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Transformation of indica rice through particle bombardment: factors influencing transient expression and selection

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Abstract

Embryogenic, friable and small (*ca*. 3 mm) calli showed optimum *gus* expression and were best suited for selection during genetic transformation of rice through particle bombardment. Through prolonged culture of mature seeds on original callus induction medium, this type of calli could be produced in large numbers across several elite rice genotypes. To minimize the non-transformed escapes 50 mg dm^{-3} hygromycin and 8 mg dm^{-3} glufosinate ammonium were found to be critical during selection. Addition of selection marker during regeneration was essential. Regular and frequent (every 15 d) transfer of calli to fresh selection medium for three cycles was also important. A simple and economic procedure for screening large number of putative resistant plants was described.

Additional key words: embryogenic calli, regeneration, selection.

Introduction

Gene transfer techniques like direct DNA delivery through particle bombardment and *Agrobacterium* mediated transformation are increasingly applied in cereal crop improvement. Though many successful reports were described in rice as well as other cereals, routine transformation of choice cultivar is yet difficult due to genotype specific response *in vitro* culture. Among the rice genotypes those belonging to subspecies japonica and javanica were found to be more responsive to tissue culture than indica genotypes (Abe and Futsuhara 1984, 1986). Rice genotypes cultivated in India belong to the subspecies indica. Since Indian subcontinent has different agroclimatic regions within, specific genotypes are grown suited to that climate. Thus improvement in rice production and productivity is possible only when genotype specific barriers of genetic transformation methods are overcome. Most of the successful reports on transgenic production are limited to japonica, javanica and a few responsive indica rice genotypes like, IR 72, IR 64 and a few aromatic genotypes as Pusa Basmati-1, Basmati 370. In the present study we focussed on several other indica genotypes with a perspective of genetic transformation through particle bombardment. Comparative study of japonica and indica genotypes for *in vitro* response revealed the key differences between the two (Visarada *et al.* 2001, Visarada and Sarma 2002). Based on that the present objective is to *1*) develop simple and easy method for large scale production of friable embryogenic calli, *2*) post bombardment selection of transformed tissues, and *3*) simple method to screen large number of putative transgenic plants.

Materials and methods

Plants: Mature seeds were collected from popular indica cultivars Vibhava, Seshu, Rasi, Nagarjuna, Sonasali, Swarna, Vikas, Mahsuri and the japonica genotype, T309 of *Oryza sativa* L. Mature dehusked seeds were surface sterilized for 3 min with 70 % ethanol followed by 3 min in 0.1 % mercuric chloride. After several washes with sterile distilled water the seeds were blotted on sterilized paper towels and plated on solidified MS medium

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Abbreviations: BAP - benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; Na_2EDTA - ethylenediaminetetraacetic acid disodium salt; T 309 - Taipei 309.

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containing 3 mg dm^{-3} 2,4-D and 0.2 mg dm^{-3} BAP supplemented with the organic additives 50 mg dm^{-3} tryptophan and 300 mg dm^{-3} casein hydrolysate (enzymatic) (Murashige and Skoog 1962). The seeds were placed in such a way that half of the embryo was in contact with the medium and the other half facing upwards. The cultures were incubated in dark at 25 ± 2 °C for callus induction. Callus was produced from rachilla as described by Visarada *et al.* (1998).

Production of target tissues: Callus was induced from mature dehusked seeds and rachilla on NBKNB medium (Sivamani *et al*. 1996, Visarada *et al*. 1998). Primary callus dissected from seeds at 15 d and 25 d was used directly as target tissues. Seed derived calli were grown continuously on original callus induction medium for 60 d without subculturing. Embryogenic calli (type I and II) were selected microscopically from mature seed derived calli and 25 d old callus from rachilla as target tissues (Visarada *et al*. 2002). Small callus pieces (*ca*. 3 mm) were sub-cultured on the same medium 4 d prior to bombardment. Robust embryogenic calli were selected and arranged on osmotic medium (NBKNB containing 30 g dm^{-3} mannitol and 30 g dm^{-3} sorbitol) 4 h prior to transformation.

