Theor Appl Genet (1984) 67: 377–382

Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture

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Received September 23, 1983
Communicated by G. Melchers

Summary. In a winter barley breeding program for barley yellow mosaic virus (BaYMV) resistance, the resistant six-rowed cv. Franka was crossed to 17 susceptible and two resistant cultivars, three of which were two-rowed. A total of 233,445 anthers of the 19 hybrids and their parents were cultured and 831 green plants regenerated. Anther culture responsiveness varied greatly between genotypes, and the responsiveness of F_1-hybrids was generally related to that of the more responsive (high) parent. On average, 3.6 green plants were recovered from 1,000 cultured anthers, almost twice as many as in comparable spring barley experiments. Androgenetic green plants were tested for their reaction to mechanical inoculation of BaYMV. In crosses of resistant parents, all the cross progeny proved to be resistant, which indicates that both parents carry identical gene(s). In the crosses of the resistant cv. Franka to susceptible parents, an average of 62% of the androgenetic progenies were resistant, which indicates that probably more than one gene is responsible for Franka's BaYMV-resistance. From the crosses of Franka to two-rowed cultivars, 282 androgenetic plants were produced. When 132 of these were tested for their reaction to BaYMV, 79 (59.8%) were resistant, and 30 of the latter were shown to be two-rowed recombinant lines. Doubled haploid lines are field-tested for other agronomic characters including grain yield and its components.

Key words: *Hordeum vulgare* – Barley yellow mosaic virus-resistance – Androgenetic responsiveness – Doubled haploids – Breeding

Introduction

Due to its high yield and earlines, winter barley is increasingly cultivated in Europe, e.g. in West Germany the cultivation area increased from 689,000 ha in 1975 to 1,299,000 ha in 1981. Correspondingly, increasing efforts of breeding are needed, especially for improving disease resistances. Recently, it became obvious that large areas of winter barley growing are infested by soil-borne barley yellow mosaic virus (BaYMV) in Germany (Huth 1981) and England (Hill and Evans 1980). Most of our present varieties, especially the highest yielding ones like Gerbel (six-rowed) and Igri (two-rowed) are highly susceptible to this virus (Huth 1982). New, resistant and high-yielding breeding lines are therefore urgently needed.

It had been demonstrated in an earlier program with spring barley hybrids (Foroughi-Wehr et al. 1982), that anther-culture can be efficiently used for producing doubled haploid lines, besides the "bulbosum-technique" (see e.g. Simpson and Snape 1981). It was subsequently tried to apply the anther culture technique in a winter barley breeding program for BaYMV resistance. The time required for establishing resistant lines could be substantially reduced by applying the recently developed test of mechanical inoculation of BaYMV (Friedt 1983).

Materials and methods

The following winter barley (*Hordeum vulgare* L.) cultivars were used as anther donors: (a) BaYMV-resistant/six-rowed: Barbo, Birgit, Franka, (b) BaYMV-susceptible/six-rowed: Arma, Bosquet, Corona, Freya, Gerbel, Hasso, Largo, Leuta, LP 1.676 (Illia), LP P.61444, Mammut, Marko, Robur, Thibaut, (c) BaYMV-susceptible/two-rowed: Alpha, Igri, LP 8.34218. Additionally, anthers of the 19 F_1-hybrids of Franka were cultured. All seeds were kindly provided by Dr. E. Rehse, F. v. Lochow-Petkus GmbH, D-3410 Northeim 13 (Wetze).

Because of technical reasons, the donor materials had to be grouped into three experiments, where each one third of the hybrids and their corresponding parents were cultured during subsequent seasons in 1982, i.e. February-June, April-July and August-December. Within each experiment, donor plants were treated identically, so that their anther culture responsiveness could be compared, cleared up for environmentally induced variation.

