Genetic (in)stability in tomato

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Stellingen

1) Het is onwaarschijnlijk dat de instabiliteit van het *yv*-locus in de cultivar Condine Red van tomaat veroorzaakt wordt door een transpositie element.
Dit proefschrift

2) Hoewel de aktiviteit van transpositie elementen belangrijk wordt geacht voor de evolutie van het plantegenoom, blijft hun rol daarin vooralsnog onbewezen.
McClintock B. (1956) Cold Spring Harbor Yearbook 21: 197-216
Schwartz-Sommer Z. and Saedler H. (1987) Mol. Gen. Genet. 209: 207-209

3) Onderzoek naar instabiele mutaties in planten zal kunnen leiden tot de identifikatie van epigenetische faktoren die gen expressie beïnvloeden.
Reuter G. and Spierer P. (1992) BioEssays 14: 605-612
Dit proefschrift

4) De door de "Gene List Committee" voor tomaat opgestelde regel het wild type allel van een recessief mutant gen aan te duiden met een kleine letter en een + - teken is onlogisch.
TGC Report (1979) 20: 3-5

5) Het progressieve verlies van expressie van een transgen in opeenvolgende generaties van *Arabidopsis* leert ons, dat niet alle mutante phenotypes na één ronde van zelfbevruchting zichtbaar zullen worden.
Kilby et al. (1992) Plant Mol. Biol. 20: 103-112

6) Transpositie elementen zoek je niet, die vind je.

7) Het getuigt van weinig realiteitszin te veronderstellen dat Nederlanders minder racistisch zouden zijn dan Duitsers.

8) Het onvermogen van politici in te grijpen in de situatie op de Balkan lijkt verdacht veel op onwil.

Stellingen behorende bij het proefschrift "Genetic (in)stability in tomato".
Ellen Wisman, Wageningen 17 september 1993.

NN08201,1658

Errata

- In Chapter 5, page 84, the 8th line of the section results should be: "seedlings, 9 carried green cotelydons, 20 variegated and 6 pure yellow ones. Similar to".

- In Chapter 5, the tables 3 and 5 on page 88 and 91, respectively, should be:

Table 3. Segregations into variegated and yellow plants in the selfed progenies of "green"and variegated plants carrying different number of fragments

Parent			Progeny			
Plant number	Phenotype	Number of fragments	Variegated (yv^{mut})	Yellow (yv^{ms})	% yv^{ms}	mean % yv^{ms}
3	variegated	0	174	142	45	
5	variegated	0	554	200	27	
11	variegated	0	176	225	56	
25	variegated	0	224	180	45	
40	variegated	0	237	162	41	
						43
4	variegated	1 (1s)	207	227	52	
23	variegated	1 (1s)	246	169	41	
						47
1	variegated	2 (2s)	190	142	43	
6	variegated	2 (1m+2s)	554	200	27	
13	variegated	2 (1l+1s)	239	195	45	
15	variegated	2 (1l+1s)	246	169	41	
27	variegated	2 (2s)	146	194	57	
29	variegated	2 (1l+1s)	222	225	50	
31	variegated	2 (1l+1s)	200	198	50	
						45
10	variegated	3 (3l)	185	287	61	
14	variegated	3 (3s)	276	94	25	
24	variegated	3 (2m+1s)	192	230	55	
26	variegated	3 (1l+2s)	170	138	45	
30	variegated	3 (1m+2s)	344	84	20	41
6	green	0	97	115	54	
8	green	0	358	122	25	
38	green	0	205	82	29	
						36
39	green	1 (1m)	211	201	49	
41	green	1 (1s)	287	105	27	
						38
21	green	2 (1l+1S)	318	159	33	
						33
9	green	3 (1m+2s)	312	131	30	
32	green	3 (2m+1s)	374	164	30	
						30

l=large fragment; m=medium sized fragment; s=small fragment

please turn over

Table 5. Distribution of several *Aps-1* alleles among green, variegated, and yellow F_2 plants derived from the cross N118 x Yv^{ms} (A) and L41 x Yv^{ms} (B)

A	*Aps-1*			
yv	1/1	+/1	+/+	Total
yv+/.	44	36	0	80
yv^{mut}/.	0	0	6	6
yv/*yv*	0	0	3	3
Total	44	36	9	89

B	*Aps-1*			
yv	3/3	+/3	+/+	Total
yv+/.	15	19	0	34
yv^{mut}/.	0	0	4	4
yv/*yv*	0	0	7	7
Total	15	19	11	45

Contents

Voorwoord

Dr. Ramanna heeft aan de basis gestaan van het project 'genetische instabiliteit in tomaat' waarvan dit proefschrift verslag doet. De vele discussies die ik met hem voerde hebben mij geholpen inzicht te krijgen in de moeilijke materie van de genetische instabiliteit. Hij wist zich altijd weer artikelen te herinneren die waardevol bleken voor het beantwoorden van de vragen opgeroepen door het onderzoek. Zijn enthousiasme voor de wetenschap werkte aanstekelijk en heeft me gestimuleerd om dit experiment af te ronden.

Maarten Koornneef is de beste begeleider waar men op zou kunnen hopen. Hoewel in het begin slechts zijdelings betrokken bij mijn project heeft hij mij altijd alle aandacht geschonken en daarnaast nog kasruimte beschikbaar gesteld ook. Het zal vreemd zijn om hem niet meer even te kunnen bellen voor advies.

De steun van Ab van Kammen gedurende deze jaren heb ik als zeer positief ervaren. Hoewel hij claimde geen expert op het gebied van genetica te zijn, hebben zijn scherpzinnige opmerkingen bijgedragen aan het helder formuleren van ingewikkelde genetische processen zowel in mijn gedachten als op papier. Hiervoor ben ik ook veel dank verschuldigd aan Pim Zabel die mij veel heeft bijgebracht op het gebied van mondelinge en schriftelijke rapportage. Dit resulteerde regelmatig in een lovend commentaar van de mensen die mijn artikelen beoordeelden. Ik hoop ook zonder Pim dit niveau in de toekomst te kunnen halen. Pim zorgde er dikwijls voor dat de koffie al stond te pruttelen als ik 's ochends wel erg vroeg op de vakgroep aankwam. Ook heeft hij mij, samen met Els Hulsebos, begeleid op mijn eerste schreden in de moleculaire biologie.

Waardevolle discussies heb ik gevoerd met vele mensen waarvan ik Jac Hille, Tom Gerats, Prof. Hagemann en Andy Pereira met name wil noemen. Zonder de hulp van mijn collega's op de vakgroepen plantenveredeling, moleculaire biologie en erfelijkheidsleer was het werk nooit af gekomen. Ook hebben zij ervoor gezorgd dat ik het uitstekend naar mijn zin heb gehad in mijn OIO periode. Al deze collega's wil ik dan ook hartelijk danken en in het bijzonder Hilda Kranendonk, Marian Bergervoet, Luuc Suurs, Jan Hontelez en Ruud Verkerk die het meeste werk hebben verzet. Ook de studenten Marcel Bruins, Ester Blok, André Drent, Teun de Vries en Guus Paardekoper hebben hun steentje bijgedragen. De planten zijn op uitstekende wijze verzorgd door de tuinmannen Jan van Schaik en Sjaak Jansen van de vakgroep plantenveredeling en Henk Kuiper, Willem van Blijderveen en Jan Laurens van de vakgroep erfelijkheidsleer. Tenslotte wil ik Corrie Hanhart nog noemen met wie ik altijd zo gezellig kon kletsen.

Scope

In the present study tomato lines carrying unstable alleles of the loci *yv* or *sulfurea* were characterized. In addition, we aimed at the isolation of an endogenous transposable element supposedly active in the unstable lines. Since the unstable loci were not cloned, we developed a transposon target system that enabled us to select for putative insertion mutants of the *Adh-1* gene. While investigating the genetic characteristics of the Yv^{ms} and Sulfurea line it became apparent that the instability of their marker loci was not necessarily related to the activity of transposition elements. Also, the instability could not be related to the activity of tomato sequences homologous to transposable elements from related species. This invited speculation about the stability of DNA sequences in tomato. It is proposed that mechanisms which are known to underlie genetic instability in other organisms, have a role in the unstable expression of *yv* and *sulfurea*. These alternative mechanisms have been summarized in Chapter 1. Chapter 2 describes the isolation of an EMS induced *Adh-1* null mutant and its use in crosses with the unstable tomato lines. In the resulting F_1 populations additional *Adh-1* null mutants were selected through the resistance of seeds or pollen grains to allyl alcohol (Chapter 4). The cloning of the entire *Adh-1* gene allowed the molecular analysis of the *Adh-1* null mutants (Chapter 3). Chapter 5 describes the cytogenetic and genetic characterization of the tomato line Yv^{ms}. The characteristics of a newly isolated unstable allele of the *sulfurea* locus are presented in chapter 6. Finally, chapter 7 describes the hybridization of tomato DNA to alien transposable element probes. These experiments emphasize the stability of transposable element-like sequences in the tomato genome.

Chapter 1

Genetic instability in plants

Introduction

The notion favored in the first half of this century that genomes evolve slowly, with mutations and recombination as the only source of variation, is more and more being challenged by new observations. Molecular studies on the organization and function of genomes have shown that they are rapidly being restructured by the rearrangement and transfer of DNA molecules (Shapiro 1992). A role of transposable elements in generating genetic diversity was first pointed out by McClintock who demonstrated that the instability of pigmentation alleles in variegated maize cobs was due to the activity of transposable elements (McClintock 1948). More recently, additional mechanisms directing genomic change have been identified. It appears that the amplification of single copy or repeated sequences is a common means of creating mutations in a single generation (Schneeberger et al. 1991, Rennie 1993). In more exceptional cases, DNA reprogramming systems are being employed to serve very specific purposes in the life-cycle of organisms as diverse as bacteria and mammals (for review see a special issue of Trends in Genetics, december, 1992).

Additional studies of genetic instability revealed mechanisms to exist that regulate gene expression by influencing chromatin structure, as opposed to alterating DNA sequence (Henikoff 1990, Rennie 1993, Porter 1991). The study of these so called epigenetic mutations has shown that it is unlikely that a single mechanisms is employed to ensure a stable inheritance of gene activity patterns (Jablonka et al. 1992). Interestingly, epigenetic mutations occur more frequently than would be expected for gene mutations affecting DNA sequence (Holliday 1989, Rennie 1993). Also in plants, a functional role for epigenetic factors in regular gene expression has recently been postulated (Meyer et al. 1992, 1993).

These examples illustrate means of altering the genome more rapidly than was previously thought. Some scientists have speculated that periods of elevated mutation rates lies at the root of the formation of new species (Rennie 1993), an idea that has been put forward several times earlier in this century (Lewis and Raven 1958). Also McClintock (1978, 1984) favoured the concept of rapid speciation on the basis of the observation that genomic change is inducible by any environmental condition perceived as stress by the genome.

Now that mechanisms directing genomic change have been identified, the question of how genomic change is controlled becomes pertinent. One method of addressing this question is to study the molecular basis of unstable gene mutations and subsequently identify genes capable of modifying their expression. Among the modifier genes thus identified in yeast and *Drosophila* were genes essential for the survival of the organism. In traditional mutagenetic studies such genes could not be recognized because the mutants were not viable. Similarly, genes without a mutant phenotype were identified (Arkipova and Ilyin 1992, Winston and Carlson 1992). Unstable gene mutations are often recognizable by the appearance of variegated patterns on somatic tissue. Instability is also recognized through the appearance of mutant progeny in non-Mendelian ratios. In this chapter, the results of the molecular investigations as

to the causes and regulation of genetic instability in plants will be reviewed in comparison to animal systems.

Transposable elements

Variegation, a common and notable phenomenon in both animals and plants, has attracted attention for a long time. As early as the twelfth century the beautifully striped rose *Rosa Gallica* L. *Versicolor* was introduced in England (Belder 1984), while Colombus introduced the variegated Indian ceremonial corn into Europe in the sixteenth century (see Gerats 1985). In the beginning of the century, de Vries (1900) reported many cases of instability, including mosaic patterns in flower color, seed pigmentation, and leaf color. In addition, unstable characters such as grain-, flower- and leaf morphology, branching and sterility were noted. The study of variegated mutants in maize led McClintock (1948) to postulate the presence of elements which are capable of moving in the genome. On the basis of genetic criteria, Nevers et al. (1986) prepared a list of unstable gene mutations in 35 different plant species, which were most likely caused by transposable element insertions. In particular, the appearance of stable revertants turns out to be an important genetic feature of insertion mutations.

Maize is a favorable genetic system for studying transposable elements, since their activity can be readily observed and analysed. For example, the maize endosperm characters are easily scorable and often indicate the phenotype of the kernel's embryo (Fincham and Sastry, 1974). Thus, rare mutations occurring in endosperm characters were not necessarily lost from the progeny and are availabe for genetic analysis. Many years after McClintock (1948) had drawn attention to the presence of mobile elements in the maize genome comparable elements were found in other organisms ranging from bacteria to mammals, indicating that transposable elements are a common component of genomes (Berg and Howe, 1989).

Molecular analysis has shown that these elements can be grouped into two main classes, according to their structural organization and mode of transposition (Finnegan 1989). The first class of elements, with the *Ac* element of maize and the *P* element of *Drosophila* as typical members, is characterized by the presence of inverted repeats flanking the element, and transposes via a "cut and paste" mechanism. These elements constitute families of autonomous and non-autonmous members. The autonomous members encode a protein (transposase) that is essential for transposition, whereas the non-autonomous members carry deletions and have lost their ability to transpose independently (Fedoroff 1989). From a number of plant species autonomous and non-autonomous elements have been isolated (Table 1).

The second class, the retrotransposons and retroposons, consists of elements that share structural similarities with retroviruses, transpose via an RNA intermediate and use reverse transcriptase. In contrast to the retroposons, the retrotransposons carry long terminal repeats in a direct orientation. In general retro(trans)posons occur in much higher copy numbers than the *Ac*-like elements (Table 2), probably as a result of their mode of transposition (Grandbastien

1992, Flavell et al. 1992, Smyth 1991).

Table 1. Characteristics of *Ac*-like transposable elements identified in several plant species

Name	Species	Size (kb)	IVRs (bp)	DRs (bp)	Type	Comments
Tam1	*Antirrhinum majus*	15	13	3	autonomous	Isolated from the unstable *niv^{rec}-53* allele*[1]
Tam2	*Antirrhinum majus*	5	14	3	autonomous	Isolated from the stable *niv-44* allele[1]
Tam3	*Antirrhinum majus*	3.5	12	5-8	autonomous	Isolated from the unstable *niv^{rec}-98* and *pal^{rec}-2* alleles[1]
Spm	*Zea mays*	8.3	13	3	autonomous	Isolated from the unstable *waxy-844* and *A* alleles[2]
Bg	*Zea mays*	4.9	5	8	autonomous	Isolated from the mutable *waxy-m32* allele[3]
Ac	*Zea mays*	4.6	11	8	autonomous	Isolated from an unstable *waxy* allele[2]
rDt	*Zea mays*	0.7	14	8	non-autonomous	Isolated from a stable recessive mutant of the *A1* gene[4]
dTph1	*Petunia hybrida*	0.3	12	8	non-autonomous	Isolated from an unstable *An1* allele[5]
Tst1	*Solanum tuberosum*	0.7	11	8	non-autonomous	Isolated as pre-existing insert in one of the (inactivated) copies of the patatin class II gene[6]
Ipsr	*Pisum sativum*	0.8	12	8	non-autonomous	Isolated as pre-existing insert in the spontaneous mutant wrinkled (*rr*) [7]
Tpc1	*Pisum crispum*	0.9	11	8	non-autonomous	Isolated as polymorphism between genomic clones of the *chalcone synthetase* gene[8]
Tat1	*Arabidopsis thaliana*	0.4	13	5	non-autonomous	Isolated as a polymorphisms between genomic clones of the *S-adenosylmethionine synthetase 1* gene[9]
Tag 1	*Arabidopsis thaliana*	3.3	22	6	ND	Isolated following its insertion in the *CHL1* gene[10]

Data are cited from the following references: Coen et al. 1989 (1), Fedoroff 1989 (2), Hartings et al. 1991 (3), Brown et al. 1989 (4), Gerats et al. 1990 (5), Köster-Töpfer et al. 1989 (6), Bhattacharyya et al. 1990 (7), Herrmann et al. 1988 (8), Peleman et al. 1991 (9), Tsay et al. (10). IVR=inverted repeat, DR= duplicated target site, ND=not determined

Molecular investigation of unstable alleles of maize and *Antirrhinum* has revealed many variants in gene-transposable element interactions (Fedoroff 1989, Coen et al. 1989). An insertion can affect gene expression either by disrupting the coding or regulatory sequences, or by making the gene responsive to the regulatory factors controlling the expression of the transposable element. The excision of a transposable element is often not precise, leaving the gene with footprints, such as duplications and deletions, thereby creating variant alleles (Döring and Starlinger 1984, Schwartz-Sommer et al. 1985, Nevers et al. 1986). Moreover, transposable element families have provided the genome with dispersed sites of sequence homology. Recombination between these sites has led to chromosomal rearrangements, which even included arms of different chromosomes (reviewed in Athma and Peterson 1991, Sheen et al. 1993). Since such rearrangements were not frequently observed, it was concluded that recombination between the dispersed repeats was repressed (Kricker et al. 1992). Recently, Kricker et al. (1992) proposed that an elevated mutation rate of G-C sites, present in the repeats, generated the sequence divergence necessary to bring about this repression. Presumably, this high mutation rate was achieved by the methylation of G-C sites, which are known to mutate frequently to A-T.

In maize it was shown that the *Ac* element induces recombination between two direct repeats of 15 kb length, thus adding a new type of instability to the repertoire of the *Ac* elements (Athma and Peterson 1992). In this particular example, the *Ac* element was inserted at the *P* locus, which confers a red pigmentation in several tissues. Apparently, the inserted Ac element generated with a high frequency large deletions, in which the entire 17 kb region between the two direct repeats flanking the *P* locus was removed. While the mutagenic effects of *Ac*-like elements in plants are well documented, little information is available on the impact of the numerous copies of retro-elements on gene expression and genome organization. Thus far, transposition events have been recorded only for the *Bs1* element of maize and the *Tnt1* element of tobacco, which were isolated from the inactivated *Adh1* and *nitrate reductase* genes, respectively. For other elements, insertional polymorphisms between varieties of the same species manifested their past activity (Grandbastien 1992).

As to the question of how genomic change is controlled, one must understand how the activity of transposable elements is regulated. It has been recognized for many years that transposition is induced by various types of genomic stress including chromosomal breakage, genomic wide hybridization (McClintock 1984, Mori et al. 1990), in vitro culture (Peschke and Phillips 1992) and mutagenic treatments (Walbot 1992). Moreover, increased levels of transposition were documented for the *Mu* and *Ac* elements of maize and the *P* element of *Drosophila* , when transferred to a host genome lacking these elements (Baker et al. 1985, Yoder 1990, Belzile and Yoder 1992, Engels 1989). This observation may indicate host specific regulation of transposition but may also be the result of genomic stress caused by the insertion of the T-DNA or by the propagation of plant cells in tissue culture. The same factors might have been responsible for the activation of an endogenous transposable element of

Table 2. Characteristics of (retro)transposons identified in plants

Name	Species	Size (kb)	LTRs (bp)	Copy number	DRs (bp)	Comments
Cin1	*Zea mays*		690	1000	5	Not found as part of a retrotransposon
Bs1	*Zea mays*	3.2	302	2-3	5	Isolated after transposition into *Adh1* gene after viral infection
Wis-2	*Triticum aestivum*	8.6	1755	200	5	Pre-existing insertion into glutelin gene, several related families
Tal	*Arabidopsis thaliana*	5.2	514	1-3	5	Isolated as a source of polymorphisms between ecotypes, several related families
Tnt1	*Nicotiana tabaccum*	5.3	610	>100	5	Isolated after transposition into nitrate reductase gene
dell	*Lilium henryi*	9.3	2400	>13,000	5	Detected as rapidly reannealing DNA
Tst1	*Solanum tuberosum*	5	283	1	5	Pre-existing insertion in *strach phosphorylase* gene
Tms1	*Medicago sativa*	0.4	120	ND	7	Pre-existing insertion in nodule specific gene
PDR1	*Pisum sativum*	5	156	50	5	Pre-existing insertion near *legumin* gene
IFG7	*Pinus radiata*	5.9	333	10,000	5	Isolated by cloning band of repetitive DNA
BARE-1	*Hordeum vulgare*	8.5	1733	multiple	5-6	Isolated on genomic library fragment
Cin4	*Zea mays*	1-6.5	non	50-100	3-16	Pre-existing insert in *A1* gene
p-SINE1	*Oryza sativa*	125	non	ND	10-15	Isolated as source of polymorphisms in the *waxy* gene between two rice species

The retrotransposons are listed above the line, the retroposons missing the long terminal repeats under the line. All data are cited from Grandbastien 1992, except the data for p-SINE1 (Mochizuki et al. 1992). ND = not determined, LTR=long terminal repeat, DR=duplicated target site

Arabidopsis , which was isolated in a plant that was transformed with an active copy of *Ac* (Tsay et al. 1993). The authors thought it unlikely that *Ac* had provided the transposase for the endogenous element, as no *Ac* binding site was present in the element. It is possible though, that the presence of an active *Ac* element may have indirectly triggered the endogenous element to become active (Tsay et al. 1993).

It was found that the activity of the maize elements is down-regulated at the transcriptional level by a low rate of transcription and by GpC-methylation of specific transposable element sequences (Fedoroff 1989, Chandler and Hardeman 1992, Gierl et al. 1989). This will be discussed in more detail in the section on methylation. The *Spm* transposable element encodes two proteins necessary for transposition, tnpA and tnpD, which are both derived from one precursor. Gierl et al.(1989) speculated that tnpD, being the minor product, was rate-limiting for transposition. Interestingly, in *Spm* transformed tobacco plants, it was observed that tnpA became the minor product, suggesting, again, a host specific type of regulation. Furthermore, repression of transposition by a specific *dSpm* element was thought to be caused by a truncated transposase encoded by *dSpm* , and it was speculated that competition for the binding sites between intact and aberrant molecules brought about the repression effect (Gierl et al. 1989). Gierl concluded that transposition in plant systems depends at least on the number of transposase molecules available and on the accessibility of the binding sites for the transposase molecule.

Evidence for other types of control of transposition has been obtained in bacteria, yeast, *Drosophila* and mammals (Berg and Howe 1989). Specific proteins were identified that repress the transcription of phage *Mu* elements and some *Tn* elements in bacteria. Also methylation appeared to regulate the activity of several bacterial elements. The *Ty* element of yeast is regulated by mating type (MAT) locus transcription factors. Two of the genes known to suppress *Ty* encode histones, suggesting that also chromatin structure determines the expression state of *Ty* (Winston and Carlson 1992). Another suppressor of *Ty* encodes a general transcription factor of RNA polymerase II. In *Drosophila* , the transcription of the *gypsy* element was suppressed by a protein specifically binding to the untranslated region of *gypsy* (Arkhipova and Ilyin 1992). The *copia* elements of *Drosophila* carry regulatory sequences, some of which found to function only in specific species (Arkhipova and Ilyin 1992). The activity of the *P* element in *Drosophila* is restricted to germinal tissue, due to the correct splicing of the transposase messenger in germinal tissue. Like the example of *dSpm*, defect transposase molecules encoded by truncated *P* elements interfered with transposition (Berg and Howe 1989). From these examples it is clear that regulation of transposition is not a simple process but acts on multiple levels.

Chromatin structure

Another class of variegated mutations commonly occuring in plants, show a stable recessive inheritance despite their apparent somatic instability. The variegation may indicate that the wild type function of the allele is repressed in some somatic cells. While the induction of inactivation is heritable, the inactivated state is not maintained throughout meiosis, since no stable recessives appear in the offspring. The mutants *marmorata, mottled, albescence* and *green stripe* in tomato represent examples of stable recessive gene mutations causing variegation,

none of which have been investigated at the molecular level. These mutations are possibly analogous to position effect variegation (PEV) mutations in *Drosophila*, and to the recessive alleles of autosomal genes conferring mosaic patterns in mouse coat color. PEV mutations will be discussed in more detail below. Molecular investigations of the mechanism governing the variable expression of coat color genes are limited to the chincilla-mottled (c^m) allele of mouse (Porter et al. 1991). This allele, that was induced by neutron irradiation treatment, is characterized by a rearrangement 5 kb upstream of the transcription start site. It was suggested that the instability in gene expression resulted from the separation of the coding sequences from its upstream enhancers. As evidenced by the reduced transcription and sensitivity of the chromatin to endonuclease in pigment deficient cells, this rearrangement appeared to impose a variable expression pattern upon the c^m gene by altering the chromatin conformation (Porter et al. 1991). This example demonstrates the effect of chromatin structure on the functional state of a gene, and may as well explain other cases of unstable gene expression in plants.

Other examples of epigenetic changes that influence the expression of a gene without altering its genotype were found among the position effect variegation (PEV) mutations in *Drosophila*. This type of instability has been shown to occur in approximately 70 euchromatic Drosophila genes (Wakimoto and Hearn 1990). In these instances the variable inactivation of an allele was the result of a DNA rearrangement, placing the gene in the vicinity of heterochromatin, which is distinguished from euchromatin by its highly condensed state that is maintained in interphase cells. This high degree of condensation appears to inhibit transcription. Hence, the inactivation of genes subject to PEV was thought to be the consequence of the high condensation level of heterochromatin extending into euchromatic regions (Henikoff 1990). PEV mutations are normally recessive, though exceptions have been found, including the variegated allele of the *brown* locus which imposed its inactived state on the unrearranged wild type allele (Henikoff and Dreesen 1989). Another exception is the *light* gene that becomes unstable following its translocation from its original position in heterochromatin to euchromatin (Wakimoto and Hearn 1990). The *light* gene is also remarkable in that it contains repeated sequences in its introns and responds in an opposite way to modifiers of PEV (see next section), which might indicate that the *light* gene is under a type of regulatory control different from euchromatic genes (Gatti and Pimpinelli 1992). In contrast to other PEV mutations, somatic chromosome pairing was shown to be essential for the expression of the variegated phenotypes of the *light* and *brown* alleles (Henikoff and Dreessen 1989, Wakimoto and Hearn 1990).

Using Drosophila PEV mutations as reporter genes, it was possible to identify approximately 120 loci affecting the level of variegation. Among these were loci encoding proteins needed for the formation of heterochromatin (Eissenberg et al. 1992) and enzymes modifying these proteins (Reuter and Spierer, 1992). The proteins modifying the expression of PEV acted as enhancers or suppressors, some of them in a dose-dependent way. Remarkably, several modifiers had opposite effects on the expression of the heterochromatic *light* gene.

While extra Y chromosomes and the *Su(var)* mutations were known to suppress PEV in (euchromatic) *white* alleles, these enhanced the variegation at the *light* locus. On the basis of these observations it was proposed that enhancers of variegation increased the number of proteins functional in establishing heterochromatin complexes (Henikoff 1990). The reverse was true for the repressors, which were supposed to induce a shortage in these proteins. Moreover, indirect evidence suggested that the developmental inactivation of homeotic genes was established by proteins with the same function. Hence, the regulation of (developmental) patterns of gene expression may generally rely on proteins specifying chromosomal regions for orderly transcription and replication (Eissenberg et al.1992, Reuter and Spierer 1992, Manuelidis 1990, Shaffer et al. 1993).

Thus far in plants, only in the case of *Oenothera blandina* has it been shown that the instability at the *P* locus, conferring a red pigment on flower buds, was due to a position effect. The instability was the result of an X-ray induced chromosomal translocation of the chromosome arm altering the position of the*P* locus. Evidence that the instability resulted from its altered position came from the observation that the instability was lost upon transferring, by crossing over, the unstable *P* allele to its original position (Catcheside 1947). The instability at the *sulfurea* locus in variegated tomato plants has also been associated with a position effect (Hagemann and Snoad 1971; Chapter 6). It was proposed that the inactivation of the *sulfurea* alleles in somatic cells resulted from an alteration in chromatin structure, similar to PEV alleles in *Drosophila*. Another peculiar feature of unstable *sulf* alleles is that they can inactivate the wild type *sulf*$^+$ allele in trans, in a way comparable to the trans-inactivation of the wild type *brown*$^+$ allele in response to the unstable *brown* allele in *Drosophila*. One requirement for this trans-inactivation of the *brown*$^+$ allele is somatic pairing, but, as yet, no evidence has been obtained showing somatic pairing to occur in plants (Heslop-Harrison and Bennett 1990). Another difference with dominant PEV at the *brown* locus was the appearance of stable recessives among the selfed offspring of the *sulfurea* mutant, indicating that the inactivated state is heritable. The lack of stable recessives among PEV populations is likely to be due to the different origin of germline cells in animals compared to plants, rather than to a different heritability of the inactivated state of the gene. In *Drosophila*, the germ line is set apart early in development and somatic changes occurring in other tissues will not be transferred to the offspring. In contrast, the plant cells that will eventually develop into germ cells have undergone many cell divisions and are more likely to accumulate somatic mutations. The study of PEV muations has revealed a function for chromatin structure in specifying DNA regions available for transcription. Conceivably, the analysis of unstable plant mutations may reveal a similar function of chromatin in plant gene expression.

