Theor Appl Genet (1998) 96: 385–388

J. G. Dubouzet · K. Shinoda · N. Murata

Interspecific hybridization of *Allium giganteum* Regel: production and early verification of putative hybrids

Received: 15 August 1997 / Accepted: 2 September 1997

Abstract Cut flowers of *Allium giganteum* Regel were emasculated and maintained in half-strength Murashige and Skoog liquid medium supplemented with 3% sucrose and 1000 ppm each of Agrimycin^{R} and Benlate^{R}. Wide hybridization was attempted and, through embryo rescue, putative hybrids were obtained from crosses involving *A. cernuum* Roth, *A. oreophilum* C.A. Mey. and *A. schubertii* Zucc. PCR amplification of the internal transcribed spacer of ribosomal DNA followed by digestion with *Nde*II generated restriction profiles that confirmed the hybrid nature of the *A. giganteum* × *A. schubertii* progenies. The other putative hybrids were found to be products of self pollination.

Key words *Alluium giganteum* · Interspecific hybridization · Cut Flower culture · Embryo rescue · Internal transcribed spacer · Restriction analysis

Introduction

Allium L. is a cosmopolitan genus with more than 700 species distributed in the northern hemisphere. *Allium* breeders have produced interspecific hybrids to obtain traits such as disease resistance, cold tolerance, exotic flavor components (Keller et al. 1996) or cytoplasmic male sterility (CMS) (Peterka et al. 1997), but they have focused on species in subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo and subgenus *Allium*.

Allium giganteum Regel is the most common *Allium* cut flower and is classified in subgenus *Melanocrommyum* (Webb et Berth.) Rouy. *Allium* 'John Dix' (*A. giganteum* × *A. christophii* Trautv.), released 40 years ago, is the only commercially available hybrid obtained from *A. giganteum* (Van Scheepen 1991). Recent attempts to obtain interspecific hybrids from *A. giganteum* produced a few putative hybrid seedlings which died soon after germination (Maeda et al. 1994).

A. giganteum produces more than a thousand small (approximately 6 mm wide) delicate flowers densely clustered in an umbel on 0.8- to 2.0-m-long flower scapes (Davies 1992), characteristics which hinder the emasculation and hybridization of plants growing in the field. These problems would be minimized if cut flowers could be used as mother plants. Cut-flower culture followed by embryo rescue have been shown to improve the success rate of interspecific hybridization in subgenus *Rhizirideum* (Dubouzet et al. 1994). Although the vase life of *A. giganteum* cut flowers is only 2 weeks (Armitage 1993), treatment with sugar and some mineral ions can extend the longevity of cut flowers (Salunkhe et al. 1990).

Another difficulty with *A. giganteum* breeding is that plants produced from seeds require 3–5 years to reach flowering size (Armitage 1993). Hence, a cheap and reliable method of early hybrid verification is also necessary. Dubouzet et al. (1996) used male parent-specific random amplified polymorphic DNA (RAPD) bands to identify putative interspecific hybrids in subgenus *Rhizirideum*. Recently, Keller et al. (1996) used karyotype analysis, flow cytometry and/or genomic in situ hybridization to establish the identity of interspecific hybrids in subgenus *Rhizirideum*.

Screening for presence or absence of restriction sites diagnostic for internal transcribed spacer (ITS) sequences of each putative parent species can resolve the identity of presumed interspecific hybrids (Baldwin et al. 1995). Havey (1992) reported that restriction fragments generated by 4-base cutters from polymerase chain reaction (PCR)-amplified internal transcribed regions (ITS1 and ITS2) of rDNA could be used to differentiate some *Allium* species. The small sample requirements for DNA extraction and subsequent PCR

Communicated by R. Hagemann

J. G. Dubouzet (✉) · K. Shinoda · N. Murata
Hokkaido National Agricultural Experiment Station, Hitsujigaoka 1, Toyohira-Ku, Sapporo 062 Japan

amplification allow the use of this PCR-based technique for the rapid screening of plantlets still undergoing aseptic culture.

