Ploidy Level and Molecular Phylogenic Relationship among Novel *Ipomoea* Interspecific Hybrids

Qing-He CAO[1,2], Jun TANG[1,2], Ang LI[1,2], Wolfgang GRUNEBERG[3], Kelvin HUAMANI[3] and Daifu MA[1,2]

[1]Sweetpotato Research Institute, China Academy of Agricultural Science, Xuzhou, Jiangsu, P.R. China; [2]Jiangsu Xuhuai Regional Xuzhou Institute of Agricultural Sciences, Xuzhou, Jiangsu, P.R. China; [3]International Potato Center (CIP), Lima, Peru

Abstract

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Interspecific hybridization can be used to broaden the genetic base, generate novel species, postulate genetic relationships, and to introgress elite alien genes. However, interspecific hybridizations using wild parents outside the *Ipomoea* section *Batatas* are very difficult and have not been much studied. We used an improved hybridization technology to generate three novel interspecific hybrids by crossing *Ipomoea batatas* (L.) Lam. × *I. hederacea* Jacq., *I. batatas* (L.) Lam. × *I. muricata* (L.) Jacq., and *I. batatas* (L.) Lam. × *I. lonchophylla* J.M. Black. The ploidy level of the interspecific hybrids was determined by flow cytometry. The cross, *I. batatas* × *I. hederacea*, yielded the first artificial pentaploid *Ipomoea* hybrid ever. The other two hybrids, *I. batatas* × *I. hederacea* and *I. batatas* × *I. muricata* were tetraploid. The first two hybrids showed normal storage roots, a significant improvement in the storage roots of currently existing interspecific Ipomoea hybrids. AFLP (Amplified Fragment Length Polymorphism) molecular markers were used to explore the genetic relationship of these three novel interspecific hybrids with three other natural diploid, tetraploid, and hexaploid species of the *Ipomoea* section *Batatas*. Cluster analysis of AFLP bands showed that these three new interspecific hybrids were closely related to cultivated sweet potato (*I. batatas*/L./Lam.), which indicated that these novel hybrids can be used as an interspecific bridge to transfer alien genes from wild to cultivated species.

Keywords: Amplified Fragment Length Polymorphism (AFLP); breeding; phylogeny; flow cytometry; sweet potato

Sweet potato (*Ipomoea batatas* /L./ Lam.) is one of the most important food and vegetable crops in the world, particularly in Sub-Saharan Africa, Southeastern Asia, and the Pacific Islands. It represents the only domesticated species of the genus *Ipomoea*, which contains 600–700 different species (Austin & Huaman 1996). In this genus, 13 wild species composed of *Ipomoea* section *Batatas* (Austin 1978; Mcdonald & Austin 1990; Austin & Huaman 1996). Up to date, most of the wild species parents used for interspecific hybridization with sweet potato have belonged to this section.

Actually, interspecific hybridization between sweet potato and its related wild species has been greatly limited, mainly due to cross-incompatibility and an interspecific reproductive barrier (Martin 1970; Shiotani *et al.* 1990; Kobayashi *et al.* 1994). Hybridization between *I. batatas* and *I. trichocarpa* Ell. or *I. gracilis* R. Br. resulted in the initiation of embryo development when *I. trichocarpa* was the female parent; however, the seeds produced through this cross were not viable (Wedderburn 1967). Orjeda *et al.* (1991) made over 28 000 pollinations between five *I. batatas* (6*x*) and forty-one *I. trifida* (Kunth) G. Don (2*x*) accessions to obtain 4*x* interspecific hybrids; their results indicated that most of the 4*x* progenies did not produce any storage roots or had very poor yields. Freyre *et al.* (1991) used the *I. trifida* synthetic hexaploids and triploids with 2*n* pollen to estimate their fertility and crossability with sweet

potato. And the lower percentage of seed germination in the above-mentioned hybrids indicated the existence of an interspecific barrier. Kobayashi *et al.* (1994) used ovule culture to obtain two interspecific hybridization combinations from *I. triloba* L. × *I. trifida* and (*I. triloba* × *I. lacunosa* L.) × *I. batatas* (4*x*). Somatic cell hybridization was also exploited to produce hybrids whose wild parents were among the following species: *I. triloba* (Yang *et al.* 2009), *I. lacunosa* (Liu *et al.* 1998; Zhang *et al.* 2002), and *I. cairica* (L.) Sweet (Guo *et al.* 2006). We recently reported the reproduction and characterization of two novel interspecific hybrids from *I. batatas* × *I. grandifolia* and *I. batatas* × *I. purpurea* using controlled pollination (Cao *et al.* 2009a).

