Genet. Res. Camb. (1995), **66**, *pp.* 203–212 *With 2 text-figures*

Induction of unstable alleles at the temperature-sensitive *Virescent-1* gene of maize using the transposable element *Dissociation*

SERGIO CERIOLI[1], STEFANIA BALLARINI[1], HELMUT UHRIG[2], EUGENIO SCALZOTTO[1] AND ADRIANO MAROCCO[3]*

[1] *Istituto di Botanica e Genetica vegetale, Università Cattolica del S. Cuore, 29100 Piacenza, Italy*
[2] *Max-Planck-Institut für Züchtungsforschung, D-5000, Köln 30, Germany*
[3] *Dipartimento di Agronomia ambientale e Produzioni vegetali, Università degli Studi di Padova, via Gradenigo 6, 35100 Padova, Italy*

(*Received 28 February 1995 and in revised form 13 June 1995*)

Summary

Transpositive mutagenesis was employed to prepare genetic strains useful in cloning the *Virescent-1* locus (*V1*) of maize. A stepwise approach was used based on: (1) the isolation of putative insertion phenotypes (62 cases); (2) the verification of the genetic nature of the selected events (36 *v1-m* mutant alleles induced); (3) the accurate genetic study of 11 alleles; (4) the genetic assessment that the alleles *v1-m1* and *v1-m4* are due to the insertion of *a Ds* element into the locus *V1*; (5) the proof that a *Ds*-like DNA element induces the inactivation of the wild type function in the allele *v1-m1*. The phenotype of the unstable alleles, studied by germinating and keeping maize seedlings at the temperature of 18 °C, are the following: alleles *v1-m1*, *v1-m9*, *v1-m11*, *v1-m17* and *v1-m18* showing a few revertant green sectors on their leaves; *v1-m4* exhibiting a reverse type of variegation; alleles *v1-m2* and *v1-m13* with a coarse pattern of variegation; alleles *v1-m12*, *v1-m21* and *v1-m23* frequently showing leaves part green with white stripes and part white with green stripes. For the alleles studied, in addition to somatic instability, germinal reversions also occurred. In some cases, these reversions resulted in stable derivatives with a different colour from that of the wild-type ('near green' or pale phenotypes). The results presented not only allow the *v1-m1* allele to be chosen as a starting material for cloning the *V1* locus, but also define the molecular strategy to be followed.

1. Introduction

In the *virescent* mutants of maize, the seedling is initially albino or yellow-green and then its colour gradually becomes indistinguishable from the wild-type (King, 1991). Low temperatures delay the process of greening whereas high temperatures normalize the mutant seedlings. This temperature response is to some extent mutant specific (Phynney & Kay, 1954; Millered & McWilliam, 1968; Hopkins & Walden, 1977). Several *virescent* mutants cannot survive temperatures below a specific threshold and frequently have abnormal photosynthetic attributes. In the early greening stages of *virescent 24* (*v24*) there is a reduction in the level of most of the thylakoid components, and during thylakoid biogenesis the assembly of the major chlorophyll (a/b) light harvesting complex (LHC) is retarded (Polacco, Cheng & Neuffer, 1985; Polacco *et al.* 1987). The chlorotic leaves of the mutants *v3*, *v12* and *v16* have plastids which were arrested in early development stages as well as aberrant thylakoids which lack the 70s plastid ribosomes (Hopkins & Elfman, 1984). The mutations *v16* and *v18* are marked by a lag in chlorophyll accumulation in young leaves and their mesophyll and bundle-sheath plastids are indistinguishable, exhibiting only rudimentary vesicular and lamellar components (Chollet & Paolillo, 1972; Edwards & Jenkins, 1988).

The initial greening of the *v1* mutant is only to some extent affected by temperature, and keeping mutant seedlings at low temperature does not destroy them (Hopkins & Walden, 1977; our unpublished results). The mutant evinces a reduced yet consistent level of greening when grown between 19 and 30 °C and has low but measurable levels of 23s and 16s rRNA (Hopkins & Elfman, 1984). We suggest that cloning of the *V1* gene could present a useful approach to the study of control by the nuclear genome over chloroplast development in higher plants. An experiment tagging the *V1* gene was performed using the maize

* To whom reprint requests should be sent.

Activator (*Ac*)–*Dissociation* (*Ds*) transposon system to discover the molecular basis of its particular phenotypic expression. It is known that the transposition of the *Ac* and *Ds* elements preferentially occurs to linked chromosomal sites (Brink & Nilan, 1952; Dooner & Belachew, 1989; Moreno *et al.* 1992; Weil *et al.* 1992; Alleman & Kermicle, 1993). This is why we used a line carrying an active *Ac* at the *wx-m7* allele which is linked to the *V1* gene at 7 map units on chromosome 9. The present study reports the isolation and characterization of eleven *v1-mutable* alleles derived from the transposition of an element of the *Ac–Ds* family to the *V1* locus. We have also shown that an active *Ac* element is required to induce instability of the *v1-m1* and *v1-m4* alleles and that an increase in *Ac* dosage results in a more severe mutant phenotype. Genetic and genomic Southern analyses revealed the existence of a *Ds* element segregating with the *v1-m1* mutation.

2. Materials and methods

(i) *Maize stocks*

The original *virescent-1* mutant (*v1-ref*) was supplied by the Maize Genetics Cooperative, Department of Agronomy, IL, USA. The *v1-ref* mutant was introgressed and was maintained in two genetic backgrounds (B37 and Oh43) at the University of Piacenza. The maize strains *C-I Ds* (McClintock, 1949), A69Y*wx-m7 a1-m3* (McClintock, 1951), *bz-m2(DI)* (McClintock, 1962) and *P-vv* (Brink & Nilan, 1952) were from the collection of the Istituto Sperimentale Cerealicoltura, Bergamo, Italy.

