

Gene transfer in *Nicotiana rustica* by means of irradiated pollen

V. Quantitative characters

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The inheritance of two quantitative characters is examined in the M_2 and backcross generations derived from crosses of *Nicotiana rustica* that used pollen irradiated with 20 Krad of γ -rays. All generations examined show, on average, a reduction in final height compared with their unirradiated controls. Leaf width was also reduced in almost all cases. Whilst in some parental combinations the direction of this change is compatible with the concept of restricted transfer of genes from the original pollen parent in other cases it is not. Instead the consistent trend across all generations is better explained as a consequence of the expression of deleterious mutations that lead to a general reduction in vigour. Differences between the two backcross generations provide some evidence of selection in the M_1 pollen against radiation damage, acting in general to improve vigour. It is concluded that the maternal trends previously reported in this species were entirely the result of induced mutations, reducing the expression of the characters concerned. No evidence can be found from this species for the existence of a mechanism for gene transfer by DNA fragment incorporation.

INTRODUCTION

The initial paper in this series (Caligari *et al.*, 1981) described the effects of pollen irradiation in *Nicotiana rustica* and showed that the second (M_2) generation after irradiation appeared more maternal than the F_2 both for qualitatively and quantitatively inherited characters. Although three mechanisms were proposed by these authors to explain their findings the favoured mechanism was the restricted transfer of the paternal genome, perhaps similar to one of the mechanisms proposed by Pandey (1975; 1980). The potential of this approach as a rapid plant breeding method generated enthusiasm (Davies, 1981; Mather, 1981). Subsequent work with the same inbred lines of *N. rustica*, however, has shown the consequences of pollen irradiation in this species to be less promising than originally hoped (Werner *et al.*, 1984; Werner and Cornish, 1984; 1985). Cytogenetical studies have revealed that the M_1 generation possesses numerous chromosomal aberrations and that these are inherited by subsequent generations. The radiation has also been shown to induce the loss of function of alleles controlling pigment synthesis in this species. Mutations of these kinds have regularly been reported following radiation treat-

ment and have little value in plant breeding. Snape *et al.* (1983) have, however, proposed that the maternal trends observed may result from selection in the M_1 gametophytes or the M_2 zygote against radiation damaged paternal chromosomes and that this may provide a valuable mechanism for restricted gene transfer during a plant breeding programme. Selection at this stage has indeed been observed in the *N. rustica* study, resulting in the reduced frequency of transmission of structurally aberrant chromosomes to the second generation and in disturbances of the segregations of major genes. The intensity of selection was, however, considered to be too low to be of practical value. In this, the final paper in the series, we report the effects of pollen irradiation in this material on the inheritance of quantitative characters, which being under the control of a number of genes indicate the effects of radiation across the whole genome.

MATERIALS AND METHODS

Nine quantitative characters were assessed on the same field experiment from which the qualitative results were obtained. The design and composition of this experiment were described fully in our

previous paper (Werner and Cornish, 1985). M_1 plants were produced from the pollination of V_{27} plants with irradiated V_{27} pollen at 20 Krad (treatment 20A) and with irradiated V_{12} pollen (20B) and of V_{12} plants with irradiated V_{27} pollen (20C) and irradiated V_{12} pollen (20D). The M_1 plants were then selfed (M_2) and reciprocally backcrossed ($V_{27} \times M_1 = BC$, $M_1 \times V_{27} = RBC$). The results of two quantitative characters will be reported in detail here. These are leaf width, the maximum width of the largest leaf measured 6 weeks after planting, and final height, the height reached at the end of the growing season. Leaf width was chosen for detailed presentation because owing to its genetic architecture it offers the most discriminating test of the restricted gene transfer hypothesis. Final height is a maturity character, generally considered to give consistent results over seasons in this species and was also presented in the initial study by Caligari *et al.* (1981). The results of the remaining characters are summarised here but are described in detail by Werner (1984).

