Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11786–11790, December 1995
Genetics

Arabidopsis mutants deficient in T-DNA integration

(transformation/DNA repair)

R. V. SONTI*†, M. CHIURAZZI*‡, D. WONG*§, C. S. DAVIES¶‖, G. R. HARLOW**††, D. W. MOUNT**, AND E. R. SIGNER*‡‡

*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; ¶Department of Biology, McGill University, Montreal, QC Canada H3A 1B1; and **Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721

Communicated by Maurice S. Fox, Massachusetts Institute of Technology, Cambridge, MA, August 25, 1995

ABSTRACT ***Arabidopsis thaliana*** **mutants originally isolated as hypersensitive to irradiation were screened for the ability to be transformed by *Agrobacterium* transferred DNA (T-DNA). One of four UV-hypersensitive mutants and one of two γ-hypersensitive mutants tested showed a significant reduction in the frequency of stable transformants compared with radioresistant controls. In a transient assay for T-DNA transfer independent of genomic integration, both mutant lines took up and expressed T-DNA as efficiently as parental lines. These lines are therefore deficient specifically in stable T-DNA integration and thus provide direct evidence for the role of a plant function in that process. As radiation hypersensitivity suggests a deficiency in repair of DNA damage, that plant function may be one that is also involved in DNA repair, possibly, from other evidence, in repair of double-strand DNA breaks.**

Agrobacterium strains can transfer a segment of DNA (T-DNA) into dicotyledonous plant cells, where it is eventually integrated into the plant genome (1). Agrobacterial genes involved in transfer have been characterized, and bacterial proteins are known to be bound to the transferred T-DNA. However, relatively little is known about plant functions after transfer, especially those involved in integration. Analysis of integrated T-DNA (2–4) and of enhancement of transformation by low-dose irradiation (5, 6) has suggested that plant DNA repair functions may be involved, and DNA repair mutants are often radiation hypersensitive (7). Therefore, we tested mutants of *Arabidopsis* originally isolated as radiation hypersensitive (8–10) for T-DNA transformation proficiency.

MATERIALS AND METHODS

Plant Lines. The UV-hypersensitive mutants were isolated in the laboratory of D.W.M. (8–10); mutant lines used here (*uvh1, uvh2, uvh5, uvh6*) are homozygous progeny of plants recovered from mutagenesis, and the radioresistant control (*UVH*) is the unmutagenized parent. The γ-hypersensitive mutants were isolated in the laboratory of C.S.D. (11); mutant lines used here [*rad4* (X4), *rad5* (GT73)] are M5 and M6 homozygotes, respectively, each derived from a single plant from the M4 generation, and the radioresistant control line (*RAD*) is derived from a nonmutant M4 sibling of *rad5*. All mutant lines are derived ultimately from ecotype Columbia (Col-0) line WT1B col-PRL, originally obtained from C. Somerville (Michigan State University).

Bacterial Strains and Plasmids. *Agrobacterium tumefaciens* MSU440, carrying both wild-type pRiA4 and binary vector pBI121, which contains the *NPT* gene encoding resistance to kanamycin, was obtained from C. Somerville (Michigan State University). Binary vector pKIWI105 in *Agrobacterium* LBA4404 (12) was obtained from R. C. Gardner (University of Auckland); the plasmid contains *NPT* as well as a *GUS* (β-glucuronidase) reporter gene that lacks a bacterial ribosome-binding site. Plasmid pMC14 carries the *Bam*HI/*Bgl* II fragment from *Arabidopsis* clone AT3102 (13) that originally included the entire *ADH* locus, but in pMC14 the internal 3.6-kb *Sac* I fragment that includes the *ADH* coding region is deleted and replaced with Pnos-*NPTII*-ocs3′; construction of this plasmid and the binary vector backbone are described elsewhere (14). Plasmid pMC78 also carries the AT3102 *Bam*HI/*Bgl* II fragment, but in pMC78 Pnos-*NPTII*-3′ocs is inserted at the upstream *Sac* I site without removal of *ADH* coding sequences; in addition, pMC78 carries P35S-*codA*-CaMV3′ (15), which was first inserted together with pUC18 at the upstream *Bam*HI site and then transferred, as *codA-ADH:NPT* bracketed by pUC18 sequences linearized at the *Aat* II site, into the *Sca* I site of binary vector pGA-3-*Sh* (16) from which the *NPT* and *Sh* genes had first been removed by *Bam*HI deletion (K. Smith, C. Linard, and E.R.S., unpublished work).

