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Tissue-culture-facilitated production of aneupolyhaploid *Thinopyrum ponticum* and amphidiploid *Hordeum violaceum* × *H. bogdanii* and their uses in phylogenetic studies *

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Summary. An aneupolyhaploid (2n = 36) of the decaploid *Thinopyrum ponticum* and an amphidiploid (2n = 28) of *Hordeum violaceum* × *Hordeum bogdanii* were produced through anther and inflorescence culture, respectively. Meiotic associations in pollen mother cells at metaphase I of these plants were analyzed. The aneupolyhaploid arose by direct embryogenesis from a microspore without passing through a callus phase. The mean pairing frequencies of 2.67 univalents + 0.54 rod bivalents + 8.85 ring bivalents + 2.75 trivalents + 0.17 chain quadrivalents + 0.56 ring quadrivalents + 0.65 pentavalents in the aneupolyhaploid (2n = 36) best fit the 2:2:1 model. However, the frequent multivalents (up to five trivalents, or three quadrivalents, or four pentavalents in a cell) indicated that decaploid *T. ponticum* has five sets of closely related genomes representable by the genome formula $J_1J_1J_1J_2J_2$. Colchicine treatment of inflorescence-derived *H. violaceum* × *H. bogdanii* regenerants greatly enhanced the rate of chromosome doubling, and completely doubled regenerants could be isolated. The *H. violaceum* × *H. bogdanii* amphidiploid had a mean pairing pattern of 12.53 univalents + 5.57 rod bivalents + 1.97 ring bivalents + 0.07 trivalents + 0.03 hexavalents, indicating the presence of desynaptic gene(s) in the original diploiid hybrid. Therefore, the pairing frequency in that diploid hybrid was an under-estimate of chromosome homology between the parental genomes, and additional diploid hybrids are needed to assess the genome homology between *H. violaceum* and *H. bogdanii*. These two contrasting experiments demonstrated that tissue culture techniques are useful in altering the ploidy level to produce plant materials suitable for genome analysis and phylogenetic studies.

Key words: Anther culture – Inflorescence culture – Haploidy – Amphidiploid – Genome analysis

Introduction

Thinopyrum ponticum (Podp.) Barkworth & D. R. Dewey (syn. *Agropyron elongatum* ssp. *ruthenicum* Beldie) is a decaploid (2n = 10x = 70) species with the common name tall wheatgrass. It is an important forage grass as well as a valuable gene reservoir for cereal crops, particularly the bread wheat. Therefore, knowledge of its genome constitution will be of value to both forage and cereal breeders. No mathematical models, however, are available for numerical genome analysis of plants beyond the octoploid level (Jackson and Casey 1982). In addition, the fact that a bivalentization system can cause an autopolyploid to behave like an allopolyploid (Charpentier et al. 1988; Wang and Hsiao 1989) makes the genome analysis of the polyploid misleading. Because the bivalentization system is controlled by several recessive genes, it may not be fully functional in hemizygotes, i.e., haploids. Thus the polyhaploid of *T. ponticum*, being a pentaploid, can be more reliably analyzed to determine its genomic constitution.

Anther culture provides one method of obtaining haploids. Dependent upon the culture medium and conditions, haploids may be derived through organogenesis or embryogenesis with or without passing through a cal-

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lus phase (Heberle-Bors 1985; Hu 1986; Liang et al. 1987; Marburger and Wang 1988; Miao et al. 1988). Plantlets have been obtained from anther culture of *T. ponticum*, but all were albino and no chromosome counts were performed (Marburger and Wang 1988).

Desynaptic genes reduce the level of chromosome pairing, and thereby the chiasma frequency, in species and hybrids so that they pose a problem for genome analysis (Wang 1989). When there is more than one hybrid plant, the presence of desynaptic genes can be detected by the observation of variations in chromosome pairing and chiasma frequency among hybrid plants (Wang 1984). This is not possible when only one hybrid plant is available, such as in the case of *Hordeum violaceum* × *H. bogdanii* (Wang and Hsiao 1986). Amphidiploidy offers a remedy for such cases where desynapsis is suspected, because normal pairing in an amphidiploid will be observed if desynapsis is absent in the diploid hybrid. Conversely, reduced chromosome pairing in an amphidiploid is indicative of the presence of desynapsis in the diploid hybrid.

