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Three new dominant *C1* suppressor alleles in *Zea mays*

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Summary

Three new dominant suppressor mutations of the *C1* transcription regulator gene in maize – *C1-IΔ1*, *C1-IΔ2* and *C1-IΔ3* – are described that suppress anthocyanin colouration in kernels similar to the function of the *C1-I* standard inhibitor. The *C1-IΔ* mutations were induced by imprecise excision of an *En/Spm* transposon in the third exon of the *C1* gene. These transposon footprints cause a frameshift in the *C1* open reading frame that leads to truncated proteins due to an early stop codon 30 amino acids upstream of the wild-type *C1* protein. Therefore, the *C1-IΔ* gene products lack the carboxy-terminal transcriptional activation domain of *C1*. The *C1-I* standard allele also lacks this domain and in addition differs in 17 amino acids from the wild-type *C1* allele. The new *C1-IΔ* alleles provide evidence that deletion of the carboxy-terminal activation domain alone is sufficient to generate a dominant suppressive effect on the function of wild-type *C1*.

1. Introduction

The *C1* gene product of maize is a *myb*-homologous transcription factor involved in regulation of anthocyanin synthesis in the aleurone layer of the endosperm (Paz-Ares *et al*., 1986, 1987; Cone *et al*., 1986). The 273 amino acid *C1* protein contains two domains, namely a basic amino-terminal *myb*-homologous region responsible for DNA binding and an acidic carboxy-terminal domain required for transcriptional activation (Paz-Ares *et al*., 1990; Goff *et al*., 1991). *C1-I*, a dominant inhibitor allele (in this paper referred to as the *C1-I* standard allele), suppresses anthocyanin pigmentation in the kernel (Coe, 1962). Paz-Ares and co-workers (1990) described the *C1-I* product as a truncated protein different from *C1* at 17 amino acid positions and, most prominently, lacking 21 residues of the acidic *C1* carboxy-terminal domain. An additive interplay of a mutation within the basic DNA-binding domain and the partial truncation of the acidic transcriptional activation domain is likely to be responsible for the repressor function of *C1-I* (Goff *et al*., 1991; Solano *et al*., 1995).

In the present study we describe three new *C1-I* alleles designated *C1-IΔ1*, *C1-IΔ2* and *C1-IΔ3* respectively, generated by excision events of an *En/Spm* transposon inserted in the third exon of the *C1* gene. Maize transposons such as *En/Spm* often undergo imprecise excision thereby leaving altered nucleotide sequences, termed footprints, at the site of integration. These changes can result in frameshift mutations giving rise to mutant protein products with new properties (Schwarz-Sommer *et al*., 1984; Wessler, 1988; Franken *et al*., 1994). Here, we report on three dominant *C1* suppressor alleles resulting from transposon excision. In these alleles truncation of the carboxy-terminus is sufficient to generate a suppressor from a fully functional *C1* allele.

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2. Materials and methods

(i) *Maize stocks*

The following full-colour lines, containing the wild-type *C1* allele, were used as crossing partners: *Line C* is a colour-converted W22 maize inbred line developed by Dr R. A. Brink, University of Wisconsin, from which the wild-type *C1* allele was originally cloned (Paz-Ares *et al*., 1986). *Aet* is an early-maturing colour line that originated from the Cornell Maize Coop collection. The *Super C* (^{s}C) line carries the ^{s}C allele, which is known to include a Box II in the *C1*

promoter (Hattori *et al*., 1992; Scheffler *et al*., 1994). The line *c1 sh wx* has been described as a tester for *C1* insertions (Peterson, 1978; Reddy & Peterson, 1983) and was included in crosses to isolate germinal excision revertant derivatives. The *c1-m763103* allele arose in an isolation plot (1975) that was developed to tag new *c1-m* alleles and confirmed as a *c1* mutable allele (Peterson, 1963, 1978). The *c1-m763103* allele was the source of the colourless and pale revertant derivatives that were isolated as selfed progenies.

