Plant Physiology, April 2000, Vol. 122, pp. 1003–1013, www.plantphysiol.org © 2000 American Society of Plant Physiologists

Breakthrough Technologies

Activation Tagging in Arabidopsis[1]

Detlef Weigel*, Ji Hoon Ahn[2], Miguel A. Blázquez[2], Justin O. Borevitz[2], Sioux K. Christensen[2], Christian Fankhauser[2], Cristina Ferrándiz[2], Igor Kardailsky[2,3], Elizabeth J. Malancharuvil[2], Michael M. Neff,[2,4] Jasmine Thuy Nguyen[2,5], Shusei Sato[2], Zhi-Yong Wang[2], Yiji Xia[2], Richard A. Dixon, Maria J. Harrison, Chris J. Lamb[6], Martin F. Yanofsky, and Joanne Chory

Plant Biology Laboratory (D.W., J.H.A., M.A.B., J.O.B., S.K.C., C.Fankhauser, I.K., E.J.M., M.M.N., J.T.N., Z.-Y.W., Y.X., C.J.L., J.C.) and Howard Hughes Medical Institute (J.C.), The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037; Department of Biology, University of California at San Diego, La Jolla, California 92093–0116 (C.Ferrándiz, S.S., M.F.Y.); and Plant Biology Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, Oklahoma 73402 (I.K., Y.X., R.A.D., M.J.H.)

Activation tagging using T-DNA vectors that contain multimerized transcriptional enhancers from the cauliflower mosaic virus (CaMV) 35S gene has been applied to Arabidopsis plants. New activation-tagging vectors that confer resistance to the antibiotic kanamycin or the herbicide glufosinate have been used to generate several tens of thousands of transformed plants. From these, over 30 dominant mutants with various phenotypes have been isolated. Analysis of a subset of mutants has shown that overexpressed genes are almost always found immediately adjacent to the inserted CaMV 35S enhancers, at distances ranging from 380 bp to 3.6 kb. In at least one case, the CaMV 35S enhancers led primarily to an enhancement of the endogenous expression pattern rather than to constitutive ectopic expression, suggesting that the CaMV 35S enhancers used here act differently than the complete CaMV 35S promoter. This has important implications for the spectrum of genes that will be discovered by this method.

The primary tool for dissecting a genetic pathway is the screen for loss-of-function mutations that disrupt such a pathway. However, a limitation of loss-of-function screens is that they rarely identify genes that act redundantly. The problem of functional redundancy has become particularly apparent during the past few years, as sequencing of eukaryotic genomes has revealed the existence of many duplicated genes that are very similar both in their coding regions and their non-coding, regulatory regions. A second class of genes whose entire function is difficult to identify with conventional mutagens, which primarily induce loss-of-function mutations, are those that are required during multiple stages of the life cycle and whose loss of function results in early embryonic or in gametophytic lethality.

Genes that are not absolutely required for a certain pathway can still be identified through mutant alleles, if such genes are sufficient to activate that pathway. Similarly, genes that are essential for early survival might be identified through mutant alleles if ectopic activation of the pathways they regulate is compatible with survival of the organism. The key in either case is the availability of gain-of-function mutations.

An example of the first case is the ethylene response pathway in Arabidopsis. While dominant, gain-of-function mutations in any of several His kinase genes result in constitutive repression of the ethylene response, loss-of-function mutations in individual genes cause no apparent phenotype. However, the combination of multiple loss-of-function mutations leads to progressive activation of constitutive ethylene response (Hua and Meyerowitz, 1998). An example of the second case is provided by the *Drosophila* homeotic gene *Antennapedia* (*Antp*), whose normal function is to promote the formation of thoracic segments and whose inactivation results in embryonic lethality (Denell et al., 1981). However, *Antp* was originally identified through gain-of-function mutations associated with the transforma-

[1] This work was supported by grants from the National Science Foundation (no. MCB–9723823 to D.W., no. IBN–9728402 to M.F.Y., and no. MCB–9631390 to J.C.), the National Institutes of Health (no. R01 GM52413 to J.C.), and by the Samuel Roberts Noble Foundation. This work was also supported by a Research Experience for Undergraduates Supplement to National Science Foundation grant (no. IBN–9406948 to D.W.), and fellowships from the Korea Science and Engineering Foundation and the Hoffman Foundation (J.H.A.), the Spanish Ministry of Education (M.A.B., C.Ferrándiz), the National Science Foundation (S.K.C., Z.-Y.W.), the Human Frontiers Science Program Organization (M.A.B., C.Fa.), the Swiss National Science Foundation (C.Fankhauser), the National Institutes of Health (M.M.N.), and the Kazusa DNA Research Foundation (S.S.). D.W. was a National Science Foundation Young Investigator and J.C. was an Associate Investigator of the Howard Hughes Medical Institute.

[2] These authors contributed equally to this study and are listed alphabetically.

[3] Present address: Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710.

[4] Present address: Department of Biology, Washington University, One Brookings Drive, St. Louis, MO 63130.

[5] Present address: Akkadix Corporation, 11099 North Torrey Pines Road, La Jolla, CA 92037.

[6] Present address: John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

* Corresponding author; e-mail weigel@salk.edu; fax 858–558–6379.

tion of antenna into leg in the adult fly due to ectopic expression of a normal protein product (Gehring, 1967).

Gain-of-function phenotypes can either be caused by mutations in the coding region that lead to constitutive activation of the resulting protein, as in dominant ethylene response mutants (Chang et al., 1993), or by mutations that alter levels or patterns of gene expression, as in dominant *Antp* mutants (Schneuwly et al., 1987). The traditional way to induce the latter type of mutation has been through chromosomal rearrangements or transposons that bring genes under the control of new promoters or enhancers (e.g. Chadwick et al., 1990; Smith et al., 1992; Miller et al., 1993; Kluppel et al., 1997; Brunner et al., 1999). A few years ago, a more directed way to induce such mutations was developed by Walden and colleagues (Hayashi et al., 1992), who constructed a T-DNA vector with four copies of an enhancer element from the constitutively active promoter of the cauliflower mosaic virus (CaMV) 35S gene (Odell et al., 1985). These enhancers can cause transcriptional activation of nearby genes, and, because activated genes will be associated with a T-DNA insertion, this approach has become known as activation tagging. The original activation-tagging vector has been used in tissue culture to identify a His kinase from Arabidopsis, whose overexpression can bypass the requirement for cytokinin in the regeneration of shoots (Kakimoto, 1996). A related approach, with a complete CaMV 35S promoter pointing outward from a transposable *Ds* element, has been used to identify dominant mutations at the Arabidopsis loci *TINY*, *LATE ELONGATED HYPOCOTYL* (*LHY*), and *SHORT INTERNODES* (*SHI*) (Wilson et al., 1996; Schaffer et al., 1998; Fridborg et al., 1999).

The past few years have seen dramatic improvements in our ability to generate large numbers of independently transformed Arabidopsis plants (Bechtold et al., 1993; Bechtold and Pelletier, 1998). We have taken advantage of this improvement, and have generated several large sets of Arabidopsis plants transformed with activation-tagging vectors containing CaMV 35S enhancers. The initial analysis of a subset of activation-tagged mutants has revealed several important features of this approach. We have found, for example, that the majority of overexpressed genes are immediately adjacent to the enhancers. Furthermore, in at least one case, the CaMV 35S enhancers primarily enhanced an endogenous expression pattern and did not induce constitutive ectopic expression.