Chromosome doubling can be obtained by applying cholchicine to various meristematic tissues (Jensen 1974). The conventionl method is to immerse tillers of a growing plant (species or hybrid) in the colchicine solution for a few hours after having cut the roots back to approximately 5 cm. This method may not be acceptable when the material for chromosome doubling happens to be a hybrid plant that the researcher cannot risk losing. In certain cases, when old perennial hybrid plants were cloned and colchicine treated, no success was achieved in spite of repeated attempts.

This paper reports the successful production of a green haploid of *T. ponticum* through anther culture and the enhanced efficiency of chromosome doubling by cholchicine treatment immediately following inflorescence culture that resulted in amphidiploids of *H. violaceum* × *H. bogdanii*. Chromosome pairing in these resultant plants sheds light on the genome constitution of *T. ponticum* and reopen the question on genome relationships between the two *Hordeum* species. The utilization of anther and inflorescence culture for producing suitable plant materials in phylogenetic investigations is illustrated.

Materials and methods

Anther culture of Thinopyrum ponticum

Greenhouse-grown *T. ponticum* var 'Jose' (R-11-32) plants provided with ambient light and temperature were used as the source material. Tillers were harvested in April 1988 when the anthers contained microspores at the late uninucleate stage. The flag leaf was removed from each tiller, and the tillers were placed in flasks containing tap water. The flasks were then wrapped with aluminum foil to maintain high humidity and to exclude light. The tillers were stored for 7–9 days at 5°–7 °C as a pretreatment. The spikes and upper peduncles were then surface sterilized for 2 min in 70% ethanol (v/v) followed by 10 min in a 50% (v/v) commercial sodium hypochlorite (5.25%) solution. The spikes were then rinsed four times with sterile deionized water. Each spike was aseptically excised from the leaf sheath, and the poorly developed basal and distal spikelets were discarded. Anthers were excised from the two basal florets of the remaining spikelets. One hundred and fifty anthers from three spikes were randomly placed on five media resulting in 30 anthers per plate. This experiment was replicated in time. The five media were hormonally altered formulations of the 85D12 medium previously described (Marburger and Wang 1988).

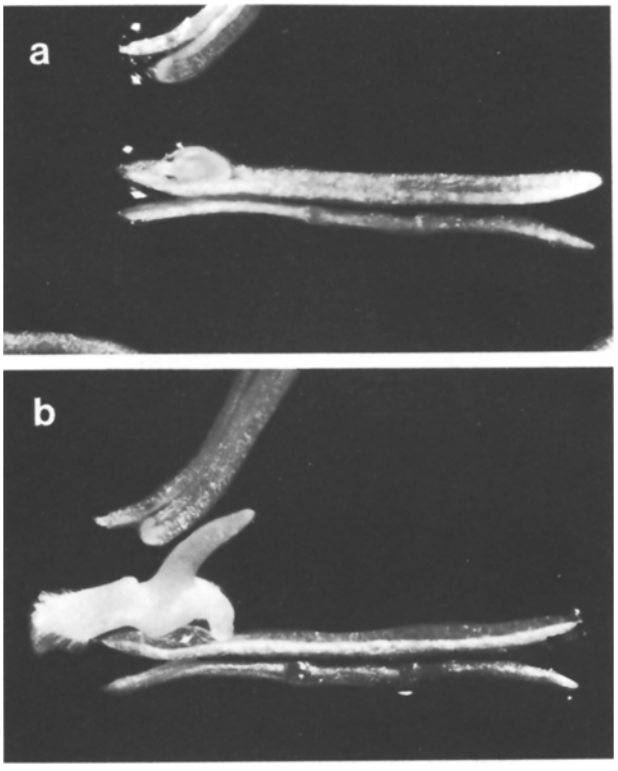

Fig. 1 a, b. Direct regeneration of a plantlet from anthers of *Thinopyrum ponticum* (2n = 70) plated on the 85D12-1 medium. **a** An embryoid developed from a pollen grain inside the anther. **b** The embryoid developed into root and shoot without any callus tissue

