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Genotypic control of pollen plant formation in *Nicotiana tabacum* L.

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Summary. Tobacco plants (*Nicotiana tabacum* L.) of four varieties ('Badischer Burley', 'White Burley', 'Techne', 'Kupchunos') were raised at different temperatures and daylengths and the effect of genotype on embryogenic pollen grain formation in situ and on pollen plant formation in anther and pollen cultures from these plants was studied. Genotype controlled embryogenic pollen grain and pollen plant formation by defining productivity under standard growth conditions (long days at 24 °C). 'Kupchunos' was the most productive variety, followed by 'White Burley', 'Techne', and 'Badischer Burley'. Furthermore, genotype defined which environmental factor was able to affect embryogenic pollen grain and pollen plant formation and also to which degree. In anther cultures, in addition to these effects, genotype controlled the formation of (an) inhibitory substance(s) in the anther wall in interaction with the plant growth conditions. In 'Badischer Burley' and 'Techne', inhibitor action could be prevented by isolation of the pollen after one week of anther culture. Finally, direct pollen cultures in 'Badischer Burley' and 'Techne' produced embryos were only when the pollen was isolated from nearly mature anthers, while in 'White Burley' and 'Kupchunos', embryos also produced at earlier stages and at higher yields. This indicated that genotype controls the time when the embryogenic pollen grains become ready to divide. The results are discussed in relation to strategies to overcome recalcitrance of species and genotypes.

Key words: *Nicotiana tabacum* – Anther culture – Pollen culture – Pollen embryogenesis – Tobacco

Introduction

Genotype is a major limiting factor for pollen plant production from anther cultures. This pertains not only to the model plant tobacco (Hlásniková 1977), but also to wheat (Picard and deBuyser 1973), rice (Guha-Mukherjee 1973), barley (Foroughi-Wehr et al. 1976), rye (Wenzel et al. 1977), potato (Jacobsen and Sopory 1978) and petunia (Mitchell et al. 1980). For this reason and because of the limited success in increasing pollen plant production by manipulations of the culture conditions, it was proposed to recombine lines with good "tissue culture ability" (Wenzel 1980) and lines of specific breeding relevance. Recent studies have therefore concentrated on a genetic rather than on a physiological strategy for pollen plant production (Foroughi-Wehr et al. 1982; Mix 1983; Lazar et al. 1984). Such a genetic strategy would, however, introduce complications into a breeding program which could annihilate the time-saving effect of doubled haploids. It would, therefore, be favourable if the genotypic limitations could be overcome by manipulation of the conditions for pollen plant formation. For this purpose, it is necessary to know how genotype controls pollen plant formation.

Evidence increases that pollen plant formation is composed of two distinct phases (Heberle-Bors 1983). In the first phase, embryogenic pollen grains (P-grains) are formed on the plants in situ, and in the second phase in vitro, these P-grains can develop into embryos and plantlets on an appropriate culture medium. Studies on genotypic effects on pollen plant production have not yet acknowledged this distinction. Therefore, in the present study, P-grain formation in situ and pollen embryogenesis in anther and two types of pollen culture were studied with pollen from plants of four tobacco varieties raised in various environments.

Materials and methods

Plant material and cultivation of plants

Seeds of *Nicotiana tabacum* L., var. 'Badischer Burley' and 'White Burley' were obtained from the Tabakforschungs-

institut Forchheim/Baden, FRG. Seeds of 'Kupchunos' were kindly provided by Dr. Sands, New York, USA, and seeds of 'Techne' from Dr. Pelletier, Paris-Orsay, France.

Seeds were started in a climate chamber under long (16 h) days at 24 °C as described earlier (Heberle-Bors 1983). After 30 days, young plants of 'Techne' and 'Kupchunos' were transferred to climate chambers with long or short (8 h) days at either 24, or 15 °C (LD24-, SD24-, LD15-, SD15-conditions) where they remained until flowering. Young plants of 'Badischer Burley' and 'White Burley' were transferred after 40 days. For the experiments, plants in full bloom, 2–3 weeks after first flower opening, were used. Faded flowers were regularly removed.

