RESEARCH ARTICLE

AMERICAN JOURNAL OF BOTANY

Phenotypic effects of allotetraploidization of wild *Arachis* and their implications for peanut domestication[1]

Soraya C. M. Leal-Bertioli[2,3,6], Márcio C. Moretzsohn[2], Silvio P. Santos[4,5], Ana C. M. Brasileiro[2], Patrícia M. Guimarães[2], David J. Bertioli[3,4], and Ana Claudia G. Araujo[2]

PREMISE OF THE STUDY: Several species of *Arachis* have been cultivated for their edible seeds, historically and to the present day. The diploid species that have a history of cultivation show relatively small signatures of domestication. In contrast, the tetraploid species *A. hypogaea* evolved into highly domesticated forms and became a major world crop, the cultivated peanut. It seems likely that allotetraploidization (hybridity and/or tetraploidization) in some way enhanced attractiveness for cultivation. Here we investigate this using six different hybridization and tetraploidization events, from distinct *Arachis* diploid species, including one event derived from the same wild species that originated peanut.

METHODS: Twenty-six anatomical, morphological, and physiological traits were examined in the induced allotetraploid plants and compared with their wild diploid parents.

KEY RESULTS: Nineteen traits were transgressive (showed strong response to hybridization and chromosome duplication): allotetraploids had larger leaves, stomata and epidermal cells than did their diploid parents. In addition, allotetraploids produced more photosynthetic pigments. These traits have the same trend across the different hybrid combinations, suggesting that the changes are more likely due to ploidy rather than hybridity. In contrast, seed dimensions and seed mass did not significantly change in response to hybridization or tetraploidization.

CONCLUSIONS: We suggest that the original allotetraploid that gave rise to cultivated peanut may have been attractive because of an increase in plant size, different transpiration characteristics, higher photosynthetic capacity, or other characteristics, but contrary to accepted knowledge, increased seed size was unlikely to have been important in the initial domestication.

KEY WORDS allotetraploid; *Arachis*; domestication; Fabaceae; hybridization; leaf morphology; leaf pigments; plant architecture; polyploidy; tetraploidization

The genus *Arachis* (Fabaceae) is endemic to South America. It is unusual among the papilionoid legumes in that the species have highly palatable seeds with a thin seed case and pod. The seeds' only protection is that they develop underground. They are rich in oils and proteins, and unlike the seeds of many legumes, they are soft and do not have significant quantities of anti-nutritional components, such as protease inhibitors and lectins. Not surprisingly, these seeds are a very attractive food source for humans. Widespread remnant populations of *Arachis* spp. near archeological sites and routes known to have been frequented by Amerindians testifies to the extensive cultivation of wild species in pre-Columbian times (Dillehay et al., 2007; Freitas et al., 2007). Most *Arachis* species are diploid, as are the vast majority of these remnant populations. Therefore, it seems notable that the cultivated peanut, *A. hypogaea*, is an allotetraploid. This species originated east of the Andes in the area comprising southeastern Bolivia and northwestern Argentina, through the hybridization of the wild diploid species *A. duranensis* and *A. ipaënsis*, followed by chromosome duplication (Kochert

[1] Manuscript received 5 August 2016; revision accepted 7 February 2017.

[2] Embrapa Genetic Resources and Biotechnology, PqEB, W5 Norte Final, Brasília, DF 70770-917, Brazil;

[3] University of Georgia, Center for Applied Genetic Technologies, 111 Riverbend Road, Athens, Georgia 30602-6810 USA;

[4] University of Brasília, Institute of Biological Sciences, Campus Darcy Ribeiro, Brasília, DF 70910-900, Brazil; and

[5] Catholic University of Brasília, Biotechnology and Genomic Sciences, SGAN 916 Módulo B Avenida W5, Brasília, DF 70790-160, Brazil

[6] Author for correspondence (e-mail: soraya.bertioli@embrapa.br, sorayab@uga.edu)

doi:10.3732/ajb.1600402

et al., 1996; Seijo et al., 2004; Bertioli et al., 2016a, b). The hybridization also gave rise to the biologically conspecific *A. monticola*, which is found in only two small areas in northeastern Argentina. While *A. monticola* persists in the wild and has kept its wild characteristics, the process of domestication transformed cultivated peanut such that it is different not only from its progenitors, but also from all diploid *Arachis* species, including those with a known history of cultivation and the independent diploid domesticate *A. villosulicarpa* (Krapovikas and Gregory, 2007; Simpson et al., 2001). It seems likely that hybridity and/or tetraploidization in some way enhanced attractiveness for domestication.