(ii) *Generation of* v1-mutable (v1-m) *and derived strains*

Using the *wx-m7* plants as the female parent, crosses were made with homozygous *v1-ref* plants. The variegated seedlings were isolated as putative insertion mutants after growing the F1 generation at a low temperature. Putative insertion mutants were selfed for 2 generations to study their segregation behaviour (Table 1). The *v1-m* revertants were selected in F2 and made homozygous by selfing. When necessary, allelism was tested by crossing *v1-m*'s with the *v1-ref* allele.

To separate the *wx-m7* allele from the *v1-m* allele by recombination, *v1-m* strains were crossed with the inbred line A69Y *wx V1* homozygote. Upon selfing the *wx-m7 v1-m/wx V1* plants, stable *virescent* phenotypes [designed as *v1-m*(nv)] were selected, self-pollinated and crossed to *C-I Ds* and to *bz-m2(DI)* strains to insure that no active *Ac* elements were present in their genome. Stable *virescent* phenotypes were crossed with the line A69Y *wx-m7 a1-m3* and with a *P-vv* strain to monitor the *Ac* response of the element putatively present at the *V1* locus in the *v1-m*(nv) phenotypes. Also the activity of the *Ac* element

Table 1. *Expected segregation ratios in F2 or test cross generations when the listed genetic situations are considered. For the F3 generation, only F2 variegated plants were considered for selfing. Genetic F2 constituents giving rise to homozygous variegated or* virescent *F3 progenies are not presented*

Plant genotype*				Expected segregation ratios (%)‡	
F1 or	F2	Generation following	A or N†	Wild-type + Variegated	*virescent*
v1-m wx-m7 / *v1 Wx*		F2	A	75	25
			N	71	29
v1-m wx-m7 / *v1 Wx*		Backcross to *v1*, *Wx* plants	A	50	50
			N	46·5	53·5
	v1-m wx-m7 / *v1 Wx*		A	75	25
	v1-m Wx / *v1 wx-m7*	F3, Class a	N	50	50
	v1-m wx-m7 / *v1 wx-m7*	F3, Class b	A	75	25
			N	75	25
	v1-m wx-m7 / *v1-m Wx*	F3, Class c	A	100	—
			N	75	25
	v1 Wx / *v1-m wx-m7*	F3, Class d	A	75	25
			N	50	50
	v1 Wx / *v1-m Wx*	F3, Class e	A	75	25
			N	71	29

* The alleles of the loci considered in coupling are written on the same row.
† A, autonomy or N, non-autonomy of the element present at *V1*.
‡ The recombination value between *V1* and *Wx* is taken in these calculations as equal to 7 cM.

https://doi.org/10.1017/S0016672300034650 Published online by Cambridge University Press

Fig. 1. Plant phenotypes of putative *Ds* insertion mutants at the *V1* locus in the presence of one copy of a linked *Ac*. The plants are from backcrosses segregating progenies grown in greenhouse at 18°C.

https://doi.org/10.1017/S0016672300034650 Published online by Cambridge University Press

present at the *Waxy* locus in the strain A69Y *wx-m7 a1-m3* was genetically assayed based on the ability of *Ac* to destabilize a *Ds* element resident at the *A1* locus (*a1-m3* allele) (McClintock, 1951). The presence of an active *Ac* in specific strains was also verified by crossing them with *bz-m2(DI)* and with *C-I Ds* strains.

All screening of seedlings were carried out at the fourth leaf stage. The plants were grown in a greenhouse at 18 °C under 80 W m^{-2} light intensity and with a photoperiod of 14 h light and 10 h dark.

(iii) *DNA extraction and Southern analysis*

Total genomic DNA was prepared from leaves of 15 day old seedlings of the appropriate genotype as described by Dellaporta, Woods & Hicks (1983). Southern analysis was performed on 8 μg DNA/sample. The genomic DNA was digested with the appropriate restriction enzymes, electrophoresed through 0·8% agarose gels and transferred to nylon membranes (Amersham, Hybond N) according to Southern (1975). The probes used in these experiments were derived from plasmid p*Ac*7B (Müller-Neumann, Yoder & Starlinger, 1984). The *Ac* probe used was the 1·6 kb *Hind*III fragment from p*Ac*7B. The external 0·74 kb *BssH*II-*Pvu*II fragment from p*Ac*7B was used to detect *Ds* elements. DNA fragments used as probes were purified by restriction enzyme digestion and electrophoresis through low melting point agarose. Gel slices containing the selected DNA fragments were excised, melted and used in the random primed labelling reaction (Feinberg & Vogelstein, 1983). Membranes were prehybridized, hybridized and washed at 65 °C according to the manufacturer's instructions (Amersham).

3. Results

(i) *Description of the unstable* virescent-1 *mutants*

Instability at the *V1* locus in the A69Y *wx-m7* line was induced in a large-scale tagging experiment. In the cross performed, the female parent was the line A69Y*wx-m7 a1-m3* carrying the elements *Ac* and *Ds* at the loci *Wx* and *A1*, respectively. The pollen donor was a *v1-ref* homozygous line. The experiment resulted in the isolation of 62 F1 seedlings with green and white sectored leaves out of 750000 seeds grown at 18 °C. These F1 plants were self-pollinated and 26 F2 progenies were found giving a ratio close to 3:1 for green and *virescent* plants, indicating that in these cases the somatic instability of their F1 progenitor plants was not heritable. In 36 cases the variegated phenotype reappeared in the F2 generation. The frequency of induction of mutable *v1* alleles was equal to $4{\cdot}8 \times 10^{-5}$. The alleles analysed further were named *v1-m1*, *v1-m2*, *v1-m4*, *v1-m9*, *v1-m11*, *v1-m12*, *v1-m13*, *v1-m17*, *v1-m18*, *v1-m21* and *v1-m23*.