Bolt Inoculation Assay. Flowering bolts of 4-wk-old plants were inoculated by stabbing with a needle, or alternatively by squeezing gently with sterile forceps, that in either case had been dipped in a saturated culture of *Agrobacterium* strain MSU440. For experiments 1 and 2 of Table 1, inoculated plants were covered with a plastic dome to increase humidity, and the Ri-induced root response was scored after 2 wk. For the remaining experiments of Table 1, light petroleum jelly was applied to the wound site immediately after inoculation, and the Ri-induced root response was scored after 3 wk.

T-DNA Transformation. For both stable transformation and transient assay, cocultivation of root explants was modified from ref. 17 as follows. Seeds were germinated on MS medium with agarose (0.8%), sucrose (3%), and Gamborg's vitamin solution (2 mg/liter) in Petri dishes placed vertically so that roots grew along the agar surface. Whole roots were excised at 3–4 wk, cultured for 4 days on callus-inducing (ARM1) medium, and cut into 1-cm explants while submerged in a saturated culture of *Agrobacterium*. The explants were blotted dry and cocultivated on ARM1 medium for 2 days and then grown for 2 days more on ARM1 medium plus antibiotic [30 parts ticarcillin (Sigma) to 1 part potassium clavulanate (SmithKline Beecham); 50 μg/ml] to prevent bacterial overgrowth. At that point, for transformation (see Table 2) the explants were transferred to shoot-inducing (ARM2) medium

Abbreviations: Kan^r, kanamycin resistance; DSB, double-strand break.

†Present address: Center for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India.
‡Present address: Istituto Internationale di Genetica e Biofisica, via Marconi 10, 80125 Naples, Italy.
§Present address: Williams College, Williamstown, MA.
‖Present address: Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721.
††Present address: Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721.
‡‡To whom reprint requests should be addressed.

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containing 25 μg of kanamycin per ml (Sigma; plant tested) plus antibiotic. Alternatively, for transient assay (see Table 3 and Fig. 2) the explants were stained for Gus activity (1 mg of indoxyl β-D-glucoside per ml/50 mM phosphate buffer, pH 7; overnight) either then (day 2) or 1 day later (day 3).

RESULTS

As a preliminary screen, we first tested transformation by the pRiA4 "hairy root" plasmid in *Agrobacterium* MSU440. When flowering bolts are inoculated, transformation results in callus and adventitious tissue at the wound site within a few weeks (Fig. 1). Table 1 shows that for this ecotype (Columbia), the radioresistant lines responded positively in at least 50% of inoculated plants, as did four of the six mutant lines tested. By contrast, response of the remaining two mutants, *uvh1* (isolated as UV hypersensitive) and *rad5* (isolated as γ hypersensitive), was clearly reduced.

We therefore tested these two mutants further for stable transformation of root explants (17) to Kan^r (kanamycin resistance) by *NPT* (neomycin phosphotransferase). Table 2 shows that over a relatively broad range of absolute frequency, with binary vectors in which the T-DNA either did or did not carry DNA homologous to the genomic *ADH* locus, both *uvh1* and *rad5* consistently gave fewer Kan^r calli per length of root explant than controls by roughly the same factor as in the pRiA4 bolt inoculation test. Thus, *uvh1* and *rad5* are clearly deficient in transformation.

In principle, the transformation deficiency could be either in transfer of the T-DNA into the plant cell or, alternatively, in integration of the T-DNA into the plant genome. To distinguish between these alternatives, we tested both mutants in a transient assay of transfer that is independent of integration (12). The assay depends on T-DNA binary vector pKIWI105, in which the *GUS* reporter gene is driven by a promoter (P35S) active in both plants and bacteria but lacks a Shine–Dalgarno sequence for bacterial ribosome attachment, so that no Gus enzyme can be produced in the bacterial cells. In this assay, T-DNA need not be integrated into the plant genome for expression, which is seen as early as 2 days after inoculation (12). Thus, any Gus activity in roots cocultivated with bacteria serves as a reporter for transfer *per se.*

