한국생물공학회지 제 10권 제 1호
Korean J. Biotechnol. Bioeng.
Vol. 10. No. 1. 89-96(1995)

고등식물의 유용 유전자 크로닝을 위한 분자적 접근

정 현 숙* · Alan B. Rose · Robert L. Last**
*조선대학교 자연과학대학 유전공학과
**미국 코넬대학교 생물공학과 및 보이스 탐슨 연구소

Molecular Approaches for Cloning of Important Higher Plant Genes

*Hyeon-sook Cheong, **Alan B. Rose and Robert L. Last
*Department of Genetic Engineering, Chosun University, Kwangju 501-759,Korea
**Boyce Thompson Institute for Plant Research at Cornell University,
Tower Road, Ithaca, NY 14853 U.S.A.

ABSTRACT

An *Arabidopsis thaliana* gene encoding phosphoribosyl anthranilate transferase is shown to be the gene that is defective in blue fluorescent trp 1 mutant plants. This gene, named PAT1, coding region is homologous to those for the phosphoribosyl anthranilate transferase from many microorganisms. This is due to a defect in tryptophan biosynthesis that leads to an accumulation of anthranilate, a fluorescent intermediate in the tryptophan pathway. PAT1 is a single-copy gene that complements all of the visible phenotypes of the different trp1 mutants. Experiments to determine the regulation of the PAT1 gene are in progress. The wild-type PAT1 promoter and several promoter deletions of PAT1 gene have been transformed into *Arabidopsis* tryptophan mutants. These constructs might identify promoter elements that control this patterns. We have isolated the homozygous lines in T_3 seeds and analyzed the protein levels using PAT antibody and PAT protein level increased two fold in pHS107. Finally, the potential of using PAT1 as a selectable marker or visible reporter of gene expression is being explored.

INTRODUCTION

Arabidopsis thaliana(L.) Heynh., a member of the *Brassicaceae*, has been used in classical plant genetics since the 1940s(1) because of the small size of the mature plant, its short generation time, and the large number of offspring normally produced after self-pollination. Due to the small size of the seeds, large scale mutagenesis is possible with this plant; thus, many mutations have been identified, studied, and mapped(2-7). The genetic map consists of five linkage groups, in concordance with haploid chromosome number of five. Multiple marker strains with mutations on each of chromosomes are available(8-10). *A. thaliana* also has been adopted as a model system for studies on the molecular genetic level(6, 7, 11). On the basis of DNA reassociation kinetics, Leutwiler *et al.*(1984)(12) have estimated that the haploid nuclear genome of *A. thaliana* contains approximately 70,000 kb which is thus the smallest genome known for higher plants so far.

Plants synthesize amino acids, purines and pyrimidines, yet plant auxotroph are extremely rare. Recently, several tryptophan auxotroph of *Arabidopsis thaliana* were uncovered(13, 14). One of the auxotrophic mutants appeared to lack the enzymatic activity corresponding to the second step in tryptophan pathway, the anthranilate phosphoribosyl(PR)-transferase. The trp1 mutant was characterized extensively because it had an easily scorable fluorescence phenotype due to the accumulation of anthranilic acid derivatives.

However, inviability of absolutely auxotrophic plants and genetic redundancy for amino acid biosynthetic enzyme genes in higher plants must be considered. Genetic redundancy, a common feature of eukaryotic genomes, can confound the search for recessive auxotrophic mutations because the presence of multiple isozymes for the same biosynthetic reaction would require multiple mutations in to produce the auxotrophic phenotype.

The tryptophan biosynthetic pathway is the source of many important compounds in plants (Fig. 1). In addition to its role in protein synthesis, this pathway provides precursors for a variety of indolic secondary products. These include the plant growth regulator auxin(IAA) (15), antimicrobial phytoalexins(16), alkaloids, and glucosinolates(17).

The hypothesis that plants will utilize the same set of reactions in microorganisms is supported by biochemical analysis(18, 19). Also, all of the existing plant tryptophan mutants have biochemical lesions in steps found in the microbial pathway(13-15).

We constructed several promoter deletions of PAT1 gene and transformed into *Arabidopsis* tryptophan mutants to determine the regulation of the PAT1 gene. These constructs are shown by genetic complementation in transgenic plants. PAT1 appears to be a single-copy gene, unlike the previously described aromatic amino acid biosynthetic genes. We have also analyzed the protein levels using PAT antibody in transgenic plants.

