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Phenotypic interactions between abscisic acid deficient tomato mutants

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Summary. A series of double mutant homozygotes have been produced from three wilty tomato mutants; *flacca, sitiens and notabilis.* The phenotypic interaction between the mutant genes has been studied. The severity of phenotype in the double mutants does not correspond to that predicted from the single mutant homozygotes. The results are discussed in relation to the probable involvement of the mutants in abscisic acid metabolism.

Key words: Abscisic acid – Mutants – Phenotypic interaction

Introduction

Three X-ray induced wilty mutants have been obtained in the tomato, *Lycopersicon esculentum* Mill. (Stubbe 1957). All three mutant alleles are recessive to the normal wild type. Plants homozygous for each of the mutations have reduced levels of the plant growth regulator, abscisic acid (ABA). Tal and Nevo (1973) estimated ABA levels using gas chromatography with a flame ionisation detector. They found that the three mutants could be ranked on the basis of endogenous ABA levels. The highest ABA concentration was found in *notabilis* (*not*) homozygotes (37 ng · g^{-1} F.Wt.) followed by *flacca* (*flc*) homozygotes (24 ng · g^{-1} F.Wt.); with the *sitiens* (*sit*) homozygote having the lowest endogenous ABA levels (17 ng · g^{-1} F.Wt.). These results correlate with the severity of the three mutant phenotypes (Tal and Nevo 1973).

The most detailed physiological research has been carried out with *flacca* mutants. Plants homozygous for *flc* can be easily identified in a segregating population by their reduced leaf area and strong tendency to wilt if subjected to even the mildest water stress (Darby et al. 1978). Wilting is due to the fact that the mutant stomata resist closure both in the darkness and during water stress (Tal 1966). It is possible to overcome the typical *flacca* symptoms and cause a dramatic phenotypic reversion by regular spray treatments with ABA (Imber and Tal 1970). The phenotypic effects of the other two mutants can also be overcome by supplementing the low endogenous ABA levels with an exogenous supply of this growth regulator (Tal and Nevo 1973). It has therefore been proposed that the primary abnormality is the ABA deficiency, and that all aspects of the wilty mutant syndrome result from this (Tal et al. 1979).

The three mutants *notabilis, flacca* and *sitiens* are clearly functionally related. However, they are non-allelic and have been mapped to three distinct loci within the tomato genome. The latest tomato linkage map (Rick 1980) shows that both the *not* and *flc* loci are situated on chromosome 7. The two genes are 19 map units apart; being located 40 and 59 units along the chromosome, respectively. The *sit* gene locus is widely separated from the other two, being 32 map units along chromosome 1. It is therefore theoretically possible to combine the mutants together to produce a series of three double mutant homozygotes ((1) *not not sit sit;* (2) *not not flc flc;* (3) *sit sit flc flc*) and conceivably also a treble mutant homozygote (*not not sit sit flc flc*).

The specific purpose of the present investigation was to produce the double mutant homozygotes and to gain some indication of their combined effects on phenotype. All three single mutant homozygotes appear to permit the accumulation of at least some ABA (Tal and Nevo 1973). The further reduction of endogenous ABA levels associated with a double genetic lesion, should ultimately provide invaluable information on the physiological role of ABA in plants.

Material and methods

A large collection of mutants have been transferred to the uniform genetic background of the tomato variety 'Alisa Craig' (Darby et al. 1978). *Lycopersicon esculentum* c.v. 'Alisa

Craig' was therefore used as the control (wild type) variety in this investigation. Near-isogenic lines of the three wilty mutants, *notabilis, flacca* and *sitiens* in this genetic background were used throughout. The procedures for the production of the three double mutant homozygotes were complex and are described below:

1 Production of notabilis/sitiens double mutants. Plant homozygous for *notabilis* were crossed with *sitiens* homozygotes. The F_1 were normal, confirming that the two mutant genes are recessive and non-allelic. Five F_1 plants (*not+sit+*) were allowed to self. As the two mutant alleles are located on different chromosomes, the F_2 was expected to show independent assortment giving an approximate 9:7 ratio of wild-type to wilty mutant phenotypes. A population of 150 F_2 plants were screened. The wild type plants were readily identified on the basis of the greater leaf area and were discarded after three weeks growth. The *notabilis* phenotypes could also be easily distinguished due to the relatively mild effects of this mutation. Plants homozygous for the *sitiens* mutation show severe wilting and a considerably reduced leaf area. However, as the severity of symptoms in any *sitiens* individual is variable, it was difficult to select the rare double mutant homozygotes (*not not sit sit*) on the basis of phenotypic differences.