MATERIALS AND METHODS

Construction of pSKI015 and pSKI074

pSKI015

A *Bgl*II/*Pst*I fragment of plasmid pSLJ2011 (Jones et al., 1992) containing a CaMV 35S promoter/glufosinate resistance (BAR)/3′ octopine synthase (*ocs*) cassette was subcloned into the *Bam*HI/*Pst*I sites of pBluescriptKS(+) (Stratagene, La Jolla, CA), resulting in pSKI001. Removal of the internal *Bam*HI site after the stop codon of the BAR gene resulted in pSKI003. The *Xho*I/*Pst*I fragment of pSKI003 containing a cassette consisting of omega translational enhancer (Ω)/BAR/3′ *ocs* was inserted downstream of the mannopine synthase (*mas*) promoter in the pCGN1547 binary vector (McBride and Summerfelt, 1990). This replaced the kanamycin resistance (*npt*II) open reading frame and 3′ *mas* sequences of pCGN1547 and yielded the binary T-DNA vector pSKI006. The 5′ *mas*/Ω/BAR/3′ *ocs* cassette of pSKI006 was subcloned into pBluescript KS(+), generating pSKI030. pSKI030 was linearized with *Bam*HI and *Hin*dIII, and ligated to the *Bam*HI/*Hin*dIII fragment of pPCVICEn4HPT (Hayashi et al., 1992), which contains the tetramerized CaMV 35S enhancer sequences, T-DNA right border, *oriV*, and T-DNA left border, yielding pSKI015.

pSKI074

The 5′ *mas*/*npt*II/3′ *mas* cassette of pCGN1547 was subcloned into pBluescript KS(+) as an *Xho*I fragment, resulting in pSKI073. pSKI073 was linearized with *Bam*HI and *Hin*dIII and ligated to the *Bam*HI/*Hin*dIII fragment of pPCVICEn4HPT.

pSKI015 and pSKI074 were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90RK), the host recommended for pPCVICEn4HPT (Hayashi et al., 1992).

Construction of pMN19 and pMN20

Sequencing of the pBAS2K plasmid rescued from the *bas2-1D* mutant, which had been generated with the pSKI074 vector, revealed a rearrangement that included insertion of about 650 bp from near the left T-DNA border of pSKI074 to the right of the partially deleted right T-DNA border. The inserted sequence included restriction sites that allowed excision of a 2-kb *Sac*I fragment spanning the tetramerized CaMV 35S enhancers along with the 5′ *mas* sequences. This fragment was inserted into the binary vector pPZP212 (Hajdukiewicz et al., 1994) in two different orientations, yielding pMN19 and pMN20. Two derivatives, pMN19-2 and pMN20-2, have lost two CaMV 35S enhancer copies through recombination in *Escherichia coli*.

Oligonucleotide Primers for PCR and Sequencing

The following oligonucleotide primers were used: T3, 5′ AAT TAA CCC TCA CTA AAG GG 3′; T7, 5′ TAA TAC GAC TCA CTA TAG GG 3′; M13 (−20), 5′ GTA AAA CGA CGG CCA GT 3′; IK007, 5′ ACC CGC CAA TAT ATC CTG 3′; IK054, 5′ ATG TGA TAT CTA GAT CCG AAA C 3′; SKI015 RB, 5′ AGA TCC GAA ACT ATC AGT G 3′; SKI015 RI, 5′ GCA AGA ACG GAA TGC GCG 3′; SKI015 Kpn, 5′ CTG GCA GCT GGA CTT CAG CCT G 3′; SKC12, 5′ TTG ACA GTG ACG ACA AAT CG 3′; and SKI074 H3, 5′ GCT CTC TCG AGG TCG ACG G 3′.

CaMV 35S Enhancers

The four CaMV 35S enhancer repeats in the activation-tagging vectors are unstable in *E. coli* and *A. tumefaciens* if stored at 4°C for extended time. We therefore used PCR

with IK007, a primer derived from the right T-DNA border, and a T7 or M13 (−20) primer to verify that the enhancer tetramer was intact in plasmids used for plant transformation. PCR with these two primers amplified a DNA fragment of 1.46 kb in the intact vectors. Note that IK007 will normally not work with transgenic plants, as the right border sequence is rarely completely transferred.

Right T-DNA Border

The sequence found between the outermost CaMV 35S enhancer and the end of the T-DNA (with the right border sequence underlined) is 5′ GAA ACT ATC AGT GTT TGA CAG GAT ATA TTG GCG GGT AAA C 3′. The base pairs preceding this sequence are not unique and are repeated downstream of the other CaMV 35S enhancers. Because of imprecise transfer of right border sequences, there was often no unique sequence at the right end of the T-DNA insertion, which made it impossible to sequence into plant DNA sequences from this end of the T-DNA. Depending on how much unique sequence is transferred, primers IK054 or SKI015 RB can be used, even though only the last five or 13 bases, respectively, hybridize to unique sequence. IK054 has worked, for example, in the *esc-1D* mutant.

Left End of Rescued Plasmids

In plasmids that had been rescued across the right T-DNA border, the following primers were used for sequencing: For mutants generated with pSKI015: T3 or SKI015 RB (from inside the *Kpn*I site), SKI015 RI (from inside the *Eco*RI site), and SKC12 (from inside the *Hin*dIII or *Pst*I sites). For mutants generated with pSKI074: T3 or SKI015 RB (from inside the *Kpn*I site) and SKI074 H3 (from inside the *Hin*dIII site).

Plant Material and Growth

Arabidopsis plants were grown at 23°C in long-day conditions (16 h of light and 8 h of dark) under a mixture of 3:1 cool-white:Gro-Lux fluorescent lights (Osram Sylvania, Danvers, MA). Our Columbia wild-type strain is available from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) as Col-7, as are pools of Col-7 seeds transformed with activation-tagging vector pSKI015. Mutants used for suppressor screens were: *phyB-4* (Koornneef et al., 1980; Reed et al., 1993), *phyA-211* (Reed et al., 1994), *det2-1* (Chory et al., 1991; Li et al., 1996), and *ga1-3* (Koornneef and van der Veen, 1980; Sun and Kamiya, 1994).

Plant Transformation and Selection

The presence of all four CaMV 35S enhancers was verified in individual bacterial colonies by PCR (see above). Bacterial cultures for plant transformation were always started from a fresh inoculum taken out of −80°C storage.

Most plants were transformed using modified versions of the vacuum-infiltration method (Bechtold et al., 1993; Bechtold and Pelletier, 1998). Some transgenic plants were generated with a spray method. *A. tumefaciens* was grown to the stationary phase, harvested, and resuspended in 2 volumes of water with 0.2% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX). The *A. tumefaciens* suspension was applied to flowering Arabidopsis plants at weekly intervals with a spray bottle. After spraying, plants were kept in covered flats for 1 d. Transformation efficiency was normally 2% of harvested seeds for both methods.

Seeds from plants infiltrated with pSKI074 were sown on Murashige and Skoog plates (Murashige and Skoog, 1962) containing 50 μg/mL kanamycin, stratified at 4°C for 2 d, and grown for about 1 week before transplanting resistant plants to soil. Seeds from plants infiltrated with pSKI015 were mixed with 0.1% (w/v) Phytagar (Gibco, Rockville, MD), incubated at 4°C for 2 d for stratification, and sown directly on soil. Commercially available Finale (AgrEvo, Montvale, NJ), which contains 5.78% (w/v) ammonium glufosinate (and is also known as Basta) was diluted 1:1,000, and herbicide-resistant plants were selected by spraying twice a week for 3 weeks.

Plasmid Rescue

The plasmid sequences in pSKI015 and pSKI074 are flanked by several restriction enzyme sites that can be used for rescue of T-DNA and adjacent plant sequences from transformed plants. The restriction enzymes *Kpn*I, *Eco*RI, and *Hin*dIII (and *Pst*I for pSKI015 only) can be used for rescue of sequences adjacent to the right T-DNA border, and *Bam*HI, *Spe*I, and *Not*I can be used for left border rescue.