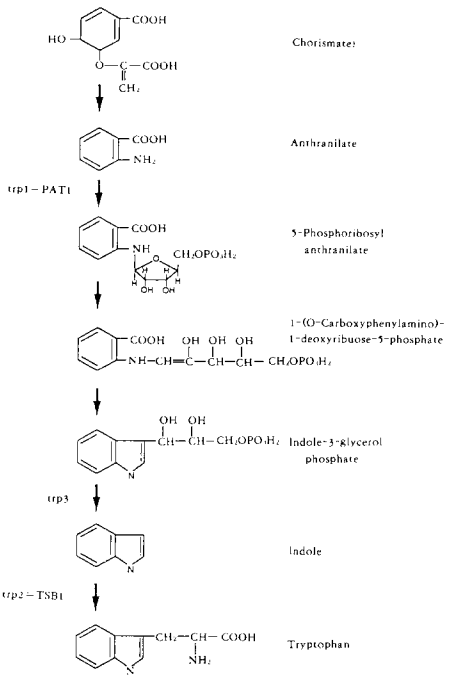

Fig. 1. The tryptophan biosynthetic pathway. The enzyme defective in trp1 mutant plants(PAT) is indicated.

The PAT1 gene, together with the trp1 plants, will add to the growing collection of molecular tools available for the manipulation and study of *A. thaliana*.

MATERIALS AND METHODS

Plant Lines and Culture Conditions

Arabidopsis thaliana lines homozygous for pgm (20), gl1-1,and trp1-100(21) was grown on agar and liquid medium or soilless mix as described (13, 14). Plant culture conditions were identical to those described(13).

DNA Manipulations

Plasmids were constructed using enzymes from New England Biolabs or Promega according to the recommendations of the manufacturers. *Escherichia coli* strains JM109 and XL-Blue were used for plasmid propagation. A genomic clone (AR1-5) that was obtained from an *A. thaliana*

library(Clonetech) in EMBL3 was provided by Alan Rose(Cornell University). Fragments that cross-hybridize to the PAT1 cDNA were subcloned as follows. pHS101, which contains a 7.8 kb *Kpn*I fragment cloned into pEND4K. This plasmid is containing PAT1 promoter deletion −30 to −184 and pHS102, 103, 104, 105, and 106 was constructed with several promoter deletions from −120 to −1112 respectively. pHS107 contains a 7.9 kb *Kpn*I fragment containing PAT1 promoter into the *Kpn*I site of pEND4K.

Plant Transformation

Plasmids containing promoter deletions of PAT1 gene were cloned into binary vector pEND4K. Each of these plasmids was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The resulting strains were used to transform trp1−100 as described(14, 22), with following modifications. Producing sterile roots were cultured in 0.5xSGM liquid medium with shaking for 7-10 days, and selection against the *A. tumefaciens* was with 50mg of kanamycin/L. Shoots were induced on SIM K50T100 and roots were formed on(RIM K50T100) rooting medium(23). Blue light induced photochemical degradation was reduced by filtration with yellow plexiglass filters(Polycast 2208, Curbell) as described(24) until the rooted shoots were transfered to soil. The kanamycin resistance of progeny seedlings was tested on PNS medium (25) containing 40 mg of kanamycin sulfate/L.

Immunoblotting

For sample preparation 0.01g of tissue(fresh) were ground up with 3 volume of 1x TBS containing 1mM PMSF, BSM and ACA. A partially purified extract separated by SDS-PAGE after protein concentration determination and transfered electrophoretically to the nitrocellulose membrane(S & S NC 0.2 um pore). The blotted proteins were subsequently identified using polyclonal PAT, PAI, TSA and TSB antibodies. These antibodies were kindly provided by Jianmin Zhao(Boyce Thompson Institute). The membrane was exposed to phosphoimager film at room temperature or a X−ray film at −70℃ with intensifying screen.

RESULTS AND DISCUSSION

Construction for Promoter Deletion of PAT1 Gene

The tryptophan biosynthetic pathway provides plants with precursors for the plant growth hormone auxin and many secondary products in addition to tryptophan. The *Arabidopsis* gene (PAT1) encoding phosphoribosyl anthranilate transferase is a single-copy gene that complements all of the visible phenotypes of the trp1 mutant(21).