In vivo determinations

Frequency of P-grains and dead pollen grains (acetocarmine staining) were determined as described earlier (Heberle-Bors 1982). The data were obtained from pollen isolated from 100 pooled anthers per treatment and variety.

Anther culture

Anthers were cultured at the early binucleate stage on a medium of Miller minerals (1963), 10^{-4} M FeEDTA ($FeSO_4 \cdot 7H_2O$ and Na_2EDTA), 2% sucrose, 0.8% Difco Noble agar, 1% activated charcoal (Merck), at pH 5.8. The cultures were placed under conditions as described earlier (Heberle-Bors 1983). Induction rate was determind after three weeks of anther culture from pollen out of 100 pooled cultured anthers per treatment and variety. Pollen plant yield was determined after 7–8 weeks of culture as percentage of plantlets to total number of cultured anthers (further 100 anthers per treatment and variety).

Pollen culture after anther preculture

Anthers were precultured on the same anther culture medium and pollen was isolated after 7 days. After two washings, the pollen was suspended in a medium of Miller minerals, 0.25 M sucrose, at pH 7 (AM-medium, also used for washing), or in a medium of the same minerals, 2% sucrose, 800 mg/l glutamine, at pH 5.8 (M1-medium) at a density of 5×10^4 grains/ml. The cultures were placed under the same conditions as for anther cultures. From pollen cultures on the AM-medium, induction rate was determined after 4 weeks of culture. The pollen suspension was centrifuged at low speed and acetocarmine added to the pollen pellet. From pollen cultures on the M1-medium, the number of pollen plantlets and macroscopically visible embryos was counted after 6–7 weeks of culture and yield expressed in percentage of the initial pollen density.

Direct pollen culture

Direct pollen cultures were performed as described earlier (Heberle-Bors and Reinert 1980) with some modifications. Flowers of various lengths were excised from SD15-plants. The anthers were aseptically removed and the pollen was squeezed out into a solution of Percoll (55 ml/100 ml) and sucrose (4%). The pollen suspension was passed through a 75-µm-nylon mesh and was centrifuged at 450 g for 30 min at 15 °C. After centrifugation, the floating pollen layer at the top of the solution was removed, diluted with a solution of Miller minerals, 4% sucrose, at pH7 and centrifuged again at 150 g for 10 min. The pollen pellet was resuspended in AM-medium and centrifuged for 5 min. This washing step was repeated twice and finally the pollen was cultured in AM-medium at a density of 5×10^4 grains/ml. The culture conditions were the same as for anther cultures. After 4–5 weeks of culture, induction rate was determined as with pollen cultures after anther preculture.

Results

Total pollen sterility (frequency of P-grains plus dead grains) in the anthers of the pollen donor plants was affected by temperature and daylength quite differently in the four varieties. Under standard LD24-conditions, pollen sterility was highest in 'Kupchunos', followed by 'White Burley', 'Techne', and 'Badischer Burley' (Table 1). Lowering the temperature under long days to 15 °C strongly increased pollen sterility in 'Badischer Burley' and 'Techne', however, only weakly in 'White Burley', while in 'Kupchunos' no clear differences could be found. Short days at 24 °C increased pollen sterility in 'White Burley', 'Badischer Burley', and 'Kupchunos'. At 15 °C, however, the short day effect in 'Kupchunos' was only weak. The higher value for P-grain frequency in this variety can be attributed to the better survival of the P-grains at low temperature (Heberle-Bors and Reinert 1981) and is therefore not an indication of increased P-grain induction. In 'Techne', no significant daylength effect could be found at either temperature.