RESULTS

At final height V_{12} was taller than V_{27} and both the F_1 and F_2 were on average taller than V_{12} (Table

1). The same relationships were found by Caligari *et al.* (1981) for this character although all their generations were on average taller than ours as a result of a seasonal difference. The 20B M_2 generation is equivalent to the 20 Krad (G) treatment of Caligari *et al.* and the two generations show the same relationship to their basic generations, being significantly shorter than their respective F_2 's. The additional generations included in this study provide a similar picture; the mean of the M_2 from the reciprocal cross, 20C, is also shorter than the F_2 ; the two irradiated self M_2 's are shorter than their respective parents although only the 20D M_2 is significantly shorter; seven of the eight backcross generations are shorter than their unirradiated equivalent, five of the differences being significant.

Now if restricted gene transfer were the cause of these trends the 20B M_2 would have inherited more maternal genome than paternal and would, therefore, be more like V_{27} , that is shorter than the F_2 . This is indeed the case as Caligari *et al.* (1981) also observed. For the same reason we would also expect the 20C M_2 to be more like V_{12} , that is a little shorter than the F_2 . It is however reduced in height beyond V_{12} and is therefore shorter than would be expected on the basis of the gene transfer hypothesis. We would expect the two backcrosses derived from the 20B M_1 's to be shorter than B

Table 1 Generation means and comparisons with their control generations for two quantitative characters. The standard errors and their degrees of freedom are derived from the between families mean squares when significant, and from the within families mean square otherwise. The approximation of Satterthwaite has been used to obtain the appropriate degrees of freedom of t' (Snedecor and Cochran, 1980). Inequalities indicated are significant at the 5 per cent level

Generation	Final Height (cm)				t' mean	Leaf Width (mm)			
	mean	s.e.	d.f.	t' test		s.e.	d.f.	t' test	
V_{27}	98.93	1.01	59		252.0	4.53	59		
V_{12}	137.52	1.60	59		201.3	4.38	59		
F_1	171.40	1.78	58		252.9	5.98	3		
F_2	152.72	2.00	159		230.2	4.10	158		
$B(F_1 \times V_{27})$	142.53	3.99	7		247.2	3.42	156		
20A M_2	90.39	7.25	3	= V_{27}	207.4	13.48	3	= V_{27}	
20B M_2	123.96	2.05	223	< F_2	183.8	5.51	11	< F_2	
20C M_2	126.29	4.33	18	< F_2	182.1	8.26	18	< F_2	
20D M_2	127.34	2.80	6	< V_{12}	167.7	8.96	6	< V_{12}	
20A BC	100.47	2.53	5	= V_{27}	253.0	5.89	48	= V_{27}	
RBC	96.29	2.35	37	= V_{27}	227.8	5.47	37	< V_{27}	
				RBC = BC				RBC < BC	
20B BC	131.75	2.23	23	< B	228.8	3.45	23	< B	
RBC	124.24	2.33	23	< B	214.3	3.16	23	< B	
				RBC < BC				RBC < BC	
20C BC	137.98	2.19	169	= B	234.7	3.47	171	< B	
RBC	128.88	2.89	15	< B	219.9	4.92	15	< B	
				RBC < BC				RBC < BC	
20D BC	161.62	1.83	26	< F_1	238.0	3.80	27	= F_1	
RBC	160.20	2.39	13	< F_1	234.8	4.71	26	< F_1	
				RBC = BC				RBC = BC	

and more like V_{27} , which they are. The two 20C backcrosses on the other hand should be more like the F_1 than B, *i.e.* taller, which they are not. Finally, we would expect none of the generations derived from the irradiated selfs to differ from their unirradiated equivalents but all three 20D generations are significantly shorter. In some generations the reduction in height can be explained in terms of the restricted transfer of the paternal genome into a maternal background. The combined results of all the generations presented here, however, are not consistent with this explanation. Instead it is clear that there is a reduction in height of all the generations regardless of the directions of the cross. Such a trend would result from the inheritance of induced deleterious mutations leading to a loss of vigour and hence to a reduction in height in all cases.

