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ORIGINAL ARTICLE

Direct regeneration of haploid or doubled haploid plantlets in cucumber (*Cucumis sativus* L.) through ovary culture

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Abstract

This paper reports a simple and effective method of directly producing cucumber plants through unfertilized ovary culture. The paper involves an experiment investigating factorial interactions between TDZ treatment, cold pretreatment, genotypes of cucumber, to improve plant induction. Cold pretreatment was effective in stimulating the ovary. The results showed that cold pretreatment for 4 days, TDZ for 0.06 mg·L^{-1}, the interaction with genotype can be used as an effective strategy to improve the efficiency of gynogenesis. The plant regeneration induction rate was highest (79.3%). In addition, we observed the process of cucumber megasporogenesis and plant regeneration. The plants obtained from ovary culture of cucumber were identified as diploid or haploid by flow cytometry, consistent with the results of chromosome counting. The diploid plants were further identified as pure doubled haploid using simple sequence repeats (SSR). The doubling treatment we used was one of the simplest and most effective methods, completed in a short time (1 h) with a doubling rate of 75%. The acclimation rate for the surviving was 70%. This work provides a basis for promoting haploid breeding in cucumber.

Keywords Cucumber (*cucumis sativus* L.) · Ovary culture · Doubled haploid · Direct regeneration

Key message

An effective technique for the production of double haploids was provided through ovary culture in cucumber (*Cucumis sativus* L.). More interesting, we observed the entire process of gynogenesis.

Introduction

Gynogenesis refers to the process of embryo regeneration from unfertilized female gametophytes in vitro or in vivo (Gémes-Juhász et al. 2002). In contrast to somatic embryogenesis, which results in clonal propagation of a specific genotype, gynogenic plants exhibit the product of meiotic segregation. They have the important characteristic of containing only one set of chromosomes and are therefore haploid plants (Maraschin et al. 2005). Homozygous double haploids obtained by colchicine-induced or spontaneous doubling are used in breeding.

The process of inducing female gametophytes to form embryos is difficult to sustainable observe, because the embryo sac is embedded in somatic cell tissues, making it difficult to isolate a single embryo sac for culture; therefore,

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the technique for obtaining haploid plants through in vitro gynogenesis is less than perfect. However, in vitro gynogenesis has unique value, especially in the cases of male sterile lines and dioecious plants, while haploids induced by means of androgenesis are not successful or their induction rates are too low (Bhat and Murthy 2007). Years of sustained effort have yielded some successes in gynogenetic induction in the *Cucurbitaceae* (Metwally et al. 1998; Gémes-Juhász et al. 2002; Shalaby 2007; Diao et al. 2009; Li et al. 2013; Plapung et al. 2014; Wang et al. 2015; Tantasawat et al. 2015), but there are still many problems related to haploid induction via gynogenesis. The culture process was complex, preculture, induction culture and regeneration culture were carried out, low embryo production and low plant regeneration rates remain major problems. In our study, combined with one-step culture method, low temperature pretreatment and the addition of TDZ in the medium were studied to improve the regeneration rate.

Cucumber is a monoecious, open-pollinated plant in which breeding cultivars by traditional methods requires 6–8 years (Tugce et al. 2017). The performance of parental lines is often affected by the environment, and cucumber resistance to pathogens is regulated by recessive genes, making breeding work difficult (Gémes-Juhász et al. 2002). DHLs (doubled haploid lines) can be obtained in 1–2 years by using unpollinated ovary cultures, and this approach is potentially highly valuable for discovering novel genetic recombinations (Chen et al. 2011). In addition, DHLs are ideal materials for genetic analysis and gene functional analyses in cucumber.

Studies of gynogenesis in cucumber (*Cucumis sativus* L.) are not rare, but gynogenetic regeneration rates have been low due to lack of established methods, hindering the application of this technique for breeding. A recent study has shown that the maximum frequencies of gynogenesis and plant regeneration are 18.4% and 7.1%, respectively (Tugce et al. 2017).

Previous studies have shown that successful production of haploids and double haploids in ovary or ovule culture depends on several factors, including genotype, pretreatment methods, embryo sac developmental stage, media composition and cultural conditions, and has been conducted as a complex process consisting of three steps, induction, differentiation and regeneration culture (Gémes et al. 2002; Diao et al. 2009; Li et al. 2013; Plapung et al. 2014; Wang et al. 2015; Tugce et al. 2017). It is difficult to control problems in the complex process of culture, leading to low regeneration rates.

Ploidy identification of regenerated plants from ovary culture is very important to the determination whether plants can be used directly in breeding. Determining the ploidy level involves flow cytometry, based on the fluorescence intensity of nuclei stained with DNA-selective dye, which is highly accurate (Loureiro et al. 2010). Flow cytometry has been used to determine the ploidy level of members of the *Cucurbitaceae* (Yetisir and Sari 2003; Lotfi et al. 2003; Claveria et al. 2005). The chromosome counting method can more accurately identify plant ploidy, but it is always difficult to use root tips in tissue culture. However, flow cytometry can be used for ploidy identification using a small number of leaves.

With respect to diploid plants, those originating from female gametocytes are homozygotes and can be utilized directly in breeding, while those originating from somatic cells are heterozygous and are usually considered undesirable products of ovary culture. Thus, it is important to identify the origin of diploid plants in cucumber ovary culture. Simple sequence repeats (SSRs), known as microsatellites, are short stretches of tandemly repeated DNA (1–5 nucleotides long). They are highly polymorphic and somatically stable and are inherited in a codominant manner. SSRs have been used as a tool for distinguishing between diploids and DHLs to identify homozygosity in cucumber (Diao et al. 2009), coconut (Perera et al. 2008), and melon (Malik et al. 2011), meaning that homozygosity assessment via SSRs is effective.

The goal of the present work was to simplify culturing procedures and to devise an effective method for the direct regeneration of haploid or doubled haploid plantlets through ovary culture. For this purpose, we investigated (1) tolerance times for low-temperature treatment at 4 °C, (2) the effects of TDZ (thidiazuron) concentrations in the medium, (3) histological and cellular morphological obsevation on gynogenesis, (4) plant ploidy identification, which was analyzed using flow cytometry and counting of chromosome number, and (5) the use of SSRs to identify homozygous origins from heterozygous genotypes in ovary culture.

Materials and methods

Plant material

Genotypes of 30 heterozygous cucumber (*Cucumis sativus* L.) were examined for preliminary screening experiments, including 12 from North China, 12 from South China and 6 European greenhouse types (Table 1). Seven gynotypes (SG11, SG22, SG33, SG34, SG64, SG25, SG35) have been selected for optimizing experiments. Among them, SG11, SG22, SG33, SG34, SG64 belong to North China type. SG25, SG35 belongs to South China type and European greenhouse type, respectively. SG33 is easy to produce embryo genotype, SG25, SG35 is low embryo genotype, SG22 is recalcitrant genotype. The plants were grown in a greenhouse at the Institute of Horticulture, Guizhou Academy of Agricultural Science, China. Ovaries of donor plants

Table 1 Genotype for screening experiments

No.	Genotype	Type
1.	SG 02	North China type
2.	SG 11	
3.	SG 22	
4.	SG 31	
5.	SG 33	
6.	SG 34	
7.	SG 42	
8.	SG 49	
9.	SG 64	
10.	SG 14	
11.	SG 15	
12.	SG 03	
13.	SG 88	South China type
14.	SG 25	
15.	SG 23	
16.	SG 61	
17.	SG 50	
18.	SG 111	
19.	SG 101	
20.	SG 115	
21.	SG 98	
22.	SG 96	
23.	SG 95	
24.	SG 78	
25.	SG 92	European greenhouse type
26.	SG 35	
27.	SG 93	
28.	SG 83	
29.	SG 89	
30.	SG 75	

were selected one month after the second female flower appeared. Unfertilized ovaries were harvested 6 h before anthesis, at this point, the color of the corolla is yellow-green, and most ovaries had nearly mature embryonic sacs (Gémes-Juhász et al. 2002) that served as optimal explants for inducing in vitro gynogenesis (Li et al. 2013). The ovaries were either fresh or pretreated in a refrigerator at 4 °C.