In the in vitro experiments, pollen plant formation in anther cultures of the four varieties was first studied. In anther cultures of plants under LD24-conditions, induction rates (frequency of early embryos) and pollen plant yields were highest in 'Kupchunos', followed by 'White Burley', 'Techne', and 'Badischer Burley' (Table 2). Short days had an enhancing effect on 'Kupchunos', 'White Burley' and 'Badischer Burley', but not on 'Techne'. With cultured anthers from plants under LD15-conditions, the order of the varieties was different from plants under LD24-conditions and pollen plant yields did not always parallel induction rates. 'Kupchunos' was again the most productive variety, but there was no increase when compared to cultures from plants under LD24-conditions. In 'Badischer Burley', induction rate was increased when compared to LD24-conditions, even above the 'Kupchunos' values, while

Table 1. P-grain and dead pollen grain frequency (%/%) in the tobacco varieties 'Kupchunos' (K), 'White Burley' (WB), 'Techne' (T), and 'Badischer Burley' (BB) as affected by daylength and temperature for the donor plants (LD = long days, SD = short days, number following LD/SD = temperature in °C)

	LD24	SD24	LD15	SD15
K	0.2/6.3	0.8/8.5	0.7/ 6.7	1.8/ 6.5
WB	0.3/5.6	0.7/7.2	2.3/14.0	5.2/18.1
T	0.3/4.3	0.4/4.8	4.2/ 7.8	4.6/ 9.9
BB	0.2/3.6	0.4/4.5	7.2/13.4	12.9/22.8

Table 2. Induction rates (IR, %) and pollen plant yields (PPY, % per cultured anther) in anther cultures of four tobacco varieties, as affected by daylength and temperature for the anther donor plants (for abbreviations, see Table 1). The values are the means of five experiments with SE

	LD24		SD24		LD15		SD15	
	IR	PPY	IR	PPY	IR	PPY	IR	PPY
K	5.5±0.2	15.1±1.2	6.7±0.4	18.8±1.4	4.3±0.4	14.7±1.3	3.8±0.3	13.3±1.0
WB	4.8±0.3	13.3±0.9	5.9±0.4	17.2±1.0	2.4±0.2	7.9±0.8	1.3±0.2	6.1±0.9
T	3.5±0.3	9.5±1.1	3.2±0.3	9.3±1.3	1.6±0.2	4.8±0.9	1.4±0.2	4.4±0.6
BB	2.3±0.2	8.1±1.1	2.7±0.3	10.2±0.8	6.2±0.3	9.5±0.9	9.1±0.5	8.8±0.9

Table 3. Induction rates (IR, %) and pollen plant yields (PPY, % per cultured pollen) in pollen cultures after anther preculture of four tobacco varieties, as affected by temperature of the pollen donor plants (for abbreviations, see Table 1). The values are the means of five experiments with SE

	SD24		SD15	
	IR	PPY	IR	PPY
K	5.0±0.4	0.039±0.003	4.1±0.3	0.023±0.002
WB	4.2±0.6	0.035±0.002	1.9±0.2	0.011±0.002
T	2.9±0.3	0.028±0.002	9.1±0.5	0.112±0.007
BB	2.1±0.3	0.025±0.002	8.3±0.6	0.086±0.006

Table 4. Induction rates (%) in direct pollen cultures (with density centrifugation) of four tobacco varieties, as affected by flower length (mm). The values are the means of five experiments with SE

	21	30	40
K	9.1±1.9	15.3±2.3	3.2±0.9
WB	17.9±2.7	28.7±3.1	8.1±1.7
T	–	–	4.5±1.1
BB	–	–	9.5±1.7

pollen plant yield remained unchanged. In 'White Burley' and 'Techne', both induction rates and pollen plant yields were reduced. Short days at 15 °C increased the induction rate in 'Badischer Burley', decreased it in 'Kupchunos' and 'White Burley', while it remained unchanged in 'Techne'. Pollen plant yields were reduced by short days in 'Kupchunos' and 'White Burley', but remained unchanged in the other varieties.

