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ARTICLE

Development of alien addition lines from *Cucumis hystrix* in *Cucumis sativus*: cytological and molecular marker analyses

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Abstract: Transferring desired genes from wild species to cultivars through alien addition lines (AALs) has been shown to be an effective method for genetic improvement. *Cucumis hystrix* Chakr. (HH, 2*n* = 24) is a wild species of *Cucumis* that possesses many resistant genes. A synthetic allotetraploid species, *C. hytivus* (HHCC, 2*n* = 38), was obtained from the cross between cultivated cucumber, *C. sativus* (CC, 2*n* = 14), and *C. hystrix* followed by chromosome doubling. *Cucumis sativus* – *C. hystrix* AALs were developed by continuous backcrossing to the cultivated cucumbers. In this study, 10 different types of AALs (CC-H01, CC-H06, CC-H08, CC-H10, CC-H12, CC-H06+H09, CC-H06+H10, CC-H06+H12, CC-H08+H10, CC-H01+H06+H10) were identified based on the analysis of fluorescence in situ hybridization (FISH) and molecular markers specific to *C. hystrix* chromosomes. And the behavior of the alien chromosomes in three AALs (CC-H01, CC-H06+H10, CC-H01+H06+H10) at meiosis was investigated. The results showed that alien chromosomes paired with *C. sativus* chromosome in few pollen mother cells (PMCs). Further, disomic alien addition lines (DAALs) carrying a pair of *C. hystrix* chromosome H10 were screened from the selfed progenies of CC-H10. Chromosome pairing between genomes provides cytological evidence for the possible introgression of alien chromosome segments. The development of AALs could serve as a key step for exploiting and utilizing valuable genes from *C. hystrix*.

Key words: Cucumis hystrix Chakr., alien addition lines, FISH, molecular markers, chromosome pairing.

Résumé : Le transfert de gènes d'intérêt d'une espèce sauvage à des cultivars via des lignées d'addition (AAL) s'est avérée une voie efficace en amélioration génétique. Le *Cucumis hystrix* Chakr. (HH, 2*n* = 24) est une espèce sauvage de *Cucumis* et possède plusieurs gènes de résistance. Un allotétraploide synthétique, *C. hytivus* (HHCC, 2*n* = 38), a été obtenu en croisant le concombre cultivé *C. sativus* (CC, 2*n* = 14) avec le *C. hystrix* et en doublant les chromosomes. Les lignées AAL *C. sativus* – *C. hystrix* ont été développées au terme de rétrocroisements répétés avec le concombre cultivé. Dans ce travail, 10 types d'AAL (CC-H01, CC-H06, CC-H08, CC-H10, CC-H12, CC-H06+H09, CC-H06+H10, CC-H06+H12, CC-H08+H10, CC-H01+H06-H10) ont été identifiées au moyen de l'hybridation in situ en fluorescence (FISH) et de marqueurs moléculaires spécifiques des chromosomes du *C. hystrix*. Le comportement à la méiose des chromosomes étrangers dans trois lignées AAL (CC-H01, CC-H06+H10, CC-H01+H06+H10) a été examiné. Les résultats ont montré que les chromosomes étrangers s'appariaient avec des chromosomes du *C. sativus* dans certains androsporocytes. De plus, des lignées d'addition disomiques (DAAL) portant une paire de chromosomes H10 du *C. hystrix* ont été identifiées au sein des progénitures issues de l'auto-fécondation des lignées CC-H10. L'appariement chromosomique entre génomes fournit des évidences cytologiques de la possible introgression de segments chromosomiques étrangers. Le développement de lignées AAL pourrait servir d'étape clé pour exploiter et utiliser des gènes utiles en provenance du *C. hystrix*. [Traduit par la Rédaction]

Mots-clés : Cucumis hystrix Chakr., lignées d'addition, FISH, marqueurs moléculaires, appariement des chromosomes.

Introduction

The genus *Cucumis* contains more than 50 species distributed mainly in Asia, Africa, and Australia, including two cultivated species: cucumber (*Cucumis sativus* L., CC, 2*n* = 2*x* = 14) and melon (*C. melo* L., 2*n* = 2*x* = 24) (Sebastian et al. 2010). As an important member of the family Cucurbitaceae, cucumber is a valuable vegetable crop around the world (Jeffrey 1980). However, the genetic base of cucumber has gradually become narrow and the genetic variation of cucumber is relatively limited (Qi et al. 2013). Furthermore, the cucumber is susceptible to pathogens, including downy mildew (caused by *Pseudoperonospora*

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cubensis) and gummy stem blight (caused by *Didymella bryoniae*), which can result in considerable quality decline and yield loss (L. Lou et al. 2013; K. Zhang et al. 2018; Olczak-Woltman et al. 2011). *Cucumis hystrix* (HH, $2n = 2x = 24$), as a sister species of cucumber, possesses numerous agriculturally resistant genes that can be utilized for cucumber genetic improvement (Wan et al. 2010; Yu et al. 2015; Zhou et al. 2008). Introducing these valuable characteristics of *C. hystrix* and broadening the narrow genetic base of cucumber through interspecific hybridization would be significant.

Interspecific hybridization, involving the crossing of cultivated crops to its wild relatives, can be used as an effective way for genetic improvement of crops by transferring resistant genes from wild species and increasing the genetic variation (Jena et al. 2016). Due to the existence of cross incompatibility, only one report with regard to the successful interspecific hybridization between *C. sativus* and *C. hystrix* has been used for crop improvement in species of *Cucumis* (Chen et al. 1997). And the allotetraploid new species *Cucumis* × *hytivus* J.-F. Chen and J.H. Kirkbr. (HHCC, $2n = 4x = 38$), deriving from interspecific hybridization between *C. sativus* and *C. hystrix*, was restored fertility after chromosome doubling (Chen et al. 2003*a*; Chen and Kirkbride 2000). The full fertile synthetic allotetraploid has served as a valuable germplasm resource for the creation of chromosome translocation, substitution, and addition lines and the analysis of genetic relationships among species of *Cucucmis* (Chen et al. 2003*b*, 2004; Zhuang et al. 2004).

Alien addition lines (AALs) containing alien chromosomes from wild species and a complete genome of the recipient species have been developed from interspecific hybridization followed by continuous backcrossing to cultivars (Ariyanti et al. 2015). AALs are valuable bridge species and genetic materials for comparative genomics research, enriching the genetic diversity by transferring desirable genes of wild species into cultivated species, and analyzing the introgression mechanism (Kong et al. 2018; H. Li et al. 2016). In particular, monosomic alien addition lines (MAALs) can be used to facilitate the chromosomal mapping of genes, study chromosomal evolution of species, and construct specific physical maps of chromosomes (Cho et al. 2006; Heneen et al. 2012). The research of AALs has extensively involved a series of crops, including onion, wheat, rice, maize, rape, and cotton (Ariyanti et al. 2015; Budahn et al. 2008; Chen et al. 2014; Kong et al. 2018; Multani et al. 2003; Rines et al. 2009). However, compared with other crops, the development and application of AALs in species of *Cucumis* remains limited due to the difficulty of distant hybridization. So far, there is only one report regarding research of AALs in species of *Cucumis*, in which two MAALs of *C. sativus* – *C. hystrix* were created during the process of allotriploid chromosome doubling (Chen et al. 2004). However, the identities of alien chromosomes were not determined in these two MAALs, and also no further research was reported. Up till now, the combination of genomic in situ hybridization (GISH) and molecular markers technique has become an effective strategy in characterizing the identities of alien chromosomes in AALs (Kang et al. 2014; Liu et al. 2017; Wang et al. 2016; Zhang et al. 2017). With the development of molecular cytogenetic techniques, high-resolution chromosome analysis technology provides a more intuitive strategy for the identification of *C. hystrix* chromosomes in the background of *C. sativus* (Bi et al. 2019).

