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A genetic analysis of a tomato (*Lycopersicon esculentum*) genotype with a high frequency of twin spots

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Abstract Among pale-green tomato plants heterozygous for the *xanthophyllic 2* (*xa-2*) mutation that were transformed with a T-DNA harbouring the NPTII and GUS gene, a plant with a high frequency of green/white twin spots was found. The genetic analysis of this plant indicated that the occurrence of these twin spots was caused by a genetic defect located at the distal end of chromosome 10S, where *xa-2* also is located. The genetic analysis of green plants regenerated from leaf explants of this twin-spot plant revealed that the green sectors derive from non-disjunction of the $xa\text{-}2^{+}$ allele. In an analysis of mitotic chromosome behaviour bridges were observed in approximately 5% of the anaphases, providing arguments that a breakage-fusion-bridge-cycle caused by a tissue culture-induced genomic instability is the most likely cause of this aberrant behaviour of chromosome 10.

Key words Genetic instability · Somaclonal variation · Twin spots · *Lycopersicon esculentum* · Tomato

Introduction

Genetic instability is known to be caused by a number of different mechanisms, including transposable element activity, paramutation and mitotic chromosome non-disjunction. Plants that are heterozygous for a co-dominant cell-autonomous chlorophyll mutation are useful for the detection of this phenomenon, since the loss or inactivation of both wild-type and mutant alleles, and the simultaneous occurrence of these events can be monitored by the formation of twin spots. Twin spots originate mainly through mitotic crossing-over (somatic recombination) or are the result of chromosomal non-disjunction (Carlson 1974). Examples of co-dominant chlorophyll mutations are the *sulfur* locus in tobacco (Evans and Paddock 1976; Carlson 1974; Lörz and Scowcroft 1983), the y_{11} locus in soybean (Vig 1969) and several *xanthophyllic* loci in tomato (Gröber 1963; Peterson and Yoder 1993; Seeni and Gnanam 1981). Genotypes heterozygous for such loci have been exploited to investigate genetic instability induced by tissue culture (Lörz and Scowcroft 1983; Seeni and Gnanam 1981), chemical compounds (Vig 1973), irradiation (Carlson 1974; Evans and Paddock 1980) and transposable element activity (Peterson and Yoder 1993). In most organisms the level of instability in somatic cells is low; however, there are genotypes in some species that display a high level of instability. Such genotypes with a heritable high genetic instability are of interest either as a source of active transposable elements or for their higher frequency of mitotic recombination (e.g. in *Aspergillus nidulans*, Parag 1977). Genotypes with a very high frequency of twin spots have been observed among *Su/su* tobacco protoplast regenerants (Lörz and Scowcroft 1983), among *Nicotiana* somatic hybrids (Evans et al. 1983), after X-ray irradiation of a tomato $xa\text{-}3^{+}/xa\text{-}3$ heterozygote (Gröber 1963) and after the introduction of a maize Ac element into tomato (Peterson and Yoder 1993). For all these treatments the occurrence of plants with a high frequency of twin spots was exceptional.

In the present paper we report on the genetic and cytogenetic analysis of a transgenic tomato plant that is heterozygous for the *xa-2* locus and has a high frequency of twin spots. The analysis of plants regenerated from twin-spot sectors allowed us to distinguish between somatic recombination and chromosomal non-disjunction as the cause of this high level of instability (Carlson 1974).

