Rachilla: An additional source for embryogenic callus induction and transformation in *Oryza sativa* L.

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Rachilla, the portion joining sterile glumes to palea and lemma, in the rice spikelet has been found to be an additional source of embryogenic callus and transformation. Induction of callus from rachilla was distinct in inflorescences of size 3 cm or above. Inflorescence length ranging between 3 and 5 cm was found to be ideal for induction of callus from rachilla. The response was optimum when the florets were of 2–5 mm. The callus induced from rachilla is lustrous, friable and embryogenic, and facilitated much faster establishment of uniform embryogenic suspension cultures (3–4 weeks) compared to seed-derived calli (6–8 weeks). Though the frequency of callus induction from rachilla is low compared to mature seed-derived calli, the regeneration potential of the former is higher. The rachilla callus could be used for bombardment either directly or after establishment of suspension cultures. The calli derived from rachilla did show advantage over seed-derived calli by hastening the process of establishing suspension cultures or for genetic transformation.

THE prospects of genetic improvement of crops through nonconventional breeding strategies are increasing with the advent of recombinant DNA technologies and improved methodologies in cell and tissue culture systems. Success in the application of genetic transformation techniques largely depends on the ability to select transformed cells and regenerate fertile plants from them. It is difficult to obtain normal fertile regenerants from single cells in monocots in general and cereals in particular. Molecular genetic improvement is, therefore, directed through the use of protoplasts and through bombardment of embryogenic calli till *Agrobacterium* is used as a routine method. Transformation of rice by direct delivery of DNA into protoplasts or bombardment of suspension calli has been reported recently by many laboratories[1,2]. Establishment of embryogenic cell suspensions for isolation of protoplasts is a tedious and time-drawn process of, 12–16 weeks. Development of methods for induction of embryogenic calli and use of different explant sources for the purpose has been enumerated in rice[3]. Of the several explant sources reported. mature seeds are the most frequently used due to their ready availability and ease in handling. Immature embryos and inflorescence are also often used due to their potential for production of embryogenic callus. Additional explant sources and the development of methods for formation of embryogenic callus cultures will enhance transformation efficiency. We report here the induction of callus from rachilla, the portion joining sterile glumes to palea and lemma (Figure 1 *a*) and enumerate the relative advantages of this new source of embryogenic callus for genetic transformation.

Immature inflorescences enclosed in boots were collected from the glasshouse-grown plants of eight elite rice varieties viz. Rasi, Vikas, Seshu, Jaya, Annada, Rasi, IR72 and Vibhava. The boots were surface-sterilized for 30 min in 0.1% mercuric chloride followed by ethanol. Inflorescences ranging in length from 1–10 cm were tried. The panicles were dissected out aseptically and cultured on MS medium[4] supplemented with 3 mg l^{-1} 2,4- dichlorophenoxyacetic acid (2,4-D), 0.2 mg l^{-1} benzylaminopurine (BAP), 50 mg l^{-1} tryptophan and 300 mg l^{-1} casein enzymatic hydrolysate. Five-to-six-week-old calli from rachilla were regenerated on

