Induction, identification, and characterization of autoallo-dodecaploid barnyard grass (*Echinochloa crus-galli* L.) using in *vitro* colchicine treatment

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Abstract

Polyploidization is a major trend in plant evolution that has many advantages over diploid. Barnyard grass (*Echinochloa crusgalli* L.) has many good characteristics, but has not been fully utilized until now. In this study, we report for the first time the *in vitro* induction of autoallo-dodecaploid *E. crus-galli* by colchicine treatment. Calli derived from young panicles were transferred to liquid medium containing different concentrations of colchicine (0.01, 0.05, or 0.1% w/v) and incubated for 24, 48, or 72 h. Treatment with 0.05% colchicine for 48 h was the most effective condition for producing polyploid plants, yielding 42.9% dodecaploids. The relative DNA content of the induced dodecaploids was twice that of wild-type hexaploids. The chromosome number of dodecaploids was $2n = 12x = 108$, whereas that of hexaploids was $2n = 6x = 54$. Compared with the hexaploids, the dodecaploids had larger individual stomata, but a lower stomatal density. There were significant differences between dodecaploid and hexaploid plants in terms of morphological variables, such as plant height, leaf length, panicle length, and grain size. Dodecaploid plants showed the obvious “gigas” effects of polyploid organs, as well as significantly reduced seed set. The nutritional concentrations of crude protein, crude fat, crude ash, and nitrogen-free extract in the dodecaploid were higher than those in the hexaploid, whereas the concentration of crude fiber in the dodecaploid was lower. Compared with the hexaploid, the concentrations of calcium, iron and some free amino acids in dodecaploid plants were significantly higher than in hexaploids. The dodecaploid *E. crus-galli* had been obtained successfully by treating calli with colchicine. And *E. crus-galli* has the potential to be developed as a new type of high quality forage crop for cultivation under stress conditions, especially the dodecaploid with its greater nutritional value.

Key Message

The autoallo-dodecaploid *Echinochloa crus-galli* L. was induced by in vitro colchicine treatment and some characteristics were identified.

Introduction

Barnyard grasses (genus *Echinochloa*) belong to the subfamily Panicoideae (family Poaceae) (Giussani et al. 2001) and include about 50 annual or perennial species that are widely distributed throughout the world (Aoki and Yamaguchi 2008; Luo and Min 1990). These species are mainly regarded as weeds that compete with crops and cause reductions in crop yields (Chauhan and Johnson 2011; Rao et al. 2007; Xuan et al. 2006), but two species, *Echinochloa utilis* and *Echinochloa frumentacea* have been cultivated as food and forage crops for a long time (Luo and Min 1990; Yabuno 1987). Of the *Echinochloa* species, common barnyard grass (*Echinochloa crus-galli*) is the most widespread (Guo et al. 2017). Research has shown that *E. crus-galli* plants have a high nutritional value and good palatability to many herbivorous animals and fish. Moreover, *E. crus-galli* can grow on low fertility or saline-alkaline soils and produce high yields, unlike other cultivated grasses. But *E. crus-galli* has not been subjected to conscious or unconscious domestication or improvement until now. Making full use of *E. crus-galli* would allow the development of a novel crop for poor soils and improve the availability of human food and animal feed in inhospitable parts of the world (Sun et al. 1989).

Polyploidization, one of the most important evolutionary events in plants, can increase genetic diversity, introduce new genetic combinations, foster adaptation to different environments, and increase plant vigor (Comai 2005; Fang and Morrell 2016; Yu et al. 2021). Studies have shown that all angiosperm species may have experienced one or more polyploidization events during their evolution (Jiang et al. 2013; Jiao et al. 2011; Otto 2007). The development of polyploids is currently one of the most effective plant breeding techniques available for obtaining useful characteristics, such as vigorous growth, larger organs and biomass, more nutrients, and higher levels of resistance to abiotic stresses, pests, and diseases (Chen 2007; Comai 2005; Dhawan and Lavania 1996; Hilu 1993; Niu et al. 2016; Shao et al. 2003). Autopolyploidization (chromosome duplication) can be achieved through natural polyploid formation (Zhu et al. 1998) or by artificial induction (Tang et al. 2010). Artificial induction is vital for polyploid breeding (Kang 2003), and colchicine treatments are widely used to induce polyploids artificially under both *in vivo* and *in vitro* conditions (Dhooghe et al. 2011; Tang et al. 2010). Colchicine treatment on proliferating shoots, bud tips (Gu et al. 2005; Roy et al. 2001; Shao et al. 2003) or calli (Gao et al. 2002; Song et al. 1997; Tang et al. 2010) has been successfully performed to achieve *in vitro* induction and accelerated multiplication of autopolyploids.

Previous studies had suggested that *E. crus-galli* is a natural allohexaploid, arising from hybridization and polyploidization between tetraploid *Echinochloa oryzicola* (the paternal donor) and an unknown diploid species (as the maternal donor) (Aoki and Yamaguchi 2008; Guo et al. 2017; Yabuno 1966). In the present study, autoallo-dodecaploid plants of *E. crus-galli* were successfully induced by treating calli with colchicine. The polyploidy was identified by stomatal characteristics, flow cytometry, and confirmed by chromosome counts, and morphological characters and plant nutrient concentrations were compared between dodecaploid and hexaploid plants.