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Material and methods

In attempts to generate tomato genotypes containing selectable markers such as nitrate reductase deficiency, antibiotic resistances and morphological markers with good regeneration capacity the genotype XC15 was isolated. This genotype was obtained by crossing MsK9 (Koornneef et al. 1987a) with a *xa-2*$^+$/*xa-2* heterozygote having the genetic background resembling that of cv 'Ailsa Craig'. The *xa-2* mutation (Persson 1960) inherits partially dominant and was located at the distal end of chromosome arm 10S (Tanksley 1993). The mutation leads to viable pale-green heterozygotes and yellow/white homozygotes, which die within 2 weeks after germination. MsK9 is a second backcross progeny of a *Lycopersicon esculentum* × *L. peruvianum* hybrid with *L. esculentum*, selected for its superior regeneration capacity in tissue culture (Koornneef et al. 1987a, 1993). The progeny derived from the MsK9 × *xa-2*$^+$/*xa-2* cross was screened for regeneration capacity using the root assay described by Koornneef et al. (1993), and a selected plant (X15) was subsequently crossed with the nitrate reductase deficient mutant C42 (Schoenmakers et al. 1991). Using the transformation procedure described by Koornneef et al. (1987b) we transformed 1 pale-green progeny plant with good regeneration capacity with *Agrobacterium tumefaciens* strain C58, which contains plasmid pZ707C that harbours the kanamycin resistance gene NPTII and the glucuronidase (GUS) reporter gene (Jefferson et al. 1987). One transformant, designated XC15, showing a high frequency of twin spots, was analysed as described below. A genotype carrying the chromosome 10 markers *hy*, *alc*, *h* and *ag* was obtained from Dr. M. Mutschler (Cornell, USA). The estimation of recombination frequencies, correcting for reduced transmission of particular male gametes (certation), was performed with the computer programme RECF2 (Koornneef and Stam 1992). Segregation for kanamcyin resistance was tested at the plant level using the spraying assay of Weide et al. 1989. Shoots were regenerated from explants of in vitro-grown shoots of XC15 as described by Koornneef et al. (1989). The analysis of mitosis in root-tip cells was performed as described by Wolters et al. (1993).

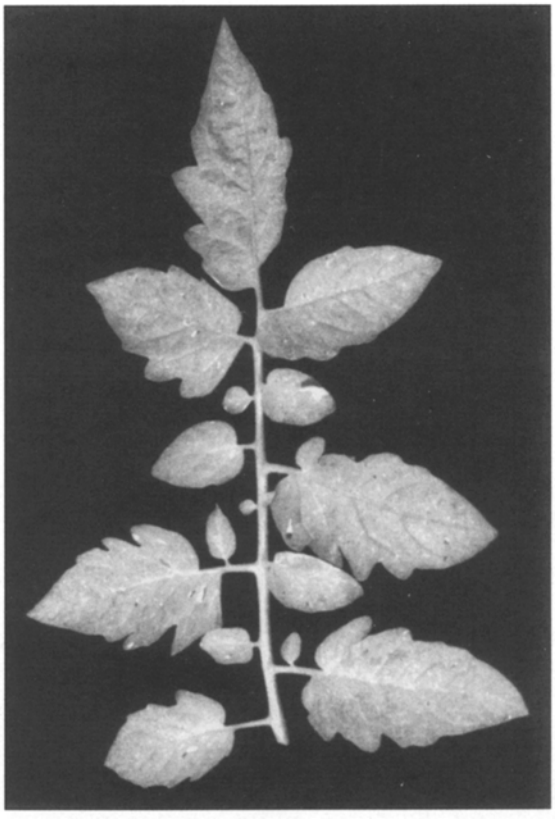

Fig. 1 Mature leaf of plant XC15 showing a high frequency of twin spots

Results

Among the five transformants obtained from the *xa-2*$^+$/*xa-2* heterozygote, 1 plant displayed a high frequency of green and white spots on pale-green leaves, most of them occurring as twin spot sectors of variable size (Fig. 1). Although this plant, designated as XC15, had a reduced fertility in comparison with that of the parental genotype, it could be crossed with other genotypes, and some selfed seeds could be obtained. The segregation ratios shown in Table 1 confirm the monogenic inheritance of the partial dominant *xa-2* mutation in both the original *xa-2* material and the XC15 material. Segregation of the lethal nitrate reductase-deficient mutation apparently did not affect the segregation ratio of *xa-2*. The progeny obtained by selfing XC15 and by backcrossing it to both the cultivar 'Moneymaker' (= wild type for marker genes except for the uniform ripening locus *u*) and to a normal *xa-2*$^+$/*xa-2* heterozygote showed that twin spots are present mainly in the pale-green plants carrying the wild-type *xa-2*$^+$ allele originating from the XC15 plant. The spotted plants in the progeny of XC15 × WT are an exception to this observation. In general, the spot frequency in these plants was lower than that found in the pale-green progeny plants of selfed XC15. Among the 40 plants from the cross XC15 × WT that were tested for kanamycin resistance, 11 pale-green plants were kanamycin-resistant and 6 were kanamycin-sensitive, whereas among the 23 green plants 7 were resistant and 16 kanamycin-sensitive. These data indicate monogenic inheritance for kanamycin resistance and suggest loose linkage with the *xa-2* locus ($\chi^2 = 4.64$; $0.01 < P < 0.05$ and recombination percentage 32.5 ± 7.5). Since kanamycin-sensitive twin-spot plants were obtained in this population and in later generations the high frequency of twin spots is likely not caused by the insertion of the T-DNA.

