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Analysis of fertility in somatic hybrids of *Nicotiana rustica* and *N. tabacum* and progeny over two sexual generations

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Summary. Somatic hybrid plants, produced between *Nicotiana rustica* and *N. tabacum* by heterokaryon isolation and culture and also by mutant complementation, were examined regarding their ability to set seed. From a total of seventeen independent somatic hybrids, three were found to be partially self-fertile while the others did not set seed. Differences regarding the methods of hybrid selection, parental varieties and chloroplast composition of hybrids did not appear to be significant regarding the ability of plants to set seed. Much variation in fertility was observed in subsequent generations and by recurrent selection of the most fertile, over two generations, it was possible to increase the level of self-fertility in some of the progeny. One R2 derivative possessed approximately a tenfold higher level of self-fertility than it's somatic hybrid parent. The presence of genetic markers from both parents were observed in all progeny indicating their hybrid nature.

Key words: Interspecific Somatic hybrids – *Nicotiana* – Fertility

Introduction

Fusion of protoplasts, and the subsequent production of somatic hybrid plants, may be beneficial to agriculture by facilitating gene flow from wild species into the genomes of agricultural crops (Gleba and Evans 1983; Pental and Cocking 1985). Somatic hybrid plants must be entered into plant breeding programmes in order to develop their potential and for this it is important that the somatic hybrids possess some fertility.

In some cases interspecific hybrids have been fertile (Power et al. 1978; Schieder 1980), while others have been sterile (Maliga et al. 1978; Power et al. 1980; Uchimiya 1982). Recently we reported the production of somatic hybrid plants between *Nicotiana rustica* and *N. tabacum* by heterokaryon isolation and culture (Hamill et al. 1984) and also by mutant complementation (Pental et al. 1984). Such hybrids may be agronomically important as *N. rustica* is resistant to several diseases which affect *N. tabacum* (tobacco) (Radhakrisha Murty and Swaminathan 1957; Stavely 1979) and also possesses a higher alkaloid content than *N. tabacum* (Legg and Mann 1961). Somatic hybrids between *N. rustica* and *N. tabacum* have been reported previously. A total of seventeen somatic hybrid plants were produced and characterized and were almost totally self-sterile with only six viable seeds produced from two lines (Douglas et al. 1981). However, Iwai et al. (1980) reported that one plant, of four somatic hybrids produced between *N. rustica* and *N. tabacum*, was fertile although no information was given regarding the extent of the fertility which was observed.

In the present study we report on the self-fertility of somatic hybrids between *N. rustica* and *N. tabacum* reported previously (Hamill et al. 1984; Pental et al. 1984) and on the progeny over two sexual generations. It was also possible to assess the presence of parental genetic markers among the progeny which served as indicators of hybridity.

Materials and methods

Plant material

The source of parental *Nicotiana rustica* and *N. tabacum* varieties and the production and characterization of somatic

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hybrid plants between these species have been reported (Hamill et al. 1984; Pental et al. 1984) and will be briefly described for the sake of clarity.

Somatic hybrid plants from six independent lines (lines 5, 6, 9, 11, 12 and 13) were derived from an experiment involving the fusion of leaf mesophyll protoplasts of wild type *N. tabacum* var 'Xanthi' and cell suspension protoplasts of wild type *N. rustica* var 'V27'. Heterokaryons were isolated and cultured and subsequently gave rise to somatic hybrid plants. Plants will hereafter be designated HS (heterokaryon selected, HS5, HS6, HS9, HS11, HS12m and HS13. Plant HS6 contained the chloroplast of *N. tabacum* while the others contained the chloroplast of *N. rustica* as determined by Fraction 1 protein analysis (Hamill et al. 1984).

Somatic hybrid plants, from 11 independent lines (lines 1–11), were derived from an experiment involving the fusion of leaf mesophyll protoplasts of a streptomycin resistant and nitrate reductase deficient mutant of *N. tabacum* DM with protoplasts from a cell suspension of wild type *N. rustica* var 'V12'. *N. tabacum* DM is streptomycin resistant and nitrate reductase deficient (Hamill et al. 1983). Somatic hybrid plants were selected in medium containing nitrate as sole source of nitrogen and also containing streptomycin and will hereafter be designated DMS (double mutant selected), DMS1–DMS11. All DMS plants contained the chloroplast of *N. tabacum* (Pental et al. 1984).

Plant material was grown as described previously (Hamill et al. 1984; Pental et al. 1984).