The unstable alleles obtained can be grouped into four phenotypic classes (Fig. 1). The first comprises *v1-m1*, *v1-m9*, *v1-m11*, *v1-m17* and *v1-m18*, which show in F1 a phenotype with few revertant green sectors. In the F2 progenies of these five mutants, variegated phenotypes were shown by progeny-testing to be linked to the *wx-m7* allele which carries an *Ac* copy at the *Wx* locus. The segregation ratios for the F2 generations of *v1-m1*, *v1-m9*, *v1-m11* and *v1-m17* fit acceptably a ratio of 3 variegated plus wild-type: 1 *virescent*, and support the assumption that the mutations are either due to the interaction of two genetic factors tightly linked on the same chromosome – one of them residing at the *V1* locus – or to an autonomous element inserted into the *V1* locus (Table 2). A segregation ratio differing significantly from the expected ones was found for *v1-m18*; there was a lower than expected number of variegated seedlings. Wild-type phenotypes were found in all F2 of this first class of *v1-m* alleles, albeit at differing frequency. The highest fraction of green plants was detected in the progenies derived from *v1-m11* (27·4%), followed by those from *v1-m17* (12·8%), *v1-m9* (9·9%), *v1-m18* (8·6%) and *v1-m1* (2·3%). 'Near green' seedlings which showed up in the F2 of *v1-m1*, *v1-m11* and *v1-m18* have been included in the wild-type fraction. These 'near green' phenotypes are not variegated; their colour is a slightly paler green than the wild-type. They may represent reversions to wild-type with only a partial restoration of the *V1* function. Allelism tests carried out against the standard *v1-ref* allele showed that all of the class 1 variegated mutants were alleles of the *V1* locus. Segregation ratios found in these allelism tests were very close to 1 variegated plus green: 1 *virescent*, as expected for a progeny of an F1 plant with the genotype *v1-m/v1-ref* crossed to the strains B37*v1-ref* or Oh43*v1-ref* (Table 3). Thus *v1-m1*, *v1-m9*, *v1-m11*, *v1-m17* and *v1-m18* are unstable mutants of the *V1* gene, putatively derived from the transposition of an element to *V1*. Based on the segregations reported in Tables 2 and 3, it is, however, not possible to assess if an autonomous element, like *Ac*, or a non-autonomous element, like *Ds*, is now present at the locus. The wild-type phenotypes reported in Table 3 appeared with frequencies lower than those recorded in Table 2, albeit with the same genotypic order, i.e. 8·6%, 5·7%, 2·7%, 2·5% and 1·2% for the *v1-m11*, *v1-m17*, *v1-m9*, *v1-m18* and *v1-m1*, respectively.

A test for establishing the autonomous or non-autonomous nature of the element present at *V1* locus in the mutants *v1-m1*, *v1-m9*, *v1-m11*, *v1-m17* and *v1-m18* was carried out by selfing randomly chosen variegated F2 plants. Table 1 gives the expected segregation ratios of these families when still two or three phenotypic classes are segregated: wild-type, variegated and *virescent* or wild-type and *virescent*. According to those ratios, the occurrence of F3 families with 50% variegated plus wild-type phenotypes, and 50% *virescent* seedlings is proof of the non-autonomy of the element present at the *V1* locus in the

https://doi.org/10.1017/S0016672300034650 Published online by Cambridge University Press

Table 2. *Segregation ratios found in F2 progenies of 11 independent unstable* virescent-1 *mutants. At the* V1 *locus, the F1 plants had the putative genetic constitution* v1-m/v1-ref

Mutant	Number of plants			$X^2_{3:1}$†	$X^2_{71:29}$‡
	Wild-type	Variegated	*virescent*		
v1-m1	2‖	63§	23	0·1	0·3
v1-m2	4	63	30	1·8	0·2
v1-m4	69‖	—	27	0·5	0·0
v1-m9	8	60§	16	1·6	3·9*
v1-m11	32‖	42§	28	0·3	0·1
v1-m12	14	38	24	1·7	0·3
v1-m13	16	58	26	0·1	0·4
v1-m17	10	48§	20	0·0	0·4
v1-m18	6	36§	28	8·4**	4·2*
v1-m21	28	42	16	1·9	4·4*
v1-m23	4	68	20	0·2	2·2

† This segregation (75% wild-type + variegated and 25% *virescent* seedlings) is expected when an autonomous element has transposed to the *V1* locus generating a *v1-m* allele.
‡ This segregation (71% wild-type + variegated and 29% *virescent* seedlings) is expected when on the chromosome 9 an *Ac* element still is resident at the locus *Wx* and a second non-autonomous element has been transposed into *V1*.
‖ Pale green phenotype not variegated.
§ Around 20% of the seedlings show a phenotype with reduced number or size of somatic reversions.
* Significant at $P = 0{\cdot}05$.
** Significant at $P = 0{\cdot}01$.

mutable alleles (classes aN and dN in Table 1). The data in Table 4 supports the theory of the existence of classes aN and dN for the alleles *v1-m1*, *v1-m9*, *v1-m11*, *v1-m17* and *v1-m18* generated by insertions of non-autonomous *Ac* responsive elements (= *Ds*).

The second class of unstable virescent mutant only included the *v1-m4* strain. The phenotype of this mutant in the F2 was indistinguishable from the wild-type, except for the rare presence of very small white sectors on its leaves. This mutant corresponded to the phenotype described as a 'reverse' type of variegation (McClintock, 1951; Nevers, Sheperd & Saedler, 1986). In the F3 generation, plants occasionally appeared with an extremely variegated phenotype. The F3 progenies of variegated F2 plants showed, moreover, a broad range of variegated phenotypes, going from almost green seedlings, as in the F2, to a variegation similar to the one described for *v1-m1*. There were also easily recognizable stable green phenotypes (either pale or fully green) from the F2 of *v1-m4*. According to the data of Table 4, and based on the same rationale used for the mutants of the first class of *v1-m* alleles, *v1-m4* is also a result of the insertion of a *Ds*-like element into *V1*.

The third class of induced mutable alleles was represented by *v1-m2* and *v1-m13*, which exhibit a coarse pattern of variegation (Fig. 1). The phenotype was noted in the F1, F2, F3 and test-cross generations of the two mutants. Reversions to wild-type appeared in F2 and test-crosses with a frequency of 4% and 6% for *v1-m2* and *v1-m13*, respectively (Tables 2 and 3). As noted above, the results reported in Table 4 suggest that the *v1-m2* and *v1-m13* mutations are due to a cis responsive non-autonomous element inserted at the *V1* gene.

