima, S.; Kobayashi, A. & Fukui, K. (2002) A novel gene delivery system in plants with calcium alginate micro-beads. *J Biosci Bioeng,* 94, 87-91.

Song, J; Bradeen, J.M.; Naess, S.K.; Helgeson, J.P. & Jiang, J. (2003a) BIBAC and TAC clones containing potato genomic DNA fragments larger than 100 kb are not stable in Agrobacterium. *Theor Appl Genet,* 107, 958-964.

Song, J.; Bradeen, J.M.; Naess, S.K.; Raasch, J.A.; Wielgus, S.M.; Haberlach, G.T.; Liu, J.; Kuang, H.; Austin-Phillips, S.; Buell, C.R.; Helgeson, J.P. & Jiang, J. (2003b) Gene RB cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight. *Proc Natl Acad Sci USA*, 100, 9128–9133.

Tai, T.H.; Dahlbeck, D.; Clark, E.T.; Gajiwala, P.; Pasion, R.; Whalen, M.C.; Stall, R. E. & Staskawicz, B. J. (1999) Expression of the Bs2 pepper gene confers resistance to bacterial spot disease in tomato. *Proc Natl Acad Sci USA*, 96,14153–14158.

Turnbull, K.M.; Turner, M.; Mukai, Y.; Yamamoto, M.; Morell, M.K.; Appels, R. & Rahman, S. (2003) Theorganization of genes tightly linked to the Ha locus in *Aegilops tauschii*, the D-genome donor to wheat. *Genome*, 46, 330-338.

Tzfira, T. & Citovsky, V. (2006). Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. *Curr. Opin. Biotechnol.*, 17, 147-154.

Van, E. J.M.; Blowers, A. & Earle, E. (1995) Stable transformation of tomato cell cultures after bombardment with plasmid and YAC DNA. *Plant Cell Rep*, 14, 299-304.

Wada, N.; Kajiyama, S.; Akiyama, Y.; Kawakami, S.; No, D.; Uchiyama, S.; Otani, M.; Shimada, T.; Nose, N.; Suzuki, G.; Mukai, Y. & Fukui, K. (2009) Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes. *Plant Cell Rep,* 28, 759-768.

Wada, N.; Kajiyama, S.; Cartagena, J.A.; Lin, L.; Akiyama, Y.; Otani, M.; Suzuki, G.; Mukai, Y.; Aoki, N. & Fukui, K. (2010) The effects of puroindoline b on the ultrastructure of endosperm cells and physicochemical properties of transgenic rice plant. *J Cereal Sci*, 51,2, 182-188.

Wada, N.; Kajiyama, S.; Khemkladngoen, N. & Fukui, K. (2011a) A novel gene delivery system in plants with calcium alginate micro-beads, in *Plant Transformation Technologies*, Stewart Jr., C. N., Touraev, A., Citovsky, V., and Tzfira, T., pp. 73-82, Wiley-Blackwell Publishers.

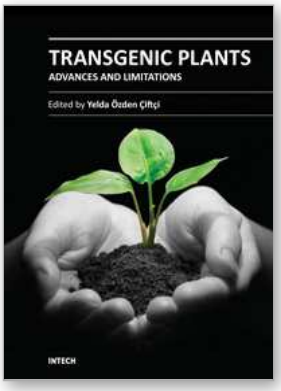
Wada, N.; Cartagena, J.A.; Khemkladngoen, N. & Fukui K. (2011b) Bioactive beads-mediated transformation of plants with large DNA fragments, in *Transgenic Plants; Method and Protocols*, Dunwell, J. & Wetten, A., Springer, in press.

Wang, Y.; Xue, Y.& Li, J. (2005) Towards molecular breeding and improvement of rice in China. *Trends Plant Sci*, 10, 610–614.

Yu, W.; Han, F.; Gao, Z,.; Vega, J. M. & Birchler, J. (2007) Construction and behavior of engineered mini-chromosomes in maize. *Proc Natl Acad Sci USA*, 104, 8924–8929.

Zhou, Y.; Kajiyama, S.; Masuhara, H.; Hosokaw, Y.; Kaji T & Fukui K (2009). A new size and shape controlling method for producing calcium alginate beads with immobilized proteins. *JBiSE*, 2, 287-293.

Zhou, Y.; Kajiyama, S.; Itoh, K.; Tanino, T.; Fukuda, N.; Tanaka, T.; Kondo, A. & Fukui K (2009). Development of an enzyme activity screening system for beta-glucosidase-displaying yeasts using calcium alginate micro-beads and flow sorting. *Appl. Microbiol. Biotechnol.*, 84, 375-382.



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