Based on the synthetic allotetraploid *C. hytivus*, two backcrosses with a diploid cultivar were carried out to create *C. sativus* – *C. hystrix* AALs. For the BC_2 progenies, GISH technique was used to distinguish alien H chromosomes in the background of the C genome. Then, a set of H chromosome specific markers and FISH probes were applied to further determine the identities of alien chromosomes. Based on these analyses, five MAALs and five multiple addition lines (MALs) were obtained among the BC_2 progenies. Further, also identified was a disomic alien addition line (DAAL) with a pair of *C. hystrix* chromosome H10 from BC_2F_2 progenies by molecular marker and GISH analysis. In addition, H-chromosome pairing behavior during meiosis was also investigated in three different types of AALs, which will provide cytological evidence for the possible introgression of alien chromosome segments.

Materials and methods

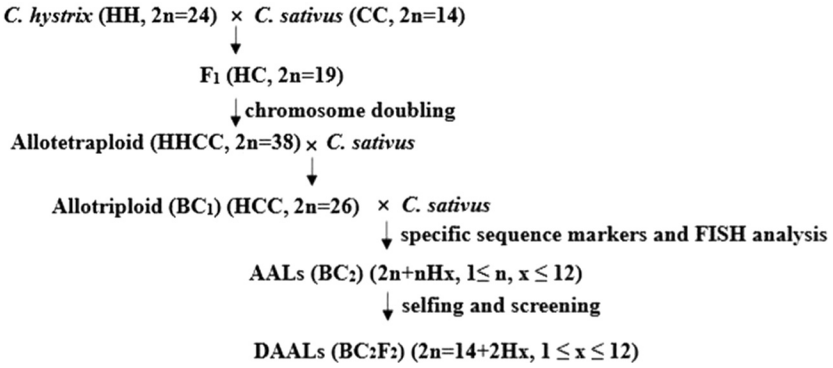
Plant materials

The diploid species *C. sativus* 'Beijing jietou' and a fertile synthetic allotetraploid species of *Cucumis* (*C. hytivus*, (HHCC, $2n = 38$)), provided by the Lab of Cucurbit Genetics and Germplasm Enhancement, Nanjing Agriculture University, obtained by interspecific hybridization and chromosome doubling, were used for this research (Chen et al. 2003*a*). *Cucumis hytivus* was used as the maternal parent and backcrossed continuously with the recurrent parent, the cultivated cucumber *C. sativus* 'Beijing jietou' (CC, $2n = 14$), to develop BC_1 and BC_2 progenies. BC_2 progenies were further self-pollinated to obtain BC_2F_2 plants. The scheme for developing *C. sativus* – *C. hystrix* AALs is shown in Fig. 1. All seeds of BC_1, BC_2, and BC_2F_2 were germinated and grown in the greenhouse of Baima Cucumber Research Station, Nanjing Agricultural University.

Specific sequence markers

Genomic DNA of *C. sativus* 'Beijing jietou', *C. hystrix*, allotriploid, and AALs (BC_2 and BC_2F_2 progenies) were extracted from healthy leaves according to the CTAB method (Murray and Thompson 1980). Based on the sequencing date of the *C. hystrix* genome (unpublished date), chromosome-specific sequences were selected to identify each chromosome of *C. hystrix*. A set of 12 chromosome-specific sequences selected from 12 chromosomes

Fig. 1. Scheme for the development of alien addition lines.



of *C. hystrix* were used to identify alien chromosomes in *C. sativus* – *C. hystrix* AALs. The polymerase chain reaction (PCR) for molecular marker experiments was essentially the same as previously described by Hechanova et al. (2018) with some modifications. The amplification procedure was as follows: initial denaturation at 94 °C for 5 min, then 29 cycles for denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were separated on 8% polyacrylamide gels or 2% agarose gel by electrophoresis.

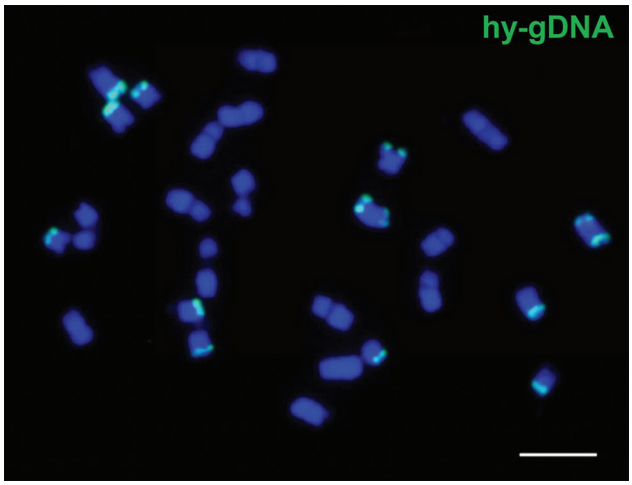
Chromosome preparation

Root tips and young flower buds of *C. sativus* – *C. hystrix* AALs were gathered and fixed in Carnoy's fixative solution for at least one day for mitotic and meiotic chromosome preparation. The procedure of chromosome preparation was basically the same as previously published protocols with minor modification (Bi et al. 2019; Q.F. Lou et al. 2013). The root tips and anthers were digested with enzyme mixtures, including 4% cellulose R-10 (Yakult), 2% pectinase (Sigma–Aldrich), and 1% pectolyase Y-23 (Yakult) in 0.01M citrate solution (pH = 4.8), for 50 min and 2 h at 37 °C, respectively. The digested root tips and anthers were dispersed on slides in Carnoy's fixative solution. The slides with well-spread chromosomes were obtained with "flame dried" methods as described previously (Lou et al. 2014). Following, to remove cytoplasm, the slides were treated with pepsin for 40–60 s at 37 °C. The procedure of pepsin treatment was performed as described by Zhao et al. (2019). Finally, the slides with well-spread chromosomes were used for FISH and GISH experiments.

Probe labeling and fluorescence in situ hybridization

The probes from genomic DNA of *C. hystrix* and satellite DNA sequences, 45S rDNA and TypIII, were applied to distinguish chromosomes in FISH experiments (Wang et al. 2017; Zhang et al. 2016). The oligo probes of C1, C4, C5, and C7, covering the whole cucumber chromosomes, were synthesized according to the reported procedure (Bi et al. 2019; Zhao et al. 2019). The oligo probe of C3-a, a segment of cucumber chromosome C3, was synthesized as described by Bi et al. (2019) and designed to anchor to *C. hystrix* chromosome H06.