For plasmid rescue, 1 g of plant tissue was harvested and genomic DNA was prepared with a DNA extraction kit (Phytopure, Nucleon Biosciences, Glasgow, UK). The extracted DNA was resuspended in 300 μL of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0). After extracting purified DNA twice with phenol-chloroform, 10 μL of genomic DNA was digested overnight with the appropriate restriction enzyme in a 50-μL reaction. After phenol-chloroform extraction, samples were ligated overnight at 14°C in a total volume of 250 μL. Ligated DNA was precipitated, and one-fifth was transformed by electroporation into recombination-deficient *E. coli* SURE cells (Stratagene), to maximize stability of the multimerized CaMV 35S enhancers.

RESULTS

New Activation-Tagging Vectors

The original activation-tagging T-DNA vector, pPCVICEn4HPT, confers resistance to the antibiotic hygromycin (Hayashi et al., 1992). Because we found hygromycin selection of Arabidopsis seedlings difficult, and because of hygromycin's toxicity to humans, we developed two new activation-tagging vectors. pSKI015 (Fig. 1A) confers resistance to the herbicide glufosinate and is most useful for selection of transgenic plants on soil. pSKI074 (Fig. 1A) confers resistance to the antibiotic kanamycin and is most useful for selection of transgenic seedlings on artificial

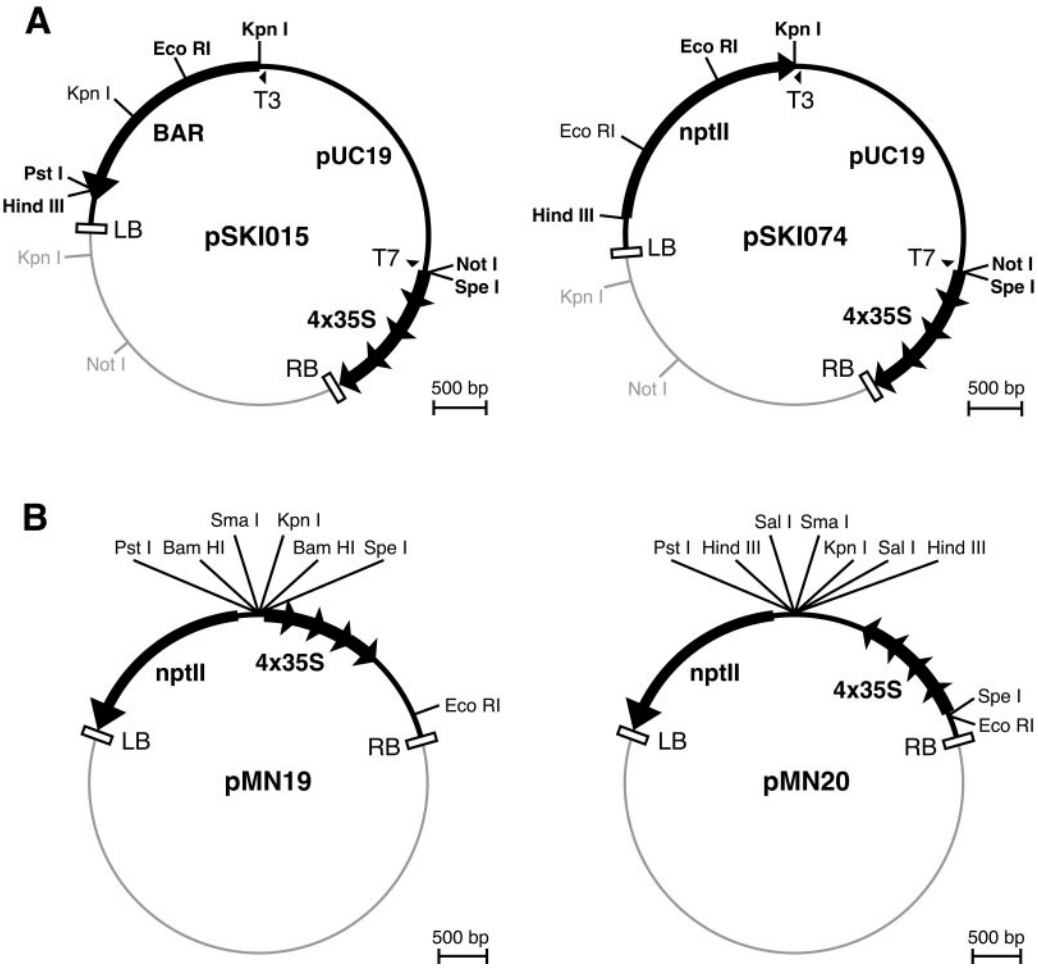

Figure 1. Vector diagrams. A, Activation-tagging vectors pSKI015 and pSKI074. Restriction enzyme sites in bold indicate limit of T-DNA internal sequences after plasmid rescue. The complete sequences of pSKI015 and pSKI074 have been deposited in GenBank (accession nos. AF187951 and AF218466). pSKI015 and pSKI074 are identical except for the *Kpn*I/*Hin*dIII fragment that contains the selection marker including promoter and transcriptional terminator sequences. pUC19, pUC19 sequences; BAR, Basta selection marker; nptII, kanamycin selection marker; T3, T3 RNA polymerase promoter; T7, T7 RNA polymerase promoter; LB, left T-DNA border; RB, right T-DNA border. B, pMN19 and pMN20 vectors used for recapitulation of mutant phenotypes.

substrates such as Murashige and Skoog (1962) medium. The CaMV 35S enhancers in these vectors correspond to nucleotides −417 to −86 relative to the transcription start (Guilley et al., 1982; Odell et al., 1985; Kay et al., 1987; Ow et al., 1987; Benfey and Chua, 1989; Fang et al., 1989). The T-DNA in pSKI015 and pSKI074 contains pUC19 sequences (Yanisch-Perron et al., 1985) with a bacterial origin of replication and an ampicillin resistance gene for plasmid rescue.

Storage at 4°C of *A. tumefaciens* carrying the activation-tagging vectors led to progressive loss of the CaMV 35S enhancer copies, since *A. tumefaciens* is recombination proficient. After 1 to 2 weeks at 4°C, the activation-tagging vectors had lost on average one copy of the CaMV 35S enhancers, with only a single copy remaining after 1 month. Therefore, the number of CaMV 35S enhancer copies in individual bacterial colonies was determined by PCR with flanking oligonucleotide primers (see "Materials and Methods").

Generation of Activation-Tagged Mutants

We used the vacuum-infiltration method and a spray method to generate several sets of transgenic plants with pPCVICEn4HPT and with new activation-tagging vectors (Table I). Two sets were in a wild-type Columbia background, and four sets were in *phytochrome A-211* (*phyA-211*) (Columbia), *phytochrome B-4* (*phyB-4*) (Columbia), *deetiolated 2-1* (*det2-1*) (Columbia), or *gibberellin deficient 1-3* (*ga1-3*) (Landsberg *erecta*) mutant backgrounds. Primary transformants generated by vacuum infiltration of *Agrobacterium tumefaciens* are non-chimeric and hemizygous for T-DNA insertions (Bechtold et al., 1993; Bechtold and Pelletier, 1998; Ye et al., 1999). We therefore screened primary transformants after selection for antibiotic or herbicide resistance directly for mutant phenotypes, since dominant mutations, as expected for activation tagging, will be already apparent in the hemizygous state. Certain phenotypes such as sterility, reduced apical dominance or pale-

Table I. *Activation-tagging screens*

Screen	Strain	Vector	No. of Transformants	Phenotype	Confirmed Dominant Mutants
1	Columbia	pPCVICEn4HPT, pSKI015, and pSKI074	25,000	Flowering time; leaf, shoot, flower, and fruit morphology	23
2	Columbia	pSKI015	5,000	Disease resistance; pigmentation	3
3	*phyB-4*	pSKI074	3,000	Hypocotyl length	3
4	*phyA-211*	pSKI074	3,000	Hypocotyl length	1
5	*det2-1*	pSKI074 and Derivatives	10,000	Plant size	1
6	*ga1-3*	pSKI015	3,000	Flowering time	3

ness were very frequent among primary transformants (about 1 in 100).