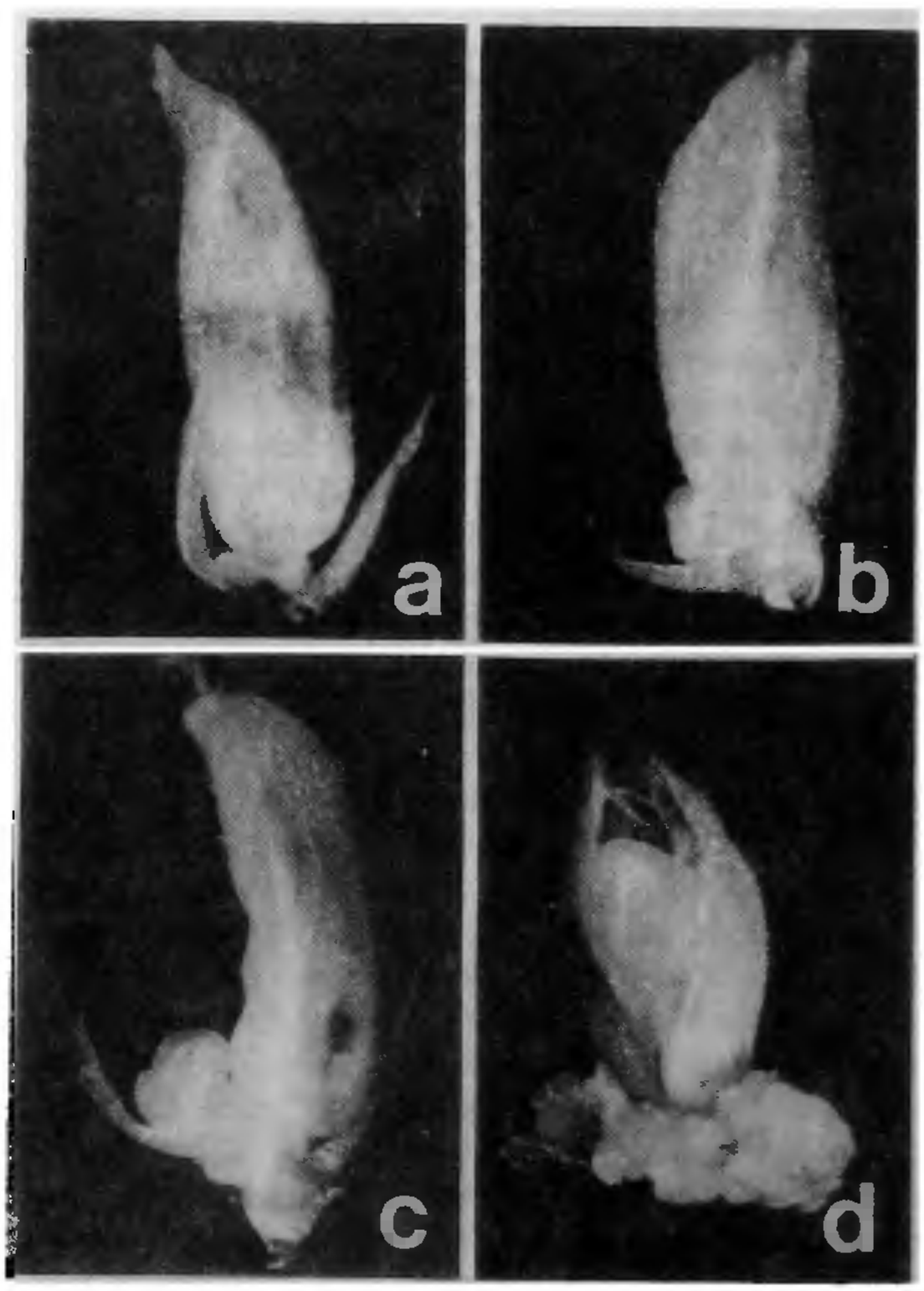

Figure 1. *a*, Rice floret; *b, c, d*, Gradual stages of callus induction from rachilla.

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solidified MS basal medium supplemented with 2.5 mg l^{-1} BAP, 1 mg l^{-1} kinetin, 0.5 mg l^{-1} napthaleneacetic acid (NAA), 500 mg l^{-1} of casein enzymatic hydrolysate and 1 g l^{-1} of proline. Calli from rachilla were also used for initiation of suspension cell cultures in N_6 liquid medium[5], supplemented with 3 mg l^{-1} 2,4-D, 5 mM proline and 20 g l^{-1} maltose as carbon source.

For standardization of the transformation protocol, callus from rachilla was induced on NBKNB medium[6] supplemented with 3 mg l^{-1} 2,4-D. The plasmid construct pCAMBIA 1301, provided by Dr R. A. Jefferson from CAMBIA, Australia, was used for transformation. This construct carries a β-glucuronidase (*gus*) gene with catalase intron and hygromycin-resistance (*hpt II*) gene for selection of plant tissues both driven by CaMV35s promoter. Plasmid DNA was isolated following the protocol given in the laboratory manual by Dr H. Czosnek (unpublished). DNA was dissolved in TE buffer (pH 8.0), checked for its purity at 260 nm and 280 nm, and the final concentration was adjusted to 1 µg/µl for ready use. The target calli were transferred to osmotic NBKNB medium, supplemented additionally with 30 g l^{-1} each mannitol and sorbitol 4 h prior to bombardment. 30 mg of 1/1.6 µ gold particles (BioRad) were presterilized and suspended in sterile distilled water. To a 50 µl (3 mg) aliquot of gold suspension, 5 µl of plasmid DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine were added in the same order while vortexing. After precipitating in 250 µl of chilled ethanol, the particles were re-suspended in 100 µl of the same. An aliquot of 7.5 µl × 2 was loaded on to six macrocarriers each. Bombardment of the calli was done at 1100–1300 psi helium pressure using BioRad PDS 1000 He delivery system. The calli were transferred to osmotic-free medium (NBKNB) 18 h after bombardment. After 24 h the calli were transferred to selection medium NBKNB containing 30 mg l^{-1} hygromycin. Subculturing was done at 10–15 d interval. Transient *gus* expression in the calli was tested after 48 h and stable expression after 3 cycles of selection. The calli were incubated at 37°C in *gus* assay mixture containing 0.5 mg/ml X-gluc Na salt (Biosynth) in 100 mM sodium phosphate buffer pH 7.0 and 10 mM Na_2 EDTA. The frequency of transient *gus* expression was observed 12 h after treatment with gus stain. The stable transformants were visible 2 h after treatment with the gus stain.