Polyploidy is often associated with changes in plant anatomy, morphology, physiology, and biochemistry (Ramsey and Schemske, 1998; Tal, 1980). Moreover, nucleotypic effects of polyploidy, also known as the Gigas effect, are well documented and are reflected in modifications such as larger cells and overall plant size and increased chlorophyll content per cell (Ni et al., 2009; Acquaah, 2012; Coate et al., 2012). Also, heterozygosity and novel genomic interactions in allopolyploids may induce a range of phenotypic variations, such as increased size of harvested organs and growth vigor, bringing advantages during artificial selection by man (Gepts, 2003).

Because peanut was a spontaneous allotetraploid, probably from a single event of hybridization followed by genome doubling, the change in ploidy level caused genetic isolation between the diploid wild ancestors and the cultigen kept genetic diversity low (Halward et al., 1991; Burow et al., 2001; Bertioli et al., 2011). Peanut is susceptible to a number of agricultural pests and environmental constraints, while wild species are an important font of resistance alleles. To make these wild alleles available for breeding, artificially induced allotetraploids (also known as amphidiploids) are produced from several wild diploid species of *Arachis* (Simpson et al., 1993; Stalker and Lynch, 2002; Mallikarjuna et al., 2011; Fávero et al., 2015; Leal-Bertioli et al., 2015). In this work, we used six induced allotetraploids, their parental diploids and the spontaneous allotetraploid *A. hypogaea* to study the effects of ploidy level increase in *Arachis.* Genetic and nucleotypic effects and the role of tetraploidy in peanut along evolution/domestication have been discussed.

MATERIALS AND METHODS

Plant material—Seeds of wild diploid *Arachis* species were obtained from the Active Germplasm Bank of Embrapa Genetic Resources and Biotechnology (Cenargen, Brasília, Brazil) and from the USDA-ARS Germplasm Bank (http://www.ars-grin.gov/; Griffin, Georgia, USA) and bulked up in a greenhouse. Genotypes used are listed in Table 1. Primary crosses, hybrid identification, and allotetraploid production were performed as described by Leal-Bertioli et al. (2015). Seeds were germinated on germitex paper with 2% w/v ethrel (2-chloroethylphosphonic acid) to break dormancy and treated with 0.05% w/v thiram to prevent fungal contamination. All plants measured were grown in the greenhouse. For the most part, data were obtained for all induced allotetraploids (also called synthetics or amphidiploids), but in a few cases, not all plants were available for measurements. Brazilian peanut cultivars Runner IAC-886, IAC-Caiapó and IAC-Tatu were used as controls.

Plant architecture—The main stem height (MSH) of 18-wk-old wild and induced allotetraploid plants was measured on at least six individuals of each genotype. Cultivated peanut plants were measured at 12 wk after germination. (Seeds were sown 6 weeks after seeds of the wild and induced allotetraploids because cultivated plants grow faster.) For assessing canopy area (CA), all leaves of eight individuals of each genotype were collected, scanned, and leaf area was calculated using the software QUANT V.1.0.1 (Vale et al., 2001). Aerial and root biomass (AB and RB) were assessed on 8-wk-old plants. Plants were harvested, washed, and dried for 72 h at 80°C. Aerial parts and roots were weighed separately. The first fully expanded leaf from the apex of at least eight different plants of each genotype was scanned and leaf areas estimated using QUANT V.1.0.1 (Vale et al., 2001).