Plasmids: Plasmid constructs p113Act1gus and pPatgus (provided by Dr. P. Anand Kumar, IARI, New Delhi, India) and pCambia1301 (provided by Dr. R. Jefferson, CAMBIA, Australia) were used. The *gus* gene encoding β-glucuronidase, is driven by rice actin1 promoter in p113Act1gus and by CaMV35S in pCambia1301 and pPatgus. The plant selection markers are *hph* encoding hygromycin phosphotransferase (HPT) and *bar* gene, encoding phosphinothricin acetyl transferase (PAT). Plasmid pPatgus contained *bar* gene, whereas, pCambia1301 contained *hph* gene.

Isolation and purification of DNA: Plasmid DNA was isolated by alkaline lysis method (Sambrook *et al*. 1989). DNA was purified by polyethylene glycol method or by passing through sepharose *CL-4B* column. DNA samples with the ratio of absorbency (260/280) between 1.7 - 2.0 were only used for coating. Plasmid DNA was stored in TE buffer (pH 8.0) at 1g dm^{-3} concentration for ready use.

Preparation of gold particles and DNA mix: Sterilization of gold particles and preparation of bombardment mix were done according to Datta (1995). Approximately 30 mg of Gold particles (size 1 and 1.6 µm, *Biorad*, Hercules, USA) were sterilized by washing three times each in absolute ethanol and sterile distilled water. The particles were suspended in 0.5 cm^3 of sterile water and stored as 0.05 cm^3 aliquots. Bombardment mix was prepared by adding, 5 µg of DNA, 0.05 cm^3 of 2.5 M autoclaved $CaCl_2$ and 0.02 cm^3 0.1 M spermidine free base (filter sterilized) to 0.05 cm^3 of gold suspension, vortexed for 3 min and incubated at room temperature for 10 min followed by centrifugation for 1 min at 30 *g*. The supernatant was removed and tested for the presence of DNA to ensure total coating of the DNA added. The pellet was washed in chilled absolute ethanol and resuspended in 0.1 cm^3 of ethanol. An aliquot of 0.0075 cm^3 was loaded twice on the surface of each macrocarrier.

Particle bombardment: Plates containing the target tissue were placed 6 or 9 cm below the stopping mesh. Particle acceleration was done using *Biorad PDS-1000/He* device at 1100, 1350 and 1550 psi under partial vacuum and each Petri plate was shot twice likewise. After 18 h following bombardment the target tissues were transferred to osmotic free NBKNB medium.

Standardization of kill curves: Control (untransformed) calli were cultured on NBKNB medium containing 30 and 50 mg dm^{-3} hygromycin (*Sigma*, Missouri, USA), and 4, 6 and 8 mg dm^{-3} glufosinate ammonium (*Wako Pure Chemical Industries*, Osaka, Japan). All the surviving calli were microscopically separated and subcultured every 15 d. Percentage of surviving calli were recorded before every subculture and also the percentage of calli regenerating on selection medium.

Selection of transformants: Calli were transferred to NBKNB medium containing 50 mg dm^{-3} hygromycin or 8 mg dm^{-3} glufosinate ammonium after 72 h of bombardment. All the surviving calli were aseptically separated by observation under a stereomicroscope and transferred every 15 d to fresh medium containing the selection agent. Putative hygromycin or glufosinate ammonium resistant calli were separated into small (3 mm or less) pieces for selection. After 3 cycles the resistant calli were transferred to RN medium containing hygromycin (50 mg dm^{-3}) or glufosinate ammonium (8 mg dm^{-3}) and incubated under light in all except Nagarjuna (Sivamani *et al.* 1996). Emerging shoots or surviving calli were transferred after 30 d to fresh RN medium with the selection agent. Regenerants were transferred to half strength MS medium for root development.

Selection of regenerants: Once the plants were established they were maintained in 5 cm^3 of Yoshida solution for a week or two and the solution was changed after every 3 d (Yoshida *et al.* 1976). For selection against hygromycin the antibiotic (30 mg dm^{-3}) was added directly to Yoshida solution. In case of *bar* gene, 0.1 % glufosinate ammonium solution was prepared in water and a drop of *Teepol* was added. Leaf samples from the putative transformants were dipped twice in the solution with half an hour interval. The treatment was repeated after a week.