In general, most of the interspecific hybridizations reported above employed a limited number of wild parents and most of those belonged to *Ipomoea* section *Batatas*. Furthermore, most of the interspecific hybrids obtained seldom produced storage roots or resulted in poor quality or yield. However, there is a large number of wild species in the genus *Ipomoea* having elite biotic and abiotic stress resistance and good quality traits, which remain to be explored and utilized.

In the present study, three new interspecific hybrids were synthesized whose wild parents were beyond the *Ipomoea* section *Batatas*. Ploidy levels of these hybrids, genetic variations and molecular phylogenetic relationships were investigated. Furthermore, AFLP was used to explore the correlation between the number of amplified DNA bands and the ploidy level.

MATERIAL AND METHODS

Plant material. Xushu 18 (*I. batatas* /L./ Lam., 2*n* = 6*x* = 90), a famous and widely grown sweet potato cultivar in China, was chosen as the female parent (Table 1). Plants of Xushu 18 were pollinated with pollen from three wild species, *I. hederacea* Jacq. (2*n* = 2*x* = 30, PI618970), *I. muricata* (L.) Jacq (2*n* = 2*x* = 30, PI279698) and *I. lonchophylla* J. M. Black (2*n* = 2*x* = 30, Grif11879), which are not members of *Ipomoea* section *Batatas* and genetically distant from Xushu18. Among the three wild species, *I. hederacea* and *I. muricata* are drought tolerant (Q.H. Cao, personal communication), and *I. lonchophylla* is resistant to the stem nematode disease (Cao *et al.* 2009b). A diploid *I. trifida*, an old natural tetraploid interspecific hybrid *I. tabascana* J.A. McDonald & D.F. Austin (Srisuwan *et al.* 2006), and a hexaploid cultivar Xushu18 were selected as controls for both phylogeny and ploidy studies. All the above wild species were introduced from the sweet potato program of Louisiana State University, USA.

Interspecific cross. As previously described (Cao *et al.* 2009a), we adjusted the plant hormone concentrations from 100 mg/l GA3 + 50 mg/l 6-BA to 120 mg/l GA3 + 60 mg/l 6-BA and applied the solution to the stalks of the pollinated flowers. The treatment was conducted for ten consecutive days to obtain better fruits and seed set.

Flow cytometry analysis and chromosome counting. Relative fluorescence intensity of PI (Propidium Iodide)-stained nuclei was analysed using a flow cytometer (FACSCalibur, BD Company, San Diego, USA) according to the method of Doležel *et al.* (1989). For ploidy analysis, the scale was calibrated using the young leaf samples of *I. trifida* as the diploid reference (standard). The flow cytometer was adjusted so that the peak representing the G1 nuclei of *I. trifida* was set at channel 50. Other samples were characterized by the relative positions of their G1 peaks. Data were analysed using the ModFit LD software, referred to the ModFit LT user guide.

Chromosome counting was done on the three newly obtained hybrids according to the procedure described by Cao *et al.* (2009a).

