Production of agronomically superior transgenic rice plants using *Agrobacterium* transformation methods: Present status and future perspectives

Malabika Roy*,†, Rajinder Kumar Jain‡,, Jai Singh Rohila* and Ray Wu***

*Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA
†Present address: Department of Botany, Bose Institute, Calcutta 700 009, India
‡Department of Biotechnology and Molecular Biology, CCS Haryana Agricultural University, Hisar 125 004, India

Almost ten years have elapsed since the initiation of experiments on rice transformation using *Agrobacterium tumefaciens*. Chen *et al.*[1] successfully transformed rice using this method, but transformation efficiency was low. After Hiei *et al.*[2] reported high efficiency of *Agrobacterium*-mediated production of fertile and heritable transgenic rice plants, this technology was adopted by many laboratories and is now in widespread use. *Agrobacterium*-mediated transformation has several advantages, including higher transformation efficiency, ability to transfer large pieces of DNA with minimal rearrangement, integration of relatively lower number of transgene copies, and low experimental costs. The success and future prospects of this technique for the genetic improvement of rice with respect to several agronomically important traits are addressed in this article.

AGROBACTERIUM tumefaciens is a soil bacterium that can genetically transform plant cells by transferring a defined piece of DNA (known as T-DNA) from its tumour-inducing (Ti) plasmid into the genome of infected plants. Virulence (*vir*) genes on the Ti-plasmid code for the functions required for processing and transfer of T-DNA, which are induced by low-molecular-weight phenolic compounds produced by the wounded plant cells[3,4]. The rapid development of rice transformation technology not only provides a valuable method for introducing useful genes into rice to improve important agronomic traits, but also helps in studying gene function and regulation[5,6]. A large number of suitable vectors have been developed for *Agrobacterium*-mediated transformation. This method also provides an opportunity to transfer large segments of DNA that may contain more than ten genes without any rearrangement[7], which is suitable for studying their cumulative, interactive effects on complex polygenic traits. Rice is considered a model system for studying gene regulation and crop improvement in monocots, similar to *Arabidopsis* and tobacco in dicots[8,9]. In this article, we have reviewed the progress made towards the development, as well as application, of the *Agrobacterium*-mediated transformation methods in producing superior transgenic rice varieties.

**For correspondence. (e-mail: rkjain@hau.hry.nic.in)

The basic methodology

A. tumefaciens causes a neoplastic plant disease, 'crown gall', by the transfer and integration of T-DNA into the host genome with a set of expressible *vir* genes[10,11]. Approximately 25 *vir* genes are arranged into seven operons[12] and are located on the Ti-plasmid. The detailed mechanism of transfer of DNA from *A. tumefaciens* to plant cells has been reviewed by many authors[3,10,13]. The process of crown gall induction consists of several discrete and essential steps. The wounding of plant tissue allows the entry of bacteria into host cells, and wound-induced synthesis of phenolic compounds induces *vir* gene transcription. The bacteria develop tumours on wound sites as a result of the transfer of certain genes into the plant chromosome. These genes, called *iaaH*, *iaaM* and *ipt*, code for the enzymes necessary for auxin and cytokinin biosynthesis and result in cell division[14]. In addition, T-DNA has genes necessary for the synthesis and excretion of opines, which are consumed by the infecting bacteria[15]. The border sequences flanking the T-DNA are essential *cis*-acting elements involved in genetic transformation of plants by *Agrobacterium*[16]. The transfer of T-DNA into plant chromosomes appears to be a polar process initiating at the right border, progressing leftward and terminating at the left border[17,18].

