Gene Transfer in Cereals

EDWARD C. COCKING AND MICHAEL R. DAVEY

Until recently, gene transfer in plants was achieved only by sexual hybridization. Now, in addition, plant genetic manipulation, with the use of both recombinant DNA and protoplast fusion technology, is being applied to an increasing range of plants. The soil bacterium *Agrobacterium tumefaciens*, with its associated plasmid, is used as a vector for introducing DNA into the genomes of dicotyledonous plants, but it has not proved suitable for cereals. Instead, the direct uptake of plasmid DNA into cereal protoplasts is being used for the transformation of cells in rice, wheat, and maize. Transformation efficiencies, in some cases, are becoming comparable to those obtained in dicotyledons with *Agrobacterium*. In rice it is now possible to regenerate efficiently whole plants from protoplasts, and this capability may soon be extended to the other cereals. By means of direct interaction of cereal protoplasts with plasmids, coupled with improved procedures for the regeneration of plants from their protoplasts, gene transfer in the cereals is becoming established at the frontiers of recombinant DNA technology.

AT THE BEGINNING OF THIS DECADE THERE HAD BEEN NO substantiated reports of the expression of a foreign gene in a genetically engineered plant (*1*). Since then the situation has changed dramatically, and the development of plasmid vectors carrying selectable markers has facilitated numerous studies on gene expression and regulation in plants. Some of these studies have focused on the suppression of symptoms due to viral infection after expression of tobacco mosaic virus (TMV) gene sequences as nuclear genes in transgenic tobacco plants (*2*), and also the expression of the firefly luciferase gene in transgenic tobacco plants (*3*). Although some dicotyledonous crop plants respond well to the natural gene transfer system of the soil bacterium *Agrobacterium tumefaciens*, the cereals, which constitute the most important group of plants for human nutritional needs, have remained unresponsive. Until recently, the opportunities to apply recombinant DNA technology to any of the cereals seemed remote. However, a number of advances have now made these plants accessible to manipulation by gene transfer techniques, with improved knowledge of their cell biology and tissue culture providing a bridge between molecular biology and plant breeding.

Transferring Genes into Plants Other Than Cereals

When one introduces new DNA sequences into plants, the quantity of foreign protein produced will depend on several factors, including the rates of transcription and translation and the stability of messenger RNA (mRNA) and synthesized protein. Studies on vector construction have identified strong promoters that control the function of certain genes.

Those promoters that have been studied in most detail include the nopaline synthase (*nos*) promoter from the transfer (T) DNA region of the tumor-inducing (Ti) plasmid of *A. tumefaciens* (*4*), and the 35*S* transcript promoter of cauliflower mosaic virus (CaMV). By combining such promoters upstream of the coding sequences of bacterial antibiotic resistance genes, it is possible to produce chimeric genes that function in plants. An example of a chimeric gene is the bacterial aminoglycoside (neomycin) phosphotransferase type II gene from the transposon Tn5 under the control of the *nos* or CaMV 35*S* promoter. The neomycin phosphotransferase (NPTII) gene encodes resistance to the antibiotic kanamycin through its ability to detoxify the drug by phosphorylation, and this gene can be used as a dominant marker to select transformed plant cells. A variety of vectors based on the *Agrobacterium* Ti plasmid are now available; the most widely used are disarmed vectors in which the oncogenicity (onc) genes of the T-DNA are deleted and replaced with a chimeric gene or other genes of interest (*5*). Expression of bacterial genes in plant cells is influenced by the promoter used. Thus, in studies with *Petunia*, the CaMV 35*S* promoter-driven expression of NPTII was at least an order of magnitude higher than when the *nos* promoter was used (*6*).

In other studies, the bacterial chloramphenicol acetyltransferase (CAT) coding sequences were used as a reporter gene (conferring resistance to the antibiotic chloramphenicol). The results showed that the activity of the CaMV 35*S* promoter in transgenic tobacco and *Petunia* is almost 30 times stronger than the *nos* promoter, and that it is constitutively expressed in all organs of the transgenic plants (*7*).

In these studies, the procedure of leaf disk transformation-regeneration was used in which surface-sterilized leaf explants were inoculated with appropriate *A. tumefaciens* strains, combining gene transfer and plant regeneration into a single process. This technique is applicable to those species that respond to *Agrobacterium* infection and that also produce plants readily from leaf explants, particularly members of the Solanaceae, including *Petunia*, tobacco, and tomato (*8*).

