

# HYBRIDIZATION AND MOLECULAR CHARACTERIZATION OF F<sub>1</sub> *ALLIUM CEPA* × *ALLIUM ROYLEI* PLANTS

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We made interspecific crosses to facilitate the introgression of desirable traits of *Allium roylei* into the *Allium cepa* genome. After hand-pollination, 906 interspecific F<sub>1</sub> *Allium cepa* × *A. roylei* plants were obtained by in vitro culture via embryo rescue. Nuclear DNA analysis showed that 97.6% of the regenerants were interspecific F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids. Genomic in situ hybridization (GISH) showed that each hybrid had 16 chromosomes, eight of which were identified as *A. cepa* and eight as *A. roylei* chromosomes.

**Key words:** *Allium cepa*, *A. roylei*, embryo rescue, genomic in situ hybridization (GISH), interspecific hybrids, molecular markers.

## INTRODUCTION

The genus *Allium* comprises about 750 species including *A. cepa*, *A. fistulosum* and *A. roylei*. Onion (*Allium cepa* L.) is a valuable vegetable crop preceded on the list of worldwide cultivated vegetable species only by tomato (FAOSTAT, 2011). Onion breeding programs currently focus mainly on improvement of existing cultivars. According to Kik (2002), cultivated and wild *Allium* species possess many desirable traits (disease and pest resistance, cytoplasmic male sterility, bulb quality) which might be useful for genetic improvement of the bulb onion. Of all the species belonging to *Allium*, *A. cepa*, *A. vavilovii*, *A. galanthum*, *A. roylei*, *A. fistulosum*, *A. altaicum*, *A. pskemense* and *A. oschaninii* have been recognized as the most important gene pools of onion (Shigyo and Kik, 2008).

According to Peffley and Hou (2000), Kik (2002) and Yamashita et al. (2005), *Allium fistulosum* possesses many traits agronomically useful for onion. Due to its resistance to onion leaf blight, pink root, anthracnose and onion fly, as well as its earliness, high dry matter content and winter-hardiness, Japanese bunching onion has been used to expand the genetic variation of onion since the last century. Studies of interspecific hybridization between *A. cepa* and *A. fistulosum* initiated by Emsweller and Jones (1935) have been continued over the years by Dolezel et al. (1980), van Raamsdonk et al.

(1992), van der Valk et al. (1991a,b), Khrustaleva and Kik (1998), Peffley and Hou (2000) and Mangum and Peffley (2005). Unfortunately, subsequent backcrosses of the obtained F<sub>1</sub> *A. cepa* × *A. fistulosum* hybrids to *A. cepa* have proved problematic. The difficulties observed in genome transmission in advanced backcross generations have been explained by the sterility of F<sub>1</sub> *A. cepa* × *A. fistulosum* hybrids probably resulting from one of three possible mechanisms: stylar incongruity (van der Valk et al., 1991a), nuclear cytoplasmic incompatibility (Ulloa et al., 1995) or central cell nuclear cytoplasmic incongruity (Mangum and Peffley, 2005). Due to problems in introgression of Japanese bunching onion traits into onion, so far only four new varieties of *A. fistulosum* have been developed: Beltsville Bunching, Delta Giant, Top Onion and Wakegi Onion (Kik, 2002).

*Allium roylei* Stearn is a wild species originating from the Indian subcontinent which harbors disease resistance against downy mildew (*Peronospora destructor*), leaf blight (*Botrytis squamosa*) and anthracnose (*Colletotrichum gloeosporioides*) (van der Meer and de Vries, 1990; de Vries et al., 1992; Galvan et al., 1997; Scholten et al., 2007; Kohli and Gohil, 2009). This species has been used to expand the genetic variation of onion since 1960 (Fritsch and Friesen, 2002). The first crosses between *A. roylei* and *A. cepa* were made in 1985 to achieve introgression of downy mildew resistance into onion