Some early developments

Between 1990 and 1992, transformation of plants belonging to the Gramineae family using *Agrobacterium* strains was attempted by several investigators[19,20], but success was limited and remained controversial until 1993 when

Chen *et al.*[1] obtained several transgenic japonica rice plants by inoculating immature embryos with *Agrobacterium*. The inheritance of the transferred DNA to the progeny was ascertained in only one plant by Southern blot hybridization. Major advances in the rice transformation methodology using *Agrobacterium* were reported by Hiei *et al.*[2] who described an efficient transformation protocol for japonica cultivars Tsukinohikari, Asanohikari and Koshihikari. Of the various explants tested, 3-week-old scutella-derived embryogenic calli proved to be the most suitable material for infection by *Agrobacterium*. The calli were co-cultivated with LBA4404 (pTOK233, pIG121Hm) and EHA101 (pTOK233, pIG121Hm) for 3 days in the presence of 100 μM acetosyringone. The observed transformation efficiency was 23%. They documented the stable integration, expression and inheritance of the reporter gene, β-glucuronidase (*GUS*), and plant selectable marker gene, hygromycin phosphotransferase (*Hpt*), in R_1 and R_2 transgenic plants. The transformation procedure of Hiei *et al.*[2] was subsequently used for the production of genetically stable transgenic plants in indica rice varieties with a transformation efficiency of 22 to 27% (refs 21–25). Aldemita and Hodges[26] and Park *et al.*[27] used immature embryos and seedling shoot apices, respectively, for *Agrobacterium* infection and reported the stable integration and inheritance of the *GUS* reporter gene in japonica and/or indica rice. Toki[28] reported a rapid procedure for the production of transgenic japonica rice plants within a period of only two months.

Factors affecting *A. tumefaciens*-mediated transformation in rice

The critical factors that determine the efficiency of *Agrobacterium*-mediated transformation in rice[5] are briefly summarized here.

Agrobacterium vectors and strains

The development and use of super-virulent binary vectors containing virulence genes (*VirB*, *VirC* and *VirG*) from pTibo542, the Ti plasmid of the supervirulent *Agrobacterium* strain A281, have been the keys to the success of monocot transformation, including rice[5,29,30]. Further, small-size (11–15 kb) hybrid vectors were developed for cloning the gene of interest. Recombinant vectors were transferred into an *Agrobacterium* strain using a helper plasmid through triparental mating[31] and used for rice transformation. Toki[28] developed a new pSMABuba binary vector for rice transformation, which is a modified version of pSAMB70[32]. Ku *et al.*[33] used EHA101 strain containing the pSMABuba vector for high-efficiency rice transformation of an intact maize *phosphoenolpyruvate carboxylase* gene (8 kb). A Bin19-derived binary vector, pKGH4, was used by Cheng *et al.*[34] to transfer the *CryIA(b)* and *CryIA(c)* genes into rice through *Agrobacterium* (LBA4404 and EHA105) transformation. Sakamoto *et al.*[35] constructed ChlCOD and CytCOD plasmids in the pIG121Hm superbinary vector, which contain the 35S CaMV promoter, the first intron of rice *SodCc* and the chimeric fusion gene encoding choline oxidase (COD) with or without a transit peptide sequence. *Agrobacterium* strain EHA101 was used to transfer the *COD* gene to produce glycinebetaine[35]. Goto *et al.*[36] transferred a soybean ferritin gene into rice using a modified binary vector, pGPTV-35S-bar[37]. Chin *et al.*[38] constructed *Ac* and gene-trap *Ds* vectors and introduced them into rice through *Agrobacterium* transformation; the *Ac*/*Ds*-mediated gene-trap system can be used for analysis of gene function. Ye *et al.*[39] developed pB19hpc, pZPsC and pZLcyH vectors carrying the genes of provitamin A (β-carotene) biosynthetic pathway enzymes; these vectors were electroporated separately into *Agrobacterium* strain LBA4404. The resultant strains were successfully used for rice transformation and to engineer the provitamin A biosynthetic pathway into rice endosperm.

In conjunction with an improved system for *Agrobacterium*-mediated plant transformation, a new binary bacterial artificial chromosome (BIBAC) vector has been developed that is capable of transferring at least 150 kb of foreign DNA into the tobacco nuclear genome[40]. The BIBAC vector has the minimal origin of replication of both *E. coli* and *A. rhizogenes* Ri plasmids, and it can replicate as a single-copy plasmid in both *E. coli* and *A. tumefaciens*. The large BIBAC T-DNAs, in conjunction with the helper that carries additional copies of virulence genes, *VirG* and *VirE*, have been used for high-frequency transformation in dicots. A modified BIBAC vector (BIBAC4) containing 60-kb or 90-kb rice BAC clone has been similarly used for rice transformation[41]. The ability to introduce high-molecular-weight DNA into plant chromosomes should accelerate gene identification and could lead to new approaches for studying genome organization[7].