Callus could be induced from immature inflorescences in two-to-three weeks of culture on MS medium with supplements as mentioned. Proliferation of callus was observed from all floral parts including ovary, rachilla and the excised ends of the inflorescence rachis. Juvenile inflorescences < 3 cm long produced a mass of callus and it was not possible to distinguish at this stage the floral part from which the callus had been derived (Figure 2 *b*). However, it was possible to delineate clearly in inflorescences > 3 cm long, the particular floral parts giving rise to callus and most distinguishable among them being the rachilla-derived callus (Figure 2 *c*). In the inflorescence of 3–6 cm length, callus induction from rachilla was more clearly visible (Figures 1 *a, b, c, d*). With further increase in length of the inflorescence, the enlargement of the ovary was more prominent than the rachilla callus. Callus induction

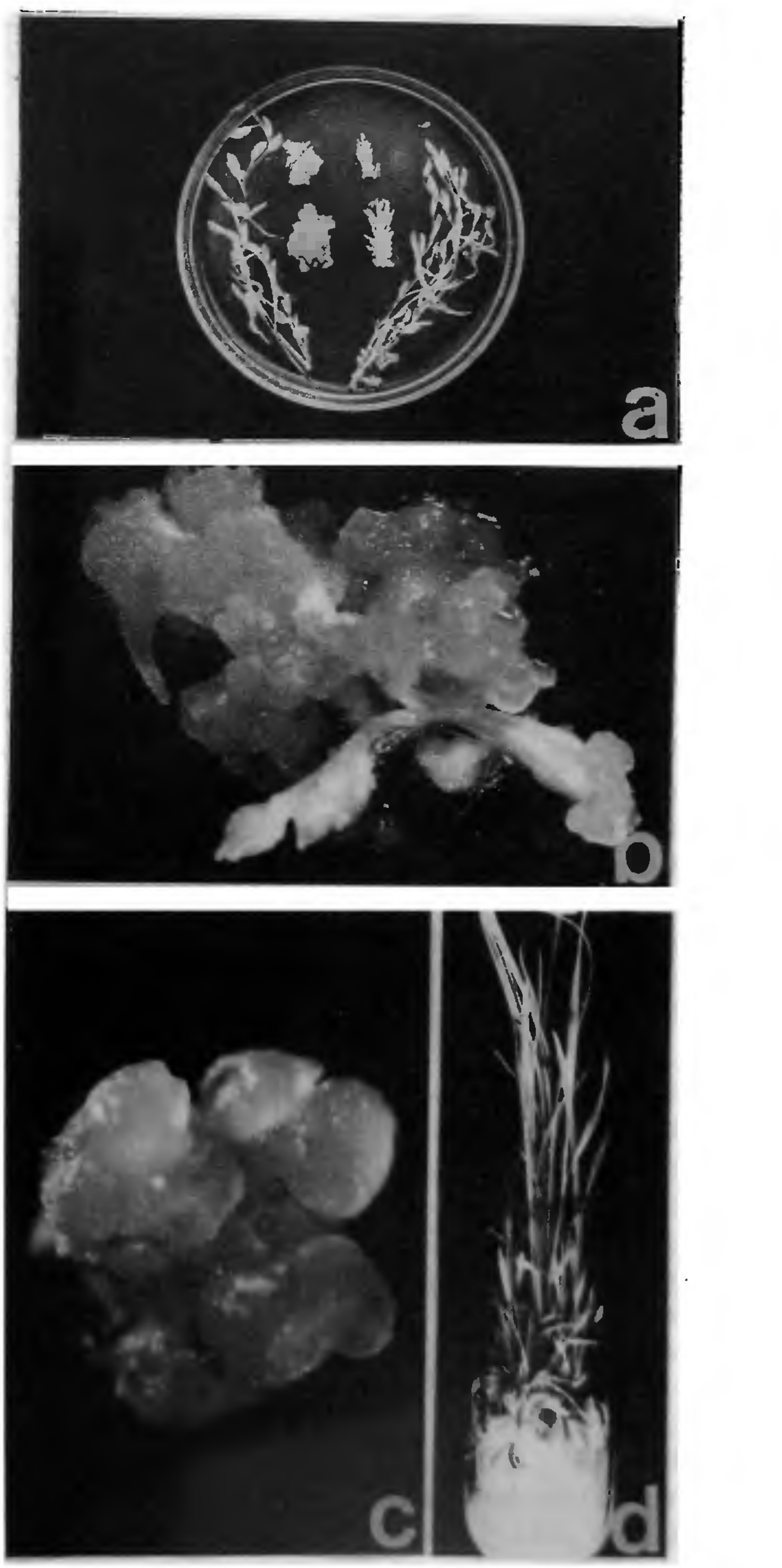

Figure 2. *a*, Callus induction from inflorescences of different sizes; *b*, Mass of callus from < 3 cm inflorescence; *c*, Embryogenic organization of rachilla callus; *d*, Regeneration from rachilla.

Table 1. Frequency of callus induction from rachilla (%)

Variety*	Floret size (mm)				
	2	3	4	5	6
Vikas	9.8 ± 1.8	21.3 ± 10.7	16.9 ± 9.0	0	–
Seshu	ND	26.5 ± 0.9	47.9 ± 12.2	32.6 ± 13.0	13.3 ± 1.9
Jaya	–	–	29.4 ± 7.5	29.2 ± 4.2	–
Ravi	–	–	–	–	18.4 ± 0.4
Rasi	ND	58.5 ± 1.5	60.4 ± 4.5	56.3 ± 6.3	29.3 ± 0.7
Vibhava	16 ± 9	0	17.0 ± 2.9	–	–
IR72	5.1 ± 0.3	–	–	–	–
Annada	37.2 ± 12.5	25.9 ± 6.2	19.2 ± 8.1	4.7 ± 6.7	0

–, Data not recorded; ND, Not distinguishable; 0, No response.
*Mean of three replicates.

Table 2. Regeneration of callus from the rachilla and seed