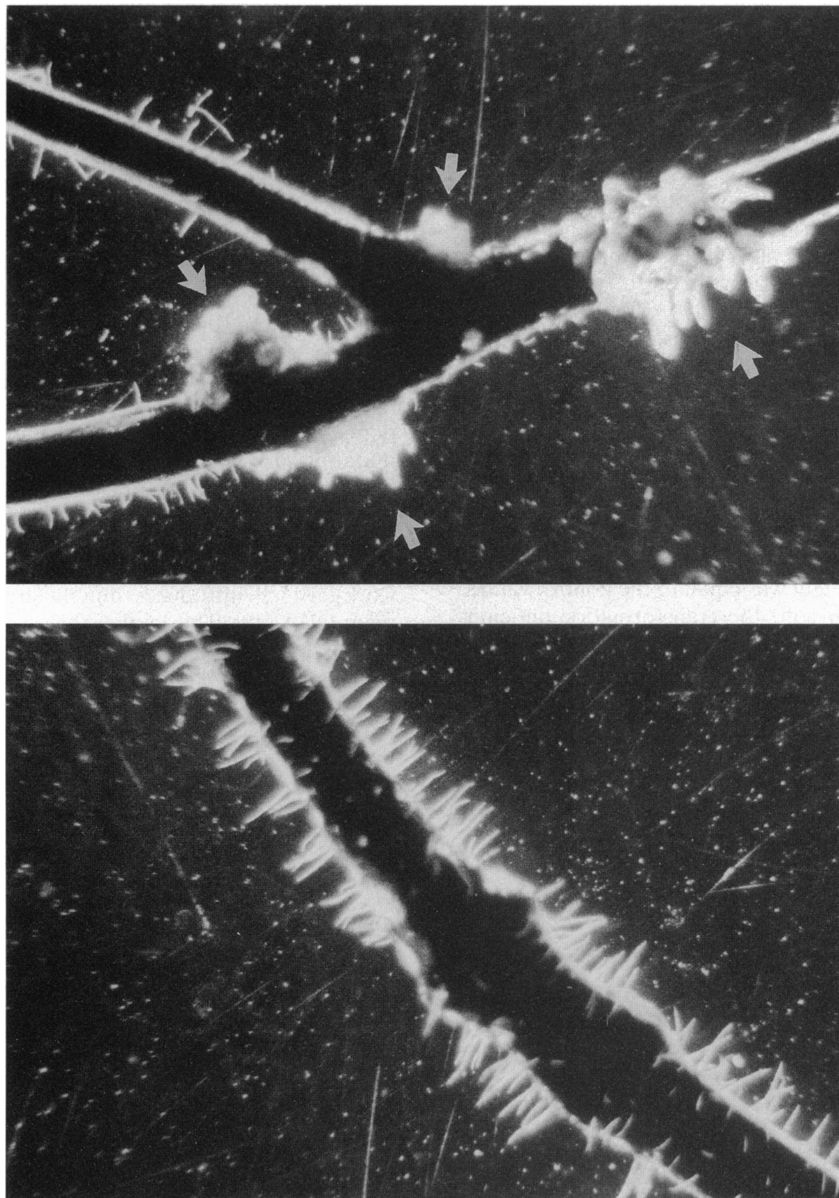

FIG. 1. Bolt inoculation assay for transformation by pRiA4 T-DNA. Callus and root formation at the wound site indicate successful transformation (*Upper*). Mock inoculation (no bacteria) leads to slight swelling only (*Lower*).

Table 1. Ectopic root transformation by plasmid pRiA4 in independent experiments

	UV	γ	Fraction of inoculated bolts responding with ectopic root tissue					
			Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
UVH	r	r	18/35 = 0.51	15/26 = 0.58	30/40 = 0.75	21/37 = 0.57		
uvh1	s	s	0/35 < 0.03	2/75 = 0.03	6/43 = 0.14			
uvh2	s	NT				18/25 = 0.72		
uvh5	s	NT				23/37 = 0.62		
uvh6	s	NT				11/23 = 0.48		
RAD	r	r					30/33 = 0.91	12/26 = 0.46
rad4	r	s					23/26 = 0.88	
rad5	r	s						1/36 = 0.03

r, Resistant; s, hypersensitive; NT, not tested.

Table 3 and Fig. 2 show that in this assay both mutants were essentially as competent for transfer as the controls (possibly excepting a slight reduction for *uvh1*, the significance of which is not clear). It is especially striking that, even though there was an absolute variation of 100-fold in the assay for *rad5* and *RAD* (presumably owing to uncontrolled variables), the relative values obtained in each experiment were similar for the two. Thus, the transformation deficiency of both *uvh1* and *rad5* appears to be in T-DNA integration rather than in transfer. Consistent with this interpretation, some of the sectors from cocultivation with the controls, but not the two mutants, appeared elongated. This can be accounted for by proliferation, during the experiment, of cells in which the T-DNA integrated early during cocultivation.