Following the anther culture experiments, pollen cultures after anther preculture were performed from plants growing under SD24- and SD15-conditions. In pollen cultures from plants under SD24-conditions, the order of the varieties was the same as in anther cultures from these plants, that is, 'Kupchunos', 'White Burley', 'Techne', 'Badischer Burley' (Table 3). Induction rates were, however, slightly lower than in anther cultures. In pollen cultures from plants under SD15-conditions, the order of the varieties was different from anther cultures of these plants. 'Techne' was the most productive, induction rate being much higher than in the anther cultures. Pollen plant yield paralleled this increase. In 'Badischer Burley', induction rate was slightly decreased as compared to the anther cultures, but nearly as high as in 'Techne'. In 'Kupchunos' and 'White Burley', induction rates were much lower than in the former varieties, but slightly higher than in the anther cultures of these plants.

Direct pollen culture was done only with plants under SD15-conditions since they produced the highest P-grain frequencies in situ in all four varieties. Flowers were excised from the plants at a length of 21±0 mm, 30±1 mm, and 40±2 mm. At each of the flower lengths, the developmental stage of the normal pollen was practically the same in all four varieties (as judged after acetocarmine staining), although the length of mature flowers differed to some extent in the four varieties. At 21 mm, the binucleate pollen grains had a weakly staining cytoplasm. At 30 mm, they had a densely staining cytoplasm, containing a vegetative nucleus with a prominent nucleolus and a few starch grains. At 40 mm, the pollen grains were packed with starch grains.

In 'Kupchunos' and 'White Burley', embryos were formed with flowers of all stages. Best yields were obtained with 30-mm-stage flowers (15.3 and 28.7%, resp., Table 4). In 'Techne' and 'Badischer Burley' pollen isolated from 21- or 30-mm stage flowers did not produce embryos. Only with 40-mm-stage flowers, embryos were produced in yields of 4.5 and 9.5%, respectively.

Discussion

The comparison of P-grain frequencies in the present study is complicated by the fact that many P-grains die during in vivo development – particularly at 24 °C – so that they are counted as dead grains. Since the P-grains are functionally sterile pollen grains and since most of

the dead pollen grains seem to be degenerated P-grains (Heberle-Bors 1982), it seems to be justified to base the present comparison upon total pollen sterility (frequency of P-grains plus dead grains).

Genotype exerted its effect on P-grain formation in different ways. Firstly, it affected P-grain frequency under standard conditions (LD24). Secondly, genotype defined the type of environmental control (no daylength effect in 'Techne') and thirdly it defined the degree to which the environmental factors were able to affect P-grain frequency.

The results on induction rates and pollen plant yields in anther and pollen cultures from plants at 24 °C closely paralleled the results on P-grain frequency in situ. Induction rates in anther and pollen cultures never exceeded total pollen sterility and the order of the varieties was the same for pollen sterility, induction rate, and pollen plant yield. Furthermore, short days enhanced pollen plant formation in 'Kupchunos', 'White Burley', and 'Badischer Burley', in parallel to pollen sterility, while in 'Techne', pollen plant formation and pollen sterility were not affected by daylength. These results are in line with the concept that the pollen grains which are able to form embryos are predetermined before culture by the growth conditions of the pollen donor plants (Horner and Mott 1979; Heberle-Bors and Reinert 1981; Heberle-Bors 1982).