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**Fig. 1.** Obtaining  $F_1$  *Allium cepa*  $\times$  *A. roylei* plants through in vitro culture. (a) Emasculated *A. cepa* umbel, (b) Flowering *A. roylei* umbel, (c) Enlarged ovaries after pollination, (d) Ovules plated on medium, (e) Embryo formation, (f) Plant regeneration, (g)  $F_1$  *Allium cepa*  $\times$  *A. roylei* plants acclimatized to ex vitro conditions

(van der Meer and de Vries, 1990). Subsequent studies on reciprocal *A. cepa* and *A. roylei* hybridization (Kofoet et al., 1990; Kofoet and Zinkernagel, 1990) led to the conclusion that resistance to *Peronospora destructor* is determined by a single dominant gene, *Pd1*. Later, AFLP marker technique combined with SCAR analysis confirmed this (van Heusden, 2000a,b). A long-term investigation on interspecific hybridization and downy mildew resistance led Scholten et al. (2007) to obtain  $F_1BC_5$ -generation plants expected to be resistant to *Peronospora destructor*.

In this study we sought to obtain and characterize interspecific  $F_1$  *Allium cepa*  $\times$  *A. roylei* hybrids. To our knowledge this is the first report of the use of embryo rescue technique in vitro to produce interspecific  $F_1$  *Allium cepa*  $\times$  *A. roylei* hybrids. To identify the obtained hybrids we generated a system of three novel independent molecular markers. We also developed a genomic in situ hybridization (GISH) protocol in order to determine the cytological characteristics of the generated  $F_1$  hybrids.

## MATERIALS AND METHODS

### PRODUCTION OF $F_1$ *A. CEPA* $\times$ *A. ROYLEI* PLANTS VIA EMBRYO RESCUE

In 2004–2007 we crossed six different onion breeding lines (A1–A6) and two cultivars (Bila, Kutnowska) with two *Allium roylei* ecotypes (333, 334). The onion breeding materials came from three Polish seed companies: PlantiCo Zielonki, Spojnia Nochowo and KHNO Polan. *A. roylei* plants were kindly provided by Dr. T. Kotlinska of the Plant Genetic Resources Laboratory, Research Institute of Vegetable Crops (RIVC), Skierniewice, Poland.

*Allium cepa* and *A. roylei* plants were grown in the greenhouse from February to June or in the field from April to August. The interspecific crosses were performed in a screen-covered cage in the greenhouse from June to August. Flowering plants from maternal stocks were subsequently emasculated (Fig. 1a) and manually pollinated with *A. roylei*

pollen (Fig. 1b). At 7–14 days after pollination, ovaries (Fig. 1c) were harvested and sterilized in 70% ethanol for 1 min followed by 20% chloramine B for 20 min. The material was then washed three times in sterile water. Ovules were aseptically excised from ovaries and placed on two different induction media (Fig. 1d). Medium A was based on B5 basal medium (Gamborg et al., 1968) with 20 g/l sucrose and 7.0 g/l agar, pH 6.0; medium B was based on BDS basal medium (Dustan and Short, 1977) with 20 g/l sucrose and 7.0 g/l agar, pH 6.0. The media were autoclaved at 121°C for 20 min. Twenty ovules were cultured on each 60  $\times$  10 mm Petri dish. The dishes were sealed with parafilm and kept at 25  $\pm$  2°C in the dark. After ca. one month, at the stage of leaf and root formation, developing embryos (Fig. 1e) were transferred to MS medium (Murashige and Skoog, 1962) with 30 g/l sucrose and 7.0 g/l agar, pH 6.0, and kept in glass tubes in a growth chamber at 25  $\pm$  2°C under a 16 h photoperiod and light intensity 55  $\mu\text{ms}^{-1}\text{m}^{-2}$  (Fig. 1f). The developed plantlets were placed in soil and acclimatized to ex vitro conditions in a chamber with regulated temperature and humidity. After acclimatization,  $F_1$  *A. cepa*  $\times$  *A. roylei* plants (Fig. 1g) were cultivated in the greenhouse.

