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Progeny tests of barley, wheat, and potato regenerated from cell cultures after in vitro selection for disease resistance

G. Wenzel and B. Foroughi-Wehr
Federal Biological Research Center for Agriculture and Forestry, Institute for Resistance Genetics, D-8059 Grünbach, FRG

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Summary. Because plant cells cultured in vitro express genetic variability and since they can be regenerated into functional plants, procedures have been designed to use this system for the production of plants with new important agronomic characteristics, particularly for disease resistance. For barley, wheat, and potato somaclones have been found that were less susceptible to a toxin of *Helminthosporium*, fusaric acid, *Fusarium coeruleum*, *F. sulphureum*, or *Phytophthora infestans*, when screened in the first in-vitro-derived generation. Here the progeny of such somaclones is evaluated after natural and artificial infection, using greenhouse-grown or field material. The progenies of the same somaclones did not express detectable differences, which indicated that no heterozygous mutations occurred. Most lines and clones differed in their level of susceptibility to the pathogen compared to the level of the starting material, but these data were in no instance significant. It is discussed here whether this lack of significance is due to a lack of genetic differences or whether the test procedures are in adequate for detecting and securing the slight, probably quantitative, alterations.

Key words: *Helminthosporium – Fusarium – Phytophthora – Hordeum vulgare – Triticum aestivum – Solanum tuberosum*

Introduction

There has been a controversy for the last 10 years over the use of spontaneous or induced variability of cells cultured in vitro, i.e., somaclonal variation. Either cell culture was judged as a new but not very useful mutagen (e.g., Hoffmann et al. 1982) or it was viewed as a useful technique for intracultivar improvement (e.g., Larkin and Scowcroft 1981). In the latter case, it was assumed that the genetic changes appearing during in vitro culture are stable and are expressed sexually or are at least vegetatively transmitted in plants regenerated from de- and redifferentiated cells. Some early encouraging findings are summarized in several reviews (Shepard 1981; Wenzel 1985; Evans and Sharp 1986). The reasons for the changes are not at all understood, but under applied aspects such an understanding might not be a prerequisite for using the procedure. It soon became obvious that somaclonal variation should start from single cells to prevent the formation of chimaeras, and should rely on a powerful selection system to make the somaclonal variation superior to classical mutation breeding, if its of any use at all. Of particular interest are selection systems, applicable in vitro, to enrich the population carrying the desired genotype. Plants regenerated from the selected fraction can then be further evaluated under field conditions.

In an in vitro screening system with different lines of barley or wheat and heterozygous clones of potato, cells from suspension cultures and/or protoplasts were screened in vitro, and selected soma- or protoclones that showed less susceptibility to toxic fractions of *Helminthosporium*, *Fusarium*, or *Phytophthora* were regenerated (Wenzel et al. 1988). In the case of barley, besides the toxin selection, fusaric acid was applied as the selective agent. Significant levels of the tolerance and resistance of regenerated plants from the first in-vitro-derived generation have been reported for the systems followed up here: barley and wheat (Chawla and Wenzel 1987a, b), potato (Wenzel et al. 1987), but also from a number of other groups working with different host/pathogen combinations (e.g., Sacristan 1985; Buiatti 1989). This paper gives the results of the sexual or vegetative progeny genera-

tions of the tolerant plants under field conditions with artificial and natural infection pressure by the corresponding diseases over a 2- to 3-year period.

Material and methods

Barley

For barley, tolerant or resistant regenerants from embryogenic suspension cultures of the spring variety Dissa were used after selfing in two subsequent generations. During regeneration, either 0.8 m*M* fusaric acid or 100 units of a standardized extract from *Helminthosporium sativum* were used as a selective agent (Chawla and Wenzel 1987a, b). As a result of the screening experiments, 18 lines were obtained that were not susceptible to *H. sativum*, and 14 clones of a total of 8 lines (I–VIII) were not affected by the fusaric acid.

The 18 *Helminthosporium*-tolerant lines were selfed and the progeny was analyzed with the same selection systems under greenhouse conditions as the original regenerated lines. For the test, five plants from each selfed progeny were taken randomly and inoculated in the four- to five-leaf stage with a suspension of *H. sativum* spores (concentration 5×10^5 spores/ml). The inoculation was set at two different leaves (Chawla and Wenzel 1987a). The lesion size was measured in square millimeters 4 days after inoculation. The infected leaves were removed and after a growth period of 4 weeks the inoculation was repeated on the same plants.

