Acta Societatis Botanicorum Poloniae

SHORT COMMUNICATION Acta Soc Bot Pol 83(1):75–79 DOI: 10.5586/asbp.2013.037
Received: 2013-05-22 Accepted: 2013-12-12 Published electronically: 2014-03-18

Production of intergeneric allotetraploid between autotetraploid non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) and autotetraploid radish (*Raphanus sativus* L.)

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Abstract

Intergeneric hybrids between non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino; 2n = 4x = 40) and radish (*Raphanus sativus* L.; 2n = 4x = 36) were obtained through ovary culture and embryo rescue. Some hybrid embryos (0.11 per ovary) were produced, but only 4 of them germinated. As most hybrid embryos failed to develop into plantlets directly, plants were regenerated by inducing shoots on the cultured cotyledon and inducing roots on the root induction medium. All hybrid plants were morphologically uniform. They resembled the non-heading Chinese cabbage in the long-lived habit, the plant status, the vernalization requirement and the petiole color, while the petiole shape, leaf venation pattern and flowers were more similar to those of radish. Upon examination of the flowers, these were found to have normal pistil, but rudimentary anthers with non-functional pollen grains. The somatic chromosome number of F1 plants was 38. Analysis of SSR banding patterns provided additional confirmation of hybridity.

Keywords: intergeneric allotetraploid; embryo culture; simple sequence repeat

Introduction

It is estimated that 47% of all flowering plants and 95% of all pteridophytes are polyploids and that the majority of these are allopolyploids [1,2], which is a widespread and major force of evolution in plants [3]. Allopolyploidy is often accompanied by major structural, cytogenetic, epigenetic and functional changes to the genome, leading to new phenotypes and to reproductive isolation [4,5]. In addition, the permanent heterozygosity fixation of the allopolyploid [6] has the potential to offer a substantial heterozygote advantage. Despite these potential benefits, allopolyploid is an enormous challenge with the orchestration of gene expression, DNA replication, and chromosome pairing. For these reasons, investigation of allopolyploids is very important. The newly synthesized allopolyploid is an ideal model system since it can offer an opportunity to study the response to this genomic change from defined parents.

Allopolyploid formation can occur by two main pathways, the so-called "one-step" and "two-step" models [5]. In the one-step model, the allopolyploid arises directly from an interspecific cross by the fusion of either two unreduced (2n) gametes from diploid parents or two normal (n = 2x) gametes from tetraploid parents. By contrast, in the two-step model, an interspecific F1 hybrid is first formed and the polyploidy is derived from it either from a fertile shoot generated by meristematic tissues having experienced a somatic doubling or by fusion of two 2n gametes produced by the F1 hybrid itself [6]. The production of 2n gametes appears to occur at a surprisingly low rate. Ramsey and Schemske estimated its frequency at 0.56% [7]. Although with the use of colchicine the frequency of the somatic doubling has increased a lot, the effective diploidization rate is still very low (10.5%) [8]. In addition, problems with chimeras, abnormal phenotypes and sterility also occur [9]. Little attention, however, has been focused on the use of this method, although a "synthetic" allotetraploid had been obtained by crossing a tetraploid *Arabidopsis thaliana* (2n = 4x = 20) and *A. arenosa* (2n = 4x = 32) [10].

In the crop *Brassica*, breeders have resorted to varying degrees of hybridization involving close relatives of it in their search for novel traits in developing new and improved varieties [11]. The non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) is a main vegetable, which grows in south of China, and it has a long history of cultivation in our country. Radish (*Raphanus sativus* L.) is cultivated worldwide. It possesses desirable agronomic characters, such as resistance to white rust (*Albugo candida*) [12], BCN (*Heterodera schachtii*) [13,14] and culbroot (*Plasmodiophora brassicae*) [15], as well as resistance to pod shattering [16]. Besides, various related wild species have attracted research attention as potential germplasms

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Handling Editor: Beata Zagórska-Marek

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for improvement of *Brassica* crops [17]. Therefore, it can be used as a gene donor for the modification of the non-heading Chinese cabbage. Here, we report the successful production of allotetraploid by intergeneric hybridization between autotetraploid non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) and autotetraploid radish (*Raphanus sativus* L.) aimed at enriching the gene pool of the non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) and creating useful research material for further understanding of the relationship and the genomic structure between the two genera.