The specific objectives of the study presented here were (1) to evaluate the feasibility of using *A. giganteum* cut flowers for interspecific hybridization and (2) to characterize the putative hybrid population by restriction analysis of the internal transcribed spacers of rDNA.

Materials and methods

Cut-flower culture, hybridization and embryo rescue

Inflorescences of field-grown *A. giganteum* were cut at the base of the scape at the start of anthesis. Only mature flowers expected to open within 3 days were emasculated; the rest were discarded. The cut flowers were maintained in half-strength Murashige and Skoog (1962) liquid medium (MS) 3% sucrose, and 1000 ppm each of Agrimycin^R (streptomycin) and Benlate^R (benomyl). At the start of stigmatic receptivity, the emasculated flowers were pollinated for 3 consecutive days with pollen from the male parents listed in Table 1.

Embryo rescue was performed 20–25 days after the start of pollination. Embryos were cultured for at least 1 month on 0.7% agar containing haf-strength MS, 3% sucrose, 5 ppm α-naphthaleneacetic acid (NAA), and 0.5 ppm 6-benzylaminopurine (BA) at pH 5.8. The resulting plantlets were subsequently transferred to hormone-free half-strength MS medium in 0.8% agar.

Hybrid verification

Minute (< 50 mg) leaf or callus tissue samples from 4 selfed plants, 30 *A. giganteum* × *A. schubertii* 'hybrids' and the rest of the putative hybrid plantlets shown in Table 1 were collected from plants growing in the greenhouse and those still in aseptic culture. DNA was extracted using the procedure reported by Dubouzet et al. (1997). The DNA samples were adjusted to 2 ng/ μL based on UV spectrometry.

The amplification reaction consisted of 5 μl DNA extract, 1.6 μl 25 m*M* $MgCl_2$, 1.31 μl ddi water, 1 μl 10 × Buffer II, 0.8 μl 10 m*M* dNTP mix, 1.1 p*M* ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) primers (Hsiao et al. 1994) and 0.05 μl AmpliTaq DNA polymerase (Perkin Elmer, USA). Amplification was performed in a Perkin Elmer 9600 using 'Touchdown' PCR (Don et al. 1991). A 3-min incubation at 94°C was followed by 10 cycles of 94°C for 15 s, 65°C for 30 s (annealing temperature was reduced by 1°C at each subsequent cycle), 72°C for 2 min. This was followed by 35 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 2 min and a final elongation step at 72°C for 5 min.

A 2.5-μl aliquot of the PCR product was mixed with 1 unit of a restriction enzyme (*Hha*I or *Nde*II), 1 μl of their respective 10 × buffers and enough water to make a 10-μl reaction. The reaction solution was incubated for 1.5 h at 37°C, and the restriction products were separated in 2% agarose by electrophoresis in TAE buffer.

Results and discussion

Cut-flower culture, hybridization and embryo rescue

Emasculation and pollination of *A. giganteum* cut flowers were readily performed in the laboratory. Dubouzet et al. (1994) suggested the use of pulsing solutions to prolong the vase life. In the present study, the main objective of cut-flower culture was to support the development of the hybrid embryos to a stage at which embryo rescue would become feasible. Cut-flower culture was considered as the in vivo stage of embryo rescue so the cut flowers were continuously maintained on liquid culture medium (1/2 MS + 3% sucrose).

Benlate^R and Agrimycin^R reduced but did not prevent microbial growth. Because of microbial infection, the holding solution had to be changed, and the flower stalks had to be cut regularly. Future research should reevaluate the utility of pulsing the cut flowers with sugar-containing solutions for short periods, as suggested by Dubouzet et al. (1994).

All pollinator parents induced ovarian swelling in *A. giganteum*, as shown by the number of ovaries harvested for embryo rescue (Table 1). Despite fungal infection, cut-flower culture for at least 3 weeks after pollination enabled some embryos to develop beyond the torpedo stage for subsequent embryo rescue. Only three of the seven cross combinations produced putative hybrid embryos (Table 1). The number of putative hybrid embryos obtained from the *A. giganteum* × *A. schubertii* Zucc. cross was remarkably high.