For testing the resistance of lines selected against fusaric acid, leaves of three randomly selected plants from each progeny were cut and placed in glass vials containing 4 ml of either 0.6 or 0.8 m*M* fusaric acid (Chawla and Wenzel 1987b). The reaction to the toxin was recorded over a 7-day period by yellowing and wilting of the leaves. For scoring, a scale of 0 to 3 was used, equivalent to none, slight, medium, and heavy effects. From the three leaves tested, the scores were added each time and recorded in Table 1. This test was repeated twice with greenhouse-grown leaves (gg); in one repetition the leaves were taken from field-grown material (f). Unselected lines were used as control. As a total score, the average of all data from the two greenhouse-grown replicates, from all field-grown leaves or from all these data (total score), is given.

The variety Dissa is susceptible to *H. sativum* although *Helminthosporium* is not a major disease. Dissa shows an average reaction against fusaric acid compared to other spring barley varieties.

Wheat

From embryogenic suspension cultures of the spring wheat varieties Atys and Pitic 62, a total of six lines (three from each variety) have been regenerated under selective concentrations of *H. sativum* extracts (Chawla and Wenzel 1987a). From three lines, up to three somaclones were used. These lines were selfed and the progeny was tested under greenhouse conditions, using *Helminthosporium* spores as artificial inoculum as described in barley.

Pitic is about twice as resistant to the artificial infection with *H. sativum* spores as Atys.

Potato

Potato protoplasts of the two dihaploid clones H 78.551/1 (=M 3) and H 75.1207/7 (=M 4) were regenerated in the presence of selective concentrations of extracts from *Fusarium sulphureum* or *F. coeruleum*. Both clones were rather resistant against *F. coeruleum* and medium resistant to *F. sulphureum*. The clones were chosen because they had the best regeneration capacity at the time these experiments were started. After regeneration of the surviving calli, a significantly higher number of protoclones proved not to be affected by the toxin in secondary callus or in a leaf test, compared to regenerated protoclones that had developed without selection pressure (Wenzel et al. 1987). From some of the selected protoclones, tubers could be harvest-

Table 1. Reaction of selfed progenies from somaclones of barley selected in vitro with different concentrations of fusaric acid. Leaves were taken from greenhouse-grown plants (gg) or from the field (f) (for further explanation see 'Material and methods')

Fusaric acid m*M*			Chlorophyll defects			Wilting			Score	
Line	Clone	Replicate	0	0.6	0.8	0	0.6	0.8	gg f	Total
Dissa	1	gg 1	4	5	4	0	0	3	3	2
	1	gg 2	0	6	5	0	0	4		
	1	f	1	1	2	0	0	0	1	
I	1	gg 1	3	3	8	0	5	9	5	5
	1	gg 2	0	7	9	0	4	9		
	1	f	2	9	9	–	–	3	6	
I	2	gg 1	1	3	9	0	3	9	4	4
	2	gg 2	0	6	7	0	3	6		
	2	f	5	7	9	0	0	3	4	
II	1	gg 1	0	1	2	0	2	2	2	2
	1	gg 2	0	6	3	0	1	4		
III	1	gg 1	0	0	3	0	0	0	2	2
	1	gg 2	0	7	9	0	4	6		
	1	f	0	6	8	0	0	1	2	
III	2	gg 1	0	3	4	0	5	3	3	3
	2	gg 2	0	5	7	0	0	6		
	2	f	2	4	7	0	0	0	2	
IV	1	gg 1	0	2	5	0	3	6	2	2
	1	gg 2	0	0	2	0	0	0		
	1	f	2	6	5	0	3	0	3	
V	1	gg 1	1	1	1	0	3	3	3	3
	1	gg 2	1	5	7	0	3	7		
	1	f	7	9	9	0	0	0	4	
VI	1	gg 1	0	4	9	0	3	9	3	3
	1	gg 2	0	4	2	0	2	0		
	1	f	3	9	8	0	0	0	3	
VII	1	gg 1	2	3	7	0	4	9	4	4
	1	gg 2	0	6	6	0	3	4		
VII	2	gg 1	0	0	1	0	0	3	2	2
	2	gg 2	0	1	7	0	3	4		
	2	f	6	9	9	0	0	0	4	
VII	3	gg 1	0	2	5	0	2	3	2	2
	3	gg 2	2	3	7	0	0	5		
VII	4	gg 1	2	3	3	0	2	6	2	3
	4	gg 2	0	2	4	0	0	4		
	4	f	7	9	7	0	0	0	4	
VII	5	gg 1	1	1	5	0	6	9	3	3
	5	gg 2	4	7	6	0	1	1		
VIII	1	gg 1	6	6	6	0	9	5	6	5
	1	gg 2	4	9	9	0	9	9		
	1	f	1	8	9	0	0	0	3	

