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ORIGINAL PAPER

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# *Cucumis* monosomic alien addition lines: morphological, cytological, and genotypic analyses

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**Abstract** *Cucumis hystrix* Chakr. (HH, 2*n*=24), a wild relative of the cultivated cucumber, possesses several potentially valuable disease-resistance and abiotic stress-tolerance traits for cucumber (*C. sativus* L., CC, 2*n*=14) improvement. Numerous attempts have been made to transfer desirable traits since the successful interspecific hybridization between *C. hystrix* and *C. sativus*, one of which resulted in the production of an allotriploid (HCC, 2*n*=26: one genome of *C. hystrix* and two of *C. sativus*). When this genotype was treated with colchicine to induce polyploidy, two monosomic alien addition lines (MAALs) (plant nos. 87 and 517: 14 CC+1 H, 2*n*=15) were recovered among 252 viable plants. Each of these plants was morphologically distinct from allotriploids and cultivated cucumbers. Cytogenetic and molecular marker analyses were performed to confirm the genetic constitution and further characterize these two MAALs. Chromosome counts made from at least 30 meristematic cells from each plant confirmed 15 nuclear chromosomes. In pollen mother cells of plant nos. 87 and 517, seven bivalents and one univalent were observed at diakinesis and metaphase I; the frequency of trivalent formation was low (about 4–5%). At anaphase I and II, stochastic and asymmetric division led to the formation of two gamete classes: *n*=7 and *n*=8; however, pollen fertility was relatively high. Pollen stainability in plant no. 87 was 86.7% and in plant no. 517 was 93.2%. Random amplified polymorphic DNA analysis was performed using 100 random 10-base primers. Genotypes obtained with eight primers (A-9, A-11, AH-13, AI-19, AJ-18, AJ-20, E-19, and N-20) showed a band common to the two MAAL plants and *C. hystrix* that was absent in *C. sativus*, confirming that the alien chromosomes present in the MAALs were derived from *C. hystrix*. Morphological differences and differences in banding patterns were also observed between plant nos. 87 and 517 after amplification with primers AI-5, AJ-13, N-12, and N-20, suggesting that these plants may contain different *C. hystrix* chromosomes.

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## Introduction

The genus *Cucumis* consists of about 30 species, two of which, *C. sativus* L. (CC, 2*n*=14) and *C. melo* L., are cultivated as major vegetable crops (Kirkbride 1993). The genetic variation in cultivated cucumber (*C. sativus* L.) is relatively limited (Kupper and Staub 1988). Resistance to several agriculturally important diseases and pests has been located in wild *Cucumis* species, including powdery mildew caused by *Sphaerotheca fuliginea* (Schlechtend.: Fr) Pollacci, downy mildew caused by *Pseudoperonospora cubensis* (Berk. and M.A. Curtis) Rostovzev, anthracnose caused by *Colletotrichum orbiculare* (Berk. and Mont) Arx, and fusarium wilt caused by *Fusarium oxysporum* Schlechtend.: Fr. (Kirkbride 1993; Leppik 1966; Lower and Edwards 1986; Thomas 1986). Because these diseases severely affect commercial production, there is an urgent need to broaden the gene pool of cucumber by introgressing economically important resistance genes from diverse sources to meet the challenges facing future cucumber production.

Interspecific hybridization represents an important strategy for broadening the genetic base of cucumber (Nijs and Custer 1990), especially for resistance breeding.

Gene introduction from wild species has suffered, however, due to strong interspecific crossing barriers and poor vigor in interspecific hybrids (Deakin et al. 1971; Franken et al. 1988). Monosomic alien addition lines (MAALs) that carry only one chromosome from wild species can improve the process of gene introduction to elite germplasm (Gao et al. 2001; Khush 1973). Gene introgressions through MAALs have been accomplished in a number of crops such as wheat (Knott 1961; Sears 1956), rice (Jena and Khush 1989), and sugar beet (Gao and Jung 2002). In addition, the potential use of MAALs includes chromosomal assignment of molecular markers or dominant plant traits (Chen et al. 1992; Peffley et al. 1985; Suen et al. 1997), molecular mapping of alien genes (Jung et al. 1992), the construction of chromosome-specific libraries (Schmidt et al. 1990), and production of disomic addition lines.