Primary transformants with abnormal morphological phenotypes were either allowed to self-pollinate, or, if self-sterile, backcrossed to the untransformed parental strain, and progeny was tested for heritability of the phenotype. The original phenotype reappeared in the progeny of about half of all putative mutants that we pursued further, while the progeny of the other half appeared wild type. Two examples of dominant, heritable mutants with adult defects, *escarola-1D* (*esc-1D*) and *jaw-1D*, are shown in Figure 2. *esc-1D* mutants flowered late in long days, with an average of 27 leaves as compared to 13 leaves in wild type. Their leaves were wavy instead of flat; and darker green, larger, and rounder than those of wild type. Both leaf petioles and stem internodes were shorter than those of wild type. *jaw-1D* plants had deeply serrated leaves, and the severity of the leaf phenotype increased in an acropetal fashion. This phenotype was reminiscent of that seen in transgenic plants that overexpress the KNAT1 homeo domain protein or the UFO F box protein (Lincoln et al., 1994; Lee et al., 1997). *jaw-1D* mutants were slightly late flowering, with an average delay of five leaves in long days. Similarly to those of *35S::KNAT1* plants, *jaw-1D* petals were light green.

Both *esc-1D* and *jaw-1D* were completely dominant, whereas other mutants, such as *constitutive disease resistance 1-1D* (*cdr1-1D*) (Table II), were semidominant. The overall frequency of heritable, dominant mutations varied for different phenotypic criteria. Because of the large population screened, we mostly pursued plants with particularly conspicuous phenotypes. In our first screen of approximately 25,000 Columbia plants, we confirmed 23 dominant mutants with dramatic morphological phenotypes (Tables II and III). Interestingly, the frequency of suppressor mutations (Table I) found in *phyB-4* and *ga1-3* backgrounds was on the same order of magnitude as those of ethyl methanesulfonate-induced suppressors of *phyB* (Reed et al., 1998) or *ga1* (M.A. Blázquez and D. Weigel, unpublished data). On the other hand, we failed to recover certain classes of mutants in the Columbia background, such as floral homeotic mutants.

Structure of Insertion Sites

To determine whether the mutant phenotypes were caused by overexpression of adjacent genes, we characterized the T-DNA insertions in several lines in which the selectable marker and the mutant phenotype cosegregated (Table II). First, we digested genomic DNA isolated from the mutants with the restriction enzymes available for plasmid rescue in the activation-tagging vectors. Southern-blot hybridization with a vector probe identified restriction enzymes that cut plant DNA within less than 20 kb from the T-DNA insertion. Plant sequences adjacent to the T-DNA insertions were recovered by plasmid rescue and analyzed by restriction mapping, DNA sequencing, and comparison with the genomic sequence in GenBank. The extent of plant sequences isolated by plasmid rescue ranged from a few hundred base pairs to over 10 kb.

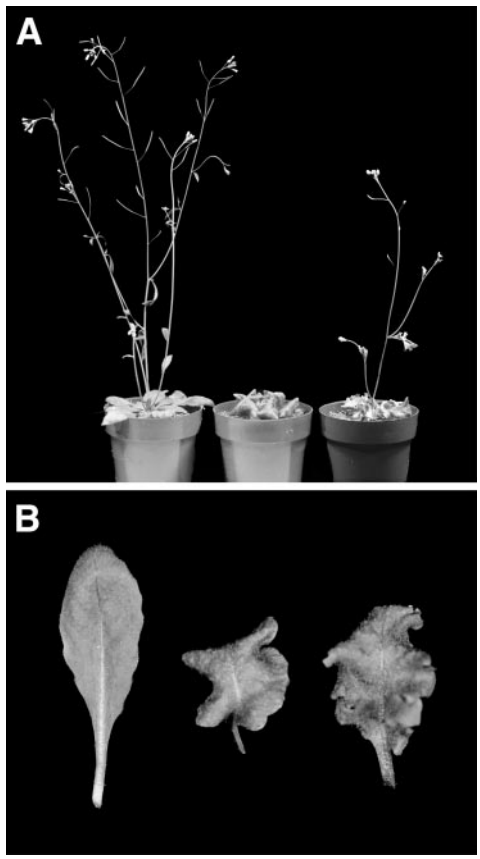

Figure 2. Two examples of dominant, activation-tagged mutants. A, Adult phenotype of *jaw-1D* (right) and *esc-1D* (center) compared with that of wild type (left). Plants were grown under long-day conditions for 5 weeks. B, Upper surface of an adult rosette leaf of *jaw-1D* (right) and *esc-1D* (center) compared with that of wild type (left).

Table II. *Activation-tagged mutants that have been characterized molecularly*

See Table I for definition of screens.

Screen	Mutant	Phenotype	Overexpressed Gene[a]	Recapitulation of Phenotype		
				Mutant DNA[b]	35S::cDNA/35S::gDNA[c]	Enhancer vector[d]
1	*ft-9D*	Early flowering	Y	+[e]	+	n/d[f]
1	*esc-1D*	Late flowering; leaf growth	Y	n/d	+	+
1	*jaw-1D*	Late flowering; leaf growth	Y	n/d	n/d	+
1	*jaw-2D*	Late flowering; leaf growth	Y	n/d	n/d	see *jaw-1D*
1	*yuc-1D*	Elongated hypocotyl and internodes	Y	See *yuc-2D*	n/d	n/d
1	*top1-1D*	Abnormal silique	Y	n/d	+	n/d
1	*lab1-1D*	Late flowering	Y	−[g]	n/d	n/d
1	*jba-1D*	Enlarged shoot meristem; abnormal leaf polarity	N	n/d	n/d	n/d
2	*cdr1-1D*	Disease resistant	Y	+	+	n/d
2	*pap1-1D*	Increased anthocyanin production	Y	n/d	+	+
2	*cds1-1D*	Disease sensitive	Y	n/d	n/d	n/d
3	*bas1-1D*	Short hypocotyl	Y	+	+	n/d
3	*bas2-1D*	Short hypocotyl	Y	+	n/d	n/d
3	*bas3-1D*	Short hypocotyl	Y	n/d	n/d	n/d
4	*yuc-2D*	Elongated hypocotyl and internodes	Y	+	n/d	n/d

[a] Y, Detected next to the right T-DNA border; N, was not detected. [b] Plants were retransformed with plant DNA and adjacent CaMV 35S enhancers rescued from the original mutant. [c] Transformation with cDNA (all except top1-1d) or genomic DNA spanning the overexpressed transcription unit fused to the CaMV promotor. [d] Transformation with genomic DNA cloned into pMN19 or pMN20 containing CaMV 35S enhancers (see Fig. 1B). [e] +, Confirmed. [f] n/d, Not determined. [g] −, Not confirmed.

We found it useful to rescue, when possible, both short and long genomic fragments. The activation-tagging vectors contain only a very short stretch of unique sequence between the right T-DNA border, which is commonly lost upon integration, and the repeated CaMV 35S enhancers. Whenever there was no unique vector sequence at the right end of the T-DNA insertion, it was impossible to sequence into plant DNA from this end. In contrast, during plasmid rescue, the distal end of plant DNA is ligated to T-DNA internal sequences, which do not vary. One can therefore use T-DNA internal primers to sequence easily into plant DNA from this end. If a rescued plasmid contains only a short stretch of plant DNA, one can rapidly sequence through the plant DNA into CaMV 35S enhancer sequences, thereby quickly determining the exact insertion point of the T-DNA.