Fig. 2. GISH analysis of allotriploid *Cucumis* with 26 chromosomes. FISH signal distribution of hy-gDNA (green) on somatic allotriploid chromosomes. Scale bar = 5 μm.



The FISH experiment and chromosome painting were performed based on chromosome-specific probes as previously published (Bi et al. 2019; Lou et al. 2014). After the first FISH experiment, some slides were reused by removing the coverslips and dehydrating in ethanol series (70%, 90%, 100%) for the second FISH to locate other probes. The procedure for reusing slides was the same as previously described by Cheng et al. (2001). Finally, images were captured by a SENSYS (http://www.photometrics.com) CCD camera attached to an Olympus (http://www.olympus-global.com) BX51 microscope. The CCD camera was controlled by Applied Spectral Imaging FISH view 5.5 software (Applied Spectral Imaging Inc., http://www.spectral-imaging.com). Images were processed using Adobe Photoshop 6.0 (Adobe Systems, http://www.adobe.com).

Results

Development of *Cucumis sativus* – *C. hystrix* AALs

Using allotetraploid *C. hytivus* pollinated with the recurrent parent *C. sativus*, we obtained an allotriploid BC_1

Table 1. Comparison of the number of fruits, seeds produced, fruit setting, seeds per fruit, and seed germination rate with different hybrid combination.

Cross combination	No. of fruit	No. of seeds	Fruit setting (%)*	Seeds per fruit	Germination rate of seeds (%)†
CC×CC	20	3302	100 (20/20)	165.10	98
HHCC×CC	20	595	34.48 (20/58)	29.75	32.68
HCC×CC	561	232	64.48 (561/870)	0.41	73.22

*%fruit setting = $\frac{\text{Number of fruits obtained}}{\text{Number of flowers pollinated}} \times 100\%$.

†%germination rate of seeds = $\frac{\text{Number of seeds germinated}}{\text{Number of seeds selected}} \times 100\%$.

Table 2. *Cucumis hystrix* chromosomes-specific sequence markers used in this study.

Specifical sequence	Forward primer (5′–3′)	Reverse primer (5′–3′)
ss H01	CACATTTCCCACCACTTG	TTTTGTTCGGCTTTTTCC
ss H02	ATGTTCTTATCCACACCTTC	ATTTTTGACCCACCTGAC
ss H03	GTGGGTGATATGGGTTGT	CGCTAATGTATTTGGTGTG
ss H04	GAACCCTCTAATCTCAACG	CAACATAATACTTCCCCG
ss H05	TCCAAGTTCCATTCACCA	CTTTCTCCCCTTACCCAC
ss H06	TTTGCTCATTGTTGTTCG	AGGACTTTACCTGCCCAT
ss H07	GGGAGTTTGGTTTCTGTC	AGGTCTATCGTTGGGTTT
ss H08	GGAGCCGTTACACCTTAG	CTTGTGCATTTCAATACCT
ss H09	GATTGATGACCCACTCCA	GTATAACACCTTCCCGCT
ss H10	CCCTACAACTTCCCCTAT	TTCCTCTTCTTGGCTAAT
ss H11	ATTGGCTTTCACTCCCTC	CACATTTTTCTCCCTTGG
ss H12	TTCAAGAACTTTCCAGGC	GAATCAATAACTCAACCACC

hybrid with 26 chromosomes. GISH analysis by hy-gDNA (*C. hystrix* genomic DNA) as probe displayed the allotriploid contained the complete genome of *C. sativus* and 12 chromosomes of *C. hystrix* (green signals) (Fig. 2). Subsequently, the allotriploid was backcrossed with *C. sativus* to generate 561 fruits and 232 seeds in two years. The average seed number per fruit was 0.41, which was far less than the number of seeds produced by the crossing of allotetraploid and diploid *C. sativus* with 29.75 seeds per fruit (Table 1). BC_2 progenies were screened for AALs using GISH analysis. Finally, 28 plants were identified as AALs, which contained one or several *C. hystrix* chromosomes. Among the 28 plants, 14 plants were MAALs carrying individual H chromosomes, 13 plants had two H chromosomes, and one plant had three H chromosomes. And the number of chromosomes in these AALs varied from $2n = 15$ to 17.

Identification of alien chromosomes in AALs by chromosome-specific sequence markers and oligo-FISH

Further, the chromosome composition of BC_2 progenies was determined by chromosome-specific sequence markers and oligopaint FISH techniques. A set of 12 HH chromosome-specific sequence markers distributing in 12 chromosomes of *C. hystrix* were selected based on the genome sequence data of *C. hystrix* (unpublished data). The detailed information of the primer sequences of these markers is listed in Table 2. These *C. hystrix* chromosome-specific sequence markers produced specific band patterns on *C. sativus* – *C. hystrix* AALs, which were consistent with *C. hystrix* and the allotriploid, but these bands were nonexistent in *C. sativus*. This indicated that the plant carried *C. hystrix* chromosomes. Finally, the results showed that 14 plants amplified individual *C. hystrix* chromosome-specific bands of H01, H06, H08, H10, and H12, which were determined as MAALs. Meanwhile, 14 plants amplified two or three different bands with different chromosome-specific primers, which were identified as CC-H06+H09, CC-H06+H10, CC-H06+H12, CC-H08+H10, and CC-H01+H06+H10 plants. Therefore, 28 AALs were divided into 10 different types with different chromosome composition by using these chromosome-specific sequence markers (Fig. 3).

Due to the existence of chromosome synteny and colinearity between *C. sativus* and *C. hystrix*, the identities of alien chromosomes in *C. sativus* – *C. hystrix* AALs can be discriminated through cross-species hybridization with a series of cucumber-specific oligo probes. Herein, a set of developed cucumber chromosome oligo probes were applied to identify the H-chromosome in *C. sativus* genome background. Based on the corresponding relationship between chromosomes of *C. sativus* and *C. hystrix* (Table 3), combining with the karyotype of *C. hystrix*, 45S rDNA probe and cucumber chromosome C1-, C3-, C4-, C5-, and C7-specific oligo probes were used to characterize the alien chromosomes of AALs following GISH analysis in this research (Wang et al. 2017; Yang et al. 2014). Chromosome H01 of *C. hystrix* was directly distinguished by oligo C7 as probe based on the complete syn-

Fig. 3. *Cucumis hystrix* chromosome-specific sequence markers used for the identification of alien chromosomes in alien addition lines. CC, *C. sativus*; HH, *C. hystrix*; HCC, allotriploid; 1, CC-H01; 2, CC-H06; 3, CC-H08; 4, CC-H10; 5, CC-H12; 6, CC-H06+H09; 7, CC-H06+H10; 8, CC-H06+H12; 9, CC-H08+H10; 10, CC-H01+H06+H10. ss H01 – ss H12 are *C. hystrix* chromosome-specific sequence markers, where ss represents specific sequence.