Acetosyringone: A potent inducer of virulence genes

Wounded dicot tissues are known to exude phenolic compounds, such as 4-acetyl-2,6-dimethoxyphenol (acetosyringone), which activate *vir* genes present in Ti plasmids[42]. Monocots either do not produce these compounds or, if they do, the levels are insufficient to serve as a signal for *vir* gene induction[19]. Chen *et al.*[1] used potato suspension culture cells for *Agrobacterium*-mediated transformation of rice. Potato suspension culture cells are good sources of phenolic compounds, such as acetosyringone and sinapic acid, which activate *vir* genes. The addition of acetosyringone is essential for successful and higher-frequency transformation, but the concentration of acetosyringone in co-cultivation medium may vary between different cultivars of rice[2,5,43].

Competent rice tissue

Attachment of the bacterium to the host plant cell is an initial step in the process of infection[44]. Attachment can be affected by plant or tissue age, cell type, cell cycle stage and other physiological parameters[45]. A distinct correlation exists between the wound-induced divisions of cells and the competence of such cells to be transformed by *A. tumefaciens*[46]. In many monocots, cells at wound sites tend to be lignified or sclerified without apparent cell division[47]. Therefore, most monocotyledons are poor hosts for *A. tumefaciens*[48,49]. Leaf extract from flowering rice plants can strongly induce *vir* genes, which in turn are known to influence transfer of T-DNA into plant cells[50]. Vijayachandra *et al.*[43] reported variations among the different tissues of indica rice in their ability to induce *vir* genes and T-strand generation. An analysis of rice leaf segments revealed that they neither induced *vir* genes nor inhibited *vir* gene induction. Of the different parts of rice plants analysed, only scutellum tissue from four-day-old rice seedlings induced *vir* genes and generation of T-strands. In scutellum tissue, *vir* gene induction was greatly enhanced in the presence of 60 μm acetosyringone. Success in rice transformation has been reported by the use of actively growing embryogenic tissue/cells, which may be derived from seed scutellum[2,22,25,43], immature embryos[26] and shoot apex[27]. Long-term culture cells and liquid suspension culture rice cells have been reported to be unsuitable for *Agrobacterium*-mediated transformation[2,43].

Genotype, medium composition and co-cultivation conditions

Optimization of culture conditions for co-cultivation of rice tissue with *Agrobacterium* is important to obtain high-transformation efficiency and it may vary with the genotype and tissue/explant used. Hiei *et al.*[2] used a modified N6 medium containing acetosyringone, 2,4-D and casamino acids for co-cultivation of rice calli with *Agrobacterium*. Other important factors for co-cultivation and *vir* gene induction are acidic pH, incubation temperature of 28°C or less[51] and high osmotic pressure[24,52]. Solid medium is better for co-cultivation than liquid medium[5]. Several media and treatments for tissue culture and high-frequency regeneration of transformed tissues of japonica, javonica and indica rice cultivars have been reported[52]. Gandhi and Khurana[24] reported that water stress treatment (use of 1.6% agar instead of 0.8% agar for medium solidification) resulted in higher-frequency regeneration from transformed indica rice calli in the absence of hormones. Inclusion of a selection agent in the regeneration medium has been reported to favour the production of a large numbers of genetically stable transgenic rice plants[26,53].

Promoters and plant selectable marker genes

A promoter drives the expression of a gene and may be a key factor in determining the success of a particular transformation experiment. Constitutive promoters direct the expression of genes in almost all tissues and are independent of any environmental or developmental conditions. The rice Act1 (refs 54, 55), maize *ubiquitin 1* (ref. 56) and chimeric maize *emu*[57] are strong promoters for gene expression in rice and have been used widely for the production of transgenic rice plants with higher-level, constitutive expression of transgene(s)[28,34,35,39,58,59].