For several dicotyledons, including *Petunia* and tobacco, transformed calluses and transformed plants regenerated from such calluses can also be obtained by cocultivating protoplast-derived cells, or cell suspensions, with *A. tumefaciens* (*9*). These procedures exploit the natural infectivity of *Agrobacterium*.

Plant cells are converted into protoplasts when treated with cell wall–degrading enzymes. Although protoplasts can be isolated readily from suitable cell suspension cultures of most cereals and can be induced to regenerate new cell walls and undergo sustained division to form calluses, there is no evidence that these cereal protoplast-derived cells can produce transformed calluses after cocultivation with *A. tumefaciens*. Even the use of compounds such as

The authors are with the Plant Genetic Manipulation Group, Department of Botany, University of Nottingham, Nottingham NG7 2RD, United Kingdom.

acetosyringone and α-hydroxyacetosyringone, which stimulate the activation of Ti virulence (vir) genes and T-DNA transfer from *Agrobacterium* to dicotyledonous cells (*10*), has apparently not elicited any transformation response in rice (*11*). It has been reported that maize seedlings can be transformed by virulent octopine and nopaline strains of *A. tumefaciens* and that opines can be detected in these transformed seedlings (*12*). However, substantiation of this report must await T-DNA analysis, in view of the recent findings that opine synthesis is not specific to transformed tissues (*13*).

The failure to achieve *Agrobacterium*-induced transformation in cereals has led to increased interest in assessing other transformation methods, including direct uptake of DNA, the fusion of spheroplasts (bacterial protoplasts) with plant protoplasts, liposome-mediated DNA delivery, and microinjection of DNA. Indeed, direct interaction with DNA was a logical development because there seemed to be no molecular biological reason why new DNA sequences that were suitably introduced into cereal cells should not undergo transcription and translation. The cell walls of monocotyledons, including those of cereals, differ in composition from those of dicotyledons, particularly in relation to matrix cross-linking polymers (*14*). They might thus constitute a major interaction barrier that one would expect to be bypassed when a technique coupling protoplasts with direct gene transfer was used.

Transferring Genes into Cereals

The use of protoplasts. Earlier studies on the uptake of DNA by plant protoplasts, including those of barley, demonstrated uptake of chromosomal, plasmid, and phage DNA, as well as association of some of the DNA with nuclei. It was also shown that polycations such as poly-L-lysine and poly-L-ornithine (PLO) enhanced the uptake process (*15*). Later, the direct interaction of Ti plasmid, isolated from *A. tumefaciens*, with *Petunia* protoplasts in the presence of PLO brought about transformation (*16*), thereby demonstrating that only the plasmid of *Agrobacterium* was required. This finding set the stage for direct gene transfer into protoplasts. However, the transformation frequency only reached a maximum of about 1 in every 10^5 protoplasts originally plated. A similar transformation frequency was reported for polyethylene glycol (PEG)–induced Ti plasmid transformation of tobacco protoplasts (*17*). Subsequent comparison of plasmid delivery methods confirmed that PEG was the chemical of choice in stimulating plasmid delivery to protoplasts (*18*). Because the procedure necessitated the use of protoplasts and resulted in a low transformation frequency, this gene transfer system was seldom used for dicotyledons. Even though it was possible to obtain kanamycin-resistant plants, again at low frequency, by such direct gene transfer into protoplasts (*19*), this approach failed to match the use of *A. tumefaciens*. However, the repeated failure of *Agrobacterium*-mediated gene transfer in the cereals, either by direct interaction of bacteria with the wounded plant or with explants, or by cocultivation with protoplast-derived cells and suspension cultures, has led to a reevaluation of direct DNA uptake for transforming cereals.

Evidence for stable transformation of cereal cells resulted from direct gene uptake into protoplasts of Einkorn (diploid) wheat (*Triticum monococcum*). The pBR322-derived plasmid used contained the maize transposable element *Ac*, the *nos* promoter, and the NPTII gene. Protoplasts isolated from a cell suspension were incubated with plasmid and PEG. After culture for several days, dividing cells were embedded in agarose-solidified medium, and colonies were selected that were resistant to 100 μg of kanamycin per milliliter. These selected colonies possessed NPTII enzyme activity (*20*). However, the frequency of transformation was only about 1 in every 10^6 protoplasts originally plated and was comparable to that previously obtained for the transformation of *Petunia* and tobacco protoplasts with Ti plasmid (*16, 17*). Moreover, the protoplasts of this cereal suspension culture, although capable of forming small calluses, were unable to regenerate plants.