The leaf width character provides an even clearer illustration of this effect. The relationship between the parents is now reversed since V_{27} has wider leaves than V_{12} whilst the F_2 is intermediate. Again in almost all generations the radiation has significantly reduced leaf width as compared with the control generations. Instead of the maternal trend towards wider leaves in the 20B M_2 that would result from restricted gene transfer we observed considerably narrower leaves than the F_2 . The 20B and 20C backcrosses also have thinner leaves contrary to gene transfer expectations and the 20D M_2 progeny have on average the thinnest leaves of any generation. Whilst leaf width was not described by Caligari *et al.* (1981), because in their experiment the parents were not significantly different from each other, it was measured

(Ingram, 1982) and a marked, dose-related reduction in width was observed similar to the trend described here. The expression of deleterious induced mutations would appear, therefore, to be the overriding phenotypic consequence of the irradiation on this character also.

Similar results were obtained for the remaining seven quantitative characters measured on these plants (table 2). Six of these, including earlier height measurements and leaf and flower dimensions, showed a consistent reduction in expression independently of the direction of the cross although not all the reductions were significant at the 5 per cent level. The remaining character, which showed consistent trends towards increased expression, was flowering time, but an increase in the time taken for a plant to flower can also be interpreted as a reduction in vigour as a result of deleterious mutations. The radiation treatment also affected the variation within many of the M_2 and backcross families. Taken over all nine characters more than one third of the generations had average within family variances significantly greater than within their equivalent unirradiated generations. Similar effects were found by Ingram (1982). Induced mutations should indeed inflate the variation within families. The restricted transfer of the paternal genome on the other hand would be expected to lead to a reduction in this variation since fewer loci would be segregating in the second generation.

Further information about the effects of the radiation can be obtained from a comparison of the BC and RBC generations. The genetical expectations of these two generations are identical

Table 2 Significant relationships (5 per cent level) between treatment means and control means; from Students *t'* tests. The characters are: FT, flowering time; FL, flower length; FW, flower width; LL, leaf length; LW, leaf width; RUF, ruffled leaf index; HFT, height at flowering time; H50, height at 50 per cent flowering; FH, final height

Generation	FT	FL	FW	LL	LW	RUF	HFT	H50	FH	Control generation
20A M_2	=	<	=	<	=	=	=	=	=	V_{27}
20B M_2	=	=	=	<	<	=	<	<	<	F_2
20C M_2	>	=	<	<	<	=	<	<	<	F_2
20D M_2	=	=	<	<	<	=	=	=	<	V_{27}
20A BC	=	=	=	=	=	=	=	=	=	V_{27}
20A RBC	=	=	=	<	<	=	=	=	=	V_{27}
20B BC	=	=	=	<	<	=	=	<	<	B
20B RBC	>	=	=	<	<	=	=	<	<	B
20C BC	=	=	=	=	<	<	=	=	=	B
20C RBC	>	<	<	<	<	<	=	<	<	B
20D BC	=	=	=	=	=	=	=	=	<	F_1
20D RBC	=	=	=	=	<	=	=	<	<	F_1

unless there are maternal effects or unless the rates of transmission of damaged chromosomes differ in the male and female gametophytes. It can be seen in table 1 that the scores of the RBC generations are in all eight cases lower than their BC generations, six of the differences being significant. It is possible that the reduced vigour of these RBC generations is a maternal effect since the seed parents in these crosses were M_1 plants and may not have produced as healthy seed as normal V_{27} plants. However, reduced seed quality would be expected to have a greater effect on characters measured earlier in the growing season than final height but this was not the case. A more likely explanation is that selection was acting, in the M_1 pollen that produced the BC generations, against damaged chromosomes of paternal origin, whilst less selection or no selection was acting in the ovules that produced the RBC generations. Since damaged chromosomes on the whole lead to a loss of vigour the BC generations, having inherited less damaged material, are more vigorous both for final height and leaf width. Note that the direction of this effect on quantitative characters is independent of the direction of the cross; selection does not produce a more maternal phenotype but a more normal phenotype. Only selection against mutations at loci for which the parents originally differed would be expected to produce a maternal bias. Selection against mutations at loci for which the parents are identical on the other hand would restore normal expression. It would appear that the latter kind of mutation predominates in these two characters and in most of the other characters that showed significant differences. Selection would also be expected to reduce the within family variances of the BC generations relative to their RBC generations; in 10 of the 36 comparisons over the nine characters significant differences were observed and in all 10 cases the BC generations had smaller variances.

