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Production and Characterization of Transgenic *indica* Rice Plants Carrying Maize *Ac-Ds* Elements Introduced by Particle Bombardment

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Abstract

Following a few reports on *indica* rice transformation, we demonstrated the recovery of transgenic *indica* rice cultivars carrying potentially useful genes through co-transformation with a selectable marker using a particle bombardment method (biolistic process). Scutellum-derived calli and embryos of 3 selected *indica* rice cultivars were used as explants. Co-transformation with selectable *bar* gene was employed to isolate transformed callus lines that are resistant to the herbicide bialaphos. Subsequently, bialaphos-resistant calli were regenerated and 29% of R_0 plants were shown to possess *Ac* and *Ds* by PCR analysis. About 50% of the putative R_0 transformants showed transmission of the transgene based on PCR and Southern blot analyses, indicating that a large number of primary transformants represented chimeric individuals, or that elimination of the transgenes eventually occurred during meiosis/mitosis. PCR-positive R_0 plants were self-pollinated to produce R_1 lines. Single and multiple integrative events were detected and R_1 progenies from 3 transgenic lines exhibited the expected Mendelian inheritance pattern. Although a few rearranged copies of the transgenes were noted, a majority of the copies remained intact after integration. Expression of the CaMV35S-driven transposase gene was revealed by the presence of the transposase transcript in Northern blots. The procedure described here provides an applicable tranformation system that can be further improved to generate more *Ac* and *Ds* lines towards establishing a functional transposon mutagenesis system in *indica* rice.

1. Introduction

Production of transgenic rice plants was first achieved by protoplast transformation systems that employed DNA uptake mediated by electroporation and polyethylene glycol [1-3]. These methods have been routinely applied to transform *japonica* subspecies of rice. However, they have not gained wide acceptance because preparation of protoplasts requires sophisticated and time-consuming process, and it is generally difficult to regenerate fertile plants. This has led to the development of particle bombardment method (biolistic process) [4] that is reproducible, rapid, technically simple and less genotype-dependent. Recently, stable transformation of rice has been achieved by *Agrobacterium*-mediated system [5], providing another alternative procedure to the protoplast transformation.

Indica rice or *indica*-type rice [6] constitutes a larger portion of the world's rice production. In contrast to *japonica* rice, *indica* rice is generally more difficult to regenerate due to its recalcitrance in cell culture response [7, 8], which is a major limitation in *indica* rice transformation. Recently, recovery of transgenic *indica* rice plants has been achieved using different transformation methods [9-13], but mainly with selectable markers and reporter genes, and still restricted to a few responsive cultivars. A few reports are available on the transformation of *indica* rice with agronomically valuable or potentially useful genes [14-16].

We have attempted to introduce the maize transposable elements, *Ac* and *Ds*, into *indica* rice by particle bombardment, following reports of their successful introduction into a number of heterologous hosts where they have retained their transpositional activity [17-22]. It has been shown that *Ac* and *Ds* can be used to isolate plant genes, even without knowledge of their products and functions, through a procedure called transposon tagging [23-27]. While none of the currently available transformation sys-

tems are ideal for *indica* rice transformation, we have chosen to employ the particle bombardment system, owing to its simplicity and applicability to a wide array of target explants.

The development of *Ac-Ds* transposon tagging in heterologous systems must be accompanied by efficient genetic transformation procedures. Transposon-induced mutagenesis in rice, which lacks well characterized endogenous transposons, offers the possibility of rapid isolation of genes controlling important agronomic traits. Here, we report the production and initial characterization of transgenic *indica* rice plants transformed with *Ac* and *Ds* through particle bombardment. The procedure demonstrated in this study can be further improved to provide a speedy and efficient transformation system for production of *Ac* and *Ds* lines to hasten the development of transposon mutagenesis system in *indica* rice.

2. Materials and Methods

2. 1 Plant materials and tissue culture methods

Out of 22 *indica* rice varieties that were screened for regeneration ability, the three most regenerable ones, *i.e.* Nona Bokra, 63-83 and IR54, were selected for transformation (data not shown). Bombarded explants were either scutella of excised seed embryos or 3 week-old scutellum-derived calli cultured on Linsmaier and Skoog (LS) basal medium [28] containing 2 mg liter^{-1} of 2,4-D. Following particle bombardment, to be described later, embryogenic calli were transferred to regeneration medium containing LS inorganic salts, 1 mg liter^{-1} of NAA, 2 mg liter^{-1} of BA, 2 g liter^{-1} of casein hydrolysate, 30 g liter^{-1} of sucrose, 30 g liter^{-1} of solbitol and 1 g liter^{-1} of MES buffer (pH 5.8).