Although radiation-hypersensitive mutants often grow poorly (7), measurement of several parameters indicated that the apparent integration deficiency of the mutants is not simply an artifact of poor transformant growth. Over a 40-day period on callus-inducing (ARM1) medium (17), where cocultivated root explants are initially grown, the transformation-deficient UV-hypersensitive mutant *uvh1* grew as well as the radioresistant control (g fresh weight of roots). Over an 8-day period on ARM1 followed by 37 days more on shoot-inducing (ARM2) medium (17), where transformed calli are selected, *uvh1* did grow less well than the control but only slightly (nearly 3-fold less fresh weight of roots) compared with the transformation deficiency (≈20-fold), and spectrophotometrically measured accumulation of chlorophyll a or b, protochlorophyllide, and carotenoids per g fresh weight was equal to the control values. Over a 10-day period on soil, the transformation-deficient γ-hypersensitive mutant *rad5* grew as well as the radioresistant control (g fresh weight of seedlings).

DISCUSSION

The two mutant lines *uvh1* and *rad5* clearly can accept T-DNA from *Agrobacterium* (Table 3 and Fig. 2). Nevertheless, they appear deficient in integration of that DNA into the *Arabidopsis* genome (Tables 1 and 2 and Fig. 1). This deficiency has been observed with four different vectors (Table 2), two carrying genomic homology and two not, which shows it is a general property of transformation in these lines.

T-DNA is usually integrated essentially at random, with only a very few ($<10^{-4}$) events targeted to homology (18). However, models can be considered in which a block in random integration shifts the plant response in the direction of homologous integration. Nevertheless, Table 2 shows that the frequency of transformation with T-DNA carrying genomic homology is roughly comparable to that with heterologous T-DNA, and by Southern analysis none of 10 *uvh1* and 1 *rad5* Kan^r transformants with a vector carrying homologous T-DNA (pMC78) resulted from homologous integration (data not shown). Thus, the residual transformants appear still to reflect random integration, and there is no indication that these mutations increase homologous integration to a readily observable level. However, given the low frequency of homologous integration to begin with, even an increase as large as 100-fold would not have been detected with this experimental design.

Radiation hypersensitivity suggests deficiency in repair of DNA damage, and T-DNA integration is a form of genetic recombination. In other organisms, defects in repair and recombination are often associated (7). Nevertheless, whether the deficiencies in repair and integration stem from a single mutation in either *uvh1* or *rad5* is not yet known. If the deficiencies in repair and integration do stem from a single mutation in *uvh1* and a single mutation in *rad5*, then, as *uvh1* is both UV and γ hypersensitive, whereas *rad5* is γ hypersensitive only, two separate genes might be involved.

Elsewhere, mutant *uvh1* has also been shown to be hypersensitive to bleomycin (G.R.H. and D.W.M., unpublished work), which introduces double-strand breaks (DSBs) into plant DNA (19). This implies that *uvh1* is deficient in repair of DSBs. Moreover, preliminary experiments with a well-characterized tandem repeat genomic insert (20) suggest further that *uvh1* may be elevated in somatic homologous recombination (T. S. Pittalwala, M. E. Jenkins, C.S.D., and D.W.M., unpublished work). DSBs are thought to occur spontaneously as well as by irradiation and are known to promote homologous recombination in yeast and phage λ (21) as well as in plants (M.C., A. Ray, R. J. Perera, J.-F. Viret, A. W. Lloyd, and E.R.S., unpublished data). Therefore, one possibility is that the *uvh1* mutant lacks an enzymic function necessary for both integration of T-DNA and repair of DSBs by one means or another and that the DSBs instead persist in such a fashion as

Table 2. Stable transformation (17) by various plasmid vectors in independent experiments

	UV	γ	Kan^r calli per cm of root explant				
			Heterologous T-DNA			Homologous T-DNA	
			pKIWI105		pGA*Sh-tk*	pMC14	pMC78
UVH	r	r	31/111 = 0.28	4/143 = 0.03	31/200 = 0.16	7/200 = 0.04	22/153 = 0.14
uvh1	s	s	1/95 = 0.01	0/138 < 0.01	0/200 < 0.005	0/200 < 0.005	4/157 = 0.03
RAD	r	r	12/78 = 0.15	78/164 = 0.47			37/168 = 0.22
rad5	r	s	1/80 = 0.01	1/163 = 0.006			1/169 = 0.006

r, Resistant; s, hypersensitive.