Methylation

A considerable amount of data has been gathered correlating the methylation of GpC sites in DNA to the functional state of a gene (Bird 1992, Pescke and Phillips 1992). Although direct evidence for the regulatory function of GpC methylation in gene expression is missing, the correlations observed are very suggestive. In plant transformation experiments, it has been demonstrated that the failure of introduced genes to become expressed was accompanied by an increase in number of methylated GpC sites (Renckens et al. 1992, Meyer et al. 1992). DNA methylation may thus represent one of the epigenetic factors regulating gene expression. Other data correlating gene function with methylation were in large part derived from studies of the activity state of transposable elements. As to the three best studied maize elements, *Ac*, *Spm* and *Mu*, it was shown that the activity of the element correlated with a reduced methylation at specific sites within the elements (Chandler and Walbot 1986, Chandler and Hardeman 1992, Fedoroff 1989). Also the activation of cryptic elements in plants derived from tissue culture correlated with a lower level of methylation (Peschke and Phillips, 1992). Furthermore, the reversibility and developmental dependence of changes in the activity state of *Ac* and *Spm* elements supported the notion that epigenetic alterations underlie these changes (Wessler 1988, McClintock 1958).

The *Spm* element has been found to exist in three interchangeable activity states (Fedoroff 1989). Fully active copies were sparsely methylated in the regulatory region, fully inactive copies were methylated to a high degree, whereas the copies that cycle between an inactive and active state were methylated at an intermediate number of sites. Fedoroff observed that, in reponse to the introduction of an active *Spm* element, silent elements were activated, and reactivation of the most densely methylated elements occurrred at a lower frequency (Fedoroff 1991). This observation contradicts the results with *Ac*, for which a higher copy number suppressed transposition (Fedoroff 1989), indicating that methylation is not the only factor determining the activity state of transposable elements.

Unstable gene expression associated with methylation has been also noticed in transgenic petunia plants, where in several transformants the expression of the introduced *A1* gene of maize, encoding dihydroflavonol 4-reductase, became unstable or inactivated (Linn et al. 1991). The (variable) inactivation of the transgene was found to be correlated with methylation of the 35 S promoter (Linn et al. 1990, Meyer et al. 1992). In this study, it was shown that the methylation status of the promoter not only depended on the integration site, but also on copy number and environmental conditions (Linn et al. 1990, Meyer et al. 1992). Interestingly, when a particular inactivated allele was combined with an active allele in a heterozygous plant, the inactivated *A1* gene imposed its expression state on the wild type *A1* allele, as inferred from the appearance of a high number of white and variegated plants in a cross between white and brick-red flowered plants (Meyer et al. 1993). The repressed state of the *A1* allele persisted in the next generation. This type of interaction is highly analogous to the

dominant position effect variegation described for the *brown* locus in *Drosophila*. While the inactivation of the *brown* allele was due to an extension of the condensed chromatin structure of heterochromatin into the *brown* gene, the inactivation of *A1* correlated with an increase in methylation. As the *A1* gene is integrated close to a repetitive region, the authors speculated that an extension of methylation from this region into the transgene caused the inactivation by altering the chromatin structure. Apparently, the methylation pattern was then imposed on the second *A1* allele (Meyer et al. 1993). This example thus demonstrated a position effect, acting on the A1 gene, in which methylation was identified as a mechanism which caused position effects, in addition to the above discussed mechanism of chromatin condensation.

Although a correlation between transposable element activity and the methylation level has been established, repression by methylation may follow complex patterns and is not always absolute. For example, an *Ac* element in the hypermethylated state was found to be active in cells of maize grown in vitro (Peschke and Philips 1992). Furthermore, in *Arabidopsis*, methylation defective mutants have been described that show no phenotypic changes (Richards et al. 1992). In addition, it has not been resolved whether demethylation is a result or a cause of activity, since methylation patterns are known to adapt to the current transcriptional state (Bird 1992). Generally, it is assumed that methylation interferes with the binding affinity of the DNA strand to trans-acting regulatory factors necessary for transcription or transposition (Jablonka et al. 1992). It can be envisaged that strong promoters and enhancers may overcome the repressed state. An argument for this assumption may come from the observation of genes that are heavily methylated but nevertheless constitutively expressed, such as the *zein* genes in maize (Antequera et al. 1985). In addition, the level of methylation of an introduced DNA segment is possibly dictated by its site of integration, since highly repeated sequences and heterochromatic regions are heavily methylated (Manuelidis 1990). In the previous section it was discussed how heterochromatic regions can control the expression of genes when these genes are integrated close to such a region.

Other instances of instability

The examples of unstable gene mutations discussed thus far demonstrated different means in bringing about the inactivation of a gene. Transposable elements alter gene expression by disrupting the coding sequence of the gene, and epigenetic changes interfere with the regulation of gene expression. Other examples of instability in plants are due to factors altering the sequence of the gene, or to the removal of the entire gene from the cell. This occurs through gene conversion, including paramutation (=somatic gene conversion), non-disjunction of chromosomes, chromosome breakage, translocations, deletions, inversions, duplication, and somatic and unequal crossing-over (Harrison and Carpenter 1977, Lesley and Lesley 1963 and 1967, Brink 1973, Sudupak et al. 1993). These phenomena can occur at a very low frequency in the genome, but, for some reason or other, the frequency noticably increases in some

genotypes. The underlying mechanisms may be diverse, even for one type of genetic event. For example, somatic gene conversion (paramutation), in which one allele imposes a new expression state on the other allele, correlated with the presence of a transposable element in the *nivea* allele of *Antirrhinum* (Krebbers et al. 1987), but also with an altered methylation status of the *R* locus in maize (Dooner and Robbins 1991) and the *A1* locus in transgenic petunia plants (Meyer et al. 1993). Recently, it was demonstrated in yeast that mitotic gene conversion was the result of recombination between a reverse transcript of a particular RNA sequence and its chromosomal allele (Derr and Strathern 1993).

In rice, a high rate of meiotic gene conversion was thought to account for the mutability of the *waxy* allele (Bureau et al. 1991). Wild type, mutant and revertant *waxy* alleles differed by a few base pairs within a transposon-like element inserted in the *waxy* gene. Because of the presence of a number of homologous copies of this element in the rice genome, the authors speculated that one copy of the element was regularly converted into another copy with a slighty different sequence (Bureau et al. 1991).

Typical for plants are the variegated phenotypes due to chlorophyll deficiencies. Many of these green-white/yellow variegated patterns are caused by the segregation of defective plastids, and not necessarily related to genetic instability. Nuclear mutations that induce heritable changes in the plastid genome can also give rise to variegated phenotypes (Kirk and Tilney-Bassett 1978, Prina 1978). To date, only one such nuclear gene has been cloned, the *iojap* gene of maize which is associated with a white striped phenotype. From biochemical studies it was known that the chlorosis was due to improper development of the plastids in a local and position dependent manner on the leaves (Han et al. 1992). The presence of an *Ds* element in the gene could explain the occurrence of revertants but not the variegated patterns on the leaves. Also the iojap protein sequence did not give any clue as to its role in the variegation. In contrast, an example of variegation in *Arabidopsis*, which was caused by the segregation of defective plastids, could be related to genetic instability (Martínez-Zapater et al. 1992). These authors showed that in this mutant the chloroplast mutator (CHM) locus induced specific rearrangements in mitochondrial DNA .

Genetic instability in tomato

Within *L. esculentum*, surprisingly little genetic diversity has been found as compared to, for example, the maize genome. This was previously inferred from the limitid genetic variation at isozyme loci (Tanksley and Orton 1983) and more recently from the use of RFLP markers (Tanksley et al. 1992). One such RFLP study revealed that of 263 loci tested, less than 3% were polymorphic within the group of old tomato cultivars (Williams and Clair 1992). In modern cultivars, the majority of the polymorphisms detected were shown to result from introgression of traits from wild species (Beek et al. 1992). In contrast, the level of variation within the *L. esculentum* var. *cerasiforme* group, the immediate ancestor of the cultivated

Table 3. Features of several unstable alleles of tomato

Name (Allele)	Chromosome	Parental genotype	Phenotype	Inducing agent	Genetic behaviour	Cause
ghost (gh)	11	San Marzano, Stokesdale	plant starts out green but turns to yellow and white, green sectors on white tissue*1)	spontaneous in both genotypes	recessive, stable	unknown
Woolly (Wo)	2	T1075	irregular patterns of pale and dark green sectors and sectors with increased number and types of hairs2)	spontaneous	dominant,aberrant inheritance	duplication of *Wo* in combination with gene *vgr* which caused the infrequent loss of chromosome 2 in somatic cells
flaked (fl)	6	Canary Export	green-white variegation and hairy-non hairy variegation3)	32_P	recessive, aberrant inheritance	loss of extra chromosomal fragment
yellow virescent masculosterilis (yv^{ms})	6	Condine Red	green-yellow sectored leaves4)	X-rays	gives rise to yellow male sterile offspring in aberrant ratios	unknown
sulfurea (sulf)	2	Lukullus	green-yellow-white mottled5)	X-rays	gives rise to yellow offspring that is lethal, paramutant	unknown

* The data were cited from the following references: Rick et al. 1956 (1), Lesley et al. 1963 and 1977 (2), Lesley et al. 1961 (3), Hagemann 1962 (4), and Hagemann 1958 (5).

tomato, was 39 % (Miller and Tanksley 1990, William and Clair 1992). The high uniformity of the L. *esculentum* genomes is thought to be related to its self-pollinating nature and its history of domestication. Most likely the entire population of modern European and US cultivars derives from the sample of tomatoes introduced in Europe in the 16th century (Rick 1976).

As in other plant genomes, tandem repeats and interspersed repeats have been distinguished in the tomato genome (Zabel et al. 1985, Ganal et al. 1988). Thus far, a telomeric (TEL) and subtelomeric (TGRI) repeat sequence have been shown to be hypervariable, allowing cultivars to be discriminated (Broun et al. 1992). They proposed that unequal crossing over underlies this variability. In addition, the use of simple sequences revealed poylmorphisms between tomato cultivars (Vosman et al. 1992). Other sequences displaying a high level of polymorphisms have not been detected in the tomato genome. In contrast, in maize, repeated and low copy sequences are known to be polymorphic, which has been attributed to the activity of transposable elements (Döring and Starlinger 1984, Schwartz-Sommer et al. 1985, Nevers et al. 1986). The low level of variation in the tomato genome may suggest that active transposable elements are not present in tomato. Because in maize and *Antirrhinum* it has been shown that the occurrence of unstable gene mutations is diagnostic for active transposable elements, an investigation as to the cause of the genetic instability observed in tomato genotypes was carried out in the present study.

The *L. esculentum* genome has not provided many examples of instability. However, in crosses between *L. pennellii* and *L. esculentum,* the mutation rate appeared to be 100 times higher than the spontaneous mutation rate of the parents (Rick 1967). Unlike the mutations generated by the insertion of a transposable element, the expression of the newly obtained mutations was stable. According to Rick the increased mutation rates in *L. pennellii* and *L. esculentum* crosses were related to small differences in distribution and amount of heterochromatin, although he did not exclude the possible involvement of transposable elements (Rick 1967). Other tomato loci showing somatic or germinal instability are summarized in Table 3. One of these mutants, *gh*, appeared sensitive to the presence of extra heterochromatin, as inferred from a significant shortage of *gh* plants detected in segregating progenies which were derived from plants carrying extra heterochromatic chromosomes (Quirós 1976). In two other variegated mutants which are conditioned by the nuclear genes, flecked dwarf (*fd*) and pseudo-m (*pmo*), variegation was enhanced in response to an extra dosage of heterochromatin (Quirós 1976). The occurrence of twin-spots revealed somatic instability for the semi-dominant marker *Xanthophyll* (*Xa*; Seeni and Gnanam 1980). Plants heterozygous for the marker *Xa* are pale green while homozygous plants are yellow/white. The appearance of green-yellow twin-spots on a pale green plant derived from in vitro culture has been postulated to be the result of somatic crossing or chromosome breakage (Seeni and Gnanam 1980). Remarkably, the same mutation appeared in an independent in vitro culture experiment (Koornneef pers communication). A notable feature of some cultivars of northern Europe is the appearance of "rogue" plants in their offspring. Rogues are distinguished by narrow leaf segments, increased growth of side shoots

and early flower development. Although it has been established that there is some inherited predisposition determining its frequent appearance in European cultivars, the genetic behaviour of the character was, as yet, too variable to understand its inheritance (Grimly 1986).

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Chapter 2

Genetic and molecular characterization of an *Adh-1* null mutant in tomato

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Summary

Treatment of tomato seeds with ethyl methanesulphonate (EMS) followed by allyl alcohol selection of M_2 seeds has led to the identification of one plant (B15-1) heterozygous for an alcohol dehydrogenase (*Adh*) null mutation. Genetic analysis and expression studies indicated that the mutation corresponded to the structural gene of the *Adh-1* locus on chromosome 4. Homozygous *Adh-1* null mutants lacked ADH-1 activity in both pollen and seeds. Using an antiserum directed against ADH from *Arabidopsis thaliana*, which cross-reacts with ADH-1 and ADH-2 protein from tomato, no ADH-1 protein was detected in seeds of the null mutant. Northern blot analysis showed, that *Adh*-1 mRNA was synthesized at wild type levels in immature seeds of the null mutant, but dropped to 25 % in mature seeds. Expression of the *Adh-2* gene on chromosome 6 was unaffected. The potential use of the *Adh-1* null mutant in selecting rare transposon insertion mutations in a cross with mutable *Adh-1*$^+$ tomato lines is discussed.

Introduction

Gene mutations that affect biochemical pathways and that can be readily recognized are powerful tools in biochemical and genetic studies. Unfortunately, however, such mutations are rather rare among higher plants. Two classes of mutants have been identified so far. The first class comprises mutants which are recognizable by their altered phenotype. Examples include mutants in loci affecting seed morphology and flower colour, such as the *shrunken* (*Sh*) locus (Chourey and Nelson 1976), the *waxy* (*Wx*) locus (Sprague et al. 1943) of maize and the *chalcone synthase* (*nivea*) locus of *Antirrhinum* (Kuckuck 1936, Sommer and Saedler 1986). The second class comprises mutants which can be selectively isolated on the basis of their resistance to toxic chemicals or drugs. Mutations blocking the synthesis or activity of adenine phosphoribosyl transferase (APRT) (Moffatt and Somerville 1988), nitrate reductase (NR) and alcohol dehydrogenase (ADH) are typical examples of the second class of biochemical mutants. When cells containing ADH are subjected to a treatment with allyl alcohol, the substrate is converted to the highly toxic aldehyde acrolein which causes cell death (Freeling 1976). In contrast, individuals which lack ADH and as a result do not produce acrolein, survive, as has been shown for maize pollen (Schwartz and Osterman 1976; Freeling and Cheng, 1978) *Arabidopsis* seeds (Jacobs et al. 1988) and *Nicotiana plumbaginifolia* cells (Widholm and Kishinami, 1988). This differential [illegible]yl alcohol offers the possibility of screening large numbers of individuals for the [illegible] null mutants simply by killing all the wild type individuals. In maize, this selection scheme has been successfully applied to the isolation of rare insertion mutants induced by transposable elements (Döring et al. 1984[a], Sutton et al. 1984). Similarly, resistance to chlorate was used to identify a transposable element of tobacco after its transposition into the *nitrate reductase* gene (Grandbastien et al. 1989).

In tomato, mutable lines are known in which the genetic instability of various distinct mutant alleles is likely to be caused by the activity of transposable elements (Ramanna et al, 1985; unpublished results). As endogenous transposable elements have not yet been isolated from tomato, these mutable lines may provide a useful source of active elements, provided a system is available that permits the selection of rare mutations caused by the insertion of an active element.

The molecular identification of endogenous transposable elements in plants is primarily based on the accidental visitation of an element to a gene of which the activity can be monitored and for which a molecular probe is available. In maize, several transposable elements could thus be isolated by virtue of their presence in genes that had been cloned already, such as the *Sh-1* gene (Geiser et al. 1982, Döring et al. 1984b), the *Wx* gene (Fedoroff et al. 1983; Schwartz-Sommer et al. 1984) and the *Adh-1* gene (Sutton et al. 1984; Döring et al. 1984a) Similarly, in *Antirrhinum majus*, the *Tam1* element could be isolated due to its insertion in the *nivea* gene (Weinand et al. 1982; Bonas et al. 1984). In tomato, however, none of the mutable loci has ever been cloned. Consequently, the molecular identification and cloning of the putative tomato element(s) has remained elusive. We have, therefore, chosen to develop a transposon target gene system that would combine the possibility of readily screening very large numbers of individuals for the visitation of the transposon in the target gene, with the possibility of subsequently cloning the element out of the inactivated target gene. As outlined above, the *Adh* gene is such an appropriate target gene.

In tomato, two *Adh* genes have been described, *Adh-1* on chromosome 4 (Tanksley 1979) and *Adh-2* on chromosome 6 (Tanksley and Jones 1981), the expression of which is developmentally regulated. The *Adh-1* gene is constitutively expressed in pollen and at rather late stages of seed development, whereas the *Adh-2* gene is mainly expressed during early stages of seed development and under anaerobic conditions (Tanksley 1979; Tanksley and Jones 1981).

A feasible approach to identify the anonymous transposable element would be to cross the mutable lines, which are homozygous for the $Adh\text{-}1^{+}$ allele, to a line carrying a homozygous *Adh-1* null mutation and to subsequently identify a rare allyl alcohol-resistant insertion $Adh\text{-}1^{o}/Adh\text{-}1^{o}$ mutant among the heterozygous $Adh\text{-}1^{+}/Adh\text{-}1^{o}$ progeny. Since this approach requires an *Adh-1* null marker line and no such genotype is available in tomato, we have isolated one for this purpose. Here, we report the isolation and the genetic and molecular characterization of an EMS-induced *Adh-1* null mutant.

Materials and Methods

Plant material. Mutant selection was performed in the progeny of plants grown from mutagenized seeds of the homozygous genotypes GT (a tomato mosaic virus - resistant pure line provided by De Ruiter Seeds, Bleiswijk, The Netherlands) and cv. Moneymaker (MM).

The line LA 889 (Khush and Rick, 1967) carrying the recessive yellow leaf marker *venosa* and the line LA 2416 carrying the *Adh-1*^{1} allele were used for genetic analysis and were provided by Dr. C. M. Rick.

Mutant selection. In order to induce mutations, approximately 1000 MM and 5000 GT seeds were submerged in a freshly prepared, unbuffered 60 mM ethyl methanesulphonate (EMS) solution for 24 h in the dark at 25 °C, then rinsed in water and sown in soil. The resulting M_1 plants (749 MM and 1752 GT plants) were grown in pots in the greenhouse to allow production of M_2 seeds that were harvested per group of 10 fertile M_1 plants. From each pool, 50 M_2 seeds were pre imbibed in water for 24 h and then soaked for 3 h in 70 mM allyl alcohol, using a slight modification of the procedure developed for *Arabidopsis* (Jacobs et al. 1988). After rinsing in water, the seeds were germinated in the dark in Petri dishes on wet filter paper at 25 °C for at least 12 days. Germinated seeds were grown to plants (M_2) in the soil. The genotype of the M_2 plants was determined by analysing individual M3 seeds from the selfed progeny for ADH activity using starch gel electrophoresis.

ADH activity tests. i) Pollen staining. Pollen was stained for ADH activity using the method of Freeling (1976) with some minor modifications. Pollen was collected in tubes and 5 ml of 0.1 M Tris-HCl buffer (pH 7.5) was added. The samples were frozen overnight at -20 °C. After thawing at room temperature for 3 h, the buffer was replaced by 5 ml of ADH staining solution (6 ml ethanol, 30 mg NAD (ß-nicotineamide adenine dinucleotide), 20 mg MTT (dimethyl thiazol tetrazolium-bromide) and 4 mg PMS (phenazine methosulfate) in 100 ml 0.1 M Tris-HCl buffer, pH 7.5 (Tanksley, 1979). Pollen was stained in the dark for 1 h at 30 °C and was directly scored using a microscope. ii) Starch gel electrophoresis. We have never been able to detect ADH-2 activity following polyacrylamide gel electrophoresis (PAGE) or ultrathinlayer iso-electrofocusing (UTLIEF). Therefore, ADH-1 and ADH-2 activity were separated on starch gels. Mature seeds were removed from the ripened fruits, soaked in 1N HCl for 1 h, rinsed with water and dried on air on filter paper. They were then soaked in water for 18 h to facilitate extraction, and individually ground in 50 µl of 0.1 M Tris-HCl buffer (pH 7.5) containing 1% β-mercaptoethanol. Immature seeds were removed from fruits at different ripening stages, briefly dried on filter paper to remove the mucilaginous substance and each seed was directly ground in 50 µl sample buffer. Roots were ground at a 1:1 ratio of tissue weight and sample buffer volume. Homogenates were absorbed in paper wicks and directly subjected to starch gel electrophoresis. Horizontal starch gels consisted of 12 % hydrolyzed starch (Connaught Lab. Ltd.) dissolved in 9/10 volume gel buffer pH 8.3 (70 mM Tris and 8 mM citric acid), and 1/10 volume electrode buffer pH 7.9 (25 mM lithium hydroxide and 180 mM boric acid) (Selander et al., 1971). Gels were run at 4 °C for 30 min at 4 V/cm. After removing the paper wicks, the gels were run for 2 1/2 h at 4 °C at 10 V/cm. The gels were transversally sliced in two parts and stained in ADH- staining solution (as described for pollen) for 2 h at 30 °C in the dark.

Gels were fixed in a solution containing 14:6:1 volumes of distilled water:methanol:acetic acid.

RNA analysis. Total RNA was isolated (de Vries et al. 1988) from roots grown in hydroculture, and from mature (dry and for 18 h imbibed) and immature seeds collected at different times after pollination. Intact seeds, prepared as described for starch gel electrophoresis, were stored at - 80 °C until extraction. Poly $(A)^+$ RNA of roots was selected by oligo dT-cellulose chromatography (Maniatis et al. 1982). Denatured RNA samples, 8 µg total RNA or 0.08 µg poly$(A)^+$ RNA, were run on formaldehyde denaturating gels (Maniatis et al. (1982) and transferred to Gene-Screen membranes (New England Nuclear) in 20 x SSC buffer. For dot blots, a series of total seed RNA (0, 0.6, 1.25, 2.5, 5 and 10 µg) and root poly$(A)^+$-RNA (0, 6, 12.5, 5 and 100 ng) was denatured in a total volume of 25 µl as described by Maniatis et al. 1982. The denatured RNA samples were diluted with 10 x SSC to a total of 200 µl and were spotted on Gene Screen membrane, using the Minifold vacuum filtration system of Schleicher & Schuell. Each slot was then rinsed with 200 µl of 3 x SSC. Northern and dot blot hybridizations were carried out for 18 h at 42 °C in 50 % formamide, 5x SSC, 1 x Denhardt's solution, 0.02 M sodium phoshpate pH 6.8, 5 mM EDTA and 100 µg/ml denatured salmon sperm DNA. After hybridization, the filters were subsequently washed for 15 min each in 2 x SSC, 0.1 % SDS at 42 °C and 0.1 x SSC, 0.1 % SDS at 65 °C.

Hybridization probes. (i) *Adh-1*. A tomato (*L. esculentum*) Sau3A partial library was constructed in lambda phage EMBL 4 (4×10^5 total recombinants) and screened by plaque hybrization at low stringency (26 °C, 50% formamide, 5 x SSC, 5% dextran sulfate, 250 µg/ml carrier DNA, 0.1 % SDS, 10 x Denhardt's solution) using the maize *Adh* cDNA clone pZmL.793 (Dennis et al. 1984) as a probe. One of the Adh-positive lambda clones (TAdh1) which contained a 17 kb insert was selected for further analysis. Restriction mapping and Southern analysis showed that TAdh1 contained 4 *Eco*RI fragments, one of which (8.0 kb) reacted with the maize Adh probe. Following sub cloning of this *Eco*RI fragment and Southern analysis, the tomato sequences homologous to the maize probe were found to be contained within an 1.8 kb *Bgl*II fragment. This *Bgl*II fragment was sub cloned into pUC 8 and M13mp10. Restriction mapping showed an eccentric *Hinc*II site, but no sites for *Sma*I, *Acc*I, *Sph*I. Comparison of the sequence of a portion of the *Bgl*II fragment with Adh sequences of maize (Gerlach et al. 1982) and pea (Llewellyn et al. 1987) confirmed the *Adh*-identity of the cloned tomato fragment. More recently, the *Bgl*II fragment was recovered from M13mp10 RF by an *Eco*RI-*Sal*I digestion and sub cloned in the corresponding sites of pTZ18R (Pharmacia). All Southern and Northern hybridizations presented in this chapter were carried out with the 1.8 kb *Eco*RI-*Sal*I fragment derived from pTZ18R/Adh-1 following purification by agarose gel electrophoresis. From the following observations we conclude that the cloned genomic 1.8 kb *Bgl*II fragment represents (a portion of) the *Adh-1* gene on chromosome 4. First, using high stringency hybridization and washing conditions, the 1.8 kb fragment did not cross hybridize

with cDNA clones pC3-7, pCB25E6 and pAdh31 (see below) that are known by expression studies (Staraci et al. 1987) and RFLP linkage analysis (Weide et al. 1993) to represent the *Adh-2* gene on chromosome 6. Second, the 1.8 kb fragment does hybridize strongly with RNA from (ripe) seeds which contain a high level of ADH-1 activity and a very low level of ADH-2 activity, whereas hybridization with the clones pC3-7, pCB25E6 and pAdh31 show a weak signal. (ii) *Adh-2*. For *Adh-2* three cDNA clones (pCB25E6, pAdh 31 and pC3-7) were available (Chase and Williams. 1986), which encompass the entire *Adh-2* coding region. Clone pC3-7 contains the first 197 bases of the coding region as well as 65 bases 5' to the start codon. Clone pCB25E6 carries a 435 bp insert from the central coding region and pAdh31 a 688 bp segment of the 3' terminal region.

Protein analysis. Antiserum raised against highly purified *Arabidopsis* ADH enzyme (Jacobs et al. 1988) was kindly provided by Dr. R. Dolferus, Free University of Brussels, Belgium. Native starch gels were blotted on nitrocellulose filters in transfer buffer (0.01 M Tris-HCl pH 7.0, 0.05 M Nacl, 0.002 M EDTA and 0.1 mM dithiothreitol). After transfer, the filters were incubated for 1 h in 100 ml of 10 mM Tris-HCl buffer pH 7.5, 0.350 M NacL and 0.5% (w/v) gelatin. The filter was then incubated for 4 h with anti ADH-serum (0.4 µl/ml) in RIA buffer (10mM Tris-HCl, pH 7.5, 0.150 M NaCl, 1 % (v/v) Triton X-100, 1% gelatin). The unbound serum was removed by washing three times with 50 ml RIA buffer (Zabel et al. 1982). The filters were incubated for 2 h with ^{125}I-labelled protein A (0.5-1 µCi) in 25 ml RIA buffer. After washing off the unbound protein A with RIA buffer (3 x 10 min incubations in 50 ml RIA buffer), the filters were rinsed in distilled water, air dried and autoradiographed at -70 °C using an intensifying screen .

Results

Isolation of an allyl alcohol resistant mutant.

In order to select for *Adh* mutants resistant to allyl alcohol, approximately 4600 Moneymaker M_2 seeds from 730 M_1 plants, and 4600 GT M_2 seeds from 920 M_1 plants were treated with 70mM allyl alcohol. Pilot experiments showed that this concentration was detrimental to both *L. esculentum* cv. Moneymaker and GT wild type seeds. From this treatment, 32 resistant plants were recovered. After selfing, the M_3 seeds of these putative mutants were tested for ADH activity using starch gel electrophoresis. In the progeny of one M_2 plant, B15-1, individuals lacking ADH-1 activity segregated, indicating that plant B15-1 was heterozygous for an *Adh-1* null mutation. Out of this selfed population plants homozygous for the *Adh-1* null mutation were selected.

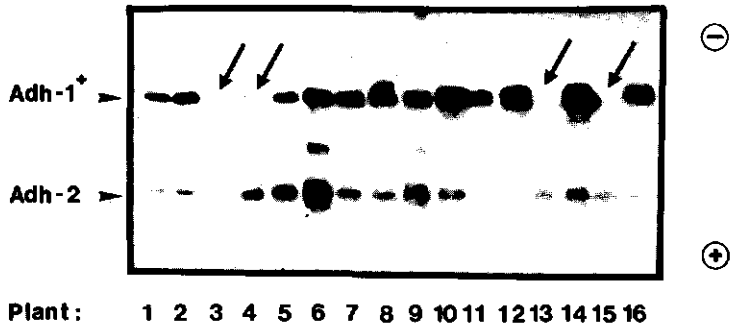

Fig. 1. Zymogram of the selfed progeny of B15-1(*Adh-1*$^{+}$/*Adh-1*^{0}). Mature seeds were imbibed for 18 h, individually ground, subjected to starch gel electrophoresis and stained for ADH activity. Note that seeds from the homozygous *Adh-1*^{0}/*Adh-1*^{0} plants 3, 4, 13 and 15 lack ADH-1 activity (indicated by an arrow). The middle band represents the heterodimer formed by the polypeptides encoded by the *Adh-1*$^{+}$ and the *Adh-2*$^{+}$ alleles. The variation in staining intensity of this band corresponds to the variation detected in the amount of ADH-1 and ADH-2 activity.