2. 2 Plasmids

Plasmids pDM302, pCKR532 and pCKR234 respectively contain *bar* gene which confers resistance to herbicide bialaphos, a transposase-coding region of *Ac*, and an *Ac*-derived *Ds* element (**Fig. 1**). Derivation of these plasmids were previously described [29, 30]. The 3.2 kb *Ac*, under the control of the cauliflower mosaic virus (CaMV) 35S promoter, is an immobilized version of the autonomous *Ac*, derived by deleting its terminal inverted repeats. The 3.0 kb *Ds* element was cloned between CaMV35S promoter and hygromycin resistance gene (*hph*) which serves as an excission marker. Bacterial culture, plasmid DNA isolation and purification by CsCl/EtBr density centrifugation were performed as described by Maniatis *et al.* [31].

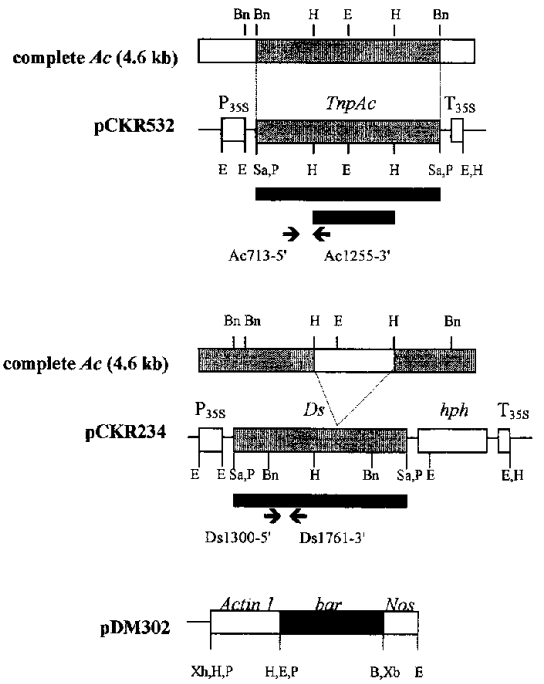

Fig. 1 Map descriptions of plasmids used in transformation.
Abbreviations: E=*Eco*RI; H=*Hin*dIII; Bn=*Ban*II; Sa=*Sal*I; P=*Pst*I; B=*Bam*HI; Xh=*Xho*I; S=*Sac*I; Xb=*Xba*I; *TnpAc*=transposase coding region of *Ac* element; *hph*=hygromycin B phosphotransferase gene; P_{35S}=CAMV35S promoter; T_{35S}=CAMV35S polyadenylation site; *Actin* 1=rice actin 1 promoter; *Nos*=nopaline synthase polyadenylation site; *bar*=phosphinotricin acetyl transferase gene. The *TnpAc* is an immobilized version of the autonomous *Ac*, derived by deleting its terminal inverted repeats. *Ds* was derived after internal *Hin*dIII deletion from the 4.6 kb *Ac* gene. The *hph* gene serves as *Ds* excision marker since the 3.0 kb *Ds* was inserted within the untranslated leader sequence of *hph*. Arrows denote the PCR primers used to amplify 542 bp *Ac* and 461 bp *Ds* regions. Solid bars denote the fragments used as probes in Southern and Northern blot analyses.

2. 3 Particle bombardment and selection of transformed calli and transgenic plants

Plasmid DNA was precipitated onto gold particles having diameter of 1.6 μm, according to the protocol described for the PDS-1000/He Particle Delivery System (Bio-Rad, Richmond, CA). For co-transformation of *bar* and *Ac/Ds*, 10 μg of each component plasmid was mixed and coated onto gold particles. About 50 pieces of calli (1-2 mm diameter) were placed 5 cm from the stopping screen and bombarded twice under partial vacuum (28 mm Hg) using disks

that were ruptured at a pressure of 1100 psi. One week after bombardment, rice calli were separated into smaller pieces and plated onto LS medium containing 5 mg liter^{-1} of bialaphos for selection of transformed cell lines. Generation of calli from bombarded embryos was initiated on non-selective medium for two weeks and then subcultured on selective LS medium. Bialaphos-resistant callus lines were regenerated on non-selective LS regeneration medium. Each primary regenerant or R_0 plant was self-pollinated, giving rise to R_1 seeds.

