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Pollen genotype selection for a simply inherited qualitative factor determining resistance to chlorsulfuron in maize

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Abstract Pollen genotype selection for genes expressed in both the haploid and diploid phases of the plant life cycle can lead to correlated responses detectable in the sporophyte. A pollen selection was conducted in two genetic backgrounds of maize (*Zea mays* L.) for chlorsulfuron resistance, conferred by the *XA17* allele. Plants of two backcross (BC) lines segregating 1 (heterozygote, resistant) : 1 (homozygote, susceptible) for chlorsulfuron resistance were used as pollen donor. Selection treatment was applied during microspore development, and tassels were cut about 10 days before anthesis and placed into a liquid medium with or without 40 mg l^{-1} of chlorsulfuron. Pollen was used to fertilize an unrelated susceptible genotype (tester). The resulting testcrosses (TC) were evaluated in the greenhouse by spraying seedlings with chlorsulfuron at 23 g ha^{-1}. Non-selected TC progenies derived from heterozygous BC plants showed a proportion of resistant and susceptible plants close to the expected 1:1 ratio, while non-selected TC progenies derived from homozygous BC plants showed susceptible plants only. Selected TC progenies derived from heterozygous BC plants showed a frequency of resistant plants ranging from 89% to 100%. BC plants homozygous for the susceptible allele, subjected to selection treatment, gave poor seed set, and no resistant plants were found in their TC progenies. Resistant TC plants obtained through pollen genotype selection were selfed, and the proportion of resistant plants was close to 3:1 in all selfed families, in accordance with the hypothesis that all of them inherited the *XA17* allele through selection. In this study, pollen genotype selection was extremely effective, and its effect persisted in the second sporophytic generation.

Keywords Pollen genotype selection · Herbicide resistance · Sulfonylurea · Imidazolinone · *Zea mays* L.

Introduction

Overlap in gene expression between the haploid gametophyte and the diploid sporophyte has proven to be a wide phenomenon in higher plants (Ottaviano and Mulcahy 1989). The haplo-diploid gene expression implies that selection exerted at the gametophytic level can lead to changes in allelic frequencies that can be detected also at the sporophytic level. Pollen genotype selection can produce a rapid change in allelic frequencies due to the large population size and to the expression of recessive alleles in the haploid cells. Selection applied to pollen genotypes has proven to be effective in several cases (see for reviews Hormaza and Herrero 1992; Sari Gorla and Frova 1992), including tolerance to herbicides such as chlorsulfuron (Sari Gorla et al. 1989), alachlor (Sari Gorla et al. 1994), and acetochlor (Frascaroli et al. 1994). However, the natural occurring variability, utilized in most pollen genotype selection studies conducted so far, was likely due to the action of several minor genes, and this could explain the limited responses to selection obtained in some cases (Ottaviano and Mulcahy 1989). Selection for simply inherited qualitative traits should permit the achievement of a more substantial and predictable response, hence allowing a better understanding of pollen genotype selection potentiality (Touraev et al. 1995).

Even though pollen genotype selection has been widely investigated, the persistence of the response in the second sporophytic generation after selection has been examined in only a very few studies. In some cases

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(Mulcahy et al. 1978; Sari Gorla et al. 1994; Frascaroli et al. 1995) a persistence of the response throughout generations was proven, but in one case (Schliching et al. 1990) it was not. This discrepancy led to the question of whether the response to pollen genotype selection was always due to a preferential fertilization by gametes expressing the desired allele or if the response could also be due to transient epigenetic effects.

From *in vitro* cell culture, Anderson and Georgeson (1989) obtained a maize cell line with a single factor *(XA17)* conferring cross resistance to imidazolinone and chlorsulfuron (Newhouse et al. 1991). The resistance was due to decreased herbicide sensitivity of the common site of action of both herbicides, i.e., the acetohydroxyacid synthase [AHAS, also called acetolactate synthase (ALS)].

The objectives of the investigation reported here were to evaluate, in two different genetic backgrounds, the response to pollen genotype selection for resistance to chlorsulfuron conferred by the *XA17* allele and to assess whether the response also persists in the second sporophytic generation after selection.