Culture medium composition

The basal medium was MS (Murashige and Skoog 1962) media supplemented with 3% sucrose and 7% agar. Two different concentrations of TDZ (0 $mg{\cdot}L^{-1}$ or 0.06 $mg{\cdot}L^{-1}$) were used as plant growth regulators, and hormone-free was controlled. Rooting medium was MS + 0.05 $mg{\cdot}L^{-1}$ 1-Naphthylacetic acid (NAA) + 30 $g{\cdot}L^{-1}$ sucrose + 7 $g{\cdot}L^{-1}$ agar. The pH of the medium was adjusted to 5.9 before autoclaving at 116 °C for 30 min.

Ovary culture process

After removing the papilloma, 6–8 ovaries were surface sterilized, first in 75% ethanol for 60 s and then in 1% sodium hypochlorite for 20 min, after which they were rinsed three times in sterile distilled water. The ovaries were sliced into 2 mm cross-sections under sterile conditions and then placed on 40 ml of solid medium in 150 ml glass petri dishes.

The cultures were incubated for 2 d at 33 °C in the dark, then transferred to 25 °C conditions with a 16/8 h (light/dark) photoperiod (cool-white fluorescent light, 3500 lx) for direct formation of plantlets. Plant was formed after 5 months of culture. A diagram illustrating the method was provided (Fig. 1).

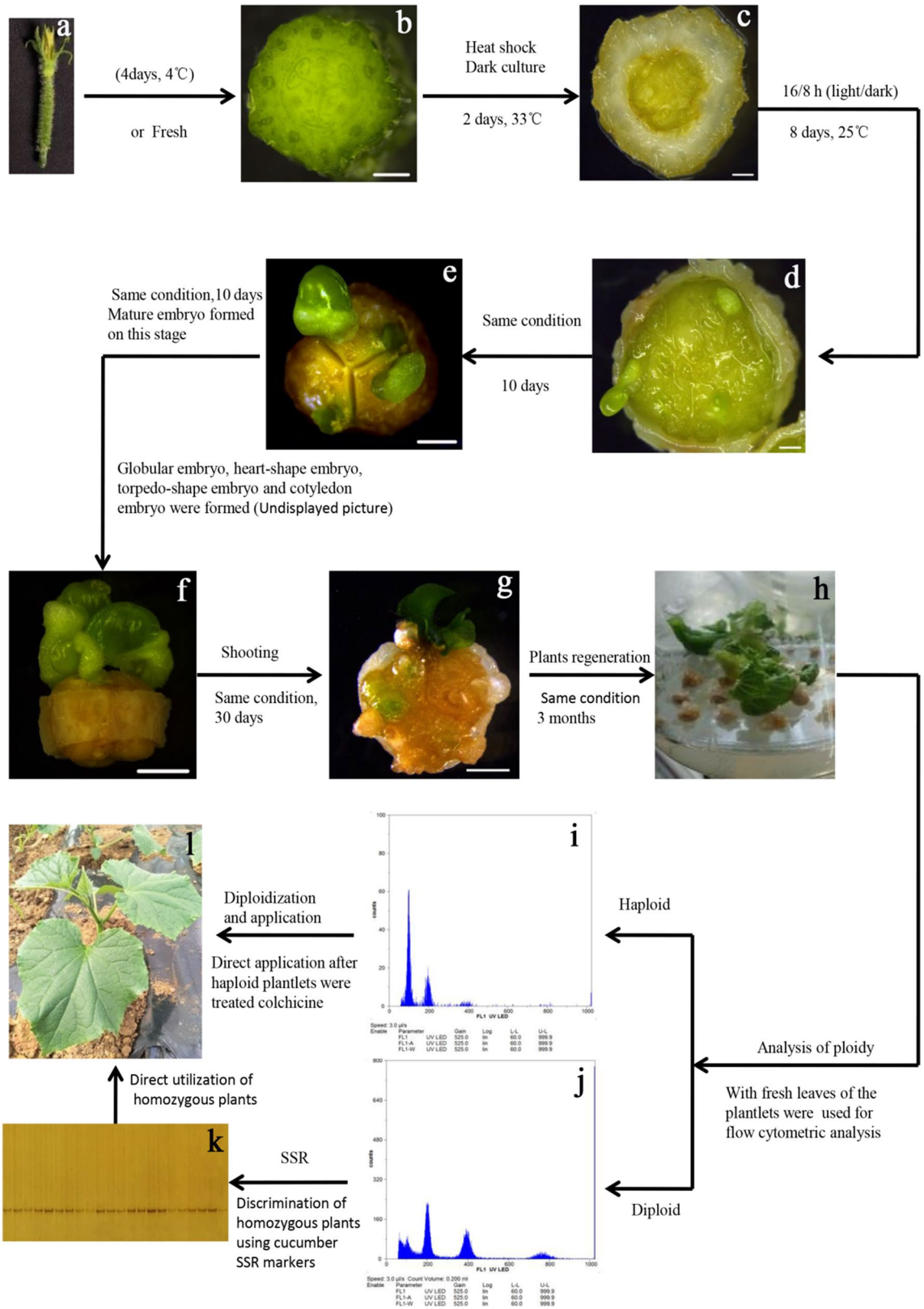

Fig. 1 Flow chart of plant regeneration via in vitro gynogenesis in cucumber. The solid black arrows show the transition between steps. The solid white lines represent 2 mm

Preliminary screening experiments

Ovary culture was carried out for 30 genotypes for preliminary screening experiments. Ovaries treated at 4 °C for 4 days before culture. MS + 0.06 mg·L^{-1}TDZ + 7% agar + 3% sucrose. Fifty ovary sections were collected for each genotype. The embryo rate was determined after 30 d of culture, and the plant regeneration rate was determined after 5 months of culture.

Optimization experiment

The main experiments was carried out in a completely randomized factorial design (i.e. seven genotypes × two pretreatment × two hormonal treatments = 28 treatments) with three replicates. Each replicate consisted of 5 ovaries (50 slices).

Observations

The observation of embryonic sac formation was based on ovaries 2 d, 1 d and 6 h before flowering of SG 33, SG35 and SG22 from 3 different geographical origins (north, south China and Europe). Sixty embryo sacs were observed at each developmental stage of each genotype, and the number of mature embryo sacs were counted. Ovarian fragments before and after culture of SG33, SG35 and SG22 were collected for resin sections to observe the development of early embryos. Fifty ovarian fragments were collected from 0 d, 2 d, 4 d, 8 d and 10 d, respectively. All materials were fixed in formalin: glacial acetic acid: 70% ethanol (FAA) in a ratio of 1:1:18 at room temperature for 48 h. The materials were then dehydrated using a graded ethanol series and embedded in spurr resin (Li et al. 2013). Nine-micrometer-thick sections were cut, stained with safranine and fast green and observed using a microscope (Leica DM2500, Wetzlar, Germany). After 20 d of culture, the embryos' morphology was evaluated using a stereomicroscope (Leica M165 C, Wetzlar, Germany).

Chromosome ploidy identification of gynogenetic plants

Partec CyFlow Space flow cytometer

Fresh, nonfixed samples were examined to determine their ploidy level using a Partec CyFlow Space flow cytometer (Partec, Münster, Germany). First, approximately 50 mg of leaf tissue was placed in a plastic petri dish; then, 0.5 ml of cell lysates (Partec, Gorlitz, Germany) was added. The leaf tissue was chopped with a sharp razor blade to release cell nuclei; then 1.5 ml of 4′,6-diamidino-2-phenylindole (DAPI, excitation wavelength of 360 nm) was added and the sample incubated in the dark at room temperature for 5 min. The sample was then filtered through a 50 µm CellTrics® disposable filter (Code No. 04-0SG042-2317). Afterward, the sample was analyzed in a Partec CyFlow Space flow cytometer with UV excitation to estimate the absolute value of DNA content for each sample. This process was repeated three times for each sample.