2. 4 Polymerase chain reaction (PCR) and hybridization analyses

Total genomic DNA was isolated from fresh leaves by the slightly modified potassium acetate method [32]. The following primer sets were used for PCR analysis: Ac713 (5′-ATT TGA TGT TGA GGG ATGC-3′) and Ac1255 (5′-TTT GGA GCT GAA GGA CTAC-3′) which amplifies a 542 bp *Ac* fragment, and Ds1300 (5′-CAT CAC CAT CAT CAT CAA CA-3′) and Ds1761 (5′-AGG CTA ACC ACT TCA TCG TA-3′) which yields a 461 bp *Ds* fragment. 50 μl PCR reaction mixture contained 60 ng DNA, 8 μl dNTPs (2.0 mM each), 2 μl of each primer (25 μM), 0.2 μl Taq polymerase (5 units), 5 μl 10X Taq polymerase buffer and 4 μl of 25 mM $MgCl_2$. Thirty five cycles of PCR was carried out in a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT) using the following thermocycle profiles: denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute and DNA synthesis at 72°C for 1 minute. Southern blots [33] were prepared from PCR-amplified products and total genomic DNA digested with *Dra*I or *Pst*I. Hybridization with labelled 1.6 kb *Ac* and 3.0 kb *Ds* probes, derived respectively from *Hin*dIII and *Sal*I fragments of pCKR532 and pCKR234, was performed using the ECL Labelling and Detection Kit (Amersham, England). Total RNA extraction and Northern blot analysis of R_1 *Ac* transgenic lines were performed as described by Maniatis *et al.* [31].

3. Results

3. 1 Transformation frequency among the primary regenerants

In this study, we employed the principle of co-transformation, since the plasmids of interest do not carry any selectable marker gene. Two separate plasmids were mixed - one harboring *Ac* or *Ds*, and one containing *in vitro* selectable *bar* gene that confers resistance to herbicidal compound bialaphos, to allow selection of genetically transformed callus lines. Using our selection protocol, resistant cell colonies emerged on selection medium 5 to 6 weeks after bombardment. Bialaphos-resistant colonies were isolated and gave rise to regenerated plantlets 8 to 10 weeks after bombardment. Since we employed separation of callus clumps during subculture, it is probable that some regenerants originated from the same cell lines. Regeneration frequency ranged from 14% to 38% as shown in **Table 1.** Initial screening for *Ac*- and *Ds*- transformed R_0 plants was done by PCR amplification of the transgenes using primers specific to *Ac* and *Ds* that amplified 542 bp and 461 bp products, respectively. In putative transformants, the expected products were amplified which were identical to those of the control plasmids, as shown in **Fig. 2.** The identity of the PCR products was established by Southern hybridization (**Fig. 2**, lower panels) and confirmed the presence of the transgene in 20 out of 68 R_0 plants. *Ac*- or *Ds*-positive plants isolated from bialaphos resistant calli (co-transformation frequency) ranged from 17% to 50% with an average of 29% (**Table 1**).

3. 2 Transmission and inheritance of the transgene in R_1 plants

We further analyzed 20 R_1 lines derived by self-pollination of 20 putatively transformed R_0 plants, but obtained only 10 lines (3 *Ac* lines and 7 *Ds* lines) that could amplify the transgenes by PCR. The low trans-

Table 1. Results of tranformation of *indica* rice with maize *Ac* and *Ds* by particle bombardment.

Transgene	Cultivar	Explant	Bombarded Filter*1	Bialaphos-Resistant Colony	Regenerated Plant*2	PCR-positive	Co-transformation Frequency (%)
bar+*Ac*	Nona Bokra	callus	5	80	24 (30)	4	17
	63-83	callus	3	36	5 (14)	2	40
	IR 54	embryo	2	21	4 (19)	1	25
bar+*Ds*	Nona Bokra	callus	2	34	10 (29)	2	20
	63-83	callus	4	52	20 (38)	10	50
	IR 54	embryo	2	32	5 (16)	1	20
Total			18	224	68 (30)	20	29

*1 Filters contained 40-50 pieces calli or embryos.

*2 Values inside parentheses indicate the percentage of regenerated bialaphos-resistant calli. Regenerated plants are not necessarily independent due to the separation of callus clumps during selection.