Inheritance of the EMS-induced Adh-1 null allele

Starch gel electrophoresis of 261 individual seeds revealed that 58 seeds of the selfed offspring of B15-1 (*Adh-1*$^{+}$/*Adh-1*^{0}) lacked ADH-1 activity, indicating monogenic and recessive inheritance of the *Adh* null allele. The zymograms of a representative set of 16 seeds is shown in Fig. 1. Although expression of the *Adh-2* gene has been reported to decline during seed

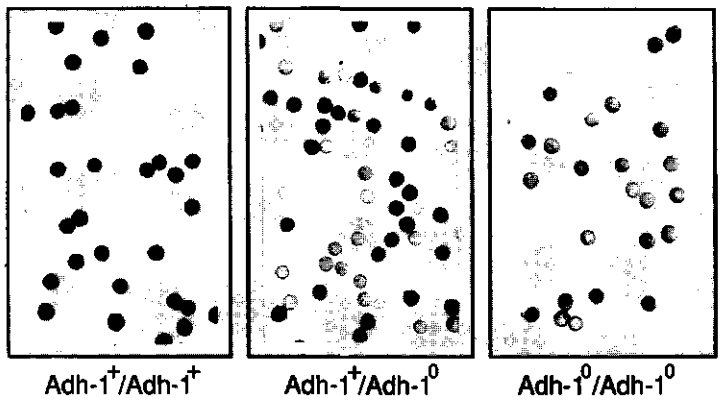

Fig. 2. Pollen histochemically stained for the presence of ADH in wild type GT (*Adh-1*$^{+}$/*Adh-1*$^{+}$), B15-1 (*Adh 1*^{0}/*Adh-1*$^{+}$) and the *Adh-1* null mutant (*Adh-1*^{0}/*Adh-1*^{0}). *Adh-1*$^{+}$ pollen grains stain blue, *Adh-1*^{0} pollen grains remain yellow or pink.

maturation (Tanksley and Jones 1981) a substantial but variable amount of ADH-2 activity was detected in mature seeds of the progeny of B15-1 (Fig. 1) that was not related to the amount of ADH-1 activity. The Mendelian inheritance of the mutated locus in B15-1 was further confirmed by staining the pollen grains for ADH activity (Freeling 1976). Pollen grains from wild type plants stained blue, while pollen grains collected from the *Adh-1* null mutant remained yellow or pink. The pollen preparation of the heterozygous plant B15-1 consisted of blue and yellow pollen at a ratio of 1:1 (Fig. 2).

Table 1. Segregation data for F_2 linkage analysis between the loci *venosa* and *Adh-1*

	Adh-1			
venosa	+/+	+/o	o/o	Total
ven+/.	1	52	22	75
ven/ven	25	0	0	25
Total	26	52	22	100

X^2=97.97, p<0.005

It could be argued that the *Adh-1*^{o} phenotype is not the result of a mutation in the structural gene but in a regulatory gene essential for expression and located at a different chromosomal position. This possibility was tested by crossing the *Adh-1* null mutant with a tomato line (LA 2416) carrying the *Adh-1*^{1} allele that encodes an isozyme with a different electrophoretic mobility. If a regulatory gene had been mutated, both polypeptides encoded by the *Adh-1*$^+$ and *Adh-1*^{1} alleles would be expected to be formed in the *Adh-1*^{o}/*Adh-1*^{1} heterozygote, due to the presence of an active regulatory gene contributed by the *Adh-1*^{1} parent. The *Adh-1*^{o}/*Adh-1*^{1} and *Adh-1*$^+$/*Adh-1*^{1} heterozygotes should then give the same isozyme pattern consisting of two homodimers and one heterodimer (see Fig. 3, lane 1). However, only the homodimer band derived from the *Adh-1*^{1} allele was detected in the *Adh-1*^{o}/*Adh-1*^{1} heterozygote (Fig. 3, lane 3), indicating that the structural *Adh-1* gene and not a regulatory gene had been mutated. Furthermore, linkage analysis in the F_2 derived from a cross of LA889 (*ven/ven, Adh-1*$^+$/*Adh-1*$^+$) x *Adh-1* null mutant (*ven*$^+$/*ven*$^+$, *Adh-1*^{o}/*Adh-1*^{o}) demonstrated that the *Adh-1* null mutation, like the wild type *Adh-1* locus, mapped on chromosome 4, at a position approximately one map unit from the *venosa* locus on position 40 (Table 1).

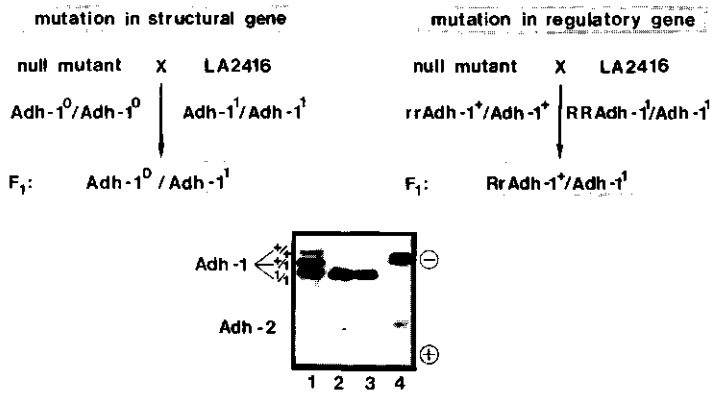

Fig. 3. The *Adh-1*^{0} phenotype is the result of a mutation in the structural gene and not in a regulatory gene. The homozygous *Adh-1* null mutant was crossed with the tomato line LA 2416 carrying the *Adh-1*^{1} allele. The ADH isozyme pattern of the F_1 seeds was determined by starch gel electrophoresis. Note that the *Adh-1*^{1}/*Adh-1*^{0} heterozygote was not capable of forming active ADH-1^{+} subunits. Lane 1: F_1 wild type GT x LA 2416 (*Adh-1*$^{+}$/*Adh-1*^{1}). Lane 2: LA 2416 (*Adh-1*^{1}/*Adh-1*^{1}). Lane 3: *Adh-1* null mutant x LA 2416 (*Adh-1*^{0}/*Adh-1*^{1}) Lane 4: wild type GT (*Adh-1*$^{+}$/*Adh-1*$^{+}$).

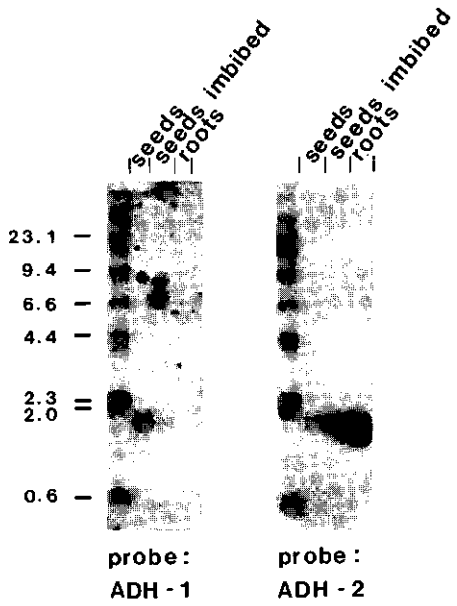

Fig. 4. Northern blot analysis of root and seed mRNA. Denatured RNA samples (8 µg total RNA (dry and imbibed seeds) or 0.08 µg poly (A)$^{+}$RNA (roots)) were run on formaldehyde denaturing gels, transferred to Gene Screen membranes and probed with the *Adh-1* clone or the *Adh-2* (pCB25E6) clone as indicated.

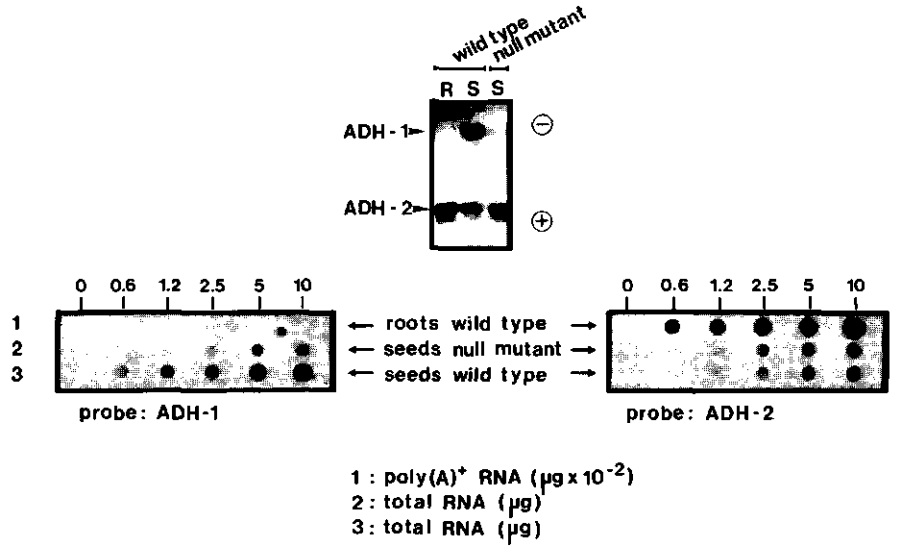

Fig. 5. Comparison of the appearance of ADH isozymes (shown on the starch gel at the top of the figure) and ADH mRNA's (shown on the dot blots at the bottom of the figure) in roots and mature dry seeds of wild type GT and the *Adh-1* null mutant. Different amounts of total RNA (as indicated) from mature seeds (rows 2 and 3) and poly(A)$^+$RNA from roots (row 1) were spotted on Gene Screen membrane and probed with the *Adh-1* clone or the *Adh-2* (pCB25E6) clone.

Molecular characterization of the Adh-1 null mutant

i) *Expression of the Adh-1 and Adh-2 genes in wild type and Adh-1 null mutant.* The expression of the two *Adh* genes was tested in seeds and roots. For that purpose, two identical Northern blots prepared from mRNA's extracted from dry and imbibed mature seeds and from roots grown in hydroculture were probed with the genomic *Adh-1* clone and the *Adh-2* cDNA clone pCB25E6. The *Adh-1* and *Adh-2* messengers were both about 1800 bp long (Fig. 4), which is in the same size range found for the *Adh* messengers in maize (1650 and 1750 bp; Gerlach et al. 1984), rice (1600 bp; Xie and Wu 1989) and pea (1400 bp; Llewellyn et al. 1987). The amount of *Adh-1* mRNA detected in mature seeds was considerably higher than the amount of *Adh-2* mRNA, whereas the reverse was seen in imbibed seeds. In roots grown in hydroculture, only *Adh-2* mRNA could be detected (Fig. 4). The Northern blot also showed that the mere imbibition of seeds was sufficient in reducing the amount of *Adh-1* mRNA. The concomitant increase of *Adh-2* mRNA in imbibed seeds may be due to the semi-anaerobic conditions developed during imbibition. In conjunction, the results demonstrated that the amount of *Adh* messengers, as detected in the respective tissues by the genomic *Adh-1* and *Adh-2* cDNA probe, coincided with the ADH enzyme activity found on starch gels (Tanksley

1979; Tanksley and Jones 1981).

Having established the patterns of expression of the two *Adh* genes in wild type (GT) tissues, we then compared the *Adh-1* mRNA level in mature seeds of wild type GT and the *Adh-1* null mutant by probing RNA dot blots with the genomic *Adh-1* clone. Poly(A)$^+$ RNA isolated from roots grown in hydroculture was used as a control to show that no cross-hybridization occurred with *Adh-2*. The amount of *Adh-1* messenger detected in mature seeds of the *Adh-1* null mutant was at least 75% lower than the amount found in wild type GT (Fig. 5). When using the *Adh-2* cDNA probe, equal amounts of transcript were detected in the *Adh-1* null mutant and GT seeds (Fig. 5), indicating that the difference in *Adh-1* mRNA levels observed between GT and the *Adh-1* null mutant reflected differences in transcript produced.

In an additional experiment, the amount of *Adh-1* mRNA was determined in seeds of GT and the *Adh-1* null mutant harvested at different maturation stages. A RNA dot blot probed with the *Adh-1* genomic clone (Fig. 6) showed that in unripe seeds the amount of *Adh-1* mRNA detectable in GT and the null mutant were comparable, in contrast to the different amounts found in mature seeds.

ii) *No ADH-1 protein was formed in the Adh-1 null mutant.* To test whether antibodies raised against the *Arabidopsis* ADH enzyme were reactive against tomato ADH enzymes and could thus be applied to detect the presence of ADH protein in the *Adh-1 null* mutant, starch gels run with proteins extracted from seeds of the *Adh-1*^{1} marker line and the wild type (GT) were

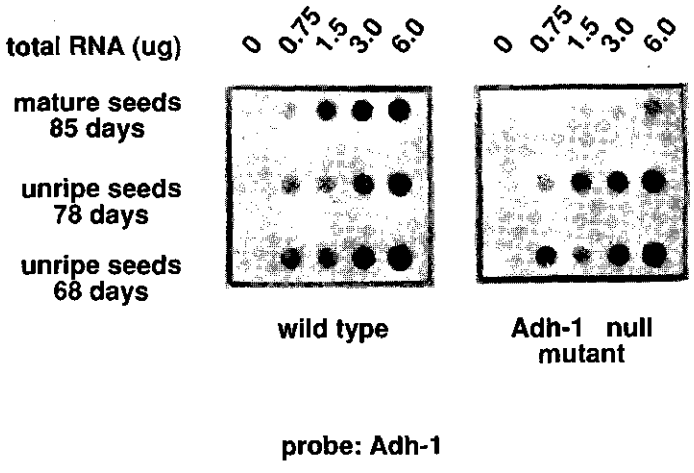

Fig. 6. *Adh-1* expression in wild type GT and the *Adh-1* null mutant during seed development. RNA was extracted from seeds of mature green fruits (68 days after pollination), orange fruits (78 days after pollination), and ripe fruits (85 days after pollination). Different amounts of total RNA (as indicated) were spotted on Gene Screen membrane and probed with the *Adh-1* clone.

subjected to isozyme staining followed by Western blot analysis (Jacobs et al. 1988). Indeed,

immuno-reactive tomato bands were detected, the number and position of which closely corresponded with the ADH-1^{+}, ADH-1^{1} and ADH-2 isozyme bands (Fig. 7). In seeds of the *Adh-1* null mutant, however, only ADH-2 and no ADH-1 protein were found (Fig. 7). To further verify that the anti-ADH serum did identify ADH proteins, a Western blot of an SDS-polyacrylamide gel loaded with proteins from GT mature seeds was probed with the *Arabidopsis* anti ADH-serum. Under these conditions a single polypeptide band reacted, the molecular mass (43 kD) of which corresponded with the mass detected for the subunit of purified tomato ADH-2 (Bicsak et al. 1982).

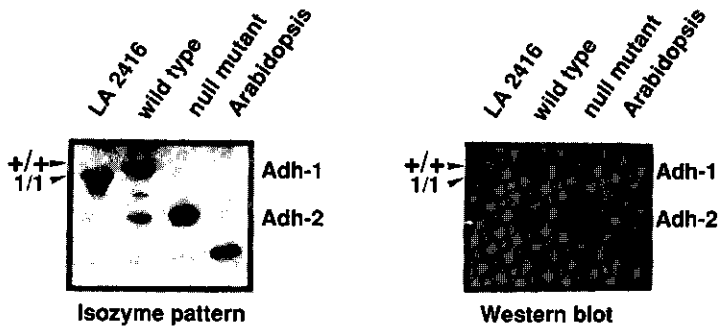

Fig. 7. Immunoautoradiographic detection of ADH-1 and ADH-2 isozymes in seeds from several tomato *Adh* genotypes and *Arabidopsis* , using *Arabidopsis* anti-ADH serum. Seed extracts were prepared from LA 2416 (lane 1), wild type GT (lane 2) , the *Adh-1* null mutant (lane 3) and *Arabidopsis* (lane 4), resolved by electrophoresis on a 12% starch gel (left gel) and blotted on nitro cellulose filters. Protein blots were incubated with *Arabidopsis*-anti ADH serum. Note that only ADH-2 and no ADH-1 protein was detected in the *Adh-1* null mutant.

Discussion

Adh null mutants have been reported for a number of plant species, including maize (Gerlach et al. 1985), *Arabidopsis* (Jacobs et al. 1988) and *Nicotiana plumbaginifolia* (Rousselin et al. 1990), but as yet not for tomato. In this chapter we describe the isolation of an *Adh-1* null mutant of tomato, that has been induced by treatment with ethyl methanesulfonate (EMS), a mutagen which is highly effective in inducing point mutations in plants. Genetic linkage analysis showed that the mutation is located approximately one map unit from the *venosa* locus at position 40 on chromosome 4 (Khush and Rick 1967). Taking into account the inaccuracy that is inherent with mapping loci by classical linkage analysis, these data strongly suggest that the *Adh* null mutation corresponds to the *Adh-1* locus at position 36 on chromosome 4 as

determined by Tanksley (1979). This conclusion is further supported by the molecular analysis showing that expression of *Adh* in the null mutant was only impaired in those tissues which are known to express the *Adh-1* but not the *Adh-2* gene (Tanksley 1979; Tanksley and Jones 1981).

Like EMS-induced *Adh* mutants of *Drosophila* (Martin et al. 1985) and maize (Gerlach et al. 1985), the tomato *Adh-1* null mutant described in this chapter was unable to synthesize ADH polypeptides at levels of the *Adh-1* transcript that were comparable to wild type, particularly in the immature seed stage. Apparently, modification(s) in the RNA sequence, such as a point mutation or a short internal deletion give rise to abortive protein synthesis. That no major deletions and/or rearrangements in the mutated *Adh-1* gene are involved was suggested by Southern blot analysis. Using eight different restriction enzymes to digest DNA from the wild type and the *Adh-1* null mutant, no differences in size were detected between the respective restriction fragments hybridizing to the *Adh-1* probe (data not shown).

Surprisingly, the original M_2 plant that had been selected for its allyl alcohol resistance was a heterozygote, displaying the typical Mendelian segregation, that is associated with a recessive mutation. Allyl alcohol, an ADH substrate which is converted in wild type cells to the highly toxic compound acroleïn, has been proven to be an effective means for selecting ADH-deficient mutants in plants (Gerlach et al. 1985; Jacobs et al. 1988; Widholm and Kishinami 1988). Recent data by Rousselin et al. (1990) indicate that ethanol can be applied for the same purpose. In this case, the ADH-catalyzed conversion of ethanol to acetaldehyde is the selective reaction, discriminating wild type and mutant cells. In considering the selection principle, the recovery of a heterozygous *Adh-1* null mutant that is resistant to allyl alcohol was not to be expected. The recovery of a heterozygous *Adh-1* null mutant might be explained by a dosage effect, implying that the overall physiological condition of the mutant seed is impaired (due to other mutations) and that the amount of enzyme encoded by the $Adh\text{-}1^+$ allele in the heterozygote is not sufficient to produce detrimental concentrations of the toxic acrolein. The absence of an homozygous *Adh-1* null mutant among the allyl alcohol resistant M_2 seedlings may simply be a consequence of sampling error, in that a rather small seed sample was used amongst which germination was poor and the yield of viable plants was low.

Seeds of the B15-1 mutant germinated poorly. This character was not related to the mutation in the *Adh-1* gene. Upon further back crossing to wild type plants, *Adh-1* null mutants with normal germination characteristics were readily recovered, as were *Adh-1* null mutants (M_3) in the selfed offspring. To test the reproducibility of the selection method we have recently back crossed the M_3 *Adh-1* null mutant lines to wild type, selfed the BC_1 and subjected the resulting F_2 seeds to allyl alcohol treatment. So far, we have not been able to establish an allyl alcohol concentration that allows the selection of heterozygous *Adh-1* null F_2 seeds. This may be due to other mutations that are independent from the *Adh-1* null mutation but impair with the overall physiological condition of the seeds. It remains to be shown whether *Adh-1* null heterozygotes can be directly selected in the selfed or hybrid progeny of the

mutable lines. Currently, *Adh-1* null mutants are further back crossed to wild type to develop the appropriate genotypes to be used in optimizing the allyl alcohol selection procedure of seeds.

Our major objective was to develop a system which allows us to identify and isolate transposable elements that are active in the tomato genome. With the *Adh-1* mutant available, it should be feasible now to select for rare, insertion mutants in the progeny of the *Adh-1* null mutant (*Adh-1*^{o}/*Adh-1*^{o}) that has been crossed to the mutable (*Adh-1*$^{+}$/*Adh-1*$^{+}$) tomato lines.

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Chapter 3

Molecular cloning of the *Adh-1* gene of tomato and its expression in transgenic tobacco.

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This chapter has been accepted for publication in Plant Molecular Biology

Summary

An 11 kb *Eco*RI genomic fragment containing the alcohol dehydrogenase (*Adh-1*) gene has been cloned. Cross-hybridization with three *Adh-2* cDNA clones suggested that the entire coding region of the *Adh-1* gene was contained on a 6.2 kb *Xba*I/*Hind*III subfragment. Using RFLP linkage analysis the genomic clone was mapped on chromosome 4 between the markers TG182 and TG65, in a position corresponding to the *Adh-1* locus, previously mapped on the basis of isozyme analysis. To further confirm the *Adh-1* origin of the genomic clone, tobacco plants were transformed with the 6.2 kb *Xba*I/*Hind*III genomic subfragment. Isozyme analysis demonstrated that in transgenic tobacco plants functional, tomato specific ADH-1 homodimers were synthesized, as well as heterodimers composed of tobacco and tomato subunits.

Introduction

ADH catalyzes the conversion of aldehydes into ethanol utilizing NAD^+ as cofactor. The tomato genome carries two ADH-encoding genes, *Adh-1* on chromosome 4 (Tanksley 1979) and *Adh-2* on chromosome 6 (Tanksley and Jones 1981). *Adh-1* is expressed exclusively in unripe seeds and in pollen grains, while *Adh-2* is expressed during early stages of seed development and under anaerobic conditions (Tanksley 1979, Tanksley and Jones 1981). Mutations blocking the production of ADH can be selectively isolated by virtue of their resistance to allyl alcohol (Schwartz and Osterman 1976, Jacobs et al. 1981, Widholm and Kishinami 1988). This differential response has been successfully applied to the isolation of three independent *Adh-1* null mutants in tomato (Chapter 2 and 4). The molecular analysis of these *Adh-1* null mutants required a genomic *Adh-1* clone. This chapter reports the isolation of such a genomic clone, which encompasses the entire *Adh-1* gene and its upstream sequences.

Material and methods

Cloning. In order to clone the *Adh-1* gene, which is contained on a single 11 kb *Eco*RI fragment, 400 ug DNA of the tomato line GT carrying the *Adh-1* wild type allele was digested to completion with *Eco*RI, loaded in a broad slot of a 0.6% agarose gel and separated by gel electrophoresis. The region of the gel between 8 and 15 kb was cut into 8 strips and DNA from each strip was isolated using the freeze-squeeze method. A sample containing 1/10 volume of the DNA of each fraction was electrophorised, transferred to a GeneScreenPlus membrane and hybridized to a genomic clone of 1.8 kb (TAdh1) containing the middle part and 3' end of the *Adh-1* gene (Wisman et al. 1991). DNA of the fraction containing the highest concentration of *Adh-1* was ligated to the lambda-DASHII vector, cut

with *Eco*RI, and subsequently packaged to produce recombinant clones, as recommended by the manufacturer (Stratagene). Phage colonies were screened by plaque hybridization using 25 ng of the TAdh1 clone, labelled (10^8-10^9 dpm/ug) by random priming, as a probe (Beek et al. 1992). Ten out of 20,000 recombinant phage plaques gave a positive hybridization signal with the TAdh1 clone as probe. Restriction enzyme analysis of DNA extracted from 5 purified positive plaques indicated that they were identical.

Physical map. The 11 kb *Eco*RI insert from one of the positive clones was sub cloned into pUC 18. Two ug of the recombinant plasmid was digested with several different combinations of restriction enzymes. Southern analysis using the TAdh1 clone and the three *Adh-2* cDNA clones, which encompassed the entire coding region (Wisman et al. 1991), revealed the position and orientation of the *Adh-1* gene.

Southern analysis, hybridization conditions and mapping. All standard DNA methodologies were carried out as described by van der Beek et al. (1992). F_2 plants derived from the inter specific cross between *Lycopersicon esculentum* cv. Allround x *Lycopersicon pennellii* LA 716 were used as mapping population. DNA was extracted from 84 individual F_2 plants, digested with *Dra*I, run on agarose gels, transferred to Gene Screen membranes and probed with the 1.8 kb *Bgl* II subfragment. The results were analysed using the computer program JOINMAP (Stam, 1993).

Starch gel electrophoresis. ADH-1 activity in wild type and transformant plants was separated on starch gels according to the method described in Wisman et al. (1991).

Transformation. A 6.2 kb *Xba*I/*Hind*III sub fragment of the 11 kb genomic *Eco*RI clone was inserted in the *Xba*I/*Hind*III site of the plasmid pBI 121 and subsequently transformed to *Agrobacterium tumefaciens* strain LBA 4404. Three weeks old cotyledons of *Nicotiana tabaccum* SR1 were transformed according to the procedure of Visser (1991) with the following modifications: Explants were pre cultured on M100 medium lacking thiamine-HCl, pyridoxine-HCl, nicotine acid and $FeSO_4.7H_2O$. The recombinant *A. tumefaciens* strain was cultured for two days in LB medium and directly used to immerse the explants for 15 seconds. Two days after transformation the explants were transferred to the MS 30 selection medium containing 1mg/l zeatin, 11mg/l cefotaxime, 100mg/l vancomycin and 100mg/l of the selectable agent kanamycin. Every 2-3 weeks the explants were transferred to fresh medium which resulted in the recovery of 36 calli with green shoots. One shoot per callus was then rooted on standard M13 medium which gave rise to 26 kanamycin resistant transformed plants.

Results and discussion

Cloning

In order to elucidate the molecular basis of the *Adh-1* null mutants, a clone of the *Adh-1* gene was required. Although a cloned 1.8 kb *Bgl*II fragment (TAdh1) containing *Adh* sequences (Wisman et al. 1991) was already available, its use was not satisfactory, because part of the structural gene was missing. Therefore, we aimed at cloning the entire *Adh-1* gene and its upstream regulatory sequences. Southern blot analysis using the TAdh1 probe indicated that the entire structural *Adh-1* gene was contained on an 11 kb *Eco*RI fragment. This fragment

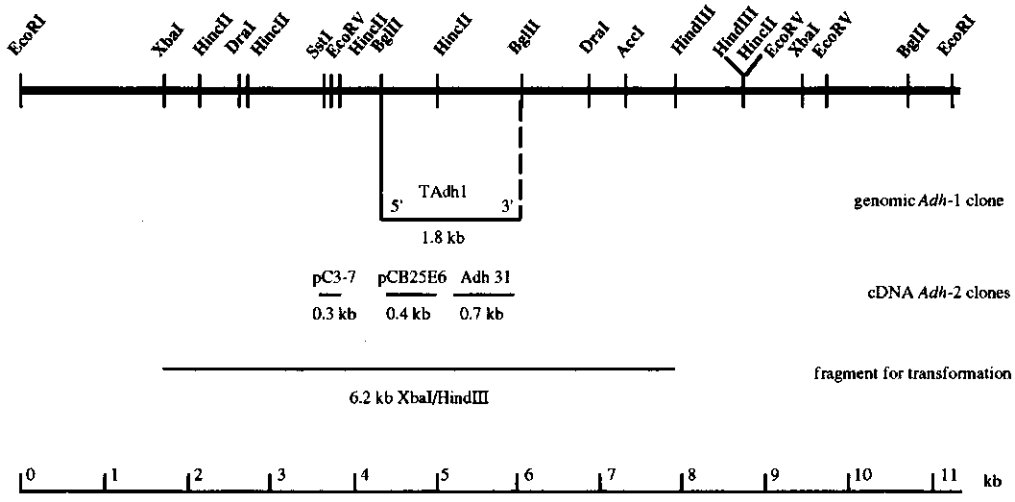

Fig. 1. Physical map of the *Adh-1* gene. The map shows the position of the *Adh-1* gene on a 11 kb genomic *Eco*RI fragment that was sub cloned into pUC18. Two ug of the recombinant plasmid were subjected to restriction mapping using the restriction enzymes indicated. Southern analysis using the three *Adh-2* cDNA clones, pC3-7, pCB25E6 and Adh 31, revealed the position and orientation of the *Adh-1* gene. The position of the TAdh1 clone, the three cDNA *Adh-2* clones and the 6.2 kb *XbaI/Hind* III sub fragment used for transformation are indicated separately.

was purified from the tomato cultivar GT by preparative gel electrophoresis, ligated to the lambda DASHII vector which was cut with *Eco*RI, and subsequently packaged to produce recombinant clones. Upon screening approximately 20,000 recombinant phage plaques using TAdh1 as a probe, 10 positive clones were identified. Restriction enzyme analysis of DNA from 5 purified positive plaques showed these to contain the 11 kb insert. One 11 kb *Eco*RI

insert was sub cloned into pUC18 to serve in the construction of a physical map (Fig. 1.). Cross-hybridization with three cDNA clones encompassing the entire coding region of the *Adh-2* gene (Wisman et al. 1991) revealed the position and orientation of the *Adh-1* gene (Fig. 1).