Chromosome counting

For chromosome preparation, root tips were collected from field-grown gynogenic plants. The procedure for chromosome preparation is essentially the same as published protocols (Chen et al. 2003). Chromosomes were stained with DAPI and subjected to fluorescence in situ hybridization (FISH) for counting those in mitotic metaphase (Lou et al. 2013).

Diploidization

Haploid shoot apical meristems were soaked by 0.1% colchicine (filter sterilization) 1 h on super-clean worktable. Next, them were washed with sterile water 3 times and inoculated in MS medium. The ploidy of plant was performed by flow cytometry after culturing 30 days.

Rooting and acclimation

The green shoots inoculated in rooting medium for 15 days, the bottle cap was lifted and placed in the culture chamber for 2 days. The rooted seedlings were carefully removed from the solidified media to minimize damage to the roots. Next, they were potted in 250 ml of peat, perlite and vermiculite (3:1:1) and were covered with plastic cap to prevent dehydration. At last, the plants were transplanted into the field after 15 days of acclimation in the light incubator.

SSR analysis

The diploid plants obtained from ovary culture were self-pollinated, and the next generation was field planted. Young leaves of approximately 20 plants were randomly selected from DHLs and mother plants (F2, second progeny of SG 33) in order to extract DNA. Genomic DNA was extracted using the cetyltrimethylammonium bromide method (Murray and Thompson 1980). DNA concentration and quality were measured with a Qubit® 2.0 fluorometer (Invitrogen-Molecular Probes, Eugene, OR) and by 1% agarose gel electrophoresis with standard lambda DNA and an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Fifteen cucumber SSR primer pairs were designed using the method of Diao et al. (2009) (Table 2), and the primers

Table 2 Primer sequences used for SSRs in this paper

Primers	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
CMCCA145	GAGGGAAGGCAGAAACCAAAG	GCTACTTTTGTGGTGGTGG
CMAG59	TTGGGTGGCAATGAGGAA	ATATGATCTTCCATTTCCA
CMGA104	TTACTGGGTTTTGCCGATTT	AATTCCGTATTCAACTCTCC
CMCTT144	CAAAAGGTTTCGATTGGTGGG	AAATGGTGGGGGTTGAATAGG
CMTC47	GCATAAAAGAATTTGCAGAC	AGAATTGAGAAGAGATAGAG
CMAT141	AAGCACACCACCACCCGTAA	GTGAATGGTATGTTATCCTTG
CMTC123	CGGATTGTACTTATTGCCAAG	CATGTGCATGTGTGCATGTAC
CMTA170a	TTAAATCCCAAAGACATGGCG	AGACGAAGGACGGTTAGCTTT
CMCT170b	ATTGCCCAACTAAACTAAACC	CACAACACAATATCATCCTTG
CMGA165	CTTGTTTCGAGACTATGGTG	TTCAACTACAGCAAGGTCAGC
CMTCl60a + b	GTCTCTCTCCCTTATCTTCCA	GATGGTGCCTTAGTTGTTCCG
CMCT505	GACAGTAATCACCTCATCAAC	GGGAATGTAAATTGGATATG
CSCTTT15a	GTTTGATAATGGCGGATTGT	GTAGAAATGAAGGTATGGTGG
CSAT214	TTGAGTACCATTGTCATAGAT	TTAGTTTAATTTCATCTCTGT
CSAT425	TAGGGCAGGTATTATTTCAG	ACGGACTGATTTAGTATAGGC

were synthesized by Invitrogen (Shanghai, China). Each DNA amplification was carried out in a 20 µl reaction mixture (1.0 µl of template DNA, 1.0 µl of each SSR primer pair, 10.0 µl of Taq mix, 7.0 µl of ddH_2O). PCR was performed as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The PCR products were separated on 7.2% polyacrylamide gels via PAGE, then subjected to silver staining (Gresshoff 1991).

Statistical analysis

The frequency of induced plant regeneration was calculated as the quotient of the total number of regenerated plants and the total number of treated ovaries. The data were subjected to analysis of variance (ANOVA), and the means were compared using Duncan's new multiple range test at the 5% level of significance ($p < 0.05$) to check for significance between groups. IBM Statistics 20.0 software (IBM company) was used for the statistical analysis.

Results

The present paper provides an efficient method for doubled haploid cucumber production through in vitro ovary. Ovaries were subjected to cold pretreatment (4 ℃) for 4 days and then they were compared with freshly cultured ovaries.

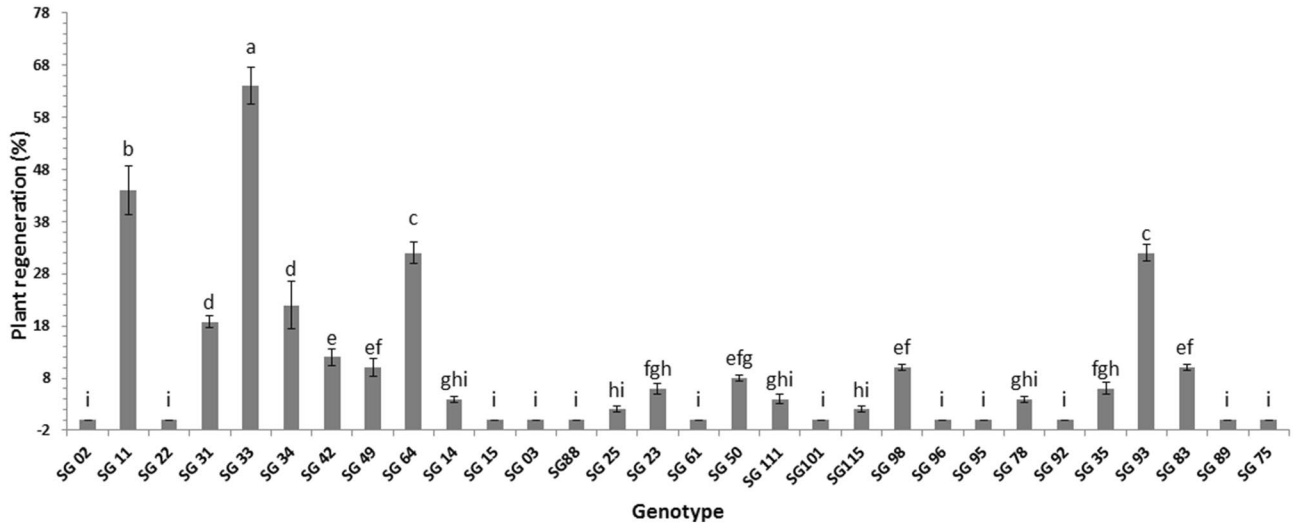

Fig. 2 Effect of preliminary screening experiments

The ovaries were cultured on TDZ to increase the doubled haploid plant induction rate. Thirty genotypes were used in the screening experiment, seven genotypes of which be used for optimization experiment. Seven genotypes' responses to cold pretreatment and TDZ application were investigated. The chromosome sets of the haploid and doubled haploid were analyzed by flow cytometry and chromosome counting. The diploid plants were further identified as pure doubled haploid using SSR. Finally, the doubled haploid plants were field (Fig. 1).

The results obtained corroborated that cold pretreatment could efficiently induce more regeneration plants in our experiment. Higher rates of doubled haploid plant emergence were achieved using appropriate concentrations of TDZ. Plant regeneration showed high genotype dependency. TDZ interaction with cold pretreatment generally induced more regeneration plants. The genotypes responded differentially to TDZ or cold treatments, hence the statistically significant interaction between genotype and TDZ or cold treatments. The three-way interaction of genotype × cold pretreatment × TDZ treatment was optimum in regeneration plant induction.