Material and methods

Plant material and intergeneric hybridization

The plant material consisted of the two autotetraploid cultivars, non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino; maternal parent, 2n = 4x = 40) and radish (*Raphanus sativus* L.; paternal parent, 2n = 4x = 36). Seeds of two cultivars were grown on experimental fields of Jiangpu Farm, Nanjing Agricultural University. Flowers were protected from foreign pollen two days before anthesis and intergeneric crosses were made by hand.

Embryo culture

The ovaries were excised 5–10 days after pollination and sterilized with 70% ethanol for 30 s followed by a sodium hypochlorite solution containing 1% active chlorite for 10 min. After washing in sterile distilled water three times, the ovaries were cultured on MS [18] hormone free solid medium containing 500 mg/l casein hydrolysate. Fifteen days later, the embryos were isolated from the ovaries and transferred to hormone-free MS medium supplemented with 5% coconut milk and 500 mg/l casein hydrolysate. As most embryos failed to develop directly into plantlets [19], cotyledons were cut off and cultured on MS medium containing 2 mg/l 6-benzyladenine (BA) and 0.1 mg/l NAA and 5% coconut milk in order to induce shoot regeneration. After the shoots regenerated, they were transplanted to hormone-free ½ MS medium supplemented with 0.2 mg/ml NAA for root induction. All cultures were incubated at 25°C in a 16-hour photoperiod. The embryo-rescued plants with well-developed root system were hardened for 4–8 days at 10–12°C, 14-hour photoperiod and then transferred into pots with soil for normal growth under glasshouse conditions [20].

Characterization of hybrids

Hybrid identity of F1 plants was confirmed by morphological examination, chromosome analysis and further characterize by simple sequence repeat (SSR) analysis.

Chromosome counts were carried out on root tips from hybrid plants and pretreated with 0.002 M 8-hydroxyquinoline for 4 hours. Material was fixed in 3:1 alcohol-glacial acetic acid, hydrolyzed in 1 N HCl 60°C for 6 min and stained with leuco-basic fuchsine 30 min, and then squash preparations were made using 45% acetic acid [21]. About 0.3 g fresh leaves were used to extract genomic DNA using the cetyl-trimethyl-ammonium bromide (CTAB) method [22]. An Eppendorf protein and nucleic determine instrument was used for determining DNA concentration. Primer sequences for SSR markers obtained from various sources [23–25] were used (Tab. 1). The SSR reactions were performed in a 20 μl volume containing 60 ng DNA, 0.5 μmol/l forward and reverse primer, 0.2 mmol/l dNTPs, 1.0 mmol/l $MgCl_2$, and 0.5 U Taq DNA polymerase. The PCR procedure was programmed at 95°C for 2 min, 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles, and then 72°C for 10 min. The products were separated on 5% vertical polyacrylamide gel. The gel was run at a 150 V constant voltage for 1 to 1.5 h before silver staining.

For cytological studies young anthers were fixed in Carnoy solution and squashed in 1% acetocarmine [26].

Results

Embryo culture

After 1–2 weeks of culture, the ovaries were observed turgid. Of all the 100 ovaries only 11 embryos developed to the mature cotyledonary stage after 3 weeks in culture (Tab. 2). Of 11 cultured embryos, 4 embryos germinated (germination rate = 36.3%) with the help of embryo culture in vitro. The 4 embryos germinated halted their development when their shoots were 1 cm in length, and did not lead to whole plants. Regeneration plantlets were induced from the cotyledon sections of the 4 germinated embryos on MS

Tab. 1 Primers for SSR marker assays.

Primer name	Forward primers	Reverse primers
FITO 137	ATGGGTAAGTCTCGTAAATG	AAACCGAATAAACCGAAA
Na10F06	CTCTTCGGTTCGATCCTCG	TTTTTAACAGGAACGGTGGC
Na12H09	AGGCGTCTATCTCGAAATGC	CGTTTTTCAGAATCTCGTTGC
Ni4A03	ACACAGAAACATCAAACATACC	GGACCGGTTTTATTTGTTCG
Ol09A06	TGTGTGAAAGCTTGAAACAG	TAGGATTTTTTTGTTCACCG
Ol10F11	TTTGGAACGTCCGTAGAAGG	CAGCTGACTTCGAAAGGTCC
Ol12F11	AAGGACTCATCGTGCAATCC	GTGTCAGTGGCTACAGAGAC
Na14D09	GATCAACGTAAGGTCGCCTC	GAATCCAACGGATCAGAAGC

Tab. 2 Embryo production from ovary culture in hybridizations between *B. campestris* ssp. *chinensis* Makino and *R. sativus*.