Mapping

In order to verify whether sequences contained on the 11 kb *Eco*RI fragment indeed originated from the *Adh-1* gene, a 1.8 kb *Bgl* II sub fragment (Fig. 1) was mapped using RFLP linkage analysis in a segregating F_2 population of a *Lycopersicon esculentum* cv. Allround x *Lycopersicon pennellii* LA 716 cross (Odinot et al. 1992). Southern analysis of 84 F_2 plants of the segregating population showed that the genomic clone was located on chromosome 4, between the RFLP markers TG 182 and TG 65, close to TG 272B (Fig. 2 and 3). No linkage was obtained with TG 22, a marker located on the distal part of chromosome 4. The position of the 1.8 kb *Bgl* II fragment corresponded to the *Adh-1* locus shown on the high density molecular linkage map constructed by Tanksley and coworkers (1992; Fig. 3).

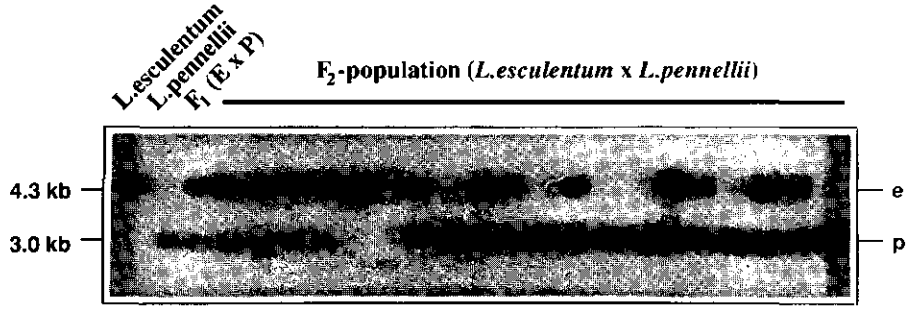

Fig. 2. Hybridization patterns of *Adh-1* in a F_2 mapping population derived from a cross between *Lycopersicon esculentum* cv. Allround x *Lycopersicon pennellii* (LA 716). DNA was extracted from 84 individual F_2 plants, digested with *Dra*I, run on agarose gels, transferred to Gene Screen membranes and probed with the 1.8 kb *Bgl*II sub fragment. The upper band of 4.3 kb (e) is the *L. esculentum* allele; the lower band of 3.0 kb (p) is the *L. pennellii* allele. A sample of 19 F_2 individuals is shown.

Adh-1 expression in transgenic tobacco plants

In order to confirm the presence of the *Adh-1* gene on the 11 kb *Eco*RI fragment, a 6.2 kb *Xba*I/*Hind*III sub fragment (Fig. 1) was inserted in the plasmid pBI 121 and subsequently introduced in cotyledons of tobacco SR1 using the *Agrobacterium tumefaciens* strain LBA 4404 (Visser et al. 1991). A total of 26 transgenic kanamycin resistant tobacco plants were

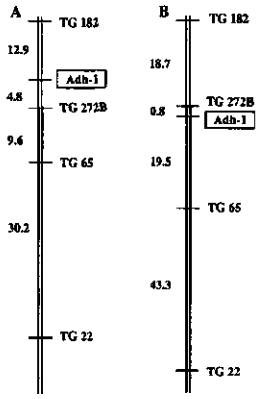

Fig. 3. The position of *Adh-1* on chromosome 4 (A) as calculated with the linkage program JOINMAP in comparison to (B) the map constructed by Tanksley and coworkers. Only the relevant portion of chromosome 4 is depicted

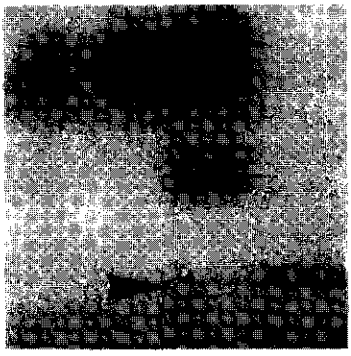

Fig. 4. ADH zymogram of (1) *Nicotiana tabaccum* SR1, (2) Tc 4-1, a kanamycin resistant tobacco plant transformed with an empty vector, (3) Ta 11-1, a kanamycin resistant tobacco plant transformed with the 6.2 kb *Xbal*/*Hind*III fragment and (4) *L. esculentum.* cv. GT. Pollen samples of each individual were ground, subjected to starch gel electrophoresis and stained for ADH activity . The *L. esculentum* homodimer in lane 3 is indicated by an arrow.

recovered. Starch gel electrophoresis of pollen grains collected from individual tobacco transformants showed tomato specific ADH-1 activity in 6 of the 14 transformants tested. The isozyme pattern of wild type tobacco SR1 consisted of three bands (Fig. 4, lane 1), suggesting a heterozygous condition for the *Adh* gene, probably as a result of the amphidiploid origin of tobacco. In contrast, in wild type tomato plants a single *Adh-1* band was obtained that migrated more slowly (Fig. 4, lane 4). Thus, when tomato *Adh-1* is expressed in the transgenic tobacco plants one would expect six isozyme bands: three from tobacco, a single band from tomato and two heterodimers composed of the tobacco and tomato subunits. Among the six transgenic plants expressing tomato *Adh-1*, only one transformant, Ta 11-1 (lane 3 in Fig. 4), showed all six bands. In the five remaining transformants the tomato

homodimer was not visible (data not shown), most likely as a result of the low expression of the tomato *Adh-1* gene as compared to the tobacco *Adh* gene. In none of the eight control plants (tobacco plants transformed with an empty vector) were isozyme bands other than those of tobacco detected (figure 4, lane 2).

Having established the isozyme patterns in transformed and wild type tobacco plants, we then screened the DNA of transformed tobacco plants for the presence of the 6.2 kb *Xba*I/*Hind*III fragment. For this purpose, a Southern blot was prepared from DNA isolated from two transformants, Ta 11-1 and Ta 10-2, which showed a high and low tomato *Adh-1* expression, respectively, and from several control plants. Upon digestion with *Hinc*II (Fig. 5), *Bgl*II (Fig. 5) and *Xba*I/*Hind*III (data not shown) fragments with an approximate length of 1.4, 1.8 and 6.2 kb were detected. The fragments corresponded to those of *L.esculentum* (lane 4 in Fig. 5) and were thus diagnostic for tomato *Adh-1*. The second 3.5 kb *Hinc*II band of *L.esculentum* was absent in the transformed tobacco plants, because this particular *Hinc*II site was not contained on the *Xba*I/*Hind*III fragment (Fig. 1). No hybridization signal was detected in the control and in the wild type tobacco plant. In conclusion, the data presented show that a functional *Adh-1* gene of tomato has been cloned, which is located on chromosome 4 and directs the synthesis of ADH-1.

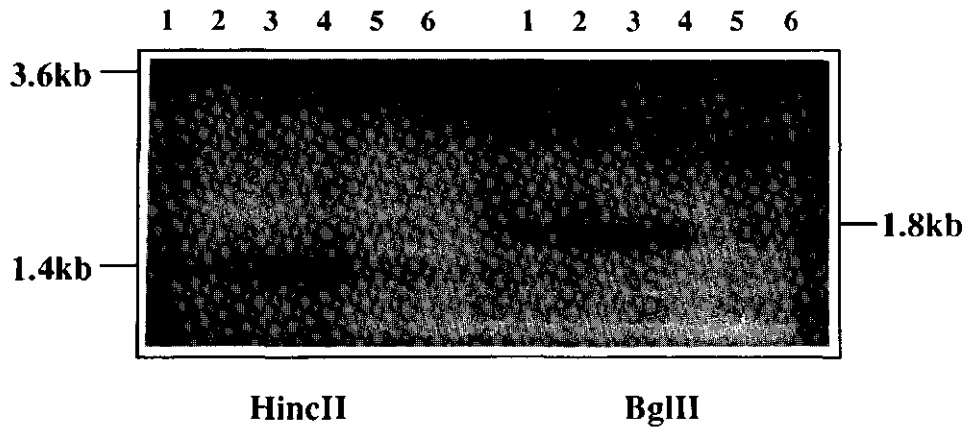

Fig. 5. Identification of tomato *Adh-1* DNA in transformed tobacco plants following Southern analysis and hybridization with TAdh1. Lane 1: Ta 11-1, tobacco plant transformed with *Xba*I/*Hind*III fragment; lane 2: Ta 10-2, a tobacco transformant with a lower level of *Adh-1* expression; lane 3: *L. esculentum* ; lane 4: Ta 11-1; lane 5: Tc 4-1, a tobacco plant transformed with an empty vector; lane 6: tobacco SR1. Note that the tomato specific *Adh-1* bands are missing from SR1 and Tc 4-1.

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Chapter 4

Isolation of two independent allyl alcohol resistant *Adh-1* null mutants in tomato following selection of pollen and seeds.

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Summary

In an attempt to trap anonymous transposable elements in the alcohol dehydrogenase (*Adh-1*) gene of tomato (*Lycopersicon esculentum*), the genetically unstable (mutable) tomato lines Yv^{ms}, Sulfurea and 2s-Iso were crossed to the *Adh-1* null mutant B15-1-8. Treatment of 158,500 F_1 seeds with allyl alcohol led to the identification of a new *Adh-1* null mutant (OYO 424). Similarly, fertilization of B15-1-8 with allyl alcohol-treated pollen collected from the mutable lines resulted in the recovery of another *Adh-1* null mutant (BOY 421). Like the mutant B15-1-8, OYO 424 and BOY 421 were found to lack *Adh-1* activity in both pollen and seeds. Genetic analysis showed that the mutations in both mutants were allelic to the *Adh-1* locus on chromosome 4. Southern analysis, using as a probe *Adh-1* genomic clones, spanning the entire coding region and 2 kb of the upstream region suggested that the mutations of the *Adh-1* alleles in B15-1-8, OYO 424 and BOY 421 were not due to insertional inactivation but rather to small alterations. Remarkably, the mutant allele of B15-1-8 was highly unstable, reverting to the wild type allele at a frequency of 5×10^{-4}.

Introduction

In plants, the molecular identification of an active, endogenous transposable element depends on its accidental visitation to a gene for which a molecular probe is available. As the frequency of insertion rarely exceeds the spontaneous mutation rate (Döring 1989), very large numbers of individuals must be screened for a change in phenotype which might be associated with the insertional inactivation of the target gene. In that manner, endogenous transposable elements have been isolated from the *waxy* and *shrunken* genes of maize (Fedoroff et al. 1983, Döring et al. 1984), the *nivea* gene of *Antirrhinum* (Bonas et al. 1984), and the *nitrate reductase* gene of tobacco (Grandbastien et al. 1989).

From tomato, no endemic transposable elements have been isolated as yet. Previously, we opted for using the *Adh-1* gene as a trap for transposable elements supposedly active in the genetically unstable (mutable) lines Yv^{ms} (Hagemann 1962), Sulfurea (Hagemann 1958) and 2s-Iso (Ramanna et al. 1985). This gene is constitutively expressed in pollen and at rather late stages of seed development (Tanksley 1979; Wisman et al. 1991). A second *Adh* gene which is present in tomato, *Adh-2*, is mainly expressed during early stages of seed development and under anaerobic conditions (Tanksley 1979 ; Tanksley and Jones 1981; Wisman et al. 1991).

Mutants deficient in ADH activity can readily be isolated on the basis of their resistance to allyl alcohol, which is toxic to wild type cells as a result of the ADH-catalyzed conversion of allyl alcohol to acrolein (Schwartz and Osterman 1976). Thus, the *Adh-1* gene

provides a transposon target system that combines the possibility of screening large numbers of individuals (seeds, pollen) for rare insertional mutations with the possibility of cloning the element out of the inactivated target gene. As discussed previously (Wisman et al. 1991), a feasible approach for the identification of an anonymous element would be to cross the mutable lines (*Adh-1*$^+$/*Adh-1*$^+$) to a marker line carrying a homozygous *Adh-1* null mutation, and then to subsequently select a rare allyl alcohol resistant *Adh-1* null mutant among the heterozygous (*Adh-1*$^+$/*Adh-1*^{0}) progeny. Such a marker line was then developed for this purpose (Wisman et al. 1991). In this paper we report the application of this line in crosses with the mutable lines in order to identify a new *Adh-1* null mutant. In a second approach, pollen of mutable lines selected for resistance to allyl alcohol was used to fertilize the *Adh-1* null mutant and wild type plants. This yielded another independent *Adh-1* null mutant.

Materials and methods

Plant material and growth conditions

Genetically unstable lines. Three tomato lines, Yv^{ms} (Hagemann, 1962), Sulfurea (Hagemann 1958) and 2s-Iso (Ramanna et al.1985), in which the genetic instability was possibly due to the activity of transposable elements, were used. Typical features of these lines include: i) variegation in leaf colour (*yellow virescent masculosterilis* (*yv* ^{ms}) and *sulfurea*), ii) non-Mendelian inheritance of the marker loci (*yv* ^{ms} and *sulfurea*) and iii) a relatively high frequency of mutations for various other loci resulting in dwarfism, deficiencies in chlorophyll, waxy pollen, a failure in chromosome pairing in meiotic cells (*desynapsis*) and a high incidence of chromosome breakage (2s-Iso; Ramanna et al. 1985).

Adh-1 null mutant. The *Adh-1* null mutant (B15-1-8) and its progenitor line (GT) have been described previously (Wisman et al. 1991). To eliminate the partial male sterility associated with B15-1-8, the mutant was back crossed to GT, and to the tomato line LA 889, which carries the mutation *venosa* (Khush and Rick, 1967), that is associated with yellow leaves with dark green veins. Among the selfed progeny of the F_1's, two homozygous *Adh-1* null mutants, GTO and VO, were selected. All F_1 plants were grown in pots in the green house under 18 hours light and a night temperature of 18 °C.

Allyl alcohol selection

Seeds. Allyl alcohol sensitivity of seeds from the genotypes GT (*Adh-1*$^{+}$/*Adh-1*$^{+}$), GT/GTO (*Adh-1*$^{+}$/*Adh-1*^{0}) and GTO (*Adh-1*^{0}/*Adh-1*^{0}) was tested using a modification of the methods of Jacobs et al. (1988) and Wisman et al. (1991). Fifty seeds of each genotype were soaked for three hours in a solution containing either 0, 10, 40, 80, 120, 160 or 200 mM allyl alcohol, under continuous aeration to suppress *Adh-2* induction. After rinsing the treated seeds for two hours in luke warm water, they were incubated on wet filter paper in Petri dishes in the dark at 25 °C. After one week the germinated seeds were counted. This treatment was replicated three times. During the course of our studies it was noticed that the response to allyl alcohol depended on the genotype and the physiological conditions of the seeds. Therefore, each seed-lot was treated with a different concentration of allyl alcohol, which was established by method of trial and error, allowing approximately 1% of the seeds to germinate (see Table 2).

Pollen. Pollen grains were collected in Petri dishes and subjected to allyl alcohol treatment in a chromatography chamber covered with filter paper, essentially according to the method of Schwartz and Osterman (1976). After wetting the filters with a solution of 15 ml distilled water containing 200 µl allyl alcohol, the pollen grains were placed in the chamber for 0, 20, 40 or 60 minutes. All treatments were done in triplicate. To assess their viability, pollen grains were germinated on 0.7% agar containing 3mM H_3BO_3, 1.7 mM $Ca(NO_3)$ and 10% sucrose, and these pollen samples were used to pollinate 15 emasculated flowers of GT or B15-1-8. In the resulting fruits, the number of seeds was determined. In addition, allyl alcohol-treated pollen of the hybrid GT/GTO (*Adh-1*$^{+}$/*Adh-1*^{0}) was used to pollinate B15-1-8 (*Adh-1*^{0}/*Adh-1*^{0}), and the genotype of the resulting progeny was determined using starch gel electrophoresis.

Use of mentor pollen. By applying stringent selection conditions, it was expected that the number of mutant pollen grains surviving would be too low to accomplish fertilization (population effect). Therefore, following pollination with pollen that had been treated for 60 minutes with allyl alcohol, a second source of pollen, the mentor pollen, was applied to serve as carrier. This method was adapted from experiments in which irradiated pollen grains were applied as a second pollinator to overcome incompatibility barriers in genomic wide crosses in cottonwood (Stettler 1968). Three different lines were used for this purpose, the features of which are summarized in Table 1. Pilot experiments showed that the use of these lines as second pollinator did not interfere with the capacity of the first pollinator to form seeds. Using the dominant *Cu* mutant (Maxon-Smith and Ritchie 1983) as a source of mentor pollen, the majority of the F_1 seedlings will show the *Cu* (curled leaf) phenotype amongst

which the seedlings derived from pollen grains of the mutable lines are recognized by their wild type phenotype. Similarly, rarely occurring true *L.esculentum/L.chilense* hybrids can be phenotypically distinguished from hybrids with the mutable lines.

Table 1. Features of mentor pollen lines

Species	Germination on tomato stigma's	Fertilization of tomato egg cells	Formation F_1 seeds in crosses to *L.esculentum*	Phenotype F_1 plants
Solanum verrucosum[1)]	+	-	-	-
Lycopersicon esculentum *Cu* mutant[2)]	+	+	+	*Cu*
Lycopersicon chilense	+	+	very few due to embryo abortion	hybrid with *L. chilense*

1) diploid *Solanum* species 2) *Cu* = dominant mutation, curled leaves

Electrophoresis and ADH activity assay. The procedure for starch gel electrophoresis and the ADH activity assay for pollen grains have been described previously (Wisman et al. 1991). In order to determine the number of revertant pollen grains, the following modifications of the ADH pollen assay were necessary. Pollen grains were collected in tubes followed by the addition of 10 ml 0.1 M Tris HCl. After a thorough shaking, the pollen mixture was immediately frozen in liquid nitrogen for a maximum of 24 hours. The samples were then thawed under continuous shaking for 2 hours at room temperature and subsequently stained for ADH activity (Tanksley 1979).

Estimation of reversion frequencies. Samples of 15 µl of pollen mixture were evenly layered on a slide and were counted under incident light at a magnification of 16 x, using a graticule with a field of 100 squares divided in a 10 by 10 pattern. The number of pollen grains was determined in one row of 10 squares and multiplied by 10 to estimate the total number of pollen. The error of this method was estimated to be 5%. All pollen grains of normal size that stained dark blue were scored as ADH^+ revertants. Light blue pollen grains were counted separately and were not included in the number of revertants.

DNA isolation, Southern transfer and hybridization. Genomic DNA from young tomato leaves was isolated according to Van der Beek et al. (1992). Three µg of restriction enzyme-digested DNA were separated on gels containing 0.6% or 1.5% agarose(w/v). After a 1 minute treatment with short wave UV to accomplish nicking, the DNA was transferred to GeneScreen Plus membrane, using the procedure as recommended by the manufacturer (DuPont). DNA was then cross-linked to the membrane by irradiation with UV for 1 minute and the filters were baked at 80 °C for 2 hours. The following restriction enzymes were used: *Aac* I, *Ava* I, *Alu* I, *Bam* HI, *Bcl* I, *Dde* I, *Dra* I, *Eco* RI, *Eco* RV, *Hae* II, *Hae* III, *Hha* I, *Hin* CII, *Hin* DIII, *Hin* FI, *Hpa* II, *Kpn* I, *Mbo* I, *Mbo* II, *Mlu* I, *Msp* I, *Nco* I, *Nde* I, *Nru* I, *Pst* I, *Pvu* I, *Pvu* II, *Rsa* I, *Sau* 3a, *Sst* I, *Stu* I, *Taq* I, *Xba* I, *Xho* I. Hybridization was performed overnight in a solution containing 50 mM Tris-HCl pH7.5, 1 M NaCl, 1%(w/v) SDS, 10% (w/v) dextran sulphate, 0.1 mg/ml salmon sperm DNA at 65 °C, using as a probe 25ng of the *Adh-1* genomic clones, labeled (10^8-10^9 dpm/µg) by random priming (Feinberg and Vogelstein, 1983). Blots were washed at a stringency of 0.5 x SSC at 65°C. Autoradiography was performed at -80 °C for 1-7 days using Kodak XAR film and an intensifying screen.

Hybridization probes. Two *Adh-1* genomic clones were available (Fig. 3). Clone TAdh1-XB was 2.7 kb long and contained the 5' region and upstream sequences (Chapter 2). Clone TAdh1 was 1.8 kb long and contained the central and 3' region (Wisman et al. 1991).

Results

Dose response curves

Since the vast majority of F_1 seeds, resulting from crosses between the between the *Adh-1* null mutant (B15-1-8) and the mutable lines (*Adh-1*$^+$/*Adh-1*$^+$), should be heterozygous for the *Adh-1* allele, it was essential to use allyl alcohol at concentrations lethal for the bulk of the heterozygotes, but non-lethal for the rare, homozygous *Adh-1* null mutants. To this end, a series of control experiments was carried out in which seeds from GT (*Adh-1*$^+$/*Adh-1*$^+$), GTO (*Adh-1*^{0}/*Adh-1*^{0}) and the F_1 (GT/GTO *Adh-1*$^+$/*Adh-1*^{0}) were treated with allyl alcohol at various concentrations. Germination of GT seeds and of GT/GTO F_1 seeds was completely inhibited by allyl alcohol at concentrations of 80 and 120 mmol respectively, whereas seeds of the *Adh-1* null mutant (GTO) survived treatments of up to 160 mmol (Fig. 1). Similar tests performed on pollen grains showed that *Adh-1*^{0} pollen collected from GTO were resistant to the treatments with allyl alcohol vapour that were detrimental to *Adh-1*$^+$ pollen grains (Fig. 2A). When allyl alcohol treated *Adh-1*^{0} pollen grains were used in crosses, normal seed set occurred (Fig. 2B), which indicated that the *in vitro* germination of pollen grains

corresponded to their ability to achieve fertilization.

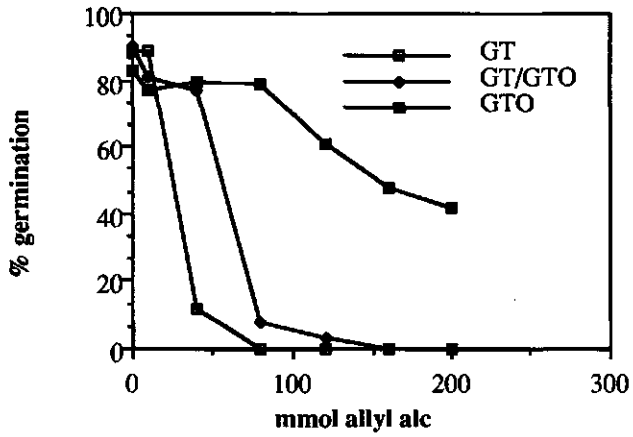

Fig. 1. Response of seeds to allyl alcohol. Fifty seeds of GT (*Adh-1*$^+$/*Adh-1*$^+$), GT/GTO (*Adh-1*$^+$/*Adh-1*^{0}) and GTO (*Adh-1*^{0}/*Adh-1*^{0}) were treated in triplicate for 3 hours with allyl alcohol concentrations of 0, 10, 40, 80, 120 and 160 mM, respectively, and allowed to germinate on filter paper for one week.

A 40-60 minute treatment of pollen grains collected from heterozygous *Adh-1*$^+$/*Adh-1*^{0} plants showed that approximately 50 % were capable of germinating *in vitro*, a figure that corresponded to the percentage of *Adh-1*^{0} pollen grains present in the sample (Fig. 2A). Despite this viability, the seed set, obtained using the sample that had been treated for 60 minutes, was reduced to 10 % (Fig. 2B). In order to examine whether *Adh-1*^{0} or *Adh-1*$^+$ pollen grains had remained functional in the allyl alcohol treated pollen mixtures, pollination's were made to *Adh-1* null mutant plants. The isozyme analysis of 30 individual seeds of each F_1 progeny showed that, from samples that had been treated for 40 and 60 minutes, only the *Adh-1*^{0} pollen had contributed to the offspring (Fig. 2C). In conclusion, it has been shown that *Adh*^{0} pollen grains were resistant to allyl alcohol treatments of up to 60 minutes and remained capable of achieving fertilization.

Isolation of an Adh-1 null mutant through selection of seeds

The three mutable tomato lines, homozygous for the wild type *Adh-1* $^+$ allele, were crossed to the *Adh-1* null mutant B15-1-8. Upon germinating the 158,500 F_1 seeds in a solution containing 120-170 mmol allyl alcohol, 2481 (1.56%) resistant seeds were obtained and

grown to mature plants without further selection. In order to identify homozygous *Adh-1* null mutants among this population, pollen was collected from each plant and stained for ADH activity. This resulted in the final identification of one *Adh-1* null mutant (OYO 424; Table 2) in a population derived from a cross between B15-1-8 and Yv^{ms}.

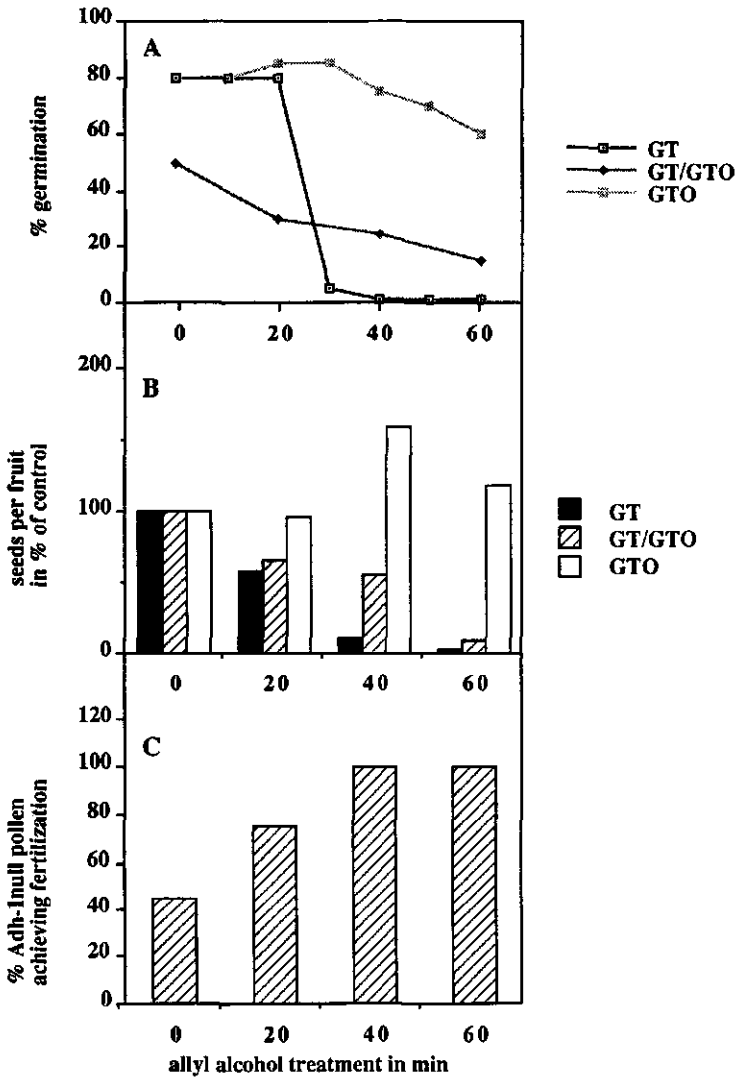

Fig. 2. Response of pollen grains to allyl alcohol.

A: Pollen of the genotypes GT (*Adh-1*$^+$/*Adh-1*$^+$), GT/GTO (*Adh-1*$^+$/*Adh-1*^{0}) and GTO (*Adh-1*^{0}/*Adh-1*^{0}) were treated with allyl alcohol for the times indicated and allowed to germinate on agar for two hours.

B: The treated pollen samples from A were used to pollinate flowers of wild type and *Adh-1* null mutant plants. The average number of seeds per fruit is expressed as a percentage of the average number of the untreated control.

C: Percentage *Adh-1* null pollen grains achieving fertilization on *Adh-1* null mutants. The seeds obtained are stained for *Adh-1* activity using starch gel electrophoresis.