We constructed plasmids to determine the regulation of the PAT1 gene. Several promoter deletions of PAT gene were used for characterizing PAT regulatory elements(Fig. 2). A DNA fragment containing the regulatory element digested with the suitable restriction enzymes to generate a serial deletion of promoter region. pHS 101 is a 7.8 kb fragment from pAR 189(from Alan Rose) containing PAT1 promoter deletion −30 to −184 in *Kpn*I site of pEND4K. pHS 102 is 7.7 kb fragment containing deletion −120 to −350 into pEND4K and pHS 103 is 7.8 kb containing −196 to −350 promoter deletion. pHS 104 is 7.6 kb containing −235 to −606 promoter deletion and pHS 105 containing promoter deletion −442 to −846 and pHS106 containing −738 to −1162 deletion. pHS 107 is a 7.9 kb fragment containing PAT1 promoter.

Upon examination of constructed DNA and transfered DNA into *A. tumefaciens* LBA4404 incited by these plasmids, we observed that same internal *EcoR*I fragments from *E. coli* and from *Agrobacterium* had undergone a deletion. Fig. 3 shows *EcoR*I digests of the original clones, pHS101, pHS102, pHS103, pHS104, pHS105, pHS106 and pHS107 (lane 2, 5, 8, 11, 14, 17 and 20), and the derivative which was retrieved from *Agrobacterium* (lane 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21 and 22). Slightly the original con-

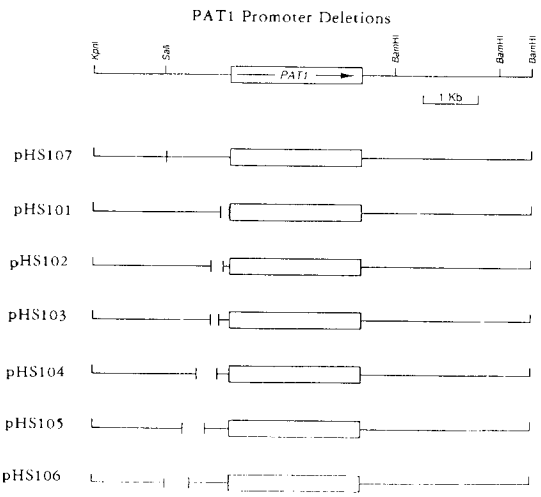

Fig. 2. Promoter deletions of PAT1 gene. pHS101, 7.8kb containing PAT1 promoter deletion −30 to −184 in the *Kpn*I site of pEND44; pHS102, 7.7kb containing −120 to −350 deletion; pHS103, 7.8kb containing −196 to −350 deletion; pHS104, 7.6kb containing −235 to −606 deletion; pHS105, 7.5kb containing −442 to −846 deletion; pHS106, 7.5kb containing −738 to −1162 deletion; pHS107, 7.9kb containing PAT promoter.

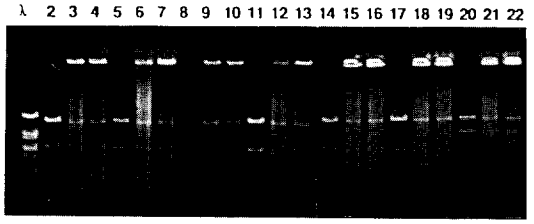

Fig. 3. Comparison of pHS101~pHS107 plasmids isolated from *E. coli* and *Agrobacterium* Purified plasmid DNA was digested to completion with *E. co*RI and separated by agarose gel electrophoresis.

structs into *E. coli* are different between them into *Agrobacterium* because their salts strengths. The binary vectors contain none of the oncogenic functions generally undesirable for transformed plants but instead utilize kanamycin resistance as a selectable marker. Selection for resistance of transformed plants cells to kanamycin has been useful(26). The vectors described here confer resistance to at least 40μg/ml kanamycin on *Arabidopsis* trp1-100 mutants.