Effect of preliminary screening experiments

ANOVA confirmed that difference among the genotypes was significant ($p < 0.05$).

In the thirty genotypes the ovary response of SG 33 was statistically significant and it produced the highest rates of plant regeneration (64%). SG11was statistically and it produced the higher rates of plant regeneration (47%). SG64 and SG93were statistically similar and they produced the higher rates of plant regeneration (28 and 29%, respectively), whereas the ovary response of 12 genotypes were recalcitrant (Fig. 2).

Effect of TDZ treatment

ANOVA results confirmed that the effect of TDZ concentration on plant regeneration rate was significant ($p < 0.05$). The rate of plant regeneration increased with increases in TDZ concentration. The plant regeneration rate was the highest when the TDZ concentration was 0.06 mg·L^{-1}(58.3%). No regenerated plant was produced on a hormone-free medium. (Fig. 3a).

Effect of cold pretreatment

Significantly different rates of plant regeneration were induced from fresh and 4 days cold pretreated ovary at 4 ℃ ($p < 0.05$). The higher regeneration rate was found in 4 d treatment (70%) (Fig. 3b).The result indicated that optimally timed low-temperature treatment of ovaries was effective for the direct formation of regenerated plants.

Effect of genotypic variation

Seven genotypes were selected from different geographical origins and cultured again to confirm the effect of genotype on plant regeneration. The ANOVA results indicated that among these seven genotypes, the ovary response of SG 33 produced the highest rates of plant

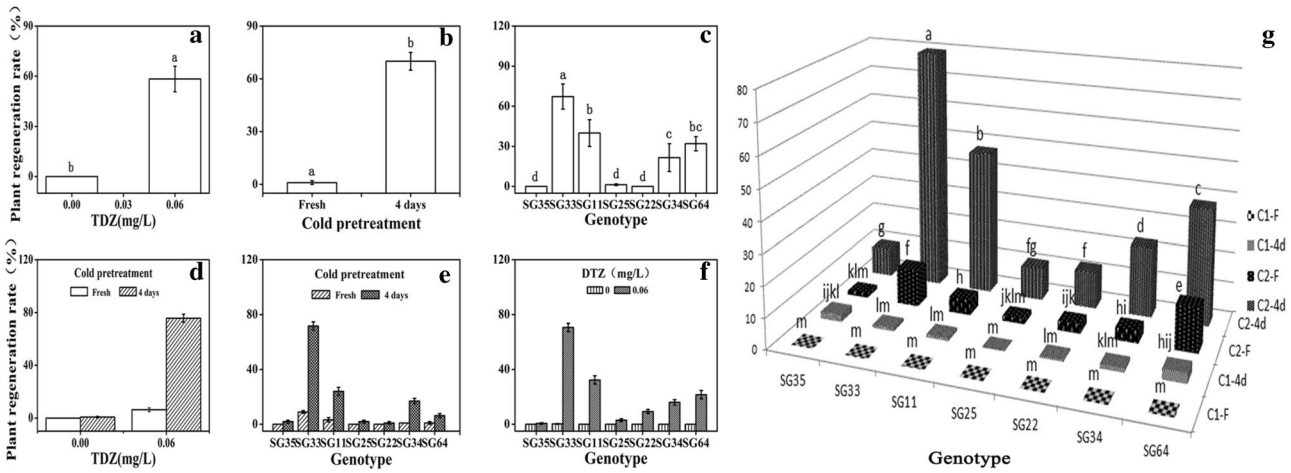

Fig. 3 Percentage of plant regeneration in ovary culture. **a** Main effects of TDZ. **b** Main effects of 4 days cold pretreatment(4 °C) or fresh culturing treatment on plant induction. **c** Main effect of genotype on plant induction. **d** Effect of cold pretreatment and TDZ treatment interaction on plant induction. **e** Effect of cold pretreatment and genotype interaction on plant induction. **f** Effect of TDZ treatment and genotype interaction on plant induction. **g** Effect of three-way-interaction of cold pretreatment × TDZ concentration × genotype on plant induction. C1: 0 mg·L^{-1}. C2: 0.06 mg·L^{-1}. *F* fresh. *d* days

(67.3%) ($p < 0.05$). The results were consistent with the initial screening experiment. SG 22 and SG35 were all the same recalcitrant (Fig. 3c).

Effect of cold pretreatment and TDZ treatment interaction

Interaction of 0.06 mg·L^{-1} TDZ with 4 days cold pretreatment ($p < 0.05$) induced the highest rates of plant regeneration among the genotypes (75.6%), freshly ovary on media containing 0.06 mg·L^{-1} TDZ produced the lowest(0.6%) amounts of plants. Whereas fresh/cold pretreated ovary cultured on hormone-free medium wasn't produced plant (Fig. 3d).

Effect of cold pretreatment and genotype interaction

The interaction of genotype × cold pretreatment was statistically significant ($p < 0.05$). Cold pretreatment for 4 days increased the plant means of all the genotypes as compared with freshly cultured ones. This increase was more noticeable for the recalcitrant genotype (SG22, SG35), such that cold pretreatment induced (1% and 2%, respectively) plant through comparison with freshly cultured ones with no response (0.0%) (Fig. 3e).

Effect of TDZ treatment and genotype interaction

The interaction of genotype × TDZ was statistically significant ($p < 0.05$). Seven genotypes were response on medium with 0.06 mg·L^{-1} TDZ. The highest plant regeneration percentages among the genotypes were recorded for SG33 (70.6%). Next, SG11, SG34 and SG64 were 32.3, 21.6% and 9.3%, respectively. SG25, SG22, SG35 were least (Fig. 3f). However, on the hormone-free medium, none of the other genotypes except SG33 responded.

Effect of three-way interaction of cold pretreatment × TDZ concentration × genotype on plant induction

The three-way interaction of genotype × cold pretreatment × TDZ treatment was statistically significant ($p < 0.05$). The interaction of 4 days cold pretreatment and 0.06 mg·L^{-1} was the best combination and induced the highest rate of plants from the genotypes, but this rate varied between different genotypes. The interaction of fresh ovaries with hormone-free medium cannot induce plants. Except for SG25, all other genotypes induced the plants on the medium of 0.06 mg·L^{-1}. The best records of plants were seen for SG33, SG11, SG64, SG34, SG22, SG25 (79.3%, 47.3%, 38%, 23%, 14%,12%, respectively) (Fig. 3g).

Megasporogenesis and embryo sac formation in cucumber

In this study, samples were collected at three time points (2 d, 1 d, and 6 h before anthesis) base on SG33, SG35 and SG22.The ovaries have 3 locules and inverted ovules (Fig. 4a, b). In the nucellus of the ovule, a relatively large sporogenous cell with a large nucleus, thick cytoplasm and low vacuolation was differentiated (Fig. 4c), with a volume greater than those of surrounding cells, developing directly into a megasporocyte without division. The cell's volume increased before meiosis (Fig. 4d). This megasporocyte underwent meiosis to form a dyad (Fig. 4e, f), then underwent a second cycle of meiosis to form a longitudinally arranged tetrad (Fig. 4g), that is, four single-nucleated megaspores. Three of the four megaspores near the micropylar end degenerated, while the megaspore near the chalazal end continued to develop, its nuclear volume increasing to form a functional megaspore. At this time, a mononuclear embryonic sac was formed (Fig. 4h) and continued to develop, enlarging in size and dividing mitotically three times in succession. After the first mitotic division, two nuclei migrated to the micropylar and chalazal ends (Fig. 4i), and the cytoplasm then divided to form a binuclear embryo sac (Fig. 4j). The binuclear embryo sac underwent two subsequent cycles of mitosis to produce a mature embryo sac with four nuclei near the micropylar end and chalazal end (Fig. 4k). Two nuclei moved to the central part of the embryo sac (Fig. 4 l) and finally fused to form a central cell with two nuclei (Fig. 4m). The remaining three nuclei formed three oocyte organelles at the micropylar end, including one egg cell and two synergids. The egg cell was larger and relatively far from the micropylar end. The synergids were located close to the micropylar end, on both sides of the egg cell, forming an inverted triangle (Fig. 4 n). At the chalazal end, three nuclei converged to form antipodal cells (Fig. 4 o), forming a mature embryo sac. Figure 4 c–h, i–l, m–n come from samples of 2 d, 1 d and 6 h before anthesis, espectively. Embryo sac of 6 h before anthesis were matured, which were cultuered to obtainning regenerated plants easily.Observation of embryogenesis and shoot formation.