Table 2. Selection of allyl alcohol resistant F_1 seeds

Cross	Number of F_1 seeds tested	Number of F_1 plants surviving	Number of *Adh-1* null mutants	Allyl alcohol concentration treatment
B15-1-8 x 2s-Iso	1,000	29	0	140
2s-Iso x B15-1-8	2,400	122	0	160
B15-1-8 x Yv^{ms}	32,000	633	1	140-170
Yv^{ms} x B15-1-8	73,000	878	0	140
B15-1-8 x Sulfurea	6,100	76	0	120
Sulfurea x B15-1-8	44,000	746	0	160
Total	158,500	2484	1	

The F_1 seeds resulted from reciprocal crosses between the *Adh-1* null mutant B15-1-8 ($Adh\text{-}1^0/Adh\text{-}1^0$) and the mutable lines Yv^{ms}, Sulfurea and 2s-Iso, each being homozygous $Adh\text{-}1^+$.

Isolation of an Adh-1 null mutant through selection of pollen grains

In addition to selecting putative insertion mutants at the seed level, an attempt was made to obtain mutants via direct selection of pollen grains collected from the mutable tomato lines using the conditions as described above. To this end, over 3000 flowers were pollinated with pollen from the mutable lines, that had been treated with allyl alcohol for 30 or 45 minutes. Similarly, over 6500 flowers were pollinated with pollen that had been treated for 60 minutes (Table 3). To accomplish effective fertilization after the last treatment, a second round of pollination was carried out within 24 hours using mentor pollen (Table 3). The 1564 seedlings derived from these crosses were grown to mature plants. ADH activity tests of the pollen grains collected from these plants showed that one plant, BOY 421, which was derived from a pollen grain from the line Yv^{ms}, was homozygous *Adh-1* null.

Table 3. Selection of allyl alcohol resistant pollen grains

Pollen parent		Number of flowers pollinated	Number of F_1 plants			
Tomato lines	Mentor pollen		Total	Derived from mentor pollen	Derived from pollen of mutable lines	*Adh-1* null mutants
2s-Iso	-	1000	537	-	537	0
Sulfurea	-	1000	204	-	204	0
Sulfurea	*Cu* mutant	200	1910	1910	0	0
Sulfurea	*Solanum verrucosum*	2000	97	0	97	0
Yv^{ms}	-	1100	130	-	130	1
Yv^{ms}	*Cu* mutant	750	8099	7946	153	0
Yv^{ms}	*Lycopersicon chilense*	600	33	14	19	0
Yv^{ms}	*Solanum verrucosum*	3100	428	0	428	0
Total		9750	11,438	9870	1568	1

Genetic characterization of the new Adh-1 null mutants OYO 424 and BOY 421

The isolated *Adh-1* null mutants both originated from the cross B15-1-8 (*Adh-1*^{0}/*Adh-1*^{0}, *Yv*$^+$/*Yv*$^+$, *u*/*u*) x Yv ^{ms} (*Adh-1*$^+$/*Adh-1*$^+$, *yv*/*yv*, *u*$^+$/*u*$^+$) and not from unwanted selfings, as inferred from the segregation of the parental markers *uniform ripening* (*u*) and *yellow virescent* (*yv*) in the selfed progenies of the mutants, OYO 424 and BOY 421. Therefore, the mutants, OYO 424 and BOY 421, carried the *Adh-1* null allele from B15-1-8, that was induced by EMS-treatment, as well as the newly isolated null allele. It could be argued that the *Adh-1* null phenotype of OYO 424 and BOY 421 was not the result of a mutation in the *Adh-1* gene but due to a dominant mutation in a regulatory gene. This possibility was tested by screening individual seeds of the selfed progenies of OYO 424 and BOY 421 for *Adh-1* activity, using starch gel electrophoresis. In addition, pollen grains collected from the selfed plants were stained for *Adh-1* activity. In none of the 32 F_2 individuals of OYO 424 and BOY 421 thus tested, *Adh-1* activity was detectable, indicating that, indeed, new null alleles from the *Adh-1* locus had been isolated.

As genes associated with an active transposable element commonly show a high mutation rate, we screened the genotypes, OYO 424 and BOY 421, and the lines, GTO and VO, which both carry the EMS-induced *Adh-1* null allele in homozygous condition, although in different genetic backgrounds, for the occurrence of revertant pollen grains. To this end, the number of dark, blue staining, revertant pollen grains containing ADH, were counted in samples that otherwise contained colourless ADH^0 pollen grains. As a control to correct for false positives, part of the pollen samples was stained in the absence of ethanol, the substrate for ADH (Table 4).

Table 4. ADH-1^0 to ADH-1^+ reversion frequencies in *Adh-1* null mutants

Adh genotype	Total number of pollen grains screened	Number of ADH^+ pollen grains	Reversion frequency x 10^{-4} (± SD)
GTO	200,130	57	2.9 ± 0.4
VO	172,030	68	4.8 ± 0.5
OYO 424	242,822	18	0.7 ± 0.2
BOY 421	221,692	27	1.2 ± 0.2
Control[1)]	159,900	1	0.006

[1)] Pollen of the *Adh-1* null mutants stained in the absence of the substrate ethanol

Among the reversion frequencies thus determined, the mutants VO and GTO, homozygous for the EMS- induced null allele, showed the highest rates of 3 - 5 x 10^{-4} (Table 4). As the mutants, OYO 424 and BOY 421, carried one copy of this highly unstable allele, no conclusion could be drawn as to the instability of the second *Adh-1* null allele present in OYO 424 and BOY 421. In addition, the isolation of three revertant plants derived from crosses of wild type (*Adh-1*$^+$/*Adh-1*$^+$) to B15-1-8 (*Adh-1*^{0}/*Adh-1*^{0}) plants, show that the reversion events were heritable. These revertants were shown to contain a molecular marker (a specific *Eco*RI fragment homologous to the retrotransposon *Tst1*, see Chapter 7) as well as the phenotypic marker (*uniform ripening*), diagnostic for the presence of the GTO genome in the revertants (data not shown). In conclusion, these data showed that the EMS-induced *Adh-1* null mutation was highly unstable, and therefore might have been a good candidate to carry an insertion.

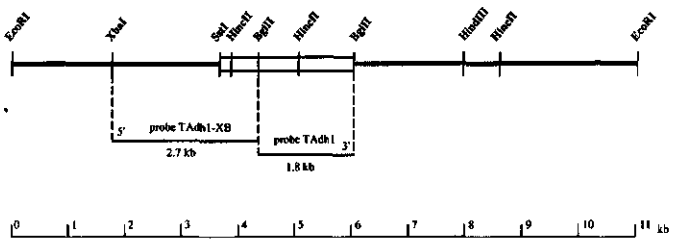

Fig. 3. Representation of the position of the *Adh-1* gene on a 11 kb *Eco*RI fragment cloned from an genomic library prepared from DNA of the tomato line GT in the Lambda DASH II vector.

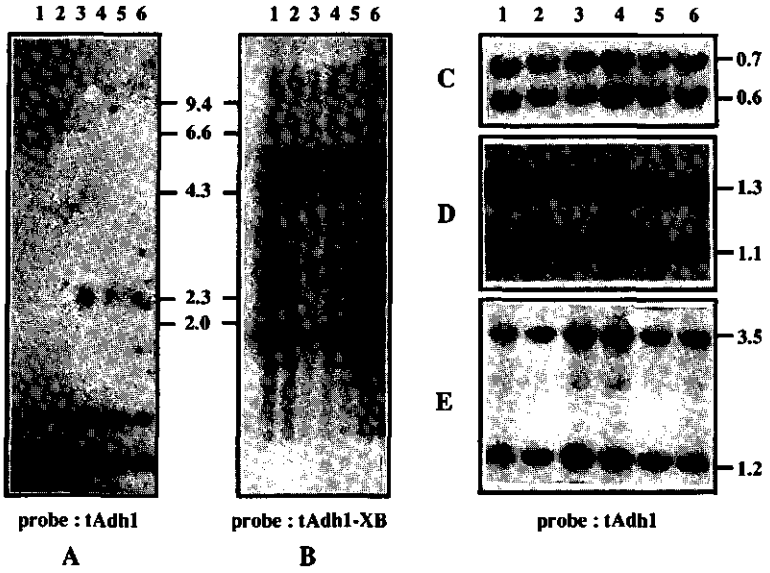

Fig. 4. Southern analysis of the *Adh-1* gene. Southern blots of *Hae* III (A+ B), *Alu* I (C), *Sau* 3A (D) and *Hinc* II (E)- digested DNA from the *Adh-1* null mutants and their wild type parents were hybridized to TAdh1 and TAdh1-XB Wild type plants: GT (lane1), Condine Red (lane 5), Yv^{ms} (lane 6) *Adh-1* null mutants: B15-1-8 (lane 2), OYO 424 (lane 3) and BOY 421 (lane 4).

Molecular analysis of the Adh-1 null mutants

The molecular basis of the three mutations at the *Adh-1* gene was examined by Southern blot analysis using two *Adh-1* specific genomic clones which span the entire gene together with approximately 2000 bp of the upstream region (Fig. 3). Although the analysis of the 5' region of the *Adh-1* gene was somewhat complicated by a moderately repetitive DNA sequence

present in the upstream region, no major restriction fragment length polymorphisms were detected between the *Adh-1* null mutants and their parental lines, B15-1-8 and Yv^{ms}, following digestion with 34 restriction enzymes (Fig. 4). Apparently, the isolated *Adh-1* mutations were due to point mutations or small rearrangements and not to a transposon insertion, as had been sought for.

Discussion

Following selection for allyl alcohol resistant seeds and pollen grains, two independent *Adh-1* null mutants were obtained, which were allelic to the EMS-induced *Adh-1* null allele (B15-1-8) previously isolated (Wisman et al 1991). As no restriction fragment length polymorphisms were detected, which could have pointed to the presence of a transposable element, the mutations were assumed to be the result of point mutations or small internal deletions.

Surprisingly, the EMS-induced *Adh-1* null allele was highly unstable, reverting at a frequency of 3-5 x 10^{-4}. This frequency was at least 100 times higher than the frequency observed in EMS-induced *Adh* null mutants in maize (Freeling 1976). As already noted by Freeling, any particular revertant can be the result of back mutations, gene conversion, suppression, activation of other genes and/or derepression of *Adh-2*. These phenomena usually occur at very low frequencies (Freeling 1977). Using starch gel electrophoresis, we demonstrated wild type ADH-1 protein activity in revertants from B15-1-8 and their offspring, showing that the derepression of the *Adh-2* gene in pollen grains is not a likely explanation for the occurrence of revertants (data not shown).

It has been suggested (Wisman et al. 1991) that the mutant B15-1-8 was incapable of synthesizing ADH proteins from its transcripts, probably as a result of a small alteration in the RNA sequence. This view is now further supported by our finding showing no major rearrangements to be associated with the mutation. In considering these characteristics of the B15-1-8 mutation, the occurrence of a high number of revertants was not expected. Usually such high reversion frequencies are associated with the excision of a transposable element and not with the occurrence of point mutations (Ralston et al. 1987). Now that the mutant and revertant alleles are available, it should be feasible to determine the DNA alterations causing the mutation and reversion. This may provide a clue as to the mechanism underlying the instability observed in this mutation.

The original objective of our studies was to identify endogenous transposable elements in tomato through the insertional inactivation of the *Adh-1* gene. The success of such an approach depends on various factors including i) the availability of genotypes in which transposable elements are active, ii) the frequency at which a transposable element visits the target gene and iii) the efficiency of the selection scheme. Although the seed selection

scheme by itself was successful in yielding a new *Adh-1* null mutant, the selection method was rather inefficient. First, the response of seeds to allyl alcohol was variable for each genotype and dependent on the growing conditions of the plants from which the seeds were harvested. Second, a variable amount of ADH-2 activity was detected in the F_1 (*Adh-1* $^{+}$/*Adh-1* ^{0}) seeds. Since the ADH-2 activity reduced the resistance of *Adh-1* null mutants to allyl alcohol, rather mild selection conditions had to be applied, which rendered the seed selection rather inefficient. For this reason, the selection of allyl alcohol resistant pollen grains is to be preferred for the analysis of large populations in the absence of ADH-2 activity.

From gene tagging experiments in maize lines containing active transposable elements, it is known that the frequency at which a particular locus is visited by a transposable element usually varies between 10^{-4} (for *Mu* elements) and 10^{-6} (Döring 1989). As no such lines were available in tomato, we made use of the mutable lines Yv^{ms} (Hagemann 1962), 2s-Iso (Ramanna et al. 1985), and Sulfurea (Hagemann 1958) assuming that their genetic instability was caused by the activity of transposable elements. Because we have not been able to detect an transposable element, this hypothesis may be challenged. It remains to be investigated whether the relatively low number of *Adh-1* mutants recovered is due to the low frequency at which the *Adh-1* gene is visited by a transposable element.

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Chapter 5

A re-investigation of the instability at the *yv* locus in tomato

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Summary

The instability at the *yv* locus on chromosome 6 of *Lycopersicon esculentum* has been re-investigated. Cytogenetic analysis of green, green-yellow variegated and yellow plants showed that the instability is not caused by the somatic segregation of extra chromosomal fragments as was proposed earlier by Hagemann. Instead, on the basis of the following observations it is postulated that the variegated line carries an unstable recessive allele of *yv* (yv^{mut}) that mutates frequently from dominant green to recessive yellow: (i) variegated plants produced variegated and yellow offspring in aberrant ratios, and (ii) yellow male sterile plants appeared in populations derived from crosses of the Yv^{ms} line with wild type plants and the line LA 780, being recessive *yv*. Using the isozyme marker *Aps-1* linked to *yv*, it was shown that the deficit in variegated and yellow mutants, which frequently occurs in F_2 populations derived from crosses between the variegated mutant and wild type yv^+ plants, was due to certation. From the analysis of these F_2 populations, it is concluded that the instability at *yv* is likely to be controlled autonomously. No evidence was obtained showing germinal reversion events to occur, as inferred from the absence of green plants among the selfed offspring. Variegated offspring were produced by a yellow plant which is interpreted to represent a somatic reversion event from yellow to variegated. Remarkably, when variegated plants were crossed to wild type plants, F_1 plants with a mutant phenotype appeared. On the basis of analogies with mutable alleles from maize and *Drosophila*, alternative mechanisms underlying the mutability of *yv* are discussed.

Introduction

Genes interrupted by transposable elements commonly show a high instability due to the frequent excision of the element out of the gene. When cell-autonomous traits such as pigmentation or chlorophyll production are involved, somatic instability gives rise to the variegated patterns so often noticeable in flowers, kernels and leaves (Nevers et al. 1986). In fact, variegation is one of the important features through which insertion mutations have been recognized. In addition to transposable elements, variegation has also been attributed to several other causes, including chimerism, virus infection, instability of (additional) chromosomal fragments, nutritional deficiencies (Kirk and Tilney-Bassett 1978) and monogenic mutations (Martínez-Zapater et al. 1992).

Nevers et al. (1986) formulated a set of general rules to allow insertion mutations to be identified on the basis of genetic criteria. These include: i) usually wild type spots appear on a recessive background, indicating that the phenotypic switch is from recessive to dominant, ii) segregation of the unstable character obeys the laws of Mendel, although stable recessives and wild type revertants frequently arise as a result of the (im)precise excision of the element, iii) new alleles are generated with heritably altered phenotypes, iv) in some instances a second

factor is required for the instability. Examples of the latter are to be found among the two-component systems such as *Ac/Ds* and *Spm/dSpm* in *Zea mays*, where the autonomous element is capable of transactivating the non-autonomous element integrated at the locus responsible for the variegated phenotype. A number of specific mutants are known in which variegation is associated with transposable element insertions, although the phenotypic switch is from dominant to recessive (reverse variegation) (Nevers et al. 1986). In some of these instances it has been shown that wild type expression of genes carrying an insertion of a transposable element occurred, due to the splicing of the element from the transcript. Null sectors on wild type background can then appear following the incorrect excision of the element (Wessler 1988, Menssen 1990).

In tomato, no endogenous transposable elements have been isolated thus far, but tomato lines which carry unstable genes have been recorded (Burdick 1956, Seeni and Gnanam 1980, Rick et al. 1959, Lesley and Lesley 1961 and 1963, Hagemann 1958 and 1962, Gill 1983). In none of these cases could the variegated character be unequivocally attributed to the presence of transposable elements. The green-yellow variegated mutant detected by Hagemann (1962) in the X_2 (=M_2) generation following irradiation of tomato seeds appeared to be a case of reverse variegation. This so called eversporting mutant was shown to be allelic to the *yellow virescent* (*yv*) locus, which is a nuclear gene affecting chloroplast development (Hagemann 1962, Herrmann and Hagemann 1967). The *yv* locus has been mapped previously on the long arm of chromosome 6 (Robinson and Rick 1954) in the proximity of the heterochromatic region bordering the centromere (Khush and Rick 1968). The mutant plants were distinguishable by the appearance of yellow sectors of variable size on the otherwise green cotyledons and leaves, and by the occurrence of green, variegated and yellow offspring. Because the yellow plants were male sterile, Hagemann has called this mutant allele *yellow virescent masculosterilis* (yv^{ms}). Typically, green seedlings became variegated during plant growth. In summary, three phenotypic classes of *yv* mutants have been recorded including variegated plants, yellow plants being male sterile (Hagemann 1967, Herrmann and Hagemann 1967) and yellow plants with normal fertility (Robinson and Rick, 1954).

On the assumption that endogenous transposable elements are involved in the instability at the *yv* locus, this case has been re investigated. Formerly, the somatic instability was explained in terms of the frequent loss of a centric fragment of chromosome 6 carrying the wild type yv^+ allele or a complementary factor which determined, together with yv^{ms}, a green leaf colour (Hagemann 1963, Herrmann and Hagemann 1967). According to this hypothesis, the presence of a fragment in an otherwise recessive homozygote yv^{ms} gives rise to green tissue, whereas the loss of this fragment in some cells and their clonal offspring produces yellow sectors or yellow progeny. In this study, cytogenetic evidence is presented that argues against the role of a centric fragment in the variegation. Furthermore, we report new genetic data on the unstable mutation, including its paramutagenic behaviour, and discuss the possible mechanisms underlying the instability.

Material and methods

Plant material. The seeds of the ever-sporting mutant (552184I6 and 552185I6) were kindly provided by Prof. R. Hagemann. The features and the origin of the tomato genotypes used are summarized in Table 1. The *yv* allele, described originally by Robinson and Rick (1954), has been indicated in the present article as yv^{st}, in order to emphasize the difference between yv^{ms} and *yv*. All plants were grown in the greenhouse with standard cultivation methods. Genetic segregation ratios were determined by germinating and growing the seedlings in boxes filled with potting soil. The seedlings were scored approximately 3 weeks after sowing.

Table 1. The features and the origin of the tomato genotypes

Code	Relevant marker genes	Origin	Reference
2501/49, Yv^{ms}	yv^{vg}, yv^{mut}	induced by X-rays in the variety Condine Red	Hagemann 1962
LA780	yv^{st}	spontaneous in asynaptic line of the variety San Marzino	Robinson and Rick 1954
N118	$Aps\text{-}1^{1}$	selected from a cross of the nematode resistant line USDA773175-1 to a susceptible line UCx99m-1	Ho et al. 1992
L41	deletion of a block of heterochromatin on the long arm of chrom 6, $Aps\text{-}1^{3}$	doubled monohaploid derived from a pollination of *L.esculentum* L121 with irradiated pollen of *L.pimpinellifolium*	Ecochard et al. 1969

Cytological techniques. For mitotic chromosome studies, the root tips were collected from young seedlings (5-6 weeks old) grown in small plastic pots. The root tips were treated for 4 hours with 0.002M 8-hydroxyquinoline. The root tips were then fixed in a 3:1 solution of ethanol and acetic acid, hydrolysed in 1N hydrochloric acid for 8 minutes at 60 ^{0}C, stained in Feulgen's reagent and gently squashed in a drop of 2% acetocarmine. Both mitotic and meiotic chromosomes were Giemsa stained according to the procedure of Ramachandran and Ramanna (1985). In each plant 50-100 somatic and pollen mother cells were analysed. The number of fragments was scored in well spread metaphase stages in mitotic cells, while in pollen mother cells, pachytene, metaphase I and anaphase I cells were analysed.

Acid phosphatase isozyme analysis. Leaf samples were assayed for acid phosphatase (APS) isozyme activity according to Aarts et al. 1991.

Results

Cytological investigations

Distribution of fragments

As to the nature of the instability at the *yv* locus in the so-called ever-sporting mutant, Hagemann proposed that the appearance of the yellow sectors on the green leaves was the result of the frequent loss of a centric fragment of chromosome 6 carrying a wild type yv^+ allele or another factor necessary for the expression of yv^{ms} (Hagemann 1963). According to this hypothesis green tissue but not yellow tissue is expected to carry the centric fragment. To verify this hypothesis we have determined the number of centric fragments in root tips and pollen mother cells (pmc) from 35 plants originating from the original mutant. Among these 35 seedlings, 8 carried green cotyledons, 19 variegated and 9 pure yellow ones. Similar to Hagemann's (1962, 1967) observations, all the seedlings with green cotelydons eventually became variegated.

Table 2. The number of plants with and without centric fragments determined in a selfed population of the Yv^{ms} line. The seedlings possessed either green, variegated, or yellow cotyledons.

	Green		Variegated		Yellow	
	Present	Absent	Present	Absent	Present	Absent
root tip cells	7	2	16	4	3	3
pollen mother cells	6	3	15	5	2	4

When examining root tip cells, most plants carried fragments in addition to the normal diploid chromosome number (2n=2x=24), their number varying between 1-6 (Table 2). Upon closer examination, however, it became clear that sometimes two pairs of macro and micro satellites of chromosome 2 were detached from the main bodies of the chromosomes giving the false impression of the presence of fragments (Fig. 1) . The chromosome 2 origin of the macro and micro satellites was further confirmed through Giemsa staining of the somatic chromosomes (Fig. 2). In pollen mother cells, the satellites were not detached from the

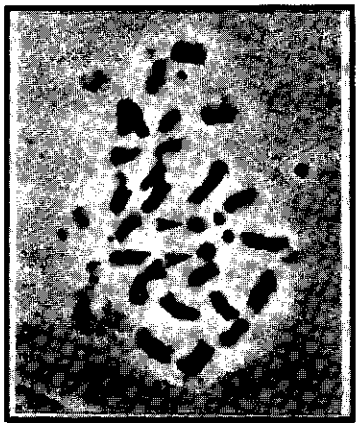

Fig. 1

Fig. 2

Fig. 3

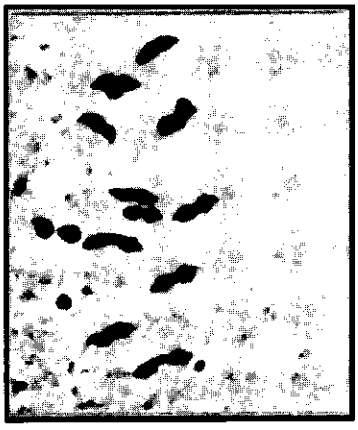

Fig. 4

Fig. 1. Feulgen stained metaphase stage in a root tip cell of the eversporting mutant Yv^{ms} showing a pair of nucleolar chromosomes (arrows) each having a large terminal satellite preceded by a smaller one.

Fig. 2. Giemsa stained late prophase stage in a root tip cell of the eversporting mutant showing four darkely stained fragments (arrows) which are in fact detached satellites.

Fig. 3. Metaphase I stage in a mutant showing 3 fragments, two of which are already dividing at this stage (arrows). One fragment (above) need not be a centric fragment, as it is not certain whether it will divide like the others.

Fig. 4. Metaphase I stage of a variegated plant showing a large and a medium sized fragment in additon to 12 normal bivalents.

chromosomes, and centric fragments were identified as such by their capacity to divide during late metaphase I or anaphase-I (Fig. 3). Following the study of pollen mother cells, it was established that the number of centric fragments present in green, variegated and yellow plants varied between zero and three (Table 2). A notable feature was the considerable variation in the size of the fragments ranging from 2-3 μM to the size of small tomato chromosomes (5-7μM). Thus, regardless of their phenotype, the majority of the plants appeared to possess fragments. Moreover, the number of fragments present in these plants was not related to the proportion of yellow plants detected in their offspring (Table 3). These findings argue against the hypothesis of Hagemann, who predicted that yellow plants are devoid of fragments and that an inverse relation exists between the number of fragments in a given parent plant and the percentage of yellows in their offspring.

Origin of the centric fragments

In order to determine whether the origin of the centric fragments could be assigned to chromosome 6, C-banding patterns of the centric fragments and all tomato bivalents were studied in pachytene cells of plants with fairly large centric fragments (Fig. 2). Giemsa staining of chromosome 6 showed one prominently C-banded region on its short arm adjacent to the centromere (Fig. 5a), while the two heterochromatic blocks on the long arm were not Giemsa stained (Fig. 5b and 5c). In contrast, the centric fragments revealed no C-banded regions although they usually were highly condensed and devoid of any euchromatin. The absence of any C-banded region among the centric fragments suggested that either the centric fragments

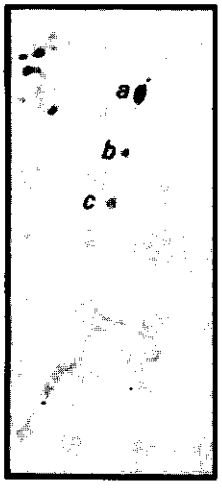

Fig. 5

Fig. 6

Fig. 5. Pachytene bivalent of chromosome 6 pair, showing a c-band on the short arm of chromosome 6 (a). Other heterochromatic regions on the long arm are not Giemsa stained (b and c).

Fig. 6. Pachytene bivalent of chromosome 5, showing the fragment paired with the centromere of this bivalent. Note: there are no euchromatic parts in the fragment.

lacked the C-banded region of chromosome 6 or that the fragments were not derived from chromosome 6 at all. The notion that the centric fragments appeared to de devoid of chromosome 6 specific sequences was further supported by the ability of the centric fragments to pair with centromeres of any of the twelve pachytene bivalents (Fig. 6). Taking into account that the centric fragments probably do not originate from chromosome 6, and that no correlation exists between the phenotype of the mutant plants and the occurrence of centric fragments in their genome, it was concluded that the centric fragments present in the original mutant played no role in the instability of this line.

Genetic analysis

Mutability of the yv^{mut} allele and genotype of variegated plants

To explain the conversion from green to yellow in the eversporting mutant we postulated the presence of an unstable *yv* allele (yv^{mut}) which frequently mutates to yv^{ms}. This assumption has originally been put forward by Hagemann before he switched to favouring the fragment hypothesis (1962). The occurrence of yellow male sterile plants in the offspring of variegated plants indicated that the newly generated yv^{ms} allele was stably transmitted to the next generation (Table 3). The percentage of yellow plants in the selfed progenies of variegated plants varied between 20 and 61 and usually differed from a 3:1 Mendelian ratio (Table 3). This variation was most likely due to the chimeric character of the parent plants. As the yv^{mut} allele frequently mutated to yv^{ms}, yv^{mut}/yv^{ms} and yv^{ms}/yv^{ms} sectors were expected to arise in the original yv^{mut}/yv^{mut} mutant. In cases where all gametes were derived from yv^{mut}/yv^{ms} sectors the maximum percentage of yellow plants among the selfed offspring should be 25%. The selfed progenies consisted, however, of significantly higher percentages, up to 61 %, of yellow plants. From this observation it was inferred that, unlike the male sterility of the yv^{ms}/yv^{ms} plants, the yv^{ms}/yv^{ms} sectors contributed to the germinal tissue. Alternatively, the relative high proportion of yellow plants could be the result of mutation events in meiotic tissue.