The total effectiveness of  $F_1$  *A. cepa*  $\times$  *A. roylei* embryo development is expressed as the number of developed embryos per number of ovules plated on media, and the total effectiveness of  $F_1$  *A. cepa*  $\times$  *A. roylei* plants through in vitro culture is expressed as the number of acclimatized plants per number of ovules cultured.

### MOLECULAR MARKERS

The hybrid nature of all of the obtained  $F_1$  *Allium cepa*  $\times$  *A. roylei* regenerants was verified with three different molecular markers (Tab. 1). SIR, ACS and A markers were developed on the basis of three *A. cepa* sequences available in the NCBI (2005). SIR-F/SIR-R primers were based on the *A. cepa* sulfite reductase gene. ACS-F/ACS-R primers were designed on the basis of the *A. cepa* anthocyanidin synthase gene. A-F/A-R primers synthesized accord-

TABLE 1. Primer sequences based on three onion cDNA clones

cDNA clone	Marker	Primers	Primer sequence (5'-3')
AY753557	SIR	SIR-F SIR-R	AGTCACTGTGCCAACTGACAACCTCAG TAACCCAAATGCTCAGCCAAACGAGG
AY585678	ACS	ACS-F ACS-R	AAGCGTGATAATTGCACCACCAGCTCC ATGCCAGATGTGGCTGGGGGCATTTTG
L48614	A	A-F A-R	TGGGTACTCCAATAGCCAGTG TGCAACCTTCGGAGAACAG

ing to van Heusden et al. (2000a,b) were based on the *A. cepa* alliinase gene.

Total genomic DNA was isolated from young leaves of *A. cepa*, *A. roylei* and all the F<sub>1</sub> regenerants with the DNeasy Plant Maxi Kit (Qiagen, Germany). PCR was performed in a 20 µl reaction containing 0.05 U Taq DNA polymerase (recombinant, Fermentas), 10 × Taq Buffer with KCl (Fermentas), 2 mM MgCl<sub>2</sub>, 0.25 mM dNTP Mix (Fermentas), 0.5 µM of each primer and 10 ng plant DNA. DNA was amplified in a Master Gradient (Eppendorf) thermal cycler programmed as follows: 94°C/2 min; 35 cycles of [94°C/30 s, 58°C/60 s, 68°C/120 s] for SIR-F/SIR-R primers, [94°C/30 s, 65°C/30 s, 68°C/90 s] for ACS-F/ACS-R primers or [94°C/30 s, 51°C/60 s, 68°C/120 s] for A-F/A-R primers; and 68°C/5 min. Products of amplification with SIR-F/SIR-R and A-F/A-R primers were resolved for 2 h in 1% agarose gel in 1 × TBE buffer. PCR products obtained after amplification with ACS-F/ACS-R primers were resolved for 4 h in 6% polyacrylamide gel in 1 × TBE buffer. After electrophoresis the gels were stained with ethidium bromide, visualized under UV transillumination and photographed.

#### CHROMOSOME PREPARATION AND GENOMIC IN SITU HYBRIDIZATION (GISH)

Twenty-four F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids were analyzed in terms of genomic in situ hybridization (GISH). Chromosome composition was examined in hybrids originating from three different cross combinations: F<sub>1</sub> *A. cepa* cv. Kutnowska × *A. roylei* ecotype 333, F<sub>1</sub> *A. cepa* cv. Bila × *A. roylei* ecotype 333, and F<sub>1</sub> *A. cepa* A5 breeding line × *A. roylei* ecotype 333.

Young roots of the hybrids were treated for 8 h in 0.03% aqueous solution of 8-hydroxyquinoline at 15°C and fixed in a 3:1 solution of methanol and glacial acetic acid. The root tips were rinsed three times in sterile water and incubated in 20 µl aqueous enzyme mixture containing 4% Onozuka cellulase (Serva Electrophoresis, Heidelberg, Germany) and 2% Pectolyase Y-23 (Sigma Aldrich), pH 4.8, for 1 h at 37°C (Plađer et al., 1998). The macerated root tips were spread by dissecting and squashing them

on grease-free slides and cooled to -20°C in a drop of fixative solution. Under a phase-contrast microscope, slides with a full set of chromosomes in well-spread metaphases (2n=16) were selected for GISH experiments.