(ii) *PCR*

Genomic DNA was isolated by the method of Dellaporta *et al*. (1983) with the modification of a RNase digestion step (20 μg per sample; 30 min; 37 °C) and a subsequent phenol extraction prior to the last precipitation. One microgram served as template in a reaction mixture containing 2 μM of each primer, 250 μM dNTPs and 2·5 U *Taq* polymerase (Boehringer, Mannheim). The 10× reaction buffer was prepared with 0·67 M Tris/HCl (pH 8·8); 0·167 M $(NH_4)_2SO_4$; 0·1 M β-mercaptoethanol and 0·067 M $MgCl_2$. The PCR was initiated with a hotstart and carried out for 30 cycles (1 min, 94 °C; 30 s, 60 °C; 2 min, 72 °C). In order to determine the insertion site of the *En1* transposon (Pereira *et al*., 1985) in the *c1-m763103* plant, primers were used that bind to one end of the transposon or to different regions of the *C1* locus. Finally, a 380 bp fragment containing the *En1* border sequences was amplified with the following primer pair: pEn3′: 5′-AGGTAGCTTACTGATGTGCGC-GC-3′ (8024–8046) and pC3: 5′-GTACTTGTTGTC-TACGCAAGCTGC-3′ (1062–1085). Sequence positions correspond to the *En1* (Pereira *et al*., 1985) and to the wild-type *C1* allele (Paz-Ares *et al*., 1987). The amplification of footprint sequences in the *c1-m763103* revertant derivatives was performed with two primer pairs in two steps. Firstly, a PCR was performed with the following primer pair: pC1: 5′-ACCGGCCAGAATAGCGCCGCTCATC-3′ (694–718); pC4: 5′-CTCGTGCTTATTGGACATCTAT-ACGTG-3′ (1085–1111). The sample was diluted 1:1000 and 1 μl served as a template in a second PCR with the nested primer pair pC2 (nested to pC1): 5′-GGACTCTTCTTCTTCCACCGGGACA-3′ (799–823) and pC3 (nested to pC4): 5′-GTACTTGTTG-TCTACGCAAGCTGC-3′ (1062–1085). The resulting 286 bp fragment was purified with Quiaquick spin columns (Qiagen, Hilden).

(iii) *Sequencing*

Sequences were determined by ABI Cycle Sequencing protocol on a ABI 377 sequencer. A 20–40 ng PCR fragment served as substrate in the linear amplification reaction (30 s, 94 °C; 30 s, 50 °C; 4 min, 60 °C).

3. Results

(i) *The origin of* cl-m763103 *and its relation to* En/Spm

The mutable *C1* allele, *c1-m763103*, was isolated as a single kernel event among progeny in an isolation plot for *C1* mutants where the parents were *C1/C1 En* × *c1 sh1 wx* (Peterson, 1978). Plant 763103 was crossed by a *c1* tester (*c1 sh wx*) to clarify whether the new mutation is indeed an allele of *C1* (*c1-m*), which was confirmed by the resulting mutable progeny (coarse pattern, Fig. 1*A*, facing page). Subsequent genetic analysis showed that *c1-m763103* is autonomously mutable, very probably representing an integration of the transposable element *En/Spm* into *C1* (data not shown).

(ii) *Origin of dominant negative revertant derivatives of* c1-m763103

In the cross of *c1-763103* by *line C* (cross 1) a number of kernels appeared with colourless aleurone sectors on a coloured background in the endosperm (Fig. 1*B*), as well as a few entirely colourless and pale kernels (not shown in Fig. 1*B*). Since the endosperm genotype of kernels from cross 1 was *c1-m763103/c1-m763103/C1*, the kernels were expected to be fully coloured and the observed colourless sectors were unexpected due to the anticipated dominance of *C1* (Fig. 1*B*).

Cross 1:

c1-m763103/c1-m763103 × *C1/C1* ⇒
(genotype of endosperm)
c1-m763103/c1-m763103/C1.

Cross 2:

C1/C1 × *cl-m763103/c1-m763103* ⇒
C1/C1/c1-m763103.