Variety	Rachilla		Seed	
	Frequency (%)	Plants/unit culture*	Frequency (%)	Plants/unit culture*
Seshu	88.9 ± 7.9	15–20	49.3 ± 7.9	5–10
Jaya	45 ± 5	1–7	43.5 ± 3.5	1–5
Rasi	73.8 ± 13.8	15-25	13 ± 5.1	3–7
IR64	75 ± 0	8-20	56.2 ± 12.9	2–7

Mean ± S.E.; *No. of plants regenerated from equal quantity of callus ca. 30 mg.

from rachilla appears to be varied, depending not only on the size of inflorescence but also on the variety represented by different cultivars. In the variety Vikas, callus induction from rachilla was observed in inflorescences of size > 5 cm whereas in Rasi, Seshu, Vibhava and Annada rachilla callus was distinguishable even in inflorescences of 3 cm size. In IR72, the response was less and inconsistent. The varieties Jaya and Rasi also responded positively for callus induction from rachilla in the inflorescence 5–6 cm size.

The relationship between the size of the floret and the rachilla callus response was examined (Table 1). The response was maximum when the floret size was in the range 2–5 mm. Callus from rachilla could not be distinguished from the tiny florets of 2 mm or less from other explant sources such as rachis and cut ends of inflorescence axis in the varieties Rasi and Seshu. In varieties IR72, Annada, Vikas and Vibhava the callus from rachilla could be distinguished even in florets of 2 mm size. Callusing decreased with the increase in the size of the floret beyond 5 mm. In rice panicles the florets are arranged in basipetal succession. This is probably why the point of origin of callus from basal florets of the inflorescence was indistinguishable and maximum distinguishable response was seen from the rachilla of the middle florets (2–5 mm).