Materials And Methods

In vitro **colchicine treatment and plantlet regeneration**

The *E. crus-galli* was collected from the experimental field of Hubei University in Wuhan City (30°34'N, 114°20'E). Young panicles, 1–2 cm long, of hexaploid *E. crus-galli* were used as explants. After disinfection, the young panicles were cut into 1- to 2-mm sections and transferred to solid N_6 (Chu et al. 1975) induction medium supplemented with 2 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg L^{-1} 6-benzyladenine (BA), 1 mg L^{-1} 1-naphthaleneacetic acid (NAA), 5% (w/v) sucrose, and 0.75% (w/v) agar (pH 6.0) to induce callus formation in the dark at 25°C. Approximately four weeks later, vigorous calli were transferred to the liquid N_6 induction medium containing 0.01, 0.05, or 0.1% (w/v) colchicine and incubated at 25°C on an orbital shaker (110 rpm) for 24, 48, or 72 h. Medium without colchicine (0.0%) was used as the control. Each treatment consisted of 30 calli. The colchicine-treated calli were rinsed three times with sterile distilled water and cultured onto fresh solid colchicine-free N_6 induction medium to re-start growth. After 7–10 days, the calli were transferred to MS (Murashige and Skoog 1962) differentiation medium supplemented with 1 mg L^{-1} BA, 2 mg L^{-1} kinetin (KT), 0.3 mg L^{-1} NAA, 3% (w/v) sucrose, and 0.75% (w/v) agar (pH 6.0) and cultured in the light at 25°C to differentiate. Shoots regenerated from calli were transferred to rooting medium containing half-strength MS supplemented with 0.3 mg L^{-1} BA, 0.5 mg L^{-1} NAA, 2% (w/v) sucrose, 0.75% (w/v) agar, and 0.03% (w/v) activated carbon (AC) (pH 6.0). Roots were prevalent after approximately 7–10 days, at which time the plantlets were acclimated for three days in the culture room, then washed in tap water, and transplanted to the open field.

Ploidy analysis by flow cytometry

Flow cytometry analysis was performed as follows. Approximately 0.5 cm^2 of young leaf tissue from *in vitro* grown plantlets was chopped with a sharp razor blade in 0.5 mL of Partec HR-A buffer (Partec high-resolution nuclei extraction solution), in a plastic Petri dish. The sample was filtered through a 30-μm Partec CellTrics cell strainers directly into the sample tube, with 2 mL of HR-B buffer (Partec high-resolution DAPI staining solution). The sample was stained for 5 min at room temperature, and the relative fluorescence of total DNA was measured by Sysmex CyFlow Ploidy Analyser II (Sysmex Partec GmbH, Münster, Germany) instrument.

Chromosome counts

After the plantlets had been transferred to the field for about two weeks, root tips were excised from the plants and pretreated with 2 mM 8-hydroxyquinoline for 2 h at room temperature and then fixed in fresh Carnoy's fixative [methanol/acetic acid, 3:1 (v/v)] overnight. Then, the root tips were rinsed in 75 mM KCl for 30 min at room temperature, digested in an enzyme mixture containing 2% cellulase and 2% pectinase at 28°C for 4 h, washed three times in distilled water, and incubated in distilled water at room temperature for 20 min. These root tips were then placed on precooled slides and squashed in the presence of the fixative. The slides were heated over an alcohol flame to dry the fixative, stained with 5% Giemsa in Sorensen's buffer at room temperature for 1 h, washed under a stream of tap water, and then dried at room temperature. The chromosomes were observed under an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan) and photographed.

Stomatal observation

Stomatal measurements were made on completely developed flag leaves of field-grown plants. The lower epidermis was observed under a light microscope. The length and width of each stoma and the corresponding guard cells were measured with a micrometer. The stomata numbers within the grid squares were then counted to calculate the stomatal density. 30 stomata sizes and densities were measured randomly on three leaves for each population.

Morphological observations

Morphological characteristics of dodecaploid and hexaploid plants, namely plant height, tiller number, flag leaf length and width, leaf thickness, panicle length, anther length, grain length and width, total grain number, filled grain number, and seed set were measured. Total grain number, filled grain number, and seed set were measured on 10 panicles, and the other characteristics were measured on 30 biological replicates for each population.

Determination of plant nutrient concentrations

The nutrient concentrations of the leaves of dodecaploid and hexaploid plants, such as concentrations of water, crude protein (CP), crude fat (ether extract, EE), crude fiber (CF), crude ash (CA), and nitrogen-free extract (NFE), were determined in hexaploid and dodecaploid plants sampled two months after planting. In addition, the nutrient concentrations of total carotene (TCA), calcium, phosphorus, iron, and free amino acids were also determined. The samples were weighed (fresh weight) then dried at 105°C for 30 min, before being dried at 80°C for 8 h. The water contents of the samples were obtained from fresh weight minus dry weight, relative to fresh weight. The nutritional concentrations were determined according to Zhang (2016): CP was determined by the Kjeldahl method, EE was determined by the Soxhlet extraction method, CF was determined by the acid-alkali washing method, and CA was determined by the dry ash method. NFE was calculated using the equation: NFE (%) = Dry matter (%) - [CP (%) + EE (%) + CF (%) + CA (%)]. TCA was determined by extraction with acetone and colorimetric (λ = 450 nm) determination. For elemental analysis, the samples were digested with concentrated nitric acid-perchloric acid at 200°C, and the concentrations

of calcium, phosphorus and iron were determined using an inductively coupled plasma-optical emission spectrometer (ICP–OES) (Thermo Fisher Scientific, USA). Free amino acids concentrations were determined by high-performance liquid chromatographic (HPLC) (Agilent, USA). The concentration of each nutrient was determined on three biological replicates for each sample.