To avoid this problem, five F_2 plants showing the easily identifiable *notabilis* phenotype were retained and allowed to self. F_3 populations derived from each of the five parents were examined individually. Three of the F_3 populations segregated approximately 3:1 for individuals showing respectively the *notabilis* phenotype alone and the double mutant homozygotes which showed very severe wilting and retarded growth. Five of these double mutants (*not not sit sit*) were sprayed daily with 5 mg l^{-1} aqueous solution of (±) 2-cis-4-trans ABA (Sigma Ltd.) to induce phenotypic reversion. Only in this way could a homozygous F_4 population be derived from these slow growing double mutants. Bulked F_4 seed was used as the pure breeding double mutant stock for subsequent experiments.

2 Production of notabilis/flacca double mutants. The procedure used here was slightly different from that described above due to the added complexity of linkage. Problems were likely to be encountered in identifying rare recombinant genotypes in the F_2. The sideshoot suppressing mutant, *lateral suppressor* (*ls*), was, therefore, used as a marker gene to allow single recombination events to be detected. The *ls* gene has recently been assigned to position 59 on chromosome 7 (Taylor and Rossall 1982) and is tightly linked to the *flc* gene locus. A recombinant genotype homozygous for both *notabilis* and *lateral suppressor* had already been produced for an earlier experiment (Taylor, unpublished data). This double mutant (*not not ls ls+ +*) was therefore crossed with a *flacca* homozygote (*+ + + +flc flc*) to give a normal F_1 heterozygote (*not+ls+flc+*). Five F_1 plants were allowed to self and a population of 150 F_2 plants were sown. The *notabilis* phenotypes were selected after three weeks growth and remainder of the population discarded. Most of these *notabilis* phenotypes also showed the *lateral suppressor* character and were rejected. However two plants had normal sideshoots indicating that recombination had occurred between the *not* and *ls* gene loci. This would simultaneously have resulted in the transfer of the *flc* mutant allele to a chromosome containing the *not* mutant allele. The two F_2 plants were allowed to self and both F_3 populations segregated approximately 3:1 for individuals showing respectively the *notabilis* phenotype alone and the double mutant homozygotes which showed very severe wilting and retarded growth. Five of these double mutants (*not not flc flc*) were phenotypically reverted by ABA spraying, as described previously, to produce a bulked F_4 seed stock. The *ls* gene was lost automatically from this population due to repulsion.

3 Production of sitiens/flacca double mutants. The mutant homozygotes were crossed to give a normal F_1 heterozygote. Five F_1 plants (*sit+flc+*) were allowed to self. A population of 150 F_2 plants were screened giving an approximate 9:7 ratio of normal to wilty mutant phenotypes. The range of phenotypic expression in both *flacca* and *sitiens* and the overall similarity between them made selection difficult. Five wilty mutants were selected at random from the F_2 and allowed to self to give five F_3 populations. A sample of five plants from each of the F_3 populations were testcrossed with the original parental *flacca* and *sitiens* lines to establish genotype. The second F_3 population tested confirmed that its F_2 parent had the genotype *sit sit+flc*. All testcrosses involving the *sitiens* parent resulted in wilty mutant progeny. However, the five F_3 plants varied in genotype at the *flc* locus. Three of these plants segregated 1:1 for the wilty phenotype in backcrosses to *flacca* homozygotes and where therefore *sit sit+flc*. One plant produced all normal phenotypes in the *flacca* backcross and was *sit sit+ +*. The remaining plant gave 100% wilty mutant progeny in backcrosses to either *flacca* or *sitiens* homozygotes and therefore had the genotype *sit sit flc flc*. This plant was selfed to give the double mutant homozygous line.