Callus induction and regeneration of plants from immature inflorescences have been reported earlier by Chen *et al.*[7], Wang *et al.*[8], and Ling and Komamine[9]. They observed callus induction from immature inflorescences of < 3 cm size. The present study also confirms the induction of callus from juvenile inflorescence as small as 3 cm or less. It was, however, difficult at this stage to clearly pinpoint the origin of callus. Since we studied several varieties with varying sizes of inflorescences as well as florets, it was possible to trace the exact origin of callus in the floral organs. The callus induced from rachilla is creamy coloured and well organized into embryoids. The size of the embryoids is smaller than those of seed-derived callus. Further, the rachilla callus is lustrous, friable and embryogenic (Figure 2 *c*) and upon transfer to regeneration medium the rachilla callus proliferated rapidly giving rise to nodular structures covered with fuzzy hairs. These structures transform into shoots (Figure 2 *d*). The regeneration frequency from calli induced from rachilla and seed embryos was compared (Table 2). The regeneration pattern of calli from these different explants could be classified into two types: (i) lustrous green spots covered with fuzzy hairs that gave rise to leaf primordia, and (ii) pale green spots with waxy coat giving rise to green structures but not leaf-like structures. Calli induced from seed embryos showed both types of regeneration whereas the rachilla callus showed predominantly the latter type. Furthermore, the regeneration frequency was far higher in rachilla callus compared to seed-derived callus in the varieties Rasi, Seshu and IR64, but it was only slightly superior in Jaya (Table 2). Also, the range of plants regenerated per unit culture was greater from rachilla callus than from mature seed, with the exception of Jaya (Table 2).

Embryogenic suspension cultures offer the advantage of mass production as well as for use in transformation mediated through protoplasts or biolistics. Poonspaya *et al.*[10] observed that the technique of transferring the callus by removing the nonembryogenic callus has facilitated the embryogenic callus to grow further and thereby allowing more embryogenic callus for regeneration. However, the seed-derived calli from indica type are compact and difficult to separate and turned brown

when removed mechanically. The young callus from rachilla is friable and is an excellent material for initiation of suspension cultures. Using the rachilla callus, homogeneous embryogenic suspension cultures could be established within 3–4 weeks. The callus from seed embryo, due to its compact nature, needed 3–4 subcultures (6–8 weeks) to get a friable callus suitable for initiation of suspension cultures. It also involved tedious microscopic separation of embryogenic callus to initiate suspension cultures. The suspension cultures developed from rachilla callus were observed to be qualitatively better than those from seed-derived callus, with easily disassociating cell clumps.