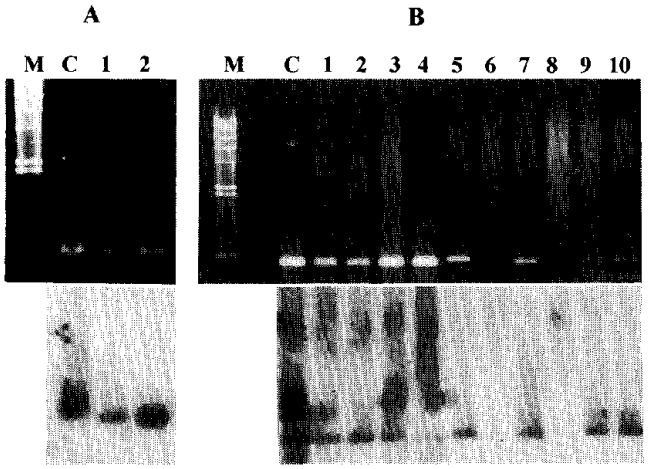

Fig. 2 PCR amplification of *Ac* and *Ds* elements from putative transformants of *indica* rice. PCR products of (A) 542 bp *Ac* and (B) 461 bp *Ds* were blotted onto nylon membrane and hybridized with *Ac* and *Ds* probes, respectively. Amplified products of putative transformants (lanes 1-10) were identical to that of the control plasmids, pCKR532 and pCKR234 (lane C on panels A and B, respectively). Southern blots, confirming the identity of the PCR products, are shown on the lower panels. Lane M is λ*Hin*dIII DNA marker.

mission of the transgenes to the progeny of the putative transformants suggests that many primary regenerants were chimeric individuals or eventual elimination of the hemizygous transgenes took place during mitosis/meiosis. Furthermore, R_1 progenies from *Ac* and *Ds* lines of Nona Bokra and 63-83 were subjected to PCR analysis to test the presence of the transgenes. **Table 2** shows 3: 1 segregation among 72 R_1 seedlings tested from 2 *Ds* lines of 63-83, indicating Mendelian inheritance of a single *Ds* locus. *Ac* line BA15 apparently exhibited 15: 1 segregation, indicating that the gene was integrated in two unlinked loci. *Ac* line BA9, bearing a single copy of *Ac*, failed to segregate in a 3: 1 ratio. In fact, the observed segregation ratio fitted to 1: 1 which indicated either that the transgene was transmitted exclusively through male or female gametes [36], or that elimination of the transgene occurred during meiosis.

3. 3 Copy number, modification and expression of the transgenes

In order to determine the copy number of the transgenes integrated into the *indica* rice genome, total DNA was extracted from PCR-positive R_1 plants and digested with *Dra*I or *Pst*I. Genomic Southern blot analysis, performed with 3. 2 kb *Ac* and 3. 0 kb *Ds* as probes, revealed both single and multiple copy integration of the transgenes (complete data not shown). In some cases, different *Ds* lines revealed identical patterns of hybridizing bands, indicating that these lines did not arise from independent transformation events but represented single callus line-derived transformants that were separated during selection. Southern blot analysis performed with *Pst*I-digested genomic DNA was expected to liberate 3. 2 kb *Ac* and 3. 0 kb *Ds* fragments. However, there were at least five bands that hybridized with *Ac* and *Ds* probes, consisting of intense 3. 2 kb and 3. 0 kb bands corresponding to the intact *Ac* and *Ds* (**Fig. 3**). Few minor hybridizing fragments, up to 16 kb in size, were also detected in the Southern blots. This indicates that while a majority of the transgene copies remained intact after integration into *indica* rice chromosomes, a few copies have undergone alteration in at least one of the *Pst*I restriction sites of the plasmid during integration process. Despite the presence of multiple integrative events and rearranged

Table 2. Inheritance of *Ac* and *Ds* in R_1 transgenic lines of Nona Bokra and 63-83.