Table 3. Transient T-DNA transfer by plasmid pKIWI105 (12) in independent experiments

			Gus$^+$ foci per cm of root explant					
			Exp. 1	Exp. 2	Exp. 3	Exp. 4		Exp. 5
	UV	γ	(day 2)	(day 2)	(day 2)	Day 2	Day 3	(day 2)
UVH	r	r	303/74 = 4.1	340/60 = 5.7		145/36 = 4.0	212/39 = 5.4	182/37 = 4.9
uvh1	s	s	306/71 = 4.3	195/60 = 3.3		105/55 = 1.9	95/46 = 2.1	33/6 = 5.5
RAD	r	r	392/97 = 4.0		29/98 = 0.3	189/41 = 4.6	848/80 = 10.8	
rad5	r	s	152/55 = 2.8		10/102 = 0.1	140/35 = 4.0	664/56 = 11.8	

r, Resistant; s, hypersensitive. Day indicates day of staining for Gus activity.

to promote homologous recombination of a tandem repeat. Further work will be necessary to test this possibility.

Comparable data for *rad5* are not yet available. In other work, however, this mutant has also been connected to homologous recombination, albeit indirectly. Mutant *rad5* has been shown to be deficient in induction by γ-irradiation of expression of a cloned *Arabidopsis* gene highly homologous to *RAD51*, a *recA* homolog that in yeast is essential for efficient homologous recombination and DSB repair (K. Smith and E.R.S., unpublished data).

In summary, these integration-deficient mutants directly implicate plant functions in the integration of T-DNA. Although *uvh1* and *rad5* differ in details of phenotype, in both lines the mutations appear to affect repair of DNA damage and integration of T-DNA and possibly processes associated with homologous recombination as well. Thus, these results suggest