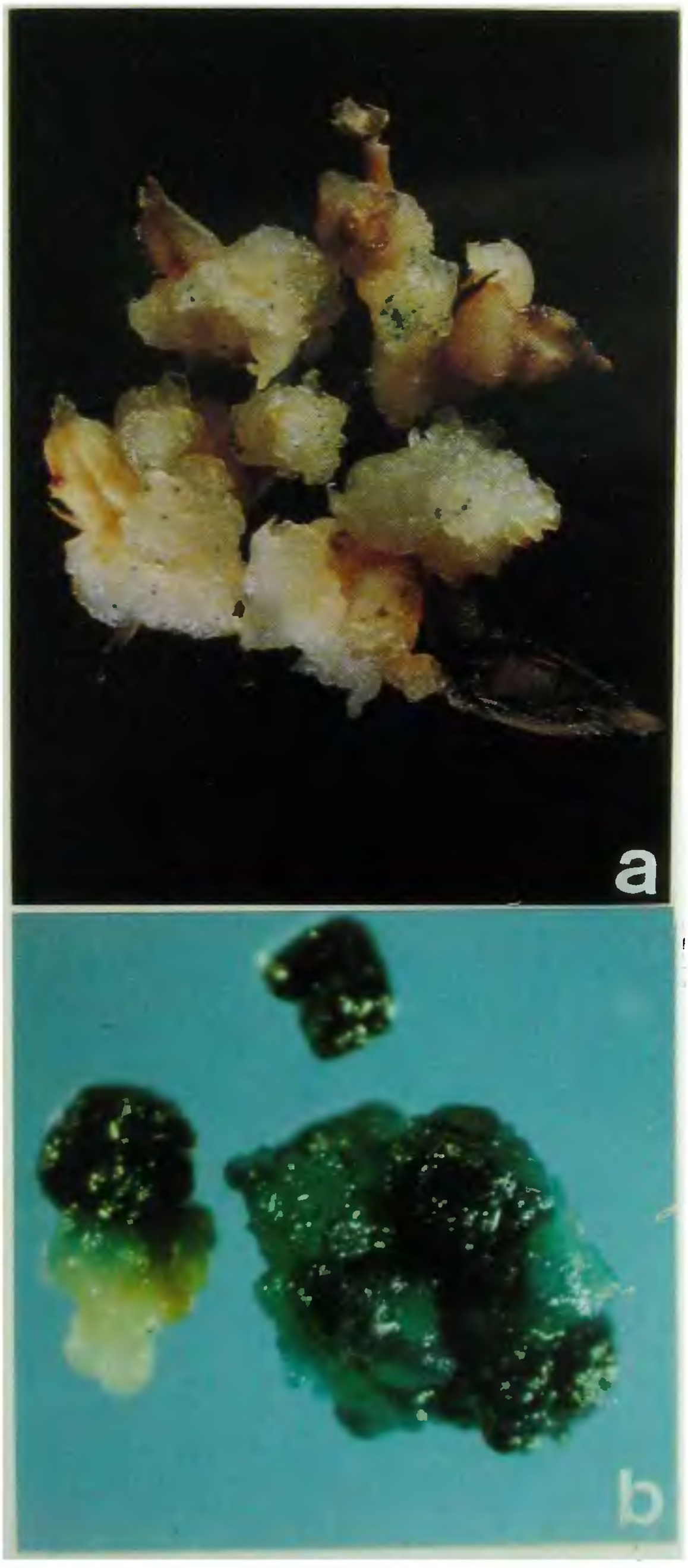

Figure 3. *a*, Transient *gus* expression in rachilla callus; *b*, Stable *gus* expression after 3 cycles of selection.

Immature embryos and suspension cell clumps are most frequently preferred as the target material for transformation through particle bombardment for obvious reasons. Nevertheless, isolation of immature embryos is tedious and laborious and may not be available round the year. Embryogenic suspension calli consisting of cell clumps with relatively less number of cells have advantage of greater exposure to plasmid DNA and selection agents and thereby minimizing the nontransformed escapes during selection[11]. Owing to highly embryogenic organization, the callus from rachilla provides excellent material for use in biolistic transformation. The friability of the rachilla callus had a distinct advantage over seed-derived calli for direct bombardment and selecting transformed cells. Six-to-seven-week-old rachilla-derived callus when bombarded directly showed transient *gus* expression (Figure 3 *a*). Observations on transient *gus* expression indicate that the japonica variety Taipei 309 is the one with the highest recipient calli (83%). Among the indica varieties tested, Seshu (70%) and Vikas (67%) showed highest followed by Vibhava (54%) and Rasi (28%) (Table 3). After transfer to the selection medium, these calli could be cut into small pieces to enhance the permeability of the selection agent, leading to efficient selection of transformed sectors and minimizing the escapes. Selection for two-to-three cycles of 10–15 d each were sufficient to eliminate most of the non-transformants. The selection was confirmed by gus histochemical assay after 3 cycles of selection (Figure 3 *b*). The frequency of transformed calli was 23.5% and 32% after 2nd and 3rd cycle of selection respectively in rachilla-derived callus, whereas it was only 8.6% in seed-derived calli after 3 cycles of selection. Direct use of rachilla-derived callus for transformation reduced the duration of transgenic production. The

Table 3. The *gus* expression in rachilla callus after 48 h of bombardment

Variety	Total calli bombarded	% Calli with blue foci
Taipei-309	70	83
Vibhava	67	54
Vikas	87	67
Rasi	62	28
Seshu	74	70

compact nature of seed-derived calli in indica varieties and problems associated with it can be circumvented by direct bombardment of rachilla-derived callus. The increased efficiency and rapidity of selection of transformants coupled with its superior regeneration makes the rachilla a potential explant source for tissue culture as well as transformation studies. Further, for protoplast-mediated gene transfer, rachilla-derived calli have an additional advantage as the time required for establishing the uniform embryogenic suspension cultures is reduced. With the availability of an additional source of embryogenic callus and the attendant advantages, our efforts are focussed on further enhancing the frequency of callus induction from rachilla for use in genetic transformation either through direct bombardment or development of embryogenic cell suspensions to isolate protoplasts for PEG-mediated transformation or electroporation. Because of its superior regeneration potential, rachilla callus can also be used for maintenance and multiplication of male sterile lines in plant breeding programmes.