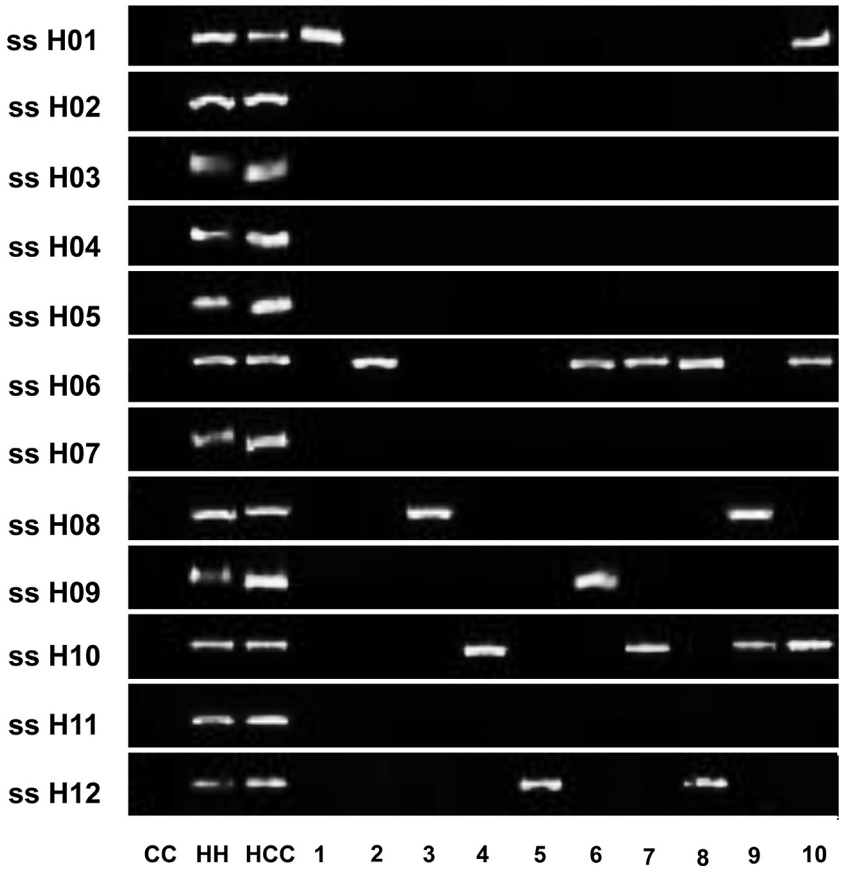


Table 3. The syntenic relationships between cucumber and *Cucumis hystrix* chromosomes.

Cucumber chromosome	Synteny to *C. hystrix*
C1	H02+H08+H12
C2	H03+H05+H11
C3	H04+H06
C4	H05+H07+H08
C5	H09+H10
C6	H02+H03+H05+H08+H11
C7	H01

teny between *C. hystrix* chromosome H01 and *C. sativus* chromosome C7 (Figs. 4*a* and 4*b*). Oligo C3-a segment was anchored to chromosome H06 of *C. hystrix* when we designed, and can be explicitly applied to determine chromosome H06. Therefore, we identified a plant with *C. hystrix* chromosome H06 by oligo C3-a as probe (Figs. 4*c* and 4*d*). Based on comparative painting, the signals of oligo C4 displayed synteny with three *C. hystrix* chromosomes, H05, H07, and H08 (Bi et al. 2019). In *C. hystrix* chromosomes, 45S rDNA signals located on chromosomes H08, H10, and H12 of *C. hystrix*. Thus, we identified chromosome H08 of *C. hystrix* by 45s rDNA and oligo C4 as probes (Figs. 4*e* and 4*f*). Oligo signals of chromosome C5 were detected on chromosomes H09 and H10 of *C. hystrix* after comparative painting, which were identified by a 45S rDNA signal present in chromosome H10 and absent in chromosome H09 (Figs. 4*g*, 4*h*, 4*k*, and 4*l*) (Zhao et al. 2019). Chromosome C1 is syntenic to three chromosomes, a part of chromosome H02, the whole chromosome H12, and a small portion of chromosome H08 (Yang et al. 2014). Chromosome H12 of *C. hystrix* in MAALs was determined by oligo C1 signals distributing on the whole chromosome H12 and the presence of 45S rDNA locus (Figs. 4*i* and 4*j*). Consequently, using the same identification method, five types of MALs were also intuitively and quickly confirmed as CC-H06+H09 (Figs. 4*k* and 4*l*), CC-H06+H10 (Figs. 4*m* and 4*n*), CC-H06+H12 (Figs. 4*o*–4*q*), CC-H08+H10 (Figs. 4*r* and 4*s*), and CC-H01+H06+H10 (Figs. 4*t*–4*w*) by oligo C3-a, oligo C1, oligo C4, oligo C5, and oligo C7 as probes.

In our research, the chromosome compositions of 28 AALs were successfully identified, and 14 BC_2 plants were MAALs with one *C. hystrix* chromosome of H01 (2 plants), H06 (1 plant), H08 (3 plants), H10 (7 plants), and H12 (1 plant). And the remaining 14 BC_2 plants were multiple addition lines with two or three H-genome chromosomes

Fig. 4. Chromosome identification of *Cucumis sativus – hystrix* alien addition lines based on FISH analysis using chromosome-specific probes. (*a*, *b*) Identification of chromosome H01 by 45S rDNA (red), hy-gDNA (green), and oligo C7 (green) as probes. (*c*, *d*) Oligo C3-a (red) and hy-gDNA (green) as probes to identify chromosome H06. (*e*, *g*, and *i*) Confirm the possible range of alien chromosomes by hy-gDNA (green) and the presence of 45S rDNA signal (red) on alien chromosomes. (*f*, *h*, and *j*) After determining the existence of 45S rDNA signal on alien chromosomes, chromosomes H08, H10, and H12 were characterized by oligo C4 (green), oligo C5 (red), oligo C1(green) as probes, respectively. (*k* and *l*) One alien chromosome was identified as chromosome H06 with oligo C3-a (red) as probe, and the other alien chromosome with no 45S rDNA signal (red) was confirmed as chromosome H09 by oligo C5 (green) as probe. (*m*, *n*) One alien chromosome H06 was characterized by oligo C3-a probe (red), another alien chromosome, carrying 45S signal (red), was identified as chromosome H10 by oligo C5 (green). (*o*–*q*) One alien chromosome was identified as chromosome H06 by oligo C3-a (red), another alien chromosome with 45S rDNA signal (red) was characterized as chromosome H12 by oligo C1 (red) signals distributing on the whole alien chromosome. (*r*, *s*) Two alien chromosomes both carrying 45S rDNA signals (green) were confirmed as chromosomes H08 and H10 with oligo probes C4 (red) and C5 (red). (*t*–*w*) Two alien chromosome with no 45S signal were characterized as chromosomes H01 and H06 by oligo C7 (green) and oligo C3-a (red), and the remaining alien chromosome with 45S signal (red) were identified as chromosome H10 by oligo C5 (green). Scale bar = 5 μm.