In this experiment we used total genomic DNA extracted from young *A. roylei* (ecotype 333) and *A. cepa* (cv. Kutnowska) leaves as probe and blocking DNA, respectively. DNA isolation was done with the DNeasy Plant Maxi Kit (Qiagen). To prepare the probe, *Allium roylei* DNA was autoclaved for 4 min, which generated 100–1500 bp fragments, and labeled with digoxigenin-11-dUTP using a Nick Translation Kit (Roche Diagnostics, Germany). To prepare the block, isolated onion DNA was replicated with the REPLI-g Midi Kit (Qiagen) to increase the DNA concentration and autoclaved for 12 min, yielding 100–500 bp fragments.

The hybridization mixture (30 µl per slide), denatured before hybridization for 10 min at 80°C, contained 50% deionized formamide, 10% sodium dextran sulfate, 20 × SSC, 50 ng/µl probe DNA, 3.5 µg/µl blocking DNA and 5.5 ng/µl DNA from herring sperm (Sigma Aldrich). Slides with metaphase spreads were denatured in 70% formamide for 1.5 min at 80°C, washed in an ethanol series (70%, 90% and 100% for 5 min each) and air-dried. Hybridization was performed overnight in a humid chamber at 37°C. Steps of posthybridization washes and detection were performed according to Dong et al. (2000) with some minor modifications. Digoxigenin-labeled probes were detected with 1% fluorescein isothiocyanate (FITC)-conjugated antidigoxigenin antibody (Roche Diagnostics). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in ProLong Gold antifade reagent (Invitrogen). Slides were examined with an Axio Imager M1 microscope equipped with separate excitation filter sets for DAPI and FITC (Carl Zeiss MicroImaging). Images were captured digitally with an AxioCam and Axio Vision Release 4.6.3. (Carl Zeiss MicroImaging). We used Adobe Photoshop ver. 9.0.2 (San Jose, California, U.S.A.) to adjust the images and optimize the contrast and background of the presented figures.



TABLE 2. Production of *Allium cepa* × *Allium roylei* plants through in vitro culture

Maternal form ( <i>Allium cepa</i> )	No. of <i>A. cepa</i> inflorescences pollinated with <i>A. roylei</i> pollen	No. of ovules plated on media (A+B)	Embryo development	Plant regeneration	Plant acclimatization
A1	28	3455	130	87	83
A2	10	1166	15	7	6
A3	43	3923	176	97	95
A4	18	1306	7	1	1
A5	81	8175	387	236	226
A6	88	7703	424	209	197
'Bila'	52	5623	309	151	137
'Kutnowska'	50	4529	320	185	161
Total			1768	973	906
Mean (%)	370	35880	4.9	55.0	93.1

## RESULTS

### PRODUCTION OF F<sub>1</sub> ALLIUM CEPA × A. ROYLEI PLANTS

During the four years of the studies we pollinated 370 *A. cepa* inflorescences with *A. roylei* pollen (Tab. 2) and plated 35,880 ovules on regeneration media, from which 1768 (4.9%) embryos started to develop. Analysis of F<sub>1</sub> embryo development revealed that cv. Kutnowska was the most embryogenic (7.1%), and the least embryogenic (0.5%) was the A4 breeding line. The mean effectiveness of F<sub>1</sub> plant regeneration in vitro was 55.0%. After acclimatization to ex vitro conditions, 906 (93.1%) F<sub>1</sub> *Allium cepa* × *A. roylei* plants were produced via embryo rescue (Tab. 2).

### CONFIRMATION OF HYBRIDITY

PCR analysis with SIR-F/SIR-R, ACS-F/ACS-R and A-F/A-R primers was performed for 906 F<sub>1</sub> *Allium cepa* × *A. roylei* regenerants.