Besides constitutive promoters, tissue-specific promoters and inducible promoters have been used for rice transformation. Yokoi *et al.*[60] reported an anther-specific *Osg6B* promoter that directed tapetum-specific expression of the *GUS* gene in rice. Cheng *et al.*[34] used a pollen-specific (*Bp10*) promoter to transform nine different japonica cultivars for high-level and tissue-specific expression of lepidopteran-specific δ-endotoxins. The endosperm-specific seed-storage protein gene glutelin promoter, *GluB-1*, was used to drive the expression of the soybean ferritin transgene specifically in rice seeds to increase the iron content [36]. Similarly, phytoene desaturase (*crtI*) gene was driven by the endosperm-specific glutelin promoter for provitamin A synthesis in rice grain[39].

Constitutive expression of some transgenes may severely affect the normal growth and development of plants. Su *et al.*[61] developed an abscisic acid (ABA)-inducible promoter for rice transformation. This ABA-inducible promoter was used to express the polyamine biosynthetic transgene(s) in rice[53].

Several marker or reporter genes have been used to monitor transformation efficiency. Such marker genes have also been used to analyse the expression potential of different promoters in transformed tissues. The *GUS* gene[62] encoded by the *uidA* locus of *E. coli*, has been the most commonly used reporter gene in rice transformation. The *GUS* gene with an intron in the N-terminal region of the coding sequence is more useful, because the chimeric *GUS* gene is expressed only in the plant cells and not in the cells of *A. tumefaciens*[2,5]. The green fluorescent protein gene (*gfp*) has also been used as a reporter gene in rice transformation experiments[63].

Identification and selection of transformation events generally rely on the differential growth of transformed versus non-transformed tissues in the presence of a selection agent. The election process may take 6 to 10 weeks and requires the use of different levels of the selection agent to eliminate escapes. A high level of the antibiotic cefotaxime is used to eliminate *Agrobacterium*, and several other antibiotics/herbicides are used to select transformed cells during various stages of the entire process. Different rice varieties exhibit different sensitivities towards selection agents. The hygromycin phosphotransferase (*hpt*) gene has been widely used as a selectable

marker gene in *Agrobacterium* transformation experiments[2,33,34,39]. Herbicides are also potential selective agents. Genes have been isolated for their resistance to various commercially important herbicides. Among them, the *bar* gene, which encodes phosphinothricin acetyltransferase, is the most commonly used selectable marker gene in rice[4,64]. The *bar* gene confers resistance to L-phosphinothricin (PPT), glufosinate (an ammonium salt of PPT) and bialaphos (a derivative of PPT). The *bar* gene has been successfully used in *Agrobacterium*-mediated transformation to select transgenic calli and rice plants[27,28,36,56].

Fertility, genetic stability and transgene expression in transgenic plants

Stable inheritance and expression of foreign genes are of critical importance for the successful application of genetically engineered rice in agriculture. Most of the transgenic rice plants obtained by using *Agrobacterium* transformation methods have been reported to show normal morphology, and 70% of the plants were fertile[2,25,34]. The number of copies of integrated genes ranged from one to six, but approximately 30% of the transformants contained one copy of the transgene[5,25]. The problem of gene silencing resulting from the presence of multiple transgene copies may be partially overcome by using *Agrobacterium*-mediated transformation[6,20].

Production of marker-free transgenic plants

Selectable marker genes are always included in transformation systems, but they are not usually required once transgenic plants have been produced. The commercialization of transgenic plants has generated a debate regarding the desirability of the transgenic products containing selectable marker genes[65–67]. Several transformation techniques/strategies for eliminating marker genes have been developed. They include (1) co-transformation, (2) a site-specific recombination system, and (3) intra-genomic relocation of transgenes via transposable elements. The co-transformation system appears to be the simplest among them and is widely used in *Agrobacterium*-mediated transformation[67].