For vernalization, seeds were sown into sterilized sand in glass-petridishes (12 cm high), where they were kept for four weeks at 3–4 °C and 10 h light (4,000 lux). Afterwards, seedlings were transferred to soil into pots and grown in a growth cabinet at 10–12 °C (10/14 h night/day) and 18–20,000 lux light intensity. After tillering, these plants were transferred to a greenhouse where they were grown under natural conditions, i.e. ca. 16 °C and 16 h light (min 10–12,000 lux) during winter and ca. 20–30 °C and natural light during summer. Anthers were collected for culture in the uninucleate stage of microspores, and were subsequently cultured as principally described for spring barley by Foroughi-Wehr et al. (1976). Opposite to earlier experiments, where Erlenmeyer-flasks were used, most of the cultures were carried on in glass petridishes (50 mm diameter) on 10 ml of solid medium.

Green plantlets emerging from calluses were transferred to a Murashige and Skoog medium supplemented by 5 $g \cdot l^{-1}$ activated charcoal to promote root development. After these plantlets had developed 2–3 leaves, they were vernalized in vitro at 5 °C and 10 h daylength (2,800–3,000 lux) for 6 weeks. Subsequently, the green plants were removed from the agar medium and potted into soil and grown in a growth chamber or greenhouse until maturity.

For chromosome number determinations, root tips of vigorously growing potted plants were pretreated in 1-bromonaphthalene for 5 h, fixed in alcohol-acetic acid (3–1) and stained by the standard Feulgen technique.

Tests for BaYMV resistance were performed by mechanical inoculation. For inoculum preparation, plants of the susceptible winter barley cv. Gerbel were grown in pots in the greenhouse in BaYMV-infested soil collected at Sunstedt east of Braunschweig. When these plants were kept under temperatures below 15 °C, some of them showed BaYMV-symptoms and were subsequently used for extraction of plant sap which was diluted in 0.1 M K_2HPO_4-buffer (1:2 w:w) (Friedt 1983). Inoculations were carried on, when androgenetic green plants had developed 2–3 tillers with 3–5 leaves each. The two youngest, fully emerged leaves of each tiller were rubbed off with the inoculation fluid by a sponge as described in detail by Friedt (1983). BaYMV symptoms usually became visible 3–4 weeks after inoculation.

Most of the androgenetic plants had spontaneously doubled or multiplied chromosome numbers (Table 4), and they were all grown to maturity. Haploid plants were doubled by colchicine (root treatment: 0.1% colchicine + 2.0% DMSO for 4 h). Mature plants were evaluated for their morphological characters, especially of the spike.

Results

A total of 233,445 anthers were cultured, 159,366 of the F_1-hybrids and 74,079 of their parents, respectively (Table 1). Altogether 1,525 albino and 831 green plantlets were regenerated. Of the latter, 335 were derived from the parents and 496 from the hybrids. On average, 3.6 green individuals were recovered from 1,000 cultured anthers. The average ratio of green to albino regenerated plants was 1:1.8.

Since not all of the donor plants could be grown at the same time, they were grouped into three experiments. Androgenetic responsiveness in these three groups cultured in different seasons was different. Especially in experiment 2, cultured during summer 1982,

Table 1. Overall results of winter barley anther culture, experiments 1 to 3

Materials	No. of anthers cultured	No. of calluses	No. of plants		(‰)[a]
			Total	Green	
Parents (20)	74,079	2,118	909	335	(4.5)
Hybrids (19)	159,366	3,946	1,447	496	(3.1)
Totals	233,445	6,064	2,356	831	(3.6)