Following PCR with SIR-F/SIR-R primers, the presence of a monomorphic 400 bp product was revealed for *A. cepa*, *A. roylei* and all examined F<sub>1</sub> plants (Fig. 2). For paternal plants, the SIR marker produced an additional amplicon of 1.2 kb. The presence of this product in the profiles confirmed that the examined regenerants were interspecific *Allium cepa* × *A. roylei* hybrids.

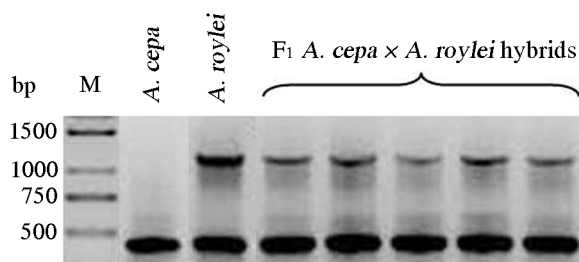
For the A marker, amplification resulted in three different fragments (Fig. 3). Two of them (350 bp, 750 bp) were characteristic of the maternal plants, while the third amplicon (800 bp) was characteristic of *A. roylei*. This product, present in electrophoretic profiles of the regenerants, confirmed their hybridity.

Use of ACS-F/ACS-R primers yielded a fragment of 760 bp, observed for both *A. roylei* ecotypes (Fig. 4). Additionally, the ACS marker generated five different amplicons (770–780 bp) characteristic of five maternal genotypes (A3, A4, A5, A6 lines, cv. Kutnowska). The profiles of F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids had two products characteristic of the maternal and paternal plants.

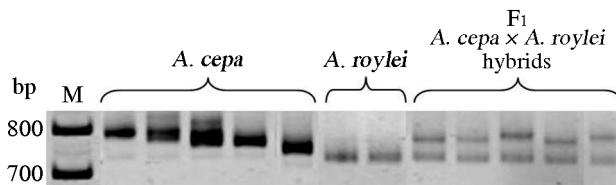
Molecular analysis carried out with SIR, A and ACS markers revealed amplicons specific to both *A. cepa* and *A. roylei* plants in 97.6% of the examined regenerants. These results confirmed that 884 of 906 plants were true F<sub>1</sub> interspecific *Allium cepa* × *A. roylei* hybrids.

### GENOMIC CONSTITUTION

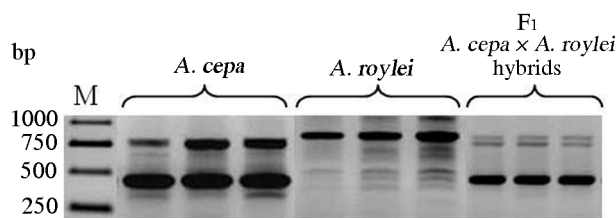
To determine their chromosome composition, we used genomic in situ hybridization (GISH) to analyze 24 interspecific F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids. Both parental species have 16 chromosomes (2n). GISH revealed that all the F<sub>1</sub> hybrids also had 16 chromosomes (2n). No differences in chromosome morphology, deletions or additional chromosomes were observed in the spread metaphases of any of the examined plants (Fig. 5a–c). We employed total genomic DNA from *A. roylei* as probe together with *A. cepa* as blocking DNA to determine the parental origin of the chromosomes of F<sub>1</sub> *A. cepa* cv. Kutnowska × *A. roylei* ecotype 333, F<sub>1</sub> *A. cepa* cv. Bila × *A. roylei* ecotype 333, and F<sub>1</sub> *A. cepa* A5 breeding line × *A. roylei* ecotype 333 hybrids (Fig. 5a–c). In GISH profiles of every analyzed hybrid plant, eight chromosomes hybridized with *A. roylei* probe DNA and showed green fluorescence; the other eight chromosomes displayed blue fluorescence, confirming their maternal origin. No recombinant chromosomes were observed in the examined metaphase spreads (Fig. 5a–c).