The ovaries were harvested 6 h before anthesis and pretreated at 4 °C for 4 d. The ovaries were inoculated on MS medium supplemented with 0.06 mg·L^{-1} TDZ. After 2 d of heat shock culture at 33 °C, the ovaries were transferred to 25 °C for direct induction of plant regeneration. Ovary sections cultured for 2d, 4d, 8d and 10 d were observed in resin sections. After 2 d of heat shock culture, the ovule had noticeably enlarged (Fig. 1c), the cells had undergone mitosis to form a binuclear structure (Fig. 5a). The cultures of ovary was then transferred to 25 °C, with 16 h of light and 8 h of darkness for 2 d (fourth day).The cells carried out multiple cycles of mitosis to form multicellular

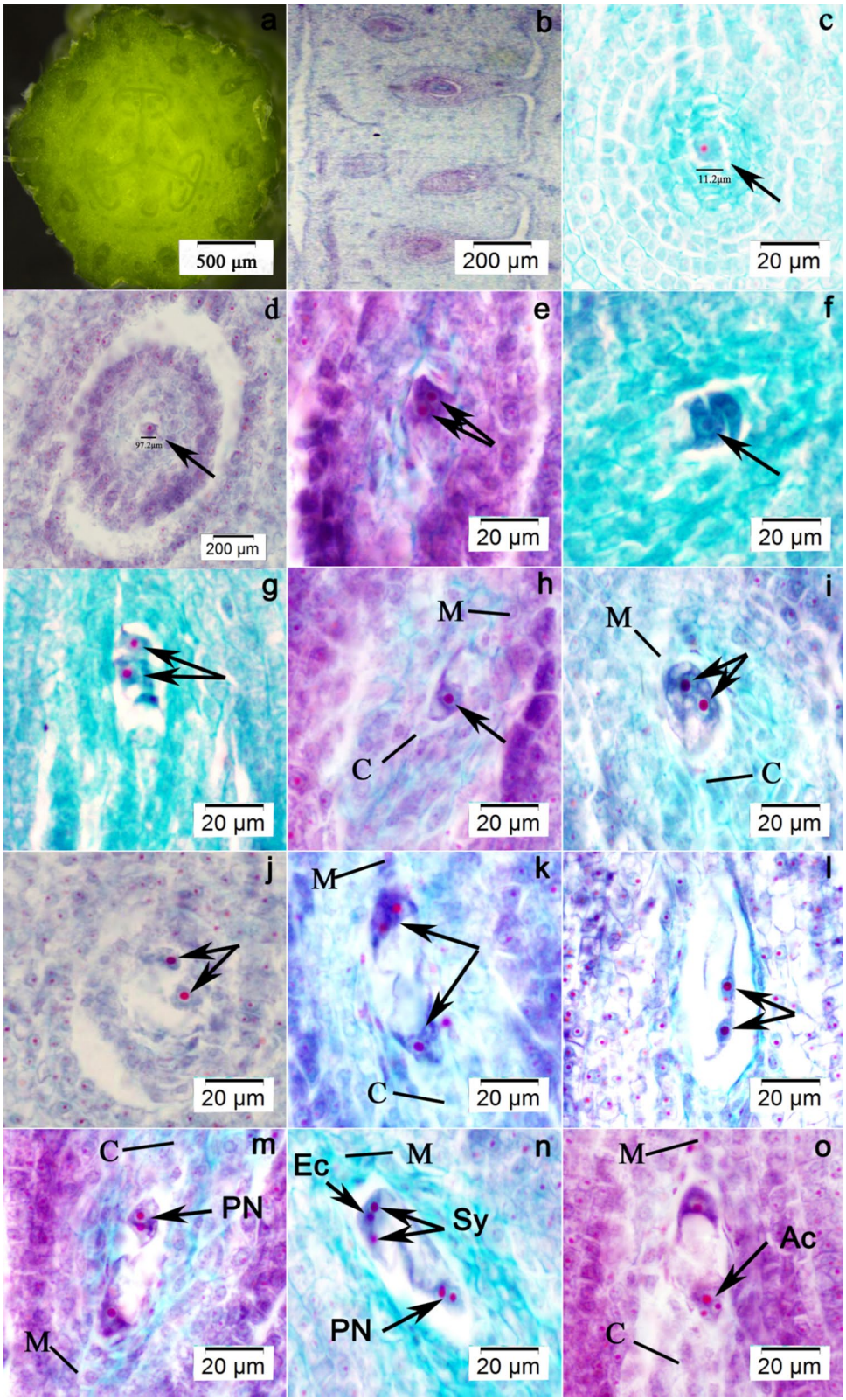

Fig. 4 Megasporogenesis and female gametophyte formation in cucumber. **a** Ovary cross-cutting. **b** Carpel longitudinal incision. **c** Sporogonial stage. **d** Megasporocyte. **e** The early stage of dyad. **f** Dyad period. **g** Tetrad period. **h** Monocyte embryo sac. **i** The early stage of dicaryotiinucleated embryo sac. **j** Binuclear embryo sac. **k** Eight nuclei embryo sac. **l** Two nuclei move toc embryo sac. **m** Binuclear central cell. **n** Mature embryo sac. **o** Antipodal cells. *M* Micropylar. *C* Chalzal. *EC* Egg cells. *Sy* Synergid. *PN* Polar nucleus. *AC* Antipodal cells

structure with multiple distinct nuclei (Fig. 5b, c, d). On the eighth day, the cells divided to form a cell clusters structure (Fig. 5e). On the tenth day, the cells divided and formed embryoid (Fig. 5f).

Some mature embryos came into being in the integument. As shown in Fig. 5f, there were complete embryos in the ovule, and the integument remained intact; however, most ovules continued to develop after protruding from the other

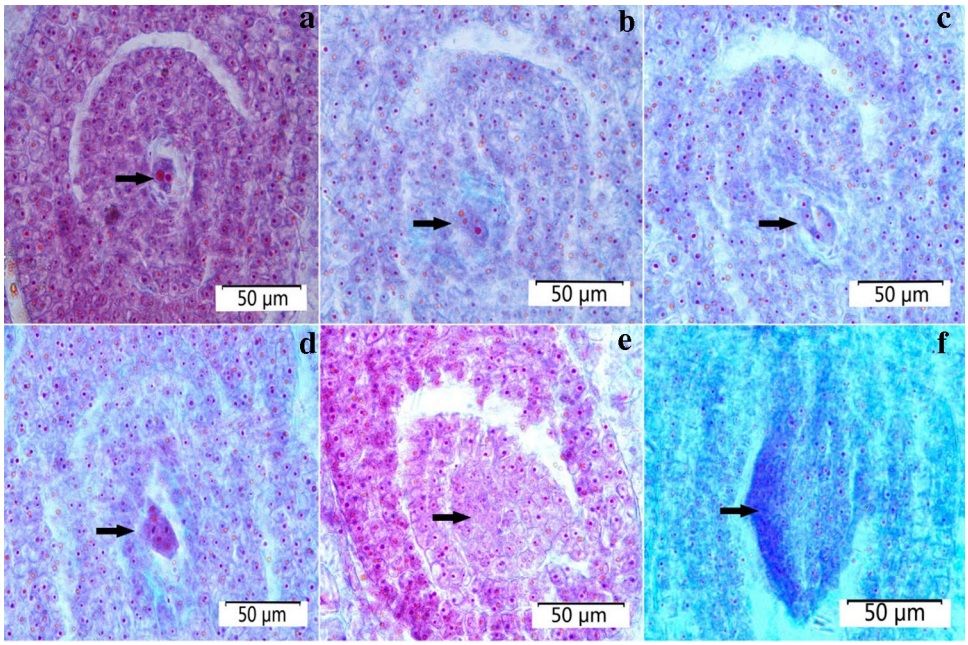

Fig. 5 The process of embryo development in cucumber ovary culture. **a** After 2 days of culture, two cells. **b**–**d** After 4 days of culture, 4–6 cells. **e** After 8 days of culture, multicellular clusters. **f** After 10 days of culture, embryoid