Plants that were heterozygotes for other cell-autonomous chlorophyll markers were obtained by crossing XC15 with the recessive mutants *h*omogeneous *y*ellow (*hy*), located on chromosome 10, and *y*ellow *v*irescent (*yv*), located on chromosome 6. Only green plants *xa-2*$^+$/*xa-2*$^+$) in the progeny of the cross with *hy* showed a high frequency of yellow-green (*hy*) spots, indicating that the loss of the wild-type (*hy*$^+$ and *xa-2*) alleles in these sectors is chromosome 10-specific. The pattern of inheritance is in agreement with a dominant mutation causing this effect and linked to the *xa-2*$^+$ allele. The few twin-spot plants in the pale-green progeny of XC15 × WT could have resulted from recombination between this locus and *xa-2*. To distinguish as to whether this locus leads to a high frequency of somatic

Table 1 Segregation of the *xa-2* locus and twin spot occurrence in the progeny of XC15

Cross	Number of plants in progeny with phenotype:				
	Green	Pale-green	(ts[a])	White	Total
$xa2^+/xa2$ selfed	158	340	(0)	159	657
XC15 selfed	10	27	(27)	11	48
XC15 × $xa2^+/xa2$	18	56	(23)	21	95
XC15 × WT[b]	75	72	(8)	–	147
XC15-self-gr[c] × $xa2^+/xa2$	15	23	(23)	–	38
[XC15 × WT]-gr[d] × $xa2^+/xa2$	12	24	(9)	–	36

[a] (ts) = number of plants with twin spots among pale-green plants
[b] WT = wild type = cv 'Moneymaker'
[c] Green progeny obtained by selfing XC15
[d] Green progeny from the XC15 × WT cross

recombination or has some other effect (e.g. on chromosome segregation during mitosis), we checked the genetic composition of the sectors by regenerating plants from leaf explants of XC15. Out of a total of 189 regenerated shoots that could be rooted, 12 plants (6.4%) turned out to be green, 163 pale green with many twin spots and 4 (2.1%) were yellow with small green spots. These yellow plants differ from the lighter and (seedling) lethal *xa-2/xa-2* homozygotes.

To establish if the regenerated green plants were the result of somatic recombination, we crossed them both with a wild-type and a normal $xa\text{-}2^+/xa\text{-}2$ heterozygote (Table 2). If a green regenerant should arise from a XC15 cell that had undergone mitotic crossing-over, it should contain only two wild-type $xa\text{-}2^+$ alleles. However, the observation that pale-green plants are obtained after crossing with a wild type and that white plants are present in the progeny of the cross with $xa\text{-}2^+/xa\text{-}2$ heterozygotes indicated that all 6 tested green regenerants still contain a mutant *xa-2* allele.

Assuming that the leaf colour depends on the dosage of the $xa\text{-}2^+$ and *xa-2* alleles, as was shown for *Xa-1* by Peterson and Yoder (1993), the green sector within the twin spot probably has a genetic constitution of two $xa\text{-}2^+$ alleles and one *xa-2* mutant allele, whereas the white sector contains only a mutant *xa-2* allele (green: $xa\text{-}2^+$ $xa\text{-}2^+/xa\text{-}2$; white: $-/xa\text{-}2$). The inheritance pattern indicates that the two $xa\text{-}2^+$ alleles in the green plants are closely linked. Since no progeny could be obtained from the very weakly growing yellow plants no genetic analysis could be performed. However, if we assume dosage sensitivity and take the green sectors as an indication that $xa\text{-}2^+$ is present, a possible genotype for the yellow plants is $xa\text{-}2$ $xa\text{-}2/xa\text{-}2^+$.