Materials and methods

Plant material and pollen selection

The *XA17* allele had been previously transferred through six backcrosses (BC) into two unrelated Pioneer susceptible maize inbred lines, hereafter indicated as A and B (the Malecot's coefficient of parentage between them is 0.032). In both genetic backgrounds, plants homozygous for the wild-type allele were susceptible to chlorsulfuron (applied as Glean at 23 g ha^{-1} a.i. by spraying seedlings at the three-leaf stage), while plants heterozygous were resistant. Throughout the backcrossing procedure it was thus possible to apply selection against the homozygotes for the recessive susceptible allele. As a result, both A and B BC_6 lines, obtained after crossing heterozygous plants from the BC_5 generation with the recurrent parent, were expected to segregate 1:1 for resistance to chlorsulfuron, half of them being heterozygous and the others homozygous for the recessive susceptible allele derived from the recurrent parent.

The BC_6 lines (hereafter indicated as BC lines) were used to carry out the pollen genotype selection. Because chlorsulfuron can persist up to several months and it is active at a very low concentration (Strek 1998), the preliminary sporophytic selection against homozygotes for the recessive susceptible allele (as was made during the backcrossing procedure) was not made on plants to be used for pollen genotype selection in order to prevent possible chlorsulfuron effects on plant and/or pollen tissues prior to selection. Because plants heterozygous and plants homozygous for the recessive susceptible allele are not distinguishable from each other in the absence of the herbicide, a random sample of both resistant and susceptible plants of each BC line A and B was grown under greenhouse conditions. The pollen genotype selection in favor of the *XA17* allele was applied during microspore development following the procedure described by Sari Gorla et al. (1989). Fifteen tassels of BC line A and 11 of BC line B were cut about 10 days before anthesis, and the cut ends were placed into a liquid medium (Polowick and Greyson 1982) containing 40 mg l^{-1} of chlorsulfuron. Another sample of 5 tassels of BC line A was subjected to the same treatment without herbicide, so as to have a non-selected control. Pollen produced from each tassel was used to fertilize the unrelated tester *Hi-II* (A188 × B73 derivative, Armstrong et al. 1991) homozygous for the recessive susceptible allele.

Evaluation of response to selection

Testcross (TC) progenies obtained after the treatments described above were evaluated for resistance to chlorsulfuron in a controlled environment (greenhouse). For each BC line, two sets of TC progenies were evaluated in two subsequent analogous experiments, arranged as randomized block designs with seven and five replications (trays), respectively, in order to control possible variation due to environmental conditions and/or herbicide application. Before herbicide treatment, the plants were examined to check whether they were uniformly developed: approximately 1 out of every 50 plants was not as developed as the others and was eliminated from further testing. Chlorsulfuron was applied as Glean at 23 g ha^{-1} a.i. by spraying plants at the three-leaf stage. Two trays for each set of TC progenies were left untreated to have a control for verifying herbicide application effectiveness. Fourteen days after treatment plants were classified as damaged (plants that had stopped growing immediately after treatment) or undamaged. No intermediate herbicide damage was observed.

Because no appreciable variation was observed among the replications and experiments, all data of each TC were pooled. TC progenies showing susceptible plants only were assumed to derive from pollen mother plants homozygous for the recessive susceptible allele. Due to the lack of genetic variability in the pollen population produced by homozygous plants, response to selection was not expected for these progenies. TC progenies showing resistant plants were assumed to derive from heterozygous pollen mother plants; therefore, response to pollen genotype selection was investigated in these progenies only. The null hypothesis that selection was not effective (i.e., that expected ratio 1 (resistant) : 1 (susceptible) was not changed) was then tested by a χ^2 test.

Evaluation of resistance in the second sporophytic generation

Two TC progenies of line B that had shown response to selection in the previous experiment (*Hi-II* × B1 and *Hi-II* × B2) were grown in the field and selfed; 23 selfed ears were harvested for *Hi-II* × B1 and 22 for *Hi-II* × B2. Two TC progenies that had shown no herbicide resistance (*Hi-II* × B7 and *Hi-II* × B8) were also grown and selfed; one random selfed ear was harvested for each of them. These latter two selfed families were produced in order to have a susceptible control with the same inbreeding coefficient and, hence, similar vigor as the selfed families obtained for *Hi-II* × B1 and for *Hi-II* × B2.