Concurrent with this finding was the successful transformation of cells of the forage grass *Lolium multiflorum* (Italian ryegrass), again by direct gene transfer. This transformation was brought about by the interaction of protoplasts with a chimeric plasmid pABD1 that carried the NPTII gene as a selectable marker under the control of promoter, terminator, and translation initiation signals derived from CaMV (*21*). Before it was used, the plasmid was linearized by restriction with the endonuclease Sma I. The PEG uptake procedure was basically that used for Ti transformation of tobacco protoplasts, but with the addition of a heat shock treatment (45°C for 5 minutes followed by 10 seconds at 0°C). The transformation frequency was still low (about 1 in every 10^3 protoplasts originally plated), and again the transformed colonies were incapable of plant regeneration. Other workers have also found this pABD1 construct with PEG uptake to be successful; for instance, sugarcane protoplasts have been transformed and colonies have been selected at a frequency of about 1 in 10^7 on 80 μg of kanamycin per milliliter. These colonies also expressed NPTII enzyme activity (*22*). However, inclusion of a heat shock in the experimental protocol did not improve the transformation frequency in this sugarcane system.

Protoplasts isolated from rice cells grown in suspension have also been reported to be amenable to PEG-induced plasmid uptake. By means of a construct carrying the *nos* promoter, the NPTII gene, and the terminator region from CaMV, transformed protoplast-derived colonies were selected on kanamycin (100 μg/ml). The transformation frequency was significantly higher in this system, reaching approximately 2% (*23*). NPTII activity was detected in the transformants, and Southern blot hybridization indicated that such colonies also possessed the foreign gene, which was probably chromosomally integrated, at high copy number. Although the plasmid used in these studies contained the *nos* gene from the Ti plasmid T-DNA, evidence presented for nopaline synthesis was equivocal. However, this is not surprising in view of the recent indication that opines can be produced in untransformed callus as a result of arginine metabolism (*13*).

Gene transfer by electroporation. In several ways protoplasts resemble cultured animal cells in their basic biological properties. Electroporation, a procedure in which cells are subjected to a high-voltage electric pulse to induce a reversible permeability change in the cell membranes, is now being used successfully for both the transient and the stable transformation of cereals. Electroporation had been used with considerable success to introduce cloned genes into mammalian cells (*24, 25*).

In several recent studies, some including the use of cereal protoplasts, electroporation has been used to introduce DNA before monitoring transient gene expression. The efficiency of gene transfer into carrot protoplasts was assessed 24 to 48 hours after electroporation by measuring the CAT activity that resulted from expression of chimeric plasmids with the *nos* and CaMV 35*S* promoters. The gene transfer efficiency was related to DNA concentration, the amplitude and duration of the electric pulse, and the composition of the electroporation medium. The gene transfer conditions, as optimized with the carrot system, were also effective with tobacco and maize protoplasts; the highest levels of CAT activity in maize were obtained with the construct carrying the CaMV promoter (*26*).

In a somewhat similar study (*27*), the promoter of the *shrunken* gene of maize was fused to the NPTII gene from the transposon Tn5 to study this promoter function in protoplasts isolated from wheat cell suspensions. Maximum NPTII activity was detected 4

days after electroporation. This study also indicated that the configuration of the introduced DNA changed from supercoiled molecules to open circular and linear forms within the plant cells, and that only the linear form remained after 10 days of protoplast culture. In this protoplast system the majority of introduced DNA was thought to remain extrachromosomal.

Evidence suggests that linear single-stranded DNA and double-stranded circular molecules may participate in the transfer of DNA from *Agrobacterium* to plant cells (*10*). Therefore, it may be worthwhile to explore the feasibility of using single-stranded DNA for direct gene transfer into cereal protoplasts. Protoplasts of rice, wheat, and sorghum have also been used to study expression of the CAT gene fused to either the CaMV 35*S* promoter or the copia long terminal repeat promoter of *Drosophila* (*28*). Gene transfer was evaluated by means of electroporators in which the high-voltage pulses could be applied either directly or indirectly, avoiding direct anode contact, to the protoplast medium. The indirect method was more rapid and resulted in higher protoplast viability.