Among the selfed population of the original variegated mutant, early and late spotting plants were identified, the latter by their green cotelydons. These different patterns of expression may indicate a difference in genotype of the variegated plant. In principle, a variegated plant can be composed of two genotypes: yv^{mut}/yv^{mut} or yv^{mut}/yv^{ms}. Taking into account that in homozygous yv^{mut} plants, two simultaneous mutations are needed for the formation of yellow sectors, it is likely that the late spotting plants represented the homozygous yv^{mut} plants. To test this hypothesis, an attempt was made to deduce the genotype of the variegated plants from the proportion of yellows in their offspring, on the assumption that yv^{mut} homozygotes are likely to produce yellows in a frequency lower then 25 % (the percentage expected for a recessive character) and yv^{mut}/yv^{ms} heterozygotes in a frequency

Table 3. Segregations into variegated and yellow plants in the selfed progenies of "green"and variegated plants carrying different number of fragments

Parent			Progeny			
Plant number	Phenotype	Number of fragments	Variegated (yv^{mut})	Yellow (yv^{ms})	% yv^{ms}	mean % yv^{ms}
3	variegated	0	174	142	43	
5	variegated	0	554	200	27	
11	variegated	0	176	225	56	
25	variegated	0	224	180	45	
40	variegated	0	237	162	32	
						41
4	variegated	1 (1s)	207	227	52	
23	variegated	1 (1s)	246	169	41	
						47
1	variegated	2 (2s)	190	142	43	
6	variegated	2 (1m+2s)	554	200	27	
13	variegated	2 (1l+1s)	239	195	45	
15	variegated	2 (1l+1s)	246	169	41	
27	variegated	2 (2s)	146	194	57	
29	variegated	2 (1l+1s)	222	225	50	
31	variegated	2 (1l+1s)	200	198	50	
						48
10	variegated	3 (3l)	185	287	61	
14	variegated	3 (3s)	276	94	26	
24	variegated	3 (2m+1s)	192	230	55	
26	variegated	3 (1l+2s)	170	138	45	
30	variegated	3 (1m+2s)	344	84	20	41
6	green	0	97	115	54	
8	green	0	358	122	25	
38	green	0	205	82	29	
						36
39	green	1 (1m)	211	201	49	
41	green	1 (1s)	287	105	27	
						38
21	green	2 (1l+1S)	318	159	33	
						33
9	green	3 (1m+2s)	312	131	30	
32	green	3 (2m+1s)	374	94	30	
						30

l=large fragment; m=medium sized fragment; s=small fragment

higher than 25%. The proportion of yellows, however, exceeded 25% in all but one progeny. Apparently, the mutation rate was too high to distinguish parental genotypes by this criterion. Alternatively, the occurrence of early and late spotting plants may point to the presence of newly arisen yv^{mut} alleles which activated the mutation process early or late in development. These so called "changes in state" have been shown to be a characteristic feature of certain alleles carrying a transposable element insertion (Mc Clintock 1948, Fedoroff 1989). Analysis

of this possibility, however, was seriously hampered because of the impossibility to assign the *yv* genotype, yv^{mut}/yv^{mut} or yv^{mut}/yv^{ms}, to individual seedlings.

Direction of mutation

Two types of crosses were made to confirm the direction of mutation in variegated plants. First, variegated plants were crossed to wild type plants to produce either yv^{+}/yv^{mut} or yv^{+}/yv^{ms} heterozygotes. Upon selfing the yv^{+}/yv^{mut} heterozygotes, a variable number of yellow plants segregated in addition to the wild type green and variegated plants. This observation was compatible with yv^{mut} having mutated to yv^{ms} during plant development. Second, a variegated plant was crossed to LA780 (yv^{st}/yv^{st}) to produce a variegated yv^{mut}/yv^{st} heterozygote. This heterozygote was then used to pollinate yellow yv^{ms}/yv^{ms} plants. In the progeny, variegated yv^{mut}/yv^{ms} and yellow yv^{ms}/yv^{st} plants were expected to segregate, the latter being male fertile since yv^{st} is dominant over yv^{ms}. Among the yellow progeny, however, we found 18.5 % male sterile plants (yv^{ms}/yv^{ms}), which indicated that the yv^{mut} allele had mutated to yv^{ms}.

Reduced transmission of the mutant alleles yv^{mut} and yv^{ms}

Previously, Hagemann had observed that F_2 populations originating from crosses between wild type yv^{+} tomato lines and variegated plants, displayed a marked deficit of yellow and variegated plants (Hagemann 1962). That is, wild type green plants occurred at a higher rate than the 75 % expected for a dominant character. Similarly, we observed a deficit of mutant phenotypes in most F_2 populations derived from crosses with ten different tomato yv^{+} genotypes. This finding suggested that the instability at the *yv* locus was not autonomously controlled, but that a second factor, needed for the expression of the instability, was segregating in these F_2 populations. Under this assumption, the original variegated mutant should be homozygous for this factor as wild type green plants have not been found after selfing. Accordingly, individuals having a green phenotype, should appear due to the absence of the second factor, although their genotype is homozygous yv^{mut}. It was not possible to test this assumption directly in the respective F_2 populations as no means were available to determine the genotype of the individual plants. Instead, three variegated plants were crossed to LA780 (yv^{st}/yv^{st}) and their backcross and selfed progenies screened for the occurrence of stable green plants (Table 4). Since such green plants were not found, it seems that yv^{mut} was autonomously controlled and that the deficit in mutant phenotypes was not the result of the segregation of an additional factor. An alternative possibility would be that the yv^{st} was also homozygous for the postulated autonomous element and segregation does not occur in the F_2 populations.

Table 4. Segregations into variegated and yellow plants in F_1, F_2, BC_1 and BC_2 populations derived from crosses between three variegated plants and LA780

Cross		F_1 progeny		F_2 progeny		BC_1 progeny		BC_2 progeny	
Parents female x male	Phenotype	Variegated	Yellow	Variegated	Yellow	Variegated	Yellow	Variegated	Yellow
LA780 x VG32	yellow x variegated	58	54	1492	955 (5)*	105	955 (2)*	85	85 (1)#
LA780 x VG40	yellow x variegated	19	26	313	535 (2)*	114	519 (1)*		
BOY149 x LA780	variegated x yellow	137	137	1743	1728 (9)*	6	43 (1)*		

( )* Number of variegated F_1 plants that have been selfed or backcrossed to LA780 using LA780 as female parent

( )# Number of variegated BC_1 plants that have been crossed to LA780 using LA780 as female parent

VG32 and VG40 = variegated plants described in Table 3. BOY149 = variegated plant described in Table 6.

Following these observations, the question arose as to which mechanism induced the shortage in mutant phenotypes in F_2 populations. For example, competition between gametes carrying wild type and mutant *yv* alleles (certation) would result in a shortage of mutant phenotypes. Also, a reduced viability of *yv* seedlings would show the same effect. To test these possibilities, the *Aps-1* isozyme marker, which is absolutely linked to the *yv* locus on chromosome 6 (Medina-Filho and Tanksley 1983, Weide et al. 1993), was used as an aid for identifying the different *yv* alleles in two crosses. The first F_2 population to be analysed for its *Aps-1* genotype was derived from a cross between a variegated plant (*Aps-1*$^+$/*Aps-1*$^+$, *yv*^{mut} /.) and a tomato genotype N118 (*Aps-1*^{1}/*Aps-1*^{1}, *yv*$^+$/*yv*$^+$; Ho et al. 1992). This population showed a marked deficit in mutant phenotypes. The second F_2 population was derived from a cross of a variegated plant (*Aps-1*$^+$/*Aps-1*$^+$, *yv*^{mut} /.) to L41 (*Aps-1*^{3}/*Aps-1*^{3}, *yv*$^+$/*yv*$^+$), a tomato genotype in which a large block of heterochromatin, that is positioned close to the centromere of chromosome 6, is deleted (Ecochard et al. 1969). In this population mutant phenotypes segregated in a normal ratio. From the latter cross, two backcross populations with LA780 (*Aps-1*$^+$/*Aps-1*$^+$, *yv*st/*yv*st) were also analysed. In both F_2 populations, green plants carried at least one copy of the *Aps-1*^{1} allele (cross A in Table 5) or the *Aps-1*^{3} allele (cross B in Table 5), indicating that, as expected, the wild type *yv*$^+$ allele was present. Furthermore, the

Table 5. Distribution of several *Aps-1* alleles among green, variegated, and yellow F_2 plants derived from the cross N118 x Yv^{ms} (A) and L41 x Yv^{ms} (B)

A	*Aps-1*			
yv	1/1	+/1	+/+	Total
yv$^+$/.	44	36	0	80
yv^{mut}/.	0	0	6	6
yv/*yv*	0	0	3	3
Total	44	36	9	52

B	*Aps-1*			
yv	3/3	+/3	+/+	Total
yv$^+$/.	18	23	0	41
yv^{mut}/.	0	0	6	4
yv/*yv*	0	0	3	7
Total	18	23	11	52

frequency of the *Aps-1*$^+$ allele among the green plants was consistent with the ratios estimated from the frequency of variegated and yellow F_2 plants. Similarly, in the 57 plants of the two backcross populations (*yv*$^+$/*yv*^{mut}, *Aps-1*^{3}/*Aps-1*$^+$ x *yv*/*yv*, *Aps-1*$^+$/*Aps-1*$^+$) the 39 green plants carried the *Aps-1*$^+$ and the *Aps-1*^{3} allele, while the 18 yellow plants were all homozygous *Aps-1*$^+$. These observations excluded the possibility that a reduced viability of mutant seedlings caused the distorted segregation ratios (Table 5), as the failure of mutant seeds to develop in plants does not influence the frequency of mutant alleles produced by the parent. In other words, two third of the green plants will carry a mutant allele, irrespective of

the number of mutants in a population. Clearly this was not the case in the progenies tested, so we conclude that certation caused the distorted segregation data. Finally, these data argued again against the possibility that a second factor was needed to express the variegation.

Reversion

As insertion mutations usually produce wild type revertant alleles through the precise excision of the element, we have screened progenies of variegated plants for the occurrence of stable green offspring. Selfed progenies consisting of approximately 7000 seeds derived from 27 yellow *yv^{ms}/yvst* heterozygotes were examined for the same purpose. Among the selfed progenies derived from variegated plants wild type green plants were not identified. In contrast, the progeny of one yellow *yv^{ms}/ yvst* plant consisted of 125 variegated and 79 yellow seedlings, originating from seeds of a cluster in a higher part of the plant, whereas the lower clusters only produced yellow progeny. Apparently, during a particular stage of development a reversion from *yv^{ms}* to *yv^{mut}* had occurred in the germinal layer.

The mutant phenotype is occasionally expressed in F_1 plants

Surprisingly, one variegated (BOY149) and three yellow male sterile plants were observed among a population of 1671 F_1 plants originating from crosses between a wild type *yv$^+$* genotype carrying an *Adh-1* null mutation (B15-1-8; Wisman et al. 1991) and variegated plants. Their true hybrid nature was demonstrated by the presence of the maternally derived *Adh-1* null allele and a 5 kb polymorphic *Eco*RI fragment (Chapter 7). From these findings it was speculated that the mutant alleles *yv^{mut}* and *yv^{ms}* are capable of changing the *yv$^+$* allele to *yv^{mut}* or *yv^{ms}* when present in the same nucleus. Alternatively, a mitotic over crossing event that had occurred very early in embryo development could explain this phenomenon.

Once the first yellow sectors had appeared on the third and fourth leaf of the variegated F_1 plant BOY149, its phenotype was indistinguishable from other variegated plants. Three cuttings derived from the variegated part of plant BOY149 were grown to flowering plants. When selfed or back crossed to LA780, segregation into variegated and yellow male sterile plants occurred indicating that the change at the *yv$^+$* locus was heritable (Table 6). The segregation ratio was not significantly different from 3:1 in the selfed population, or from 1:1 in the backcross population, as if BOY149 was a *yv^{mut}/yv^{ms}* heterozygote in which the *yv^{mut}* allele did not mutate to *yv^{ms}*. A comparable low mutation rate of the *yv^{mut}* allele was also observed in the selfed offspring of six variegated BOY149 descendants, in which the proportion of yellow male sterile plants was either 25% or was slightly higher than 25%.

Table 6. Segregations into variegated and yellow plants in F_2, F_3 and backcross populations with LA780 obtained from the variegated F_1 plant BOY149

Parent	Selfed progeny			BC_1 progeny		
	Variegated (yv^{mut})	Yellow (yv^{ms})	% yv^{ms}	Variegated (yv^{mut})	Yellow (yv^{ms})	% yv^{ms}
BOY149[1)]	257	111	30	298	281	49
BOY149-1	152	88	37*	78	100	56
BOY149-2	167	113	40*	47	92	66*
BOY149-3	127	46	27	106	93	47
BOY149-4	270	187	41*	89	108	55
BOY149-5	52	30	37	127	137	52
BOY149-6	116	90	44*	119	196	62*

[1)] 6 different variegated F_2 plants of BOY149 were selfed or back crossed to LA780 using LA780 as male parent
* segregations significantly different from 3:1 in selfed progenies or from 1:1 in backcross progenies, $P < 0.01$

Discussion

Previously, the appearance of yellow spots on a green background was explained by the loss of an extra chromosomal fragment (Hagemann 1963, Herrmann and Hagemann 1967). By analysing the selfed offspring of the original mutant, we have now shown that no relation exists between the phenotype of a plant and the presence of extra chromosomal fragments. Furthermore, cytogenic data argued against Herrmann's assumption that these centric fragments carry a wild type yv^+ allele or a complementary factor necessary for the expression of yv^{ms}. For these reasons, it appears incorrect to ascribe the mutability of the mutant line to the frequent loss of the centric fragments (Hagemann 1963, Herrmann and Hagemann 1967). Furthermore, in Hagemann's model it is quite difficult to explain how a reversion from yellow to variegated can take place, an event we have recorded in our material.

The phenotypic switch from green to yellow in the eversporting mutant Yv^{ms}, and the appearance of yellow plants in its offspring, suggested the presence of an unstable allele (yv^{mut}) that frequently mutates to recessive yellow (yv^{ms}). That the direction of mutation was from yv^{mut} to yv^{ms} was confirmed by the appearance of yellow male sterile plants in populations derived from yv^+/yv^{mut} and yv^{mut}/yv^{st} heterozygotes. The mutation rate appeared

to be autonomously controlled and very high as was inferred from the high percentage of yellow offspring

The unusually high frequency of mutation invited speculation about the possible involvement of transposable elements in the instability. Since the direction of mutation was from dominant green to recessive yellow, the mutant line represented an example of reverse variegation. This less common phenomenon has been earlier associated with transposable elements (Nevers et al. 1986). An example is the unstable mutation (*Y18m*) of soybean, which showed similarities in phenotype and genetic behaviour to the unstable *yv* mutant. In case of *Y18m*, the patterns of reverse variegation were attributed to the activity of a transposable element (Peterson and Weber 1969, Chandlee and Vodkin 1989). One important difference with the unstable *yv* mutation was the appearance of stable wild type revertants among the selfed offspring of *Y18m*. Such revertants have not been found in yv^{mut} offspring. Moreover, another characteristic feature of transposable element insertions apparently missing from the unstable *yv* mutant, was the production of alleles with new phenotypes. The yv^{mut} allele mutated frequently to yv^{ms}, but it seemed that the new alleles were identical in conditioning male sterility (yv^{ms}), a character that is not necessarily associated with a yellow virescent condition. The yv^{ms} allele appeared to condition a more extreme phenotype than the yv^{st} allele, as seed set can be poor on yv^{st} plants which are in less favourable conditions. Hence, the male sterility might be a pleiotropic effect of the yv^{ms} allele.

The observation that the phenotype of the recessive *yv* alleles was sometimes expressed in F_1 hybrids may indicate that a transposable element present in the mutant *yv* allele inserted into the homologous wild type yv^+ allele, a phenomenon that seems to occur rarely in petunia insertional alleles (Bianchi et al. 1978). Assuming that the frequent transposition of an element from a nearby site into the *yv* locus gave rise to yellow tissue, the observed stability of the yv^{ms} allele should not be expected, as the yv^{ms} allele is supposed to carry the insertion. To date only one example of a comparable situation has been described in maize, where in the absence of *Ac*, wild type expression of the *waxy* gene occurred, although a *Ds* element was inserted in the upstream sequences. When *Ac* was present, however, mutant sectors arose as a result of intragenic transpostion events, thereby generating stable and unstable germinal derivatives (Weil et al. 1992). Thus, although the involvement of a transposable element in the instability at *yv* cannot be ruled out other mechanisms deserve serious attention.

Thus far, the molecular basis of instances of genetic instability other than those implemented by transposable elements is hardly known. Examples in plant and animal systems do show that instability can be explained in terms of the reversible alteration in chromatin structure rather than in DNA sequence (Reuter and Spierer 1992, Jablonka et al. 1992). It is recognized that the components of chromatin and their conformation influence the functional state of the gene. Variable factors are the timing of replication during the S-phase, the DNAse sensitivity and the degree of condensation and methylation (Jablonka et al 1992). The latter two, which are probably not independent from each other, have been put forward as

mechanisms underlying variegation. In *Drosophila* the variegated expression of genes was thought to be the result of the extension of the highly condensed state of heterochromatin into the gene (Henikoff 1990). The same model was proposed to explain the instability at alleles of the *sulfurea* locus of tomato which is located in the heterochromatic region of chromosome 2 (Hagemann 1969, Chapter 6). In transgenic tobacco plants the variable expression of the maize *A1* gene correlated with the methylation of its promoter sequences (Meyer et al. 1992). It can be envisaged that the instability at *yv* is caused by a similar mechanism affecting chromatin structure because of its position in a heterochromatic region. Moreover, the *yv* mutation was induced by X-ray treatment that is known to cause chromosomal rearrangements. Hence, a change in the position of *yv* relative to heterochromatin could be responsible for its unstable expression.

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Chapter 6

Isolation of a new paramutagenic allele of the *sulfurea* locus in the tomato cultivar Moneymaker following in vitro culture

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Summary

A new allele, SC148, of the *sulfurea* locus in *Lycopersicon esculentum* was detected in a line derived after repeated selfing of plants that had been regenerated from tissue culture. Like the original *sulf* mutant, SC148 displayed two mutant phenotypes: green-yellow speckled plants in which the $sulf^{vag}$ allele was present and pure yellow plants homozygous for the $sulf^{pura}$ allele. Although the mutant alleles are recessive to wild type, an unpredictable number of variegated and pura plants appeared in F_1 progenies derived from crosses between SC148 and wild type tomato plants. The presence of the wild type $sulf^{+}$ allele in these variegated heterozygotes was demonstrated using a cytological marker that is linked to *sulf*. It is concluded that the mutant *sulf* allele of SC148 imposes its variegated expression state on the wild type $sulf^{+}$ allele present in $sulf^{+}/sulf^{vag}$ heterozygotes. This behaviour, known as paramutation, was also described for the original *sulf* allele. The SC148 allele, however, appeared to induce changes at an earlier stage in development. The analogy of this paramutagenic system to dominant position effect variegation in *Drosophila* is discussed.

Introduction

A puzzling genetic phenomenon, termed paramutation, involves the unusual interaction of alleles when brought together in a heterozygote. In this interaction, one allele that is said to be paramutagenic, imposes its expression state on the other allele present. Since the altered expression state is maintained in the next generation the change is considered to be heritable. The resulting shift in phenotype, which often occurs from wild type to mutant, is observed either directly in the heterozygote or in its offspring. Paramutation was first noticed for the rabbit ear rogue mutation in *Pisum sativum* in 1915 (Bateson). Another example is the *sulfurea* mutant in tomato which was recovered after X-ray treatment of seeds of the variety Lukullus (Hagemann 1958, 1969). Two distinct mutant phenotypes, both recessive to wild type, were documented: *sulf-variegata,* recognizable by the appearance of yellow/green speckled leaves, and *sulf-pura* with yellow/white cotyledons. The homozygous pura plants die in seedling stage but can be rescued for genetic analysis by grafting the seedlings on wild type plants.

Both the *variegata* and *pura* allele were found to be paramutagenic, as judged from the proportion of $sulf^{+}/sulf^{vag}$ and $sulf^{+}/sulf^{pura}$ heterozygotes developing variegated leaves and branches on the otherwise green plants. Upon selfing, an excess of variegated progeny was obtained which was proportional to the extent of variegation on the parent, indicating that the alteration of the wild type allele was heritable. Hagemann (1969) compared the level of paramutagenicity of the $sulf^{pura}$ and $sulf^{vag}$ alleles in crosses with various tomato lines. The variable results indicated that two groups of alleles existed. Within the $sulf^{pura}$ group, the $sulf^{pura}$ alleles induced variegation in 0.5 - 100% of the F_1 ($sulf^{+}/sulf^{pura}$) plants, whereas in

the *sulf*^{vag} group the maximum level was 12%. Remarkably, in hybrids with *L. hirsutum* and *L. pennellii* the level of paramutagenicity did not exceed 2% (Hagemann 1969).

Other cases of paramutation, summarized by Brink (1973) and later by Hagemann and Berg (1977), involved loci in tomato (*C-6*), *Oenothera* (*cruciata*), *Celosia* (*ap*), *Malva* (*laciniata*), *Antirrhinum* (*deficiens* and *nivea*) and maize (*R* and *B*). Molecular investigations, although thus far limited to three cases of paramutation, have suggested that the underlying mechanisms may be diverse (Krebbers et al. 1987, Dooner and Robbins 1991). In a R_4 population, derived after repeated selfing of a plant that was regenerated from a tissue culture, a plant was recovered which carried variegated sectors on its leaves. In this study we show that this new mutant is an allele of the *sulfurea* locus of tomato and that the mutant allele is paramutagenic, like the *sulfurea* allele isolated previously.

Material and methods

Plant material. The *sulfurea* mutant SC148 was recovered in an experiment designed to assess somaclonal variation in tomato plants that had been regenerated from leaf explants (van der Bulk et al. 1990). The selfed progenies (R_2 lines) of more than 900 regenerated plants of the tomato cultivar Moneymaker were screened for variant phenotypes. In a relatively large number of these R_2 lines, a chlorotic semi-dwarf phenotype designated "type 57" was detected (van der Bulk et al. 1990). This "57" dwarf phenotype was transmitted to the progeny, though no true breeding lines could be obtained. In an R_4 line derived from repeated selfing of the "57" dwarf type, two out of 21 plants (plant 900333 and 900334) developed variegated sectors on their leaves. Other variegated plants were not observed among the 23 R_2 and 19 R_3 plants derived from the original R_1 line. The progeny of plant 900333 consisted of 10 wild type plants, while the progeny of plant 900334 segregated into 11 wild type plants, two plants with variegated sectors and one uniformly variegated plant. This uniformly variegated R_5 plant, designated SC148, was further analysed.

Seeds of the original *sulfurea* mutant were kindly provided by Prof. Hagemann. This mutant was recovered in the X_2 (=M_2) generation following X-ray treatment of seed of the tomato variety Lukullus (Hagemann 1958). One variegated R_6 plant, which was selected from the selfed progeny of SC148, was crossed to the following four tomato genotypes: Condine Red, which carries long satellites, Red Cherry, a small fruited tomato cultivar, GT, a tomato mosaic virus resistant pure line, kindly provided by DeRuiter seeds Bleiswijk, The Netherlands, and 2s-Iso, a tomato line which carries an extra chromosome consisting of two heterochromatic short arms of chromosome 2, kindly provided by Dr. C.M. Rick (Ramanna et al. 1985). The phenotype of the seedlings were scored 3-4 weeks after sowing.

Cytological techniques. The length of the satellite on chromosome 2 was determined in meiotic pachytene cells using the cytological techniques described previously (Ramanna and Prakken, 1967).

Results

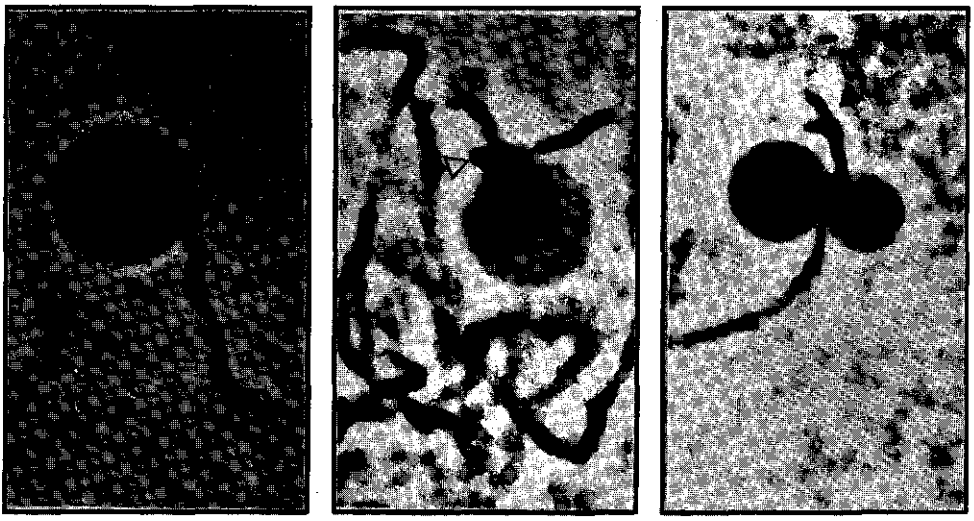
The newly isolated mutant SC148 was recognized by a green speckled phenotype which was very similar to the *sulfurea* mutant previously isolated by Hagemann (1958). Like the original *sulfurea* mutation, the selfed progeny of SC148 segregated in a non-Mendelian ratio into variegated and yellow seedlings (puras), with the latter dying in seedling stage (Table 1). On testing the new mutant for genetic complementation with the original *sulf* locus, all F_1 plants showed clearly a green-speckled phenotype, which, under the assumption that the mutation is recessive (see below), strongly suggest that the new mutant is an allele of the original *sulf* locus (Table 1).

Table 1. Segregations into green, variegated and pura plants in selfed, F_1 and F_2 progenies of SC148

Cross female x male	Phenotype of parents	Plant generation	Number of plants			% mutant [1] (vag + pura)
			Green	Variegated (vag)	Pura	
SC148 selfed	variegated	I_1	-	71	13	100.0
SC148 x Sulfurea	vag x vag	F_1	-	56	12	100.0
SC148 x Red Cherry	vag x green	F_1	535	162	16	25.0[a]
SC148 x GT	vag x green	F_1	123	52	11	33.9[b]
SC148 x Condine Red	vag x green	F_1	545	266	56	37.1[b]
SC148 x 2s-Iso	vag x green	F_1	583	542	26	49.3[c]
SC148 x Condine Red	green	F_2	47	28	15	47.8[a]
SC148 x Condine Red	green	F_2	18	72	3	80.6[b]
SC148 x Condine Red	green	F_2	58	12	27	40.2[a]
SC148 x Condine Red	green	F_2	73	21	0	22.3[c]

[1] Within F_1 and F_2 populations a different letter (a, b or c) indicates a significant difference in number of mutants when tested with a X^2 test, ($P<0.05$).

Because the mutant SC148 was identified among a population of 11 wild type plants, the mutation was likely to be recessive to wild type (see material and methods). This assumption was further tested by crossing the mutant to the tomato lines Red Cherry, Condine Red, GT and 2s-Iso, which are homozygous *sulf*+. The majority of the F_1 seedlings were wild type green, suggesting that the SC148 mutation indeed was recessive. However, as the F_1 seedlings developed further, variegated sectors appeared on the leaves of a variable number of plants (see F_1 populations in Table 1). In addition, homozygous *pura* plants were found among the F_1 plants. The number of mutant offspring (variegated and pura) ranged from 25% for crosses with Red Cherry to nearly 50 % for crosses with the 2s-Iso line. These observations suggested that, like the original *sulfurea* mutant of Hagemann, the SC148 allele is paramutagenic, changing the phenotype of the wild type locus into *variegata* or *pura* in somatic cells.



A B C

Fig. 1. Light microscope images of the satellites of chromosome 2 in pachytene cells of Moneymaker (A), Condine Red/SC148 heterozygote (B) and Condine Red (C). Note the short satellites in Moneymaker (open arrowhead), the long satellites in Condine Red (bold arrowhead) and the long and short satellites in the heterozygote.

To confirm that the wild type *sulf*+ locus indeed was present in the variegated hybrids, the genotype of the variegated *sulf*+/SC148 hybrids was determined using a cytological marker linked to the *sulf* locus. The *sulf* locus is located near or within the heterochromatic region bordering the centromere of chromosome 2, the nucleolar chromosome which carries the satellite. Since no crossing over has been observed between this satellite and the centric heterochromatin of chromosome 2, the *sulf* locus was thought to be absolutely linked to the

satellite (Hagemann and Snoad 1971). The length of the satellite varies significantly among tomato genotypes (Hagemann and Snoad 1971, Ramanna and Prakken 1967). A rather short satellite is present in the SC148 mutant as in its parental genotype Moneymaker, whereas the satellite of Condine Red is 5-6 times longer (Fig. 1). Thus, the long satellite provides a useful diagnostic marker for the wild type *sulf*$^+$ allele in crosses between SC148 and Condine Red. As shown in Fig. 1, both the short and long satellites were present in SC148/Condine Red heterozygotes with a variegated phenotype, indicating that the wild type *sulf*$^+$ allele was also present. From this result it was concluded that the wild type *sulf*$^+$ allele changed to variegata in SC148/Condine Red heterozygotes in response to the presence of the mutant *sulf* allele.

The variable percentage of mutants present in the F_1 populations (Table 1) suggested that the level of paramutagenicity depended on the genotype to which the SC148 was crossed. A difference in genetic background could not be the only cause of the variable paramutagenic activity of the SC148 allele. This was inferred from the appearance of green and variegated plants in one particular F_1 population which should consist of F_1 plants with the same genotype. Therefore it was concluded that the paramutagenic activity depended on other unknown, factors.