Fertility of somatic hybrid plants and analysis of offspring

Pollen viability was determined by incubating freshly dehisced pollen in a few drop of 2% w/v acetocarmine solution (Collins 1979). Seed set was determined by securing paper bags over flower buds before they opened and seed pods were collected about one month afterwards. Fertility values were calculated according to the following criteria. It was found that seed pods of the *N. tabacum* varieties used in the present study contained 400–500 seeds while those of *N. rustica* typically contained 250–300 seeds. A hypothetical value of 400 seeds per pod was assumed in a 100% fertile hybrid and actual self-fertility values of somatic hybrids and offspring were calculated relative to this value. (Thus, an average of 20 seeds per pod corresponds to a self-fertility value of 5%). Actual self-fertility values were calculated to the nearest 0.5%. Metaphase chromosome spreads were counted using young root tips pretreated in a solution of 0.03% hydroxyquinoline (Sigma) for 6 h at 18 °C. Chromosomes were visualized by Feulgen staining. Isoelectric focusing of esterases and the subunits of Fraction 1 protein were as described (Hamill et al. 1984; Pental et al. 1984).

Terminology of hybrid plants and offspring follows the recommendations of Chaleff (1981). The term R represents the regenerated somatic hybrid plants. The R_1 generation refers to the first sexual generation produced upon selfing the somatic hybrids. The R_2 generation was produced upon selfing the R_1 generation. Designation of particular R_1 and R_2 plants is described in the text.

Genetic markers in somatic hybrids and offspring

In addition to electrophoretic markers (isoelectric focusing of leaf esterases and the subunits of Fraction 1 protein), there were genetically defined markers available to assess the extent of hybridity in somatic hybrids and offspring. *N. rustica* (var 'V12') possessed dominant alleles for a gene causing a black-walled ovary which was expressed in all DMS somatic hybrids (Pental et al. 1984). In addition, nitrate reductase proficiency (chlorate sensitivity) was due to the presence of *N. rustica* nitrate reductase genes in these plants (Pental et al. 1984). *N. rustica* var 'V27' is genetically recessive for yellow stem and leaves (Jinks et al. 1981). This feature was found to be recessive in the HS series of somatic hybrids which all possessed the green colour of the *N. tabacum* parent (Hamill et al. 1984).

In all hybrids the intermediate nature of the flowers with respect to corolla length and shape was also a good indication of hybridity.

Results

Fertility of somatic hybrids and offspring

The fertility of somatic hybrids of both HS and DMS series was assessed and is summarized in Table 1.

It is evident that the fertility was non-uniform in the population. Many of the plants produced copious amounts of stainable pollen but produced empty pods containing shrivelled seeds. Only three plants (plants HS5, HS6 and DMS8) from a combined total of seventeen somatic hybrids, produced significant numbers of seeds and even then these possessed only 1–2% fertility of the parents with each pod also con-

Table 1. Analysis of *N. rustica* + *N. tabacum* somatic hybrids

Species and Somatic hybrid line no.		Chromosome no.[a]	Pollen viability	% Self-fertility
N. tabacum var 'Xanthi'		48	95	100
N. tabacum DM		48	98	100
N. rustica var 'V12'		48	100	100
N. rustica var 'V27'		48	100	100
N. rustica + *N. tabacum* Somatic hybrid				
Line no.	HS5	74–77	21	1.0
Line no.	HS6	82–87	38	1.5
Line no.	HS9	73–76	17	0
Line no.	HS11	75–79	43	0
Line no.	HS12	72–79	11	0
Line no.	HS13	nd	4	0
Line no.	DMS1	81–86	62	0
Line no.	DMS2	nd	31	0
Line no.	DMS3	nd	3	0
Line no.	DMS4	72–74	48	0
Line no.	DMS5	nd	2	0
Line no.	DMS6	nd	7	0
Line no.	DMS7	63–67	40	0
Line no.	DMS8	76–79	78	2.0
Line no.	DMS9	nd	12	0
Line no.	DMS10	68–72	2	0
Line no.	DMS11	63–67	17	0

[a] Actual numbers were difficult to ascertain in somatic hybrids and thus a range is presented. The actual number lay within this range; nd = not determined

taining large numbers of shrivelled seeds. However, it is clear that the method of hybrid selection of chloroplast composition of the hybrids did not determine the ability of hybrid plants to set seed. Also, all plants analysed possessed a chromosome number less than the summation of that of both parents (Table 1) demonstrating that aneuploidy did not preclude self-fertility.

Seeds of plants HS5, HS6 and DMS8 were sown and showed 64%, 58% and 76% germination, respectively, when sown in compost.

Ten R_1 plants of each fertile hybrid (designated R_1 a–j) were grown to the flowering stage and self-fertility was assessed. All R_1 plants derived from hybrid HS5 (HS5 R_1 a–j) were self-sterile. Three R_1 plants from hybrid HS6 possessed 1–2% fertility (namely HS6 R_1 b, HS6 R_1 c, HS6 R_1 g) while the others were self-sterile. Four R_1 plants from hybrid DMS8 possessed 1–2% fertility (namely DMS8 R_1 b, DMS8 R_1 c, DMS8 R_1 e and DMS8 R_1 f) while the others were self-sterile. Seeds of plants HS6 R_1 c, HS6 R_1 g, DMS8 R_1 e and DMS8 R_1 f were collected and sown. They showed 92%, 87%, 78% and 94% germination, respectively.