In contrast to the results with plants at 24 °C, the results on induction rates and pollen plant yields in anther and pollen culture from plants at 15 °C did not always parallel the increase in P-grain frequency in situ, and the frequencies of early embryos (induction rates) did not always parallel the final pollen plant yields. These discrepancies can be resolved on the basis of an inhibitor present in higher quantity in anthers from plants at 15 °C as compared to 24 °C (Heberle-Bors and Reinert 1979) and on differences in the timing of P-grain development. In 'Badischer Burley' anther cultures from plants at 15 °C, this inhibitor does not affect the formation of early embryos, since induction rate is not affected, but inhibits embryogenesis at a later stage. In 'Techne' anther cultures, the strongly reduced induction rate indicates that the inhibitor already blocks the first sporophytic divisions in the P-grains. One can therefore assume that in 'Techne' the inhibitor is present in higher amounts or that a further inhibitor is present. In both varieties, however, the inhibitor is removed by isolation of the pollen after anther preculture and divisions can start in the P-grains resulting in yields corresponding to the increase in P-grain frequency in anthers from plants at 15 °C as compared to plants at 24 °C. In anther cultures of 'Kupchunos' and 'White Burley', the inhibitor also blocks the first sporophytic divisions, but its deleterious effect is not eliminated by isolation of the pollen after anther preculture. The results on direct pollen cultures showed that in these latter two varieties, the P-grains become ready to divide earlier than in the former (see below). As a further indication, in 'White Burley', the first sporophytic divisions take place in less than 7 days (Sunderland 1971), but in 'Badischer Burley' in more than 7 days (Heberle-Bors and Reinert 1979; Dollmantel and Reinert 1980). Therefore, the P-grains of 'White Burley' and presumably also of 'Kupchunos' are likely to divide during the 7 days of anther preculture and the inhibitor is able to block or kill these P-grains, so that isolation of the pollen occurs too late in order to prevent inhibitor action.

The nature of the postulated inhibitor is still unknown. 7-azaindole, an inhibitor of auxin synthesis, has been shown to increase P-grain frequency after anther preculture and pollen plant yield in subsequent pollen cultures (Dollmantel and Reinert 1980) indicating that supra-optimal auxin concentrations might be deleterious for P-grain development during anther preculture.

The genotypic effect on P-grain maturation allows to give an explanation for the gross and unexpected differences in embryo formation from direct pollen cultures in the four varieties. During in vivo flower development, many P-grains die as the anther matures (Heberle-Bors and Reinert 1981; Heberle-Bors 1982). In pollen cultures of 'White Burley' and 'Kupchunos', from 40-mm-stage flowers, therefore, less viable P-grains are present and less embryos are produced than in cultures from flowers of earlier stages, and in 'Badischer Burley' and 'Techne', where embryos are formed only in cultures from 40-mm-stage flowers, embryo yields are much lower than should be expected from the initial P-grain frequency.

In conclusion, three major effects of genotype could be distinguished, i.e., P-grain formation in situ, formation of an inhibitor in the anther wall, and timing of P-grain maturation. This distinction allows a precision of the term "tissue culture ability" (Wenzel 1980) which may be useful for the development of strategies to overcome recalcitrance of genotypes.

From a plant breeder's point of view, genotypic limitation of pollen plant formation is undesirable. He wants to obtain haploids in high frequency from any genotype. In the light of the present results it seems, however, that one has to accept that P-grain formation can be manipulated only within the limits imposed by genotype. Unless more knowledge on the fine-regulation of plant development accumulates and more specific substances can be found to influence plant development (Heberle-Bors 1983), the only meaningful strategy is to utilize as efficiently as possible the few P-grains present in situ. This points to the efficient selection of the P-grains out of the mass of normal pollen grains (Heberle-Bors and Reinert 1980). On the

other side, the reduction of "tissue culture ability" to its major compound P-grain frequency provides the breeder with a simple method for a screening for high yielding genotypes since, in most species, P-grains can be easily distinguished from normal pollen grains in nearly mature anthers in vivo (Heberle-Bors and Odenbach, in press). This might be useful for recombination of such lines with lines of special breeding relevance.

In contrast to the genotypic effect on P-grain formation, the other two compounds of "tissue culture ability" can be eliminated by appropriate experimental conditions. The inhibitor effect can be avoided by adoption of different pollen culture systems, as in the present study. For such pollen cultures, the genotypic effect on P-grain maturation is crucial. In direct pollen cultures of the present type, where P-grain development takes place entirely in vivo, in pollen cultures after anther preculture or after chill-treatment (Rashid and Reinert 1981), P-grain maturation affects the time of pollen isolation. In direct pollen cultures of the type as performed by Imamura et al. (1982) and Gandhimathi (1982), it should affect the time of medium change. Inadequate consideration of this effect might be one of the reasons that pollen cultures are still not a routine method of pollen plant production.

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