In addition to these short-term experiments, electroporation has also been used for the stable transformation of protoplasts that were derived from maize cells grown in suspension with supercoiled plasmids carrying the CaMV 35*S* promoter and the NPTII gene. Transformed, dividing cells were resistant to 100 μg of kanamycin per milliliter, and clones expressing NPTII enzyme activity were selected at a frequency of about 1 in 10^4 of the protoplasts originally plated (*29*).

A systematic study of the factors affecting the efficiency of stable transformation of plant protoplasts, when interacting directly with plasmids, resulted in a significant increase in transformation frequency (*30*). When protoplasts of tobacco and the pABD1 construct linearized by Sma I treatment were used, the efficiency of direct gene transfer was 1000 times as great as that obtained previously with a procedure combining chemical and physical treatments. Thus, the application of heat shock to the protoplasts, optimization of the PEG concentration, addition of PEG to the protoplasts after the DNA, and attention to the initial field strength during electroporation elevated the transformation frequency of the tobacco protoplasts to about 2% of the protoplast-derived cell colonies. It seems likely that, with additional refinements in electroporation apparatus design and circuitry, these combined treatments applied to a range of cereal protoplasts should also increase transformation frequency.

Use of liposomes and bacterial spheroplasts. Although attention has been focused on chemically induced plasmid uptake, electroporation, or a combination of these approaches, basic studies have also been conducted on the delivery of plasmid DNA to cereal protoplasts by means of artificial lipid vesicles (liposomes) encapsulating DNA. This method has been shown to result in the transformation of tobacco protoplasts (*31*). Spheroplasts containing suitable plasmids are being used in a somewhat similar manner, and these studies are leading to an improved understanding of the optimal conditions required for gene transfer (*32*). When this approach was evaluated in rice, about 1 in 10^4 of the colonies derived from the fusion of protoplasts from cell suspensions with *Agrobacterium* spheroplasts were claimed to synthesize T-DNA specific opines (*33*).

Microinjection of DNA and other methods of DNA delivery. Microinjection of DNA into the nuclei of protoplasts has also developed recently as an efficient method of gene transfer, with intranuclear microinjection of alfalfa protoplasts giving transformation frequencies of up to 26% (*34*). This result is encouraging since alfalfa has proved difficult to transform with *A. tumefaciens*. Because the microinjected protoplasts were handled individually, selectable markers were not required to recover the transformants.

A significant development has been the use of electric field pulses to introduce foreign nucleic acids into plant cells. Thus, after the introduction of TMV RNA directly into isolated tobacco mesophyll cells, as many as 50% of the cells expressed TMV RNA after 24 days (*35*). This "electroinjection" technique, which bypasses the necessity to isolate protoplasts, may be useful for cereal suspension cells.

According to a recent study, whole maize plants developed symptoms of viral infection if inoculated with strains of *Agrobacterium* carrying tandomly repeated copies of genomes of gemini maize streak virus in their T-DNA (*36*). This finding that *Agrobacterium* can transfer maize streak virus DNA to maize questions the present limited-host range of *Agrobacterium*. It is now possible to use *Agrobacterium* carrying repeated copies of other gemini virus DNA genomes in its T-DNA for similar studies on other cereals. Gemini viruses are not, however, seed-transmitted, and this may limit their use in transformation assessments utilizing *Agrobacterium*-mediated virus infection.

Another promising approach for the transformation of cereal plants, which also does not involve tissue culture techniques, is the injection of chimeric plasmid DNA into rye floral tillers (*37*). It has been postulated that DNA is subsequently transported by the plant vascular system to the rye germ cells, which can take up the DNA if they are at a "competent" stage. The observed transformation frequency is low (about 0.07%), and it has yet to be determined if a similar competent stage occurs in other cereals. Self-pollination of maize plants with pollen mixed with DNA from a donor plant results in nearly 10% of the kernels per ear having transformed endosperm, but this frequency was greatly reduced in subsequent generations (*38*).

Utility of Transformed Cereal Systems

Transformed cereal systems would permit studies of the regulation of foreign genes in cereals, comparable to those of the expression of maize genes encoding heat shock proteins in *Petunia* (*39*) and the phytochrome-controlled expression of a wheat gene in transgenic tobacco seedlings (*40*). For rice, a genomic library in a phage lambda vector has been constructed, and attempts have been made to identify and clone DNA sequences that might enable the construction of artificial chromosomes (*41*).