Twenty R_2 plants of each selected R_1 family (designated R_2 1–20) were grown to the flowering stage and their self-fertility was assessed (Table 2). It is evident that the fertility of the R_2 generation in each of the four families studied was very variable and possessed members with lower and also higher pollen viabilities and actual self-fertility values than those observed in the R (somatic hybrid) and R_1 (first sexual generation) populations. Also, many plants produced pods containing a large number of shrivelled seeds. The overall trend was towards a higher incidence of self-fertility in each R_2 family than in their respective R_1 families although some self-sterile plants were observed in each R_2 family.

In one of the R_2 families in particular (HS6 R_1 g R_2 1–20), four plants, of 20 studied, possessed self-fertility values of 10% or greater. The most fertile plant in this family (designated HS6 R_1 g R_2 19) possessed a self-fertility value of 16%.

Table 2. Analysis of second sexual generation (R_2 generation) progeny of *N. rustica* + *N. tabacum* somatic hybrids

R_2 family designation	% Pollen viability mean ± SD (range)	% Self fertility mean ± SD (range)
HS6 R_1c R_2 (1–20)	51±29 (0–82)	1.5±1.4 (0–5.5)
HS6 R_1g R_2 (1–20)	52±26 (2–81)	3.5±5.1 (0–16)
DMS8 R_1e R_2 (1–20)	67±28 (0–94)	1.1±0.9 (0–3)
DMS8 R_1f R_2 (1–20)	60±30 (0–95)	2.1±1.6 (0–5)

Analysis of hybridity in hybrid offspring

Although much variation was observed in each generation, there were no indications of the loss of any genetic markers in any offspring. All R_1 R_2 plants of the DM series possessed black wall ovaries and a hybrid flower with respect to shape and corolla length. In addition all plants possessed nitrate reductase as was evident by normal growth in compost. Nitrate reductase deficient plants were typically chlorotic when grown in compost (Pental et al. 1984). All R_1 and R_2 plants of the HS series possessed green stems and leaves and all possessed a hybrid flower with respect to shape and corolla length.

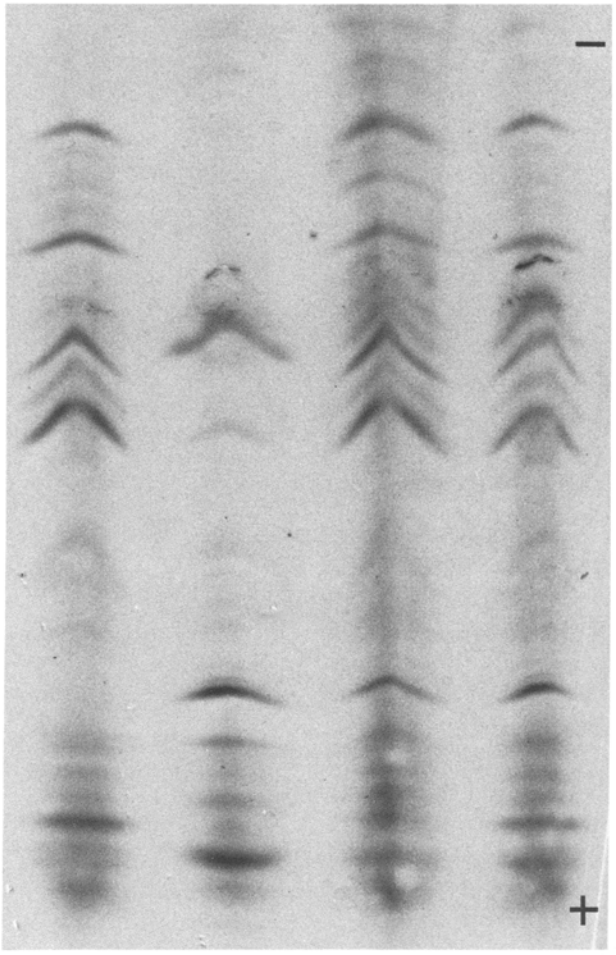

Fig. 1. Isoelectric focusing zymogram of leaf esterases. From *left to right: N. tabacum* leaf extract; *N. rustica* leaf extract; somatic hybrid between *N. tabacum* and *N. rustica* HS6 (R generation) leaf extract; second generation (R_2 generation) hybrid derivitive HS6 R_1g $R_1$19 leaf extract