In co-transformation, a plant cell is transformed with two separate DNAs, one incorporating a gene of interest and the other containing the selectable marker gene. Consequently, the gene of interest segregates from the marker gene in successive generations. Novel super-binary vectors carrying two separate T-DNAs for co-transformation have been constructed[31]. One T-DNA contains a drug-resistance, selectable marker gene and the other contains the *GUS* gene. More than 50% of the co-transformants were *GUS*-positive and drug-sensitive. Both the efficiency of co-transformation and the frequency of unlinked integration of the two T-DNAs into plants were reasonably higher through the *Agrobacterium* transformation method than a direct gene delivery method.

In site-specific recombination, the gene of interest is cloned between the asymmetric inverted repeat sequences, which are the sites for specific recombination. The recombination event is identified in the transformants by selecting the presence of cloned genes[67].

Maize transposable elements *Ac/Ds* maintain transposition competence when transferred to other plant cells and provide a useful alternative to carrying out multiple independent transformations in order to achieve optimal transgene expression. Selectable markers can be removed via transposition of the *Ds*; only the gene of interest flanked by the T-DNA repeats remains in the plant[38].

Agronomically useful genes transferred into rice via *Agrobacterium*-mediated transformation

Several agronomically important genes have been introduced into rice to improve resistance/tolerance to insect pests, bacterial diseases and abiotic stresses using the various transformation methods available[6,68]. An increasing number of scientists worldwide are now preferably using *Agrobacterium* methods to transfer agronomically useful genes into rice. The progress made in this direction is briefly reviewed here.

Biotic stress tolerance

Rice genetic engineering for insect resistance has received special attention. The transfer of truncated and suitably codon-modified δ-endotoxin genes from *Bacillus thuringiensis* (*Bt*) has been the major approach to achieve this objective. Cheng *et al.*[34] reported the transfer of synthetic *CryIA(b)* and *CryIA(c)* genes into rice using an *Agrobacterium* transformation method. The transformation efficiency was 5- to 6-fold higher than in transgenic rice plants obtained by particle bombardment[59,69]. Their reports showed that the mortality rates of striped stem borers and yellow stem borers were both 97% within five days of infection in transgenic plants compared to the non-transformed plants[34], while transgenic plants obtained by other methods showed only a 70% insect mortality rate[70].

Rice blast is a devastating plant disease. Class I chitinase genes, *cht-2* and *cht-3*, were transferred through *Agrobacterium* transformation in japonica rice cultivars Nipponbare and Koshihikari[59]. R_0 transgenic rice plants that constitutively expressed either chitinase gene under the control of the 35S CaMV promoter, showed significantly higher resistance to the rice blast pathogen *Magnaporthe grisea*. Both high-level expression of chitinase and the blast resistance characteristic were stably inherited in the next generation.

Abiotic stress tolerance

Salinity and drought-stress tolerance: Plant productivity is greatly influenced by environmental stresses. One plant response that aids in acclimation to water deficit and high salinity is the accumulation of compatible solutes/osmolytes, such as glycinebetaine[35] and polyamines[53,71]. Transgenic rice plants containing the bacterial *COD* gene, which converts choline to glycinebetaine, were found to accumulate glycinebetaine at higher levels[35,69]. These plants showed enhanced tolerance to salt and cold stresses. The *COD* gene was targetted to both the chloroplast and cytosol in rice cells. The cytosol-targetted transgenic rice plants accumulated glycinebetaine at levels 4- to 5-fold higher than the chloroplast-targetted plants. Both cytosol-targetted and chloroplast-targetted transgenic plants showed better vegetative growth, fewer wilting symptoms, less chlorophyll loss and better protection of photosystem II in comparison to non-transformed plants under salt and low-temperature stresses.

Changes in polyamine metabolism under different stress situations have been reviewed thoroughly[68]. The accumulation of polyamines under different stress situations provides protection to the membrane structure[72,73]. Two major polyamine biosynthesis genes, arginine decarboxylase (*ADC*) and S-adenosylmethionine decarboxylase (*SAMDC*), have been transferred through *Agrobacterium*-mediated transformation into japonica rice using an ABA-inducible promoter[53]. A large number of R_0 and R_1 transgenic lines have been obtained, and R_2 plants are currently being analysed for stress tolerance.