The wild relative of cultivated cucumber, *C. hystrix* (HH, 2*n*=24), is a potentially valuable source of many economic traits, including resistance to the root knot nematode (Chen and Kirkbride 2000), tolerance to low irradiance and temperature (Qian et al. 2002; Zhuang et al. 2002), and resistance to downy mildew (unpublished data). During past years, progress has been made towards achieving gene introgression from *C. hystrix*, following synthesis of a new fully fertile amphidiploid species *C. hytivus* (HHCC, 2*n*=38) (Chen and Kirkbride 2000; Chen et al. 2003b) and the production of partially fertile allotriploids hybrids from a mating between *C. hytivus* and *C. sativus* (HCC, 2*n*=26) (Chen et al. 2003a). Nevertheless, clear evidence of the introgression of *C. hystrix* chromosomes into a *C. sativus* background is still lacking. The creation and strategic development of MAALs for cucumber breeding could be a key step in realizing gene transfer into this economically important vegetable crop species. In this paper, we report and characterize two MAALs in *Cucumis* that originated from somaclonal variation during in vitro culture and colchicine treatment. The ultimate aim of this work will be the transfer of genes from *C. hystrix* to cultivated cucumber and enhanced understanding of the genetic and genomic structure of the genus.

## Materials and methods

### Plant materials

In February 2002, shoots of *Cucumis* allotriploid (HCC) plants were treated with different concentrations (0.0%, 0.2%, 0.4%, and 0.8%) of colchicine for 1, 2, and 3 days. At least 30 shoots were used in each treatment. The treated shoots were rinsed with sterilized water and transferred to MS media (Murashige and Skoog 1962) +6-BA 0.2 mg/l for shoot elongation. Subsequently, explants were cultured on 1/2MS+IBA 0.2 mg/l to root. Ten days later, well-rooted shoots were rinsed with sterilized water to remove residual rooting media, and then shoots were transferred to 128-plug trays (55.0×27.5×5.0 cm, Agricultural Developing Company of Lide, Beijing, China) containing a sterilized mixture of sand and soil (3:1=v:v) for acclimatization in greenhouse conditions.

To reduce transpiration, a clear, 0.05-mm-thick, polyvinyl plastic cover was placed over each plug tray, and trays were placed in a controlled environment chamber held at 25±1°C with relative humidity at 80% and irradiance at 200 μmol $m^{-2}s^{-1}$. Plastic covers were gradually removed when most of the plantlets exhibited new vegetative growth (~7 days). Acclimatized plants (5–8 cm in length) were transferred to a greenhouse after the cover had been completely removed (about 2 weeks after initiating acclimatization). All surviving plants which had been treated with colchicine, and all other genotypes used in this study, were transplanted to a research farm managed by Nanjing Agricultural University, Nanjing, China in April 2002. Fifteen plantlets were planted in the field to represent each genotype. *C. hystrix*, *C. sativus* cv. Beijing jietou, and *C. hytivus* were germinated from seeds. The *Cucumis* allotriploid (*C. hytivus* × C. *sativus* cv. Beijing jietou) was from plantlets generated in vitro.

### Cytogenetic identification

#### *Mitotic observation*

Young actively growing tendrils (1–3 cm long) from growing plantlets were used to determine chromosome number of plants derived from colchicine treatment according to the methods of Chen and Qian (2002). Tendrils were pretreated in 8-hydroxyquinoline supplemented with 2–3 drops of *p*-dichlorobenzene for 2 h at 18°C. Tendrils were then fixed in Carnoy's solution (3 glacial acetic acid:2 chloroform:5 ethanol=by vol.) for 24 h, and stored at 4°C in 70% (v/v) alcohol until examination. Tendrils were hydrolyzed in 1 N HCl for 10 min at 60±1°C and then stained with acetocarmine. Stained tendrils were squashed in 45% acetic acid. At least 30 meristematic cells with well-spread chromosomes were observed in each tendril examined.

#### *Meiotic observation*

For meiotic analysis, flower buds 1.0–2.5 mm in length were selected, fixed in Carnoy's solution for 24 h, and stored in 70% ethanol at 4°C until analysis. The anthers were cut into two parts with a razor blade and then stained with acetocarmine. Analyses of meiotic chromosomes were performed at pachytene, diakinesis, metaphase I, anaphase I, and anaphase II. At least 100 pollen mother cells from each ploidy level were examined at different meiotic stages. Fertility was assessed by observing pollen shape and stainability. At least 1,000 mature pollen grains were stained in 1% acetocarmine and analyzed according to Momotaz et al. (1998). Images of *Cucumis* chromosomes were recorded with Kodak film using a single-lens reflex camera attached to an Olympus (BX-51) microscope system at 1,500× magnification.