The fourth class of mutants comprises *v1-m12*, *v1-m21* and *v1-m23*. They frequently showed leaves part green with white stripes and part white with green stripes. Such sectors were clearly visible in the variegated F2 and F3 plants (Tables 3 and 4); reversions to the green phenotype appeared with a high frequency. The genetic segregation data in the selfed F3 families which gave a ratio of 1 wild-type plus variegated: 1 *virescent* plant indicates that *v1-m12*, *v1-m21* and *v1-m23* alleles represent new insertions of a non-autonomous genetic element (Table 4).

(ii) *The genetical basis of the* v1-m1 *and* v1-m4 *alleles*

All the F1 plants selected as putative *v1-m* phenotypes produced a proportion of F2 progenies having variegated *waxy* kernels, with segregation ratios close to 3*Waxy*: 1 *wx-mutable*. The new induced *v1-m* alleles were, in fact and as expected, linked on chromosome 9 to *Ac*. In such situation, it was difficult either to test the genetic nature of the element present at the *V1* locus, or to assign to such element a non-autonomous or an autonomous behaviour (as discussed previously). The two alleles *v1-m1* and *v1-m4* were selected to derive chromosome 9 bearing the

https://doi.org/10.1017/S0016672300034650 Published online by Cambridge University Press

Table 3. *Segregation ratios found in progenies of 11 independent unstable* virescent-1 *mutants when F1 variegated plants with the putative genetic constitution* v1-m/v1-ref *were test crossed to two different genetic strains homozygous for the* v1-ref *allele*

Mutant	Background of the *v1-ref*, *v1 ref* parent	Number of plants			$X^2_{1:1}$†	$X^2_{46\cdot5:53\cdot5}$‡
		Wild-type	Variegated	*virescent*		
v1-m1	B37	4	76	86	0·2	0·2
	Oh43	4	23	24	1·8	0·8
v1-m2	B37	6	71	80	0·1	0·4
	Oh43	8	13	26	0·5	0·1
v1-m4	B37	5	45	48	0·0	0·8
v1-m9	B37	6	99	115	0·4	0·1
v1-m11	B37	16	80	104	0·3	0·2
	Oh43	4	17	22	0·0	0·0
v1-m12	B37	11	71	62	2·7	6·3*
	Oh43	12	14	26	0·0	0·2
v1-m13	B37	24	48	78	0·2	0·1
	Oh43	8	16	23	0·0	0·2
v1-m17	B37	9	66	65	0·7	2·8
	Oh43	5	18	16	0·9	2·0
v1-m18	B37	4	80	64	2·7	6·2*
	Oh43	9	15	18	0·6	1·5
v1-m21	B37	16	68	106	2·5	0·4
	Oh43	4	17	29	1·3	0·4
v1-m23	B37	4	106	102	0·3	2·5
	Oh43	—	25	29	0·3	0·0

† This segregation (50% wild-type + variegated and 50% *virescent* seedlings) is expected when an autonomous element has transposed to the *V1* locus.
‡ This segregation (46·5% wild-type + variegated and 53·5% *virescent* seedlings) is expected when on chromosome 9, an *Ac* element still is resident at the locus *Wx* and a second non-autonomous element has been transposed into *V1*.
* Significant at $P = 0{\cdot}05$.

Table 4. *Segregation ratios found F3 progenies of 11* virescent-1 *unstable mutants. The F1 plants had the putative genotype* v1-m/v1-ref. *F2 variegated plants were chosen at random and selfed. Only families with a clearly segregating* virescent *phenotype are considered*

Mutant	No. of progenies considered	No. of families with segregation ratios† compatible both with autonomy or non-autonomy of the element at *V1* (F3 classes aA, aN, bA, bN, cA, cN, dA, dN, eA and eN of Table 1)	Families with segregation ratios compatible only with non autonomy of the element at *V1* from a second element (F3 classes aN and dN of Table 1)					
			No.	Wild-type	Variegated	*virescent*	$\chi^2_{1:1}$	$\chi^2_{3:1}$
v1-m1	12	10	2	2	36	46	1·1 ns	37·4**
				1	34	42	0·7 ns	36·3**
v1-m2	17	14	3	8	38	48	0·0 ns	37·1**
				2	46	48	0·0 ns	32·0**
					40	52	1·6 ns	48·7**
v1-m4	7	5	2	1	46	44	0·1 ns	26·5**
				4	46	38	1·6 ns	15·5**
v1-m9	13	10	1	16	28	40	0·2 ns	22·9**
v1-m11	9	7	1	4	44	40	0·7 ns	19·6**
v1-m12	15	14	1	6	46	38	2·2 ns	14·2**
v1-m13	11	9	2	2	42	48	0·2 ns	36·2**
				18	28	42	0·2 ns	24·2**
v1-m17	11	9	1	1	32	40	0·7 ns	34·6**
v1-m18	9	8	1	4	38	40	0·0 ns	25·3**
v1-m21	12	10	1	18	12	26	0·3 ns	13·7**
v1-m23	11	9	1	4	48	58	0·3 ns	45·1**

† See Table 1 for expected segregation ratios.
ns and ** Not significant and significant at $P = 0{\cdot}01$, respectively.

https://doi.org/10.1017/S0016672300034650 Published online by Cambridge University Press

Table 5. *Segregation ratios found in F2 progenies from F1 crosses between the* v1-m1 *and* v1-m4 *stable homozygous mutant plants and the A69Y* wx-m7 *and* P-vv *strains carrying active* Ac *copies*