Cultured anthers were incubated for 44–48 days. The temperature was maintained at constant 26 °C; the illumination was 20 μmol photons/m^2 per second provided by fluorescent lights; the photoperiod was 12 h; the relative humidity was 60% – 70%. Plantlets that developed by direct regeneration were placed on a hormone-free Murashige and Skoog medium (Murashige and Skoog 1962) containing the following components (amount per liter): MS salts, 4.3 g; myoinositol, 100 mg; L-asparagine, 150 mg; glycine, 2 mg; nicotinic acid, 0.5 mg; pyridoxine-HCl, 0.5 mg; thiamine-HCl, 0.2 mg; sucrose, 20 g; and agar, 7 g. The medium was adjusted to pH 5.7 with 0.1 *N* NaOH before autoclaving. Incubation conditions for rooting were the same as for plantlet induction. Rooted plantlets were transplanted to sterile peat mix, covered with a perforated polyethylene bag, and acclimated in a growth chamber at 20°/18 °C day/night for 4 weeks. Illumination was provided by both fluorescent and incandescent bulbs at 200 μmol photons/m^2 per second for a 12-h photope-

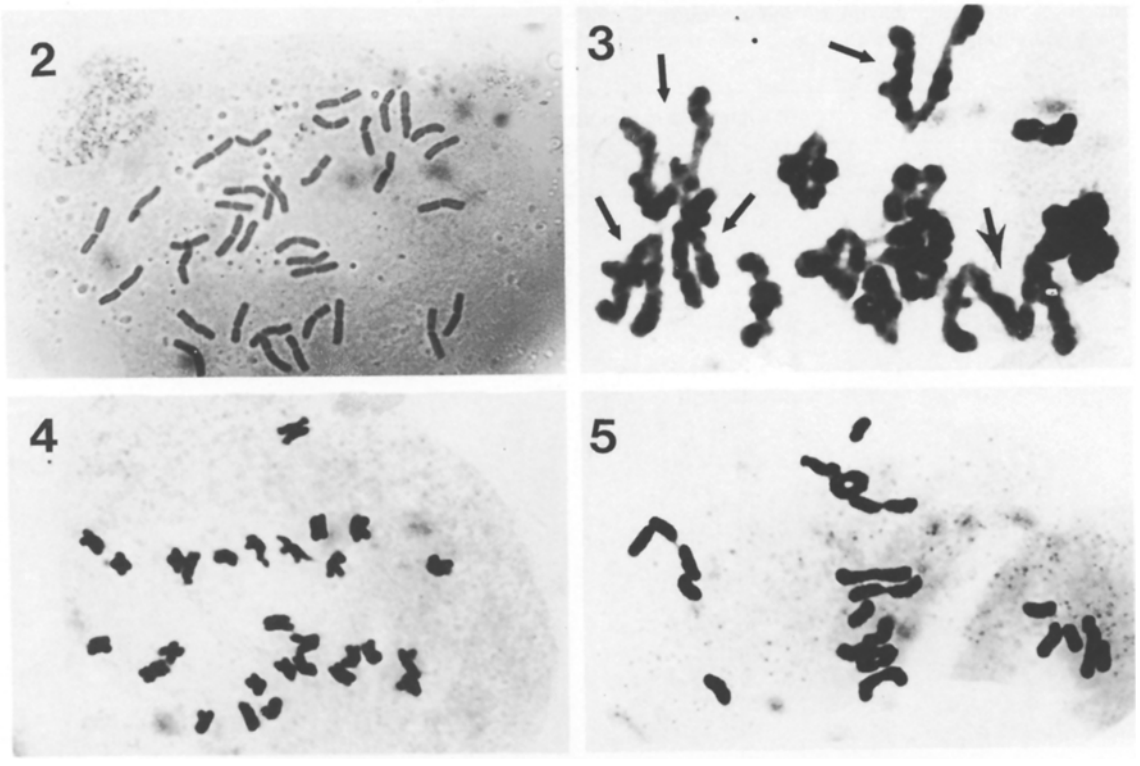

Fig. 2. A root-tip cell of the anther-culture regenerant of *Thinopyrum ponticum* (2n = 70) showing 36 chromosomes

Fig. 3. A meiotic metaphase I pollen mother cell of the aneupolyhaploid *Thinopyrum ponticum* showing 1 univalent, 9 bivalents, 4 trivalents (*small arrows*), and 1 pentavalent (*large arrow*)

Fig. 4. An anaphase I pollen mother cell from the amphidiploid of *Hordeum violaceum* × *H. bogdanii* exhibiting 28 chromosomes

Fig. 5. Meiotic pairing in the amphidiploid of *Hordeum violaceum* × *H. bogdanii* showing 14 univalents, 5 rod bivalents, and 2 ring bivalents

riod. The plants were later transferred to a greenhouse where they were maintained for 2 months until sufficient tillering occurred to allow subcloning. Three clones were placed in a vernalizer for 10 weeks at 4 °C and a 12-h photoperiod provided by fluorescent lights.