### Random amplified polymorphic DNA analysis

Total cellular DNA was extracted from 0.2 g of young leaf tissue from each genotype examined (plant no. 517 and plant no. 87, *C. hystrix*, *C. hytivus*, *C. sativus* cv. Beijing jietou, and the allotriploid) using a simplified 2% CTAB method (modified from Murray and Thompson 1980). RNA was removed with 1,000 μg/l pre-boiled RNase (Sangon Company, Shanghai, China), incubated 30 min at 37°C. DNA quality and quantity were determined by electrophoresis in 0.8% agarose gels and by spectrophotometry, then subsamples were diluted to 10 ng·$\mu l^{-1}$ for PCR (Sambrook et al. 1989).

One hundred 10-base pair primers from Sangon Company were screened for this study. The PCR was performed in a final volume of 20 μl containing 50 mM KCl, 10 mM Tris (pH=9.0), 0.1% tetramethyl ammonium chloride, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM 10-base primer, 40 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase (Sangon). Amplification was performed with a thermocycler (PTC-100; MJ Research Inc., Chatham, N.J., USA) according to the procedure described by Staub et al. (1996). Samples were denatured for 4 min at 94°C and three cycles at 94°C

for 15 s; annealed for 15 s at 35°C; extended for 75 s at 72°C; then 40 cycles at 94°C/15 s, 40°C/15 s, and 72°C/75 s; the last cycle had an extra 7 min for the extension period at 72°C followed by 4°C. All RAPD products were visualized by electrophoresis (1.0% agarose gels) and staining with 5 μg/ml ethidium bromide following the standard method (Sambrook et al. 1989). The gel was immediately photographed with a Seagull camera (Shanghai, China).

## Results

### Production and identification of putative MAALs

Of the 360 shoots treated with colchicine at different concentrations for different lengths of time, 252 survived after acclimatization. Longer treatment times and higher colchicine concentration resulted in decreased survival rates, which varied from 93.3% to 26.7% (Table 1). During early development of surviving plantlets, leaves appeared abnormal, presumably due to the colchicine treatment. These plants quickly recovered, however, and appeared similar to the control genotype (allotriploid, no colchicine treatment) except for four plants designated plant nos. 13, 43, 87, and 517. Plant nos. 87 and 517 had been treated with 0.4% colchicine for 3 days, and nos. 13 and 43 had been treated with 0.8% colchicine for 2 days and 3 days, respectively. The chromosome number of plant nos. 87 and 517 was determined to be $2n$=15; in each case, one chromosome appeared smaller than the others (Fig. 2A), suggesting that they were likely to be MAALs or trisomics. Chromosome number differed from cell to cell in plant nos. 13 and 43; therefore, they were considered mixoploid.

### Characterization of MAALs

#### *Morphological characteristics*

Both putative MAALs, plant nos. 87 and 517, grew more slowly and were easily differentiated morphologically from the allotriploids, *C. hytivus*, and *C. sativus* (Table 2). The leaf shape of these plants was palmate and hastate, respectively, in contrast to the pentagon shape observed in allotriploid and the wavy leaf edges observed in the amphidiploid *C. hytivus* (Fig. 1A). Fruit length of plant nos. 87 and no. 517 was 23.6±0.9 cm and 25.4±1.5 cm, respectively, much longer than the allotriploids (12.1±2.1 cm) (Fig. 1B, C). Both plants showed white spines, characteristic of *C. sativus*, in contrast to black spines observed in progenies of the interspecific hybrids from the female parent, *C. hystrix*; therefore, we can assume that the gene for black spines is not located on the alien chromosome in either plant no. 87 or no. 517. Both MAALs also showed a multibranching character reminiscent of *C. hystrix*.

**Table 1** Treatment of *Cucumis* allotriploid tendrils with different concentrations of colchicine

| Concentration of colchicine (%) | 0 | | | 0.2 | | | 0.4 | | | 0.8 | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment length (days) | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | – |
| No. of treated shoots | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 360 |
| No. of surviving shoots | 28 | 27 | 25 | 26 | 23 | 21 | 26 | 17 | 12 | 25 | 14 | 8 | 252 |
| Frequency of survival (%) | 93.3 | 90 | 83.3 | 86.7 | 76.6 | 70 | 86.7 | 56.7 | 40 | 83.3 | 46.7 | 26.7 | 70.0 |
| No. of MAALs (monosomic alien addition lines) and mixoploids | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2[a] | 0 | 1[b] | 1[b] | 4 |