[a] Frequency of green regenerants per 1,000 anthers

Table 2. Results of anther culture of winter barley hybrids and parents

Material	No. of anthers cultured	No. of calluses	No. of plants		(‰)[a]
			Total	Green	
Period 1 (cultured from February to June 1982, exp. 1)					
Parents					
Franka (F)	3,357	32	4	2	0.6
Arma	3,360	65	12	3	0.9
Bosquet	2,538	80	9	0	0
Corona	1,047	53	39	11	10.5
Freya	1,680	18	6	0	0
Robur	2,115	45	26	8	3.8
Thibaut	1,830	90	50	6	3.3
Hybrids					
F × Arma	7,113	281	88	24	3.4
F × Bosquet	2,880	65	18	8	2.8
F × Corona	4,938	87	25	5	1.0
F × Freya	6,309	93	16	7	1.1
F × Robur	6,825	312	111	22	3.2
F × Thibaut	2,724	223	126	51	18.7
Period 3 (cultured from August to December 1982, exp. 3)					
Parents					
Franka (F)	4,488	61	4	3	0.7
Alpha	6,606	53	4	2	0.3
Barbo	4,629	135	67	32	6.9
Birgit	2,870	19	7	3	1.0
Igri	9,138	999	512	220	24.1
Leuta	3,049	36	9	1	0.3
LP 8.34218	7,614	179	97	15	2.0
Hybrids					
F × Alpha	24,747	275	50	15	0.6
Barbo × F	17,151	249	97	37	2.2
Birgit × F	14,535	94	21	14	1.0
F × Igri	12,807	939	423	175	13.7
Leuta × F	8,259	101	19	8	1.0
F × LP 8.34218	29,280	945	375	92	3.1

[a] Frequency of green plants per 1,000 cultured anthers

responsiveness was comparatively low. This is mainly traced back to unfavourably high temperatures during high summer. In experiments 1 and 3, callus formation averaged about 3%, whereas albino and green plant frequencies were below 1% (Table 2). However, there were large differences between genotypes in their anther culture responsiveness. As an example, the results of experiment 3 are presented graphically in Fig. 1. The common parent cv. Franka showed a low callus formation rate and a particularly low plant regeneration. Other parents, like cvs. Alpha, Birgit and Leuta exhibited a similarly low level of responsiveness as Franka, and the corresponding three hybrids behaved very much alike their parents (Fig. 1). On the contrary, cvs. Barbo, LP 8.34218, and especially cv. Igri were much more responsive and were in this respect similar to their respective F_1-hybrids (Fig. 1). As a rule, the responsiveness of F_1-hybrids was closely related to that of the higher parents (HP), as demonstrated e.g. for plant regeneration rate in Table 3, where F_1- and HP-responsiveness show a highly significant positive correlation (r = +0,69). On the contrary, the responsiveness of F_1-plants was not significantly related to that of their lower parents (LP) (Table 3). The comparatively close relationship between F_1- and mid-parent (MP) responsiveness, is due to the fact, that the latter is more similar to the HP- than to the LP- value.

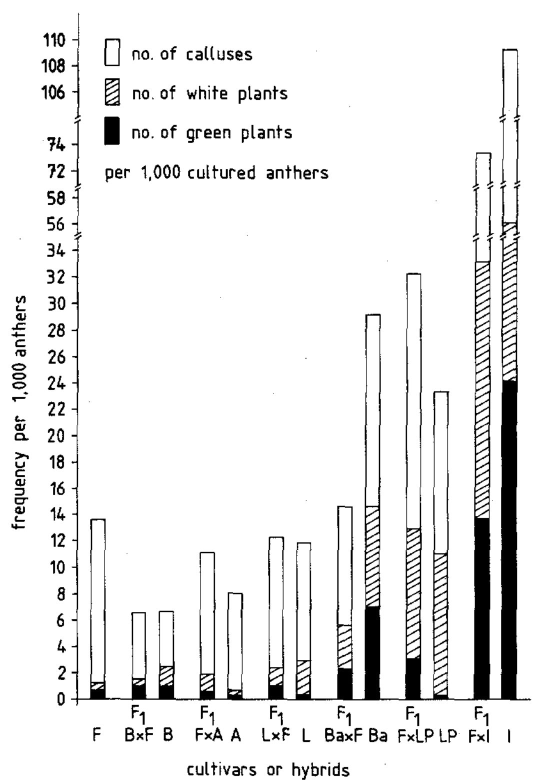

Fig. 1. Androgenetic responsiveness of six representative winter barley F_1-hybrids and their parents. *F* = Franka (common BaYMV-resistant parent), *B* = Birgit, *A* = Alpha, *L* = Leuta, *Ba* = Barbo, *LP* = LP 8.34218, *I* = Igri