Embryos are sometimes injured or broken during embryo rescue so the initial aseptic culture medium included NAA and BA to induce growth from whole or fragmentary embryos. Explants with multiple shoots were transferred to hormone-free media to allow further development and maturation. Hence, practically all the rescued embryos developed into plantlets (Table 1). Multiple shoot formation from embryos is desirable in cross combinations with a low success rate because this will facilitate subsequent multiplication. Along with leaves and roots, many of the explants continued to produce watery callus even after they were transferred to hormone-free media. Although continuous callus production facilitates clonal multiplication

Table 1 Wide hybridization of *A. giganteum*

Male parent	Number of			
	Pollinated flowers	Fruits harvested	Rescued embryos	Plantlets
giganteum	57	20	16	16
oreophilum	47	34	2	2
schubertii	57	50	71	54
cernuum 'Titan'	37	5	1	1
unifolium	33	20	0	
caesium	42	11	0	
altaicum	48	4	0	
cyathophorum var *farreri*	52	20	0	
Total	373	164	90	73

by division, the presence of callus tissue lowers survival rates after transplanting.

A year after pollination, most of the putative hybrids were still being maintained in aseptic culture to allow sufficient growth before greenhouse culture. The slow growth of putative hybrids and their high cost of maintenance underlines the need for a hybrid verification system that would enable the early elimination of plants resulting from unwanted pollination.

Hybrid verification by restriction analysis of the ITS region

The ITS primers produced an approximately ~700-bp band from *A. giganteum*, *A. cernuum* Roth and *A. oreophilum* C.A. Mey. *A. giganteum* could be differentiated from *A. cernuum* and *A. oreophilum* using restriction patterns generated by *Hha*I. *Nde*II generated restriction patterns that could distinguish *A. giganteum* from *A. cernuum*, *A. oreophilum* or *A. schubertii*.

Digestion of the PCR-amplified ITS regions of *A. giganteum* and its four selfed progenies using *Hha*I or *Nde*II generated similar restriction profiles. This implies that neither sexual reproduction nor prolonged tissue culture affected the presence of restriction sites in the ITS region of rDNA.

A restriction site for *Nde*II was found to be present in the ITS region of *A. giganteum* but absent in that of *A. schubertii* (Fig. 1). The restriction profiles of the PCR-amplified ITS regions of 28 putative *A. giganteum* × *A. schubertii* hybrids showed both parental bands. This affirms the codominant inheritance of the restriction sites in the ITS region of rDNA and confirms the hybridity of these plants. The 2 remaining *A. giganteum* × *A. schubertii* 'hybrids' showed the restriction profile of *A. giganteum*, implying that they were produced by accidental self pollination.

Digestion with *Hha*I of the PCR-amplified ITS regions of the remaining putative hybrids between *A. giganteum* and *A. cernuum* or *A. oreophilum* generated restriction profiles which were similar to those of *A. giganteum*. This meant that these 'hybrids' were selfed progeny.

A. giganteum is classified under section *Compactoprason* R. Fritsch, whereas *A. schubertii* belongs to section *Kaloprason* R. Kam. (Fritsch 1992). The relatively large number of hybrids produced between these two species (Table 1), coupled with in vitro multiplication from callus tissues, will allow rapid commercialization. Previous interspecific hybrids from these two sections include *A.* 'Globe Master' (*A. macleanii* Baker × *A. christophii*) and *A.* 'John Dix' (Van Scheepen 1991).