Crosses	No. of cultured ovaries	No. of embryos produced	No. of embryos survived	Rate of germination (%)
B. campestris ssp. *chinensis* Makino × *R. sativus*	100	11	4	36.3

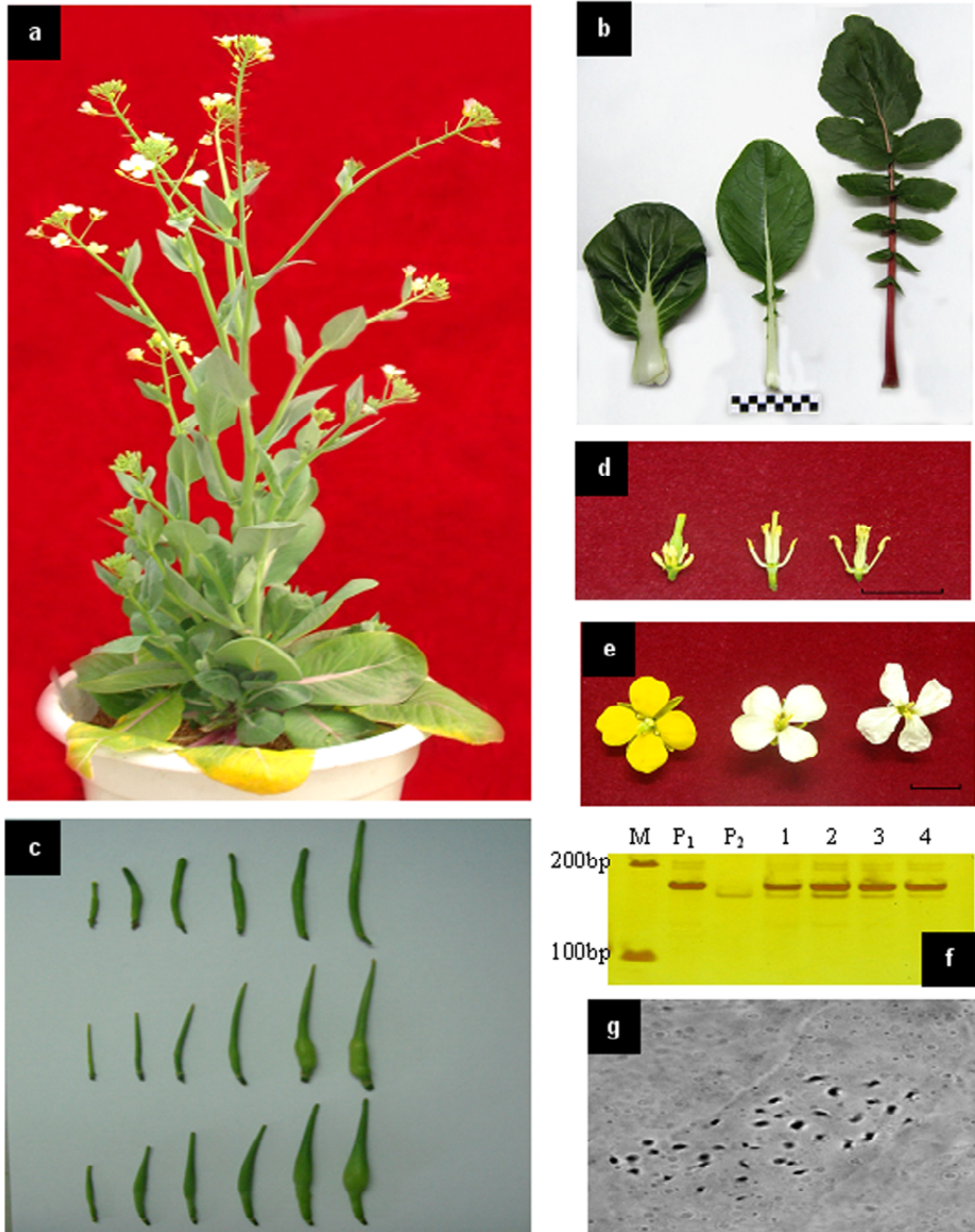

Fig. 1 Hybrid identity of F1 plants. **a** Flowering stage of F1. **b** Leaves of *B. campestris* (left), *R. sativus* (right) and F1 (middle). **c** Siliques of *B. campestris* (up), *R. sativus* (middle) and F1 (bottom). **d** Stamens and pistil of *B. campestris* (left), *R. sativus* (right) and F1 (middle). **e** Flower of *B. campestris* (left), *R. sativus* (right) and F1 (middle). **f** Amplification results of primer Na14D09 on *B. campestris* (P1), *R. sativus* (P2) and F1 (1, 2, 3 and 4). **g** Chromosome number of F1 (2n = 4x = 38; 1000×). Scale bars: **b–e** 1 cm.

medium supplemented with 2.0 mg/l 6-benzyladenine and 0.1 mg/l NAA, and then they were transplanted to the root induction medium. A total of 4 lines were obtained with several numbers of plants.

Characterization of hybrids

All hybrid plants were morphologically uniform and grew vigorously.

The hybrids were intermediate in size and shape (Fig. 1a). They resembled the non-heading Chinese cabbage in the long-lived habit, the plant status, the vernalization requirement, the petiole color (Fig. 1b). Petiole and silique shape, leaf venation pattern and flower color were more similar to those of radish (Fig. 1c–e). Upon examination of the flowers, these were found to have normal pistil, but rudimentary anthers with non-functional pollen grains (Fig. 1d).

The somatic chromosome number of the regenerated plants was counted at the middle stage of cell division. The results showed that the chromosome number of all plants tested was 38 (Fig. 1g), indicating that these regenerated plants were all true hybrids of *B. campestris* (2n = 4x = 40) ×*R. sativus* (2n = 4x = 36).

Out of 8 SSR primer pairs, only 1 pair (12.5%) SSR primers (Tab. 1) had polymorphic between parents gnomic DNA. All of the hybrids were tested by SSR analysis with 8 primer pairs. SSR analysis indicated that the hybrids had hybrid patterns containing characteristic bands from *R. sativus* in addition to the *B. campestris*, which exhibits codominant (Fig. 1f).

Discussion