Segregation for resistance to chlorsulfuron was studied in the selfed generation following the same procedure previously described, and the layout was a randomized block design with two replications. The resistance transmission was evaluated by verifying that all plants belonging to TC progenies *Hi-II* × B1 and *Hi-II* × B2, which in the previous experiment were 100% resistant, were also heterozygous for the *XA17* allele and, hence, that their selfed generations had 3:1 ratio of resistant to susceptible plants. Segregation of selfed generations was tested by a χ^2 test. The two TC progenies *Hi-II* × B7 and *Hi-II* × B8, which in the previous experiment were 0% resistant, were expected to be homozygous for the recessive susceptible allele and to give rise to susceptible plants only in the corresponding selfed generation.

Results and discussion

Evaluation of response to selection

Fertilization of *Hi-II* susceptible plants with non-selected pollen of BC line A led to a seed set ranging from 224 to 368 kernels per ear (Table 1). Two non-selected TC progenies (*Hi-II* × A1 and *Hi-II* × A2) showed segregation for resistance to chlorsulfuron, and a proportion of

Table 1 Evaluation of response to pollen genotype selection: BC line A and B, non-subjected and subjected to selection treatment (non-selected and selected, respectively), number of kernels per ear (seed set) obtained after crossing with the unrelated genotype *Hi-II*, number of plants resistant or susceptible evaluated for each testcross (TC) progeny and χ^2 test for departure from the expectation of the 1:1 ratio of resistant to susceptible plants in TC progenies. χ^2 test was performed only for TC progenies derived from pollen mother plants assumed to be heterozygous

Plant [a]	Seed set (no.)	Resistant (no.)	Susceptible (no.)	$\chi^2_{(1)}$ [b]
Non-selected				
A1	336	77	90	1.0 ns
A2	368	92	84	0.4 ns
A3	300	0	147	–
A4	224	0	160	–
A5	308	0	156	–
Selected				
A6	317	80	0	80.0**
A7	355	244	0	244.0**
A8	250	160	0	160.0**
A9	170	166	2	160.1**
A10	205	75	1	72.1**
A11	320	79	4	67.8**
A12	435	74	4	62.8**
A13	75	0	53	–
A14	20	0	16	–
A15	0	ND [c]	ND	–
A16	0	ND	ND	–
A17	0	ND	ND	–
A18	0	ND	ND	–
A19	0	ND	ND	–
A20	0	ND	ND	–
Selected				
B1	320	101	0	101.0**
B2	256	113	4	101.5**
B3	138	122	15	83.6**
B4	229	0	140	–
B5	167	0	75	–
B6	70	0	57	–
B7	290	0	140	–
B8	193	0	140	–
B9	0	ND	ND	–
B10	0	ND	ND	–
B11	0	ND	ND	–

[a] The numbers refer to the identification of the plant subjected or not to selection

[b] χ^2 test. ns; Not significant; ** $P \leq 0.01$

[c] ND: Not determined due to no seed set

resistant to susceptible plants not different from the expected 1:1 (Table 1). This finding indicates that the two pollen mother plants were heterozygous and that pollen grains carrying *XA17* and those carrying the recessive susceptible allele had the same competitive ability in the absence of the herbicide. The other three non-selected TC progenies showed no resistant plants at all, indicating that the corresponding pollen mother plants were homozygous for the recessive susceptible allele.