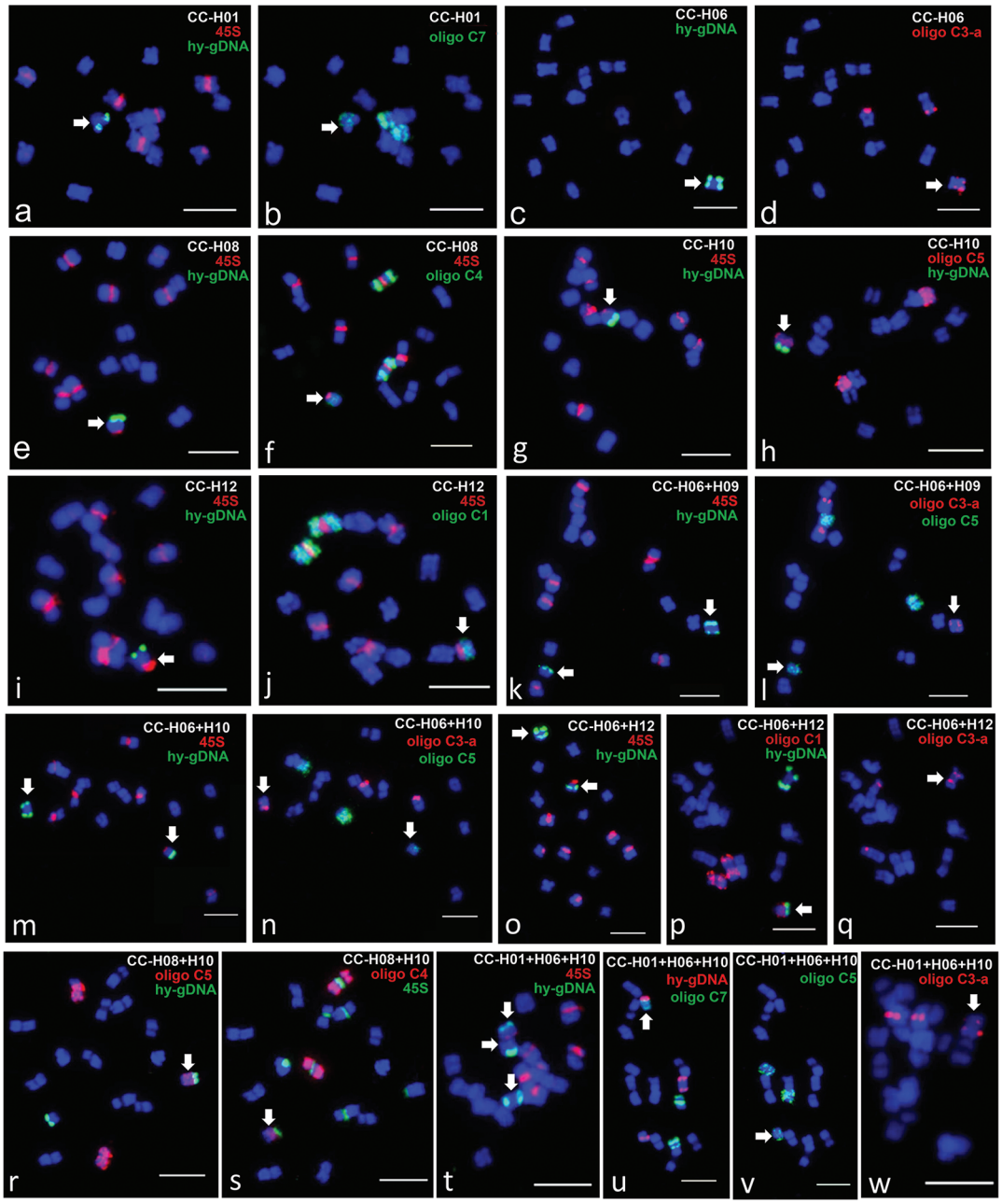


Table 4. Identification of alien chromosomes in the alien addition lines.

Chromosome component	H01	H06	H08	H09	H10	H12	No. of plants
14+1	2	1	3		7	1	14
14+2		12	1	1	10	2	13
14+3	1	1			1		1
Sum	3	14	4	1	18	3	28
Transmission rate (%)	10.71	50	14.29	3.57	64.29	10.71	

of H06+H09 (1 plant), H06+H10 (9 plants), H06+H12 (2 plants), H08+H10 (1 plant), and H01+H06+H10 (1 plant) (Table 4). The results from the cytological methods were consistent with the results of chromosome-specific sequence markers.

The observation on meiotic behavior of AALs

To analyze the meiotic behavior of alien chromosomes in background of cultivated cucumber, we selected three AALs containing one, two, and three alien chromosomes, respectively, to track chromosome pairing. A total of 134 well-resolved pollen mother cells (PMCs) at meiosis from CC-H01, CC-H06+H10, and CC-H01+H06+H10 plants were observed to analyze the behavior of chromosome pairing by FISH and GISH experiments (Fig. 5). After determining the composition of chromosomes, we used 45S rDNA, TypIII, and hy-gDNA as probes to distinguish *C. hystrix* chromosomes H01, H06, and H10. For example, in the CC-H01+H06+H10 plant, hy-gDNA probe can produce bright signal on one end of chromosome H01. Two hy-gDNA signals are at both ends of chromosome H06. And the 45S rDNA and hy-gDNA signals are at both chromosome H10 terminals (Wang et al. 2017). The chromosome configuration of the three AALs are shown in Table 5.

The results showed that, in the CC-H01 plant, one H-chromosome unpaired as a univalent (7II+1I) in 91.38% (53/58) PMCs at metaphase I (Fig. 5*a*), whereas the H01 paired with a pair of cucumber chromosomes in a trivalent configuration (6II+1III) in 5.17% (3/58) PMCs (Fig. 5*b*). Notably, we also observed that alien chromosome H01 paired with one C-chromosome to form a bivalent, while the other C-chromosome existed as a univalent (7II+1I) in 3.45% (2/58) PMCs (Fig. 5*c*). In 26 anaphase I PMCs, the alien chromosome lagged behind among 69.23% (18/26) PMCs (Fig. 5*d*), and the remaining 30.77% (8/26) PMCs showed the alien chromosome was normally segregated to one pole (Fig. 5*e*).

In 48 metaphase I PMCs of CC-H06+H10 plants, alien chromosomes H06 and H10 both remained in a univalent formation (7II+2I) in 93.75% (45/48) PMCs (Fig. 5*f*). We also observed in 4.17% (2/48) PMCs, H10 paired with a pair of C chromosomes to form 6II+1III+1I (Fig. 5*g*). Chromosomes H06 and H10 were also found to pair with two different pairs of C chromosomes as a trivalent (5II+2III) in one PMC (Fig. 5*h*). Meanwhile, we also observed one anaphase I PMC, and H06 and H10 going to different poles normally in this PMC (Fig. 5*i*).

In the CC-H01+H06+H10 plant, three univalents (7II+3I) were observed in 85.71% (24/28) PMCs at metaphase I (Fig. 5*j*), and the alien chromosome H06 paired with one C-chromosome in a bivalent configuration (7II+3I) in one PMC (Fig. 5*k*). Analysis of 10.71% (3/28) PMCs showed that alien chromosome H10 existed in the form of a trivalent (6II+1III+2I) (Fig. 5*l*). Further, we observed one anaphase I PMC, and two alien chromosomes going to one of the poles, and the remaining alien chromosome was going to the other pole in this PMC (Fig. 5*m*).

The statistical results showed that among a total of 134 meiotic metaphase I PMCs from three AALs (CC-H01, CC-H06+H10, CC-H01+H06+H10), the alien chromosomes were unpaired as univalents in 122 (91.04%) PMCs, and nine (6.72%) PMCs harbored trivalents. Interestingly, the alien chromosomes paired with one C-chromosome to form a bivalent in three (2.24%) PMCs.