Chromosome doubling of Hordeum violaceum × *H. bogdanii*

The diploid hybrid has been reported earlier (Wang and Hsiao 1986). Inflorescences of this hybrid were cultured on the MS medium supplemented with 2,4-D (2 mg/l), BA (6-benzyladenine, 0.05 mg/l), CH (Casein, acid hydrolysate, 500 mg/l), L-asparagine (150 mg/l), and sucrose (3%) to induce callus formation, and on similar media without or with decreased 2,4-D and increased sucrose (5%) for differentiation. After the shoots and roots were formed, the plantlets were transferred singly into test tubes containing slanted rooting agar medium [MS medium supplemented with L-asparagine (150 mg/l) and sucrose (2%)]. Various cholchicine treatments were applied while the plantlets were in the test tubes. These included (a) one application of colchicine (from 0.01% to 0.02%) for 3–8 days before rinses in water and transplanting into soil; or (b) double applications of colchicine at 0.1% (6 h) or 0.2% (5 h) followed by growth in water between the treatments and transplanting into soil following the second colchicine treatment. All potted plants were grown in a greenhouse. Chromosomally doubled plants were induced to flowering with a long-day (16 h) photoperiod in a growth chamber.

Mitotic and meiotic analyses

Ten root tips per plant and at least 10 cells per root tip were analyzed to determine mitotic chromosome numbers (Hsiao et al. 1986) in the regenerants. Meiotic analysis was peformed on metaphase I (MI) pollen mother cells (PMCs) squashed in acetocarmine. Chromosome association data of the aneupolyhaploid (2n = 36) *T. ponticum* were subjected to numerical analysis (Espinasse and Kimber 1981).

Results

Production and analysis of aneupolyhaploid Thinopyrum ponticum

One of the 60 anthers cultured on 85D12-1 medium (no auxin, 1.5 mg/l kinetin) produced a pollen embryo (Fig. 1 a) after 42 days. This embryo developed during the subsequent 6 days to form a shoot and root (Fig. 1 b). The shoot had a pale yellow coloration and only developed chlorophyll after the young plantlet was removed

Table 1. Distribution (%) of root tips having various chromosome numbers in regenerants from inflourescence culture of *Hordeum violaceum* × *H. bogdanii* hybrid, with or without colchicine treatment. Ten root-tips per regenerant and at least ten cells per root-tip were counted

Colchicine		Regenerants		Chromosome number							
%	Duration	Treated	Survived	14	15	28	27	26	14+27	14+28	15+28
0	0	13	12	100 (12)[a]	–	–	–	–	–	–	–
0.1	6 h										
0.2	5 h	3	3	90	–	–	–	–	–	–	10
				80	–	20	–	–	–	–	–
				70	–	20	–	–	10	–	–
0.2	5 h										
0.2	5 h	20	17	100 (2)	–	–	–	–	–	–	–
				90 (2)	–	10 (2)	–	–	–	–	–
				80	–	20	–	–	–	–	–
				80	–	–	20	–	–	–	–
				80	20	–	–	–	–	–	–
				70 (2)	–	30 (2)	–	–	–	–	–
				60	–	40	–	–	–	–	–
				50	–	40	–	10	–	–	–
				40	–	60	–	–	–	–	–
				30	–	70	–	–	–	–	–
				20 (2)	–	80 (2)	–	–	–	–	–
				20	–	60	–	–	10	10	–
				0	–	100	–	–	–	–	–
0.01	5 days	8	4	100 (2)	–	–	–	–	–	–	–
				10	–	90	–	–	–	–	–
				10	–	80	–	–	–	10	–
0.01	8 days	1	1	90	10	–	–	–	–	–	–
0.015	5 days	2	1	30	–	70	–	–	–	–	–
0.02	3 days	4	1	100	–	–	–	–	–	–	–
0.02	4 days	2	1	40	–	50	–	–	–	10	–
0.02	5 days	3	0								
0.02	6 days	3	1	100	–	–	–	–	–	–	–

[a] The numbers in parentheses are number of plants

from the anther and placed on the hormone-free MS medium. Vigorous growth of the plant facilitated its subcloning and maintenance. The roots tips of this plant had 36 chromosomes (Fig. 2); thus it is an aneupolyhaploid.