In addition to the generation means the means of the families within each generation were also compared with the control generations to determine whether any individual families behaved abnormally as a result of a rare gene transfer event. Some families for some characters departed significantly from the control generations in the opposite direction to their generation means. A few families flowered earlier than their control, for example, whilst others had significantly larger flowers. Almost all deviations in the three height characters on the other hand were towards reduced expression. The pattern of departures bore no relationship to the directions of the original crosses

but were scattered through all generations so that many of the deviant families departed in the opposite direction from that expected as a result of gene transfer. They do not, therefore, provide any evidence of the operation of a rare and unusual genetic mechanism. Instead these anomalous deviations would appear related to the genetic architectures of the characters concerned as Lawrence (1975) found in *Arabidopsis thaliana*. Flowering time for example exhibits ambidirectional dominance in *N. rustica* (Mather and Jinks, 1982). This is consistent with a history of stabilising selection for this character which would lead to the fixation both of functionally active increasing alleles and functionally active decreasing alleles. The mutation of these two kinds of loci to a functionless form would then affect the character in opposite directions. Final height on the other hand has probably been subjected in the past to directional selection for increasing expression resulting in directional dominance. Few functionally active decreasing alleles will have been fixed so that very few mutations to a functionless form will increase final height.

The results described are from the treatment of pollen with 20 Krad of gamma rays. The field experiment also included a full set of 12 generations derived from a 10 Krad treatment and the four M_2 generations from a 2 Krad treatment. The lower doses produced effects very similar in direction to those from 20 Krad, but reduced in magnitude (Werner, 1984).

DISCUSSION

The effects of pollen irradiation on the quantitatively inherited characters described here are entirely consistent with the effects observed in the preliminary study of this material (Caligari *et al.*, 1981; Ingram, 1982). In some instances the radiation induced a change in mean expression of a quantitative character that led to a greater similarity to the maternal parent. The additional generations described in this study show that this maternal trend is found only in certain parental combinations. Instead the consistent effect of the radiation has been, as a consequence of the expression of deleterious mutations, to reduce plant vigour with a concomitant reduction in all characters for which decreasing expression indicates decreasing vigour. Seed irradiation is known to have similar effects in this species (Ingram and Jinks, 1982; Ingram, 1982). This conclusion is in line with the

conclusions of the other studies in this series which provided evidence of induced chromosomal damage and of major gene mutations in this material.

Note that the magnitude of the reduction in vigour is not equal for all generations. For example, the 20B and 20C M_2 generations show a greater reduction in final height (as compared with the equivalent unirradiated generation) than do the 20A and 20D M_2 's. A similar effect was observed by Powell and Caligari (1985) who were unable to find any significant reduction in vigour in M_2 generations of barley derived by selfing even though M_2 generations derived from the same parents by crossing had shown such a reduction (Powell *et al.*, loc. cit.). This effect is a consequence of the generally recessive nature of induced mutations and is consistent with conventional mutational effects as the following simple model illustrates. Consider a quantitative character controlled by a number of segregating loci for each of which the increasing allele is completely dominant to the decreasing allele and where both alleles mutate to a recessive mutant indistinguishable from the decreasing allele. (This model describes perfectly the effect of irradiation on the major gene controlling ovary colour in this material (Werner and Cornish, 1985).) Irradiation of the decreasing allele will have no effect on phenotype. Selfing with irradiated pollen will produce M_1 plants heterozygous for the mutant allele and the dominant increasing allele. Each recessive allele will therefore influence the phenotypes of only the one in four M_2 individuals that are homozygous for the mutant allele. Crossing with irradiated pollen, on the other hand, will produce a more marked effect since when mutation occurs the M_2 generation, being derived from M_1 plants heterozygous for the decreasing allele and the mutant allele, will permit expression of the mutant allele both in the individuals homozygous for it and in the individuals heterozygous for it and the decreasing allele. On crossing, therefore, the mutant allele will influence the phenotype of three-quarters of the M_2 generation. If both parents possess some increasing alleles then the M_2 generations from both the irradiated selfs will show some decline in expression, but the equivalent irradiated crosses will show a rather greater decline. Whilst there is no reason to believe that all loci affecting a quantitative character behave on irradiation in such a simple manner the model serves to illustrate that the observed differences in response between the M_2 generations are not inconsistent with conventional mutational effects.