In order to test this possibility further, F_2 populations, that had been derived from four SC148/Condine Red hybrids with a green phenotype, were analysed for the occurrence of mutants (Table 1). These four SC148/Condine Red hybrids were thought to be genetically identical because the parental lines were homozygous. Yet, the proportion of mutants detected in their selfed progenies ranged from 22% to 81% (Table 1). In principle a *sulf*$^+$/*sulf*^{vag} heterozygote produces upon selfing 25% variegated (*sulf*^{vag}/*sulf*^{vag}) plants and 50% *sulf*$^+$/*sulf*^{vag} heterozygotes that have the potential to become variegated through the action of the *sulf*^{vag} allele. Thus, the proportion of variegated offspring can vary between 25% and 75%, when in none or all heterozygotes the expression of the wild type *sulf*$^+$ allele has been altered, respectively. Among the four F_2 progenies tested, we found one progeny with approximately 25% mutants, indicating that the SC148 allele had not been triggered to become paramutagenic. The other extreme progeny consisted of 81% mutant plants, a percentage that was not significantly different from 75%. This might also indicate that the original F_1 plant, although green, carried homozygous *sulf*^{vag}/*sulf*^{vag} sectors that apparently contributed to the offspring. The other two F_2 progenies consisted of an intermediate number of mutants, which was consistent with the expected number taking into account that in the F_1 population of this particular cross 37% of the plants became variegated (Table 1). Taken together, it was concluded that it was difficult to assign the level of paramutagenicity to a single cause. Genetic as well as random factors could influence the level of paramutagenic activity of the SC148 allele.

Discussion

It was demonstrated that a new *sulfurea* mutant SC148 was isolated following in vitro regeneration of leaf explants. Remarkably, the first variegated leaves arose in an R_4 population obtained after repeated selfing of the "57" dwarf type (see material and methods), whereas one would have expected the R_2 generation to express recessive characters. Apparently, the *sulf* mutation arose spontaneously but it cannot be excluded that the unknown mechanism responsible for the instability of "57" dwarf also induced the instability at the *sulf* locus. No other *sulfurea* genotypes had been recovered among several generations of the unstable "57" dwarf type of which at least 30 were analyzed.

The newly isolated *sulfurea* mutation possessed the same two singular features as the original *sulfurea* allele in that it gave rise to pura seedlings and was paramutagenic. The paramutagenic activity of SC148 allele was demonstrated by showing the presence of the long satellite typical for the wild type $sulf^+$allele in variegated $sulf^+/sulf^{vag}$ hybrids.

In accordance with Hagemann's (1969) observations, the percentage of variegated plants in F_1 populations derived from crosses between SC148 and various tomato lines was found to vary. Hagemann (1969) attributed the differences in paramutagenicity to the spontaneous appearance of variant alleles. In our material, however, the formation of new alleles was unlikely to explain the variable number of mutants appearing in the F_1 populations, since all crosses were made on the same SC148 plant. It was difficult to envisage that this plant passed on different alleles in every cross. On the other hand one has to take into account that SC148 consists of $sulf^{vag}/sulf^{vag}$, $sulf^{vag}/sulf^{pura}$ and $sulf^{pura}/sulf^{pura}$ sectors. When, for example, the *pura* allele has a higher paramutagenic activity, like the *pura* alleles described by Hagemann (1969), the number of mutant F_1 plants in a particular population depends on which sector had participated in the cross. In addition, the variation could reflect a genetic background effect on paramutation.

The level of paramutagenicity of the new *sulfurea* allele was much higher than that of the original $sulf^{vag}$ allele. In crosses with SC148 the maximum the number of variegated F_1 plants varied between 25% and 50 %, while in Hagemann's (1969) material this percentage varied between 0% and 12%. In comparing these percentages one has to take into account that other tomato genotypes have been used by Hagemann in the crosses. The difference in paramutagenic activity appears to be too large to be explained only by a difference in genotypes. Rather it was concluded that the SC148 allele displayed a higher paramutagenic activity.

Another observation supporting this conclusion was the appearance of *pura* plants among the F_1 hybrids. In Hagemann's material, the change from $sulf^+$ to $sulf^{vag}$ and to $sulf^{pura}$ occurred stepwise and unidirectional. If this also applied to our mutant, then, in order to form F_1 hybrids ($sulf^+/sulf^{vag}$) with a pura phenotype, the wild type locus $sulf^{vag}$ would have had to mutate to *variegata* and subsequently to *pura* between fertilization and

germination. In the same time span, the *sulf*^{vag} allele (SC148) would have had to mutate to *pura*. An alternative explanation would be, not that a *sulf*^{vag} allele, but a *sulf*^{pura} allele, had been present in the F_1 heterozygote. If this *pura* allele had altered the wild type *sulf*$^+$ allele directly into a *pura* allele, only one alteration would be needed to explain the appearance of *puras* among the F_1. Whatever the case, the new *sulfurea* mutation appeared to be active during an earlier stage in development, as compared to the original *sulfurea* mutant, in which the paramutagenic process appeared to start after seed germination (Hagemann and Berg 1978).

In considering possible explanations for the instability at the *sulf* locus, two aspects of the *sulfurea* mutation need to be distinguished. First, the variegated patterns in leaf colour suggested that the inactivation of the *sulf* allele occurred in some cells but not in others. Second, its paramutagenic behaviour required the imposition of the inactive state on the wild type *sulf*$^+$ allele. These features resemble a special case of position effect variegation (PEV) at the *brown* locus in *Drosophila* (Henikoff and Dreesen 1989). When the *brown* locus was rearranged close to heterochromatin, its expression became unstable, giving rise to variegated patterns in eye colour, similar to the variegated patterns of the *sulf* mutant. Furthermore, the unstable *brown* allele inactivated *in trans* the unrearranged wild type allele, comparable to the *in trans* inactivation of the wild type *sulf*$^+$ allele. In case of the *brown* locus the variable inactivation is thought to be the result of the condensed state of heterochromatin extending into the *brown* gene. Apparently, this heterochromatinization was then imposed on the wild type *brown* allele. It seems plausible that such a model accounts for the instability at the *sulf* locus as was also proposed by Hagemann (1969). In this context it is noteworthy that the *sulf* locus maps close to, or possibly within, the heterochromatic region of chromosome 2. Moreover, the *sulf* alleles were recovered after a treatment with X-rays or after the in vitro regeneration of leaf explants. Both treatments are known to cause chromosomal rearrangements which induced also PEV mutations in *Drosophila* (Henikoff 1990).

There are more similarities with PEV. In general the level of PEV can be influenced by the amount of heterochromatin present in the nucleus (Henikoff 1990). Such influence was also observed for the *sulf* mutation when crossed to the 2s-Iso line, which carries an extra chromosome consisting of two completely heterochromatic arms of chromosome 2. The high percentage of mutants among this F_1 population suggested that the instability at *sulf* was enhanced in response to an extra dosage of heterochromatin. If so, this response is very similar to that of the *light* gene of *Drosophila* (Devlin et al. 1990, Gatti and Pimpinelii 1992). This so called heterochromatic gene behaves in a manner opposite to that of the euchromatic genes, in that it became unstable when positioned in euchromatin. The instability of the *light* gene is enhanced by adding extra heterochromatin in the form of Y chromosomes (Devlin et al 1990, Henikoff 1990). Likewise, *sulf* may be a heterochromatic gene only correctly expressed in a heterochromatic environment. Final proof that a position effect is involved in paramutagenicity of the *sulf* locus may be obtained by translocating the gene to a euchromatic

environment, and by studying its expression at the new site, as has been achieved for the *P* locus in *Oenothera* (Catcheside 1947).

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Chapter 7

Genomic distribution of tomato elements homologous to transposable elements of tobacco and potato

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Summary

Transposable elements isolated from species closely related to tomato were used to assay the level of variation of transposable element-like sequences in tomato genotypes. By using the elements *dTst1* and *Tst1* of potato and the *Tnt1* element of tobacco as probes on Southern blots of tomato DNA, we have identified cross-hybridizing sequences which are present in multiple copies in the tomato genome. The patterns of the repeated transposable element-like sequences show considerable polymorphisms to exist between *L. esculentum* and its wild relatives, but not between stable and unstable *L. esculentum* genotypes. The results indicate that the repeated sequences thus identified have not evolved at a faster rate than low copy sequences. Upon screening an ethyl methanesulphonate treated tomato genotype with *Tst1*, three polymorphisms were detected, none of which could be unequivocally related to a transposition event. One polymorphisms was due to demethylation of a C-residue.

Introduction

Two main classes of transposable elements have been distinguished on the basis of common structural features which are thought to be pertinent to their mode of transposition. The first class with the *Ac* elements of maize as typical members, comprise families of autonomous and non-autonomous members which transpose via an DNA intermediate (Berg and Howe, 1989). The autonomous elements are capable of independent transposition and carry all DNA sequences required for transposition, including terminal inverted repeats, a transposase gene and DNA motifs to which the transposase binds. Their non-autonomous deletion derivatives lack an intact transposase gene and depend for transposition on a transposase encoded by an autonomous element (Berg and Howe, 1989).

The second class of transposable elements, the retrotransposons, transpose via an RNA intermediate and utilize reverse transcriptase. They are recognized by virtue of structural and functional similarities to retroviruses, but they have lost the ability to form infectious particles. The class of retrotransposons have been subdivided in two major categories, the *gypsy* -and *copia*-like elements which share long direct terminal repeats (LTR) of several hundreds of basepairs, and the *LINE*-like elements or retroposons which lack the LTRs and show less similarity to retroviruses (Berg and Howe 1989).

Active autonomous elements have been identified in maize, petunia and *Antirrhinum* through their association with unstable mutations showing variegated phenotypes (Fedoroff et al. 1983, Döring et al. 1984, Bonas et al. 1984, Gerats et al. 1990). Activity of plant retrotransposons is not as easily recognized since excision of the element, which leads to reversion of the mutation, is not part of the transposition process. Except for the *Tnt1* element of tobacco and the *Bs1* element of maize, retrotransposons of plants were identified fortuitously and classified as such on the basis of structural homologies with functional transposable

elements (Grandbastien 1992).

Studies on the distribution and abundance of transposable elements have revealed that transposable elements are a common component of plant genomes (Smyth 1991). Any plant may carry representatives of several transposable element families which occur in many copies dispersed throughout the genome. Usually, retrotransposons are amplified to much higher numbers than the *Ac*-like elements, a phenomenon which is likely to be the result of a difference in mechanism for transposition (Smyth 1991, Grandbastien 1992).

Taking into account the mobile nature of transposable elements and their ability to induce chromosomal rearrangements, transposable element sequences are expected to provide a source of polymorphic markers, as has been shown in maize (Johns 1990). To date, it has not been investigated whether this also applies to species in which the level of genetic variation is low, such as tomato. Sequence divergence in cultivated tomato was found to be mainly restricted to DNA segments carrying disease resistance genes that have been introduced from wild tomato species (Miller and Tanksley 1990, van der Beek et al. 1992), to subtelomeric regions (Broun et al. 1992), and to simple sequence repeats (Vosman et al. 1992).

Here we address the question as to whether transposable element-like sequences are polymorphic between tomato genotypes and contribute to the genetic instability observed in the *L. esculentum* genotypes Sulfurea, Yv^{ms} and 2s-Iso (Chapter 3). In the Sulfurea and Yv^{ms} line, instability was recognized by the appearance of green/yellow patterns on their leaves (Hagemann 1958, 1962; Chapter 6). In the 2s-Iso line, genetic instability has been related with a chromosome breakage fusion bridge cycle (Ramanna et al. 1985). Using transposable elements cloned from related species including the retrotransposons isolated from *Nicotiana tabacum* (*Tnt1*; Grandbastien et al. 1989), *Solanum tuberosum* (*Tst1*; Camirand et al. 1990) and the non-autonomous elements of *Solanum tuberosum* (*dTst1*; Köster-Töpfer et al. 1989) and petunia (*dTph1*; Gerats et al. 1990) we show that homologous transposable element-like sequences exist in the tomato genome, but are not a source of polymorphisms.

Material and methods

Plant material and transposable element probes. The sources and the characteristics of the plant material and the probes used are summarized in Table 1 and 2. The non-autonomous element (Köster-Töpfer et al. 1989) and the retrotransposon (Camirand et al. 1990) isolated from potato have both been named *Tst1*. In order to avoid confusion, we have renamed the non-autonomous element defective *Tst1* or *dTst1*, analogous to the system used for the defective *Spm* element (*dSpm*) of maize (Banks et al. 1985) and the defective element (*dTph1*) of petunia (Gerats et al. 1990).

Table 1. Origin and characteristics of genotypes of *Lycopersicon*

Species	Genotype	Source/ Accession number	Characteristics
L. esculentum	GT	de Ruiter seeds	-
	GTO	Wisman et al. 1991	*Adh-1* null mutant
	Condine Red	Hagemann 1962	parental line of Yv^{ms}
	Yv^{ms}	Hagemann 1962	variegated, unstable *yv* allele
	Sulfurea	Hagemann 1958	variegated, unstable *sulf* allele
	2s-Iso	Ramanna et al. 1985	presence of extra chromosome consisting of two short arms of chromosome 2
	83MS/83MR	Klein-Lankhorst et al. 1991, Ho et al. 1992	pair of near isogenic lines differing for the region around the nematode resistance gene *Mi*
	Moneymaker	-	-
	Sonatine	de Ruiter seeds	-
L. peruvianum	-	LA 2157	-
L. hirsutum	-	LA 1777	-
L. pennellii	-	LA 716	-

DNA isolation , Southern transfer and hybridization. Total DNA was extracted from young frozen leaves of tomato plants following the method described by van der Beek et al. (1992). Plant DNA (2.5 ug) was digested with a standard set of restriction enzymes, including *Hin*DIII, *Eco*RI, *Eco*RV, *Hae*III, *Bgl*II, *Dra*I, *Taq*I and *Xba*I. In some instances *Bst*NI was included. In addition, the restriction enzymes *Eco*RII, *Nru*I, *Pvu*II, *Hin*CII, *Stu*I, *Spe*I, *Nde*I, *Bam*HI, *Bst*NI, *Hpa*II, *Msp*I and *Aat*II were used to screen the tomato lines GT and GTO for RFLPs. Restriction fragments were separated by electrophoresis on 0.8 % agarose gels, denatured and blotted onto Gene Screen Plus hybridization membranes (New England Nuclear). Hybridizations were carried out with probes released from the vector using restriction enzymes as indicated in Table 2 and purified by agarose gel electrophoresis. The fragments were radiolabelled and hybridized as described by Klein-Lankhorst et al. (1991) at either 55 °C, 58 °C or 65 °C. Blots were washed at different stringency's as specified in the figure legends.

Table 2. Features of transposable element probes

Element			Vector	Restriction* enzyme	Fragment size	Characteristics
Name	Type	Origin				
dTph1	non-autonomous	petunia	bluescript	*Bam* HI	300 bp	complete element + 8 bp of flanking petunia DNA (1)
dTst1	non-autonomous	potato	pGUS	*Dra* I	510 bp	internal fragment without inverted repeats (2)
Tnt1	retrotransposon	tobacco	pMBC102001	*Bgl* II	339 bp	reverse transcriptase (3)
Tnt1	retrotransposon	tobacco	pMBC102001	*Eco* RV/*Xba*I	400 bp	LTR (3)
Tst1	retrotransposon	potato	bluescript	*Eco* RI/*Nco* I	3800 bp	reverse transcriptase, RNA binding site domain, protease (4)

* restriction enzymes used to release the transposon sequence from the vector
Data are cited from the following references:Gerats et al. 1990 (1), Köster-Töpfer et al. 1989 (2), Grandbastien et al. 1989 (3), Camirand et al. (4)

Results

Identification of tomato sequences homologous to dTst1 and dTph1

In order to study the occurrence of tomato elements homologous to the non-autonomous elements *dTst1* from potato and *dTph1* from petunia, Southern blots of DNA from various genetically stable and unstable tomato lines, digested with eight different restriction enzymes, were hybridized at different stringency's. Under reduced stringency (55 °C, 2 x SSC) no cross-hybridization occurred with the *dTph1* element from petunia (data not shown), whereas multiple bands hybridized to the *dTst1* potato element (Fig. 1). In all tomato lines tested, the *Dra*I digest revealed two prominent bands of 1.0 and 1.2 kb, suggesting that most copies of the sequence homologous to *dTst1* have two internal *Dra*I sites in common. The potato transposable element *dTst1* also contains two internal *Dra*I sites, though its size of 510 bp is considerably shorter than the tomato counterparts. No polymorphisms were observed among the six tomato lines tested (Fig. 1). Apparently, the repeated sequences homologous to *dTst1* are highly conserved and stable in *L.esculentum*. After washing the filter at a higher stringency (65 °C, 0.5 x SSC) all bands disappeared.

Similar to the experiments conducted with the non-autonomous elements, Southern blots of DNA of several tomato lines were probed with internal fragments of the retrotransposons *Tnt1* from tobacco and *Tst1* from potato. In this analysis, DNA of *Lycopersicon pennellii*, *L.hirsutum* and *L. peruvianum* was also included. Under reduced stringency (58 °C, 2xSSC)

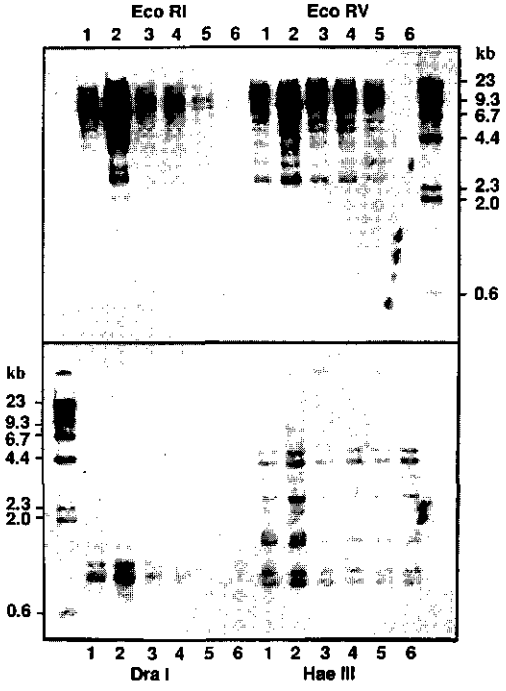

Fig. 1. Identification of tomato sequences homologous to *dTst1*. Total DNA from a nematode resistant line *L.esculentum* 83M7138 (lanes 1), a nematode sensitive line 83M7133 (lanes 2), Condine Red (lanes 3), Yv^{ms} (lanes 4), 2s-Iso (lanes 5) and from Sulfurea (lanes 6) was digested with *Eco*RI, *Eco*RV, *Dra*I and *Hae*III, separated on 0.8% agarose gels, transferred to Gene Screen Plus membranes, and hybridized with *dTst1* at 55 ^{0}C. The filters were washed at 55 ^{0}C, 2xSSC.

a family of at least 20 restriction fragments homologous to the reverse transcriptase domain of *Tnt1* was detected (Fig. 2). Among the *L. esculentum* genotypes tested no polymorphisms were apparent (Fig. 2). On the other hand, the *L. esculentum* pattern was clearly different from the patterns of *L. pennellii*, *L. hirsutum* and *L. peruvianum* (Fig. 2A). Following a wash at higher stringency (65 °C, 2 x SSC) approximately half of the bands disappeared (Fig. 2B), indicating that some fragments are more homologous to *Tnt1* than others. The most prominent bands were identical in all genotypes tested. When probing the same blot with sequences from the long terminal repeat of *Tnt1* no distinct bands were detected (data not shown). The apparent low level of homology between LTR sequences of tobacco and tomato is consistent with the reported divergence within LTR's of *Drosophila*, yeast and plant retroelements (Berg and Howe 1989, Grandbastien 1992).

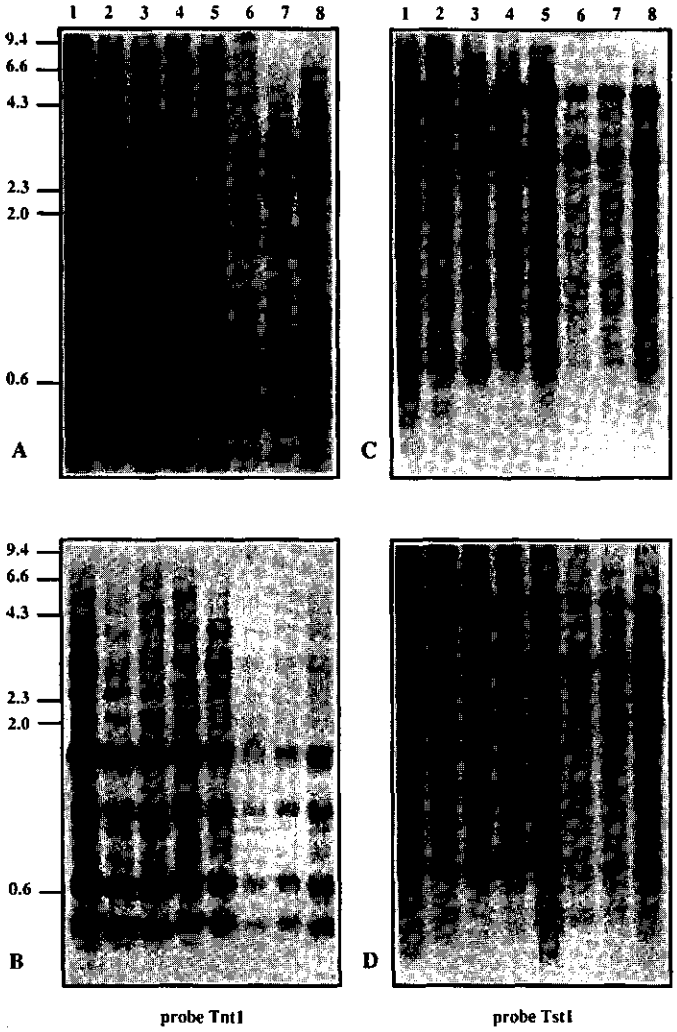

Fig 2. Identification of tomato sequences homologous to *Tnt1* (left panel) and *Tst1* (right panel). Total DNA from *L. esculentum* cv. Sonatine (lanes 1) , *L. pennellii* LA 716 (lanes 2), *L. hirsutum* LA 1777 (lanes 3), *L. peruvianum* LA 2157 (lanes 4), Yv^{ms} (lanes 5), Sulfurea (lanes 6), 2s-Iso (lanes 7) and from *L. esculentum* cv. Condine Red (lanes 8) was digested with *Bst*NI, separated on 0.8% agarose gels, transferred to Gene Screen Plus membranes, and hybridized with *Tnt1* or *Tst1* at 58 ^{0}C. The filters were washed at 58 ^{0}C, 2xSSC (A and C) or at 65 ^{0}C, 2 x SSC (B and D). Autoradiography was performed at -80 ^{0}C for 16 hours (A), 14 hours (B), 4 hours (C + D) using Kodak XAR film and intensifying screen.

Having established the hybridization patterns with *Tnt1*, the blot was then probed with a 3.8 kb internal fragment of the *Tst1* element of potato (Fig. 2C). A hybridization pattern consisting of at least 30 bands was obtained which was different from the *Tnt1* pattern. Polymorphisms were found only between the species *L. pennellii*, *L. hirsutum*, *L. peruvianum*, and *L. esculentum*, but not among the *L. esculentum* genotypes. Like the *Tnt1* pattern, the *Tst1* pattern consisted of bands with varying intensity. None of the *Tst1* bands

disappeared upon washing at higher stringency (65 °C, 2 x SSC), indicating that the potato and tomato transposable element-like sequences are highly homologous (Fig. 2D).

Detection of polymorphisms after EMS treatment

In the previous section it was shown that no polymorphisms were detectable among various tomato genotypes following digestion with the standard set of eight enzymes and hybridization to *Tst1*. An exception, however, was the tomato line GTO, showing a unique 5 kb band in a *Eco*RI digest in addition to the other bands in common with GT, the line used as a control (Fig. 3 and 4). The GTO line, which is homozygous for an *Adh-1* null allele (Chapter 4), is derived from GT following treatment with ethyl methanesulphonate (EMS). The *Eco*RI 5 kb band segregated in a Mendelian fashion independent from the *Adh-1* null mutation (Fig. 3).

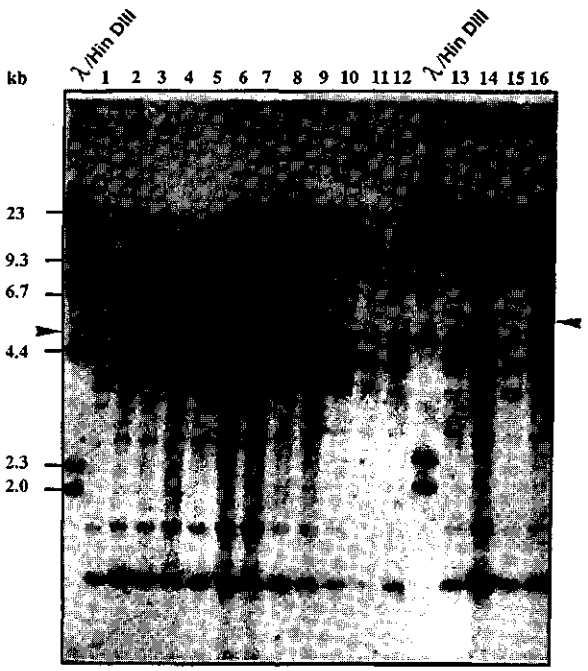

Fig. 3. Segregation of the unique 5 kb *Eco*RI band in 16 *Adh-1*$^{+}$/*Adh-1*^{0} individuals of a cross between GT/GTO (*Adh-1*$^{+}$/*Adh-1*^{0}) and *L. esculentum* 2s-Iso (*Adh-1*$^{+}$/*Adh-1*$^{+}$). DNA was digested with *Eco*RI, separated on 0.8% agarose gels, transferred to Gene Screen Plus membranes, and hybridized with *Tst1* at 65 °C. Note that the appearance of the unique 5 kb *Eco*RI band is independent from the presence of the *Adh-1*^{0} allele.

In order to test whether the *Eco*RI fragment in GTO was the result of the insertion of a new copy of a retrotransposon, two experiments were carried out. In the first experiment a search was carried out for restriction enzymes that did not cleave the unique 5 kb *Eco*RI band in a double digest with *Eco*RI and, thus, might cleave the DNA flanking the putative new retrotransposon copy. Among the 12 restriction enzymes tested, *Hpa*I, *Aat*II, *Stu*I, *Hae*II, *Pvu*II and *Nru*I were found to obey this criterion (data not shown). In the second experiment, the selected restriction enzymes were tested separately to reveal additional RFLP's which should be associated with a new copy of a retrotransposon. With the exception of *Pvu*II (see below), none of the restriction enzymes revealed a clear banding pattern, thus precluding a clear

conclusion to be made regarding the presence of a new insertion. The occurrence of a smear of fragments in digests made by methylation sensitive restriction enzymes strongly suggests that the majority of the retrotransposon-like sequences is heavily methylated and/or present in methylated chromosomal regions.

Using the restriction enzymes *Eco*RII and *Pvu*II, differences between GT and GTO involved intensities of the hybridization signal (see arrows in Fig. 4). In the *Eco*RII digest of GTO, the reduced intensity of a band of approximately 3 kb was accompanied by the appearance of a unique band that was smaller in size (see arrows in Fig. 4), indicating that an extra *Eco*RII site was generated. This polymorphism appeared to be the result of demethylation of a C-residue in the *Eco*RII site, as digestion with the isoschizomere *Bst*NI, that is insensitive to methylation, abolished the polymorphism.

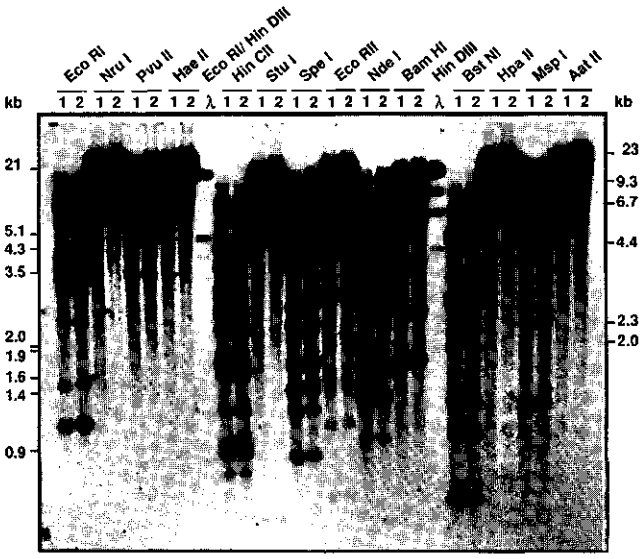

Fig. 4. Detection of polymorphisms (see arrows) following hybridization with *Tst1*. DNA was isolated from *L. esculentum* GT (lanes 1) and GTO (lanes 2), digested with the restriction enzymes as indicated, separated on 0.8% agarose gels, transferred to Gene Screen Plus membranes, and hybridized with *Tst1* at 65 ^{0}C. The filters were washed at 65 ^{0}C, 0.5 x SSC.

Discussion

We have demonstrated the presence of transposable element-like sequences in the tomato genome, using transposable elements from potato and tobacco. Since cross-hybridizing tomato sequences were highly homologous to the retrotransposon probes *Tst1* and *Tnt1*, which carry the reverse transcriptase domain that is highly conserved among eukaryotes, we conclude that the tomato genome contains retrotransposon-like sequences .