Line	Transgene	Tiller number	No. of seedlings analyzed	PCR test (+)	PCR test (-)	Expected Ratio	χ^2
BA15	*Ac*	1	42	37	5	15: 1	2.29
BA12	*Ac*	1	15	7	8	1: 1	0.07
		2	12	5	7	1: 1	0.33
		Total	27	12	15	1: 1	0.40
BD1	*Ds*	1	12	9	3	3: 1	0.00
		2	12	10	2	3: 1	0.44
		3	12	6	6	3: 1	4.00*
		Total	36	25	11	3: 1	4.44
BD3	*Ds*	1	12	8	4	3: 1	0.00
		2	12	9	3	3: 1	0.44
		3	12	6	6	3: 1	4.00*
		Total	36	23	13	3: 1	4.44

* significant at 5% level

BA15 and BA12 are Nona Bokra transgenic lines; BD1 and BD3 are 63-83 transgenic lines

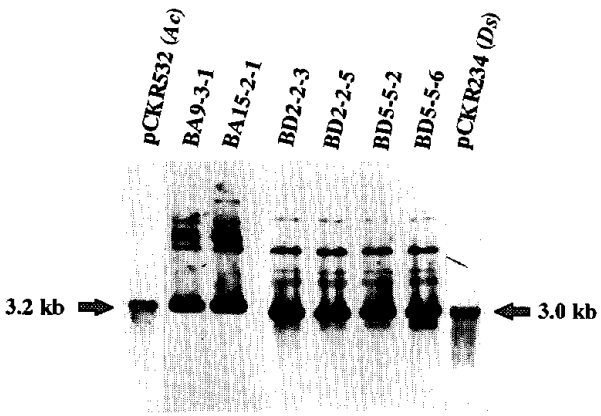

Fig. 3 Genomic Southern blots showing the hybridization patterns with *Ac* and *Ds* probes after *Pst*I digestion of total DNA (20 μg per sample) from transgenic rice lines. An arrow indicates the expected band of 3.2 kb for *Ac* (left) and 3.0 kb for *Ds* (right). Minor hybridizing bands of higher molecular sizes indicate the presence of rearranged copies of the transgene. pCKR532 and pCKR234 represent *Pst*I-digested markers. BA represents plants from two different *Ac* lines of Nona Bokra, and BD represents plants from two different *Ds* lines of 63-83. Identical hybridization patterns indicate that the lines might have originated from the same cell lines that were separated during selection.

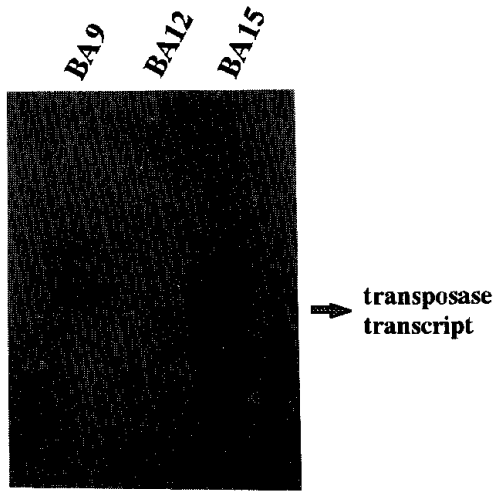

Fig. 4 Northern blot analysis of total RNA (20 μg per sample) from 3 *Ac* lines. Lines BA9 and BA15, carrying at least 5 copies of *Ac*, showed the presence of the transcript after probing the Northern blot with 1.6 kb fragment of the *Ac* transposase coding region. A line BA12 did not show the transposase transcript.

copies, the precise expression of the *Ac* transposase was observed. **Fig. 4** shows the presence of the transposase transcripts in two *Ac* lines (BA9 and BA15) after probing Northern blots with the *Ac* coding region. The transcript produced by one putative *Ac* line (BA12), carrying a single copy of the transgene, could not be detected in Northern blot.

4. Discussion

In this paper, we demonstrated the production of transgenic *indica* rice plants that carry stably inherited *Ac* and *Ds* through a simple transformation system using particle bombardment. Biolistic delivery of an *in vitro* selectable *bar* gene mixed with unselectable *Ac*/*Ds* resulted in the recovery of transformed plants carrying *Ac* or *Ds*. This co-transformation principle proved to be highly efficient in obtaining co-transformed cell lines as observed in earlier reports [34, 35]. Regeneration of fertile plants from selected callus lines was attained 8 to 10 weeks after bombardment process, attesting to the rapidity of the biolistic method in generating transformed plants. Initial evidences for integration of the maize *Ac* and *Ds* elements into *indica* rice genome were presented in PCR and genomic Southern blot data. Inheritance of the *Ac* and *Ds* loci conformed to the expected Mendelian inheritance pattern.