Histochemical assay of *Gus*: Transient *Gus* expression was tested after 24 h of bombardment. Stability of gene expression was tested after second and third cycles of

selection on hygromycin. Leaf and root portions of the regenerants were also tested for *Gus* expression. Gus assay mixture containing 0.5 mg cm^{-3} X-gluc Na salt (*Biosynth*, St. Gallen, Switzerland) in 100 mM sodium phosphate buffer pH 7.0 and 10 mM Na_2EDTA. After adding Gus stain, the samples were evacuated and incubated at 37 °C.

Results

Production of target tissues: Primary callus from 2-week-old mature seed was hard and compact. Necrosis was more when it was cut into small pieces to be used directly as target tissues or for production of large number of small calli. When the primary callus from mature seeds was cultured continuously for 60 d on original medium it produced large masses of callus with distinct phenotypes (Fig. 1). Embryogenic, which can be defined as white to cream coloured compact organized callus (type I) and yellow organized callus (type II) could easily be separated from other calli and divided into *ca.* 3 mm or less size pieces, that were subcultured 4 d prior to bombardment. Callus adjoining the attachment to seed embryo was highly embryogenic. Survival rate of these calli was high compared to subcultured callus. On the other hand, small embryogenic calli were produced from the rachilla, the portion joining the sterile glumes to palea and lemma, and were used as the target material. Thus prolonged culture of seeds and rachilla facilitated the production of large number of embryogenic calli with less effort.

15-d-old and 25-d-old mature seed calli showed low transient expression. 60-d-old seed calli and rachilla derived calli were small (3 mm) in size and showed higher expression (Table 1). Size of target tissues did influence the transient expression. Smaller calli always gave higher number of blue foci often with dark blue

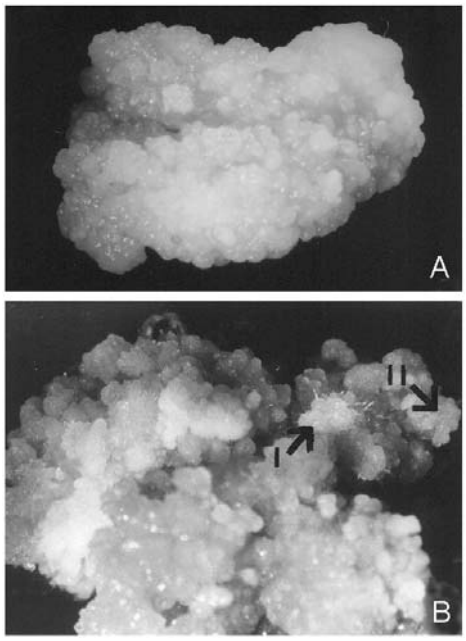

Fig. 1. Mature seed derived calli in the indica genotypes. Hard and compact 15-d-old callus (*A*) and friable callus derived from mature seeds cultured continuously on original induction medium for 60 - 70 d (*B*). I - white or cream coloured compact organized callus (type I), II - yellow organized callus (type II).

Table 1. Total number of blue foci in different explant sources transformed with pCambia1301 at 1350 psi by particle bombardment in rice genotypes. Mean ± SE.

Explant source	T 309	Vibhava	Seshu
15-d-old seed callus	98.3 ± 13.1	34.6 ± 9.1	54.0 ± 7.0
25-d-old seed callus	150.0 ± 9.9	167.6 ± 14.6	91.6 ± 10.5
60-d-old seed callus	878.3 ± 62.5	576.3 ± 101.7	764.3 ± 104.9
Suspension callus	1069.3 ± 122.1	-	-
Rachilla callus	757.3 ± 113.1	480.3 ± 112.3	841.7 ± 182.2

Table 2. Transient *gus* expression in calli of indica type rice cultivars transformed by particle bombardment. Mean ± SE.

Genotype	pPatgus calli with foci [%]	pPatgus total blue foci	pCambia1301 calli with foci [%]	pCambia1301 total blue foci
T309	82 ± 8.2	808 ± 196	76.0 ± 9.3	576 ± 212
Seshu	80 ± 6.8	264 ± 89	64.6 ± 18.0	320 ± 58
Nagarjuna	85 ± 10.5	420 ± 103	73.9 ± 10.0	288 ± 35
Rasi	50 ± 12.8	121 ± 42	-	-
Vibhava	-	-	51.5 ± 27.8	224 ± 38

patches compared to large and compact calli. Frequency of transient expression in different genotypes was tested by bombarding with the plasmids pPatgus and pCambia1301 (Table 2). The genotypes T 309, Seshu and Nagarjuna showed consistently superior expression compared to Vibhava and Rasi.