Table 3. Relationship between plant regeneration frequencies of winter barley F_1-hybrids and their parents

Hybrid (F = Franka)	Total plant regeneration per 1,000 anthers				
	F_1	Mid-parent mean (MP)	Low parent (LP)	High parent (HP)	F/M[a]
F × Gerbel	0	0.55	0	1.1	F
F × Mammut	0	1.55	1.1	2.0	M
F × Largo	0.3	1.25	1.1	1.4	M
Birgit × F	0.6	1.80	1.2	2.4	F
F × Alpha	2.0	0.90	0.6	1.2	F
Leuta × F	2.3	2.05	1.2	2.9	F
F × Marko	2.8	1.65	1.1	2.1	M
F × LP.P61444	3.0	2.20	1.1	3.3	M
F × Freya	3.4	2.40	1.2	3.6	M
F × Hasso	3.7	0.75	0.4	1.1	F
F × Corona	5.1	19.20	1.2	37.2	M
Barbo × F	5.7	7.85	1.2	14.5	F
F × Bosquet	6.3	2.35	1.2	3.5	M
F × LP1.676	9.4	4.65	1.1	8.2	M
F × Arma	12.5	2.40	1.2	3.6	M
F × LP8.34818	12.9	7.00	1.2	12.8	M
F × Robur	16.4	6.75	1.2	12.3	M
F × Igri	33.1	28.65	1.2	56.1	M
F × Thibaut	47.0	14.25	1.2	27.3	M
Correlations (r)		MP/F_1	LP/F_1	HP/F_1	
Calluses		+0.73***	+0.46*	+0.73***	
Total plants		+0.67**	+0.29[ns]	+0.69**	
Green plants		+0.55*	+0.44[ns]	+0.54*	

[a] F or M indicates, if HP was female or male parent of a hybrid

[ns], *, **, *** Not significant, and significant at P = 0.05, 0.01, and 0.001, respectively

Chromosome number determinations in the androgenetic (A_1) plants revealed, that the majority of plants was spontaneously diploid, i.e. doubled haploid (68.9%, Table 4). Another 10.6% were still haploid, so that altogether about 80% are useful for practical breeding purposes. The remainder were predominantly triploids and aneuploids, mainly trisomics ($2n = 2x + 1 = 15$) (Table 4).

Androgenetic green plants were submitted to laboratory tests for resistance to barley yellow mosaic virus (BaYMV) by mechanical inoculation (Friedt 1983). Up till now, a total of 198 plants could be tested, of which 130 (i.e. 65.7%) proved to be resistant. Among 179 progenies of Franka crossed to susceptible parents, 111 (62.0%) did not show BaYMV-symptoms after inoculation, whereas all of the doubled haploids derived from crosses of Franka to resistant parents were resistant (Table 5).

Three of the hybrids included one two-rowed parent each. In these cases, six-rowed and two-rowed doubled haploid lines were expected in about equal proportions. Actually, 30 (38%) of the resistant and 14 (26%) of the susceptible lines tested up till now were two-rowed. The former are recombinants which combine BaYMV-resistance from the six-rowed parent Franka with the two-row spike of the original two-rowed parents (Table 6).

Table 4. Chromosome numbers of androgenetic plants of winter barley (*Hordeum vulgare* L.)