Allele	Source of *Ac*	Number of F2 progenies	Number of plants		
			Wild-type	Variegated	*virescent*
v1-m1	*wx-m7*	6	590	32	164
	P-vv	5	316	61	32
v1-m4	*wx-m7*	5	408	22	96
	P-vv	5	331	64	32

newly induced *v1-m* allele but without the active *Ac* element present in *wx-m7*. The experiment was designed to obtain a more direct test for the autonomy or non-autonomy of the element present at *V1*. Using homozygous *wx-m7 v1-m* plants as the female parent crosses were made with the line A69Y *wx V1* which carries no active *Ac*. The resulting F1 plants were self-pollinated and 600 F2 seeds with a stable *wx* phenotype were selected (this phenotypic choice, however, included also some *wx-m* seeds with low level of variegation classified as *wx* stable kernels). The plants obtained were selfed and out of their F3 families, two (0·3%) were found which were homozygous *wx* and had a stable *virescent* phenotype. In crosses of homozygous *wx v1-m1*(nv) plants to *C-I Ds* teter, 28 F1 ears showed no kernels sectors with the *C sh bz wx* phenotype since *Ds* does not initiate chromosome breakage in absence of active *Ac*. The *wx v1-m1*(nv) plants were also crossed with *bz-m2(DI)* tester plants. Examination of the kernels on the F2 ears indicated that *v1-m1*(nv) carrying plants had no active *Ac* in their genome since no purple sectors on a *bronze* background appeared in non-*Bz* kernels. This result can be interpreted as deriving from: (1) The transposition without reintegration of the *Ac* element from *wx-m7*, with the generation of a *wx* stable allele; if the *v1-m* allele was generated by a *Ds*-like element, the expected phenotype at the *V1* locus would be the stable selected one, and the result will have demonstrated the non-autonomous nature of the *v1-m* allele. (2) A recombination event internal to the chromosome region *Wx-V1* generating a *wx v1-m* recombinant chromosome – this possibility supports the same conclusion as (1). (3) Generation of a *v1-m* non-autonomous allele from an autonomous one, either coupled to the loss of *Ac* from the genome while generating a *wx* stable allele, or to a recombination event in the *Wx-V1* region. This third possibility seems unlikely because it entails several independent events to generate the *wx-v1-m* chromosome.

If either (1) or (2) is the explanation for the generation of the *wx v1-m* strain, the non-autonomous element present in *v1-m1* and *v1-m4* should cause variegated phenotypes when exposed to *Ac*. The *wx v1-m* plants with a stable *virescent* phenotype (derived from the alleles *v1-m1* and *v1-m4*) were crossed with the genotype A69Y *wx-m7 a1-m3*. As expected, segregation ratios close to 75% green: 22% *virescent*: 3% variegated were found in the F2 progenies (Table 5). Only the plants directly derived from *wx*-variegated kernels, and thus having an active copy of *Ac*, showed a variegated *virescent* phenotype. In experiments similar to this one, the *Ac* element at the *P* locus was used to reactive the *wx v1-m* phenotypes. Crosses with *P-vv* again gave *virescent* and variegated phenotypes in F2.

While the *v1-m2*, *v1-m9*, *v1-m11*, *v1-m12*, *v1-m13*, *v1-m17*, *v1-m18*, *v1-m21* and *v1-m23* mutants also produced in proper crosses stable *virescent* phenotypes, they have not yet been assayed for their capacity to be transactivated by *Ac*.

(iii) *Cosegregation of the* v1-m1 *phenotype with a* Ds-*like fragment*

The genetic analysis on *v1-m1* showed that the expression of its variegated phenotype depends on the presence in the genome of an *Ac* transposable element. This was taken as evidence that in this unstable mutant a *Ds*-like element was present at the *V1* locus. Non-autonomous elements like *Ds* are not considered particularly useful for gene tagging experiments because there are so many copies of them in the maize genome (Döring, 1989; Walbot, 1992). In the search for an absolute linkage of *Ds* sequences to the mutant phenotype, this problem was circumvented by using restriction enzymes sensitive to methylation.

Southern blot analysis was performed on DNA from progenies derived from *v1-m1* by using two different molecular probes, the 0·74 kb external *Bss*H II-*Pvu* II fragment of *Ac* and its 1·6 kb internal *Hind* III fragment (Müller-Neumann, Yoder & Starlinger, 1984).

Genomic DNA from *v1-m*(nv) phenotypes not having active *Ac*'s in their genome and from the *V1 wx-m7* parental line was digested with the restriction endonucleases *Kpn* I, *Pst* I, *Sal* I, *Sst* I and compared to genomic DNA preparations of *virescent* and variegated plants. These were isolated from the F2 progenies of the trans-activation experiment and had the genotype *v1-m*(nv)/*wx v1-m*(nv)/*wx* and *v1-m*/*wx-m7 v1-m*/*wx-m7*, respectively. If a *Ds*-like element was

https://doi.org/10.1017/S0016672300034650 Published online by Cambridge University Press