Genomic DNA isolation and AFLP analysis. Genomic DNA was extracted from frozen and dried leaves of plants grown in the field. The leaf tissue was ground to a fine powder and DNA was extracted

Table 1. Description of accessions used in interspecific hybridization

No.	Primary code	Name	Source of materials	Ploidy level
1	CIP	*I. batatas* (Xushu18)	China	2*n* = 6*x* = 90
2	PI 618970	*I. hederacea*	Hongkong	2*n* = 2*x* = 30
3	PI 279698	*I. muricata*	Mexico	2*n* = 2*x* = 30
4	Grif11879	*I. lonchophylla*	Australia	2*n* = 2*x* = 30
5	PI 540729	*I. trifida*	Colombia	2*n* = 2*x* = 30
6	PI 518479	*I. tabascana*	Mexico	2*n* = 4*x* = 60

using the improved CTAB method (Huang & Sun 2000). The AFLP analysis was performed as described by Vos *et al.* (1995). DNA double-digestion was carried out using the enzyme combination of EcoRI/MseI. After ligation with oligonucleotide adapters, a pre-selective amplification was carried out with *Eco*RI+A and *Mse*I+C primers, and PCR products were then diluted 15-fold with water and used as template for selective amplifications using both *Eco*RI+3 and *Mse*I+3 primers. In total 21 primer-pair combinations were chosen to produce a high number of unambiguous polymorphisms in sets of the 10 sweet potato genotypes tested. PCR products were separated using electrophoresis on a 6% polyacrylamide gel in TBE (Tris-Boric acid-EDTA) buffer for about 1.5 h.

Data analysis. For each of the primer-pair combinations, the number of polymorphic and monomorphic fragments was counted across all six species with different ploidy levels. Only clearly readable bands with strong intensity were scored manually and included in the binary data matrix (i.e. 1 and 0 denoting the presence and absence of a band, respectively). The percentage of genetic similarity index between samples was calculated and derived according to the method of Nei and Li (1979). Phylogenetic analysis was performed using the NTSYS pc. 2.11a software and the phylogenetic tree was produced from the AFLP data matrices using the unweighted pair group method with arithmetic averages (UPGMA). To evaluate the strength of the resulting clades, the data were analysed by the bootstrap method of Felsenstein (1986). One hundred bootstrap samples were generated by random resampling of the data set (Felsenstein 1985) and were separately subjected to Wagner parsimony analysis.

RESULTS

Xushu18 (maternal parent) was pollinated with pollen from 108, 105, and 98 flowers of three wild species, *I. hederacea*, *I. muricata* and *I. lonchophylla*, respectively. To overcome the ovary development barrier, the combination of different plant growth hormones (as described in the methodology) was applied to the stalk of flowers. These crosses generated 3, 2, and 2