ed and the next vegetative field-grown generations were produced. Half of the selected protoclones died before tuber production because of heavy virus infection.

Uniform tubers from 17 protoclones of the *Fusarium* selection experiment were subjected to the *Fusarium* test described by Langerfeld (1979), using artificial infection with both *F. sulphureum* and *F. coeruleum* inocula of 30 tubers per clone tested. As scores, the infection in percent and the infection index were recorded. The percentage of infection was determined by the formula: $\frac{(TTW-UPT)}{TTW} \times 100$, where TTW is the total tuber weight and UPT is the weight of the uninfected part of the tuber. The infection index is calculated from the product of the depth and width of the infected tuber area divided by 100.

For *Phytophthora* resistance, potatoes were regenerated from protoplasts of the two homozygous dihaploid clones AH 82.4521/6 (=AH1) and AH 84.4568/6 (=AH2). AH1 had a medium general field resistance, while AH2 was highly susceptible. As in the *Fusarium* selection, toxic fractions of culture filtrates of the *Phytophthora* growth medium had been used as a selective agent (Foroughi-Wehr and Stolle 1985). Of 142 protoclones regenerated using *Phytophthora* toxin in protoplast culture, 75 could be transferred into the field. From these, the vegetatively propagated progeny of 15 protoclones were tested for their level of resistance. Besides the evaluation of the natural field infection, two different artificial infections were carried out at the tubers and at the leaves, respectively. For tuber infection, up to 60 tuber halves were inoculated according to Schöber and Höppner (1972). In the leaf test, 100 leaf pieces of the same size of each clone were inoculated with a zoosporangia-suspension as published by Hodgson (1962). The sporulation index was calculated on the mycelium growth on 100 leaf pieces and evaluated using the following formula: $\frac{m1 \times 1 + m2 \times 2 + m3 \times 3}{3}$, where m1, m2, and m3 stand for percent of leaves with either one of the three different types of mycelium growth: slight, medium, or strong, 7 days after infection. The other scores are arbitrary units from 1 (highly resistant) to 9 (highly susceptible).

For statistical evaluation, in all systems the Dunnett-test was predominantly used (Sachs 1972).

Results

Barley

Figure 1 gives the average lesion sizes of up to five lines from the selfed progenies originating from each of the 18 somaclones selected in vitro for *H. sativum* resistance, using embryogenic suspension culturs of the spring variety Dissa. Lines descending from the same somaclone, however, expressed no measurable variability, although this was expected due to the possibly heterozygous nature of the mutation leading to the somaclone formation. But the average lesion size differs from somaclone to somaclone (Fig. 1). Despite the number of repetitions and the repeated inoculation at different times, none of the phenotypically visible differences was significant. Even the three best lines and the four worst lines cannot be significantly distinguished from the average. Thus, we can speak only of a tendency for most somaclones to be more susceptible in the sexual progeny than the donor variety

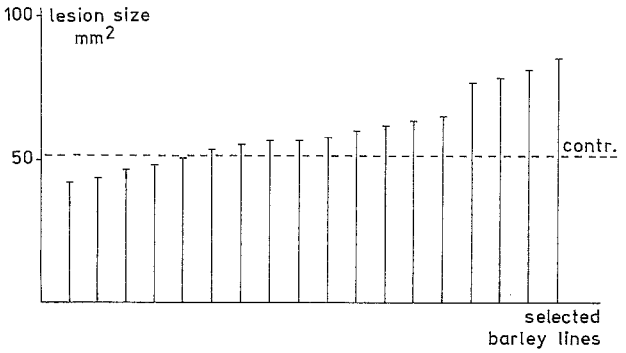

Fig. 1. Reaction of the sexual progeny of in-vitro-selected barley lines to the infection with *Helminthosporium sativum* spores

Dissa. Since the test was identical and the plants were grown under the same semicontrolled greenhouse conditions as the first in-vitro-grown generation, the lack of significance is difficult to explain. Most probably, the in-vitro-grown somaclones tested previously were not uniform enough and their number per somaclone was not large enough to give a reliable result.