Chromosome no.	No. of plants	% plants
2n = x = 7	73	10.6
2n = 2x = 14	475	68.9
2n = 2x + 1 = 15	7	1.0
2n = 3x = 21[a]	7	1.0
2n = 4x = 28[b]	125	18.1
2n = 4x + 1 = 29	3	0.4
Total	690	100.0

[a] Including 1 plant with 22 chromosomes
[b] Including 1 plant with 27 + 1 fragment chromosome

Table 5. Reaction to BaYMV infection of androgenetic winter barley lines

Type of hybrid (F = Franka)	Expected proportion of resistant lines		Reaction to BaYMV-inoculation	
	1 gene	2 compl. genes	No. of tested plants	No. of resistant plants
F × resistant parents (2)[a]	100%	–	19	19 (100%)[b]
F × susceptible parents (10)	50%	75%	179	111 (62%)
Total			198	130

In parentheses: [a] no. of hybrids, [b] percentage of resistant plants

Discussion

A total of 2,356 androgenetic plants were regenerated from 233,445 anthers collected from 19 winter barley hybrids and their 20 parents (Table 1). Therewith, an average of 10 plants were recovered from 1,000 cultured anthers, which is slightly less than in an earlier similar program with 53 spring barley hybrids, where an average of 13 plants had been regenerated per 1,000 anthers (Foroughi-Wehr et al. 1982). However, the frequency of green, i.e. practically useful plants was only 2 per 1,000 anthers in the earlier but 3.6 per 1,000 in the present program. Correspondingly, the proportions of albino to green plants were 5.5:1 in the earlier and 1.8:1 in the present work.

It could be demonstrated by Foroughi-Wehr et al. (1982) that green and albino plant formations are independent features of anther culture responsiveness. The present winter barley material seems therefore to be characterized by an increased capacity for green instead of albino plant regeneration as compared to the earlier spring barley.

Genotypically variable responsiveness was observed in the present study, similar to the genotypic effects described for spring barley by Foroughi-Wehr et al. (1982). As all the cross parents were also tested in the present program it is possible to compare hybrids and their parents over a wide range of genotypes.

The significant correlation between F_1- and HP-responsiveness (Table 4) means, that the higher anther culture responsiveness is inherited and is usually either dominant or partially dominant over non-responsiveness. This interpretation is evident at least for the hybrids Franka × Igri and Barbo × Franka (Fig. 1), where plant regeneration frequencies of the hybrids are intermediate to those of the parents. In other crosses, e.g. Franka × Arma and Franka × Thibaut, a kind of hybrid vigour is observed in the F_1-hybrids, particularly regarding green plant yield (Table 3).

Table 6. Proportions of different phenotypes derived from anther culture of F_1-hybrids of the six-rowed, BaYMV-resistant cv. Franka and three two-rowed, susceptible parents

Hybrid	No. of androgenetic lines						
	Total	Resistant			Susceptible		
		Total	6-row	2-row	Total	6-row	2-row
Franka × Alpha	13	6	5	1	7	6	1
Franka × Igri	91	53	33	20	38	27	11
Franka × LP8.34218	28	20	11	9	8	6	2
Total	132	79	49	**30**	53	**39**	14
%	100.0	59.8	37.1	22.7	40.2	29.6	10.6

Note: bold faced figures indicate recombinant lines

In the present investigation no reciprocal crosses were included. Nevertheless, the data seem to indicate, that strong maternal effects on anther culture responsiveness do not exist. Even the most responsive hybrids were derived from crosses with HP as the male parent (Table 3). This interpretation is in agreement with the findings of Foroughi-Wehr et al. (1982), which demonstrated that slight, but not substantial reciprocal effects may be found only in certain cross combinations. Likewise, in wheat (*Triticum aestivum* L.) no differences were detected between the reciprocals of three crosses (Bullock et al. 1982). It can be concluded therefore, that the transfer of androgenetic responsiveness to F_1-hybrids of wheat, barley and probably other cereals does not depend on the source of cytoplasm. It rather depends on nuclear genes, which act either (partly) dominantly or additively and are therefore substantially expressed in F_1-hybrids of barley (Foroughi-Wehr et al. 1982) and wheat (Bullock et al. 1982), as well as in corn (*Zea mays,* Sheridan 1982).