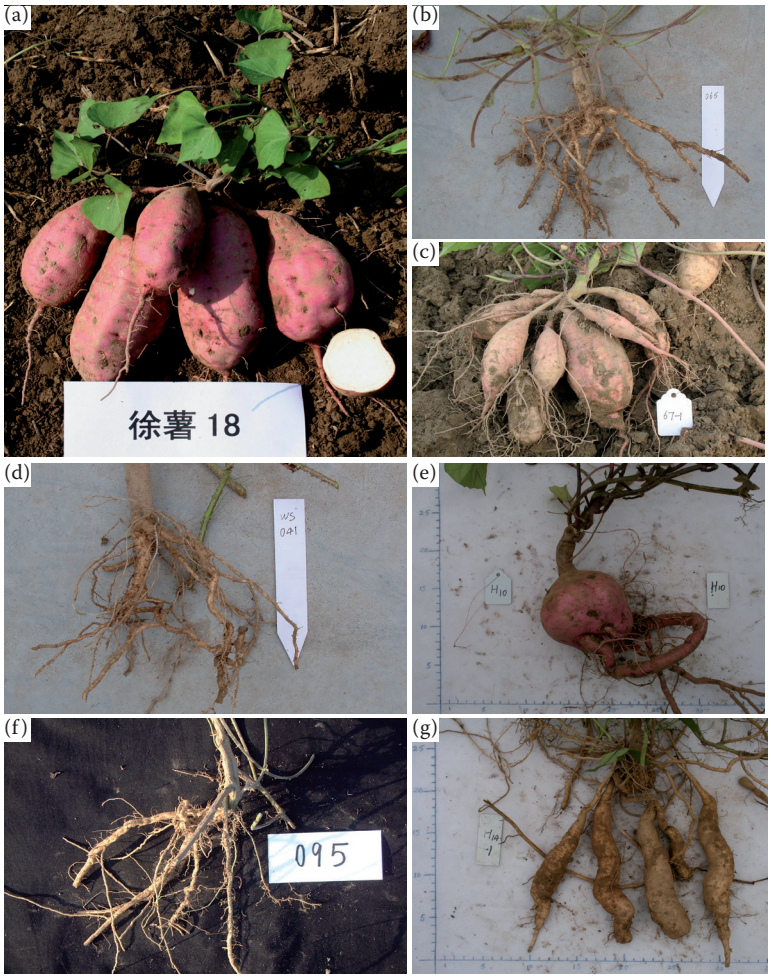

Figure1. Storage roots of the three interspecific hybrids and their parents; the female parent cultivated sweet potato Xushu18 (a), the wild relative parent *I. hederacea* (b), Xushu18 × *I. hederacea* (c), the wild relative parent *I. muricata* (d);Xushu18 × *I. muricata* (e), the wild relative parent *I. lonchophylla* (f) and Xushu18 × *I. lonchophylla* (g)

Table 2. Interspecies hybridization between sweet potato and its three related species

The cross name	No.of crosses made	No.of fruits produced	No. of seeds produced	No. of adult plants produced	Ploidy level and chromosome No.
Xushu18 × *I. hederacea*	108	3	4	1	$2n = 5x = 75$
Xushu18 × *I. muricata*	105	2	5	1	$2n = 4x = 60$
Xushu18 × *I. lonchophylla*	98	2	3	1	$2n = 4x = 60$

fruits and set 4, 5 and 3 seeds, respectively (Table 2). Due to the poor germination rates and weak growth of the seedlings, only one seedling from each cross combination was able to grow into an adult plant. The hybrid plants $H_{67\text{-}1}$ (*I. batatas* × *I. hederacea*) and H_{10} (*I. batatas* × *I. muricata*) showed larger storage roots (Figure 1) than our previous interspecific hybrids (Cao *et al.* 2009a). The larger storage roots in this cross showed a greater similarity to their maternal parent Xushu18 and it is possible to benefit from the future preservation and propagation of these accessions. The hybrid plant $H_{14\text{-}1}$ (*I. batatas* × *I. lonchophylla*) set smaller storage roots (Figure 1), and its vine was twining and spreading.

The FC analysis of PI-stained nuclei showed a dominant peak corresponding to the G1 nuclei of the materials being measured (Figure 2). The dominant peak reflected the ploidy level of each sample. The G1 peak of the reference diploid *I. trifida* was approximately at channel 50. The G1 peaks of the newly obtained interspecific hybrids $H_{67\text{-}1}$ (*I. batatas* × *I. hederacea*), H_{10} (*I. batatas* ×*I. muricata*), and $H_{14\text{-}1}$ (*I. batatas* × *I. lonchophylla*) were at channels 125, 100, and 100, respectively, suggesting that $H_{67\text{-}1}$ was pentaploid while H_{10}, $H_{14\text{-}1}$ were tetraploid. Most histograms revealed a low coefficient of variation (less than 5%) indicating the high reliability of these results. The root tip cells of the three newly obtained interspecific hybrids were squashed for chromosome counting. We found that the hybrid $H_{67\text{-}1}$ carried ~75 chromosomes and both H_{10} and $H_{14\text{-}1}$ contained ~60 chromosomes (Table 2). These results were consistent with the results from our flow cytometer (FC) analysis.