In the reciprocal cross (cross 2) with *c1-m763103* plants as male partners no colourless sectors were found among these ears. The different phenotypes of crosses 1 and 2 can be due to the different allele combinations in the aleurone layer of the triploid endosperm. One possible explanation of the findings is that *c1-m763103* generates dominant negative alleles that can suppress one dose of wild-type *C1* in the endosperm (cross 1) but not two doses (cross 2).

Seventy-eight colourless and pale germinal derivatives of the progeny of cross 1 were selected and selfed

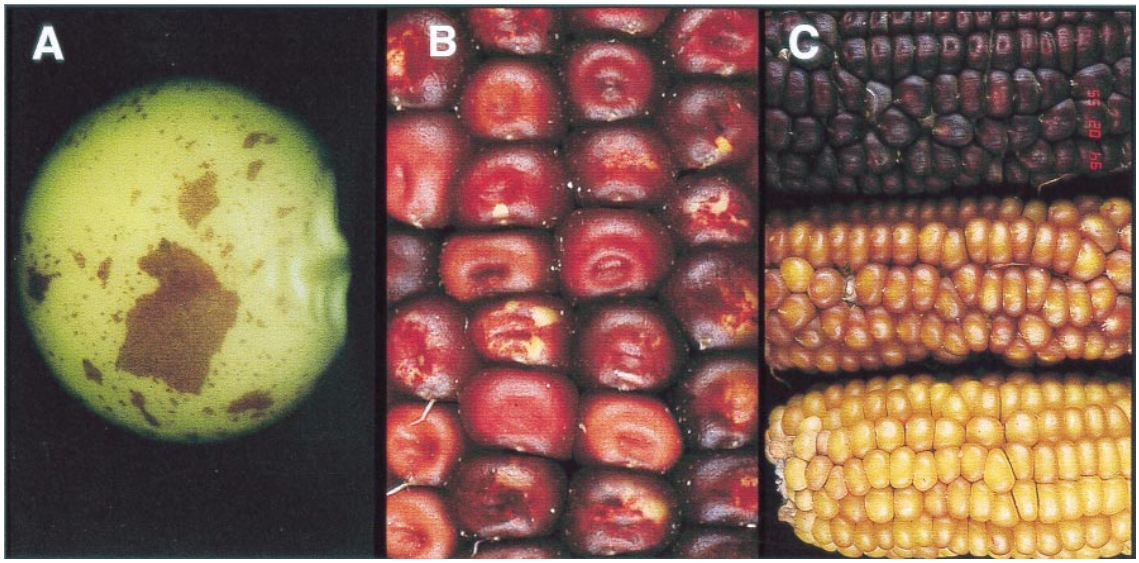

Fig. 1. Phenotypes of the *c1-m763103* allele and the colourless revertant derivatives following excision of *En/Spm*. (*A*) Kernel of the mutable *c1-m763103* allele (*c1-m763103/c1/c1*). (*B*) Kernels with colourless sectors resulting from the cross of the *c1-m763103* allele with the wild-type (*c1-m763103/c1-m763103* × *C1/C1*). The genotype of the endosperm in these kernels is *c1-m763103/c1-m763103/C1*: the colourless sectors have the genotype *C1-IΔ/C1-IΔ/C1*. (*C*) Progenies from the cross *C1-IΔ/C1-IΔ* × *C1/C1* (the male crossing partner was, from top to bottom: ^{S}C, *line C*, *Aet*).

to isolate colourless and pale revertant derivatives. Plants grown from these colourless kernels were used as female plants and as in cross 1 rested with *line C* (*C1/C1*) as well as with the other full-colour lines *Aet* and ^{S}C. The progenies showed differences in colour expression in the kernels when lines *Aet*, *line C* or ^{S}C were used as the male crossing partners (Fig. 1 *C*). The strongest suppressive effect can generally be seen with the *Aet* line as a male parent, where the kernels are colourless. A somewhat weaker suppressive effect is usually observed with the *line C* parent, where the kernels are pale-coloured but clearly distinguishable from wild-type kernels. In the cross with the ^{S}C line all kernels are coloured, indicating no suppressive effect of the *c1-m763103* revertant derivative in this combination (Fig. 1 *C*). This indicates that the suppressive capacity of the *c1-m763103* revertant derivatives is variable when combined with different *C1* wild-type alleles, but variations in colour suppression are also seen within crosses with one line – although suppression is always uniform for one ear.