[a] MAAL
[b] Mixoploid

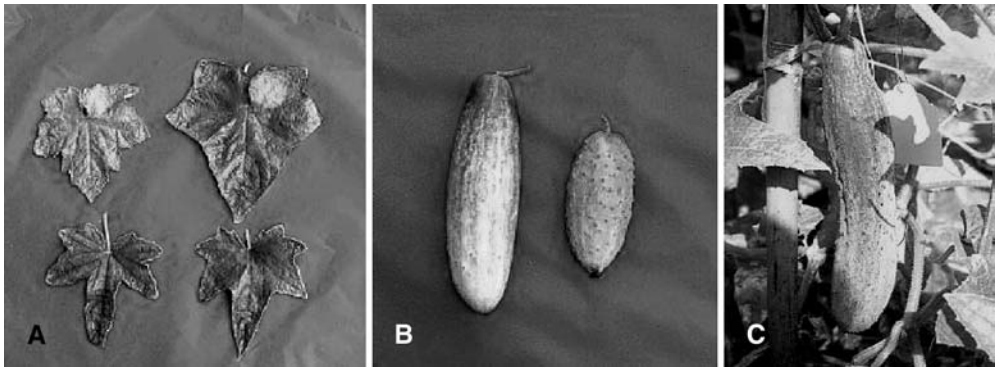


**Fig. 1A–C** Morphology of the monosomic alien addition lines (MAALs) and interspecific hybrids at different ploidy levels. **A** Shape of leaves, pentagon shape for amphidiploids *Cucumis hytivus* (left-hand, upper row) and allotriploid that derived from *C. hytivus*×*C. sativus* cv Beijin jietou (*right-hand, upper row*), palmated in plant no. 87 (*left-hand, lower row*) and hastate in plant no. 517 plants (*right-hand, lower row*). **B** Typical fruit of allotriploid (*left*) and amphidiploid (*right*). **C** Typical fruit of MAAL no. 517

**Table 2** Morphological characteristics of four *Cucumis* genotypes

| Character | Plant no. 517 | Plant no. 87 | Allotriploid | *C. sativus* cv. Beijing jietou |
|---|---|---|---|---|
| Fruit length | 25.4±1.5 | 23.6±0.9 | 12.1±2.1 | 51.7±3.5 |
| Sepal length | 0.6±0.1 | 1.1±0.2 | 0.7±0.1 | 1.5±0.3 |
| Branch number | 9.0 | 8.0 | 4.9 | 1.3 |
| Spine color | White | White | Black | White |
| Leaf color | Dark green | Yellow and ceraceous | Dark green | Dark green |