Data analysis

The data were analyzed using SPSS Statistics Version 24.0 (IBM, Armonk, NY, USA). Significant differences among the means of dodecaploid and hexaploid material were identified using the two-sample *t*-test.

Results

Survival of colchicine-treated calli

The survival rates of calli decreased with increasing colchicine concentration or treatment time. Colchicine treatment at 0.1% for 72 h proved to be highly toxic, resulting in the death of all the calli (Table 1). The colchicine concentration and treatment time also influenced the frequency of plantlet regeneration, the number of plantlets decreasing with increases in either colchicine concentration or treatment time. The results showed that the highest polyploidy rate was 42.9%, in the 0.05% colchicine concentration treatment applied for 48 h (Table 1).

Table 1
Survival and doubling rate of treated calli of *E. crus-galli* at different concentrations and durations of colchicine

Colchicine concentration (% w/v)	Duration (h)	Survival (%)[a]	No. of hexaploids obtained[b]	No. of dodecaploids obtained[b]	Doubling rate (%)[c]
0	24	100	27	0	0
	48	100	25	0	0
	72	100	20	0	0
0.01	24	90	21	0	0
	48	86.7	18	2	10.0
	72	80	14	2	12.5
0.05	24	76.7	9	3	25.0
	48	70	8	6	42.9
	72	66.7	8	3	27.3
0.1	24	33.3	4	1	20.0
	48	13.3	2	0	0
	72	0	0	0	0

Note: [a] Survival rate (%) = (no. of surviving calli /no. of treated calli) × 100.

[b] Each value represents the number of plantlets regenerated from treated calluses.

[c] Doubling rate (%) = (no. of 12*x* plantlets /no. of 6*x* and 12*x* plantlets) × 100.

Identification of ploidy level by flow cytometry analysis

The use of flow cytometry to identify ploidy level was faster than by conventional chromosome count methods. By comparison with the DNA content of G1 nuclei of hexaploid *E. crus-galli*, the ploidy level of each plant regenerated from colchicine-treated callus was determined by flow cytometry analysis. The results showed that 17 plants in all colchicine-induced populations exhibited a DNA content twice that of the control (hexaploid) plants (Fig. 1). The results confirmed the ploidy level of the colchicine-induced plants and showed that dodecaploid plants of *E. crus-galli* had been obtained successfully.

Determination of chromosome number

Chromosome counting is the most direct and accurate ploidy analysis method. The ploidy levels of the dodecaploid *E. crus-galli* determined by flow cytometry were confirmed by chromosome counts. Yabuno (1953) showed that the chromosome number for *E. crus-galli* was $2n = 6x = 54$. Through chromosome counting in root tips, we found that the chromosome number of hexaploid *E. crus-galli* plants was indeed 54, while the

chromosome number of the putatively dodecaploid plants was $2n = 12x = 108$ (Fig. 2). This showed that the dodecaploid *E. crus-galli* had been successfully isolated and recovered by treating calli with colchicine in the present study.

Stomatal characteristics

Variations in stomata length and width, guard cell length and width, and stomatal density were recorded in both dodecaploids and hexaploids (Table 2). The stomatal length and width of the dodecaploids (31.66 and 8.36 μm, respectively) were significantly larger ($P < 0.01$) than those of the hexaploids (18.16 and 6.24 μm, respectively). The guard cell length and width of the dodecaploids (46.63 and 9.86 μm, respectively) were also significantly greater ($P < 0.01$) than those of the hexaploids (31.83 and 6.99 μm, respectively), whereas the stomatal density in dodecaploid plants (115.95 mm^{-2}) was significantly lower ($P < 0.01$) than that in the hexaploid plants (175.64 mm^{-2}) (Table 2, Fig. 3). The results confirmed the tendency that stomata size increased but stomatal density decreased after polyploidization (Beaulieu et al. 2008; Gu et al. 2005; Niu et al. 2016; Tang et al. 2010), indicating that stomatal observation was a rapid and efficient method for screening for polyploids of *E. crus-galli*.

Table 2
Comparison of stomata characteristics between hexaploid and dodecaploid *E. crus-galli*

Materials	Stomata length (μm)	Stomata width (μm)	Guard cell length (μm)	Guard cell width (μm)	Stomata density/mm^2
6*x*	18.16 ± 0.92^A	6.24 ± 0.61^A	31.83 ± 1.58^A	6.99 ± 0.68^A	175.64 ± 13.66^A
12*x*	31.66 ± 2.79^B	8.36 ± 0.63^B	46.63 ± 2.29^B	9.86 ± 1.02^B	115.95 ± 9.10^B

Note: Values represent the mean ± SD. Values within the same column followed by different uppercase letters are significantly different at 0.01 probability levels.