Standardization of kill curves: Control experiments to find out the lethal doses of hygromycin revealed that 30 mg dm^{-3} hygromycin lead to a large number of escapes in all the indica genotypes tested. Necrosis of callus was complete and the entire callus turned brown when cultured on medium containing 50 mg dm^{-3} hygromycin for 2 cycles of 15 d each. In Nagarjuna, single cycle of selection was enough to kill all the control (untransformed) calli. In Seshu and Swarna 2 % of untransformed calli (control) survived after 2 cycles of selection, but failed to regenerate in contrast to their companion calli maintained without hygromycin. Sensitivity to hygromycin thus varied among the genotypes (Fig. 2*A*). Therefore, the calli bombarded with the plasmid pCambia1301 were cultured on medium containing 50 mg dm^{-3} hygromycin for only two cycles initially. Since all the regenerants turned out to be non-transformed escapes selection pressure was extended to one more cycle. A similar experiment with control calli conducted to find out the optimum dose of glufosinate ammonium, confirmed that 8 mg dm^{-3} is lethal to control calli (Fig. 2*B*). Duration and concentration of selection agent were varied according to genotype sensitivity. Necrosis and cell death was rapid and complete when hygromycin was used as a selection agent, whereas, it was diffused and graded in case of glufosinate ammonium.

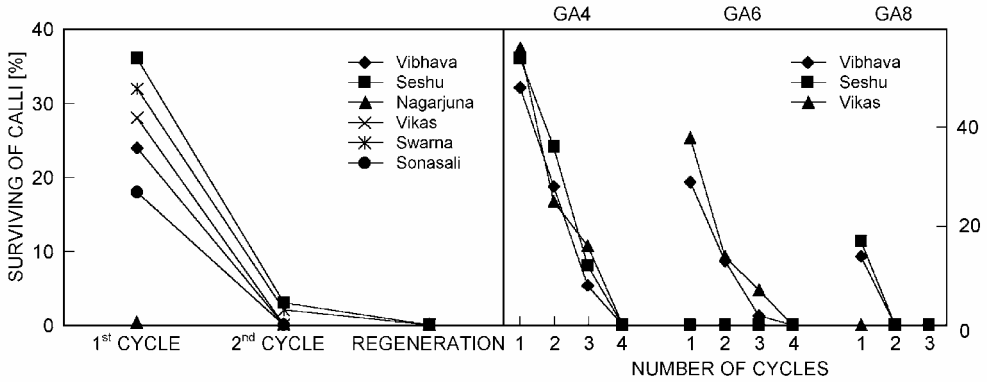

Fig. 2. Standardization of kill curves. A selection on 50 mg dm^{-3} hygromycin (*on the left*) and on 4, 6 and 8 mg dm^{-3} glufosinate ammonium (*on the right*).

Post bombardment selection: Selection, multiplication and regeneration of transformed cells are the most important steps of transformation, irrespective of the method of DNA delivery. For obtaining stable integration of the DNA, the optimized procedure was used (5 µg DNA, 1100, 1350 psi He pressure and 6 cm target cell distance, Visarada and Sarma, unpublished). Initial experiments using 15 and 25-d-old seed callus posed difficulty for selection for marker gene since these callus pieces were large and the cell clusters on the top escaped the selection agent. Further, cutting the calli lead to necrosis and cell death. Henceforth, 60-d-old seed derived calli and rachilla derived calli were used routinely for transformation. *Gus* expression of these putative transformants in the indica genotypes was tested at intervals, along with T 309, to verify the selection efficiency (Table 3). After two cycles of selection in T 309 the blue sectors expressing the reporter gene were large, whereas, it was localized to small areas in the indica genotypes. With T 309, the callus multiplication was rapid in general and also on selection medium. Transfer of transformed calli to fresh selection medium at every 15-d interval improved the selection efficiency. Small size of the callus (3 mm or less) was preferred for selection. Putative resistant calli were obtained in all the genotypes. To further eliminate non-transformants, the rachilla derived calli as well as 60-d-old mature seed derived calli were subjected to 3 cycles of selection. *Gus* expression was not observed in putative hygromycin resistant calli in Nagarjuna, Rasi and Vikas after two cycles of selection. Stable transformation of the *gus* gene was confirmed by histochemical assay after three cycles of selection in rachilla derived calli and seed derived secondary callus. However, as the histochemical assay of *gus* gene was destructive, the putative resistant calli in the succeeding experiments were regenerated.