tissues (Fig. 6a), the embryoid broke through the integument and developed into a globular embryo (Fig. 6b), a heart-shaped embryo (Fig. 6c), a torpedo-shaped embryo (Fig. 6d) or a cotyledon-shaped embryo (Fig. 6e–i), which took 20 d. The cotyledon-shaped embryos continued to grow into green embryos (Fig. 6j) and formed shoot after 30 d of culture (Fig. 6k), then formed green shoot after 3 months of culture (Fig. 6l). Abnormal embryos such as translucent embryos or embryos with multiple cotyledons were also observed during embryogenesis (not shown in this article).

Chromosome ploidy identification of gynogenetic plants

Ploidy identification of gynogenetic regenerated plants was carried out using flow cytometry to identify ploidy in young leaves and chromosome counting for the root tips of the plants. Flow cytometry and chromosome counting were very accurate for the determination of chromosome number. Of the 45 plants obtained from SG 33, 11 plants were haploid (Fig. 7 c) (2n = x = 7; Fig. 7e); 20 plants were identified as DH (Fig. 7d), which showed the number of diploid chromosomes (2n = 2x = 14; Fig. 7f); and the other 14 plants were mixed polyploid or polyploid plants (not discussed in this article). In addition to the differences in chromosome number between haploid and double haploid, different phenotypes were observed. Under the same culture conditions, haploid plants grew slowly and were smaller in size (Fig. 7a). DH plants were normal plants, and their parents were normal plants (Fig. 7b). The three methods for identifying ploidy level were closely related.

Diploidization, rooting and acclimation

On the rooting medium, the plant growth was normal and the root growth was robust (Fig. 8). Low concentration NAA was beneficial to root formation. Using a substrate of peat, perlite and vermiculite (3:1:1) to transplant, domesticated in a light incubator with a survival rate of 70%, and the survival rate of transplanting in the field is 100%.

Identification of homozygous diploid plants via SSR analysis

We determined that plantlets either originated from the embryonic sac (egg apparatus or synergids), with spontaneous doubling of chromosomes occurring during embryogenesis, or that plantlet growth was initiated from diploid somatic tissue (the nucellus and the integuments) of the ovary. Therefore, discrimination of diploid plants was performed using SSR analysis. Fifteen microsatellite primer pairs outlined in the Table 2 were screened. Three (CMTC47, CMTCl60a + b and CSGTT15a) of these primers indicated polymorphism in the mother plants. These primers were used for the detection of homozygosity. One primer pair (CMTCl60a + b) generated banding patterns that distinguished gynogenic plantlets from the mother material. Thus, SSR markers involving microsatellite CMTCl60a + b showed 100% homozygosity and confirmed that the alleles in the mother material (F2) were also present in the gynogenic plants. Marker CMTCl60a + b, could distinguish between different doubled haploid lines (DHLs) originating from the same mother material. DHL-2 was the same as DHL-3 but was different from DHL-1. Some mother (F2)

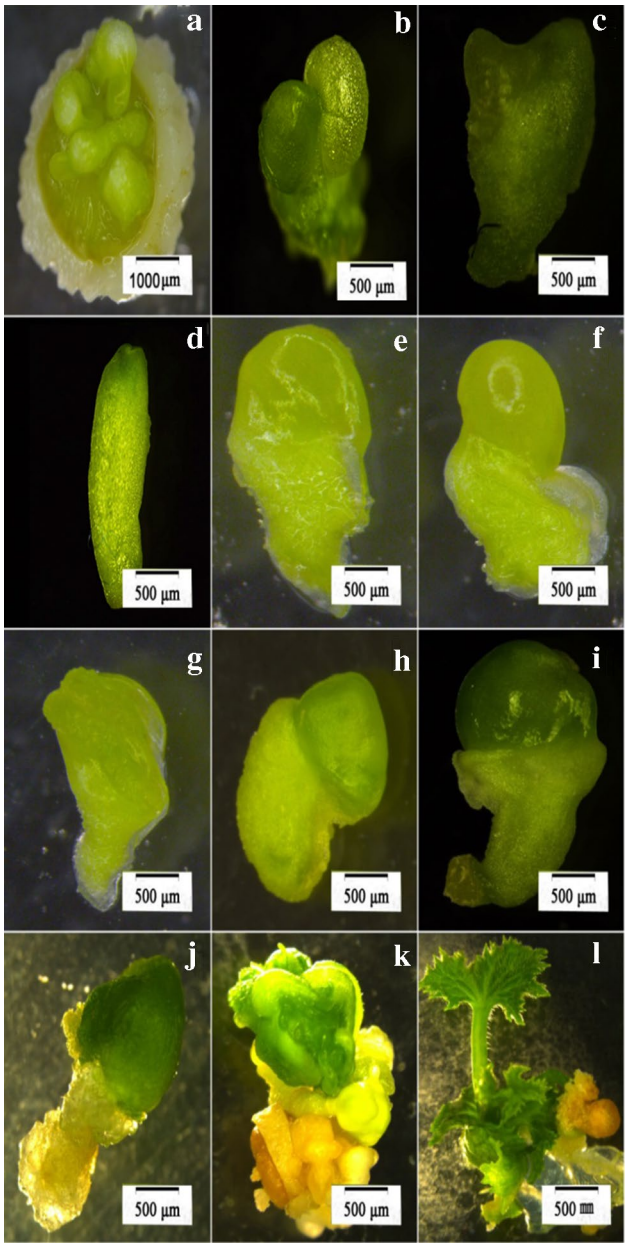

Fig. 6 The process of embryonic development after ovule protruding tissue. **a** Whole ovule protruding tissue; **b** Globular embryo; **c** Heart-shaped embryo; **d** Torpedo-shaped embryo; **e–i** Cotyledon-shaped embryo; **j** Cotyledon-shaped embryo turning green; **k** Cotyledon-shaped embryo forming shoot; **l** Normal plant

plants displayed two amplification bands, while those that originated via ovary culture showed a single band at the same locus (Fig. 9). By first investigating the loci to analyze the unknown diploids, we could efficiently eliminate the heterozygotes in a single amplification.

Discussion

Effect of cold pretreatment, TDZ and genotypes

The effect of cold pretreatment, TDZ, genotype and its interaction were investigated in the present experiment. Under natural conditions, in vivo gynogenesis depends on plant nutrition. Apart from macro- and microelements, ovarian development depends on phytohormones. In contrast, the aim of in vitro gynogenesis is to transition the female gametocyte from the gametophyte development pathway to the sporophyte pathway. In this case, plant growth regulators should be added to stimulate gynogenic development. In cucumber, TDZ has been successfully used for gynogenic induction and regeneration of embryos. When TDZ was used to induce embryos from ovary culture, the maximum frequencies of plant regeneration were 7.1% and 9.0% (Gémes-Juhász et al. 2002 and Diao et al. 2009). Gynogenesis of Chinese long cucumber was induced in unpollinated ovules cultured on cucumber basal medium (CBM) supplemented with TDZ. However, the plantlet formation frequency was 12.14% (Li et al. 2013). In addition, the embryogenesis rate was 16.92% on induction media supplemented with 0.02 mg·L^{-1} TDZ (Wang et al. 2015). Embryo-like structures had been obtained by culturing cucumber ovaries on MS basal media supplemented with TDZ (Tantasawat et al. 2015). In the present experiment, growth regulators were essential for the production of DHLs. The ANOVA results confirmed that the differences were statistically significant ($p < 0.05$). The plant regeneration rate was 58.3% on medium supplemented with 0.06 mg·L^{-1} TDZ, no regenerated plants were produced on a hormone-free medium.