The structures of 14 different insertion sites are schematically shown in Figure 3. In all cases shown, we found an overexpressed gene, as determined by RNA-blot hybridization, immediately adjacent to the multimerized CaMV 35S enhancers. In a few cases, including *jaw-1D*, *esc-1D*, and *late bloomer 1-1D* (*lab1-1D*), we also analyzed the expression levels of the gene distal to the overexpressed gene. In neither case was the distal gene overexpressed as well.

The distance between the insertion point and the overexpressed gene ranged from 380 bp to 3.6 kb. The CaMV 35S enhancers were found to be inserted both upstream and downstream of overexpressed genes, confirming that the CaMV 35S sequences in the activation-tagging vectors acted as true enhancers (Fang et al., 1989). Only in one case, *jabba-1D* (*jba-1D*) (Table II), which had a single insertion upstream of position 57,615 of P1 clone MBK5 (accession no. AB005234), did we fail to detect an overexpressed gene within 10 kb of the right border. We detected an overexpressed gene next to the left border in *jba-1D*; however, whether this gene caused the *jba-1D* phenotype has not been determined.

In contrast to T-DNA insertions reported for many loss-of-function mutants (Feldmann, 1991), the T-DNA insertions in activation-tagged mutants were never rearranged in such a way that it was difficult to recover adjacent plant DNA by plasmid rescue or to determine the structure of T-DNA insertions. This probably reflects the fact that an activation-tagged phenotype requires productive interaction of enhancer sequences on the T-DNA with adjacent plant sequences. In *phyB activation-tagged suppressor 2-1D* (*bas2-1D*) (Table II), about 650 bp from near the left T-DNA border of pSKI074 had been transposed to the right of the partially deleted right T-DNA border, but apparently did not interfere with activation of *BAS2* on the other side of the transposed DNA. In *esc-1D*, *lab1-1D*, and *constitutive disease susceptibility 1-1D* (*cds1-1D*) (Table II), two T-DNAs were inserted at the same locus, with both CaMV 35S enhancer tetramers pointing outward. Although these mutants contained two enhancer tetramers at the same locus, we detected an overexpressed gene next to only one CaMV 35S enhancer tetramer.

We found two pairs of mutants with similar phenotypes, and subsequent analysis showed each pair to be allelic. The insertions in *yucca-1D* (*yuc-1D*) and *yuc-2D* (Table II) were 2.1 kb apart, with no other gene predicted in the genomic interval between the two insertions, and the same gene was overexpressed in both mutants (Fig. 3). The same was true for *jaw-1D* and *jaw-2D*, whose T-DNA insertions were sep-

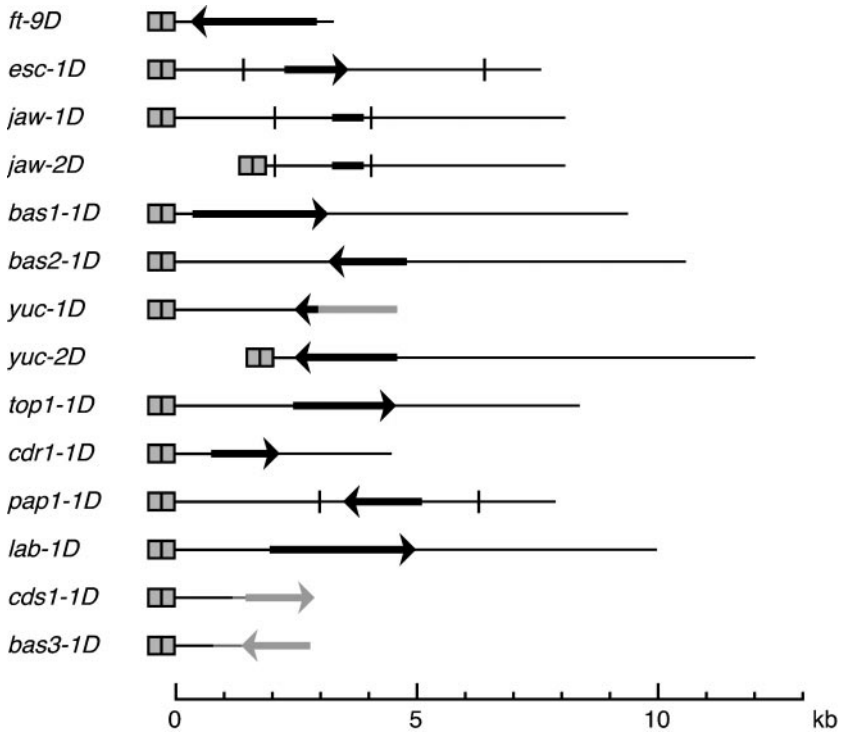

Figure 3. Activation-tagged loci. The extent of the largest plasmid rescued from each mutant is shown as a black line, with rectangles indicating the CaMV 35S enhancers. The overexpressed gene is indicated, with an arrow pointing in the direction of transcription. A cDNA for *jaw-1D* and *jaw-2D* has not been identified, and the direction of transcription is unknown. For *esc-1D*, *jaw-1D* and *jaw-2D*, and *pap-1D*, vertical lines delimit the extent of the genomic fragment cloned into pMN19 or pMN20–2 for recapitulation of the phenotype. The genomic positions of the *jaw-1D*, *jaw-2D*, and *lab1-1D* (as well as *jba-1D*) insertions are described in the text, and the *ft-9D* insertion has been published previously (Kardailsky et al., 1999). The right border of the T-DNA insertion in *esc-1D* is downstream of position 77,719 in BAC F9H16 (GenBank accession no. AC007369), and the overexpressed gene is F9H16.12, which encodes a protein with homology to AT-hook proteins (Aravind and Landsman, 1998). The insertions at the other loci will be described in detail elsewhere.

arated by 1.6 kb of genomic DNA without any other gene in the intervening sequence (Fig. 3). *JAW* RNA was more highly overexpressed in *jaw-2D*, in which the CaMV 35S enhancers were closer to the *JAW* gene, than in *jaw-1D* (Fig. 4). The exact distance of the T-DNA insertion to the activated *JAW* gene has not yet been determined, as a *JAW* cDNA has not been isolated. The *JAW* gene, which gives rise to an RNA of approximately 0.55 kb, is contained between nucleotides 69,528 and 71,501 of BAC F9D16 (accession no. AL035394). This interval encompasses the genomic fragment that could recapitulate the *jaw* phenotype (see below). Annotation of BAC F9D16 does not predict a gene for this DNA fragment, suggesting that the *JAW* open reading frame is either very short or interrupted by several introns, or that *JAW* does not encode a protein.

Figure 4. Overexpression of *JAW* RNA in *jaw* mutants. A RNA blot with 40 μg of total RNA in each lane was probed with a 2-kb *Pst*I/*Bam*HI fragment (nucleotides 69,528–71,501 of BAC F9D16 [GenBank accession no. AL035394]).

Strategies for Confirmation of Mutant Phenotypes

For nine loci corresponding to 11 mutants, we confirmed that the overexpressed gene caused the mutant phenotype by recapitulating the original phenotype in a new set of transgenic plants. For each locus, we used at least one of three different strategies for recapitulation of mutant phenotypes. In several cases, transgenic plants generated by any of the methods described below produced a phenotypic series, including even stronger phenotypes than the original activation-tagged mutants.

In five cases, we could reproduce the dominant mutant phenotype by retransforming a genomic fragment from the original mutant into wild-type plants (Table II). To this end, mutant DNA spanning both the CaMV 35S enhancer multimer and adjacent plant sequences was cloned into a conventional T-DNA vector. We normally used a fragment containing all of the CaMV 35S enhancers. A fragment containing a single CaMV 35S enhancer was used for *cdr1-1* because of convenient restriction sites, and this was sufficient to recapitulate the original phenotype. The only casein which transformation with the mutant DNA did not recapitulate the phenotype was that of the late-flowering mutant *lab1-1D*, in which the *ANTHOCYANINLESS2* (*ANL2*) gene (Kubo et al., 1999) was overexpressed, caused by insertion of CaMV 35S enhancers approximately 2 kb upstream of the start of the *ANL2* open reading frame. The mutant DNA that we retransformed into wild-type plants extended beyond *ANL2* into the next gene downstream of

Table III. *Activation-tagged mutants that have been confirmed in the second generation but not characterized in detail*

See Table I for definition of screens.