Morphological differences between dodecaploids and hexaploids

There were obvious morphological differences between dodecaploid and hexaploid plants (Table 3, Fig. 4). The leaf color of dodecaploid plants was deeper than that of hexaploid plants, and the leaf edge of dodecaploid plants was rolled, whereas that of hexaploid plants was flat. The plant height and tiller number of the dodecaploid plants were lower than the corresponding variables in the hexaploid plants, but the flag leaf length and thickness, panicle length, anther length, grain length and width of the dodecaploid plants were all significantly greater ($P < 0.01$) than the values of the hexaploid plants. The seed set of the dodecaploid plants was affected by polyploidization like many polyploids (Dhawan and Lavania 1996; Li 2008), and was only 26.27%, compared with 90.65% for the hexaploid plants.

Table 3
Morphological comparison between hexaploid and dodecaploid *E. crus-galli*

Materials	Plant height (cm)	Tiller No.	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)	Panicle length (cm)	Anther length (mm)	Grain length (mm)	Grain width (mm)	Total grain no./panicle	Filled grain no./panicle	Seed set (%)
6*x*	145.06 ± 6.63^A	18.73 ± 9.07^A	21.59 ± 2.10^A	1.56 ± 0.15^A	± 0.03^A	± 1.51^A	1.17 ± 0.06^A	3.28 ± 0.13^A	1.69 ± 0.06^A	± 18.28^A	± 16.87^A	90.65 ± 1.74^A
12*x*	138.29 ± 9.71^B	18.30 ± 10.59^A	26.22 ± 3.44^B	1.66 ± 0.24^A	0.28 ± 0.03^B	23.43 ± 1.87^B	1.45 ± 0.08^B	4.09 ± 0.27^B	2.00 ± 0.09^B	603.60 ± 17.66^A	158.80 ± 18.23^B	26.27 ± 2.43^B

Note: Values represent the mean ± SD. Values within the same column followed by different uppercase letters are significantly different at 0.01 probability levels.

Differences in plant nutrient component between hexaploids and dodecaploids

Conventional nutrient characters. The concentrations of water, CP, EE, CF, CA and NFE in hexaploid and dodecaploid *E. crus-galli* plants were determined (Table 4). Compared with hexaploid plants, the concentrations of CP, EE, CA and NFE in dodecaploid plants increased, while the concentration of CF decreased. This indicated that the dodecaploid *E. crus-galli* had better feeding value than the wild-type hexaploid form. Moreover, the CP concentrations of hexaploid (7.30%) and dodecaploid (8.18%) leaves were close to that of brown rice (8.0%~11.0%) (Chen et al. 2006) and maize grains (7.2%~11.0%) (Wang et al. 2005).

Table 4
Comparison of nutrient concentrations between hexaploid and dodecaploid *E. crus-galli*

Materials	Water (%)	CP (%)	EE (%)	CF (%)	CA (%)	NFE (%)	TCA (μg/g)	Ca (mg/g)	P (mg/g)	Fe (μg/g)
6*x*	81.85 ± 0.42[a]	7.30 ± 0.43[a]	2.24 ± 0.22[a]	35.85 ± 2.52[a]	9.59 ± 0.02[a]	45.03 ± 2.32[a]	12.82 ± 1.53[a]	5.05± 0.32[A]	4.65± 0.21[a]	169.68 ± 8.45[a]
12*x*	83.19 ± 0.88[a]	8.18 ± 0.42[a]	2.31 ± 0.26[a]	34.49 ± 1.57[a]	9.60 ± 0.02[a]	45.42 ± 1.92[a]	14.13 ± 2.49[a]	7.18± 0.44[B]	5.04± 0.33[a]	192.05 ± 2.08[b]

Note: 1. Abbreviations: CP–crude protein, EE–crude fat, CF–crude fiber, CA–crude ash, NFE–nitrogen free extract, TCA–total carotene, Ca–calcium, P–phosphorus, Fe–iron.

2. Except for water content, the other nutrient concentrations were calculated based on dry matter.

3. Values represent the mean ± SD. Values within the same column followed by different uppercase and lowercase letters are significantly different at 0.01 and 0.05 probability levels, respectively.

Concentrations of total carotene, calcium, phosphorus, and iron. The result showed that the concentrations of TCA, calcium, phosphorus, and iron in dodecaploid *E. crus-galli* plants all increased (Table 4). Moreover, the concentration of calcium and iron in dodecaploid plants were significantly higher than those in hexaploid plants ($P < 0.01$ and $P < 0.05$, respectively).

Concentrations of free amino acids. The concentrations of free amino acids in hexaploid and dodecaploid plants are shown in Table 5. It can be seen the concentrations of all amino acids in dodecaploid plants increased with different extents except for aspartic acid (Asp). Especially, the concentrations of glycine (Gly), threonine (Thr), proline (Pro), methionine (Met), arginine (Arg) and total free amino acids were significantly higher than those in hexaploid plants ($P < 0.01$ for Arg, $P < 0.05$ for the others).