For further analysis the progenies from the crosses with *line C* were classified for colour expression ranging from strong (colourless) to weak (pale) colour suppression. We hypothesized that the suppressive effect observed in *c1-m763103* revertant derivatives is due to mutations in the *C1* gene product that are caused by inaccurate excision of *En/Spm*. In order to test this hypothesis we analysed the DNA sequence of the *C1* locus in *c1-m763103* revertant derivatives. Because of the intensity differences in colour expression within the progeny of cross 1 we selected 24 individual kernels that covered the whole range from strong to weak colour inhibition for sequence analysis.

(iii) *DNA sequence determination of the insertion site and the 24* cl-m763103 *revertant derivatives*

First, the insertion site of *En/Spm* in the *c1-m763103* progenitor was mapped (see Section 2). The insertion occurred after nucleotide 970 in exon three of the *C1* sequence (Paz-Ares *et al*., 1987; Fig. 2 *A*). Primers flanking this position were used to obtain PCR fragments from all 24 *c1-m763103* revertant derivatives. Sequence analysis of these fragments showed footprints diagnostic for *En/Spm* excisions (Saedler & Nevers, 1985). Three different types of sequence alterations were detected, resulting in the insertion of one to four bases at the *En/Spm* excision site (Fig. 2 *A*). As the suppressor function of the respective gene products resembled the *C1-I* standard allele, the mutants were termed *C1-IΔ1*, *C1-IΔ2* and *C1-IΔ3*. Of the 24 *cl-m763103* revertant derivatives analysed, 20 were of type *C1-IΔ1*, three of *C1-IΔ2*, and one of *C1-IΔ3*. Each of the three mutations shifts the reading frame by one nucleotide after codon 239 (*C1-IΔ2*) or 240 (*C1-IΔ1*, *C1-IΔ3*) leading to premature translational termination at position 978 (Fig. 2 *A*). As a result of these frameshift mutations, 33 or 34 amino acids of the wild-type *C1* allele are truncated and two or three amino acids are added compared with the wild-type C1 protein (Fig. 2 *B*).

No correlation of the strength of the suppressive colour inhibition with a particular footprint sequence was found; for example, the 20 individuals grouped in the *C1-IΔ1* category originally comprised both strong- and weak-coloured kernels. Several of the individuals of the *C1-IΔ1* category (genotype *C1-IΔ1/C1*, *line C*) have been backcrossed using *line C* or *Aet* as a pollen