1. Zhang, S., Chen, L., Qu, R., Marmey, P., Beachy, R. and Faquet, C., *Plant Cell Rep.*, 1996, **15**, 465–469.
2. Jain, R. K., Jain, S., Wang, B. and Wu, R., *Plant Cell Rep.*, 1996, **15**, 963–968.
3. Christou, P., in *Rice Biotechnology and Genetic Engineering*, Technomic Publishing Co. Inc., USA, 1994, pp. 45–93.
4. Murashige, T. and Skoog, F., *Physiol. Plant*, 1962, **15**, 473–497.
5. Chu, C. C., Wang, C. C., Sun, C. S., Hsu, S. C., Yin, K. C., Chu, C. Y. and Bi, F. Y., *Sci. Sinica.*, 1975, **18**, 659–668.
6. Sivamani, E., Shen, P., Opalka, N., Beachy, R. N. and Fauquet, C. M., *Plant Cell Rep.*, 1996, **15**, 322–327.
7. Chen, T. H., Lam, L. and Chen, S. C., *Plant Cell Tiss. Organ Cult.*, 1985, **4**, 51–54.
8. Wang, M. S., Zapata, F. J. and De Castro, D. C., *Plant Cell Rep.*, 1987, **6**, 294–296.
9. Ling, D. H. and Komamine, A., *J. Tropical Subtropical Bot.*, 1994, **2**, 70–78.
10. Poonspaya, P., Nabors, M. W., Wright, K. and Vajrabhaya, M., *Plant Cell Tiss. Organ Cult.*, 1989, **16**, 175–186.
11. Jain, R. K., Jain, S. and Wu, R., *Plant Cell Rep.*, 1996, **15**, 449–454.

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A^+–C Mismatched base-pairing in DNA

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The structural aspects of an antiparallel DNA duplex with A^+–C mismatched base-pair by molecular dynamics calculations are discussed. The A^+–C mismatched base-pair which has been characterized by NMR with two hydrogen bonds namely, $A^+(6NH_2)$–C(3N) and $A^+(1NH)$–C(2O), fits into B-form duplex with little distortion in the pyrimidine strand. A in the A^+–C mispair is efficiently stacked to the flanking purine bases. Such efficient purine–purine stacking provides extra stabilization to this mismatched base-pair. On the other hand, C in the mispair is displaced from the helix axis while adjacent T (i.e. T7) is slightly off because of the enhanced base-pair twist. This supports the experimental observation of unusual chemical shifts for the H5 and H6 protons belonging to the C of the A^+–C mismatched base-pair.

MISMATCHES in DNA can arise during replication, and in genetic recombination[1–4]. If they are not corrected, the mismatches may lead to point mutations in subsequent replication. There have been several investigations by X-ray and solution NMR on purine–purine (A–A, A–G, A–I and G–G), purine–pyrimidine (A–C and G–T) and pyrimidine–pyrimidine (C–C, T–T and T–C) mismatches in oligonucleotides[5–28]. The studies on A–C mismatched base-pair support that the wobble pair is stabilized by $A(6NH_2)$–C(3N) hydrogen bond. Based on the observed short nitrogen–carbon distance in the crystal, it was suggested that the A–C mismatch pair is stabilized by a second hydrogen bond involving 1NH of protonated A and 2CO of C. Though pKa for the protonation of A in the free base is approximately 4, it was suggested to be higher when A basepairs with C in a DNA duplex[25–28].

5′TGAGGAAAGAAGGT3′
3′CCTTCTTTCCTC 5′
M1

In a recent NMR study on the molecular system M1, we have characterized the formation of a stable antiparallel duplex at neutral pH with three mismatched base-pairs, A^+–C, G–T and T–C (ref. 29). The study provided the first evidence for the protonation of A(N1) site at neutral pH leading to the formation of A^+–C mismatched base-pair with two hydrogen bonds, $A^+(1NH)$–C(2O) and $A^+(6NH_2)$–C(3N).

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