The creation and identification of DAALs

In this study, the CC-H10 plant was self-pollinated to produce BC_2F_2 progenies for obtaining DAALs. We obtained a total of 26 self-pollinated seeds from one fruit, and 19 plants survived. Out of 19 BC_2F_2 progenies, eight plants (42.11%) produced a specific band of *C. hystrix* chromosome H10 based on the molecular marker analysis (Fig. 6*a*). These BC_2F_2 plants with specific bands were further analyzed using GISH technique to determine the number of *C. hystrix* chromosomes. Finally, we obtained two BC_2F_2 progenies containing 16 chromosomes, a pair of which were chromosomes H10 of *C. hystrix* with green hybridization signals (Fig. 6*c*).

Phenotypic traits of AALs

Preliminary phenotypic observations were performed on the obtained AALs and DAALs, and it was found that there are morphological variations in different types of AALs. Compared with the normal diploid cultivated cucumber, most of AALs showed slow growth rates. In general, plants with two alien chromosomes grew more slowly than plants with one alien chromosome. The phenotypes of AALs were biased toward cultivated cucumber, but some AALs differed from the diploid cultivated cucumber in some morphology characteristics, such as leaf shape and branching. The differences in leaf shape are shown in Fig. 7.

Fig. 5. Alien chromosome behavior of *Cucumis sativus* – *hystrix* alien addition lines at meiotic metaphase I and anaphase I. TypIII (red), hy-gDNA (green), and 45S rDNA (yellow) as probes were used to distinguish alien chromosomes. (*a*–*e*) Pollen mother cells (PMCs) taken from the CC-H01 plant. (*f*–*i*) PMCs of the CC-H06+H10 plant. (*j*–*m*) PMCs of the CC-H01+H06+H10 plant. (*a*) Chromosome H01 existed as a univalent (7II+1I). (*b*) Chromosome H01 formed a trivalent with a pair of C chromosomes (6II+1III). (*c*) Chromosome H01 formed a bivalent with one C chromosome (7II+1I). (*d*) Chromosome H01 lagged behind. (*e*) Chromosome H01 was segregated to one pole. (*f*) Chromosomes H06 and H10 both unpaired as univalents (7II+2I). (*g*) Chromosome H10 paired as a trivalent (6II+1III+1I). (*h*) Chromosomes H06 and H10 existed as trivalents (5II+2III). (*i*) Chromosomes H06 and H10 going to different poles normally. (*j*) Chromosomes H01, H06, and H10 all remained as univalents (7II+3I). (*k*) Chromosome H06 paired as a bivalent (7II+3I). (*l*) Chromosome H10 paired as a trivalent (6II+1III+2I). (*m*) Two alien chromosomes going to one of the poles, the remaining alien chromosome going to the other pole. Scale bar = 5 μm.

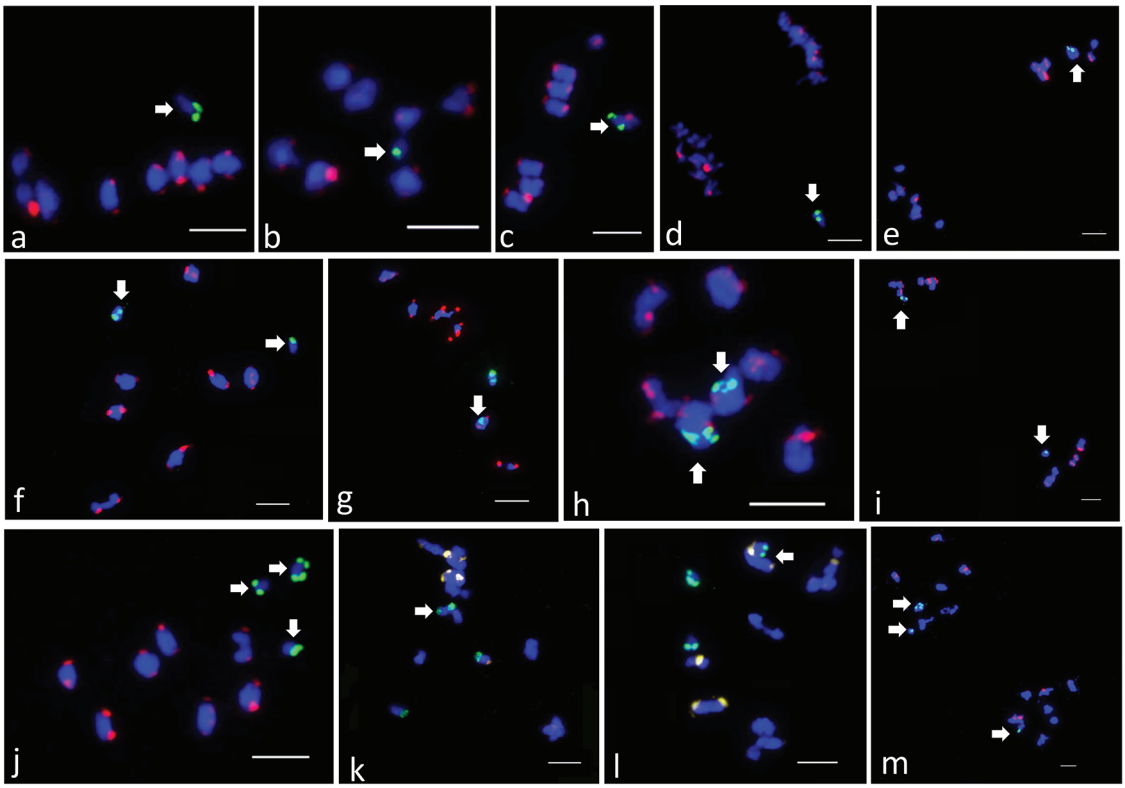


Table 5. Chromosome configuration of three alien addition lines with different alien chromosomes.

Chromosome configuration	CC-H01	CC-H06+H10	CC-H01+H06+H10	Sum
0III+7II+1I	55	—	—	55
1III+6II+0I	3	—	—	3
0III+7II+2I	—	45	—	45
1III+6II+1I	—	2	—	2
2III+5II+0I	—	1	—	1
0III+7II+3I	—	—	25	25
1III+6II+2I	—	—	3	3
Total PMCs	58	48	28	134

The leaf shapes of the CC-H01, CC-H08, and CC-H10 plants were biased toward the diploid cultivated cucumber. The growth rate of the CC-H10 plant was the fastest compared to the other AALs identified. The leaf size of the CC-H08+H10 plant was smaller than the cultivated cucumber. And the leaf blades with seven lobes were palmate and apex narrowly acute. Many central asymmetric leaves appeared on the plant of DAALs with a pair of chromosome H10 (Fig. 6*d*).

The growth rates of the CC-H06, CC-H06+H10, and CC-H01+H06+H10 plants were significantly slower than the normal diploid cultivated cucumber. The edge of the leaves was wavy, and the leaves were thicker.

The CC-H06+H09 plant grew slowly and had the strongest branching ability compared with all AALs identified. The plant blossomed in about 80 days, which was later than other AALs identified. The edge of leaves was wavy, and the leaves were slightly small.