In order to determine the phylogenetic relationship of these three hybrids with sweet potato and investigate the DNA band variations among different ploidy materials, a diploid *I. trifida*, tetraploid *I. tabascana*, and a sweet potato cultivar in *Ipo-*

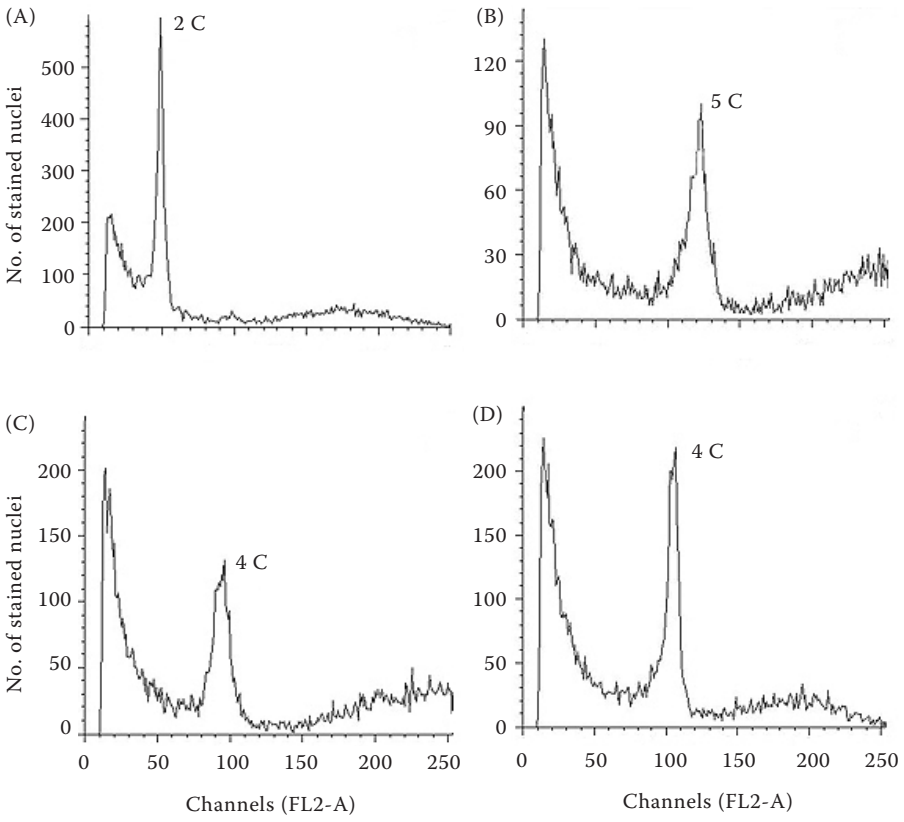

Figure 2. Ploidy analysis of the three novel interspecific hybrids using a flow cytometer: histogram of fluorescent intensity of nuclei showing G1 peaks of diploid indicator *I. trifida* (a), histogram of $H_{67\text{-}1}$ (Xushu18 × *I. hederacea*) (b), histogram of H_{10} (Xushu18 × *I. muricata*) (c), histogram of $H_{14\text{-}1}$ (Xushu18 × *I. lonchophylla*) (d)

Table 3. Interspecific similarity index (Nei and Li's coefficient) among six accessions based on AFLP data

Accesions	*I. trifida*	*I. tabascana*	H67-1	H10	H14-1	Xushu18
I. trifida	1					
I. tabascana	0.56337271	1				
H67-1	0.56045137	0.6200967	1			
H10	0.56552094	0.6369495	0.895755	1		
H14-1	0.55961331	0.7481203	0.7748522	0.7889366	1	
Xushu18	0.53866809	0.6337272	0.8280494	0.8163266	0.7599356	1

moea section *Batatas* were selected as controls for the diploid, tetraploid, and hexaploid, respectively. Through genetic similarity calculation by NTSYSpc 2.11a, differences at the DNA level among different species were determined by comparing the genetic similarity indexes for a total of 21 pairwise comparisons (Table 3). The genetic similarity indexes among all pairs of six *Ipomoea* species varied from 0.54 (between *I. trifida* and Xushu18) to 0.90 (between H_{10} and $H_{67\text{-}1}$), thus providing the evidence that all the six *Ipomoea* species were closely related.