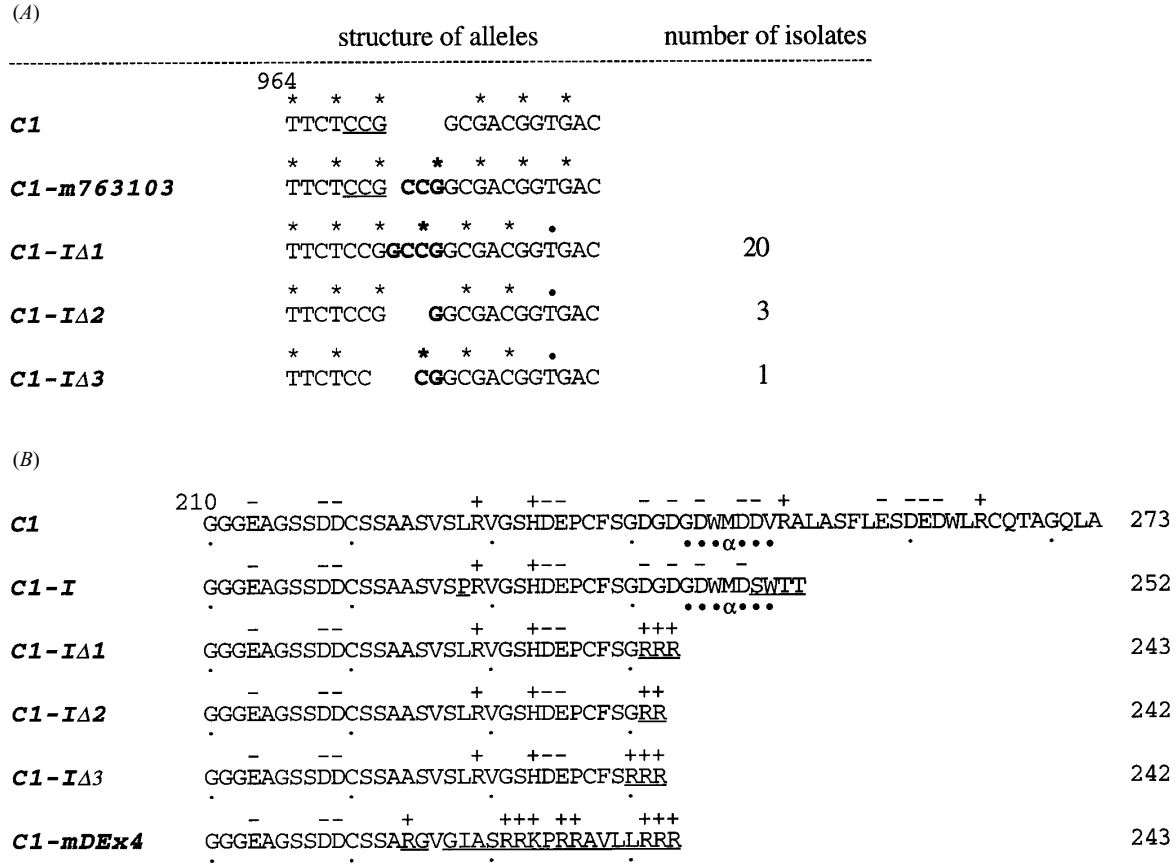

Fig. 2. (*A*) Footprints leading to the *C1-IΔ1*, *C1-IΔ2*, *C1-IΔ3* alleles generated by imprecise excision of the *En/Spm* element from the *C1* gene (additionally inserted nucleotides are in bold). The target site, generated upon integration of *En/Spm* in *C1*, is underlined. Codons are marked by stars above each sequence. Stop codons in the *C1-IΔ* alleles generated by frameshifting are indicated by dots. The number of independent excision events analysed is indicated. (*B*) *C1* protein sequence comparison. The amino acids at the carboxy-terminus are shown, starting from residue 210 of the putative gene products of wild-type *C1*, standard *C1-I*, three alleles of this work (*C1-IΔ1*, *C1-IΔ2*, *C1-IΔ3)* and *C1-mDEx4* described by Franken and co-workers (1994). Residues deviating from wild-type *C1* are underlined. The distribution of charged acidic (−) and basic (+) residues is given above each sequence, respectively. The positions of α-helices predicted by the method of Rost & Sander (1993) are marked by dots.

Table 1. *Backcross analysis of the* c1-m763103 *allele* (C1/c1-m763103 × C1/C1)

	Line C			*Aet*		
	Cl	Pale	(%) Pale	*Cl*	Pale	(%) Pale
1	128	105	45·0	116	124	51·7
2	145	139	48·9	148	146	49·7
3	80	68	45·9	183	186	50·4
4	92	68	42·5	89	91	50·5

The *Cl* (Coloured) class and the pale class are expected to segregate 1:1. (Endosperm genotypes are *C1 C1 C1* and *C1 C1-I C1-I.)*

source. The resultant ears segregated 1:1 for colour suppression (Table 1). This provides further evidence for the genetic correspondence between the colour inhibitor trait and the revertant derivatives of the *c1-m763013* allele. The colour expression on the ears was uniform; however, different ears derived from one individual had again quite varying degrees of colour suppression (data not shown). This excludes segregating genetic factors that would modify colour expression.