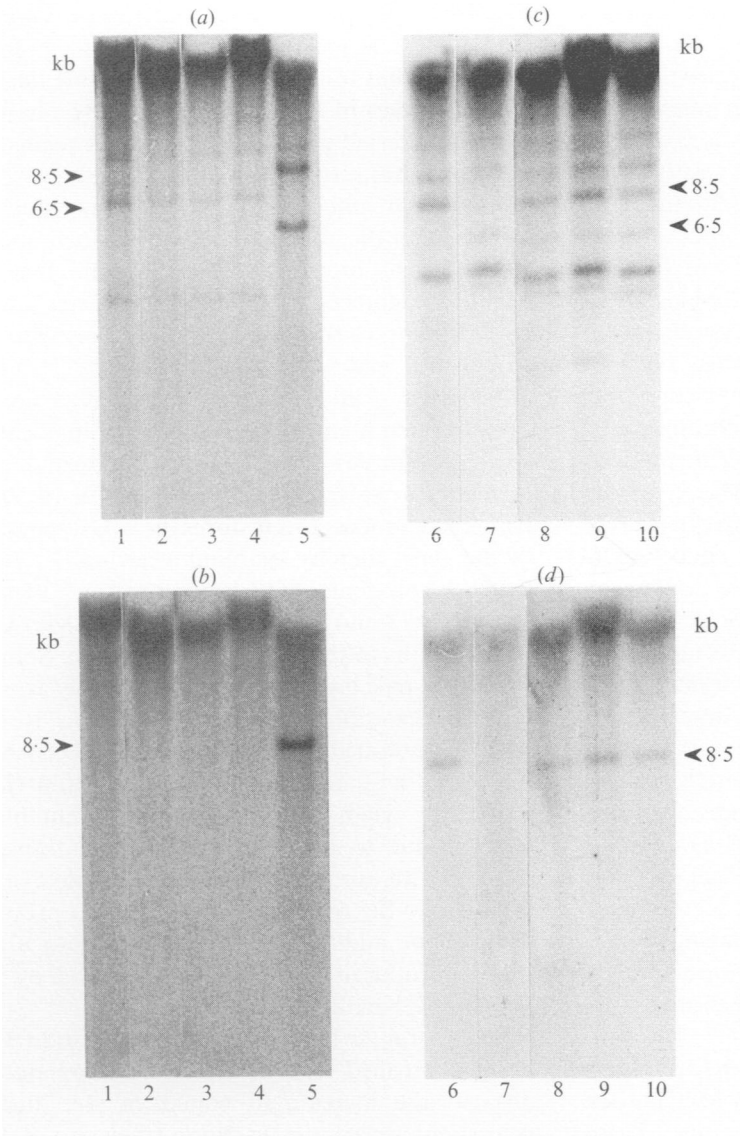

Fig. 2. Southern blot analysis of genomic DNA from: *a*, *b* – 1–4, homozygous *v1-m1*(nv) plants derived from *v1-m1* by the elimination of the active *Ac* copy present at *wx-m7* from the genome; – 5, the progenitor genotype *V1 wx-m7 a1-m3*. *c*, *d* – 6, wild-type plant, – 7, stable *virescent* plant, and – 8–10, variegated plants, selected out of the F2 of the cross *wx V1* x *wx-m7 v1-m1*. The DNA was restricted with *Sal*I and either hybridized to the 0.74 kb *Bss*H II-*Pvu* II (*a*, *c*), or to the 1·6 kb *Hind*III (*b*, *d*) *Ac* fragments (Müller-Neumann, Yoder & Starlinger, 1984).

present in the *v1-m* allele at the *V1* locus, a hybridization fragment could be expected to characterize the *v1-m*(nv) plants and not the progenitor strain *V1 wx-m7*. This turned out to be the case (Fig. 2*a*, *c*): a 6·5 kb band was present or absent in the two genotypes, respectively, when their DNA was digested with *Sal*I and hybridized to the 0·74 kb *Bss*H II-*Pvu* II 5′ end of *Ac*. In segregating F2 families from the cross *V1 wx* x *v1-m wx-m7*, the resulting *Sal*I fragment was linked to either the stable or the variegated *virescent* phenotype (25 variegated and 16 stable *virescent* F2 seedlings were tested). The 6·5 kb fragment did not, however, hybridize to the internal *Ac* probe (Fig. 2*b*, *d*), indicating that the element residing in *v1-m1* at the *V1* locus was a defective *Ac* copy. The hybridization pattern of Fig. 2*b*, *d* indicates, moreover, that a 8·5 kb band is present in *v1-m* variegated and in green genotypes, and absent in stable *v1-m*(nv) phenotypes, that is in plants without an active copy of *Ac*. This 8·5 kb corresponds both genetically and in size to the active copy of *Ac* present in *wx-m7*.

5. Discussion

Transposons are discrete genetic units that alter gene expression by inserting in or near an active gene. In maize, the genetics of several systems of transposable elements has been developed in detail (McClintock,

https://doi.org/10.1017/S0016672300034650 Published online by Cambridge University Press

1984; Peterson, 1987), and cloned transposons are now tools to isolate loci whose gene product is unknown (Walbot, 1992). The first step in this approach is the isolation by transposition mutagenesis of mutable alleles at the loci of interest.

The gene product of the *Virescent-1* locus is as yet unknown. To tag the *V1* locus we decided to move *Ac* from the allele *wx-m7* to *V1*. Both loci are on chromosome 9 separated by 7 cM, and our findings show that the *V1* locus can accept *Ds*-like DNA sequences that interfere with gene expression. In our genetic materials the frequency of this event is $4{\cdot}8 \times 10^{-5}$. This value is not particularly high compared to those cited by Döring (1989) for intrachromosomal transposition. McClintock (1952), experimented on genetic material in which *Ds* maps 3 cM apart from the *Sh* locus and obtained transposition frequencies of $3{\cdot}7$–10^{-3}. Frequencies of $2{\cdot}9 \times 10^{-3}$ are cited for *Ac* transposition at the *P-vv* locus, when the transposon integrates in the same chromosome at a distance ranging from 0 to 4·4 cM (references in Döring, 1989).

The presence of *Ds* close to or in the *V1* transcription unit induces several different effects; as described by Alleman & Kermicle (1993) where 43 mutants resulted from inserting of the transposable element *Ds* into the *R* locus. Under specific temperature conditions, gene action at *V1* is inhibited in the *v1-m1*, *v1-m9*, *v1-m11*, *v1-m17* and *v1-m18* alleles. In the presence of *Ac*, these mutants revert somatically (green sectors on a white background) and germinally, producing phenotypes indistinguishable from the wild-type (but in some cases stable 'near-green' reversions were observed; see results).

The phenotype of the mutations *v1-m12*, *v1-m21* and *v1-m23*, matches the one reported by McClintock (1948) for the *c-m2* and *wx-m1* mutants. These mutations, which can be seen at the seed level, evince adjacent sectors marked by contrasting gradations in the intensity of expression of the wild-type trait. In our experiment, *v1-m12*, *v1-m21* and *v1-m23* plants showed leaf sectors with marked colour-intensity variations, ranging from very pale to dark shades. According to McClintock, the sectors show a reverse pattern of visible mutations deriving from a gain or a loss at the locus of the gene units which control the functioning of the locus (state of the locus) during mitosis as well as the appearance of the mutation itself.