Table 5
Comparison of free amino acids concentrations between hexaploid and dodecaploid *E. crus-galli*

Materials	Asp	Ser	Glu	Gly	His	Arg	Thr	Ala	Pro
6*x*	0.07± 0.04[a]	129.70± 11.81[a]	67.23± 14.18[a]	190.34± 32.74[a]	147.23± 19.08[a]	8.91± 0.98[A]	79.22± 7.13[a]	67.32± 4.58[a]	145.08± 33.03[a]
12*x*	0.02± 0.01[a]	150.84± 28.73[a]	81.84± 0.79[a]	351.46± 56.76[b]	150.95± 23.98[a]	28.33± 1.90[B]	205.07± 48.69[b]	153.34± 48.39[a]	6581.19± 1362.72[b]

Table 5
Continued

Materials	Cys	Tyr	Val	Met	Lys	Ile	Leu	Phe	Total
6*x*	7.82± 1.62[a]	226.43± 27.16[a]	212.26± 19.58[a]	87.43± 4.51[a]	137.64± 38.30[a]	61.25± 6.19[a]	149.36± 10.76[a]	53.75± 2.72[a]	1933.46± 140.84[a]
12*x*	8.08± 1.85[a]	264.76± 37.46[a]	253.04± 53.01[a]	102.50± 6.64[b]	187.54± 39.22[a]	74.09± 11.83[a]	160.78± 22.19[a]	57.42± 8.40[a]	8984.52± 1637.56[b]

Note: 1. The unit of data was μg/g.

2. Values represent the mean ± SD. Values within the same column followed by different uppercase and lowercase letters are significantly different at 0.01 and 0.05 probability levels, respectively.

These results confirmed the trend of increased nutrient concentrations in plant polyploids (Dhawan and Lavania 1996; Li 2008), showing that polyploidization can be an important way to improve the feeding quality of crops.

Discussion

Key factors that affect the efficiency of induced polyploidy

Colchicine is frequently used for polyploid induction in plants (Dhooghe et al. 2011). In this study, we demonstrated that autopolyploid plants of the allohexaploid wild-type *E. crus-galli* plant could be obtained by treating calli with colchicine. Colchicine concentration and exposure time are two key parameters for successful polyploidization, but there is an evident interaction between them. In the present study, low concentrations or short exposure times were not successful, while excessively high doses or long exposure times were lethal. Thus, every plant requires testing to find the optimum balance between concentration and treatment duration (Dhooghe et al. 2011). In our study, the combination of 0.05% colchicine concentration for 48 h resulted in the highest rate of chromosome doubling (Table 1).

The potential value of E. crus-galli **as a forage crop**

E. crus-galli is the most widespread species in the genus *Echinochloa* (Guo et al. 2017), which reflects its broad and strong adaptability. Research has shown that *E. crus-galli* leaves and seeds have high nutritive value. The crude protein concentration of the leaves is similar to that of rice and maize grains, while the cellulose concentration is about 34%, making it very suitable for the nutritional needs of herbivores. Feeding experiments showed that the palatability of *E. crus-galli* plants was high with respect to cattle, sheep, goat, rabbit, and fish (Sun et al. 1989).

In the present study, the autoallo-dodecaploid *E. crus-galli* was successfully obtained for the first time from the allohexaploid *E. crus-galli* by *in vitro* chromosome doubling. The dodecaploid plant has a very high ploidy level (12*x*) and chromosome number (2*n* = 108) and higher plants with such high chromosome numbers are rare in nature. We found that the dodecaploid *E. crus-galli* could still grow normally and also had the common advantages associated with polyploidization. In terms of morphology, dodecaploid *E. crus-galli* showed the obvious “gigas” effects in polyploid organs, such as longer and thicker leaves, longer panicles, larger grains, larger stomata and longer stomatal guard cells. While the fertility of the dodecaploid plants was lowered, with a seed set of only 26.27% compared with 90.65% for the hexaploid; low fertility in the dodecaploid may allow for increased vegetative biomass by way of compensation and should not be a problem because barnyard grasses are not grown for their seed production. The dodecaploid plants had greater nutritional value than did the hexaploid plants. The concentrations of crude protein, crude fat, crude ash, and nitrogen-free extract in dodecaploid plants were higher, while the concentration of crude fiber was lower than those in hexaploid plants, increasing the feed quality and palatability. Moreover, the concentrations of calcium, iron and some free amino acids in the dodecaploid plants were significantly higher than those in hexaploid plants.

The high nutrient concentrations of *E. crus-galli* determined in the present study were consistent with those which had already been reported (Sun et al. 1989; Yi and Peng 1993; Zhang et al. 1992). These results highlighted the good feeding value of *E. crus-galli* once again. Moreover, *E. crus-galli* had a well-developed root system, with strong tillering and abundant leaves, and tolerance to drought, waterlogging, and saline-alkaline soils, as well as other harsh conditions, so that it can be planted on land where typical forage crops could not grow. In addition, *E. crus-galli* plants can be mowed several times once planted (Zhang et al. 1992). As a consequence of these valuable traits, attention is increasingly being paid to *E. crus-galli* by many researchers, who consider *E. crus-galli* to have the potential to be developed into a new type of high quality forage crop for stressful conditions. Such a development would be of great significance in improving the structure of animal husbandry (Zhang et al. 2018). The dodecaploid *E. crus-galli* created in this study could accelerate the development of such research because of its higher nutritional value. Although the seed set of the dodecaploid plants was approximately one-quarter that of the hexaploid, the high reproduction coefficient of *E. crus-galli* meant that one dodecaploid plant could still produce a large number of seeds, to meet the seed demand from livestock farmers for this potentially new forage crop. There is clear potential to develop hexaploid and dodecaploid *E. crus-galli* as a new type of forage crop in the future.