Measurement of phenotype

Two simple measurements were used to quantify the phenotypic effect of the various wilty mutant genotypes. The growth rate of young plants was clearly retarded by the mutant genes. Estimation of leaf area, following a fixed period of growth reflects this. Transpiration rate was also obtained as a measure of the intensity of phenotypic expression of the 'wilty' character.

In the first experiment the interaction between *notabilis* and the other two mutant genes was studied. Transpiration rates of four plants of each mutant type and the isogenic control were examined. Eight week old plants were used, in order that the slow growing double mutants could obtain a reasonable size for measuring transpiration rate. Each plant was watered to full soil water capacity and the pots enclosed in polythene bags sealed at the base of the stem. Plants were weighed hourly for 6 h at approximately 18 °C. The leaf are a of each plant was measured using a Paton Electronic Planimeter.

In the second experiment the growth rate of seven plants each of the three single mutants and the three double mutant genotypes were compared. The leaf area of each plant was measured weekly following the expansion of the cotyledons. Non-destructive area measurements were taken using a camera system linked to a Delta-T area meter.

Results

1 Phenotypic effect of the notabilis mutant allele in different genetic backgrounds

Table 1 shows that the three wilty mutant genes can be ranked in order of severity. For the two parameters used, *notabilis* has the mildest phenotypic effect. This confirms the result of Tal and Nevo (1973). Although the three mutant alleles are at separate loci, they

Table 1. Phenotypic effect of the wilty mutant genotypes

Genotype	Transpiration rate ($g \cdot m^{-2} \cdot h^{-1} \pm SE$)	Leaf area ($cm^2 \pm SE$)
+ +	21.95 ± 1.24	621.81 ± 29.92
not not	38.41 ± 3.10	432.27 ± 26.99
flc flc	53.20 ± 1.66	363.92 ± 13.78
sit sit	68.00 ± 0.94	246.68 ± 8.65
not not flc flc	88.11 ± 3.10	24.62 ± 4.02
not not sit sit	111.90 ± 9.81	9.78 ± 1.45

appear to form a quantitative series in terms of expression. This is probably related to quantitative differences in their capacity to accumulate ABA (Tal and Nevo 1973).

The production of the double mutants allows examination of the additivity of the three genes. The phenotypic effect of the relatively mild *notabilis* mutation in genetic backgrounds homozygous for either *flacca* or *sitiens* is important here. Table 2 shows that for transpiration rate *notabilis* appears to exert a similar reduction (65–75%) in both normal and mutant gene backgrounds. This may be misleading if these genotypes are nearing the physical limits of stomatal aperture and therefore of transpiration rate.

The figures for leaf area (Tables 1 and 2) show that the phenotypic effect of *notabilis* is more dramatic against a wilty mutant gene background. Only a 30% reduction in leaf area was observed due to *notabilis* alone. However, when added to either *sitiens* or *flacca* it caused a further reduction of over 90%. The wilty mutant genes are clearly not additive; the severely retarded double mutants (*not not flc flc* and *not not sit sit*) are almost qualitatively different from any of the single mutants.

2 Phenotypic effect of the double mutant genotype sit sit flc flc

In the previous section it was shown that the severity of the double mutant phenotype is better reflected in plant area measurements than in transpiration rate. Figure 1 shows the effect of each genotype on growth rate, monitored by weekly area measurements. The effect of the double mutant genotypes is accentuated by the poor light conditions prevailing in the heated greenhouse during the growing period; January/February. The double mutants grown in October/November for the previous experiment (Tables 1 and 2) performed rather better. The slight decrease in leaf area in the *not not sit sit* genotypes toward the end of the experiment was due to drying out of the tips of the cotyledons. These plants were barely able to grow at all under these conditions.