The similarity matrix obtained after multivariate analysis using Nei and Li's (1979) coefficient index is shown in Table 3. These similarity indexes were used to generate a dendrogram (Figure 3) by UPGMA analysis in order to determine the grouping of different ploidy materials. From Figure 3, *I. trifida* was

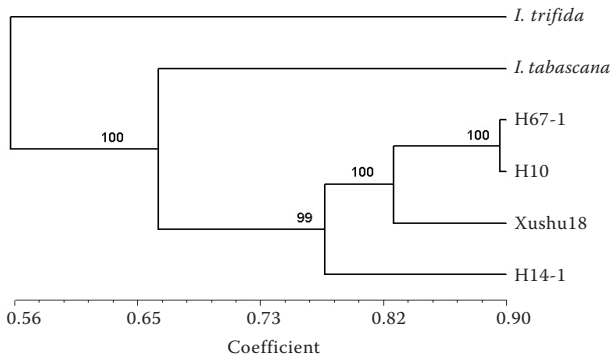

Figure 3. Phylogenetic relationship of the three novel interspecific hybrids with *Ipomoea* species at different ploidy levels based on AFLP data set by UPGMA

The three new synthetic hybrids were from the following hybridization. $H_{67\text{-}1}$: Xushu18 × *I. hederacea*; H_{10}: Xushu18 × *I. muricata*; $H_{14\text{-}1}$: Xushu18 × *I. Lonchophylla*

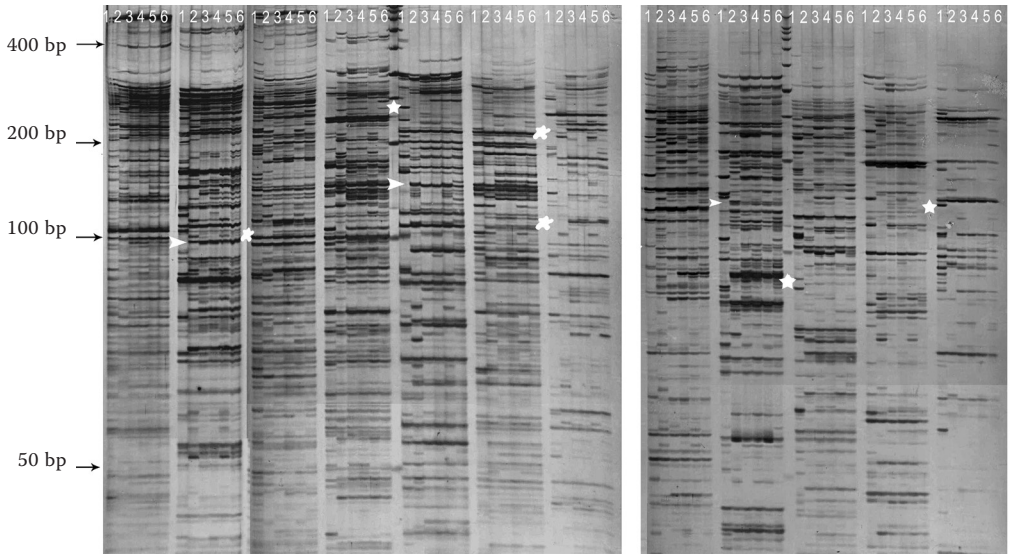

Figure 4. AFLP bands were amplified by primer-pair combinations from six accessions

Lane 1 – *I. trifida;* lane 2 – *I. tabascana*; lane 3 – Xushu18 × *I. hederacea*; lane 4 – Xushu18 × *I. muricata*; lane 5 – Xushu18 × *I. Lonchophylla*; lane 6 – *I. batatas* (Xushu18); arrow-heads show some bands absent in the diploid *I. trifida* while they are present in the other ploidy materials; five-stars show some special bands present in the diploid only while they are absent in the other polyploidy materials; the leaning five-stars show some common bands that were amplified only in the interspecific hybrids and maternal parent Xushu18

thought to be the most distant from the other five *Ipomoea* species. The natural interspecific hybrid *I. tabascana* was the second most distant in the phylogenetic tree. The newly obtained hybrids $H_{67\text{-}1}$ and H_{10} were clustered into one group at an index of 0.90, which indicated that the hybrids $H_{67\text{-}1}$ and H_{10} were the closest genetically. These two hybrids, together with *I. batatas* (Xushu18), were clustered into one group at an index of 0.82. The artificial tetraploid $H_{14\text{-}1}$ was clustered into a group with the above three accessions at an index of 0.78.