It has now been demonstrated that mixtures of different DNAs, added to tobacco protoplasts, can result in cotransformation of unlinked foreign genes. Cotransformation by direct gene transfer will provide a rapid and easy method of introducing nonselectable genes into plant genomes, including those of the cereals. In tobacco, tissues selected after the transformation of protoplasts with Ti plasmid were also found to have sequences homologous to those of the calf thymus DNA (used as carrier DNA in the uptake procedure) and Ti plasmid sequences integrated into their nuclear genomes (*42, 43*). In other experiments, up to 47% of kanamycin-resistant clones also expressed a nonselectable *nos* gene (*44*).

The challenge is to increase the transformation frequency in the cereals to that currently achieved with *Agrobacterium*-mediated transformation of dicotyledons and to be able to regenerate fertile plants efficiently and reproducibly from the transformed cereal protoplasts. Developments in vector construction have superseded developments in DNA delivery and knowledge of factors influencing the regeneration of plants from cereal protoplasts. As emphasized by Burton (*45*), plant breeding is a "numbers game," and success will be directly proportional to the volume of material handled. Successful gene transfer to cereals for breeding improvements will necessitate high frequencies of transformation combined with efficient and reproducible regeneration of transformed plants; high transformation frequencies will also be required for basic studies on promoter sequences (*46*).

At a recent Rockefeller Foundation Meeting (*47*), it was reported that a system has been developed for the efficient regeneration of rice plants from protoplasts (*47*). Protoplasts from suspensions of cells of embryo, leaf, root, or anther origin are first subjected to heat shock treatment (5 minutes at 45°C) (*48*) and then cultured in an agarose-solidified medium (*49*). The resulting colonies, when plated directly onto a hormone-free medium, produce green plants, principally through somatic embryogenesis. Plant regeneration can be obtained as rapidly as 7 weeks from the time of protoplast isolation for the japonica rice varieties Taipei 309, Fujisaka 5, and Nipponbare. The development of finely divided, fast-growing cell suspension lines was essential for efficient rice protoplast isolation, division, and subsequent plant regeneration, which occurred by means of somatic embryogenesis from 10 to 20% of the protoplast-derived colonies (*47*). It is necessary to assess cultures of maize, wheat, barley, and other cereals for sustained division of protoplasts isolated from suitable cell suspensions and to monitor plant regeneration, in a manner similar to that which has proved so successful for rice. Thus far, protoplasts isolated directly from rice and other cereal seedlings or plants have not been shown to undergo sustained division to form colonies; the ideal would be to obtain regeneration of plants from protoplasts derived directly from cereals.

As recently emphasized (*50*), successful genetic manipulation by recombinant DNA technology will depend on how readily genotype and phenotype can be related, and the ease with which the desired gene sequence can be isolated and cloned. Transfer of single gene traits, such as those for herbicide resistance, as already accomplished in certain dicotyledonous species (*51*), is now technically feasible with rice. Eventually, this capability will be extended to all the major cereals. However, most agronomically desired improvements, for instance, in yield, pest and pathogen resistance, stress tolerance, and photosynthetic efficiency, involve many genes of unknown identity. In this connection it may be possible, with the use of transposable elements, to isolate and characterize DNA surrounding the site of transposon insertion, thereby identifying genes responsible for a specific trait. Several transposable elements from maize have been characterized molecularly and could be used as specific probes for the isolation of genes from mutants induced by the integration of transposable elements (*52*). A better understanding of the control of photosynthesis, fungal pathogenicity, and solute uptake is also beginning to open up new possibilities for genetic manipulation. The recent discovery and identification of 2-carboxy-D-arabinitol-1-phosphate as an endogenous nocturnal inhibitor of photosynthesis through its effect on ribulose-1,5 bisphosphate carboxylase oxygenase (*53*) will provide a target for gene manipulations. The suggestion that plant chitinases are important antifungal agents in plants (*54*) indicates yet another target for transformation, as does the suggestion that a character that enhances potassium and sodium discrimination, nearly tenfold, is located on the long arm of the 4D chromosome of wheat (*55*). Although transfer into plant chromosomes and expression of nuclear-coded genes have been achieved for single gene traits, similar procedures are not yet applicable to organelle-coded genes.