Screen	Mutant	Phenotype
1	yozhik	Narrow leaf blade; partial loss of apical dominance, stunted inflorescence
1	9194	Reduced internode elongation
1	94A2	Narrow, buckled leaves; short inflorescence
1	C983	Dwarf
1	C915	Dwarf
1	G031	Small; reduced internode elongation
1	J305	Reduced internode elongation
1	M004	Small; round, ruffled leaves
1	Q842	Porcupine-like; short inflorescence, loss of apical dominance
1	Q790	Pale; small; loss of apical dominance
1	Q650	Compact, lobed leaves
1	M553	Long, thin rosette leaves
1	X7A0	Small; compact rosette
1	Y004	Leaves rolled upwards; short inflorescence
1	3TB4	Dark green
5	das1	Suppresses dwarf phenotype of *det2-1*
6	gAT10	Suppresses non-flowering of *ga1-3* in short days
6	gAT11	Suppresses non-flowering of *ga1-3* in short days
6	gAT14	Suppresses non-flowering of *ga1-3* in short days

ANL2, a *shaggy* kinase homolog, suggesting that the rescued DNA covered the complete *ANL2* locus.

While retransformation of mutant DNA including CaMV 35S enhancers is the most direct approach, this is not always straightforward, especially when the rescued plasmid does not completely cover both the CaMV 35S enhancer tetramer and the overexpressed gene. Therefore, we designed new T-DNA vectors, pMN19 and pMN20, which contain multiple cloning sites adjacent to the same tetramerized CaMV 35S enhancers as in the activation-tagging vectors (Fig. 1B). Two derivative vectors, pMN19–2 and pMN20–2, contain only two CaMV 35S enhancers, and are useful for moderate levels of overexpression. For three mutants, plant genomic sequences that had been cloned into pMN19 or pMN20–2 and transformed into wild-type plants reproduced the mutant phenotypes (Fig. 3; Table II).

The third strategy, which we used for six mutants, was to introduce either a cDNA or an appropriate genomic fragment under the control of the complete CaMV 35S promoter into wild-type plants (Table II).

Stability of Mutant Phenotypes

We assessed the stability of mutant phenotypes by propagating activation-tagged mutants through multiple generations. Several mutants, including *esc-1D*, *jaw-1D*, *jaw-2D*, and *production of anthocyanin pigment 1-1D* (*pap1-1D*) (Table II), could be established as homozygous lines and stably maintained, with eight being the maximum number of generations observed. However, in other mutants, including *ft-9D*, *tower of Pisa 1-1D* (*top1-1D*) (Table II), gAT10, and gAT14 (Table III), the dominant phenotype became attenuated in subsequent generations.

An interesting case is exemplified by *ft-9D*, since the *FT* loss-of-function phenotype is known to be late flowering (Koornneef et al., 1991). *ft-9D* plants did not become late flowering in subsequent generations, but rather reverted to the wild-type phenotype, indicating that reversion was caused by selective inactivation of the CaMV 35S enhancers and not of the entire *FT* locus. Silencing of CaMV 35S enhancers was not observed in transgenic SKI083 plants that had been retransformed with the *ft-9D* mutant DNA and showed a phenotype that was at least as strong as that of the original mutant. The early-flowering phenotype of these retransformed plants was stable over more than six generations.

Action of CaMV 35S Enhancers

Although recovery of multiple alleles for two different loci, *YUC* and *JAW*, suggests that saturation for activation-tagged mutants can be reached, we did not recover dominant alleles of many other genes known to have an overexpression phenotype. Therefore, we investigated the possibility that the CaMV 35S enhancers used in the activation-tagging vectors did not lead to true constitutive expression. To this end, we compared the *FT* expression profiles in wild-type plants and two different *FT* overexpressers with identical phenotypes. SKI059 plants contain an *FT* cDNA under the control of the complete CaMV 35S promoter, while SKI083 plants had been generated by transforming wild-type plants with genomic DNA of *ft-9D* including the tetramerized CaMV 35S enhancers. Both SKI059 and SKI083 plants produce terminal flowers and

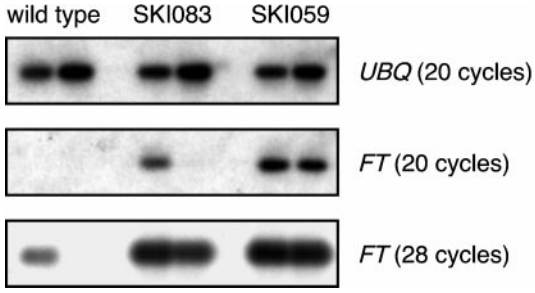

Figure 5. Overexpression of *FT* RNA in the wild type and in two different *FT* overexpressers. SKI083 is a transgenic line that contains genomic DNA from the *ft-9D* mutant, with the same configuration of CaMV 35S enhancers and *FT* DNA as the original mutant. SKI059 contains a fusion of the CaMV 35S promoter to an *FT* cDNA (Kardailsky et al., 1999). RNA was extracted from shoots and hypocotyls (left lanes) or roots (right lanes) of 8-d-old plants. Reverse transcription followed by PCR was performed as described, with a *UBIQUITIN10* (*UBQ*) fragment amplified as a control (Blázquez and Weigel, 1999; Kardailsky et al., 1999). The 28-cycle amplification of *FT* cDNA was performed with the same RT products as the 20-cycle amplification, but PCR, blotting, and hybridizing to an *FT* probe were carried out independently. Seedlings were grown under long-day conditions on Murashige and Skoog (1962) medium.

flower with four leaves, regardless of day length (Kardailsky et al., 1999).

In wild-type seedlings, *FT* RNA is much more abundant in the aerial part of the plant than in the root (Kardailsky et al., 1999; Kobayashi et al., 1999) (Fig. 5). As expected, no such difference was seen in SKI059 seedlings, in which *FT* RNA accumulated at much higher levels than in wild-type plants. In contrast, although overall *FT* RNA levels were also elevated in SKI083 seedlings, these plants showed a marked difference in *FT* RNA abundance between aboveground tissue and roots, as did wild-type plants (Fig. 5), indicating that *FT* was overexpressed in SKI083 plants but not constitutively expressed in all tissues.

DISCUSSION

We used large-scale T-DNA transformation to demonstrate the utility of enhancer activation tagging in Arabidopsis, with the frequency of dominant morphological mutants being at least 1 in 1,000. In a different type of gain-of-function screen using a *Ds* element carrying a CaMV 35S promoter, four dominant mutants were found among 1,100 lines analyzed (Wilson et al., 1996), indicating that the two approaches are largely comparable in their efficiency. We also found that although activation-tagged mutant phenotypes are not always stable, a stable phenotype can be created in transgenic plants retransformed with mutant DNA.

We isolated several interesting mutants in two small screens for mutations that suppress the long-hypocotyl phenotype of hypomorphic *phyB* mutants or the failure of gibberellin-deficient *ga1* null mutants to flower in short days. Signal transduction pathways often involve the relay of positive signals, and disrupting an upstream element will attenuate these positive signals. One can thus reason that overexpression of downstream elements will cause the enhancement of the residual signal flux in an attenuated pathway, resulting in the suppression of a mutation in an upstream element.