The present paper describes the production of a new allotetraploid by intergeneric hybridization between autotetraploid non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) and autotetraploid radish (*Raphanus sativus* L.) through ovary culture and embryo rescue. We found that the production of allotetraploid was about 4%, which compared favorably with percentages reported for *B. napus* × *D. siifoIia* (11.5%) [27] and *B. campestris* × *M. arvensis* (5%) [19]. The interspecific hybridizations we obtained confirm that crossing between tetraploid parents is a useful method in producing synthetic allotetraploid. In addition, the use of two tetraploid parents offers a good model for the study of a one-step process of polyploidization whereby, in nature, unreduced gametes of two diploid parents can yield allotetraploid progeny.

For cytological studies, no functional pollen grains have been found and no seeds were obtained by selfing and crossed with two parents, which shows that the F1 maybe male sterile.

The intergeneric hybrids between non-heading Chinese cabbage and radish were successfully produced and characterized. These will be the base material for developing the whole set of *R. sativus*-*B. campestris* additions in future for genome analysis and chromosomal localization of genes. In addition, the agronomical potential of the hybrid progenies obtained by selfing or backcross are under current evaluation on their advancement, improvement and exploitation. Furthermore, this hybrid plant offers an ideal model system to study the response to genomic changes from defined parents, such as structural rearrangements on the chromosome level [28] and sequence level [29,30], regulation of gene expression [31], activation of transposons [32], and amplification, reassortment, or elimination of highly repetitive sequences [33] and low-copy sequences [34].

Acknowledgments

Authors are grateful to Jiangsu province science and technology support program (BE201130172) for financial support.

Authors' contributions

The following declarations about authors' contributions to the research have been made: designing research: ZSN; performing experiments: ZSN, SCZ, ZJS; writing the manuscript: ZSN, SCZ, LY.

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