Vernalized clones produced spikes that were used for meiotic analysis. Forty-eight pollen mother cells were analyzed and averaged 2.67 univalents, 0.54 rod bivalents, 8.85 ring bivalents, 2.75 trivalents, 0.17 chain quadrivalents, 0.56 ring quadrivalents, and 0.65 pentavalents. Such pairing patterns best fit the 2:2:1 model of genome affinity even though the extra chromosome complicated the numerical analysis (C. Chapman personal communication). Multivalents were frequently observed (Fig. 3); up to five trivalents, three quadrivalents, or four pentavalents occurred. In one PMC, one trivalent, one quadrivalents, and four pentavalents were present. At least one pentavalent occurred in 21 out of 48 (44%) PMCs; and 54% of the PMCs contained at least one quadrivalent. All except one PMC had a minimum of one trivalent.

Production and analysis of the amphidiploid of H. violaceum × *H. bogdanii*

A total of 59 regenerants were obtained from inflorescence culture of the diploid hybrid. Plantlets still in test tubes were subjected to various colchicine treatments, and chromosome numbers in ten root tips per plant were determined when the potted plants had developed sufficient tillers (Table 1). All of the regenerants not receiving colchicine treatment had 14 chromosomes in all of their root-tip cells. All three plants that received double colchicine treatments of 0.1% for 6 h and 0.5% for 5 h were mixoploids with 28 chromosomes in at least one root-tip. Seventeen regenerants that had been treated twice with 0.2% colchicine for 5 h each had 82% chromosome doubling. Two plants remained at the diploid level and one plant had 15 chromosomes in two root tips. All of the other plants had root tips with 28 (or 27 in one case) chromosomes. One plant had been completely doubled, i.e., 28 chromosomes in all of the analyzed cells of each

root tip. Another plant had root-tips with both diploid and tetraploid cells in the same root tip. Plants that received a single colchicine treatment at lower (0.01% – 0.02%) concentrations but longer durations (3–8 days) had variable results which could not be summarized because of the small sample size.

Spikes of the completely doubled plant, the amphidiploid C_0, had nondehiscent anthers. However, all of the PMCs in this plant had 28 chromosomes (Fig. 4). Meiotic analysis of 30 cells in this amphidiploid revealed a low pairing pattern of 12.53 univalents + 5.57 rod bivalents + 1.97 ring bivalents + 0.07 trivalents + 0.03 hexavalents. Thus the *c* value (mean arm-pairing frequency; Kimber and Alonso 1981) for this tetraploid was 0.35. This low pairing (Fig. 5) was unexpected unless the diploid hybrid was desynaptic.

Discussion

The direct regeneration of plantlets without passage through a callus phase had been observed previously in anther culture of *T. ponticum* (Marburger and Wang 1988); in this case albino plants resulted. In the experiments presented in the present paper, direct regeneration was repeated, and the result was the production of a green aneupolyhaploid. Although albinism is a common problem in anther culture, it might be circumvented by improved culture conditions. The only difference between this study and the previous one was the removal of kinetin from shoot regeneration medium MCR7 (Marburger and Wang 1988) on which the plantlets resulting from direct regeneration were placed for further development. In the earlier study all three plantlets regenerated from 'Jose' on 85D12-1 were albino (Marburger and Wang 1988), while the only regenerant from the present study was green. Whether the difference is attributable to the change in medium composition requires further investigation. Nevertheless, the present study demonstrates that green polyhaploids of *T. ponticum* can be obtained by anther culture through direct regeneration, as has been reported for bread wheat (Liang et al. 1987).