Conclusions

In the present study, autoallo-dodecaploid plants of *E. crus-galli* were successfully induced by treating calli with colchicine. The polyploidy was identified by stomatal characteristics, flow cytometry, and confirmed by chromosome counts, and morphological characters and plant nutrient concentrations were compared between dodecaploid and hexaploid plants. *E. crus-galli* has the potential to be developed as a new type of high quality forage crop for cultivation under stress conditions, especially the dodecaploid with its greater nutritional value.

Abbreviations

AC Activated carbon

BA 6-Benzyladenine

2, 4-D 2, 4-Dichlorophenoxyacetic acid

KT Kinetin

MS Murashige and Skoog medium

N6 N6 medium

NAA 1-Naphthaleneacetic acid

Declarations

Authors contributions

ZS, DC conceived and designed research; XY, WW, XZ, ZF, PL, XZ performed the experiments; JW, WZ, YH, ZS analyzed the data; XY, ZS, DC wrote and revised the paper. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interests.

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Figures

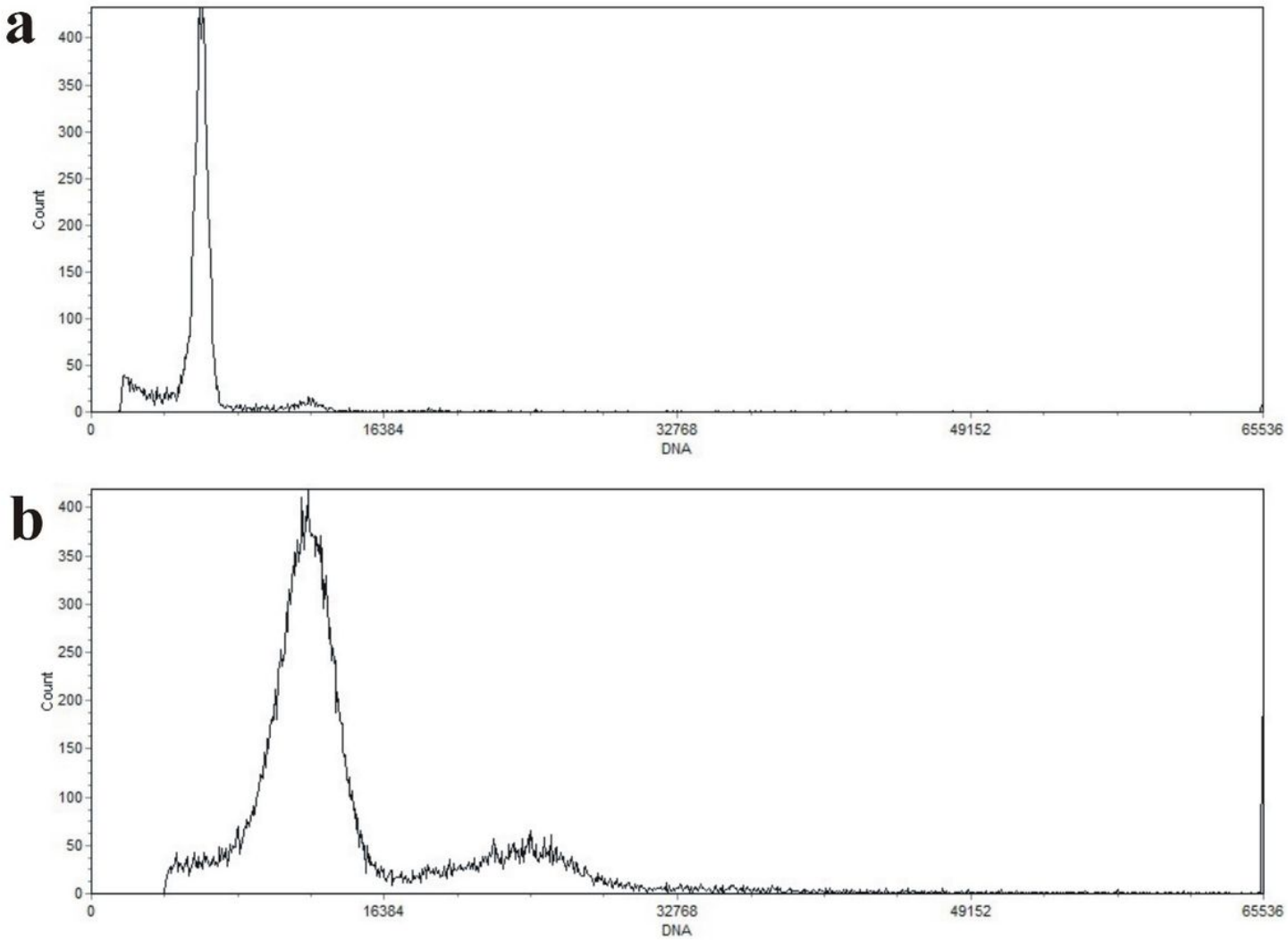

Figure 1

Flow cytometry histograms of hexaploid and dodecaploid E. crus-galli. a Hexaploid. b Dodecaploid