Chilling tolerance: The chilling sensitivity of higher plants is closely correlated with the degree of fatty acids unsaturation in the thylakoid membranes of their chloroplasts[74]. Chilling-tolerant rice plants expressing the *Arabidopsis* glycerol-*GPAT* gene have been produced via *Agrobacterium*-mediated transformation[58]. The photosynthetic apparatus in transgenic rice plants was better protected than in non-transgenic control plants under stress situations. The transgenic plants showed higher levels of unsaturated fatty acids and higher photosynthetic rates than non-transformed plants under chilling stress conditions.

Scavenging of hydroxyl radicals: Protection of sensitive metabolic reactions through maintaining the structures of protein complexes or membranes by an increased capacity for hydroxyl radical scavenging, may be an important strategy for engineering tolerance to water stress. Ferritin provides iron for the synthesis of iron proteins, such as ferredoxin and cytochromes, and prevents damage from the free radicals produced by iron/oxygen[75]. Transgenic plants producing the iron-storage protein ferritin were found to be more tolerant to oxidative damages than control plants[36].

Improving photosynthesis efficiency

Phosphoenolpyruvate decarboxylase (PEPC) is the key enzyme of C_4 plants. It catalyses the initial fixation of atmospheric CO_2, even in the presence of high concentrations of O_2. The photosynthetic pathway of C_3 rice plants was modulated by transferring the C_4 *PEPC* gene[33]. This was the first biotechnological approach to modulate a photosynthetic pathway in cereal crop plants. Atmospheric oxygen is known to reduce photosynthetic efficiency by as much as 40% through a process called photorespiration. This process increases under drought and high-temperature conditions, because the concentration of CO_2 decreases in the leaf due to closure of stomata. C_4 plants have evolved a biochemical mechanism to overcome the O_2 inhibition of photosynthesis. The C_4 photosynthetic pathway serves as a pump to concentrate the atmospheric CO_2 at the site of ribulose 1,5 bisphosphate (Rubisco), thus suppressing its oxygenase activity and the associated photophosphorylation[76]. The maize *PEPC* gene of the C_4 photosynthetic pathway, including all the exons, introns, promoter and terminator sequence, was transferred into C_3 rice through *Agrobacterium*-mediated transformation. The transgenic rice plants showed high levels of expression of the maize *PEPC* gene and accumulated high levels of PEPC protein (12% of the total leaf soluble protein). High-level expression of the *PEPC* gene was also observed in R_2 and R_3 plants. Physiologically, the transgenic plants exhibited reduced oxygen inhibition of photosynthesis compared to non-transformed plants[33]. Thus, this report demonstrates a successful strategy for transferring the key biochemical component of the C_4 photosynthetic pathway into C_3 plants.

Improving nutritional quality of rice seeds

Endosperm, the edible part of rice grains, lacks several essential nutrients, such as provitamin A. Vitamin A deficiency causes symptoms ranging from night blindness to those of xerophthalmia and keratomalacia, leading to total blindness. *Agrobacterium* transformation methods have been used to transfer genes encoding several β-carotene biosynthetic pathway enzymes into rice endosperm (carotenoid-free) in a single transformation effort using three vectors[39]. The vector pB19hpc combines the sequences from a plant (daffodil) phytoene synthase (*psy*) with the sequence coding for a bacterial phytoene desaturase (*crtI*) placed under control of the endosperm-specific glutelin (*Gt1*) and the constitutive CaMV35S promoter, respectively. The phytoene synthase cDNA contains a 5′-sequence coding for a functional transit peptide, and the *crtI* gene contains the Rubisco small subunit transit peptide (*tp*) sequence. The combination of these transgenes enabled biosynthesis of provitamin A in the rice endosperm. In