In the case of fusaric acid, eight somaclones (I–VIII) originating from suspension cultures of Dissa were selected. The selfed progeny of one somaclone showed a segregation of 50 albino mutants to 189 normal green plants in the seedling stage, which indicated that heterozygous mutations took place. But, as in the *Helminthosporium* experiment, there were no segregations of the resistance behavior observed in descents from the same somaclone, e.g., clones one to five from the original clone VII, indicating no heterozygosity in the locus of interest.

In all progenies from the selfed somaclones, the distinction of the leaf symptoms increased with increasing fusaric acid concentration, as in the very first generation. There was not, however, a high correlation among the tests with leaves taken from greenhouse- or field-grown plants. Leaves from field-grown plants showed no wilting and dried without chlorophyll degeneration or any other symptoms. The greenhouse-grown leaves wilted as expected, but not a single progeny of the selected lines was significantly less wilting in the mean of the three replications than the control plants of Dissa, and even the four drastically susceptible lines (I, 1; I, 2; VII, 1; VIII, 1) were not significantly worse (Table 1). Again, these results show that the early selection based on spontaneous somaclonal variation in vitro does not result in lines that a breeder would identify with the presently used tests as different from the starting material.

Wheat

The three best-growing and fertile somaclones each of Atys and Pitic, regenerated in the presence of selective

Table 2. Infected leaf area in square millimeters on progenies of somaclones from wheat selected in vitro for *Helminthosporium* resistance

Line	Clone	Score	
		Infection	
		1st	2nd
Pitic control	1	27.6	142.9
I	1	27.6	178.3
	2	37.4	110.1
II	1	20.9	157.9
III	1	21.8	140.4
	2	21.6	131.0
	3	33.8	131.6
Atys control	1	40.4	193.4
I	1	24.5	130.9
	2	22.9	166.4
II	1	17.2	107.5
III	1	16.0	122.5

concentrations of *Helminthosporium* toxin from embryogenic suspensions, have been tested in the two subsequent selfed generations. Although some heterozygosity due to somaclonal variation and dominant mutations was expected, in all cases studied in wheat, no significant differences were detected in lines originating from the same somaclone, just as in barley. Thus, in the tests only two to three lines from each progeny were analyzed further. The offspring of Pitic gave the same range of symptoms as the controls. The amount of additional variability was nonsignificant. For Atys, all three selected somaclonal lines looked better than the starting material (Table 2), but this difference could not be proven. As the results demonstrate, it was not possible to improve the rather high level of *Helminthosporium* resistance of Pitic. The absolute numbers indicate that the lower level of resistance of the variety Atys might have been increased. A field test with natural infection pressure should clarify this question but, as shown in potato, the differences that can be identified by normal field infections are so large that the small quantitative differences expected here will probably not be significant.

Potato

In potato, protoplasts of the two dihaploid clones M3 and M4 were screened in vitro with extracted toxins of *Fusarium culmorum* or *Fusarium sulphureum*. After different tests on the first in-vitro-derived generation that showed significantly decreased susceptibility of the selected protoclones, 6 protoclones preselected with *F. sulphureum* and 11 protoclones preselected with *F. coeruleum* were tested with the functional fungi on field-grown tubers. Tables 3 and 4 show the results over 3 years. It becomes evident that in the field-grown generations no significant improvement over the donor material is detectable.