Studies have suggested that ovary culture conditions can be modified to produce high-quality DH plants (Li et al. 2013). Some abiotic stress treatments, e.g., cold, dark/light, and starvation, exhibit stimulating effects in in vitro culture (Chen et al. 2011). Stress might not only alter the pathway of gametophyte development but led to autophagy and reuse of gametophyte gene products, thus transforming microspores into nonspecific, totipotent cells (Hosp et al. 2007). Cold treatment had been reported to be beneficial for gynogenesis (Pazuki et al. 2018) and androgenesis (Lantos et al. 2018). For example, cold (4 °C) pretreatment for 1 week is the best treatment for sugar beet ovary culture (Pazuki et al. 2018). In the present experiment, the ovaries were treated at 4 °C for 4 d and then cultured in MS media supplemented with 0.06 mg·L^{-1} TDZ, the highest rate of plant regeneration was 75.6%.

Gynogenesis (Tugce et al. 2017) and androgenesis (Castillo et al. 2015; Popova et al. 2016; Lantos et al. 2018) were highly dependent on genotype. Some genotypes are

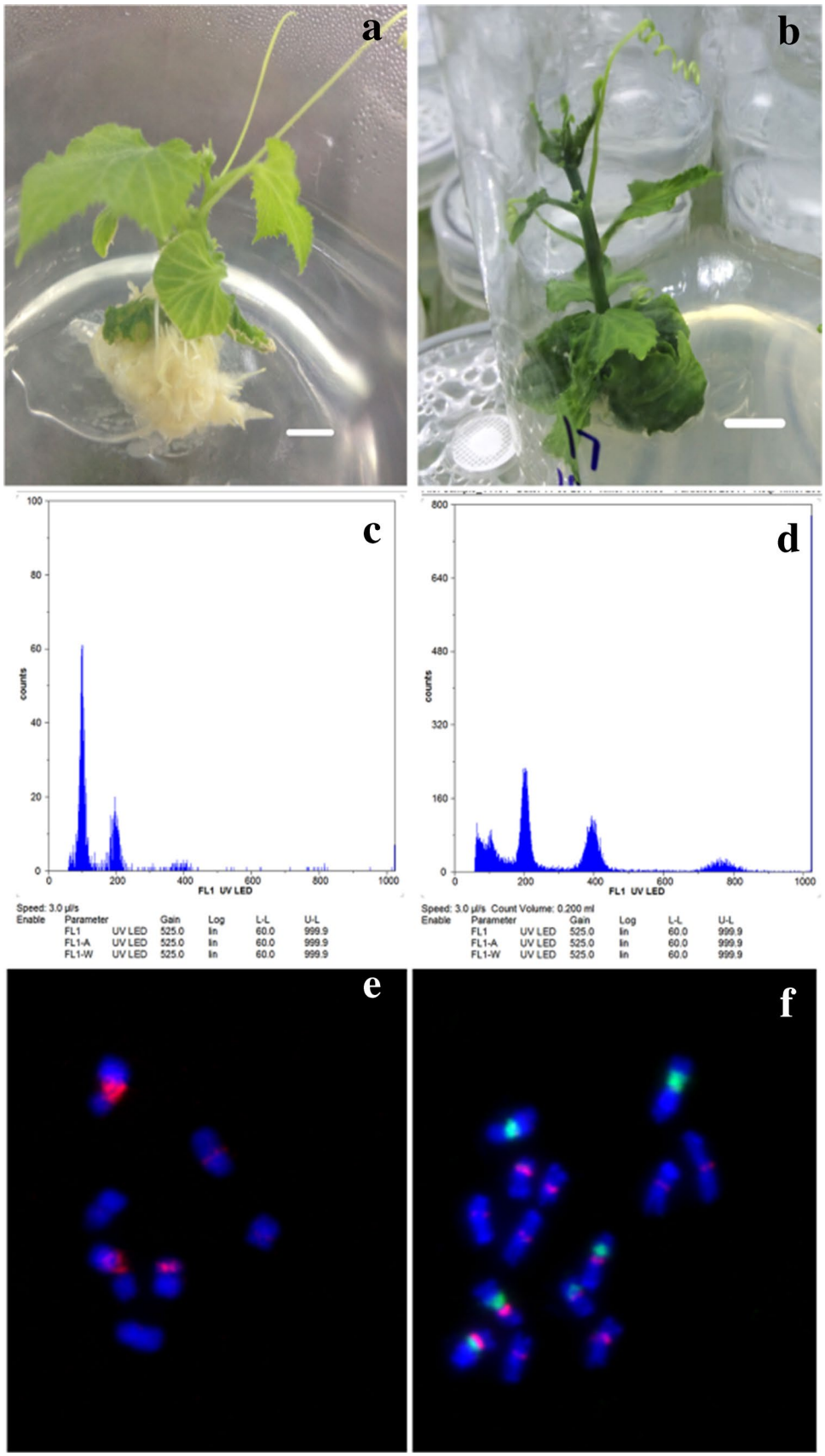

Fig. 7 Analysis of chromosome ploidy in cucumber. **a** haploid plants; **b** DH plants. **c** DNA distribution of a haploid plant; **d** DNA distribution of a diploid plant; **e** the chromosome number of haploid (n = x = 7); **f** the chromosome number of diploid(2n = 2x = 14). **c** and **d** abscissa: relative fluorescence intensity, ordinate: relative nuclear counts

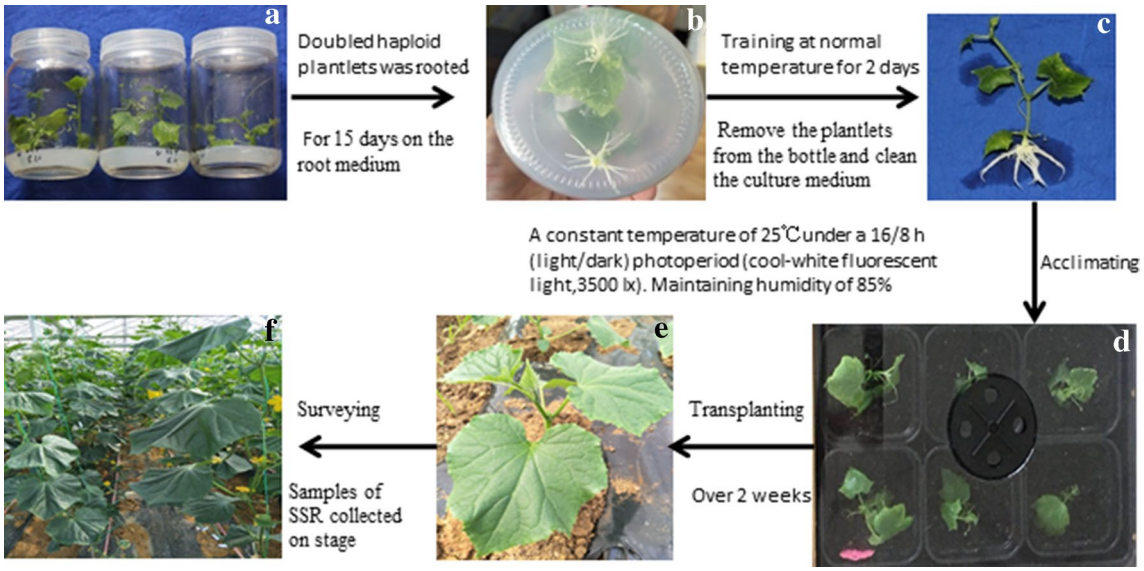

Fig. 8 Plant rooting, acclimation and cultivation **a** Doubled haploid plantlets. **b** Plant rooting. **c** Plants after acclimating. **d** Plant after cultivating. **e** and **f** Plants in the field