It may also be possible to transfer genes across sexual barriers by using nonrecombinant DNA somatic cell fusion procedures that do not rely for their implementation on a detailed knowledge of the genes involved (*50*). In this respect, protoplast fusion is now being undertaken in rice to transfer both nuclear- and cytoplasmic-encoded genes. Thus somatic hybrids might be produced between cultivated rice and wild rice species with useful traits (*56*), such as salinity tolerance and resistance to a range of fungal and virus diseases.

The transfer of cytoplasmic male sterility might also be achieved by cytoplasmic hybrid (cybrid) production resulting from protoplast fusion. Cybrids are of interest for the production of hybrid cereals and for studies on heterosis (*57*). Overall, gene transfer in cereals will require close integration of cell culture and molecular approaches if significant advances are to be made in the use of these new technologies for cereal improvement.

REFERENCES AND NOTES

1. E. C. Cocking *et al.*, *Nature (London)* **293**, 265 (1981).
2. P. P. Abel *et al.*, *Science* **232**, 738 (1986).
3. D. W. Ow *et al.*, *ibid.* **234**, 856 (1986).
4. C. H. Shaw *et al.*, *Nucleic Acids Res.* **12**, 7831 (1984).
5. R. B. Simpson, A. Spielmann, L. Margossian, T. D. McKnight, *Plant Mol. Biol.* **6**, 403 (1986).
6. S. G. Rogers, K. O'Connell, R. B. Horsch, R. T. Fraley, in *Biotechnology in Plant Science, Relevance to Agriculture in the Eighties,* M. Zaitlin, P. Day, A. Hollaender, Eds. (Academic Press, New York, 1985), pp. 219–226.
7. F. Nagy, J. T. Odell, G. Morelli, N-H. Chua, *ibid.*, pp. 227–235.
8. R. B. Horsch *et al.*, *Science* **227**, 1229 (1985).
9. L. Márton, G. J. Wullems, L. Molendijk, R. A. Schilperoort, *Nature (London)* **277**, 129 (1979); M. R. Davey, J. P. Freeman, J. Draper, E. C. Cocking, in *Plant Tissue Culture 1982*, Proceedings of the 5th International Congress on Plant Tissue and Cell Culture, A. Fujiwara, Ed. (Japanese Association for Plant Tissue Culture, Abe Photo, Tokyo, 1982), pp. 515–516; R. B. Horsch *et al.*, *Science* **223**, 496 (1984); M. DeBlock *et al.*, *EMBO. J.* **3**, 1681 (1984).
10. S. E. Stachel, E. Messens, M. Van Montagu, P. Zambryski, *Nature (London)* **318**, 624 (1985); S. E. Stachel, B. Timmerman, P. Zambryski, *ibid.* **322**, 706 (1986).
11. M. Van Montagu, in *Rice Genetics, Proceedings of the International Rice Genetics Symposium* (International Rice Research Institute, Los Baños, Philippines, 1985), p. 839.
12. A. C. F. Graves and S. L. Goldman, *Plant Mol. Biol.* 7, 43 (1986).
13. P. Christou, S. G. Platt, M. C. Ackerman, *Plant Physiol.* **82**, 218 (1986).
14. S. C. Fry, *Annu. Rev. Plant Physiol.* **37**, 165 (1986).
15. P. F. Lurquin and C. I. Kado, *Mol. Gen. Genet.* **154**, 113 (1977).
16. M. R. Davey *et al.*, *Plant Sci. Lett.* **18**, 307 (1980).
17. F. A. Krens, L. Molendijk, G. J. Wullems, R. A. Schilperoort, *Nature (London)* **296**, 72 (1982).
18. J. Draper, M. R. Davey, J. P. Freeman, E. C. Cocking, B. J. Cox, *Plant Cell Physiol.* **23**, 451 (1982); J. P. Freeman *et al.*, *ibid.* **25**, 1353 (1984).
19. J. Paszkowski *et al.*, *EMBO J.* **3**, 2717 (1984).
20. H. Lörz, B. Baker, J. Schell, *Mol. Gen. Genet.* **199**, 178 (1985).