Table 3. Scores of vegetatively propagated potato protoclones, preselected in vitro with toxic extracts from *Fusarium suphureum* after tuber infection with *F. sulphureum* (*F. s.*) or *F. coeruleum* (*F. c.*). Percent infection and infection index are explained in 'Material and methods'

	Infected with *F. s.*			Infected with *F. c.*	
	% Infection		Index	% Infection	Index
Year	87/88	88/89	88/89	88/89	88/89
Clone					
M4 control	34	9	4	4	1
1	–	20	5	23	1
2	–	15	6	4	1
3	19	16	6	0	1
4	10	13	8	8	1
5	13	9	5	23	2
6	11	14	4	0	1

Table 4. Scores (explained under 'Material and methods') of vegetatively propagated potato protoclones, preselected in vitro with toxic extracts from *Fusarium coeruleum* after tuber infection with *F. coeruleum* (*F. c.*) or *F. sulphureum* (*F. s.*)

	Infected with *F. c.*		Infected with *F. s.*		
	% Infection	Index	% Infection		Index
Year	88/89	88/89	87/88	88/89	88/89
Clone					
M3 control	4	1	20	6	4
1	20	3	–	7	3
2	8	1	10	7	3
3	4	1	14	12	4
4	0	1	12	11	4
5	4	1	20	8	3
6	4	1	–	7	3
7	0	1	14	7	4
8	4	1	–	9	4
9	6	1	–	5	2
10	0	1	–	7	3
11	0	1	–	6	3

For *F. coeruleum*, regardless of whether the selection has been performed with *F. sulphureum* or *F. coeruleum*, all tubers from protoclones tested show extreme resistance to the fungus (Table 3). In contrast to the results of the tests with *F. sulphureum* and to the results in wheat and barley, no protoclone became more susceptible.

Field-grown tubers of the protoclones selected with *F. sulphureum* and subsequently screened with the same

Table 5. Results of different tests for the resistance of the vegetative progeny of potato protoclones against *Phytophthora infestans*

Clone	Leaf			Tuber	
	Natural infection		Hodgson test	Artificial infection	
	1987	1989	1989	1987	1988
AH 1 control	5	9	41	3	9
1	8	9	70	7	9
5	7	9	71	6	8
9	7	9	53	6	8
6	6	7	75	6	9
4	5	9	52	7	9
7	7	7	97	7	7
15	7	7	97	5	8
3	5	9	84	5	8
14	7	7	83	5	9
10	6	7	82	8	5
11	6	7	82	9	5
8	5	9	80	7	7
13	7	7	71	3	7
2	3	9	64	5	7
12	5	7	37	4	7
AH 2 control	7	7	69	3	4
26	5	5	–	3	6

fungus did not express improved clones, but in several cases did express strongly susceptible protoclones. After selection of protoplasts with *F. coeruleum*, the number of clones that were less susceptible than the controls was visually improved when infected with *F. sulphureum* (Table 4). Due to the strong year-to-year differences and the limited number of repetitions, no result could be seen to be made significant.

Sixty protoclones from two different donor dihaploids (AH 1 and AH 2) were field-tested for *Phytophthora* resistance in 1987 and from these, 23 clones were again tested in 1988 and 1989. Over the 3 years, rather different natural infection pressure was expressed: in 1987 there was medium pressure for leaf and tuber blight; in 1988 we observed hardly any leaf infections but heavy tuber infections, resulting in nearly 30% clones with completely rotted tubers. The correlation coefficient between leaf and tuber blight was completely insignificant. Finally, in 1989 we had such a very heavy leaf blight infection that the plants died before the tubers were infected. Again, there was no correlation between leaf and tuber blight.

Most 2nd- and 3rd-year tests were made only with clone AH 1, since many protoclones of AH 2 expressed a very strong tuber blight, although the leaves were more resistant than the donor clone. Under the very strong natural infection pressure of 1989, one protoclone of AH 2 was clearly more resistant than the original material. For protoclones of AH 1, no clone was better in leaf blight than the starting material, however, several clones expressed rather a higher susceptibility (Table 5). Only in one protoclone was the sporulation index a bit lower (37) than in the donor material, although here the index of 41 was an average one. The results for tuber blight were so variable from year to year that we could not measure with the commonly used test the slight quantitative differences expected from the protoclonal variation.

It must be stressed that the official tests for resistance require 100 leaves for the *Phytophthora* test and 30 tubers for the *Fusarium* test, in order to attain a significant answer. Such material must be as uniform as possible, a prerequisite that is normally not fulfilled when the organs are taken from cultures started in vitro, which give rise to plants over a longer time period. Consequently, the first test of the in-vitro-grown plants is often not reliable because this material is not optimal. The high number of replicates needed for a significant answer, even with uniform field-grown material, indicates the weakness of the present tests. Thus, it is not possible to give a final answer to the question of whether the non-significant data are due to insufficient sharpness of the test procedure or to a lack of genetic diversity. To answer this question, more sensitive and more reproducible tests are needed. At present it cannot be concluded that the low correspondence of the field and the in vitro results is caused by a basically nonfunctioning in vitro selection, but is rather a consequence of test systems that are too imprecise, both in vivo and in vitro.