The presence of transgene modifications after transformation was evident from Southern blots. Despite this, the precise expression of the transposase gene was confirmed by Northern blot analysis. This suggests that a majority of the *Ac* copies have been intact and thus produce the transposase transcript. Rearrangements and fragmentation are commonly observed as post-transformation events. Such alterations in the transgene structure were also observed in *indica* rice transformed by PEG- and *Agrobacterium*-mediated systems [10, 13].

A major drawback in our procedure, however, was the high occurrence of false positives, most likely due to epigenetic or chimeric events. Due to the randomness of the biolistic process, only a small fraction of target cells may receive the transgene. In the absence of direct selection for desired transgenic cells, the probability of obtaining non-transformants and chimeric plants is high [37]. Therefore, plasmid delivery process needs to be further refined to improve the overall transformation efficiency. The presence of chimeric individuals may also be a result of elimination of the hemizygous transgenes during mitosis/meiosis via some mechanisms. The low transmission of the transgene to the progeny could be due to this phenomenon which was also speculated from the observed 1:1 segregation ratio. Distorted segregation of the transgene has been reported [11, 36] but the reason(s) remains unclear.

Not much work has been done on *indica* rice transformation with agronomically valuable or potentially useful genes. The introduction of *Ac* and *Ds* elements into *indica* rice is a prelude to our objective of establishing a transposon tagging system for isolation of important genes from *indica* germplasm. Insertion mutants will be generated by transposition and insertion of *Ds* upon trans-activation by an immobile transposase source. To separate the mobile *Ds* from the immobile *Ac*, introduction of the two transgenes were done independently on the same cultivar. Plants carrying stabilized *Ac* that actively produces transposase will be crossed to a given *Ds* starter line. In the F_1, *Ds* is expected to transpose to new sites upon excision. Excision events can be monitored by a phenotypic assay for hygromycin resistance since *Ds* was cloned within the untranslated leader sequence of the *hph* gene. Also, PCR analysis using primers specific to the CaMV35S promoter and *hph* gene can be employed to rapidly determine *Ds* excission. The site of *Ds* transposition should then be surveyed. Transformed callus lines containing both *Ac* and *Ds* are now being regenerated and analyzed for *trans*-activation. Our main objective, however, is to produce *Ds* starter lines which carry the element at different chromosomal locations. This is to exploit the tendency of *Ac* and *Ds* to transpose preferentially to linked sites on the same chromosome as reported in other species [21, 38, 39]. Future transposon tagging work can be greatly facilitated if the map positions of *Ds* among independent starter lines are known. Work is now under way to map the single *Ds* locus on the rice chromosomes using the inverse PCR technology that can generate probes from regions flanking the integrated *Ds* element [40, 41]. The starter line that has *Ds* closest to the locus to be tagged will then be selected and crossed to an *Ac* line to generate a transposon-mutagenesized population.

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References

[1] Toriyama, K., Arimoto, Y., Uchimiya, H., Hinata, K., 1988. Bio/Tech., **6**: 1072-1074.

[2] Zhang, H. M., Yang, H., Rech, E. L., Golds, T. J., Davis, A. S., Mulligan, B. J., Cocking, E. C., Davey, M. R., 1988. Plant Cell Rep., **7**: 379-384.

[3] Shimamoto, K., Terada, R., Izawa, T., Fujimoto, H., 1989. Nature, **338**: 274-276.

[4] Christou, P., Ford, T. L., Kofron, M., 1991. Bio/Tech., **9**: 957-962.

[5] Hiei, Y., Ohta, S., Komari, T., Kumashiro, T., 1994. Plant J., **6**: 271-282.

[6] Glazzman, J. C. 1987. Theor. Appl. Genet., **74**: 21-30.

[7] Lee, L., Schroll, R. E., Grimes, H. D., Hodges, T. K., 1989. Planta, **178**: 325-333.

[8] Datta, S. K., Datta, K., Potrykus, I., 1990. Plant Cell Rep., **9**: 253-256.

[9] Datta, S. K., Peterhans, A., Datta, K., Potrykus, I., 1990. Bio/Tech., **8**: 736-740.

[10] Peng, J., Kononowics, H., Hodges, T., 1992. Theor. Appl. Genet., **83**: 855-863.

[11] Cooley, J., Ford, T., Christou, P., 1995. Theor. Appl. Genet., **90**: 97-104.

[12] Aldemita, R., Hodges, T., 1996. Planta, **199**: 612-617.

[13] Rashid, H., Yokoi, S., Toriyama, K., Hinata, K., 1996. Plant Cell Rep., **15**: 727-730.