Transformation of Root Explants and Regeneration

To develop a rapid and efficient *Agrobacterium*-mediated transformation method for *Arabidopsis*, we used propagated roots from liquid culture during 10 days and incubated root explants on CIM containing 0.5mg of 2,4-D and 0.05mg of kinetin per liter. Optimal shoot regeneration was achieved when we transfered to SIM containing 0.15mg of IAA, 5mg of 2-IP, 50mg of kanamycin sulfate and 100mg of timentin. Further differentiation, transfered shoots to RIM. When initiated roots are developed after 1-3 weeks on RIM transfered GM plates kept on edge to let roots grow and transfered unrooted shoots to fresh RIM plates every 2 weeks. When good root growth was evident, transfered to soil. Two weeks after transfer to the SIM/K50/T100 green transformed calli in background of yellowish calli were formed(Fig. 4A). Using this method based on kanamycin selection, some plants showed chlorosis(Fig. 4B) and most of these green calli formed shoots after transfer to fresh SIM(Fig. 4C). The high incidence of shoot formation made this system to study initiation and development of shoots(27, 28) and we successfully used kanamycin selection to develop a reliable *A. tumefaciens*-mediated root transformation procedure(Fig. 4A, B, C).

The PAT1 transgenes of promoter deletion complement at trp1 mutations. As summarized in Table 1, some transgenic plants containing any of the promoter deletion of PAT1 are no longer blue fluorescent(Fig. 5A, B, C). The complementing activity in the transformed plants is genetically linked to kanamycin resistance. Many transformants give rise to roughly on-quarter blue-fluorescent and kanamycin-sensitive progeny, as expected if the plants are heterozygous for

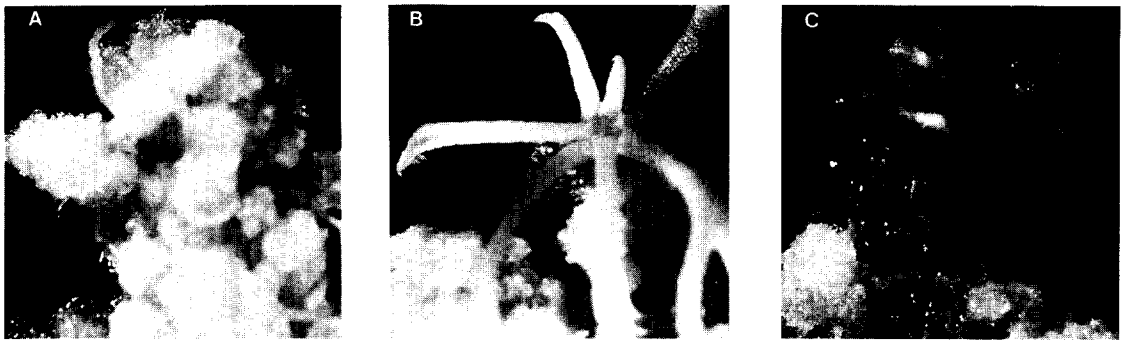

Fig. 4. The developmental stages of transgenic plants. (A), Green callus formation after two weeks of transformation; (B), Chlorosis on the PNS medium containing kanamycin; (C), Shoots induction by kanamyan selection.

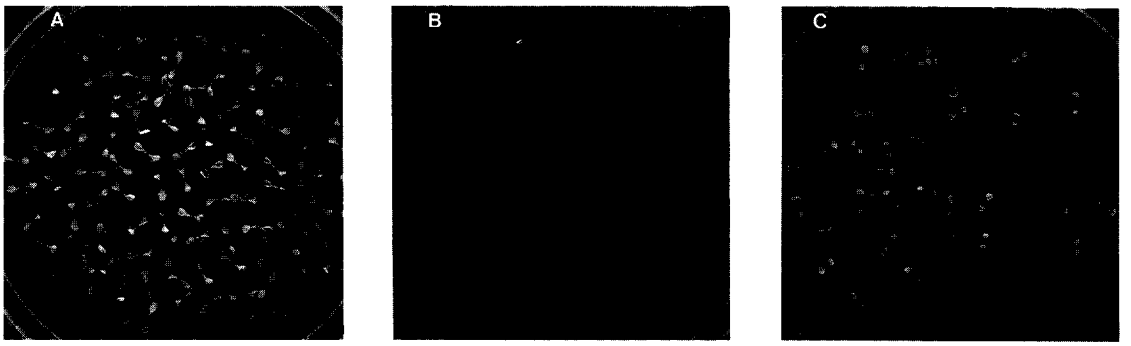

Fig. 5. PAT1 complementation of trpl. The plants were photographed under visible of shortwave UV light. Some blue-fluorescent, kanamycin sensitive individuals can be seen in segregating transgenic lines. A, Trpl-100 shows fluorescent completely; B, pHS101 can be seen in segregating; C, pHS107 shows non-fluorescent.