Table 3. Number of blue foci exhibited by rice calli transformed by particle bombardment after different intervals of selection (* - large blue sectors, ** - small blue sectors).

Genotype	24 h	1st cycle	2nd cycle
T309	1065	15	4*
Vibhava	195	5	2**
Vikas	24	2	0
Rasi	181	0	0
Seshu	390	7	3**
Nagarjuna	23	0	0

Table 4. Selection of resistant calli and regeneration of putative genetic transformants by particle bombardment in rice genotypes (+ - regenerated with selection substrate, – - regenerated without selection substrate, * - indicates 3 blue spots).

Genotype	Plasmid	Number of experiments	Number of resistant calli after 3 cycles	Number of plants regenerated
Vikas	PG	7	0	–
	1301	3	2	0^+
Vibhava	PG	3	5	22^{-}*, 0^+
	1301	8	13	259^{-}, 0^+
Seshu	PG	8	43	0^+
	1301	23	480	262^{-}, 18^+
	BB	4	20	0^+
Nagarjuna	1301	4	66	0^+
	PG	7	186	178^{-}
Swarna	PG	4	123	0^+
	1301	3	179	0^+
Sonasali	PG	2	3	0^+
	1301	3	17	0^+
Mahsuri	PG	4	143	0^+
	1301	5	40	0^+

Resistant calli upon transfer to regeneration medium turned brown. Some of the calli showed green regions after 15-20 d and others after 30 d, within the same genotype. Putative resistant calli were regenerated on medium with and without hygromycin in the genotypes Vibhava and Seshu. A large number of plants were obtained when the selection pressure was not applied. In the genotypes Vibhava and Seshu 262 and 259 plants were regenerated respectively from *hmr* calli (Table 4). Likewise 22 and 178 plants were regenerated from GA resistant calli in Vibhava and Nagarjuna, respectively. These plants were screened in Yoshida solution for resistance against hygromycin and glufosinate ammonium. All the plants were sensitive to selection. In Vibhava, two or three microscopic spots were visible in roots and leaves. Therefore, selection pressure was continued during regeneration and also while subculturing on regeneration. Thus, the selection was applied for 3 cycles of 15 d each on callus induction medium and 2 cycles of 30 d each in regeneration medium in other cultivars as Swarna, Sonasali and Mahsuri. A few resistant plants were obtained in Vibhava and Seshu on hygromycin (Table 4). Putative resistant calli regenerated after continuous selection pressure in Vibhava showed blue patches in the leaves. In Mahsuri and Sonasali, all the calli turned necrotic when regenerated on hygromycin containing medium, whereas, in Swarna, many of them did survive but did not regenerate. The surviving calli were found non-transformed when assayed histochemically for *gus*. None of the glufosinate ammonium resistant calli could be regenerated.

Discussion

More often, immature embryos are preferred as target material for ballistics and other tissue explants to a lesser extent, as they are free from hassles of long term tissue culture and associated sterility, not withstanding the isolation of immature embryos and their seasonal availability. On the other hand, the obvious advantages of mature seed as source of explant tissue are that they can be produced in large quantities and stored and ease of handling which is why seed derived calli were preferred for this study.

Though 15- and 25-d-old calli were used as target tissues in the past they showed low transient expression and were difficult to select. As these calli were large in size, they escaped selection pressure and their mechanical separation led to necrosis. Sivamani *et al.* (1996) developed a procedure for the less responsive indica genotype, TNI, in which thousands of homogenous calli derived from mature seed were generated, selectively proliferated and used as targets after testing the regeneration potential of their sibling calli. In the present study we could easily generate large number of small calli in all the seven-indica genotypes. Embryogenic calli of type I and II could be separated and used as target tissues (Visarada *et al.* 2002). The present system enables the production of friable embryogenic callus in as many as seven indica genotypes. In a similar study, Utomo *et al.* (1995) could produce embryogenic calli and establish suspension cultures in five cultivars of rice by extending the period of subculture on the original callus induction medium to 10 - 20 weeks.