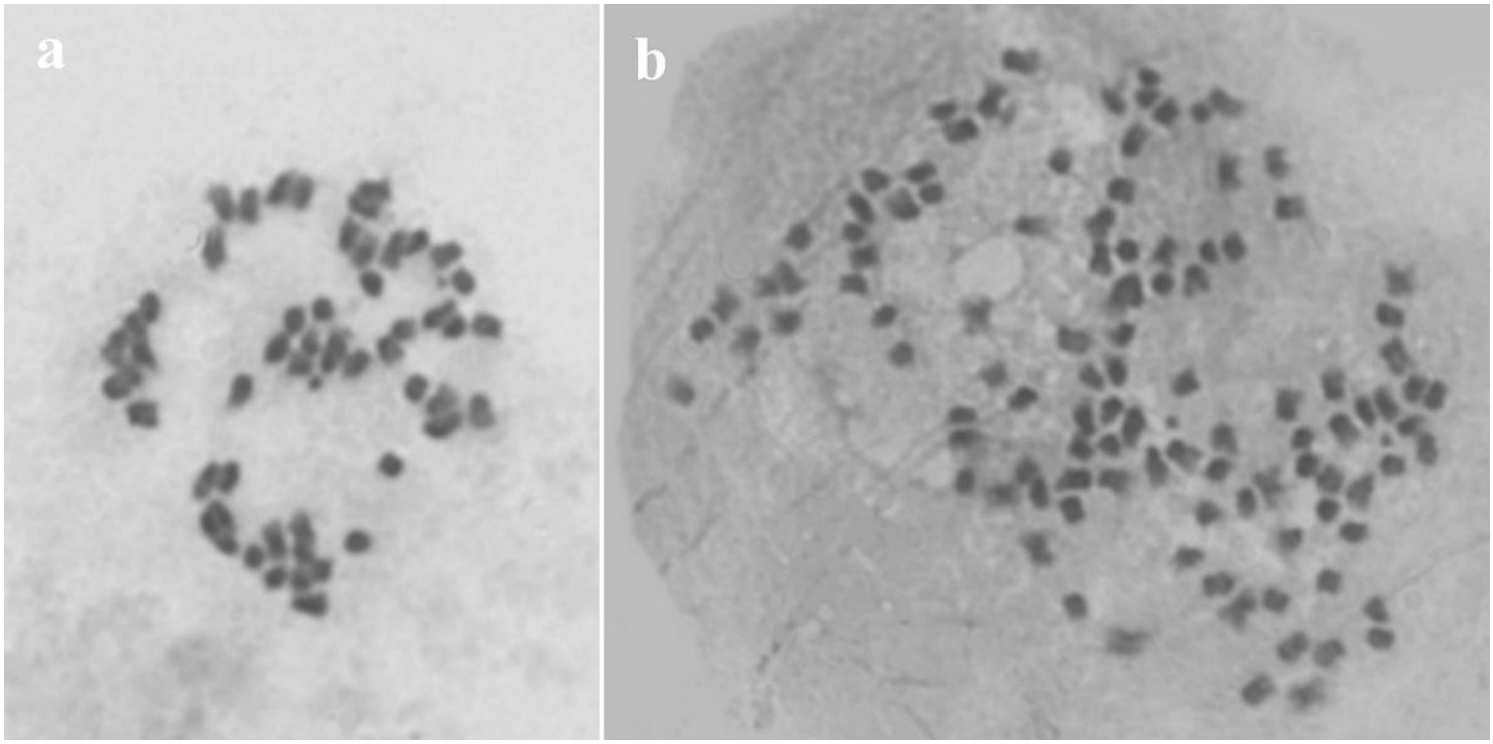

Figure 2

Chromosomes of root tip cells of hexaploid and dodecaploid E. crus-galli. a Hexaploid (2n = 6x = 54). b Dodecaploid (2n = 12x = 108)