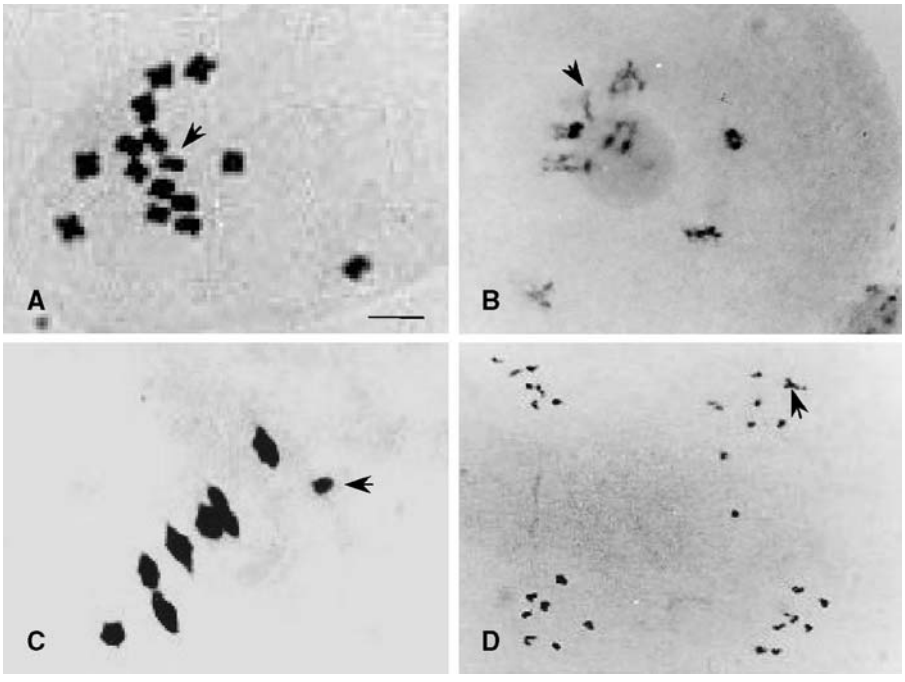


**Fig. 2A–D** Cytological analysis of two MAALs in *Cucumis* (2*n*=15) originating from *C. hystrix* (HH) × *C. sativus* (CC) ("C" represents genome from *C. sativus* and "H" represents genome from *C. hystrix.*). **A** Metaphase chromosomes in MAALs (2*n*=15=14CC + 1H), *Arrow* indicates the putative alien chromosome. **B** Diakinesis chromosomes, 7 bivalents and 1 univalent (*arrow*). **C** Metaphase I showing 7 bivalents and 1 univalent (*arrow*). **D** Telophase II showing 7 chromosomes at the two left poles while 8 chromosomes are at the two right poles (*arrow* indicates two chromosomes)

The two MAALs differed from each other with respect to several morphological characters. Plant no. 87 had yellowish leaves covered with wax, while plant no. 517 had dark green leaves. In addition, the sepals in plant no. 87 were bigger than those of plant no. 517 (Table 2). Pollen fertility was 86.7% and 94.5% for plant nos. 87 and 517, respectively, considerably higher than observed in the allotriploid, which was 9.8%.

## Cytogenetic analysis

The somatic chromosomes of *C. hystrix* are smaller in size than those of *C. sativus* (Chen et al. 2003b). In mitotic cells, one extra chromosome was present in both MAALs that was much smaller than the other 14 chromosomes (Fig. 2A), presumably derived from *C. hystrix*. The MAAL karyotypes were also confirmed by chromosome counts in PMCs at both diakinesis and metaphase I (MI). Seven bivalents and one univalent were observed at diakinesis and MI (Fig. 2B, C). The univalent chromosome was attached to the nucleolus in plant no. 517 (Fig. 2B), and was smaller than the bivalent-forming chromosomes. Trivalent configurations resulted from pairing of homologous chromosomes from *C. hystrix* and *C. sativus* was low, about 5.1% and 4.5%, respectively, suggesting plant nos. 87 and 517 were not trisomics and were correctly characterized as MAALs.

Meiotic behavior in the two MAALs was also examined at other meiotic stases. In both MAALs, meiosis was asynchronous; different meiotic results were observed in samples taken from the same anther. Leptotene in the MAAL plants was characterized by the chromatin thread contracting into a bundle, where one end of the bundle was connected to the nucleolus, similar to cucumber. Chromosome pairing observed at late diplotene in MAAL plants was well spread and the decrescent nucleolus remained visible until diakinesis. In two cases, asymmetric separation at anaphase I and II led to the formation of four different poles at telophase II (Fig. 2D). Seven chromosomes were observed at two poles and eight chromosomes at the other two poles, a configuration that would result in the two gamete classes observed.

## Molecular analysis by RAPD marker

RAPD analysis was performed with 100 random 10-base pair primers. Eleven arbitrary RAPD primers showing polymorphisms between *C. hystrix* and *C. sativus* were

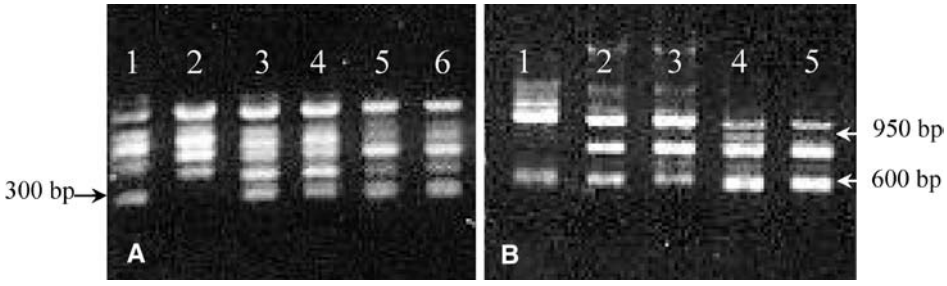


**Fig. 3A–B** MAALs in *Cucumis* verification by RAPD analysis. **A** RAPD products of different genotypes in *Cucumis* were amplified by primer E-19; *arrows* indicate bands common with *C. hystrix*. *1 C. hystrix*, *2 C. sativus* cultivated Beijing jietou, *3* amphidiploid *C. hytivus*, *4* allotriploids in *Cucumis*, *5* MAAL no. 517, *6* MAAL no. 87. **B** RAPD products of different genotypes in *Cucumis* were amplified by primer AI-5. *Arrows* indicate the characteristic bands in MAALs. *1 C. hystrix*, *2 C. sativus* cv. Beijing jietou, *3* amphidiploid *C. hytivus*, *4* MAAL no. 517, *5* MAAL no. 87