The results in Fig. 1 support those of Table 1 in the order of severity of gene expression. The effect of *notabilis* alone was relatively mild; *flacca* was more severe and *sitiens* was similar to *flacca* but slightly more extreme. All three single mutants were once again in a completely different category from the almost totally retarded double mutants, *notabilis/flacca* and *notabilis/sitiens*. The new result emerging from this experiment involved the double mutant *sitients/flacca*. This

Table 2. Genetic interaction between *notabilis* and the background genotype

Genotype comparison	Transpiration rate (% increase)	Leaf area (% decrease)
not not versus + +	74.9	30.1
not not flc flc versus + +	301.0	96.0
not not sit sit versus + +	401.0	98.0
not not flc flc versus *flc flc*	65.6	93.2
not not sit sit versus *sit sit*	64.6	96.0

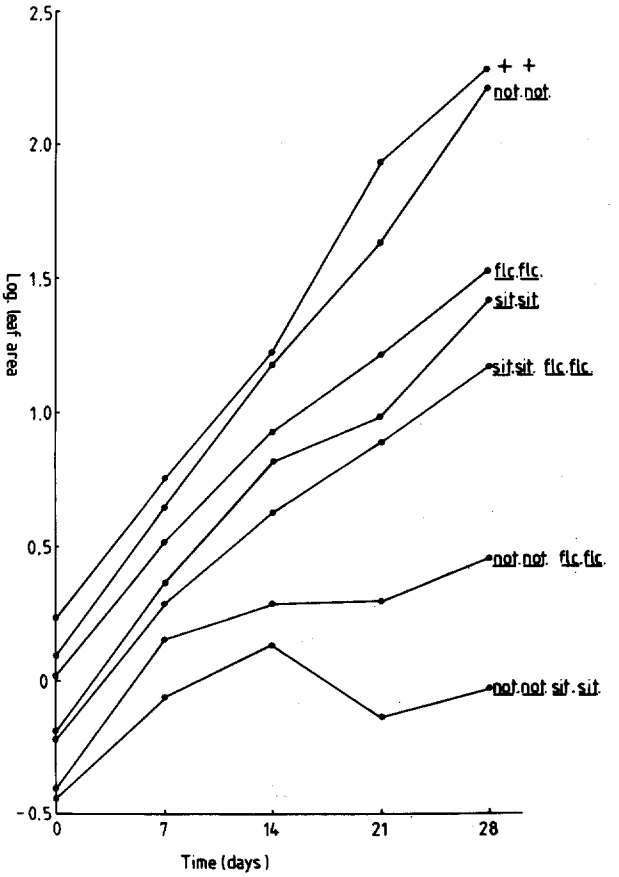

Fig. 1. Effect of wilty mutant genes on growth rate. Leaf area measurements originally in cm^2-converted to a log scale. 0 days = time at which cotyledons were fully expanded

genotype was not available for the previous experiment, as its authenticity was being confirmed by backcrossing to each of the two parental lines. Because it gave wilty mutant phenotypes following both backcrosses, this line must be homozygous for both *sit* and *flc* genes.

The phenotypic effects of combining *flacca* and *sitiens* were very interesting. It was expected that because these two mutants are both more extreme than *notabilis,* their double mutant would also have the most severe phenotype. It can be seen from Fig. 3 that this was not the case. The genotype *sit sit flc flc* was easily the most rapidly growing of the three double mutant combinations. It was closer in phenotype to the single mutant homozygotes of *sitiens* and *flacca,* than it was to the other two double mutants. Nevertheless, this double mutant did cause a greater reduction in leaf area than either *sitiens* or *flacca* alone.

It is also interesting to note from Fig. 1 that the differences between the various genotypes are apparent throughout the experiment, even in the size of the cotyledons. Genotypes therefore appear to be expressed very early in development.

Discussion

ABA deficient mutants should provide vital information concerning the role of this growth regulator in plant development. A mutation resulting in a total block of ABA biosynthesis throughout the plant would be particularly useful. Those plant processes which are ABA-dependent, or ABA-requiring could be identified.