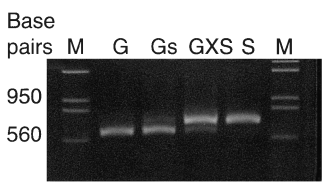

Fig. 1 Restriction profile of the PCR-amplified internal transcribed spacer regions of rDNA after digestion with *Nde*II. *M Hin*dIII/*Eco*RI double-digest marker, *G A. giganteum*, *Gs A. giganteum* selfed progeny; *G* × *S A. giganteum* × *A. schubertii*, *S A. schubertii*

With respect to future breeding of *A. giganteum*, our results indicate that evaluation of other pollinator species from section *Compactoprason* and *Kaloprason* should be prioritized. Manual emasculation can not guarantee the complete removal of the anthers, and it also often leads to unavoidable damage to the tiny and delicate flowers. Hence, artificial induction of male sterility in this species should be investigated.

The two techniques described in this report may also be useful for other wide hybridization programs in *Allium*. Cut-flower culture expedites manual emasculation and subsequent pollination of male-fertile species and assures a controlled environment free from the vagaries of nature. Mini experiments on the effects of such factors as nutrients, hormones, temperature etc. on hybridization can be easily implemented using cut flowers.

Restriction analysis of ITS regions provides the breeder with a rapid, reliable and reasonably economical method of interspecific hybrid verification. Amplification of minute DNA samples by PCR allows hybrid verification at the initial stages of aseptic culture. ITS restriction profiles are easy to interpret because codominant inheritance produces additive profiles in the hybrid.

References

Armitage AM (1993) Specialty cut flowers. Timber Press, Portland, Ore.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowskii MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence of angiosperm phylogeny. Ann Mo Bot Gard 82:247–277

Davies D (1992) *Alliums*: the ornamental onions. BT Batsford, London, UK

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Dubouzet JG, Arisumi K, Etoh T, Maeda M, Sakata Y (1994) Studies on the development of new ornamental *Allium* through interspecific hybridization. III. Hybridization of autumn-flowering species through pull-style pollination, cut flower culture and embryo rescue. Mem Fac Agric Kagoshima Univ 30:35–42

Dubouzet JG, Etoh T, Arisumi K, Yoshitake T (1996) A diagnostic test to confirm interspecific *Allium* hybrids using random amplified polymorphic DNA from crude leaf DNA extracts. J Jpn Soc Hortic Sci 65:321–326

Dubouzet JG, Shinoda K, Murata N (1997) Phylogeny of *Allium* L. subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo according to dot blot hybridization with randomly amplified DNA probes. Theor Appl Genet 95:1223–1228

Fritsch RM (1992) Infra-subgeneric grouping in subgenus *Melanocrommyum* (Webb et Berth.) Rouy. In: Hanelt P, Hammer K, Knupffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. Inst Genet Crop Plant Res, Gatersleben, Germany, pp 67–75

Havey MJ (1992) Restriction enzyme analysis of the chloroplast and nuclear 45*s* ribosomal DNA of *Allium* sections *Cepa* and *Phyllodolon* (*Alliaceae*) Plant Syst Evol 183 : 17–31

Hsiao C, Chatterton NJ, Asay KH, Jensen KB (1993) Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. Genome 37 : 112–120

Keller ERJ, Schubert I, Fuchs J, Meister A (1996) Interspecific crosses of onion with distant *Allium* species and characterization of the presumed hybrids by means of flow cytometry, karyotype analysis and genomic in situ hybridization. Theor Appl Genet 92 : 417–424

Maeda M, Dubouzet JG, Arisumi K, Etoh T, Sakata Y (1994) Effects of cold storage and staggered planting in forcing culture of spring-flowering *Allium* species. J Jpn Soc Hortic 63 : 629–638

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays within tobacco tissue cultures. Physiol Plant 15 : 473–497

Peterka H, Budahn H, Schrader O (1997) Interspecific hybrids between onion (*Allium cepa* L.) with S-cytoplasm and leek (*Allium ampeloprasum* L.) Theor Appl Genet 94 : 383–389

Salunkhe DK, Bhat NR, Desai BB (1990) Postharvest biotechnology of flowers and ornamental plants. Springer, Berlin, Heidelberg New York, p 20

Van Scheepen J (1991) International checklist for hyacinths and miscellaenous bulbs. Royal General Bulbgrowers' Assoc, Hillegom, The Netherlands, pp 6–14