[14] Datta, S. K., Datta, K., Soltanifar, M., Donn, G., Potrykus, I., 1992. Plant Mol. Biol., **20**(4): 619-629 .

[15] Lin, W., Anuratha, C. S., Datta, K., Potrykus, I., Muthukrishnan, S., Datta, S. K., 1995. Bio/Tech., **13**: 686-691.

[16] Wünn, J., Klöti, A., Burkhardt, P. K., Ghosh Biswas, G. C., Launis, K., Iglesias, V. A., 1996. Bio/Tech., **14**: 171-176.

[17] Baker, B., Schell, J., Lõrz, H., Fedoroff, N., 1986. Proc. Natl. Acad. Sci. USA, **83**: 4844-4848.

[18] Knapp, S., Coupland, G., Uhrig, H., Starlinger, P., Salamini, F., 1988. Mol. Gen. Genet., **213**: 285-290.

[19] Yoder, J. I., Palys, J., Alpert, K., Lassner, M., 1988. Mol. Gen. Genet., **213**: 291-296.

[20] Zhou, J. H., Atherly, A. G., 1990. Plant Cell Rep., **8**: 542-545.

[21] Bancroft, I., Dean, C., 1993. Genetics **134**: 1221-1229.

[22] Izawa, T., Miyazaki, C., Yamamoto, M., Terada, R., Iida, S., Shimamoto, K., 1991. Mol. Gen. Genet., **227**: 391-396.

[23] Fedoroff, N. V., Furtek, D. B., Nelson, O. E. Jr., 1984. Proc. Natl. Acad. Sci. USA, **81**: 3825-3829.

[24] Walbot, V., 1992. Annu. Rev. Plant Physiol. Plant Mol. Biol., **43**: 49-82.

[25] Aarts, M. G. M., Dirkse, W. G., Stiekema, W. J., Pereira, A., 1993. Nature, **363**: 715-717.

[26] Chuck, G., Robbins, T., Nijjar, C., Ralston, E., Courtney-Gutterson, N., Dooner, H. K., 1993. Plant Cell, **5**: 371-378.

[27] Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J., Jones, J. D. G., 1994. Science, **266**: 789-792.

[28] Linsmaier, E. M., Skoog, F., 1965. Physiol. Plant., **18**: 100-126.

[29] Cao, J., Duan, X., McElroy, D., Wu, R., 1992. Plant Cell Rep., **11**: 586-591.

[30] Shimamoto, K., Miyazaki, C., Hashimoto, H., Izawa, T., Itoh, K., Terada, R., Inagaki, Y., Iida, S., 1993. Mol. Gen. Genet., **239**: 354 - 360.

[31] Maniatis, T., Fitsch, E. F., Sambrook, J., 1982. "Molecular Cloning: A laboratory manual", Cold Spring Harbor Laboratory Press, Plainview, New York.

[32] Dellaporta, S. L., Wood, J., Hicks, J. B., 1983. Plant Mol. Biol. Rep., **1**: 19-21.

[33] Southern, E. M., 1975. J. Mol. Biol., **98**: 503-517.

[34] Li, L., Qu, R., Kochko, A., Fauqet, C., Beachy, R. N., 1993. Plant Cell Rep., **12**: 250-255.

[35] Shimada, T., Güel, F., Takumi, S., 1995. Bull. RIAR, Ishikawa Agr. Coll., **4**: 1-8.

[36] Christou, P., Swain, W. F., Yang, N. S., McCabe, D., 1989. Proc. Natl. Acad. Sci. USA, **86**: 1991-1995.

[37] Cao, J., Zhang, W., McElroy, D., Wu, R., 1991. In "Rice Biotechnology" (eds. by Khush, G. S., Toennissen, G. H.), p. 175-198, C.A.B. International, U. K.

[38] Jones, J. D. G., Carland, F. C., Lim, E., Ralston, E., Dooner, H. K., 1990. Plant Cell, **2**: 701-707.

[39] Keller, J., Lim, E., Dooner, H. K., 1993. Theor. Appl. Genet., **86**: 585-588.

[40] Triglia, T., Peterson, M. G., Kemp, D. J., 1988. Nucleic Acids Res., **16**: 8186.

[41] Knapp, S., Larondelle, Y., Roβberg, M., Furtek, D., Theres, K., 1994. Mol. Gen. Genet., **243**: 666-673.