21. I. Potrykus, M. Saul, J. Petruska, J. Paszkowski, R. D. Shillito, *ibid.*, p. 183.
22. W.-H. Chen *et al.*, *Plant Cell Rep.*, in press.
23. H. Uchimiya *et al.*, *Mol. Gen. Genet.* **204**, 204 (1986).
24. E. Neumann, M. Schaefer-Ridder, Y. Wang, P. H. Hofschneider, *EMBO J.* **1**, 841 (1982).
25. H. Potter, L. Weir, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7161 (1984).
26. M. Fromm, L. P. Taylor, V. Walbot, *ibid.* **82**, 5824 (1985).
27. W. Werr and H. Lörz, *Mol. Gen. Genet.* **202**, 471 (1986).
28. T.-M. Ou-Lee, R. Turgeon, R. Wu, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6815 (1986).
29. M. E. Fromm, L. P. Taylor, V. Walbot, *Nature (London)* **319**, 791 (1986).
30. R. D. Shillito, M. W. Saul, J. Paszkowski, M. Müller, I. Potrykus, *Bio/Technology* **3**, 1099 (1985).
31. M. Caboche and A. Deshayes, *C. R. Acad. Sci. (Paris)* **299**, 663 (1984).
32. K. Gartland, M. R. Davey, E. C. Cocking, *Cell Biol. Int. Rep.*, in press.
33. A. Baba, S. Hasezawa, K. Syono, *Plant Cell Physiol.* **27**, 463 (1986).
34. T. J. Reich, V. N. Iyer, B. L. Miki, *Bio/Technology* **4**, 1001 (1986).
35. H. Morikawa, A. Iida, C. Matsui, M. Ikegami, Y. Yamada, *Gene* **41**, 121 (1986).
36. N. Grimsley, T. Hohn, J. W. Davies, B. Hohn, *Nature (London)* **325**, 177 (1987).
37. A. de la Peña, H. Lörz, J. Schell, *Nature (London)* **325**, 274 (1987).
38. Y. Ohta, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 715 (1986).
39. D. E. Rochester, J. A. Winer, D. M. Shah, *EMBO J.* **5**, 541 (1986).
40. F. Nagy, S. A. Kay, M. Boutry, M.-Y. Hsu, N.-H. Chua, *ibid.*, p. 1119.
41. A. T. Ganesan, in *Rice Genetics, Proceedings of the International Rice Genetics Symposium* (International Rice Research Institute, Los Baños, Philippines, 1986), p. 859.
42. F. A. Krens *et al.*, *Plant Mol. Biol.* **5**, 223 (1985).
43. R. Peerbolte *et al.*, *ibid.*, p. 235.
44. R. J. Schocher, R. D. Shillito, M. W. Saul, J. Paszkowski, I. Potrykus, *Bio/Technology* **4**, 1093 (1986).
45. G. W. Burton, in *Plant Breeding II*, K. J. Frey, Ed. (Iowa State Univ. Press, Ames, 1979), p. 453.
46. G. An, *Plant Physiol.* **81**, 86 (1986).
47. E. C. Cocking *et al.*, paper presented at Rockefeller Foundation Planning Meeting on Rice Improvement, International Rice Research Institute, Los Baños, Philippines, October 1986; R. Abdullah, E. C. Cocking J. A. Thompson, *Bio/Technology* **4**, 1087 (1986).
48. J. A. Thompson, R. Abdullah, W.-H. Chen, K. M. A. Gartland, *J. Plant Physiol.* **127**, 367 (1987).
49. J. A. Thompson, R. Abdullah, E. C. Cocking, *Plant Sci.* **47**, 123 (1986).
50. D. Pental and E. C. Cocking, *Hereditas* **3**, 83 (1985).
51. L. Comai, L. C. Sen, D. M. Stalker, *Science* **221**, 370 (1983).
52. N. Fedoroff, in *Mobile Genetic Elements,* J. A. Shapiro, Ed. (Academic Press, New York, 1983), pp. 1–63.
53. S. Gutteridge *et al.*, *Nature (London)* **324**, 274 (1986).
54. A. Schlumbaum, F. Mauch, U. Vögeli, T. Boller, *ibid.*, p. 365.
55. J. Gorham, C. Hardy, R. G. Wyn Jones, L. R. Joppa, C. N. Law, *Theor. Appl. Genet.*, in press.
56. M. S. Swaminathan, *Plant Mol. Biol. Rep.* **4** (no. 1), 1 (1986).
57. A. Kumar and E. C. Cocking, *Am. J. Bot.*, in press.