The phenotype of *v1-m2* and *v1-m13* alleles corresponds to a coarse pattern of variegation already visible in F1.

The phenotype of the *v1-m4* mutation (recessive white stripes on green background) matches the one known as 'reverse variegation' (McClintock, 1968). Similar phenotypes were also described for the *o2-m1*, *ø2-m3* and *ø2-m4* mutants resulting from insertions of *Ds* at the *Opaque-2* locus (Motto *et al.* 1986), as well as for other alleles induced at several loci by the *En/Spm* system (Peterson, 1980). Nevers, Sheperd & Saedler (1986) postulate that in 'reverse variegation' the element is located in the control region of the locus and that transcription begins inside the element and continues in the locus. An inactive phase of the element inserted at the locus, or of its regulator, can interrupt transcription, thereby producing mutant sectors. An alternative assumption (McClintock, 1951) is that the insertion of *Ds* at the locus may cause chromosome breaks followed by the loss of the acentric fragments which harbor the locus, a phenomenon leading to the formation of tissues with mutant clones in a wild-type background.

Mutable *v1* alleles produce, upon reversion, green or pale green plants that are easily distinguished from variegated or *virescent* phenotypes. Green derivatives probably arise from the transposition of *Ds* away from the *V1* locus; this restores the wild-type condition of the gene, thereby enabling a normal *V1* function. Selfed F3 progenies of the mutants *v1-m2*, *v1-m11*, *v1-m12*, *v1-m13* and *v1-m21* show a very high frequency of reversions to the wild-type phenotype. Somatically stable but pale green derivatives probably arose by an imprecise excision of *Ds* from the *V1* locus. Revertants with an intermediate phenotype have been reported for *bz-m4* as a result of *Ds* transposition (Dooner, 1980). The origin of such derivatives of mutable alleles may be due to an integration of the transposable element with exons, leading, upon complete or partial excision, to the restored production of a protein with changed or additional aminoacids, or to a normal protein produced at lower levels (reviewed by Nevers, Sheperd & Saedler, 1986).

Studies on *Ac* indicate that the time and frequency of *Ac*-controlled somatic mutations correlate with its dosage; the increase in copies of *Ac*, during development, delays the mutation (excision) of *Ds*. Our data show that the *v1-m1* and *v1-m4* mutations require an active *Ac* for the expression of their somatic instability. In our experiments, we noted that heterozygous seedlings segregating in the backcross progenies of the two mutants, had a clear cut variegated phenotype. In contrast, variegated plants segregating in F2 progenies of these or other *v1-m* alleles, had a proportion of poorly variegated phenotypes; there were less reversions and they were of smaller size. If the plants are assumed to be homozygous for *Ac*, which we have not directly verified, this observation indicates that an increase in the dosage causes either a delayed or a less frequent excision of the *Ds* element. There are other examples of *Ds* insertions, such as one at *c-m1* and *wx-m1* (McClintock, 1949), in which an increase in *Ac* dosage results in a few very late-occurring *Ds* mutations.

The frequency of somatic mutation of alleles controlled by *Ds* is difficult to analyse (McClintock, 1948). Beside interference of the *Ac* dosage discussed earlier, changes in *Ac* activity or phase create special somatic patterns of instability (McClintock, 1949). A third phenomenon may involve phase changes of the

Ds element itself, causing a different reaction to *Ac* dosage (Weil *et al.* 1992).

The *Ac* activity on the element at the locus *V1* was genetically assessed for the allele *v1-m1* because of its demonstrated capacity to destabilize a *Ds* element resident at the locus. Our findings also indicate that the *Ac* resident at the locus *P-vv* activates the *Ds* at the alleles *v1-m1* and *v1-m4* in a different way to the *Ac* at *Waxy* locus on chromosome 9. This lends support to the assumption of the differential action of *Ac* elements on different *Ds* elements (Rhoades & Dempsey, 1982; Hake, Vollbrecht & Freeling, 1989).

The *Ds* elements are not seen as suitable transposons for a tagging experiment because of the high number of copies in the genome (Döring, 1989). This problem has been overcome by using the parts of the element which only hybridize with a few genomic copies for molecular probes (Wienand & Saedler, 1987), or by marking the same gene with two different transposons (O'Reilly *et al.* 1985). Moreover, *Ds* elements like *Ds1* exist that do not hybridize with the autonomous *Ac* element. In our experiments, the general approach of searching for an absolute association between a DNA fragment revealed by a transposon probe and a mutant phenotype was an effective solution even when a high number of copies of the element were present. Using this method, via the element *Ds2* isolated from allele *sh-m5933*, the gene *Bz2* (Theres, Scheele & Starlinger, 1987) and the mutation *Kn1-2F11* (Hake, Vollbrecht & Freeling, 1989) were cloned.

We are especially indebted to Francesco Salamini for encouraging support of this work and many suggestions for the final draft of the whole manuscript. We thank Carlo Lorenzoni for critical reading of the manuscript; Francesca Baruffaldi, Alberto Gianinetti and Giacomo Zapparoli for helping with some of the experiments. This research was funded by Ministero delle Risorse Agricole, Alimentari e Forestali grant 'Resistenze genetiche delle piante agrarie agli stress biotici ed abiotici'.

References

Alleman, M. & Kermicle, J. L. (1993). Somatic variegation and germinal mutability reflect the position of transposable element *Dissociation* within the maize *R* gene. *Genetics* **135**, 189–203.

Brink, R. A. & Nilan, R. A. (1952). The relation between light variegated and medium variegated pericarp in maize. *Genetics* **37**, 519–544.

Chollet, R. & Paolillo, D. J. (1972). Greening of a *virescent* mutant of maize. I. Pigment, ultrastructural, and gas exchange studies. *Zeitschrift für Pflanzenphysiologie* **68**, 30–44.