**Fig. 2.** Nuclear DNA analysis with SIR-F/SIR-R primers. *A. cepa* cv. Kutnowska (lane 1); *A. roylei* ecotype 333 (lane 2); F<sub>1</sub> *A. cepa* × *A. roylei* hybrids (lanes 3–7); M – DNA size standard (1 kb DNA Ladder Plus, Fermentas)



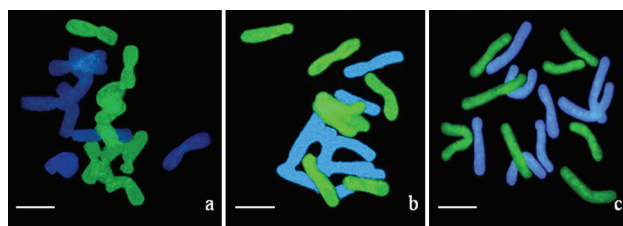
**Fig. 4.** Polyacrylamide gel electrophoresis of PCR products amplified with ACS-F/ACS-R primers. *A. cepa*: A4 breeding line (lane 1); A6 breeding line (lane 2); A5 breeding line (lane 3); A3 breeding line (lane 4); cv. Kutnowska (lane 5); *A. roylei*: ecotype 333 (lane 6); ecotype 334 (lane 7); F<sub>1</sub> *A. cepa* × *A. roylei* hybrids (lanes 8–12); M – DNA size standard (100 bp DNA Ladder Plus, Fermentas)



**Fig. 3.** Nuclear DNA analysis with A-F/A-R primers. *A. cepa*: cv. Kutnowska (lane 1); A1 breeding line (lane 2); A2 breeding line (lane 3); *A. roylei*: ecotype 333 (lanes 4, 5); ecotype 334 (lane 6); F<sub>1</sub> *A. cepa* × *A. roylei* hybrids (lanes 7–9); M – DNA size standard (1 kb DNA Ladder Plus, Fermentas)

## DISCUSSION

Embryo rescue methods have enabled successful intergeneric and interspecific hybridization of many vegetables (*Lycopersicon*, *Solanum*), cereals (*Oryza*, *Triticum*) and fruits (*Actinidia*, *Fragaria*), helping to expand genetic variability within many plant genera (Sharma et al., 1996). This technique has also helped increase the pool of genetic resources within the genus *Allium*. Crosses between onion and numerous distant *Allium* species followed by embryo development in vitro have produced a considerable number of interspecific hybrids (Gonzalez and Ford-Lloyd, 1987; Keller et al., 1996). In vitro culture has also been applied to produce interspecific hybrids within edible Alliaceae. After crossing *Allium cepa* and *Allium fistulosum* plants and culturing hybrid embryos, Dolezel et al. (1980) obtained both reciprocal interspecific *A. cepa* × *Allium fistulosum* hybrids. Nascent embryo rescue yielded interspecific hybrids between onion and fertile garlic (Ohsumi et al., 1993). Yanagino et al. (2003) used ovary culture to produce an interspecific *A. ampeloprasum* × *A. sativum* hybrid, and Peterka et al. (1997) combined ovary and ovule culture to hybridize onion with leek. Unfortunately, embryo rescue technique proved



**Fig. 5.** Genomic in situ hybridization (GISH) of root preparations of F<sub>1</sub> *A. cepa* × *A. roylei* hybrids (2n = 16). Hybridization signals on mitotic chromosomes. (a) F<sub>1</sub> *A. cepa* cv. Kutnowska × *A. roylei* ecotype 333 hybrid, (b) F<sub>1</sub> *A. cepa* cv. Bila × *A. roylei* ecotype 333 hybrid, (c) F<sub>1</sub> *A. cepa* A5 breeding line × *A. roylei* ecotype 333 hybrid. Paternal genomic probes were detected with fluorescein isothiocyanate (FITC)-conjugated antidigoxigenin antibody (*A. roylei*, green signals). The other DAPI-counterstained areas belong to *A. cepa* (blocking DNA, blue fluorescence). Bars = 10 μm

ineffective in an attempt to obtain interspecific *A. cepa* × *A. schoenoprasum* hybrids (Gonzalez and Ford-Lloyd, 1987).