Fertilization of the susceptible tester *Hi-II* with chlorsulfuron-selected pollen of BC line A was successful for 9 out of 15 plants, with a seed set that in 7 cases was comparable to that of tassels not subjected to selection treatment. Seed set was lower than 100 kernels per ear in 2 cases only. The 7 TC progenies derived from the ears with the highest seed set showed a percentage of resistant plants equal to or higher than 95%, with a significant departure from the expected 1:1 ratio of resistant plants. For 3 TC progenies (i.e., TC involving A6, A7, and A8) 100% of the plants were chlorsulfuron-resistant, indicating the complete elimination of the recessive susceptible allele during the selection process. The 2 TC progenies with a very low seed set (i.e., *Hi-II* × A13 and *Hi-II* × A14) showed no resistant plants, suggesting that the 2 pollen mother plants were homozygous for the susceptible allele and that chlorsulfuron treatment during microspore maturation was very harmful to susceptible pollen genotypes. It is therefore reasonable to assume that the 6 plants whose pollination did not seed set at all (from A15 to A20) were also homozygous for the susceptible allele. Considering together the 2 pollen mother plants A13 and A14 and the 6 from A15 to A20, 8 of the 15 pollen mother plants became highly impaired in terms of fertilization ability as a result of the selection treatment. This proportion is very close to 50%, i.e., the expected ratio of pollen mother plants homozygous for the recessive susceptible allele in the BC progeny. We were unable to assess whether the poor seed set or the failure of fertilization were only due to an effect of chlorsulfuron on susceptible pollen, as suggested by the extremely high response to selection observed in the other TC progenies, or also to an effect of the herbicide on the sporophytic susceptible tissues of the tassels. However, no obvious differences for possible injuries were observed among tassels during their maturation in the presence of the herbicide.

Fertilization of *Hi-II* plants with selected pollen produced by plants of BC line B was successful in 8 out of 11 cases (Table 1), with a seed set ranging from 70 to 320. In 3 cases (i.e., B1, B2, and B3) the percentage of resistant plants was higher than expected (from 89% to 100%), thus indicating that pollen mother plants were heterozygous and that also in this genetic background pollen genotype selection was very effective in rescuing pollen genotypes expressing the resistant allele out of a segregating pollen population. The remaining 5 TC progenies (from B4 to B8) showed no resistant individuals, suggesting that pollen mother plants were homozygous for the recessive susceptible allele. It should be noted that homozygous pollen mother plants of BC line B showed seed sets similar to those of heterozygous plants, whereas for BC lines A the pollen mother plants that were assumed to be homozygous for the recessive allele showed a seed set much lower than that of the heterozygous pollen mother plants. Moreover, homozygous susceptible plants of BC line B seemed to produce TC seed more frequently than homozygous susceptible plants belonging to BC line A (5/8 vs. 2/8). These results suggest that the ability of selection to prevent susceptible pollen genotypes from fertilizing, in the absence of competition with resistant pollen genotypes, was slightly lower in BC line B than in BC line A. This could be attributed to differences in pollen and/or mother plants vigor *per se* between the two BC lines.

Evaluation of resistance in the second sporophytic generation

Selfed families obtained from individuals belonging to susceptible TC progenies showed no resistant plants (data not presented), which was as expected because they were supposed to be homozygous for the recessive susceptible allele. This finding indicated that the evaluation of herbicide resistance was also appropriate for the selfed families and not biased by the different vigor *per se* of plants due to the different level of inbreeding. Segregation in the selfed families of resistant TC progenies *Hi-II* × B1 and *Hi-II* × B2 significantly differed from the expected 3:1 ratio in only 1 case out of 23 for the former TC progeny and 1 case out of 22 for the latter (Table 2). These frequencies are expected to occur just by chance only 1 in 20 times, given the chosen α probability level (0.05) of the Type-I error. Moreover, the 2 cases of significant departure from the expectation showed a proportion of resistant to susceptible plants in the family even higher than 3:1. Therefore, the hypothesis that all plants belonging to selected TC progenies *Hi-II* × B1 and *Hi-II* × B2 were heterozygous was not rejected. This result is in accordance with the hypothesis that response to selection was due to a preferential fertilization by gametes carrying the resistant allele, and not merely due to transient epigenetic effects, and is consistent with the results obtained by other authors (Mulcahy et al. 1978; Sari Gorla et al. 1994; Frascaroli et al. 1995) further supporting the importance of pollen genotype selection in determining the variation of allelic frequencies detectable in the subsequent sporophytic generations.