Proliferation of transformed cells and selection of transformed sectors is another important event since the transformed cells are at a disadvantage in terms of their retarded cell growth and multiplication in comparison to non transformed ones. In T 309 proliferation was faster compared to indica genotypes. Transient *gus* expression in terms of frequency as well as total number of blue foci indicates the intensity and uniformity of gene expression. A moderate and evenly distributed *gus* expression is preferred rather than intense and localized one, as the latter may cause more cell damage and mortality. A detailed gene expression pattern both in terms of frequency of calli as well as total blue foci was described by Li *et al.* (1997). Both these parameters were taken into account in the present study for assessing transformation efficiency.

Selection for extended periods was necessary in indica genotypes to obtain sufficient proliferation of the transformed cells to form somatic embryoids. On extending the selection for 3 cycles stable transformed calli could be obtained (32 % in rachilla derived callus and 8.6 % seed derived secondary callus), which supported the view that selection for 3 cycles on hygromycin at 50 mg dm^{-3} helped to obtain stable

transformed calli (Visarada *et al* 1998). Regenerated putative transformed plants that were obtained in the genotypes Vibhava, Seshu and Nagarjuna turned out to be escapes despite the large number of calli being bombarded. It could be possible that the selectable marker gene was transmitted to a few cell generations and then either silenced or eliminated (Sivaprasad 1997). Another possibility described by Christou *et al.* (1991) is that the transformed tissue is so efficient in detoxifying the selection agent that the adjoining cells also survive resulting in escapes in regeneration. Such an instance was reported by Burkhardt (1996), who could isolate a number of putative transformant calli in the indica genotype, that on further selection turned out to be non-transgenic.

A leaf assay method to identify transgenic plants expressing hygromycin and phosphinothricin gene(s) was described by Wang and Waterhouse (1997). In our studies regenerants from putative transformed calli with or without selection pressure in the regeneration medium, when screened in Yoshida's solution for resistance to hygromycin or glufosinate ammonium all plants turned out to be sensitive. This procedure seems to be effective and easy to adopt to clearly delineate the transgenics. Further, the stage of root initiation and elongation is observed to be most sensitive for application of selection pressure as reported by Casas *et al.* (1995).

Though information on procedure and protocols on bombardment using japonica test genotype, T309 was reported, a detailed investigation like the one undertaken in this present study on indica rice is not available. Genetic transformation of rice is confined to japonica, javanica genotypes, a few responsive indica genotypes like, IR 72 and IR 64; aromatic rice genotypes like Pusa Basmati-1, Basmati 370 and Karnal local that could be transformed earlier by protoplast mediated methods. Thus transgenic approach was limited to selected genotypes, which were otherwise successful even before the introduction of particle bombardment and *Agrobacterium*. Similarly, in maize A188 and its derivatives, in wheat Bobwhite lines and in barley Golden Promise and Igri are found to be most responsive for genetic transformation. Crop improvement programs involve the agronomic elevation of several cultivars that are specific to agroclimatic regions. In such cases useful genes can be introduced into receptive genotypes and further transferred to genotypes of choice through conventional means (Altpeter and Varshney 2001). In the present paper several indica genotypes, which were suited to different agroclimatic zones and which were hitherto not reported for transformation, were studied. These results indicate the possibility that though certain genotypes are good at transient expression (receiving DNA), the additional genes are either knocked out or transformed cells are unable to multiply after certain stages. If the transformed genes are eliminated after certain cell divisions, it is to be confirmed whether they sustain when introduced through conventional means. The experience reported here can be applicable to other means of transformation including *Agrobacterium* and can be directive for indica rice transformation especially under resource constraints. Production of large number of embryogenic calli for bombardment, small size of the target tissue for effective selection and application of selection pressure during regeneration are some of the steps that help contribute to success in indica transformation. Further the methodology described for screening of putative transformants not only facilitates screening in large number. It is also simple, easy and economical.

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