4. Discussion

Molecular analysis of revertant derivatives from the mutable plant designated *c1-m763103* revealed three new alleles – *C1-IΔ1*, *C1-IΔ2* and *C1-IΔ3* – that were generated by independent excision events of an *En/Spm* transposable element from the *C1* locus. These footprints lead to premature termination of the *C1* open reading frame. Like the protein encoded by the *C1-I* standard allele the gene products of the *C1-IΔ* alleles lack the acidic carboxy-terminal activation domain of wild-type *C1* (Paz-Ares *et al.*, 1990; Goff *et al.*, 1991). Suppression of anthocyanin colouration in kernels carrying the *C1-IΔ* alleles indicates that the encoded proteins act as transcriptional repressors

similar to the *C1-I* protein (for review see Franken *et al*., 1994). While in standard *C1-I* 21 amino acids of *C1* are deleted, the *C1-IΔ* proteins lack 33 or 34 residues (Fig. 2*b*). Secondary structure predictions by the method of Rost & Sander (1993) suggest an α-helix within amino acids 244–250 in both *C1* and *C1-I* but not in the shorter *C1-IΔ* alleles (Fig. 2*B*). The absence of this α-helix could result in a decrease in protein stability in the truncated *C1-IΔ* proteins. Instability of the truncated proteins could account for the observed diversity of suppressor action in the *C1-IΔ1* alleles, although mRNA instability can not be excluded. However, the differences can not be attributed to genetic background factors, since in the backcrosses homogeneous coloured ears were obtained, segregating 1:1 for suppression.

While the *C1-IΔ* proteins are probably less stable than the standard *C1-I* repressor, they are still able to act as transcriptional suppressors. In contrast *C1-mDEx4* does not exhibit a suppressive dominance effect (Franken *et al*., 1994). In the *C1-mDEx4* mutant the frameshift occurs further upstream at codon 224 leading to 20 residues at the carboxy-terminus that deviate from the wild-type *C1* sequence (Fig. 2*B*). The altered carboxy-terminal sequence in the *C1-mDEx4* allele obviously no longer permits the inhibitory function, perhaps by further decreasing protein or mRNA stability.

The suppressive potency for the *C1-IΔ* alleles is lower than that of the *C1-I* standard allele as indicated by the finding that two doses of *C1-IΔ* are necessary for suppressing one dose of wild-type *C1*. This effect may not only be due to structural differences, but could also result from an enhanced transcription level associated with the *C1-I* standard allele. It was shown that the steady-state mRNA levels of the *C1-I* standard allele are substantially higher than in the wild-type *C1* allele (Paz-Ares *et al*., 1990). Enhanced transcription was correlated with a alteration in the Box II of the promoter in the *C1-I* standard allele (Scheffler *et al*., 1995). The *C1-IΔ* alleles described here are derived from a wild-type genetic background and therefore are probably lacking the transcription-enhancing Box II in the promoter. Therefore, the *C1-IΔ* alleles would be expressed at a much lower level compared with the *C1-I* standard allele. This would explain the fact that two doses of the *C1-IΔ* alleles are required for the inhibition of one wild-type *C1* allele. Thus it appears that colour suppression is generally stronger in combination with the *Aet* line than with *line C*. Whether this indicates any differences in gene expression of the *C1* alleles in these lines is not known. In contrast, two doses of the *C1-IΔ* alleles cannot suppress one dose of the ^{S}C allele in the aleurone. The ^{S}C allele contains the same promoter mutation as the *C1-I* standard allele in its Box II and is therefore strongly transcribed. In this situation even one dose of the ^{S}C allele could make enough functional *C1* protein to get fully coloured kernels.

The sequence of the *C1-I* standard allele deviates from wild-type *C1* by 17 additional amino acid alterations besides truncation of the carboxy-terminal region (Paz-Ares *et al*., 1990). Therefore the suppressor function of the *C1-I* standard allele cannot be attributed exclusively to the deletion of the acidic domain at the carboxy-terminus (Goff *et al*., 1991). In contrast, the suppressive effect of the *C1-IΔ* alleles is due to truncation of the carboxy-terminal domain. This finding confirms the original explanation (Paz-Ares *et al*., 1990) that a transcription activator is converted into a competitive inhibitor by deletion of the acidic activation domain.