Dellaporta, S. L., Woods, J. & Hicks, J. B. (1983). A plant DNA mini-preparation: Version II. *Plant Molecular Biology Reporter* **1**, 19–21.

Dooner, H. K. (1981). Regulation of the enzyme UFGT by the controlling element *Ds* in *bz-m4*, an unstable mutant in maize. *Cold Spring Harbor Symposia on Quantitative Biology* **55**, 457–462.

Dooner, H. K. & Belachew, A. (1989). Transposition pattern of the maize element *Ac* from the *bz-m2(Ac)* allele. *Genetics* **122**, 447–457.

Döring, H. P. (1989). Tagging genes with the maize transposable elements. An overview. *Maydica* **34**, 73–88.

Edwards, G. E. & Jenkins, C. L. D. (1988). C4 photosynthesis: Activities of photosynthetic enzymes in a *virescent* mutant of maize having a low-temperature-induced chloroplast ribosome deficiency. *Australian Journal of Plant Physiology* **15**, 385–395.

Feinberg, A. P. & Vogelstein, B. (1983). A technique for radiolabelling DNA restriction nuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.

Hake, S., Vollbrecht, E. & Freeling, M. (1989). Cloning *Knotted*, the dominant morphological mutant in maize using *Ds2* as a transposon tag. *EMBO Journal* **8**, 15–22.

Hopkins, W. G. & Walden, D. B. (1977). Temperature sensitivity of *virescent* mutants of maize. *Journal of Heredity* **68**, 283–286.

Hopkins, W. G. & Elfman, B. (1984). Temperature-induced chloroplast ribosome deficiency in *virescent* maize. *Journal of Heredity* **75**, 207–211.

King, J. (1991). *The Genetic Basis of Plant Physiological Processes*. New York: Oxford University Press.

McClintock, B. (1948). Mutable loci in maize. *Carnegie Institution of Washington Year Book* **47**, 155–169.

McClintock, B. (1949). Mutable loci in maize. *Carnegie Institution of Washington Year Book* **48**, 142–154.

McClintock, B. (1951). Chromosome organization and genic expression. *Cold Spring Harbor Symposia on Quantitative Biology* **16**, 13–47.

McClintock, B. (1952) Mutable loci in maize. *Carnegie Institute of Washington Year Book* **51**, 212–219.

McClintock, B. (1962). Topographical relations between elements of control systems in maize. *Carnegie Institution of Washington Year Book* **61**, 448–461.

McClintock, B. (1968). The state of a gene locus in maize. *Carnegie Institution of Washington Year Book* **66**, 20–28.

McClintock, B. (1984). The significance of responses of the genome to challenge. *Science* **226**, 792–801.

Millered, A. & McWilliam, J. R. (1968). Studies on a maize mutant sensitive to low temperature. II. Influence of temperature and light on the production of chloroplast pigments. *Plant Physiology* **43**, 1967–1972.

Moreno, M. A., Cheng, J., Greenblatt, I. & Dellaporta, S. L. (1992). Reconstitutional mutagenesis of the maize *P* gene by short-range *Ac* transposition. *Genetics* **131**, 939–956.

Motto, M., Marotta, R., Di Fonzo, N., Soave, C. & Salamini, F. (1986). *Ds*-induced alleles at the *Opaque-2* locus of maize. *Genetics* **112**, 121–133.

Müller-Neumann, M., Yoder, J. & Starlinger, P. (1984). The DNA sequence of the transposable element *Ac* of *Zea mays* L. *Molecular and General Genetics* **198**, 19–24.

Nevers, P., Sheperd, N. S. & Saedler, H. (1986). Plant transposable elements. *Advances in Botanical Research* **12**, 103–203.

O'Reilly, C., Sheperd, N. S., Pereira, A., Schwarz-Sommer, Z., Bertram, I., Robertson, D. S., Peterson, P. A. & Saedler, H. (1985). Molecular cloning of the *a1* locus of *Zea mays* using the transposable elements *En* and *Mu*. *EMBO Journal* **4**, 877–882.

Peterson, P. A. (1980). Instability among the components of a regulatory element transposon in maize. *Cold Spring Harbor Symposia on Quantitative Biology* **45**, 447–455.

Peterson, P. A. (1987). Mobile Elements in Plants. *CRC Critical Reviews in Plant Sciences* **6**, 105–208.

Phinney, B. O. & Kay, R. E. (1954). Interaction of environment and genotype in the expression of *a virescent* gene, *pale-yellow-1*, of maize. *Hilgardia* **23**, 185–196.

Polacco, M., Chang, M. T. & Neuffer, M. G. (1985). Nuclear, *virescent* mutants of *Zea mays* L. with high levels

of chlorophyll (a/b) light-harvesting complex during thylakoid assembly. *Plant Physiology* **77**, 795–800.

Polacco, M., Vann, C., Rosenkranz, L. & Harding, S. (1987). Nuclear genes that alter assembly of the chlorophyll a/b light-harvesting complex in *Zea mays*. *Developmental Genetics* **8**, 389–403.

Rhoades, M. M. & Dempsey, E. (1982). The induction of mutable systems in plants with the high-loss mechanism. *Maize Genetics Cooperation Newsletter* **56**, 21–26.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

Theres, N., Scheele, T. & Stalinger, P. (1987). Cloning of the *Bz2* locus of *Zea mays* using the transposable element *Ds* as a gene tag. *Molecular and General Genetics* **209**, 193–197.

Walbot, V. (1992). Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. *Annual Review of Plant Physiology* and *Plant Molecular Biology* **43**, 49–82.

Weil, C. F., Marillonnet, S., Burr, B. & Wessler, S. R. (1992). Changes in state of the *wx-m5* allele are due to intragenic transposition of *Ds*. *Genetics* **130**, 175–185.

Wienand, U. & Saedler, H. (1987). Plant transposable elements: Unique structures for gene tagging and gene cloning. In *Plant DNA Infectious Agents* (ed. B. Hohn and J. Schell), pp. 205–227. Wien/New York: Springer-Verlag.