Table 1. Summary of complementation results.

Plasmid[a] Introduced	Number[b] Analyzed T_2	Kanamycin Resistant	Kanamycin Sensitive	Fluo-rescent	Non-Fluorescent
trp1-100	100	0	100	100	0
pHS101	100	80	20	20	80
pHS102	100	91	9	10	90
pHS104	100	75	25	15	85
pHS105	100	70	26	75	25
pHS107	100	85	15	0	100

[a] Genotype is trp1-100.

[b] Each T_2 line is derived from an individual regenerated primary transformant(T_1) by self pollination.

the dominant PAT1 and NPTII genes at single locus(21). The trp1-100 plants are much easier to work because even though they are blue fluorescent they grow normally and set seed well(13, 14, 21).

The PAT1 gene, in conjunction with the trp1 mutants, should prove to be useful tool for the study of *A. thaliana*. Many of the techniques that allow sophisticated modifications of yeast, microbial, and even mammalian genomes are unavailable in plant molecular genetics. The antibiotic resistance genes currently employed have some disadvantages, such as allowing a high number of individuals lacking the genes to escape selection.

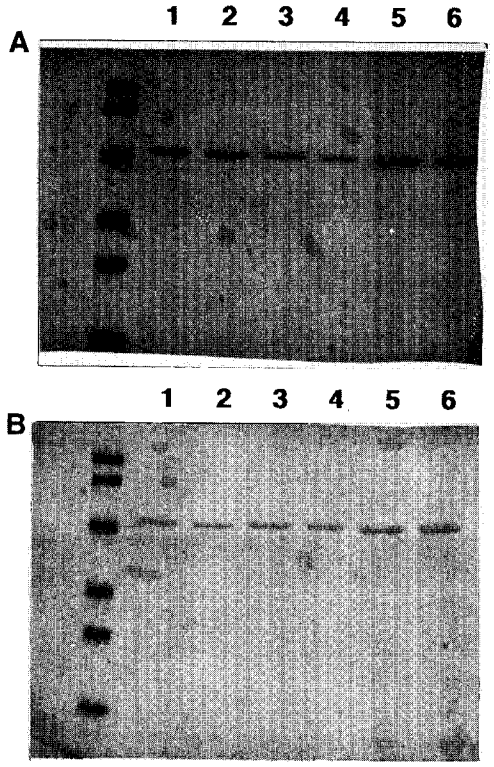

Fig. 6. A, B. Total protein of trpl-100 and transgenic plants. A, Lane 1, trpl-100; Lane 2, pHS101; Lane 3, pHS102; Lane 4, pHS104; Lane 5, pHS105, Lane 6, pHS107; B, Duplication of 6A for TSA and TSB protein levels.

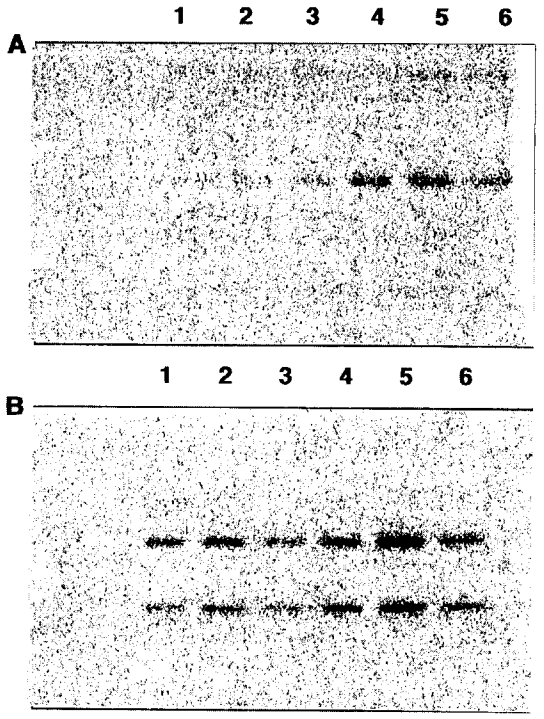

Fig. 7. Immuodetection of trpl-100 and transformants of PAT1 promoter deletions. A, PAT and PAI antibodies, Lane 1, trpl-100; Lane 2, pHS101; Lane 3, pHS102; Lane 4, pHS104; Lane 5, pHS105, Lane 6, pHS107; A, TSA and TSB antibodies, Lane 1, trpl-100; Lane 2, pHS101; Lane 3, pHS101; Lane 4, pHS104; Lane 5, pHS105, Lane 6, pHS107.