Chromosome number variation among androgenetic green plantlets was very similar to that reported for spring barley (Foroughi-Wehr et al. 1982) and rye (*Secale cereale,* Friedt et al. 1983b). About 80% or more are useful in a practical breeding program, and this rate could even be further increased, if calluses were transferred to regeneration media as soon as possible, as indicated by corresponding experiments in rice (*Oryza sativa* L.). Therewith, the probability of spontaneous doubling of chromosome number is reduced, so that a higher rate of haploid or homozygous diploid (doubled haploid) plants is recovered. In this way, Chen et al. (1982) obtained 47.7% haploid, 46.5% diploid and only 5.7% polyploid progeny. In this experiment, no trisomic individuals were regenerated, whereas in our present work 1% trisomics were found (Table 4). Such trisomics can be helpful for genetic analyses.

The present breeding program had been particularly drafted for BaYMV resistance. All of the hybrids include the six-rowed cv. Franka as a resistant parent, and three of them have another resistant variety as a second parent. The complete BaYMV-resistance of these cultivars is inherited recessively and is probably determined by one or two major genes (Friedt et al. 1983a; Friedt, unpublished). Furthermore, resistances of the parents Franka, Birgit and Barbo are obviously at least partly genetically identical (Friedt, in preparation). Under these preconditions, 100% resistant doubled haploids are expected in the crosses of Barbo and Birgit to Franka. In the other crosses, which only include Franka as a resistant parent, 50% or 75% resistant lines are expected, if either one or two resistance genes are assumed, respectively. In fact, no one susceptible progeny has been found in the former group till now (Table 5). In the second group, 111 of 179 lines tested, i.e. 62%, are resistant to BaYMV. In respective anther culture experiments of hybrid rice (*Oryza sativa* L.) heterozygous for suitable marker genes, it was demonstrated that the observed segregation fit the expected 1:1 gametic segregation ratio (Chen et al. 1982). From our own data, it can be tentatively concluded that either one or two resistance genes determine the complete BaYMV-resistance of cv. Franka.

Of particular practical interest are those crosses including one two-rowed cultivar, since two-rowed winter types become increasingly important in cultivation in Europe. Especially the two-rowed variety Igri is widely preferred in practical growing so that breeding activities also concentrate on this modern type of winter barley. Fortunately, Igri proved to possess an outstandingly high regeneration capacity in anther culture, so that a particularly large number of androgenetic lines was derived from the cross Franka × Igri. But a substantial number of lines was also recovered from the crosses of Franka to Alpha and LP 8.34218, two other two-rowed types (Table 2). Of a total of 282 androgenetic green plants out of these three hybrids, 132 were tested for their BaYMV-reactions up to now. So far, 79 (59.8%) proved to be as resistant as Franka (Table 6), and 44 (33.3%) are two-rowed. In detail, the data in Table 6 indicate, that the four discernible phenotypes, i.e. resistant/6-row, resistant/2-row, susceptible/6-row and susceptible/2-row, were not obtained in equal proportions. Similar frequencies would be expected, if the action of two independent gene-pairs for BaYMV-reaction and row-number, respectively, were assumed. The actual deviations from the expected 1:1:1:1 segregation are highly significant ($\chi^2 = 20.1$, $P < 0.01$). There is a clear excess of six-rowed over two-rowed and of resistant over susceptible phenotypes, respectively. The latter result could be explained by the action of more than one gene pair controlling BaYMV-resistance (compare Table 5).

It is clearly demonstrated by the figures in Table 6, that the proportions of recombinant lines are close to the expected values, i.e. 25% each. Therefore, reproducible proportions of new homozygous resistant and two-rowed breeding lines can be established via anther culture and subsequent mechanical inoculation of BaYMV in only one year after hybridization.

All the doubled haploid lines are further tested for other agronomic characters, including grain yield and its components in the field. Replicated field experiments are carried on in 5 to 10 m² field plots at three locations to estimate agronomic performance of resistant doubled haploids with a high degree of reliability.

Acknowledgements. We are grateful to Dr. Gerhard Wenzel for very helpful advice and support. Thanks are due to Mrs. Anna Huber, Mrs. Rosa Stein and Mrs. Erna Weber for skilful technical assistance.

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