Six different *Ipomoea* species were genotyped with 21 AFLP primer-pair combinations, which had been tested to be highly polymorphic at the International Potato Centre (CIP). Most of the AFLP bands ranged from 50 to 400 bp (Figure 4). A total of 1862 bands were scored. Among them, 558 bands (30.0%) were monomorphic in six test materials, whereas 1304 bands (70.0%) were polymorphic. For each primer pair, an average of 88 total bands and 62 polymorphic bands were detected. For each accession, the number of total bands increased following the increase in ploidy levels. For example, the total amplified DNA bands of diploid *I. trifida* were 1034, those of tetraploid *I. tabascana*, H_{10}, $H_{14\text{-}1}$ and pentaploid $H_{67\text{-}1}$ were 1192, 1330, 1315, and 1370, respectively. The hexaploid *I. batatas* had 1452 bands. The amplification results (Figure 4) showed three main phenomena: (1) some bands were absent in the diploid *I. trifida* while they were present in the other polyploidy materials; (2) some special bands were only present in the diploid and absent in polyploidy materials; (3) some bands were amplified only in the interspecific hybrids and their maternal parent Xushu18.

DISCUSSION

Among the three novel synthetic interspecific hybrids, only $H_{67\text{-}1}$, which was from a cross between hexaploid *I. batatas* and diploid *I. hederacea*, was identified as a pentaploid. It was not the expected tetraploid, indicating that 2*n* gametes might have occurred in a male wild paternal species, which needs to be confirmed in the future. The other two interspecific hybrids were likely to be produced from normal meiosis of their respective parents.

According to the results of AFLP scores and the cluster analysis, all of the three newly synthetic interspecific hybrids have a closer relationship with sweet potato than with *I. trifida* and *I. tabascana*. These results suggest that the wild parents of the three novel hybrids have a close relationship with the cultivated species to some extent. Further AFLP experiments are required to determine the genetic distance between these wild species and sweet potato. The genome of the novel synthesized interspecific hybrid was found to contain three sets of chromosomes from *I. batatas* (Xushu18) and one or two sets from the wild parental species. Theoretically, DNA from Xushu18 accounts for three quarters (like in H_{10}, $H_{14\text{-}1}$) or three fifths (like in $H_{67\text{-}1}$) of the total genomes of these hybrids. This would partially explain why the features of the three new hybrids were closer to those of the sweet potato cultivar Xushu18. These materials are useful in studying the effect of gene dosage and ploidy level variations.

The correlation between ploidy levels and amplified DNA band numbers has been studied in various species (Chen *et al*. 2004; Liu *et al.* 2004; Ma *et al.* 2010); however, it has been rarely studied in *Ipomoea*. Our results revealed some interesting facts. The number of amplified AFLP bands increased following the increase in ploidy level. The amplified AFLP bands in the novel synthesized tetraploid interspecific hybrids were similar in number, while the old hybrid *I. tabascana* had fewer bands, indicating that some DNA bands might have been lost during speciation. The sweet potato cultivar Xushu18 was found to have the highest number of amplified DNA bands. However, considering its hexaploid level, the average bands per chromosome set of sweet potato would be the lowest as compared to those of the diploid wild species and the tetraploid hybrids. These results can be partly explained by the fact that polyploidization is usually followed by a genome-wide loss of some of the redundant genomic material (Adams & Wendel 2005). Differential gene loss (i.e. loss of some duplicates but not others) following polyploidization is responsible for much of the deviation in co-linearity among closely related plants, such as cereals (Paterson *et al*. 2003).

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Corresponding author:

Dr. Qinghe Cao, China Academy of Agricultural Science, Sweetpotato Research Institute, Xuzhou, 221131 Jiangsu, P.R. China; e-mail: cqhe75@yahoo.com