The most fertile R_2 offspring (designated HS6 R_1 g R_2 19) was analysed regarding the composition of Fraction 1 protein (not shown). It was found to possess the chloroplast encoded subunit of *N. tabacum* and the nuclear encoded subunits of *N. tabacum* and *N. rustica*. In this respect it was identical to the somatic hybrid (HS6) from which it was derived (Hamill et al. 1984). Isoelectric focusing of leaf esterases showed the presence of both *N. tabacum* and *N. rustica* isozymes in this R_2 offspring (Fig. 1). Again in this respect it was identical to the somatic hybrid from which it was derived. The chromosome number of this plant was in the range of 77–79, which was slightly less than that found for the somatic hybrid parent HS6 (Table 1).

Discussion

Analysis of self-fertility in *N. rustica* + *N. tabacum* somatic hybrids (R generation) showed that three plants, from a total of seventeen hybrids produced, were partially fertile.

The same number of *N. rustica* + *N. tabacum* somatic hybrids were produced and characterized by Douglas et al. (1981) and were almost totally self-sterile with only six seeds produced from two lines. Iwai et al. (1980) reported that one *N. rustica* + *N. tabacum* somatic hybrid, of four plants analysed, was fertile although no information was given regarding the extent of self-fertility. The reasons for the difference in fertility of plants, especially those analysed in the present study and those produced by Douglas et al. (1981), are not immediately obvious. It is unlikely that parental varietal differences are important, as suggested by Douglas et al. (1981), because the present study encompassed somatic hybrids produced between two varieties of *N. tabacum* and two varieties of *N. rustica*. Similar trends in fertility were observed in somatic hybrids of either parental background. Two types of selection were employed and somatic hybrids were produced which possessed either the *N. rustica* or *N. tabacum* chloroplast. Neither of these considerations appear to have been of primary importance regarding hybrid fertility. It was suggested that aneuploidy resulted in sterility of somatic hybrids (Evans 1983). However, all *N. rustica* + *N. tabacum* somatic hybrids which were analysed in the present study were aneuploid, showing aneuploidy does not necessarily result in sterility. Other workers have also shown aneuploid somatic hybrids may possess fertility (Smith et al. 1976; Chupeau et al. 1978).

It is possible that the total length of time spent in the unorganised growth phase before plant regeneration is important regarding the subsequent fertility of somatic hybrids. Chromosome breakages and restructuring have been reported in celery as a result of growth in the unorganised phase in addition to the commonly reported phenomenon of aneuploidy in cell culture (Murata and Orton 1983, 1984). In the present study of *N. rustica* + *N. tabacum* somatic hybrids, the *N. tabacum* parental protoplasts were isolated from mesophyll tissue while those of *N. rustica* were isolated from cell suspensions which were less than three-months old. In the case of Iwai et al. (1980), protoplasts both parental species were isolated from mesophyll tissue. In the case of Douglas et al. (1981) however, the sources of both parental protoplasts were cell suspensions one of which was eighteen-months old while the other was four-months old at the time of protoplast isolation. It is possible that in this latter case, a large degree of chromosome restructuring had occurred before plant regeneration, resulting in the high level of sterility reported by Douglas et al. (1981). This may suggest that the time of unorganised growth should be kept as short as possible in order to maximise the chances of somatic hybrids being fertile.

The second point arising from the present study is that the level of self fertility of *N. rustica* + *N. tabacum* somatic hybrids could be increased over two sexual cycles by utilization of the high degree of variation in fertility which was observed in the R_1 and R_2 generations. The selection of the most fertile offspring resulted in one R_2 offspring possessing 16% self-fertility – approximately ten times higher than the somatic hybrid from which it was derived. Such fertile plants may be valuable for developing new varieties of tobacco which combine beneficial aspects of both *N. tabacum* and *N. rustica*. However, the embryo-endosperm incompatibility reaction which makes sexual hybridization difficult between *N. tabacum* and *N. rustica* (Brink and Cooper 1941; Douglas et al. 1983) is probably of some importance regarding the fertility of somatic hybrids described in the present study. The observation in each generation, of shrivelled seeds in pods of many plants may be evidence of it's presence. Therefore recurrent selection of the most fertile offspring, over a number of generations, may be necessary in order to recover highly fertile hybrids.

In conclusion, it is clear from the present study involving somatic hybrids between *N. rustica* and *N. tabacum*, that fertility was non uniform in the population. Even though only partially fertile somatic hybrids were recovered, it was possible to increase the level of fertility by recurrent selection of the most fertile offspring over two generations. It is possible that such a strategy of recurrent selection for increased self-fertility in somatic hybrids, over several generations, may be useful in allowing the detection of individuals most suitable for backcrossing with parental species in plant breeding programmes.

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