References

Coe, E. H. (1962). Spontaneous mutation of the aleurone color inhibitor in maize. *Genetics* **47**, 779–783.

Cone, K. C., Burr, F. A. & Burr, B. (1986). Molecular analysis of the maize anthocyanin regulatory locus *C1*. *Proceedings of the National Academy of Sciences of the USA* **83**, 9631–9635.

Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983). A plant DNA minipreparation: version II. *Plant Molecular Biology Reports* **1**, 19–21.

Franken, P., Schrell, S., Peterson, P. A., Saedler, H. & Wienand, U. (1994). Molecular analysis of protein domain function encoded by the *myb*-homologous maize genes *C1, Zm 1* and *Zm 39*. *The Plant Journal* **6**, 21–30.

Goff, S. A., Cone, K. C. & Fromm, M. E. (1991). Identification of functional domains in the maize transcriptional activator *C1*: comparison of wild-type and dominant inhibitor proteins. *Genes & Development* **5**, 298–309.

Goff, S. A., Cone, K. C. & Chandler, V. L. (1992). Functional analysis of the transcriptional activator encoded by the maize *B* gene: evidence for a direct functional interaction between two classes of regulatory proteins. *Genes & Development* **6**, 864–875.

Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L. C., McCarty, D. R. & Vasil, I. K. (1992). The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes & Development* **6**, 609–618.

Paz-Ares, J., Wienand, U., Peterson, P. A. & Saedler, H. (1986). Molecular cloning of the *c* locus of *Zea mays*: a locus regulating the anthocyanin pathway. *EMBO Journal* **5**, 829–833.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P. A. & Saedler, H. (1987). The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. *EMBO Journal* **6**, 3553–3558.

Paz-Ares, J., Ghosal, D. & Saedler, H. (1990). Molecular analysis of the *C1-I* allele from *Zea mays*: a dominant mutant of the regulatory *C1* locus. *EMBO Journal* **9**, 315–321.

Pereira, A., Schwarz-Sommer, Z., Bertram, I., Peterson, P. A. & Saedler, H. (1985). Genetic and molecular analysis of the Enhancer (*En*) transposable element system of *Zea mays*. *EMBO Journal* **4**, 17–23.

Peterson, P. A. (1963). Influence of mutable genes on induction of instability in maize. *Proceedings of the Iowa Academy of Science* **70**, 129–134.

Peterson, P. A. (1978). Controlling elements: the induction of mutability at the *A2* and *C* loci in maize. In *Maize Breeding and Genetics* (ed. D. B. Walden). New York: Wiley.

Reddy, A. R. & Peterson, P. A. (1983). Transposable elements of maize: genetic bases of pattern differentiation of some mutable *c* alleles of the Enhancer system. *Molecular and General Genetics* **192**, 21–31.

Rost, B. & Sander, C. (1993). Prediction of protein secondary structure at better than 70% accuracy. *Journal of Molecular Biology* **232**, 584–599.

Saedler, H. & Nevers, P. (1985). Transposition in plants: a molecular model. *EMBO Journal* **4**, 585–590.

Scheffler, B., Franken, P., Schütt, E., Schrell, A., Saedler, H. & Wienand, U. (1994). Molecular analysis of *C1* alleles in *Zea mays* defines regions involved in the expression of this regulatory gene. *Molecular and General Genetics* **242**, 40–48.

Schwarz-Sommer, Z., Gierl, A., Klösgen, R. B., Wienand, U., Peterson, P. A. & Saedler, H. (1984). The *Spm* (*En*) transposable element controls the excision of a 2-kb DNA insert at the *wx-m8* allele of *Zea mays*. *EMBO Journal* **3**, 1021–1028.

Solano, R., Nieto, C., Avila, J., Canas, L., Diaz, I. & Paz-Ares, J. (1995). Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from *Petunia hybrida*. *EMBO Journal* **14**, 1773–1784.

Wessler, S. R. (1988). Phenotypic diversity mediated by the maize transposable elements *Ac* and *Spm*. *Science* **242**, 399–405.