The small number of useful selection markers currently available restricts the number of manipulation possible in single strain(21, 29). The PAT1 gene could serve as a marker in plant transformation, allowing selection or screening for the loss of blue fluorescence in trpl−100 plants. PAT1 might also prove useful as a gene that can be selected against 5-methylanthranilate(14).

The PAT1 gene might serve as a visible reporter of gene expression because the presence or activity of PAT1 can be easily detected in whole living plants by screening for fluorescence.

Evidence for a Transformed PAT gene

In order to understand how a plant regulates the tryptophan biosynthetic pathway to pullfill these diverse functions, it is important to monitor the enzymes of the pathway at the protein level. We used polyclonal antibodies against the following enzymes of the Trp biosynthetic pathway: phosphoribosyl anthranilate transferase(PAT), tryptophan synthase a(TSA) and tryptophan synthase b(TSB). The cDNAs of PAT, PAI, TSA, and TSB were inserted into the *E. coli* overexpression vector, pGEX1 or pGEX2T to make fusion proteins with glutatione S-transferase. The fusion proteins were used to make

Table 2. The comparative quantification of protein level by immunoassay.

Object Name	Quantity
trp1-100	1.00
pHS101	1.05
pHS102	1.02
pHS104	1.57
pHS105	1.305
pHS107	2.437

polyclonal antibodies in rabbits. As shown in Fig. 6, 7, the transformants of PAT1 promoter deletions increased protein level to compare with the control(trp1-100). pHS101 and pHS102 are similar amount of protein level and pHS 104 and pHS105 are one and half times of the protein amount of the control. However, PAT protein level increased twofold in pHS107(Table 2). These results suggest pHS107 contained one set of insert with wild type PAT1 gene and promoter of pHS101 and pHS102 didn't work anymore after deletion. pHS104 and pHS105 decreased activity of PAT1. Immunoassay is rapid and comparatively simple and these antibodies will be used in analysis of the regulation and localization of their corresponding enzymes in *Arabidopsis* (Jianmin Zhao, personal communication).

The binary vector system described has been successfully to introduce a number of genes into plants(27).

요 약

*Arabidopsis thaliana*의 trp1 변이 식물체는 UV 하에서 푸른 형광 빛을 발하며, phosphoribosyl anthranilate transferase를 encoding하는 유전자가 결여되어 있다. 이 유전자를 PAT1이라고 하며, 많은 미생물에서 phosphoribosyl anthranilate transferase와 homologous하다. 트립토판 생합성에서 이 효소가 결여되면 anthranilate가 축적되며 형광 빛을 발하게 된다. PAT1의 유전자 조절을 알아보기 위하여, PAT1 유전자의 promoter를 단계별로 삭제하여 트립토판 변이 식물체의 형질전환과 재분화를 시도하였다. 이러한 유전자 조작을 통하여 이 유전자의 발현 양상을 조절하는 promoter 요소와 작용을 확인할 수 있으리라고 생각된다. 또한 PAT의 항체를 사용한 immunoassay를 통하여 형질전환체의 단백질 양의 변화를 분석한 결과 PAT1의 완전한 promoter를 가진 pHS107의 형질전환체는 대조구보다 2배의 단백질 양을 나타냄으로서 2쌍의 PAT 유전자가 발현되었음을 알 수 있었다. PAT1은 selection marker와 reporter 유전자로서 분자유전학연구에 공헌할 수 있으리라고 생각된다.

ACKNOWLEDGEMENT

This work was supported in part by the professor Training program of the Korean Government(1993) and a grant by the Genetic Engineering Research program(1994) from the Ministry of Education.