54. McElroy, D., Chamberlain, D. A., Moon, E. and Wilson, K. J., *Mol. Breed.*, 1995, **1**, 27–37.
55. Zhang, W., McElroy, D. and Wu, R., *Plant Cell*, 1991, **3**, 1155–1165.
56. Christensen, A. H., Sharrock, R. A. and Quail, P. H., *Plant Mol. Biol.*, 1992, **18**, 675–689.
57. Chamberlain, D. A., Brettell, R. I. S., Last, D. T., Witrzens, B., McElroy, D., Dolferus, R. and Dennis, E. S., *Aust. J. Plant Physiol.*, 1994, **21**, 95–112.
58. Yokoi, S., Higashi, S. I., Kishitani, S., Murata, N. and Toriyama, K., *Mol. Breed.*, 1998, **4**, 269–275.
59. Nishizawa, Y., Nishio, Z., Nakazono, K., Soma, M., Nakajima, E., Ugaki, M. and Hibi, T., *Theor. Appl. Genet.*, 1999, **99**, 383–390.
60. Yokoi, S., Tsuchiya, T., Toriyama, K. and Hinata, K., *Plant Cell Rep.*, 1997, **16**, 363–367.
61. Su, J., Shen, Q., David, Ho, T-D. and Wu, R., *Plant Physiol.*, 1998, **117**, 913–922.
62. Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W., *EMBO J.*, 1987, **86**, 3901–3907.
63. Vain, P., Worland, B., Kohli, A., Snape, J. W. and Christou, P., *Theor. Appl. Genet.*, 1998, **96**, 164–169.
64. Cao, J., Duan, X., McElroy, D. and Wu, R., *Plant Cell Rep.*, 1992, **11**, 586–591.
65. Flavell, R. B., Dark, E., Fuchs, R. I. and Fraley, R. T., *Bio/Technology*, 1992, **10**, 141–144.
66. Goldsbrough, A., *Trends Biotechnol.*, 1992, **10**, 417.
67. Yoder, J. I. and Goldsbrough, A. P., *Bio/Technology*, 1994, **12**, 263–267.
68. Jain, R. K. and Jain, S., *Indian J. Exp. Biol.*, 2000, **38**, 6–17.
69. Nayak, P., Basu, D., Das, S., Basu, A., Ghosh, D., Ramakrisnan, N. A., Ghosh, M. and Sen, S. K., *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 2111–2116.
70. Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M. F., Oliva, N., Abrigo, E., Khush, G. S. and Datta, S. K., *Theor. Appl. Genet.*, 1998, **97**, 20–30.
71. Bouchereau, A., Aziz, A., Larher, F. and Martin-Tanguy, J., *Plant Sci.*, 1999, **140**, 103–125.
72. Lee, J. K., John, S. A. and Weiss, J. N., *J. Gen. Physiol.*, 1999, **113**, 555–563.
73. Dobrovinskaya, O. R., Muniz, J. and Pottosin, I. I., *J. Memor. Biol.*, 1999, **167**, 127–140.
74. Murata, S., Sato, N., Takahashi, N. and Hamazaki, Y., *Plant Cell Physiol.*, 1982, **23**, 1071–1079.
75. Theil, E. C., *J. Biol. Chem.*, 1990, **265**, 4771–4774.
76. Hatch, M. D., *Biochim. Biophys. Acta*, 1987, **895**, 81–106.

ACKNOWLEDGEMENTS. This work was supported by research grant number RF98001-606 to R.W. from the Rockefeller Foundation. M.R. and J.S.R. were supported by post-doctoral fellowships, and R.K.J. was supported by a Biotechnology Career Fellowship from the Rockefeller Foundation.

Received 6 March 2000; revised accepted 1 July 2000

Geometrical features of a nonlinear wavefront

Phoolan Prasad
Department of Mathematics, Indian Institute of Science, Bangalore 560 012, India

We use the equations of weakly nonlinear ray theory (WNLRT), developed by us over a number of years, to study all possible shapes which a nonlinear wavefront in a polytropic gas can have. As seen in experiments, a converging nonlinear wavefront avoids folding itself in a caustic region of a linear theory and emerges unfolded with a pair of kinks. We review the work of Baskar, Potadar and Szeftel showing the way in which the solution of a Riemann problem of the conservation form of the equations of WNLRT can be used to study the formation of new shapes of a nonlinear wavefront from a single singularity on it. We also study the ultimate result of interactions of elementary shapes on the front.