The CC-H12 plant grew slowly, the margin of the leaves was serrated, and the leaves were narrow. Leaves margins of the CC-H06+H12 plants were slightly serrated, and the surface of the blades were slightly wrinkled.

Discussion

In this research, five *C. sativus* – *C. hystrix* MAALs with individual H-genome chromosomes (CC-H01, CC-H06, CC-H08, CC-H10, CC-H12) and five MALs with two or three H-genome chromosomes (CC-H06+H09, CC-H06+H10, CC-H06+H12, CC-H08+H10, CC-H01+H06+H10) were developed by consecutively backcrossing allotetraploid

Fig. 6. Identification of disomic alien addition lines by specific sequence markers and GISH analysis. (*a*) Chromosome-specific sequences marker, ss H10, used for identifying the existence of chromosome H10 in BC_2F_2 plants from a self-pollinated CC-H10 plant. Numbers 1–19 represent the 19 surviving plants. (*b*) Banding pattern of the CC-2H10 plant after PCR amplification. (*c*) Two alien H10 chromosomes identified by hy-gDNA (green) and 45S rDNA (red) as probes. CC, *C. sativus*; HH, *C. hystrix*; HCC, allotriploid. (*d*) Leaf phenotype of the CC-2H10 plant. Scale bar = 5 μm.

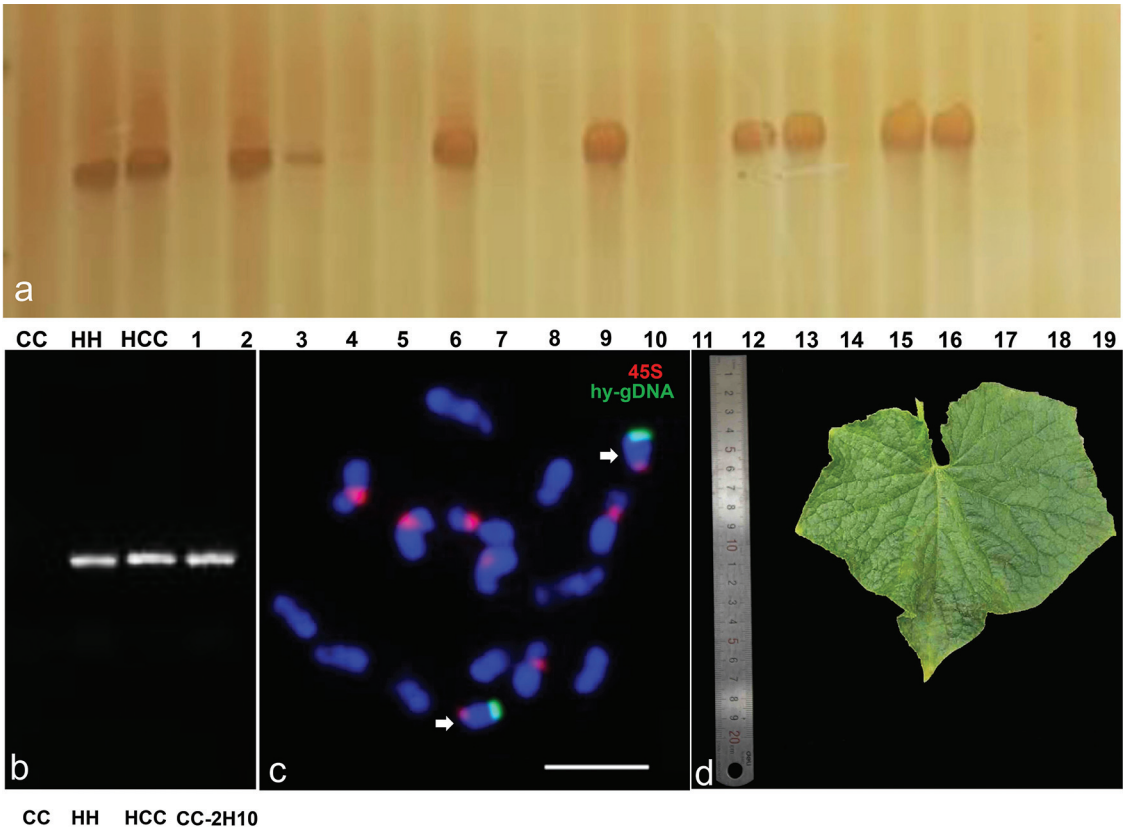
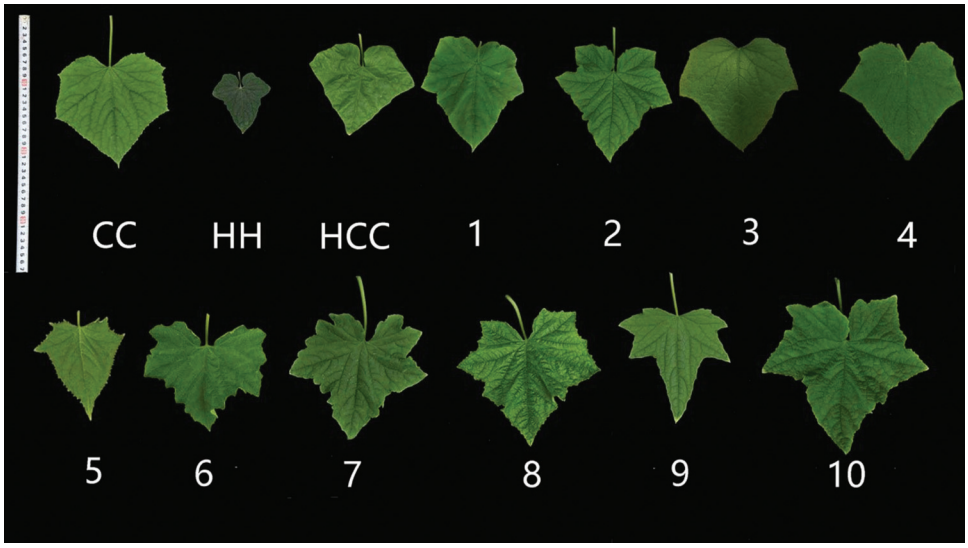


Fig. 7. Leaf phenotype of different alien addition lines. CC, *C. sativus*; HH, *C. hystrix*; HCC, allotriploid; 1, CC-H01; 2, CC-H06; 3, CC-H08; 4, CC-H10; 5, CC-H12; 6, CC-H06+H09; 7, CC-H06+H10; 8, CC-H06+H12; 9, CC-H08+H10; 10, CC-H01+H06+H10.