Surprisingly, we already found two activation-tagged alleles for two different loci, *YUC* and *JAW*, even though we did not identify dominant alleles for many other genes known to have obvious gain-of-function phenotypes. For example, floral regulators that have strong gain-of-function phenotypes when overexpressed include *CONSTANS*, *LHY*, *CIRCADIAN CLOCK ASSOCIATED 1*, *FLOWER PROMOTING FACTOR 1*, *FT*, *TERMINAL FLOWER 1*, *LEAFY*, and several of the many MADS box genes (Weigel and Nilsson, 1995; Simon et al., 1996; Kania et al., 1997; Riechmann and Meyerowitz, 1997; Ratcliffe et al., 1998; Schaffer et al., 1998; Wang and Tobin, 1998; Kardailsky et al., 1999; Kobayashi et al., 1999; Michaels and Amasino, 1999; Sheldon et al., 1999). Of these, we only recovered a dominant allele for the flowering-time gene *FT*. Similarly, only two early-flowering mutants were identified in a large set of plants generated with the pSKI015 vector by R. Amasino and colleagues (personal communication), one of them being another activation-tagged allele of *FT*. There are several possible explanations for this observation. One is that T-DNA has strong site preferences for insertion, although pronounced site preferences have not been reported for loss-of-function mutations induced by T-DNA (Koncz et al., 1989; Feldmann, 1991). An alternative possibility is that there are factors that limit the interaction of CaMV 35S enhancers with endogenous genes.

There are two lines of evidence for this second hypothesis. First, in three cases in which two activation-tagged alleles at the same locus were found, *YUC*, *JAW*, and *FT*, the CaMV 35S enhancers were inserted on the same side of the gene. Similarly, Kakimoto (1996) identified four CaMV 35S enhancer tagged alleles at the *CYTOKININ INDEPENDENT 1* (*CKI1*) locus, with all four alleles having the CaMV 35S enhancers inserted on the same side of *CKI1*. This seems unusual, as the enhancers were found in different mutants both upstream and downstream of the up-regulated gene, confirming that the CaMV 35S sequences act as true enhancers (Fang et al., 1989). A possible explanation is that the Arabidopsis genome, which is closely packed with genes (Bevan et al., 1998), contains many insulator sequences, which protect genes from the effects of adjacent enhancers or silencers (Chung et al., 1993), and that there are not many places into which CaMV 35S enhancers can insert and activate nearby genes. An alternative or additional possibility is that CaMV 35S enhancers activate only a subset of endogenous promoters because of promoter preference or promoter selectivity, a phenomenon known from other systems (Ohtsuki et al., 1998). Both scenarios are compatible with the observation that in three mutants with two linked T-DNAs, *esc-1D*, *cds1-1D*, and *lab1-1D*, overexpressed genes were found only next to one of the CaMV 35S enhancer tetramers.

Another scenario to explain the apparent selectivity of enhancer activation tagging can be inferred from our observation that in at least one case (*FT*) the CaMV 35S enhancers caused a different mode of up-regulation than the one that was achieved with a complete CaMV 35S promoter driving a *FT* cDNA. In this case, the endogenous expression pattern appeared to be enhanced by the CaMV 35S enhancers, as opposed to true constitutive and ectopic activation. The enhancement of the endogenous expression pattern could be coincidental in this case, since the CaMV 35S enhancer sequences used here (nucleotides −417 to −86 relative to the transcription start) are much more active in shoots than in roots (Benfey and Chua, 1989), similar to what has been observed for the endogenous *FT* gene (Kardailsky et al., 1999; Kobayashi et al., 1999). However, a similar enhancement of the endogenous expression pattern has been observed for *BAS1*, whose endogenous expression pattern is very different from that of *FT* (Neff et al., 1999).

An important question for any gain-of-function screen is whether the gain-of-function phenotype of a mutant reflects the normal role of an activated gene. The possibility that CaMV 35S enhancers lead primarily to an enhancement of the endogenous expression pattern is exciting, because the phenotype resulting from such an enhancement, as opposed to ectopic overexpression, would be more likely to reflect the normal role of the activated gene.

One of our motivations for conducting activation-tagging screens is to identify genes that have redundant roles and that are not easily identified by loss-of-function

mutations. With the exception of *FT* and *LAB1*, none of the other mutations led to the activation of known genes, indicating that activation tagging is successful in identifying new genes. To quickly determine the loss-of-function phenotypes of new genes, we are currently evaluating a new generation of activation-tagging vectors that includes the non-autonomous transposon *Ds* in the T-DNA. One can then exploit a well-known feature of *Ds*, preferential transposition to closely linked sites (Bancroft and Dean, 1993), to disrupt the overexpressed gene.

For *FT*, the loss-of-function phenotype is the opposite of the gain-of-function phenotype, validating that the gain-of-function phenotype reflects the normal role of *FT* in flowering (Koornneef et al., 1991; Kardailsky et al., 1999; Kobayashi et al., 1999). The case for *LAB1* is less clear. In *lab1-1D*, the *ANL2* gene (Kubo et al., 1999) is overexpressed, but the mutant DNA failed to recapitulate the *lab1-1D* phenotype. Because the *anl2* loss-of-function phenotype is unrelated to flowering (Kubo et al., 1999), we did not further investigate the cause of late flowering in *lab1-1D*.

Among the newly identified genes, the *bas1-1D* phenotype is opposite to that of *BAS1* antisense plants. *bas1-1D* plants have short hypocotyls and increased sensitivity to light, while antisense plants have long hypocotyls and reduced sensitivity to light (Neff et al., 1999), indicating that the activation-tagged phenotype reflects the normal function of *BAS1*.

In summary, we have demonstrated for whole plants the utility of activation tagging using CaMV 35S enhancers, even though it is at present unclear whether enhancer activation tagging will identify every gene that has a potential overexpression phenotype. Our experience suggests that the gain-of-function approach pioneered by Coupland and colleagues (Wilson et al., 1996; Schaffer et al., 1998) using an outward facing CaMV 35S promoter will provide a good complement to the enhancer activation-tagging method used here. In addition, an alternative to activation tagging will be the use of tissue-specific promoters to misexpress genes during specific phases of the life cycle or in specific tissues. Preliminary results using a T-DNA with an outward facing *APETALA1* promoter indicate that this approach will be successful (M. Ng and M. Yanofsky, unpublished results).

ACKNOWLEDGMENTS

We thank Rick Amasino for communicating unpublished results, and Kim Hanson, Jason Lutes, and Serena Nguyen for technical assistance. We thank Dave Patton and Danielle Friedrichsen for help with the sequence analysis of pSKI015 and pSKI074, respectively, and Jonathan Jones and Rick Walden for the gift of plasmids.

Received October 13, 1999; accepted December 8, 1999.

LITERATURE CITED

Aravind L, Landsman D (1998) AT-hook motifs identified in a wide variety of DNA-binding proteins. Nucleic Acids Res **26:** 4413–4421

Bancroft I, Dean C (1993) Transposition pattern of the maize element *Ds* in *Arabidopsis thaliana*. Genetics **134:** 1221–1229

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. CR Acad Sci **316:** 1194–1199

Bechtold N, Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol Biol **82:** 259–266

Benfey PN, Chua N-H (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J **8:** 2195–2202

Bevan M, Bancroft I, Bent E, Love K, Goodman H, Dean C, Bergkamp R, Dirkse W, Van Staveren M, Stiekema W, Drost L, Ridley P, Hudson SA, Patel K, Murphy G, Piffanelli P, Wedler H, Wedler E, Wambutt R, Weitzenegger T, Pohl TM, Terryn N, Gielen J, Villarroel R, De Clerck R, Van Montagu M, Lecharny A, Auborg S, Gy I, Kreis M, Lao N, Kavanagh T, Hempel S, Kotter P, Entian K-D, Rieger M, Schaeffer M, Funk B, Mueller-Auer S, Silvey M, James R, Montfort A, Pons A, Puigdomenech P, Douka A, Voukelatou E, Milioni D, Hatzopoulos P, Piravandi E, Obermaier B, Hilbert H, Düsterhöft A, Moores T, Jones JDG, Eneva T, Palme K, Benes V, Rechman S, Ansorge W, Cooke R, Berge C, Delseny M, Voet M, Volckaert G, Mewes H-W, Klosterman S, Schueller C, Chalwatzis N (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature **391:** 485–488