selected for further analysis. Eight primers (A-9, A-11, AH-13, AI-19, AJ-18, AJ-20, E-19, and N-20) out of the 11 initially selected showed polymorphism in the MAALs relative to *C. sativus*. The two MAALs plants, *C. hytivus*, allotriploids, and *C. hystrix* all shared a common band that was absent in *C. sativus* (Fig. 3A). This suggested that the alien chromosomes present in the MAALs were derived from *C. hystrix*. Meanwhile, banding-pattern differences were also observed between plant no. 87 and plant no. 517 after amplification with primers AI-5, AJ-13, N-12, and N-20, consistent with the inference based on the morphological differences that they contain different *C. hystrix* chromosomes.

## Discussion

The conventional approach to obtain MAALs includes three steps: (1) production of an amphidiploid through distant hybridization; (2) production of the sesquidiploid, e.g., allotriploid, by backcrossing the amphidiploid to the diploid species; and (3) production of the MAALs through repeated backcrossing of the allotriploid to the diploid species followed by selection (O'mara 1940). In this study, two MAALs were produced after treating the allotriploid with colchicine. Other researchers have shown similar results as a consequence of colchicine treatment in *Sorghum* (Kasha 1974) or 3-fluorophenylalanine in *Lolium*×*Festuca* hybrids (Zenk 1974). One possible hypothesis to account for the observation that most chromosomes from one parent are eliminated after colchicine treatment is that the different parental species show asynchrony with respect to duration of the mitotic cell cycle (Kasha 1974). In our experiment, no hexaploids have ever been obtained, perhaps as a consequence of treatment time and/or procedure.

MAALs have proven very useful in a variety of genetic studies (Brar and Khush 1997; McGrath et al. 1990). Alien chromosome addition lines harboring one single chromosome from the wild species *C. hystrix* might be used as a bridge to transfer genes of interest originating in *C. hystrix* to individual chromosomes of *C. sativus* via recombination or translocation events. Successful examples of gene introgression through MAALs have been obtained in several important crops. Examples include the transfer of leaf rust resistance from *Aegilops umbellulata* into hexaploid wheat (Sears 1956), the transfer of stem rust resistance from *Agropyron elongatum* to hexaploid wheat (Knott 1961), the transfer of mildew resistance from *Avena barbata* into hexaploid oats (Aung and Thomas 1978), and the transfer of brown rust resistance from *Oryza officinalis* into rice (Jena and Khush 1989). In beet, MAALs were used to bring nematode resistance from *Beta procumbens* into cultivated sugar beet, *B. vulgaris* (Löptien 1984; Savitsky 1978). More recently, leaf spot resistance found in *B. corolliflora* was also transferred to sugar beet using this strategy (Gao and Jung 2002).

In *Cucumis* species, the successful hybridization between *C. hystrix* and *C. sativus* (Chen et al. 1997) has provided a new opportunity for *Cucumis* breeders. Based upon the analysis presented in this paper, two MAALs have now been identified and characterized in which a chromosome from *C. hystrix* is present in a *C. sativus* background. These lines can serve as a bridge for gene transfer, reducing breeding time in transferring economically important traits from *C. hystrix* to cultivated cucumber. Further work towards a complete set of MAALs will involve backcrossing allotriploids to the diploid cucumbers. Our results indicate that additional approaches such as embryo rescue may be necessary to address issues of viability and fertility in the backcross progenies.

Classical cytological methods, including C-banding and G-banding techniques, will be necessary to accurately distinguish among addition lines. Such methods have been successfully used in many species to identify alien chromosomes. However, chromosomes in *Cucumis* are relatively small with poor stainability (Tsuchiya and Gupta 1991); hence, it is difficult to identify the alien chromosomes of MAALs or other aneuploids. Recent advances in molecular marker technology and in situ hybridization techniques have enhanced precision in detecting chromosome introgressions or introgressed segments (Brar and Khush 1997). Future work will focus both on establishing more MAALs and enhancing recombination between homologous chromosomes in

*Cucumis* by identifying gene(s) controlling homoeologous chromosome pairing.

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