Non-disjunction of chromosomes can occur in the case of chromosome breakage followed by a breakage-fusion-bridge-cycle (BFBC) (Peterson and Yoder 1993 and references herein). To obtain cytogenetic confirmation for this process 301 anaphase cells in three different twin-spot plants were analysed. In 11 cells clear bridges involving only one chromosome were observed (Fig. 2) and in 9 other cells possible bridges were observed, indicating that bridges were formed in 4–7% of the cells. This frequency does not differ very much from the number of non-pale-green plants regenerated from the leaf explants of XC15 plants. An additional indication that the green regenerants contain a mutant *xa-2* allele is the occurrence of some pale-green spots on most of these plants. Since the genetic analysis indicated that the chromosome carrying the wild-type $xa\text{-}2^+$ allele undergoes non-disjunction, one expects that this will cause a loss of genetic information in cells homozygous for this chromosome. Close inspection of the green plants from the progeny of XC15 revealed that they are all characterized by small irregular, paler green specks, which might be explained by locally reduced cell growth or even cell

Table 2 Segregation of leaf colour and twin spot occurrence in the progeny of fertile green plants (Gt) regenerated from leaf explants of XC15

Cross	Number of plants in progeny with phenotype:				
	Green	Pale-green	(ts[a])	White	Total
Gt4 × WT[b]	62	73	(20)	–	135
Gt5 × WT	24	25	(1)	–	49
Gt6 × WT	31	33	(1)	–	64
Total GT × WT	117	131	(22)	–	248
Gt2 × $xa2^+/xa2$	1	5	(2)	5	11
Gt3 × $xa2^+/xa2$	5	11	(6)	6	22
Gt4 × $xa2^+/xa2$	20	49	(27)	21	90
Gt5 × $xa2^+/xa2$	15	22	(10)	11	48
Gt6 × $xa2^+/xa2$	17	40	(20)	16	73
Gt10 × $xa2^+/xa2$	31	78	(37)	50	159
Total Gt × $xa2^+/xa2$	89	205	(102)	109	403

[a] (ts) = number of plants wth twin spots among the pale green plants
[b] WT = wild type = cv 'Moneymaker'

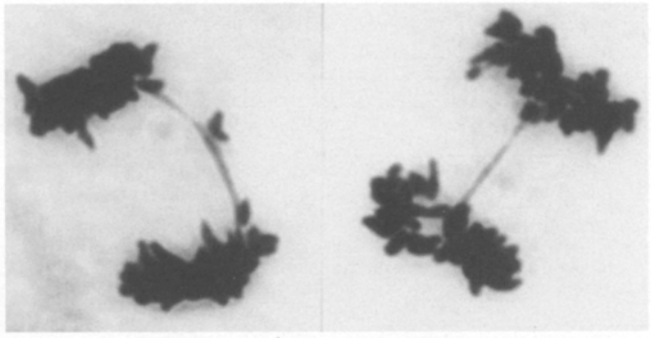

Fig. 2 Mitotic anaphases of two twin spot plants from the progeny of XC15

death. Furthermore, the green plants with such spots were reduced in fertility even after repeated backrossing. The patchy leaf characteristic was used in a linkage analysis shown in Table 3. The data indicate that this character behaves as a dominant locus located above *hy* on the top of chromosome 10, where *xa-2* is also located (Tanksley 1993). In addition, this putative locus has a reduced transmission since correcting for certation against this dominant "speck allele" results in consistent segregation ratios with linkage (Table 3) (χ^2 values with $P > 0.10$). These results suggest that the specks and twin spots originate from a common genetic cause, which is located at the top of chromosome 10.