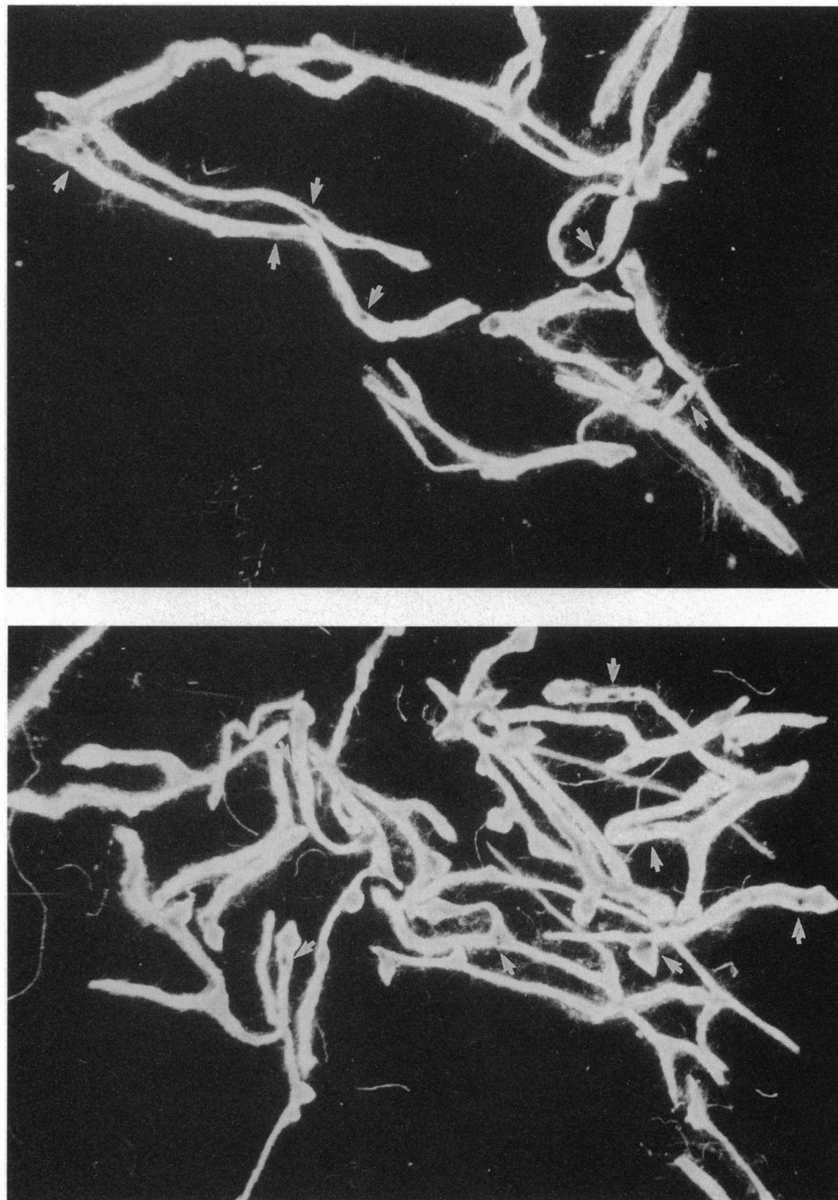

FIG. 2. Transient assay for T-DNA transfer to explanted root segments. Small blue spots against the pale root tissue background reflect staining for *in planta* Gus activity and thus indicate T-DNA transfer. (*Upper*) *UVH* (nonmutant) control. (*Lower*) *uvh1* (integration-deficient UV-hypersensitive mutant).

that elements of the enzymic machinery involved in repairing DNA damage, perhaps including DSBs that can stimulate recombination, may also be required for an obligate step in T-DNA integration.

We thank Chris Somerville and Richard Gardner for *Agrobacterium* strains and Michelle Burns for technical help. This work was supported by Grants DMB 92-06129 to E.R.S. and MCB 91-18591 to D.W.M. from the National Science Foundation and by a grant from the Natural Sciences and Engineering Research Council of Canada (C.S.D.). M.C. was supported by fellowships from the Consiglio Nazionale delle Richerche, Rome, and the North Atlantic Treaty Organization, and D.W. was supported by the Massachusetts Institute of Technology Undergraduate Research Opportunities Program.

1. Zambryski, P. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43,** 465–490.
2. Matsumoto, S., Ito, Y., Hosoi, T., Takahashi, Y. & Machida, Y. (1990) *Mol. Gen. Genet.* **224,** 309–316.
3. Gheysen, G., Villaroel, R. & Van Montagu, M. (1991) *Genes Dev.* **5,** 287–297.
4. Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Bakkeren, G., Crameri, A., Angelis, K., Redei, G. P., Schell, J., Hohn, B. & Koncz, C. (1991) *EMBO J.* **10,** 697–704.
5. Koehler, F., Cardon, G., Poehlman, M., Gill, R. & Scheider, O. (1989) *Plant Mol. Biol.* **12,** 189–199.
6. Koehler, F., Benediktsson, I., Cardon, G., Andreo, C. S. & Scheider, O. (1990) *Theor. Appl. Genet.* **79,** 679–691.
7. Friedberg, E. C. (1985) *DNA Repair* (Freeman, New York).
8. Harlow, G. R. & Mount, D. W. (1991) *J. Cell. Biochem.*, Suppl. 15A, 71.
9. Harlow, G. R., Jenkins, M. E., Pittalwala, T. S. & Mount, D. W. (1994) *Plant Cell* **6,** 227–235.
10. Jenkins, M. E., Harlow, G. R., Liu, Z., Shotwell, M. A., Ma, J. & Mount, D. W. (1995) *Genetics* **140,** 725–732.
11. Davies, C., Howard, D., Tam, G. & Wong, N. (1994) *Mol. Gen. Genet.* **243,** 660–665.
12. Janssen, B.-J. & Gardner, R. C. (1989) *Plant Mol. Biol.* **14,** 61–72.
13. Chang, C. & Meyerowitz, E. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1408–1412.
14. Chiurazzi, M. & Signer, E. R. (1994) *Plant Mol. Biol.* **26,** 923–934.
15. Perera, R. J., Linard, C. & Signer, E. R. (1993) *Plant Mol. Biol.* **23,** 793–799.
16. Perez, P., Tiraby, G., Kallerhoff, J. & Perret, J. (1989) *Plant Mol. Biol.* **13,** 365–373.
17. Marton, L. & Browse, J. (1991) *Plant Cell Rep.* **10,** 235–239.
18. Lichtenstein, C. & Barrena, E. (1993) *Plant Mol. Biol.* **21,** v–xii.
19. Hille, J., Verheggen, F., Roelvink, P., Franssen, H., van Kammen, A. & Zabel, P. (1986) *Plant Mol. Biol.* **7,** 171–176.
20. Assaad, F. F. & Signer, E. R. (1992) *Genetics* **132,** 553–566.
21. Thaler, D. S. & Stahl, F. W. (1988) *Annu. Rev. Genet.* **22,** 169–197.