Such an unambiguous conclusion can not be drawn from the hybridization experiments involving the non-autonomous elements. Homologous tomato sequences were only detected at low stringency with a transposon probe from potato but not with a probe from petunia. This is in line with the divergence of internal sequences that is typical for non-autonomous elements

(Gerats 1990). It should be added, however, that a low homology retained among non-autonomous elements was sufficient to allow the isolation of non-autonomous elements from pepper using *dTst1* from potato as a heterologous probe (Kikuchi et al. 1991). Along these lines it is plausible to conclude that the repeated sequences detected in our hybridization experiments do correspond to (non)-autonomous elements.

Considering the potential instability of transposable elements, we have tested whether transposable element clones revealed genomic variation between tomato genotypes. The cross-hybridizing transposable element-like sequences were not found to be heterogeneous in organization or distribution in the various tomato genotypes tested, including the unstable lines Yv^{ms}, Sulfurea, and 2s-Iso. Apparently, the genetic instability observed in these lines is associated either with the activity of other transposable elements or with features not related to transposition. Polymorphisms, however, were detected between *L.esculentum* and its wild relatives. These results are very similar to those obtained with single copy probes, suggesting that the transposable element-like elements have not evolved at a rate different from single copy genes.

Among the tomato lines screened with alien transposable element probes, only the GTO line, which had been mutagenized by EMS, showed DNA polymorphisms. Interestingly, these polymorphisms were not associated with a particular phenotype but visualized hidden alterations. One polymorphism could be related to demethylation of a C-residue in an *Eco*RII site, indicating that EMS treatment can induce such a change in addition to the point mutations and small deletions which are induced by alkylation of G-residues (Auerbach 1976).

The identification of retrotransposon-like elements in tomato using the *Tst1* and *Tnt1* probes is in accordance with the results of Voytas et al. (1992) and Flavell et al. (1992^{a+b}), who succeeded in amplifying retrotransposon-like elements from the tomato genome using PCR directed by primers homologous to the reverse transcriptase domain of *copia*-like retrotransposons. In most plants, several classes of *copia*-like reverse transcriptase sequences were amplified, the relationship of which showed little congruence with the evolutionary relationship of their hosts (Flavell et al.1992^{b}). Here we show that tomato contains two distinct classes of retrotransposon-like sequences which are homologous to *Tst1* and *Tnt1*, respectively but do not show such incongruency. The homology observed between tomato, potato and tobacco elements suggests that they evolved from a sequence present in a common ancestor prior to speciation. The notion that retrotransposon-like elements in plants are old relics of the genome which have usually lost the ability to transpose (Voytas et al. 1992, Flavell et al. 1992) also applies to tomato, as shown by the absence of polymorphisms of retrotransposon-like sequences between tomato genotypes.

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Chapter 8

Concluding remarks: the stability of the tomato genome

With a view of isolating transposable elements of tomato, we have explored the possibility that the instability at the *sulfurea* and the *yv* loci and in the 2s-Iso line was due to the activity of transposable elements (Hagemann 1962 and 1958; Chapter 4, 5 and 6). Since these loci have not been cloned, we have attempted to trap the putative transposable element(s) in the *Adh-1* gene using the unstable lines as potential transposon donors (Chapter 4). Indeed, we succeeded in selecting new *Adh-1* null mutants, thereby showing the feasibility of the selection system in tomato. Unfortunately, however, the mutants were found to be due to point mutations or small DNA alterations rather than to the insertion of a specific DNA sequence (Chapter 4).

The success of a transposon trapping strategy relies not only on the availability of genotypes carrying active transposable elements, but also on the frequency by which a transposable element visits the target gene and the efficacy of the selection system. A priori, evaluation of these parameters is virtually impossible. The successful selection of several *Adh-1* maize mutants carrying various transposon insertions, including *Bs1*, *Ds* and *Mu* elements suggested at least that the *Adh-1* gene should be an appropriate target gene (Freeling and Bennett 1985).

The possible involvement of a transposable element in the instability of the *sulf* and *yv* loci was further explored by genetic means. By applying genetic criteria used to indicate the role of transposable elements in the instability of maize and *Antirrhinum* alleles (Nevers et al. 1986), it became apparent that these criteria fell short in explaining the genetic instability at the respective tomato loci. Thus, the frequent insertion of a transposable element from a nearby site into *yv* provided a plausible explanation for the appearance of yellow sectors, but not for the subsequent high stability of the resulting yellow phenotype. The failure of stable wild type revertants to turn up in the progeny of variegated plants was also interpreted to argue against the possibility that a transposable element was involved (Chapter 5).

Now that it has become clear that, despite considerable efforts of our group and others (Yoder, pers. comm.), the isolation of active endogenous transposable elements remains elusive, the question arises as to whether the *Lycopersicon esculentum* genome is unusual in being genetically highly stable, as compared to other plant species, including its close relatives. Indeed, several observations point in that direction. While for many years the tomato genome has received the attention of geneticists resulting in the recovery of a large and wide variety of mutations, unstable gene mutations with phenotypes characteristic for transposable element insertions have not been found. One of the criteria applied in recognizing foregoing transposition events is the presence of DNA footprints in the genome. Upon insertion, a transposable element generates a short duplication that, usually, is partly retained after transposition (Fedoroff 1989). In maize and *Antirrhinum* such footprints have often been shown to be responsible for the genetic variation between genotypes (Dooner et al. 1985, Sommer et al. 1988, Nevers et al. 1986, Schwartz-Sommer et al. 1985). Within the species *L. esculentum*, polymorphisms are scarce (Miller and Tanksley 1990, Tanksley et al.

1992, van der Beek et al. 1992, Weide et al. 1993). Furthermore, we found no evidence for the mobility of the tomato transposable element-like sequences, as detected by transposable element sequences from tobacco and potato (Chapter 7). In this context it may be relevant to note that the molecular analysis of wild type and mutant tomato alleles did not lead to the accidental identification of inserts that are structurally related to transposable elements. In other species, it is not unusual to stumble upon such inserts or other footprints related to transposition (Chapter 1: Table 1 and 2).

Besides the lack of transposable element activity in tomato, an explanation for the limited genomic variation in *L. esculentum* might be found in its narrow genetic basis. It is believed that the domestication of tomato started in Mexico, its putative ancestor being the small fruited *L. esculentum* var. *cerasiforme* (Dun). In the sixteenth century small samples of tomatoes were introduced from Mexico into Europe by Spanish explorers. From this sample, which represented only a small portion of the germplasm, all present tomato cultivars have been derived. Furthermore, tomato made the transition from cross pollinator to self-pollinator, upon being challenged to a new environment that lacked the appropriate insects to facilitate cross pollination (Rick 1976). This transition may have reduced the effective population size even further, as only plants with styles and stamen of the same length could serve as self pollinator.

The selection of desired genotypes may have further contributed to the stability of the tomato genome. In maize, for example, it was shown that the activity of the transposable element *Uq* in the original population was lost in the inbred lines presumably in response to the selection for uniformity (Gierl et al. 1989). The reverse was seen in an inbred population that had been selected during 50 generations for low protein levels. This inbred line was devoid of any active *Uq* copies, until the selection was reversed to high protein levels. Then active *Uq* elements turned up spontaneously, possibly because variation was needed again (Gierl et al. 1989).

Whatever may be the case regarding the tomato genome, it is clear that genetic variation is limited. Active transposable elements may be absent from the genome (due to genetic drift) or may have been silenced (ir)reversibly. In considering the data available, the intriguing question remains as to which mechanisms are responsible for the instability at the *yv* and *sulfurea* alleles. Apart from transposable element insertions, alternative explanations ought to be considered in view of the striking similarities with instabilities recorded for alleles in other organisms. In *Drosophila,* for example, a gene can become unstable following relocation to a position close to heterochromatin, presumably due to the high degree of condensation of heterochromatin which extends into the gene and thus prevents transcription (Henikoff, 1990). Other examples of instability are to be found among transgenic plants in which the variable expression of the introduced gene correlates to the level of methylation (Meyer et al. 1992). Apparently, the site of integration in the host DNA is one of the factors determining the degree of methylation of the introduced gene (Linn et al. 1991). These

reports have led us to propose the involvement of factors influencing the structure of chromatin in the instability at *sulf* and *yv* loci. Other features of chromatin associated with altered gene expression are the timing of replication in S phase and the sensitivity to endonucleases (Jablonka et al. 1992, Porter et al. 1992). Both *sulf* and *yv* genes are located close to heterochromatin and were induced by X-ray treatment or in tissue culture (Hagemann 1958, 1962), that is, by treatments known to induce chromosome breaks (Chapter 5 and 6). Conceivably, the instability of the alleles could be the result of a rearrangement relocating the genes to chromatin regions missing the appropriate structural properties necessary for regular expression of these genes.

Following this line of argument, the usefulness of the unstable tomato lines in experiments aiming at the isolation of endogenous transposable elements is questionable, and may be one should rather resort to wild relatives of tomato which are known to be much more heterogeneous (Miller and Tanksley 1990). Another possibility would be to activate silent elements by UV radiation (Walbot et al 1992). For instance, it has been shown that the reactivation of silent *Mu* elements in maize increased up to 40 fold following irradiation of pollen grains. Interspecific hybridization is another means by which transposition is enhanced. Rick (1967) has found new mutations in *L. esculentum* x *L. pennellii* hybrids which are unknown in the parental species and occurred at a rate at least 100 times the spontaneous mutation frequency. He proposed that this phenomenon was either due to the different distribution of heterochromatin in the parental species, or to transposition. Finally, the choice of the selectable tissue could be critical for success, as the activity of transposable elements can be tissue specific. This was clearly demonstrated for the retrotransposon *Tst1* of tobacco which was isolated following selection of chlorate resistant protoplasts (Grandbastien 1989). Later, it was shown that the expression of *Tst1* was induced in protoplast in response to cell wall degrading enzymes (Grandbastien pers. comm.) but usually occurred in roots (Pouteau et al 1991).

In summary, in the present study it was shown that transposable elements do not play an important role in the genetic instability observed in several tomato lines. The instability at *yv* and *sulf* might be the result of epigenetic changes, such as changes in chromatin structure and methylation patterns. The *yv* and *sulf* loci could thus prove useful in studying the function of chromatin structure in gene expression, which, to date, has only been achieved in *Drosophila* and yeast.

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Summary

Samenvatting

Summary

Genetic instability has been thought to be associated mainly with the activity of transposable elements. Indeed, transposable elements, which are defined by their ability to move both inter- and intra chromosomally, are responsible for the variegated expression of many alleles in maize, *Antirrhinum* and petunia. The mutability of these alleles is the direct result of the activity of the transposable element. When an element inserts at a given locus, the expression of the gene is often abolished, but due to the frequent excision of the element, revertant wild type cells arise among the mutant tissue. Somatic instability is readily observed when the affected gene is cell autonomously expressed and contributes to a visible phenotype such as flower- and leaf colour or endosperm characters. Unstable alleles, as a result of a transposon insertion, also exist for genes controlling other aspects of plant development. In those cases the instability is recognized in the progeny through the appearance of revertants with a wild type or a new phenotype.

Although the emphasis in molecular studies has been placed on cases of instability associated with transposable elements, several other mechanisms inducing unstable gene expression have been distinguished, including highly programmed systems such as the mating type switch in yeast. Studies on the variegation in coat colour of mouse and the variegation in eye colour of *Drosophila* have revealed the importance of the conformation of chromatin for normal gene expression. Also in tomato, unstable genotypes have been recognized with variegated phenotypes. The question arose whether the mechanism(s) underlying the genetic instability observed in these particular tomato genotypes could be identified, and whether these genotypes could be used as a source for transposable elements.

Transposable elements in maize have proven to be an important tool in isolating genes, even if the gene products are unknown. To make such an approach feasible in tomato we have aimed at the identification and isolation of endogenous transposable elements in tomato using the unstable tomato genotypes Yv^{ms}, Sulfurea and 2s-Iso. The first two, which carry the unstable alleles *yv*^{ms} and *sulfurea*, were distinguished by green-yellow variegated patterns on their leaves. The instability of the 2s-Iso line had been investigated earlier. This line carries extra chromosomal fragments that were derived from the short arm of chromosome 2. The occasional appearance of variegated sectors on the leaves of this line was shown to be associated with a chromosome breakage-fusion-bridge cycle that occurs in the extra chromosomal fragments. Typical features of the three unstable tomato lines include: (i) a relatively high frequency of mutations for various characters, (ii) non-Mendelian inheritance for their marker loci (*yv*^{ms}, *sulfurea*), and (iii) a high incidence of chromosome breakage (2s-Iso).

Since none of the unstable loci have been cloned, a direct examination of the cause of instability at *sulfurea* and *yv*^{ms} was not possible. Under the assumption that the instability in these lines was indicative for the presence of active transposable elements, a transposon

target-gene system was developed, which combines the possibility of readily screening very large numbers of individuals for the rare insertion of the transposon, with the possibility of subsequently cloning the element out of the inactivated target gene. The *Adh-1* gene was chosen as target for the putative transposable element, since mutants could be selected by virtue of their resistance to allyl alcohol both at the pollen and seed level.

The seed selection approach involved the crossing of the mutable lines (*Adh-1*$^{+}$/*Adh-1*$^{+}$ to a line carrying an *Adh-1* null mutation (*Adh-1*^{0}/*Adh-1*^{0}) and the subsequent identification among the progeny (*Adh-1*$^{+}$/*Adh-1*^{0}) of an allyl alcohol resistant (*Adh-1*^{0}/*Adh-1*^{0}) mutant. This approach required an *Adh-1* null mutant which was specially isolated for this purpose following EMS treatment of seeds. Using this null mutant, 150,000 F_1 seeds were obtained amongst which one *Adh-1* null mutant was identified which did not show enzyme activity in pollen and seeds, the tissues usually expressing *Adh-1*. The pollen selection approach involved treatment of pollen of the mutable lines with allyl alcohol before pollination to wild type flowers. Thus, over 3000 wild type flowers were pollinated which resulted in the identification of one *Adh-1* null mutant among the F_1 progeny. Like the other *Adh-1* null mutant, no expression was observed in either seeds or pollen grains (Chapter 2 and 4).

In order to reveal the molecular basis of the three *Adh-1* mutants, the entire *Adh-1* gene including its upstream regulatory sequences, was cloned from the tomato genome. The *Adh-1* origin of the genomic clone was confirmed by showing that its genetic map position corresponded to the *Adh-1* locus, determined previously on the basis of isozyme markers. Moreover, tomato specific ADH-1 activity was demonstrated in transgenic tobacco plants following transformation with the genomic clone (Chapter 3). Using two *Adh-1* subclones encompassing the entire promoter and coding region, no restriction fragment length polymorphisms were detected in a Southern analysis between wild type plants and the *Adh-1* mutants, indicating that no insertions or major rearrangements in the mutated *Adh-1* alleles had occurred. Surprisingly, the EMS induced *Adh-1* null mutant appeared to be unstable: ADH null pollen grains reverted to wild type at a frequency of approximately 5×10^{-4}. Although the occurrence of reversions in such high frequencies is usually related to the presence of a transposable element in the locus, this seemed not to be the case in the EMS-induced *Adh-1* null mutant (Chapter 4).

The genetic characteristics of the Yv^{ms} and Sulfurea mutants have been described earlier by Hagemann. He proposed that the instability at *yv* was caused by the somatic segregation of an extra chromosomal fragment carrying the wild type *yv*$^{+}$ allele or another complementary factor. Accordingly, the frequent loss of the fragment from cells of a homozygous recessive *yv* plant was thought to account for the appearance of yellow leaf sectors and of yellow progeny. We have shown by cytogenetic analysis, however, that the phenotype of mutant plants was not dependent on the presence or absence of extra chromosomal fragments. Alternatively, we postulated the presence of an unstable allele,

yv^{mut}, that frequently mutates to *yv*^{ms} during plant development. The *yv*^{ms} allele appears to be stable in somatic tissue, as no revertant green spots arose on yellow leaves. Only once was a somatic reversion event recorded in a yellow plant, which all of a sudden started producing variegated offspring. Upon further genetic testing, an unusual feature of mutant *yv* alleles was detected. In a frequency of approximately 1 : 400, F_1 plants with mutant phenotypes appeared in populations derived from crosses of Yv^{ms} with wild type plants. In considering the characteristics of the Yv^{ms} line a role of transposable element in the instability was not very likely, since no stable wild type green revertants were recovered. Also the stability of the yellow mutants derived from the unstable Yv^{ms} line argued against this assumption. Because of similarities with position effect variegation in *Drosophila*, in which expression of genes became unstable when they were relocated close to heterochromatin, it is tempting to assume that the variable expression of the *yv* allele is associated with an altered structure of chromatin (Chapter 5).

The same model may apply to the *sulfurea* locus. In both instances the position of the locus in close proximity to, or within heterochromatin was taken as an argument in favour of this model. In addition, we have detected a new unstable *sulfurea* allele (SC148) among the R_4 progeny of a plant regenerated from tissue culture that, similar to the original *sulfurea* mutation, imposed its variegated expression pattern upon the wild type allele in a certain number of heterozygotes. Preliminary data indicated that this number depended upon the amount of heterochromatin available in the genome (Chapter 6).

In an alternative approach, several stable and unstable tomato lines have been screened for the presence of sequences homologous to cloned (retro)transposons from *Nicotiana tabacum* and *Solanum tuberosum* (Chapter 7). Southern blot analysis indicated that (retro)transposon-like elements do exist in the tomato genome, although they were not variable among *L. esculentum* genotypes. This observation supported the notion that the tomato genome is very stable, rarely showing polymorphisms between genotypes. The implications of the apparent stability of the *L. esculentum* genome for the isolation of active transposable elements were discussed.

Samenvatting

Ieder verschijnsel in de natuur dat afwijkt van wat men gewoon is, trekt onmiddellijk de aandacht. Illustratieve voorbeelden hiervan zijn variëteiten met bont gekleurde bloemen en bladeren die als rariteiten in tuinen staan. In de botanische tuin 'De Dreijen' zijn bijvoorbeeld de streeprozen te bewonderen die waarschijnlijk de oudste siervorm van de Franse Roos vertegenwoordigen. Het vermoeden bestaat dat deze streeproos al in de 12^{e} eeuw is meegebracht door de kruisvaarders. Een ander voorbeeld is de siermaïs die zich onderscheidt door bont-kleurige korrels. Onderzoek naar de oorzaak van de bontheid in maïs in de veertig- en vijftiger-jaren bracht een tot dan toe onbekend verschijnsel aan het licht, dat betrekking heeft op erfelijke eigenschappen. Men was er vroeger van overtuigd dat de eigenschappen (genen) op een vaste positie op het chromosoom liggen, maar nu bleek dat er een uitzondering op deze regel mogelijk was. De zogenaamde transpositie elementen, ofwel springende genen, hebben de eigenschap zich te verplaatsen op het chromosoom. Incorporeren zij toevallig op een plek waar een andere eigenschap ligt, bijvoorbeeld rode bloemkleur, dan wordt dit zichtbaar als een mutatie: de bloemkeur wordt dan wit. Als nu het transpositie element tijdens de bloemontwikkeling weer uit het gen voor rode bloemkleur springt dan wordt de mutatie ongedaan gemaakt en verschijnen er rode vlekken of strepen op de witte bloembladeren. Op deze wijze wordt het ontstaan van bonte patronen verklaard op bloemen, bladeren en maïskorrels .

Met behulp van moderne moleculaire technieken is men er in geslaagd transpositie elementen te isoleren en heeft men kunnen vaststellen dat een speciale structuur ten grondslag ligt aan de mobiliteit van deze elementen. Naast de fundamentele betekenis van dit onderzoek zijn transpositie elementen ook een belangrijk hulpmiddel gebleken in de isolatie van andere eigenschappen. Zo kan het element gebruikt worden om de eigenschap waarin het geïncorporeerd is te markeren, waarna het mogelijk is deze eigenschap via moleculaire technieken te isoleren. Dit is vaak op een andere manier niet mogelijk. Om deze methode in tomaat te ontwikkelen zou men willen beschikken over een transpositie element van tomaat. Om deze reden zijn tomatelijnen geselekteerd die afwijken van het normale type door hun bontbladigheid. Analoog aan de bontheid in maïs en andere gewassen was de verwachting dat de bontheid in bladkleur in tomaat ook door een transpositie element werd veroorzaakt.

Daarom luidde de vraagstelling van het project beschreven in dit proefschrift als volgt: wordt de instabiliteit in bladkleur veroorzaakt door een transpositie element of liggen andere mechanismen hieraan ten grondslag? Deze vraag kon niet direct beantwoord worden omdat de moleculaire hulpmiddelen om de eigenschap bladkleur (waarin we verwachten het transpositie element aan te treffen) te onderzoeken niet aanwezig waren. Om deze reden werd een systeem opgezet om het transpositie element te vangen in een andere eigenschap dat wel moleculair te bestuderen is. Deze eigenschap was alcohol dehydrogenase (*Adh*), een eiwit dat alcohol kan omzetten in een andere stof. Het idee was om een transpositie element in *Adh* te

laten springen, een gebeurtenis die te herkennen is aan de uitschakeling van *Adh*. De herkenning kon niet op het oog gebeuren omdat planten die geen alcohol dehydrogenase maken er precies hetzelfde uitzien als alle andere tomateplanten. Maar als we de zaden of stuifmeelkorrels behandelen met allyl alcohol dan worden alle zaden en stuifmeelkorrels die *Adh* bevatten gedood, omdat *Adh* allyl alcohol omzet in een giftige stof. Zaden en stuifmeelkorrels zonder *Adh* overleven deze behandeling echter wel. Op deze wijze zijn duizenden zaden en stuifmeel korrels getest op het voorkomen van *Adh*-loze mutanten en zijn er inderdaad drie mutanten gevonden. Moleculaire analyse van deze mutanten heeft echter aangetoond dat deze mutaties niet ontstaan zijn door de insertie van een transpositie element. Dit werk staat beschreven in de hoofdstukken 2 t/m 4.

In hoofdstuk 5 staat de genetische analyse beschreven van de bontbladige mutant Yv^{ms}. Door het bestuderen van de nakomelingschappen van deze lijn werd naar aanwijzingen gezocht die de oorzaak van de instabiliteit konden verklaren. De nakomelinschap van bontbladige planten was ongewoon omdat naast de verwachte bontbladige nakomelingen er ook altijd gele planten waargenomen werden in een niet voorspelbaar aantal. Er werd gekonkludeerd dat tijdens de ontwikkeling van de plant de eigenschap bontbladigheid veranderde in geelbladigheid. Hoe die verandering tot stand komt is nu nog niet te zeggen. Uit de genetische analyse bleek dat het niet waarschijnlijk is dat de verandering in geelbladigheid wordt veroorzaakt door de insertie van een transpositie element in de eigenschap bontbladigheid, hoewel in theorie de mogelijkheid blijft bestaan. In dit hoofdstuk worden ook alternatieve mechanismen besproken.

Een van die alternatieven leek aantrekkelijk om de bontbladighied in een andere tomatelijn, Sulfurea, te verklaren. In deze lijn was, evenals in de Yv^{ms} lijn, een eigenschap gemuteerd die de bladkleur beinvloedt. Sulfurea planten zijn geel-groen gespikkeld en duidelijk verschillend van de bontbladige Yv^{ms} planten die groene bladeren hebben met grote gele vlekken. Net als bij Yv^{ms}, bevinden zich onder de Sulfurea nakomelingen een onvoorspelbaar aantal geelbladige nakomelingen. Maar nog een ander ongewoon fenomeen, aangeduid als paramutatie, werd waargenomen. Wanneer men een mutant, bijvoorbeeld een geelbladige tomaat, met een groenbladige tomaat kruist, dan is normaliter de hele nakomelingschap groen. Kruist men echter een bontbladige Sulfurea plant met een groenbladige plant, dan bestaat de nakomelingschap naast de groene nakomelingen uit een bepaald percentage bontbladige Sulfurea planten. Dit percentage lijkt afhankelijk van welke groene ouderlijn gekozen wordt. Met andere woorden de kruising van Sulfurea met een gewone tomaat, een vleestomaat of een kerstomaat levert een verschillend percentage bontbladigen op in de nakomelingschap. Dit paramutageen karakter van de Sulfurea mutant is inderdaad erg ongewoon als men in aanmerking neemt dat er in het plantenrijk duizenden mutanten beschreven zijn en dat er ongeveer tien paramutageen zijn.

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In hoofdstuk 7 is geprobeerd op een andere manier aanwijzingen te krijgen dat transpositie elementen aanwezig zijn in tomaat. Daartoe is een Southern analyse uitgevoerd. Deze techniek stoelt op het feit dat eigenschappen die voorkomen in een groep van organismen elkaar kunnen herkennen. Dit gaat alleen als er niet te veel veranderingen in de eigenschap zijn opgetreden. Het is gebleken dat de eigenschappen meer op elkaar lijken in nauwer verwante gewassen. Een voorbeeld: de eigenschap *Adh* is erg geconserveerd in het plantenrijk, wat wil zeggen dat er niet veel veranderingen zijn opgetreden in *Adh* in verschillende plantensoorten. In een Southern analyse kan *Adh* van tomaat dan ook niet alleen het *Adh* van tomaat herkennen, maar ook dat van de nauw verwante soorten aardappel, tabak en petunia en het *Adh* van onverwante soorten zoals maïs en het zandraketje. Ook voor transpositie elementen is het aangetoond dat zij geconserveerd zijn in het plantenrijk. Dit maakte het mogelijk met behulp van transpositie elementen geïsoleerd uit aardappel en tabak op transpositie-lijkende elementen in tomaat aan te tonen. Tot nu toe hebben we geen aanwijzingen gevonden dat deze elementen in tomaat zich kunnen verplaatsen op het chromosoom, een eigenschap die karakteristiek is voor transpositie elementen. Nader onderzoek is nodig om met zekerheid aan te tonen dat het inderdaad om transpositie elementen gaat.

Nu is aangetoond dat de isolatie van een transpositie element in tomaat niet zo eenvoudig is, drong de vraag zich op of de cultuurtomaat niet ongeschikt was voor dit doel. Het is bijvoorbeeld aangetoond dat tomateneigenschappen op moleculair nivo zo goed als identiek zijn in de verschillende tomatencultivars. Dit zou een aanwijzing kunnen zijn dat transpositie elementen zich niet hebben verplaatst gedurende de ontwikkeling van nieuwe cultivars. Hadden zij dat wel gedaan dan zou men immers verwachten dat er meer veranderingen opgetreden waren. Een tweede punt dat mogelijk op de afwezigheid van aktieve transpositie elementen wijst is, dat er ondanks tientallen jaren onderzoek aan tomaat, nooit mutanten zijn gevonden die het typische gedrag van een, door een transpositie element veroorzaakte, mutatie vertoonden. De mogelijke afwezigheid van aktieve transpositie elementen, en de waargenomen uniformiteit van de tomatencultivars, is misschien terug te voeren op de ontstaans geschiedenis van tomaat. Men neemt aan dat alle cultivars zijn ontstaan uit een kleine populatie tomaten die in de 16^{e} eeuw in Europa geïntroduceerd is vanuit Mexico. Deze tomatenplanten hadden slechts kleine vruchten. De selektie op grotere vruchten en andere gewenste eigenschappen heeft waarschijnlijk de variatie in tomatetypes nog verder gereduceerd. Als gevolg hiervan zou het kunnen zijn dat de tomaat transpositie elementen die zich kunnen verplaatsen heeft verloren. Daarom lijkt het een goed alternatief om het zoeken naar transpositie elementen voort te zetten in de wilde verwanten van tomaat, die een grotere verscheidenheid vertonen.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 8 juni 1955 te Zeist. Aan het Rijkslyceum Schoonoord te Zeist behaalde zij in 1975 het diploma Atheneum-B. Daarna is zij drie jaren werkzaam geweest in verschillende bejaarden tehuizen. In die tijd werd het diploma voor bejaardenverzorgende behaald. In 1978 startte zij aan de Landbouwhogeschool in Wageningen en behaalde in januari 1981 het kandidaatsdiploma plantenveredeling. De ingenieursstudie, die werd afgesloten in 1985, omvatte de vakken quantitatieve genetica, plantenveredeling en cytotaxonomie. In 1986 verbleef zij één jaar in de Verenigde Staten in het gewas evolutie laboratorium van professor de Wet aan de Universiteit van Illinois, waar zij cytologisch onderzoek verrichtte aan maïs-*Tripsicum* hybriden. In 1987 werkte zij een half jaar bij de vakgroep populatie en evolutie biologie van de Rijksuniversiteit Utrecht waar aan de hand van chloroplast DNA variatie de verwantschappen in het genus *Sedum* werden bepaald. In oktober 1987 werd zij aangesteld als onderzoeker in opleiding bij de vakgroepen plantenveredeling en moleculaire biologie van de Landbouwuniversiteit in Wageningen. Dit proefschrift doet verslag van de resultaten van dit onderzoek. Sinds 1 juni 1993 is de auteur werkzaam als post-doc in het maïs evolutie project onder leiding van professor H. Saedler bij het Max Planck Instituut te Keulen.