The consistent reciprocal differences in the backcross generations can be interpreted as evidence of selection in the male gametophyte against radiation damage. This again is in agreement with our previous findings. The proposal of Snape *et al.* (loc. cit.) that selection of this kind would lead to useful maternal trends is not however supported by these results which show that selection acts in all cases to restore some of the vigour lost as a result of the radiation damage. The irradiation of *N. rustica* pollen therefore provides no evidence for the existence of a genetic mechanism that might be used for the rapid transfer of paternal genes. Whilst disturbed segregations of major genes were observed in favour of the maternal alleles such effects were slight and, in this species at least, achieved only at the expense of considerable deleterious radiation damage.

Finally, it is worthwhile to compare our findings with studies of pollen irradiation in other species. The *N. rustica* results, in common with all other reported studies, fail to provide any additional evidence to support the unusual mechanisms for gene transfer proposed by Pandey (1975; 1980; 1983). For example, Sanford *et al.* (1984a, b) and Chyi *et al.* (1984) examined a total of 87,000 potential transformation events in a range of crop species but failed to find any transformants. Furthermore, in an unpublished study of our own, attempts to transfer the dominant allele for red flower colour from *N. forgetiana* to *N. alata*, two species used by Pandey, also failed. 26 pollinations using a mixture of self-incompatible *N. alata* pollen and *N. forgetiana* pollen irradiated with 100 Krad of gamma-rays produced 251 offspring. 238 of these plants flowered but all had white flowers and appeared to be self progeny of *N. alata*. Pandey's own results, therefore, provide the only evidence that supports his theories.

The cytogenetical effects that have been described earlier in this series are entirely consistent with other published consequences of pollen irradiation including the more recent studies of Snape *et al.* and Powell *et al.* (1983). With the exception of some of the results of Powell *et al.* the behaviour of the major genes described in our previous paper is also in agreement with the results of a number of other workers. Fewer studies of the effects of pollen irradiation on quantitative characters have been made. The three quantitative characters examined by Snape *et al.* in *Triticum aestivum* show a decline in expression following irradiation in a manner similar to that reported here; for ear length this is in the maternal direction; for grain number the decline is beyond that of the

maternal parent suggesting considerable loss of fertility in the M_2 generation; for plant height this trend is in the paternal direction and is explained by the authors as "a carry-over effect of the radiation damage". Similarly four of the five characters described by Powell *et al.* and Caligari *et al.* (1984) on *Hordeum vulgare* also show a decline following irradiation but in these cases, as in the original *N. rustica* cross, the maternal parent is less vigorous. A true maternal trend again cannot be distinguished from mutational loss in this limited study. The fifth character, grain number, shows a decline at the lowest radiation dose used, increasing to normality at the highest dose, an unexpected trend. Both these studies are largely in agreement with ours in suggesting that mutational loss is the major cause of the phenotypic deviations observed. The *Hordeum* work, however, offers some evidence that may be interpreted as indicating the operation of a strong selection mechanism against radiation damage although others working with this species did not find this (Donini *et al.*, 1970; Devreux *et al.*, 1972). Firm conclusions must await a more exhaustive examination of this species.

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