The response to selection obtained in this study is much more marked than that obtained in other pollen genotype selections for tolerance to chlorsulfuron (Sari Gorla et al. 1989) or for tolerance to other herbicides (Sari Gorla et al. 1994; Frascaroli et al. 1994). This

Table 2 Analysis of selfed families of plants belonging to resistant TC progenies B1 and B2, number of plants resistant and susceptible for each selfed family, and χ^2 test for departure from the expectation of the 3:1 ratio of resistant to susceptible plants

TC progeny B1				TC progeny B2			
Selfed family	Resistant (no.)	Susceptible (no.)	$\chi^2_{(1)}$ [a]	Selfed family	Resistant (no.)	Susceptible (no.)	$\chi^2_{(1)}$ [a]
1	48	6	5.6*	1	46	6	5.0*
2	42	8	2.2 ns	2	47	9	2.4 ns
3	47	9	2.4 ns	3	46	10	1.5 ns
4	48	10	1.9 ns	4	26	6	0.7 ns
5	36	9	0.6 ns	5	24	6	0.4 ns
6	78	20	1.1 ns	6	40	10	0.7 ns
7	35	10	0.2 ns	7	44	11	0.7 ns
8	40	12	0.1 ns	8	42	11	0.5 ns
9	37	12	0.0 ns	9	49	13	0.5 ns
10	40	13	0.0 ns	10	25	7	0.2 ns
11	38	13	0.0 ns	11	46	14	0.1 ns
12	44	16	0.1 ns	12	26	8	0.0 ns
13	41	15	0.1 ns	13	41	13	0.0 ns
14	38	14	0.1 ns	14	43	15	0.0 ns
15	38	14	0.1 ns	15	37	13	0.0 ns
16	46	17	0.1 ns	16	48	17	0.0 ns
17	37	14	0.2 ns	17	36	13	0.1 ns
18	37	16	0.8 ns	18	59	22	0.2 ns
19	32	14	0.7 ns	19	33	13	0.3 ns
20	34	15	0.8 ns	20	39	16	0.5 ns
21	40	18	1.1 ns	21	74	32	1.5 ns
22	34	16	1.3 ns	22	34	19	3.3 ns
23	31	15	1.4 ns				
Total	935	312	0.0 ns		892	297	0.0 ns

[a] χ^2 test. ns, Not significant; * $P \leq 0.05$

marked response is comparable to the response expected when selection is made at the diploid level in favor of a recessive allele controlling a simply inherited qualitative trait. In this latter case, in fact, the favored allele could be fixed in only one generation, providing an efficient method for genotype identification is used (Hallauer and Miranda 1988). The very high effectiveness of pollen genotype selection for a monogenic qualitative trait was also revealed in tobacco by Touraev et al. (1995) who used a transgenic reconstruction approach and obtained fixation of the resistant allele after *in vitro* pollen maturation. In the present study, another factor could have contributed to the very high response to selection: the BC lines chosen for our experiment were both expected to produce pollen populations non-segregating for more than 98% of loci, on the average. This could have permitted a clear discrimination of pollen grains carrying the *XA17* allele by minimizing variability for the quantitatively inherited factors altering tolerance to the herbicide or controlling pollen vigor *per se* (Ottaviano et al. 1980, 1988; Landi et al. 1989).

The results presented herein indicate that pollen genotype selection was able to favor those pollen grains expressing the *XA17* allele for resistance to chlorsulfuron in two different genetic backgrounds. Effectiveness of selection was extremely high in both backgrounds, thus emphasizing the potential of this method, especially in the case of a simply inherited trait. Selection applied during microspore development *in vivo* can be utilized for selecting among pollen grains produced by the same plant. It could permit the rapid transfer of an allele conferring resistance into elite germplasm, by subjecting heterozygous plants to pollen genotype selection during the backcrossing procedure. Pollen genotype selection could also be used for selecting among pollen grains produced by different plants, for example by applying selection during *in vitro* pollen maturation (Touraev et al. 1995) or during *in vivo* pollen germination and tube growth (Sari Gorla et al. 1989; Frascaroli and Landi 1996). The feasibility of these latter approaches has not been widely investigated so far, but they seem promising for rescuing rare alleles of interest present in a bulk of pollen from different sources.

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