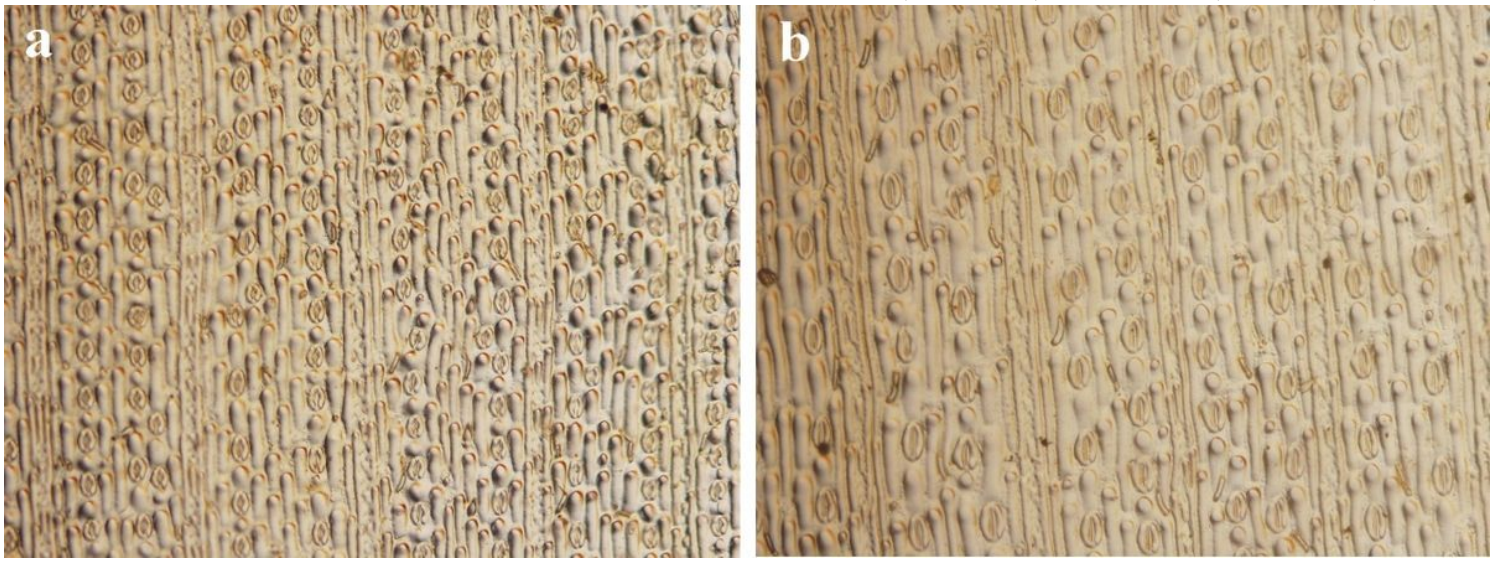

Figure 3

Comparison of stomata characteristics between hexaploid and dodecaploid E. crus-galli. a Hexaploid. b Dodecaploid

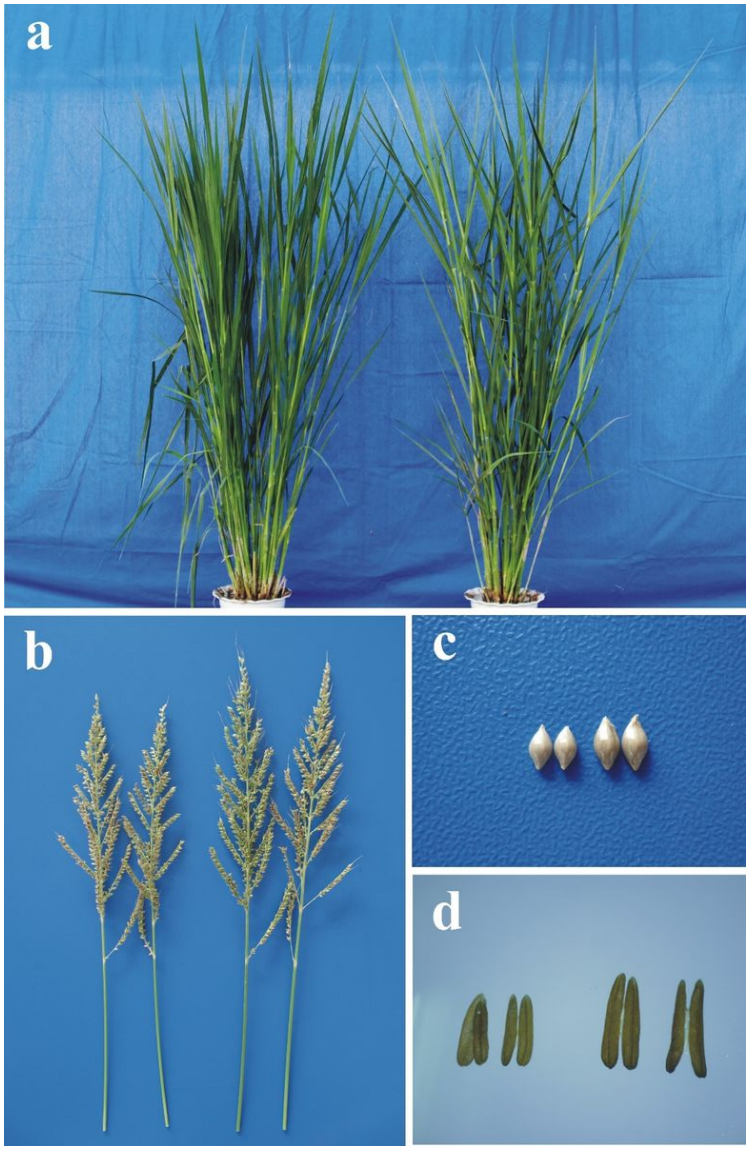

Figure 4

Morphological comparison between hexaploid and dodecaploid E. crus-galli. a Plants (booting stage; Left: 6x; Right: 12x). b Panicles (Left: 6x; Right: 12x). c Grains (Left: 6x; Right: 12x). d Anthers (Left: 6x; Right: 12x)