The three tomato mutants studied here are all 'leaky' in that they allow the production of some ABA (Tal and Nevo 1973). This may be because any mutant causing the complete absence of ABA from the plant, would be inviable. Unless a mutant of this type could be recognised and phenotypically reverted by exogenous ABA, it is perhaps unlikely that a seed stock would be formed from it. Selection may have ensured that all known ABA-deficient mutants are leaky.

Combining together two mutants may provide a way of avoiding this problem. A relatively conservative amino acid substitution in one of the enzymes of the ABA biosynthetic pathway might result in a partial loss of enzyme activity. The presence of two inefficient enzymes in the same pathway might result in an almost total block of biosynthetic capacity. A double mutant homozygote might therefore reduce ABA levels far more dramatically than either mutant alone.

The results of the present investigation are consistent with this simple model. The *sitiens* mutant does not represent the limit of expression of the wilty mutant syndrome in the tomato. The double mutants *notabilis/flacca* and *notabilis/sitiens* lead to a much more extreme phenotype (Tables 1, 2 and Fig. 1). More detailed physiological investigations of these two double mutants, including measurement of endogenous ABA levels, should provide relevant information concerning the normal role of this growth substance. Measurement of hormone levels in these mutants may present practical problems due to their extremely slow growth (Fig. 1), and the consequent lack of tissue for extraction. If the low endogenous ABA levels of these genotypes can be confirmed, the present results lead to a superficially surprising conclusion. A severe reduction in the levels of a presumed inhibitor of growth and development appears to cause a massive reduction in growth rate. The results certainly indicate that ABA is an essential requirement for the maintenance of plant growth. It is therefore not surprising that single mutants leading to a total loss of ABA have not so far been detected.

The unexpected order of severity of phenotype of the three double mutants also requires some further discussion. The results appear to indicate that *notabilis* is in some way distinct from the other two mutants. Although *flacca* and *sitiens* were the most severe of the single mutants, they did not have the expected dramatic effect when combined together (Fig. 1). If the correlation between phenotype and endogenous ABA levels in the single mutants (Tal and Nevo 1973) is assumed to hold for the double mutants, it would be predicted that *sitiens/flacca* would have more ABA than either *notabilis/flacca* or *notabilis/sitiens.* Two alternative hypotheses concerning a possible role for the mutants in ABA biosynthesis could conceivably explain this.

Firstly, it is possible that there are two major pathways which are responsible for ABA biosynthesis. A defective enzyme leading to a loss of function in one pathway would permit some ABA biosynthesis in the other. This could account for the fact that the mutants are leaky. If *flacca* and *sitiens* were assumed to involve different enzymes within the same pathway, then their double mutant would still have one unimpaired biosynthetic route for ABA. In contrast, it is possible that *notabilis* involves an enzyme in the alternative biosynthetic pathway from that affected by *sitiens* and *flacca.* Therefore the double mutants involving *notabilis* would be defective in both pathways, resulting in a much greater reduction in ABA levels and a more extreme phenotype.

The problem with this explanation is that it seems surprising that the plant would need two alternative pathways to produce a compound which is only required in very low amounts. It is also hard to envisage why the deficiency of ABA in single mutants could not be fully compensated by increased activity in the unaffected biosynthetic pathway.

The second hypothesis requires that there is only one pathway of ABA biosynthesis in the plant. All three mutants are assumed to result in a reduction in enzyme activity at some point in this pathway, resulting in a reduction of ABA levels. The phenotypic effect of the double mutants could be explained by the assumption that both *flacca* and *sitiens* essentially affect the same enzyme; whereas *notabilis* involves an enzyme controlling a different step in the pathway. The *flacca* and *sitiens* gene loci may code for different polypeptide sub-units of the same enzyme. Combining them together may result in only a small additional loss of enzyme activity.

However, the double mutants involving *notabilis* would have a dual biosynthetic block at two distinct points on the pathway. This may result in a more drastic reduction in ABA levels resulting in the extreme phenotype detected in *notabilis/flacca* and *notabilis/sitiens* double mutants.

More detailed work to establish the precise biochemical basis of the three mutants will be required before accepting either of the above explanations of the present results.

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