Discussion

The results of 2–3 years of progeny tests using natural or artificial infection on plants, preselected in vitro with embryogenic suspension cultures or protoplasts, produced phenotypic differences in the level of resistance in both negative and positive directions. None of the data, however, was significantly different from the controls. This is not in agreement with the test of the first in-vitro-derived generation, which produced significantly improved somaclones in our previous experiments (Chawla and Wenzel 1987a, b; Wenzel et al. 1987). Due to the selection approach, nonmutated and negative variants were discarded. Thus, the appearance of so many bad variants, although not significant, is not in good agreement with the results obtained on the first greenhouse-grown regenerants. The disagreement between both tests was not of the same magnitude in all cases; the rather artificial system of screening for *Fusarium* resistance using fusaric acid in barley resulted in the lowest conformity, while the *Helminthosporium* toxin selection and the *Fusarium* test on potato resulted in a better one. The improved AH 2 protoclone of potato with *Phythophthora* resistance is a clear indication that more resistant clones

can be obtained after in vitro selection. In the experimental series reported here, however, it is, an exception.

For the first screening, it is definitely important to use selective agents that are essentially correlated to the infection process as, e.g., the toxin Sirodesmin PL in the case of *Phoma lingam* selection in *Brassica* (Sjödin and Glimelius 1989 a). It should further be known whether the screening system can detect between quantitative or only between qualitative changes. For the latter case specificity is needed, while in the first case even morphological changes or mutants of the transport system will express an altered in vitro behavior that can be detected only with rather sensitive tests. The first screening normally done on in-vitro-derived greenhouse-grown plant material deals with rather small numbers and requires a laborious test procedure that might even identify the small quantitative differences. Work with field-grown plants in larger numbers can only use less sophisticated tests and thus does not differentiate slight changes. Such slight improvements are, however, of importance although the breeder can only make use of them after further combination breeding programs.

Whether the second test was identical with the first screening system, as in all cereal experiments, or whether it was a real field test did not have any influence on its reproducibility. In potato, both systems were compared but none gave any significant answers. Particularly for *Phytophthora* the annual differences were so strong that it was impossible to use the replications for a significant differentiation of protoclones. On the other hand, breeders can distinguish genotypic differences in segregating populations with the present test, and this not only for qualitatively inherited traits but also for quantitative ones. This means that the genotypic differences following in vitro mutagenesis, even if they are real, are smaller than the differences used by the breeder after meiotic segregation. The strong influence of the environment points in the direction of a quantitative nature of somaclonal variation; in breeding for resistances, such a quantitative situation is often preferred, since a more durable resistance is expected. As in classical combination breeding programs, however, this type of resistance demands a scaling up, until by combining several small resistances a level is achieved that can even be detected by the rough field tests presently used.

Although it has been proven that in vitro selection for fungal resistance works on the in vitro level, the correlation to subsequent field tests was, in all cases tested here, unreliable. This is probably not only due to the rather artificial in vitro screening and the not-too-sensitive field tests that detect only differences greater than differences coming from average somaclonal variation, but also to insignificant genetic changes, since even the direction of the response was often not in agreement with the first scoring.

For the host/pathogen combinations and the screening procedures used, it can be said that with an acceptable input, somaclonal variation will hardly result in significantly improved plant material. One actually has to know more about the actions of the pathogen or the reactions of the infected plant, to develop precise strategies enabling the detection of useful new variants. Most of these drawbacks apply as well if the variability is not a mutagenic one but is a consequence of a planned combination breeding program where, e.g., the segregating microspores of an F_1 plant or somatic hybrids can be used for the in vitro selection. As the genetic complex coding for the resistance can be incorporated in such combinations, the necessary variability can be directed and the chances of finding the desired new genotype even with a simple screening are much better, as demonstrated by Ye et al. (1987) for salt resistance in microspore-derived plants and by Sjödin and Glimelius (1989 b) for *Phoma* resistance in somatic *Brassica* hybrids.

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