참고문헌

1. F. Laibach(1943), *Bot. Arch.*, **44**, 439.
2. G. P. Redei(1975), *Ann. Rev. Genet.*, **9**, 111.
3. M. Koornneef, J. Van Eden, C. J. Hanhart, P. Stam, F. J. Braaksma and W. J. Feenstra (1983). *J. Hered.*, **74**, 265.
4. M. A. Estelle and C. R. Somerville(1986), *Trands Genet.*, **2**, 69.
5. D. W. Meinke(1986), *Oxford surv. Plant Mol. Cell Biol.*, **3**, 122.
6. E. M. Meyerowitz(1987), *Ann. Rev. Genet.*, **21**, 93.
6. J. K. Bryan(1990), In B. J. Miflin, P. J. Lea, eds, The Biochemistry of Plants, Vol. 16. Academic Press, New York, 161.
7. J. L. Bowman, M. F. Yanofski and E. M. Meyerpowitz(1988), *Oxford Surv. Plant Mol. Cell. Biol.*, **5**, 57.
8. M. Koornneef(1983). *Genetica*, **66**. 33.
9. M. Koornneef and J. H. van der Veen(1983), *Genetica*, **61**, 41.
10. M. Koornneef(1987), In "Genetic Maps 1987: A Compilation of Linkage and Restriction Maps of Genetically Studied Organisms" (S. J. O'Brian,, ed.), pp. 742-745. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

11. E. M. Meyerowitz(1989), *Cell,* **56**, 263.
12. L. S. Leutwiler, B. R. Hough-Evans and E. M. Meyerowitz(1984), *Mol. Gen. Genet.,* **194**, 15.
13. R. L. Last and G. R. Fink(1988), *Science,* **240**, 305.
14. R. L. Last, P. H. Bissinger, D. J. Mahoney, E. R. Radwanski and G. R. Fink(1991), *Plant Cell,* **3**, 345.
15. A. D. Wright, M. B. Sampson, M. G. Neuffer, L. Michalczuk, K. P. Slovin and J. D. Chohen (1991), *Science,* **254**, 998.
16. J. Tsuji, E. P. Jackson, D. A. Gage, R. Hammerschmidt and S. C. Somerville (1992), *Plant Physiol.,* **98**, 1304.
17. G. W. Haughn, L. Davin, N. Giblin and E. W. Underhill(1991), *Plant Physiol.,* **97**, 217.
18. J. K. Bryan(1990), In B. J. Miflin, P. J. Lea, eds, The Biochemistry of Plants, Vol. 6. Academic Press, New York, 161.
19. B. K. Singh, D. L. Siehl and J. A. Connelly (1991), *In* B. J. Miflin, ed, Oxford Surveys of Plant Molecular and Cell Biology, Vol7. Oxford University Press, Oxfprd, UK, 143.
20. T. Caspar, S. C. Huber and C. Somerville (1985), *Plant Physiol.,* **79**, 11.
21. A. B. Rose, A. L. Casselman and R. L. Last (1992), *Plant Physiol.,* **100**, 582.
22. D. Valvekens, M. Van Lijsebettens and M. Van Montagu(1988), *Proc. Natl. Acad. Sci. U. S. A.,* **85**, 5536.
23. B. Damm and L. Willmitzer(1988), *Mol. Gen. Genet.,* **213**, 15.
24. T. C. Stasinopoulos and R. P. Hangarter (1990), *Plant Physiol.,* **93**, 1365.
25. G. W. Haughan, C. R. Somorville(1986), *Mol. Gen. Genet.,* **204**, 430.
26. R. T. Fraley, S. G. Rogers, R. B. Horsch, P. R. Sanders, J. S. Flick, S. P. Adams, M. L. Bittner, L. A. Brand, C. L. Fink, K. S. Fry, G. R. Galluppi, S. B. Goldberg, N. L. Hoffmann and S. C. Woo(1983), *Proc. Natl. Acad. Sci. U.S.A.,* **80**, 4803.
27. A. M. Lloyd, A. R. Barnason, S. G. Rogers, M. C. Byrne, R. T. Fraley and R. B. Horsch (1986), *Science,* **234**, 464.
28. S. N. Sheikholeslam and D. P. Weeks(1987), *Plant Mol. Biol.,* **8**, 291.
29. M. R. Capecchi(1989), *Trands Genet.,* **5**, 70.