Discussion

Twin spots that occur on plants heterozygous for a semi-dominant cell-autonomous mutation are often assumed to originate from somatic recombination (Evans and Paddock 1976). In contrast, single spots are often assumed to originate from chromosomal disturbances (Vig 1969). The genotype of plants regenerated from twin-spot sectors on a tobacco *Su/su* genotype was in agreement with mitotic crossing-over in 12 out of the 13 cases tested (Carlson 1974). Using a similar approach as Carlson (1974) we concluded that most of the twin spots on tomato XC15 heterozygous for the *xa-2* locus originate from non-disjuction of the *xa-2*$^+$ allele, which appeared to be the cause of the twin spots. Most likely, this mechanism is typical for the XC15 plant, which is characterized by an extremely high frequency of twin spots. In the experiment of Carlson (1974), the occurrence of both single and twin spots was relatively rare. Probably the incomplete separation of the two chromatids during the mitotic anaphase results in breakage of the attached chromatids at a random site, which may result in one daughter cell receiving two linked *xa-2*$^+$ alleles and leave the other cell without a wild-type allele. A genetic defect at the end of chromosome 10 apparently leads to this behaviour. This cause is different from the one found for the high frequency twin-spot plant (in *xa-3*$^+$/*xa-3* background) and its progeny analysed by Gröber (1963, 1967), who observed a relation with non-disjunction of chromosome fragments (carrying the *xa-3*$^+$ allele). Our explanation is similar to that of Peterson and Yoder (1993), who obtained several genotypes with a high frequency of twin spots among plants harboring an Ac element from maize in a tomato *xa-1*$^+$/*xa-1* background. The impossibility to explain the yellow/green twin spots in some of their material by somatic recombination was their main argument for a breakage-fusion-bridge-cycle (BFBC) mechanism. An analysis as performed in our experiments could have provided additional arguments for this hypothesis. Cytogenetic arguments for the presence of BFBC phenomena in isochromosomes of tomato have been described by Ramanna et al. (1985). The presence of yellow instead of white sectors, as shown by the regeneration of yellow plants, suggests that the chromosome carrying the mutant *xa-2* allele also undergoes non-disjunction at a non-negligible frequency. These yellow sectors were not very obvious on XC15, and therefore it can not be excluded that the explant culture itself resulted in non-disjunction for this chromosome. It is also possible that the *xa-2* chromosome received a BFBC-sensitive chromosome end by mitotic recombination or by another unknown interaction with the homologous chromosome.

The genetic defect located on the top of chromosome 10 that causes the formation of chromosome bridges seems to have been generated by the tranformation

Table 3 Segregation ratios in the F_2 derived from a cross of a plant with specks (*hy*$^+$, *u*, *h*$^+$, *ag*$^+$) and a plant with the genotype *hy*, *u*$^+$, *h*, *ag*

Marker[a]	Number of plants with:				% m/m	Recombination %[b]
	Specks, m$^+$/.	No specks m$^+$/.	Specks, m/m	No specks m/m		
hy	44	15	6	33	39.8[c]	16.4 ± 3.5
u[d]	28	26	10	3	19.4	32.5 ± 8.7
h	41	28	12	17	29.6	38.4 ± 5.7
ag	45	32	8	14	22.2	57.1 ± 6.0

[a] *hy* Homogeneous yellow (5), *u* uniform ripening (19), *h* hairs absent (46), *ag* anthocyanin gainer (132). All of the marker genes are located on chromosome 10, and their map position according to Tanksley (1993) is given between parentheses

[b] Recombination percentage and standard deviation estimated with the RECF2 programme (Koornneef and Stam 1991) taking into account certation against the *hy*$^+$, *u*, *h*$^+$, *ag*$^+$ alleles

[c] Significantly different from 25% ($P < 0.05$)

[d] Many plants could not be scored for *u* because of sterility

experiment. However, it cannot be decided if somaclonal varation as such or an introduced T-DNA had a role in this. The T-DNA with the functional kanamycin resistance gene is not responsible for this aberrant behaviour of chromosome 10 in XC15. Lörz and Scowcroft (1983) found such a high frequency twin-spot plant among their protoplast regenerants. Lee and Phillips (1988) provided strong arguments that delayed replication often occurs in tissue culture and may result in chromosome breakage, which may leave instable chromosome ends that can serve as the basis of the BFBC in XC15. However, when the genetic event leading to a high frequency of twin spots is due to tissue culture it must be relatively rare. Although plants with higher (mainly non-twin) spot frequencies were found among the more than 1000 plants regenerated from leaf explants of *xa-2*$^{+}$/*xa-2* by Seeni and Gnanam (1981) and among the 1266 plants obtained in our laboratory (C. Zijlstra and H. C. H. Schoenmakers unpublished data), no plants with a high frequency of twin spots were reported. A detailed molecular and cytogenetic analysis of the telomere region of chromosome 10 in the XC15 genotype may provide insight in the nature of the genetic defect described in this report.

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