The practical usefulness of this polyhaploid of *T. ponticum* is to study its meiotic pairing patterns for insight into the genomic constitution of this decaploid species. Although the decaploid behaves like an allopolyploid with strictly bivalent formation, the haploid exhibited high multivalent frequencies, which suggests the presence of a bivalentization system in the decaploid and the ineffectiveness of this system in the hemizygous haploid state. However, the fitness of its pairing patterns to 2:2:1 instead of 4:1 or 5:0 still suggests a partial bivalentization. If 0.5 univalents and 0.5 trivalents are subtracted to adjust for the extra chromosome (assuming a trisomy), the *c* value for this pentaploid is still higher than 1.0. The high *c* value along with up to four pentavalents indicates that the five genomes are all very closely related to each other. In fact, the pairing frequencies in this 36-chromosome polyhaploid *T. ponticum* were similar to that of a 36-chromosome hybrid of *Agropyron* species that is known to have five P genomes (2.72 univalents, 1.04 rod bivalents, 6.42 ring bivalents, 3.16 trivalents, 0.34 chain quadrivalents, 0.26 ring quadrivalents, 1.24 pentavalents; C. Hsiao unpublished data). Therefore, *T. ponticum* can be regarded to be an autodecaploid that behaves as an allodecaploid due to the bivalentization system. We believe that the decaploid *T. ponticum* most likely has five sets of the J genome that may be the same or different versions of J^b and J^e (Wang and Hsiao 1989).

Conventional chromosome doubling techniques require large numbers of young seedlings or clones (tillers) of plants for colchicine treatment. This is practical when the material consists of a species with ample seed supply. When attempting to double the chromosome number in plants that are especially rare intergeneric or interspecific hybrids, researchers risk losing the material when they apply colchicine treatments. The use of tissue culture techniques can diminish this danger by multiplying the plant material.

To preserve the original hybrid(s), a tissue culture system must maintain chromosomal stability. The inflorescence culture used in this study meets this requirement, since all the regenerants in the control group, i.e. those not receiving colchicine treatment, had 14 chromosomes in all of the root-tip cells. The observed polyploidy and mixoploidy in the regenerants can only be attributed to colchicine treatments in this study, although other tissue culture systems might result in mixoploidy and polyploidy without colchicine treatment (Chu et al. 1984). Of course, tissue culture-generated mutation or structural changes (translocation, inversion, deletion, insertion, and amplification, etc.; Jorgensen and Andersen 1989), which could not be detected by chromosome counting in root-tip cells, might still have occurred in our inflorescence culture system.

Even if mutation or somaclonal variation occurred in the regenerants of the diploid hybrid *H. violaceum* × *H. bogdanii*, they could not have occurred at the gene loci controlling chromosome synapsis during meiosis in all of the regenerants that had arisen from different calli. Among all those doubled sectors of this diploid hybrid, none of the spikes contained dehiscent anthers; this is in contrast with the presence of dehiscent anthers in amphidiploids of other intergeneric hybrids produced through the same procedures (R. R.-C. Wang unpublished data). Mutations as a result of tissue culture could be ruled out as the cause for desynapsis in the amphidiploid of *H. violaceum* × *H. bogdanii*, because such a conclusion requires the assumption that a 100% mutation rate occurred at the gene locus controlling chromo-

some synapsis. Nondehiscence may have been the consequence of desynapsis or alternatively, the recessive genes for nondehiscence and desynapsis may be closely linked on one chromosome of *H. violaceum*, because both diploid parental species were dehiscent and fertile while the desynaptic diploid *H. violaceum* was nondehiscent (R. R.-C. Wang unpublished observation). Desynapsis has been detected in diploid *H. violaceum* and its triploid hybrid with *Psathyrostachys juncea* (Wang 1984). It was therefore not surprising that the diploid hybrid between *H. violaceum* and *H. bogdanii* was also desynaptic; in fact, it had been suspected (Wang and Hsiao 1986; Bothmer et al. 1986).

Chromosomal heterozygosity caused by tissue culture, if it occurred, might affect chromosome pairing in the regenerants. However, any heterozygosity in the diploid regenerants would be transformed into homozygosity upon chromosome doubling. Because every chromosome has a homologous partner to pair with in the amphidiploid, chromosomal heterozygosity as a result of tissue culture cannot explain low chromosome pairing in amphidiploids. In spite of the presence of a perfect partner for each of the two parental genomes in the amphidiploids, chromosome pairing was so low that desynapsis is the only reasonable explanation. Analysis of the amphidiploid confirmed that the diploid hybrid of *H. violaceum* × *H. bogdanii* (Wang and Hsiao 1986) indeed had a desynaptic gene and that the pairing frequency in the diploid hybrid represented an underestimate of genome relatedness. Additional diploid hybrids between *H. violaceum* and *H. bogdanii* without desynaptic genes are needed in order to adequately study their relationship.

We demonstrated in this paper that tissue culture, specifically anther and inflorescence culture, can be applied to alter plant ploidy to obtain suitable plant materials for genomic analysis. This provides valuable information on phylogeny or species relationships.

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