In this study we obtained a large population of F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids through embryo culture. To our knowledge this is the first report of production of such hybrids in culture in vitro. Others have succeeded in hybridizing *A. cepa* with *A. roylei*, but van der Meer and de Vries (1990) and Scholten et al. (2007) obtained only individual hybrid plants from seeds harvested after hand-pollination of both parental species. We achieved 2.5% overall efficiency of production of interspecific F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids through embryo rescue. This compares with 1.6% mean efficiency of production of F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids from seeds harvested after crossing *A. cepa* and *A. roylei* plants (data not shown). These results are in accord with those of Dolezel et al. (1980) and Gonzalez and Ford-Lloyd (1987), who used embryo rescue technique to increase the effectiveness of reciprocal *Allium cepa* × *A. fistulosum* hybridiza-

tion versus normal seed sowing by 40% and 70%, respectively. In vitro culture evidently can facilitate hybrid embryo development and overcome genetic incompatibility between taxonomically distant parental species and the post-fertilization barriers that hinder seed maturation in planta.

The difference in the amount of DNA between *Allium cepa* (33.5 pg DNA/2C) and *A. roylei* (28.5 pg DNA/2C) is only 5 pg (Khrustaleva and Kik, 1998). Due to the similarity of nuclear DNA content of the parental species, the hybridity of the obtained F<sub>1</sub> *Allium cepa* × *A. roylei* plants could not be examined by flow cytometry. That is why we verified the hybrid character of the obtained F<sub>1</sub> regenerants by nuclear DNA analysis. We know of only a few reports on practical application of molecular markers to confirm the hybrid nature of plants obtained from crosses within the genus *Allium* (Ohsumi et al., 1993; Bark et al., 1994; Yanagino et al., 2003), so it seemed worthwhile to develop molecular markers to differentiate *A. cepa* and *A. roylei* accessions, which would be useful tools for confirming the hybrid nature of plants obtained from crossing the two species. In this study we developed three novel, independent molecular markers which confirmed almost all (97.6%) of the regenerants as true interspecific hybrids. As expected, each marker disclosed polymorphic fragments from both parental species in the molecular profiles of F<sub>1</sub> interspecific hybrids. These molecular studies developed three nuclear DNA markers useful in assessing the level of DNA polymorphism between *A. cepa* and *A. roylei* species. The high percentage of F<sub>1</sub> plant population hybridity demonstrates that embryo rescue technique is an effective method for producing F<sub>1</sub> *Allium cepa* × *A. roylei* hybrid plants.

In this work, genomic in situ hybridization (GISH) clearly distinguished *A. cepa* and *A. roylei* chromosomes in the F<sub>1</sub> hybrids. The overriding aim of this project is to introgress the downy mildew resistance locus from *A. roylei* into the bulb onion genome. This method should prove useful for genomic constitution analysis of subsequent backcross (*A. cepa* × *A. roylei*) × *Allium cepa* generations. Previous studies on introgression of downy mildew resistance into onion based on AFLP markers and GISH analysis revealed that the *A. roylei* fragment harboring the downy mildew resistance probably is at the distal end of the long arm of chromosome 3 of the analyzed backcross progenies (van Heusden et al., 2000b; Scholten et al., 2007).

The next step of our investigation will entail advanced backcrosses of the obtained F<sub>1</sub> *Allium cepa* × *A. roylei* hybrids with the bulb onion in order to eliminate undesirable *A. roylei* traits. Preliminary studies in this area suggest that due to the genetic distance between *A. cepa* and *A. roylei*

species, the fertility of F<sub>1</sub> hybrid plants might be impaired, probably as a result of lack of homology between the parental genomes. Currently our research focuses on explaining the barriers that disallow successful (*Allium cepa* × *A. roylei*) × *Allium cepa* backcrosses.

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