IN a linear theory of wave propagation, the rays starting from various points of an initial wavefront may envelop a surface which is called caustic. For a small amplitude wave (so that linear theory is valid) propagating in a polytropic gas, which is in uniform state and at rest, the rays are straight lines orthogonal to the successive positions of the wavefront. Therefore every concave wavefront leads to the formation of a caustic and the front itself folds in the caustic region with cusp type of singularities as seen in Figure 1.

The figure represents a very interesting case of a caustic, which starts from the arête (2, 0) and ends on both sides at a finite distance at points (5, ± 2) at a time $4\sqrt{2}$, whereas the front continues to have cusp type of singularity even after the time $t = 4\sqrt{2}$ due to discontinuities in the curvature of the initial wavefront at points (1, ± 2).

Experimental results[1] showed that the caustic is resolved for a front which is a moderately strong shock front. This, of course, was predicted as early as in 1957 (ref. 2) using a theory based on heuristic arguments and has been subject of discussion by us over a number of years[3–7], see Figure 2. An important conclusion from these investigations is that (i) the resolution of the caustic by nonlinearity is accompanied by appearance of a new type of singularity on the front, *kink* (which was called shock–shock by Whitham) across which the amplitude and the normal direction of the front change discontinuously[8] and (ii) the geometrical features of a weakly nonlinear wavefront[5,9,10] and a weak shock front (moderately weak in the sense of Strutevant and Kulkarni) are qualitatively the same. Therefore, in order to study geometrical features of a shock front, we study those of a weakly nonlinear wavefront by a simpler set of conservation laws. In this process, we also review some unpublished results obtained by Baskar, Potadar and Szeftel during 1999. A study of geometrical features of a shock using the conservation forms of the equations of a weakly nonlinear shock ray theory is in progress.

Basic equations

Consider a two-dimensional pulse of small amplitude propagating in a polytropic gas, which before the arrival of the pulse, is in uniform state and at rest. Under a short wave or high frequency approximation, the pulse can be described by a one-parameter family of nonlinear wavefronts. If a shock front appears, these weakly nonlinear wavefronts ahead of and behind the shock will keep on interacting with the shock and then disappear after that. However, we ignore this interaction and trace the history of one of these nonlinear wavefronts, as it would have been without the interaction. We use appropriate nondimensionalization of dependent and independent variables and denote by m the Mach number of the wavefront. Then m is the wavefront velocity divided by the constant sound velocity of the undisturbed medium. Let θ be the

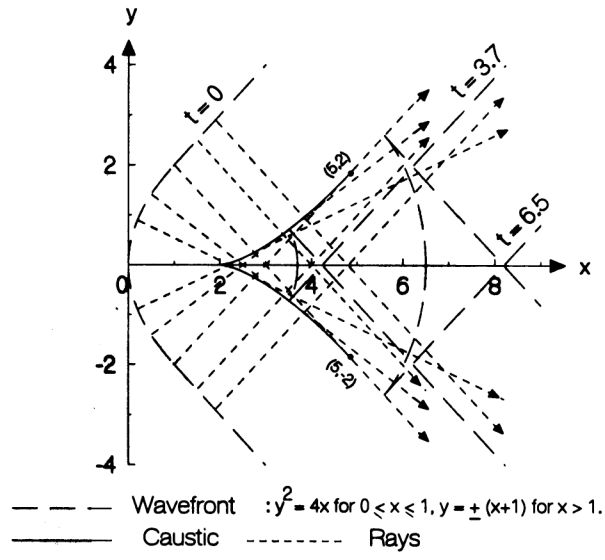

Figure 1. Linear wavefront propagation in an isotropic homogeneous medium with speed of propagation unity.

*e-mail: prasad@math.iisc.ernet.in