Blázquez MA, Weigel D (1999) Independent regulation of flowering by phytochrome B and gibberellins in *Arabidopsis*. Plant Physiol **120:** 1025–1032

Brunner E, Brunner D, Fu W, Hafen E, Basler K (1999) The dominant mutation *Glazed* is a gain-of-function allele of *wingless* that, similar to loss of APC, interferes with normal eye development. Dev Biol **206:** 178–188

Chadwick R, Jones B, Jack T, McGinnis W (1990) Ectopic expression from the *Deformed* gene triggers a dominant defect in *Drosophila* adult head development. Dev Biol **141:** 130–140

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science **262:** 539–544

Chory J, Nagpal P, Peto CA (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in Arabidopsis. Plant Cell **3:** 445–459

Chung JH, Whiteley M, Felsenfeld G (1993) A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell **74:** 505–514

Denell RE, Hummels KR, Wakimoto BT, Kaufman TC (1981) Developmental studies of lethality associated with the *Antennapedia* gene complex in *Drosophila melanogaster*. Dev Biol **81:** 43–50

Fang RX, Nagy F, Sivasubramaniam S, Chua NH (1989) Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell **1:** 141–150

Feldmann KA (1991) T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. Plant J **1:** 71–82

Fridborg I, Kuusk S, Moritz T, Sundberg E (1999) The Arabidopsis dwarf mutant *shi* exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. Plant Cell **11:** 1019–1032

Gehring W (1967) Bildung eines vollständigen Mittelbeins mit Sternopleura in der Antennenregion bei der Mutante *Nasobemia* (*Ns*) von *Drosophila melanogaster*. Arch Julius Klaus Stift Vererbungsforsch Sozialanthropol Rassenhyg **41:** 44–54

Guilley H, Dudley RK, Jonard G, Balàzs E, Richards KE (1982) Transcription of cauliflower mosaic virus DNA: detection of promoter sequences, and characterization of transcripts. Cell **30:** 763–773

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol **25:** 989–994

Hayashi H, Czaja I, Lubenow H, Schell J, Walden R (1992) Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. Science **258:** 1350–1353

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94:** 261–271

Jones JD, Shlumukov L, Carland F, English J, Scofield SR, Bishop GJ, Harrison K (1992) Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgen Res **1:** 285–297

Kakimoto T (1996) *CKI1*, a histidine kinase homolog implicated in cytokinin signal transduction. Science **274:** 982–985

Kania T, Russenberger D, Peng S, Apel K, Melzer S (1997) *FPF1* promotes flowering in Arabidopsis. Plant Cell **9:** 1327–1338

Kardailsky I, Shukla V, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer *FT*. Science **286:** 1962–1965

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **230:** 1299–1302

Kluppel M, Nagle DL, Bucan M, Bernstein A (1997) Long-range genomic rearrangements upstream of *Kit* dysregulate the developmental pattern of *Kit* expression in W^{57} and W^{banded} mice and interfere with distinct steps in melanocyte development. Development **124:** 65–77

Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T (1999) A pair of related genes with antagonistic roles in mediating flowering signals. Science **286:** 1960–1962

Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Korber H, Redeí GP, Schell J (1989) High-frequency T-DNA-mediated gene tagging in plants. Proc Natl Acad Sci USA **86:** 8467–8471

Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol Gen Genet **229:** 57–66

Koornneef M, Rolff E, Spruit CJP (1980) Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Heynh. Z Pflanzenphysiol **100:** 147–160

Koornneef M, van der Veen JH (1980) Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana*. Int Z Theor Angew Genet **58:** 257–263

Kubo H, Peeters AJ, Aarts MG, Pereira A, Koornneef M (1999) *ANTHOCYANINLESS2*, a homeobox gene affecting anthocyanin distribution and root development in Arabidopsis. Plant Cell **11:** 1217–1226

Lee I, Wolfe DS, Nilsson O, Weigel D (1997) A *LEAFY* co-regulator encoded by *UNUSUAL FLORAL ORGANS*. Curr Biol **7:** 95–104

Li J, Nagpal P, Vitart V, McMorris TC, Chory J (1996) A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science **272:** 398–401

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S (1994) A *knotted1*-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell **6:** 1859–1876

McBride KE, Summerfelt KR (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol Biol **14:** 269–276

Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11:** 949–956

Miller MW, Duhl DM, Vrieling H, Cordes SP, Ollmann MM, Winkes BM, Barsh GS (1993) Cloning of the mouse *agouti* gene predicts a secreted protein ubiquitously expressed in mice carrying the *lethal yellow* mutation. Genes Dev **7:** 454–467

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Physiol Plant **15:** 473–497

Neff MM, Nguyen SM, Malancharuvil EJ, Fujioka S, Noguchi T, Seto H, Tsubuki M, Honda T, Takatsuto S, Yoshida S, Chory J (1999) *BAS1*: a gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. Proc Natl Acad Sci USA **96:** 15316–15323

Odell JT, Nagy F, Chua N-H (1985) Identification of DNA-sequences required for activity of the cauliflower mosaic virus-35S promoter. Nature **313:** 810–812

Ohtsuki S, Levine M, Cai HN (1998) Different core promoters possess distinct regulatory activities in the *Drosophila* embryo. Genes Dev **12:** 547–556

Ow DW, Jacobs JD, Howell SH (1987) Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by the firefly luciferase gene as a reporter of promoter activity. Proc Natl Acad Sci USA **84:** 4870–4874

Ratcliffe O, Amaya I, Vincent C, Rothstein S, Carpenter R, Coen E, Bradley D (1998) A common mechanism controls the life cycle and architecture of plants. Development **125:** 1609–1615

Reed JW, Elumalai RP, Chory J (1998) Suppressors of an *Arabidopsis thaliana phyB* mutation identify genes that control light signaling and hypocotyl elongation. Genetics **148:** 1295–1310

Reed JW, Nagatani A, Elich TD, Fagan M, Chory J (1994) Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol **104:** 1139–1149

Reed JW, Nagpal P, Poole DS, Furuya M, Chory J (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. Plant Cell **5:** 147–157

Riechmann JL, Meyerowitz EM (1997) MADS domain proteins in plant development. Biol Chem **378:** 1079–1101

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carré IA, Coupland G (1998) The *late elongated hypocotyl* mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93:** 1219–1229

Schneuwly S, Klemenz R, Gehring WJ (1987) Redesigning the body plan of *Drosophila* by ectopic expression of the homoeotic gene *Antennapedia*. Nature **325:** 816–818

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES (1999) The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11:** 445–458

Simon R, Igeño MI, Coupland G (1996) Activation of floral meristem identity genes in *Arabidopsis*. Nature **382:** 59–62

Smith LG, Greene B, Veit B, Hake S (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. Development **116:** 21–30

Sun T-p, Kamiya Y (1994) The Arabidopsis *GA1* locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. Plant Cell **6:** 1509–1518

Wang ZY, Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell **93:** 1207–1217

Weigel D, Nilsson O (1995) A developmental switch sufficient for flower initiation in diverse plants. Nature **377:** 495–500

Wilson K, Long D, Swinburne J, Coupland G (1996) A *Dissociation* insertion causes a semidominant mutation that increases expression of *TINY*, an Arabidopsis gene related to *APETALA2*. Plant Cell **8:** 659–671

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene **33:** 103–119

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. Plant J **19:** 249–257