C. hytivus with cultivated cucumber. This is the first report on the accurate identities of alien chromosomes in species of *Cucumis* AALs. Though Chen et al. (2004) obtained two *C. sativus* – *C. hystrix* MAALs, the identities of alien chromosomes were not defined. Due to only one or several alien chromosomes in the background of cucumber genome, *C. sativus* – *C. hystrix* AALs could be used for the analysis of specific genes related with the corre-

sponding chromosomes of *C. hystrix* and could also be used as bridges for transferring desirable genes from the H genome to the C genome. Furthermore, *C. sativus* – *C. hystrix* AALs are useful genetic materials for elucidating the introgression mechanism of *C. hystrix* genes in C-genome background. In this study, we also created a DAAL with a pair of homoeologous chromosomes of H genome (CC–2H10), which is the first report with regard to DAALs in species of *Cucumis*. DAALs are not only valuable materials for investigating the origin and evolution of genomes, but they are also useful for chromosomal localization of elite genes (An et al. 2015; Du et al. 2014; Kong et al. 2018; Q.F. Li et al. 2016). Compared to AALs, DAALs are beneficial to maintain the stability of meiosis. The generation of DAALs will be used as ideal bridge materials and elite germplasm resources for genetic research and cucumber breeding.

The identities of alien chromosomes in AALs are usually identified through combining GISH analysis with molecular markers (Ali et al. 2001; Tan et al. 2005, 2017; Tang et al. 2018). In this study, we screened 28 AALs carrying *C. hystrix* chromosomes by GISH analysis. Subsequently, we determined the chromosome composition of AALs through *C. hystrix* chromosome-specific sequence markers. Meanwhile, following GISH and molecular marker analyses, we further identified alien chromosomes of *C. sativus* – *C. hystrix* AALs to verify the accuracy of molecular marker results by using an oligo-FISH technique, which was recently developed (Han et al. 2015; Bi et al. 2019). Here, oligo probes specific to individual cucumber chromosomes were used for FISH to identify alien chromosomes in AALs. The oligo probes covering any chromosomes/segments were designed based on a sequenced cucumber 'Chinese Long' genome. Based on the homoeologous relationships between cucumber and *C. hystrix* chromosomes, cross-species chromosome painting using cucumber chromosome-specific oligo probes could be applied to distinguish the corresponding chromosomes/segments from *C. hystrix* (Bi et al. 2019; Yang et al. 2014). The oligopaint FISH technique could be clearly applied to identify chromosomes (Albert et al. 2019; Braz et al. 2017; Han et al. 2015). In our research, the application of chromosome-specific oligo probes provides an intuitive and effective way to distinguish the alien chromosomes in AALs, and this method provides an idea for the related research of other crops AALs.

In this study, an allotriploid was pollinated using the pollens of diploid cultivated cucumber to create AALs. The low seed setting rate in allotriploid fruit might be attributed to the high proportion of abnormal female gametes generated by the allotriploid BC_1 hybrid. A similar phenomenon was also reported in rice as well as onion (Ariyanti et al. 2015; Multani et al. 2003). Here, we obtained a total of 232 seeds from 561 mature fruits in two years, and the average number of seeds per fruit was 0.41. The extremely low number of seeds from the crossing of an allotriploid and diploid cucumber makes it more difficult to develop a complete set of MAALs. Meanwhile, among BC_2 plants identified as AALs, the number of chromosomes were almost 15 or 16, and the frequency of AALs with more than three alien chromosomes was far lower. We inferred that the female gametes containing less than 10 chromosomes produced by the allotriploid are more likely to survive. The surviving female gametes were combined with normal male gametes of cultivated cucumber to form AALs with extra *C. hystrix* chromosomes. From the limited alien chromosomes transmission rate, we also distinctly found that the transmission rates of individual H-genome chromosome were different. AALs with chromosome H10 and with both chromosomes H06 and H10 appeared with high frequencies. The transmission rates of other individuals with alien chromosome were relatively low. Similar results in the obvious variation of different alien chromosome transmission rates in AALs have been involved in many crops (Chen et al. 2014; Tan et al. 2017). Many factors may affect the transmission rate of alien chromosomes, such as homoeologous pairing between genomes and the size of chromosomes. Tan et al. (2017) observed that alien chromosomes with higher transmission rates had smaller sizes in *Brassica oleracea* – *B. nigra* MAALs. In our research, alien chromosome H10 with the highest transmission frequency is the shortest chromosome of all *C. hystrix* chromosomes. We speculate that the transmission rate of alien chromosomes in *C. sativus* – *C. hytrix* AALs was probably related to the size of *C. hystrix* chromosomes.

One or several alien chromosomes in AALs are likely to pair with chromosomes of the recipient species, resulting in the possibility of recombination and exchange of chromosomes. AALs can effectively introduce chromosomal structure variation and have been extensively used as genetic resource for transferring resistant genes from wild species into cultivated species by the introgressions of alien chromosome segments (Hechanova et al. 2018; Narain et al. 2016; Song et al. 2013). In wheat, J. Zhang et al. (2018) developed a novel wheat – *D. villosum* alien introgression line conferring high stripe rust resistant genes from the backcross progenies of wheat – *D. villosum* 3V addition lines. In rice, disomic introgression lines conferring resistance to brown phanthopper, green leafhopper, bacterial blight, and blast were derived from MAALs (Jena et al. 2016). In this research, *C. sativus* – *C. hystrix* AALs were developed by interspecific hybridization followed by continuous backcrossing to cucumber, and the introgressions of alien DNA segments may occurr in the process of creating AALs due to homoeologous recombination. However, it is difficult to detect the introgression of alien DNA segments by oligo-FISH with only the 12 chromosome-specific markers used here. Therefore, the relationships between phenotype of AALs and alien chromosomes or introgression segments need to be further investigated in our next work.

AALs are useful materials for studying the mechanism of chromosome pairing and interspecific introgression. Theoretically, chromosome pairing between different genomes is the premise of recombination, and chromosomal recombination and exchange during the meiosis stage are reliable ways to obtain interspecific introgression lines (Chen et al. 2014). In this research, we observed the meiotic behaviors of AALs with 1–3 alien chromosomes. The alien chromosomes were unpaired as univalents in a majority of PMCs at meiotic metaphase. Similar results have been reported in other AAL crops (Rui-fen et al. 2002; Tan et al. 2017). Although, the pairing behaviors between H- and C-genomic chromosomes were detected at a low rate, we still observed the existence of chromosome pairing behaviors between the genomes of *C. hystrix* and *C. sativus*. The results provided possible cytological evidence for chromosomal recombination and the development of introgression lines by AALs as basic materials. In meiosis behavior analysis for the specific AALs, oligo-FISH did not produce clear signals to allow us to discriminate the individual chromosome identity of *C. sativus* and *C. hystrix*, which may be due to the signal interference of cross hybridization during homoeologous chromosome paring. Therefore, we used 45S rDNA, TypIII, and hy-gDNA as probes for FISH to determine the chromosome in *C. hystrix* AALs. Determining the identities of their paired cucumber chromosomes is of great significance for studying the interspecific introgression mechanism of alien chromosome segments. Therefore, further works will continue to improve the application of oligo-FISH during meiosis to identify *C. hystrix* chromosomes and their paired cucumber chromosomes. And combined with high-resolution chromosome analysis technology, the observation of the meiotic behavior of alien chromosome and the introgression process of alien chromosome segments will accelerate the research of interspecific introgression mechanism.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Author contribution statements

Q.L. and J.C. conceived the research and designed the experiments. M.L., Q.Z., Y.L., and W.H. performed the research. X.Q. provided the chromosome-specific sequences of *C. hystrix*. Q.L. and M.L. wrote the manuscript. M.D. participated in revising the paper. All authors read and approved the final manuscript.

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