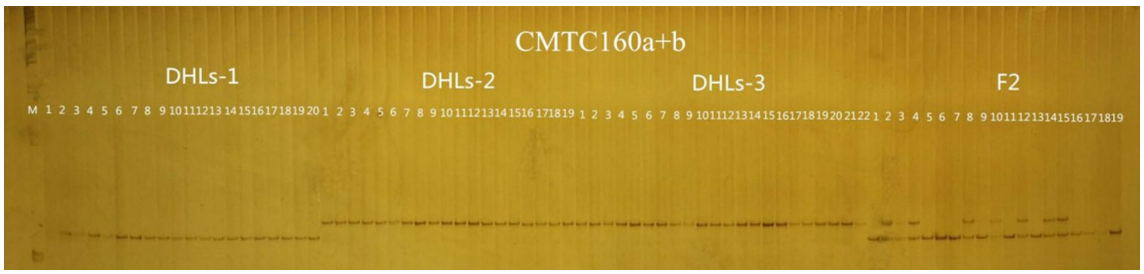

Fig. 9 PAGE of microsatellite CMTCl60a + b showing the homozygous state of DHLs from ovary culture. DHL-1: First DHLs (20) from SG 33; DHL-2: Second DHLs (19) from SG 33; DHL-3:Third DHLs (22) from SG 33; F2: second progeny of SG 33

recalcitrant, the rate of embryogenesis is low in pepper anther cultures, or reactions are not observed (Popova et al. 2016). In our study, significant effects of genotype on gynogenesis were statistically determined. Embryos were obtained from all 30 genotypes, but plants were obtained for only 18 genotypes, with the regeneration rate varying greatly, the plant regeneration rate of SG33 was 64%. The SG33 was 67.3% in the optimization experiment. Pazuki et al. (2018) published the effect of genotype × cold pretreatment × hormone treatment interaction on sugar beet ovule culture. Experiments have shown that this interaction was very effective in inducing haploid embryogenesis, although genotypes respond differently to the interaction. The three-way interaction of the independent variables(genotype × cold pretreatment × TDZ) showed statistically significant effects. SG33 × 4 ℃ cold pretreatment × 0.06 mg·L^{-1} TDZ was the optimal combination, which plant regeneration rate was 79.3%.

Megasporogenesis and embryo sac formation in cucumber

The development of the female gametophyte can be divided into two stages, megasporogenesis and female gametogenesis. The most common form of female gametophyte is composed of seven cells of four different cell types, including

three antipodal cells, two synergids, one egg cell and one central cell.

During megasporogenesis, the diploid megaspore mother cells undergo meiosis and produce four haploid nuclei. There are three main megasporogenesis patterns in angiosperms: monosporic, bisporic and tetrasporic. The main difference between the three types is whether the cell plate occurs after meiosis, which determines the number of megaspores forming after meiosis (Yadegari 2004). In this study, it was observed that the megasporogenesis of cucumber is monosporic. During the development of the female gametophyte, the megaspore was initially mitotic but with nondividing cytoplasm. Subsequently, the cytoplasm of the dikaryotic cells splits to form two monocytes, called binuclear embryo sacs. These two cells performed a second mitotic cycle to form a tetranuclear embryo sac; then, the tetranuclear embryo sac was once again mitotically divided to form a seven-cell, eight-nucleus mature embryo sac. This is consistent with the formation of female gametophytes in cucumber reported by Luo et al. (2007).

Embryogenesis and plant regeneration in cucumber ovary culture

A completely developed embryo sac is necessary to successfully produce regenerated plants. Previous studies have shown that the most sensitive ovaries contain nearly mature or fully mature embryo sacs (Gémes-Juhász et al. 2002). At the time of anthesis, seven-celled female gametophytes are formed and the embryo induction rate is high (Li et al. 2013). In the process of embryo development observed in previous reports, after one week of culture, enlarged ovules were observed on some explant surfaces. These ovaries were then cultured for 2–4 weeks to form globular embryos. After being transferred to regeneration media, the globular embryos developed into heart-shaped embryos after 4–6 weeks. The heart-shaped embryos then developed into torpedo-shaped embryos, then cotyledon-shaped embryos 6–8 weeks later (Li et al. 2013). In the present study, only one-step media was used to induce plants via ovary culture. The early stages of embryonic development should be observed using histological sections. When the ovules enlarged and protruded, cellular morphology was observed using a stereoscopic microscope. Segments of unfertilized ovaries were then cultured on plantlet induction media in the dark at 33 °C for 2 d. The color of the ovary fragments changed from green to yellow, and the initial features of the enlarged ovules could be observed on the surfaces of segments. The cells divided mitotically into cell clumps in 4–10 d. Some ovules protruded from the tissues and continued to develop. It took 20 d for the embryoid structures to form globular embryos, heart-shaped embryos, torpedo-shaped embryos and then cotyledon-shaped embryos. The cotyledon-shaped embryos subsequently differentiated into green shoots after 30 d. The shoots were ultimately cultured for 3 months to form complete plants. We observed the process from cell division to plant regeneration, which had not been conducted in previous studies.

Chromosome ploidy identification of gynogenetic plants

Flow cytometry analysis can be carried out at the seedling stage of tissue culture. It is easy to operate with a small sample of explant. Flow cytometry is used to estimate plant genome size (nuclear DNA content). Based on DNA selective dyeing, the fluorescence intensity of guard cells can be detected. The results are very accurate (Loureiro et al. 2010). It has been reported (Yetisir and Sari 2003; Lotfi et al. 2003; Claveria et al. 2005) that flow cytometry has been used to detect the ploidy level of *cucurbitaceae*. In this study, flow cytometry and chromosome counting were consistent in identifying plant ploidy level.

Identification of homozygous diploid plants by SSR analysis

Three primer pairs, CMAG59, CMGA104 and CMCTT144, were shown to identify the homozygosity of gynogenesis plants (Diao et al. 2009). SSR markers have been successfully used in other crop species to identify spontaneous DH plants and to reveal homozygosity in very early embryogenesis (Malik et al. 2011; Perera et al. 2008; Chani et al. 2000). In the present study, fifteen microsatellite primer pairs were screened. Three of these fifteen pairs exhibited polymorphism in the mother plants, and these three, CMTC47, CMTCl60a + b and CSGTT15a, were used to detect polymorphism in genotype SG 33. CMTCl60a + b, could distinguish different DHLs that originated from ovary culture and the same mother material. Those plants were genetically homozygous, derived from embryo sac cells and considered spontaneous DH cucumber plants.

Conclusion

The present paper reports a simple and effective method of directly producing cucumber plants through unfertilized ovary culture. Based on our results, cold pretreatment for 4 days, TDZ for 0.06 $mg{\cdot}L^{-1}$, the interaction with genotype can be used as an effective strategy to improve the efficiency of gynogenesis. Apart from the two–three-way interactions (cold pretreatment × TDZ treatment × genotype), the induction effect was the main variable in all experiments. The plants obtained from ovary culture of cucumber plants were identified as diploid or haploid by flow cytometry, consistent

with the results of chromosome counting. The diploid plants were further identified as pure doubled haploid using simple sequence repeats (SSR). The doubling treatment we used was one of the simplest and most effective methods, completed in a short time (1 h) with a doubling rate of 75%. The acclimation rate for the surviving was 70%. In addition, we observed the process of cucumber megasporogenesis and plant regeneration. This study provides a basis for promoting haploid breeding, and provides potential insights into the production of diploid and haploid plants through the culture of ovaries for other *cucurbitaceous* crops.

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Data availability The data sets supporting the results of this article are included within the article.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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