Leaf cell morphology—Cell features assessed on both abaxial and adaxial leaf surfaces of 12-wk-old plants included stomatal guard cell length and width (StL, StW) and densities of stomata (StD), epidermal cells (ECD) and trichomes (TD), and a stomatal index (StI) for each leaf surface. Stomatal index, the ratio of stomata to the total number of epidermal cells (including stomata), was calculated as StI = [StD/(ECD + StD)] ×100 (Cutter, 1986). The epidermis of the central portions of two leaflets of the first expanded leaf from five plants of each genotype was dissociated (Berlyn and Miksche, 1976). Eight dissociations (four for adaxial and four for abaxial surfaces observations) were mounted for each plant using glycerinated gelatin and observed using differential interferential contrast (DIC) optics and an AxiosKop microscope (Zeiss, Oberkochen, Germany). Images were captured with the AxioCam MRc digital camera using the Axiovision Rel. 4.8 software and manipulated using Adobe (San Jose, California, USA) Photoshop CS software. Stomatal density was calculated from counts of the epidermal cells and stomata in four areas of 0.33 mm^2 on each surface from leaflets of five plants.

Leaf photosynthetic pigments—To determine the chlorophyll and carotenoid content per leaf area, 1 cm diameter discs of leaf tissue were homogenized in liquid nitrogen, mixed by vortexing with 500 μL of cold 80% acetone and extracted overnight at 4°C in the dark. Tubes were then centrifuged at 18,000 × *g* for 15 min at 4°C; 100 μL of the supernatant was then added to 900 μL of 80% acetone in a 1 mL cuvette. Absorbance was determined at 663 nm, 647 nm, and 470 nm using a SpectraMax M2e multimode microplate reader spectrophotometer (Molecular Devices, San Jose, California, USA). Concentrations of chlorophyll *a* (Chla), chlorophyll *b* (Chlb), total chlorophyll (Chla+b) and total carotenoids (xanthophylls and β-carotene, X-car) were estimated using the formulas of Lichtenthaler (1987).

Seed dimensions and mass—At least 10 fresh-shelled seeds per plant were weighed and their length and width measured at the longest and widest point of each seed, respectively, using a digital caliper. For plants that produced fewer than 10 seeds, all seeds available were evaluated. Seeds from the following genotypes were measured: induced allotetraploid IpaDur1, IpaVillo1, and GregSten1; their parental diploid species; three accessions of *A. monticola*; and the cultivars IAC-Runner-886, IAC-Caiapó and IAC-Tatu (Table 1).

Data analyses—Statistical analyses were performed using the software R (R Core Team, 2014). Normality was verified with the Shapiro–Wilk test. Comparisons were performed using standard variance analyses, followed by the Tukey test of multiple

TABLE 1. *Arachis* genotypes and the induced allotetraploids used in this study.

Species Authority, Accession	Plant ID	Ploidy	Genome type	Collection site
A. batizocoi Krapov. & W.C.Gregory, K9484	Abat	2*x*	KK	Parapeti, Bolivia
A. duranensis Krapov. & W. C.Gregory, V14167	Adur1	2*x*	AA	Salta, Argentina
A. duranensis Krapov. & W. C.Gregory, SeSn2848	Adur2	2*x*	AA	Salta, Argentina
A. gregoryi C.E.Simpson, Krapov. & Valls, V6389	Agreg	2*x*	BB	Vila Bela, Mato Grasso, Brazil
A. ipaënsis Krapov. & W.C.Gregory, K30076	Aipa	2*x*	BB	Gran Chaco, Bolivia
A. stenosperma Krapov. & W.C.Gregory, V10309	Asten	2*x*	AA	Rondonopolis, Mato Grasso, Brazil
A. villosa Benth, V12812	Avillo	2*x*	AA	Bella Union, Uruguay
A. monticola Krapov. & Rigoni, PI 219824 [a] (K7264)	Amont2	4*x*	AABB	Jujuy Province, Argentina
A. monticola Krapov. & Rigoni. PI 468196 [a] (G30062)	Amont3	4*x*	AABB	Jujuy Province, Argentina
A. monticola Krapov. & Rigoni. PI 263393 [a] (K7264)	Amont5	4*x*	AABB	Jujuy Province, Argentina
A. hypogaea subsp. *fastigiata* var. *fastigiata* L.	IAC-Tatu	4*x*	AABB	Natural allotetraploid, commercial cultivar [b]
A. hypogaea subsp. *hypogaea* var. *hypogaea* L.	IAC-Caiapó	4*x*	AABB	Natural allotetraploid, commercial cultivar [b]
A. hypogaea subsp. *hypogaea* var. *hypogaea* L.	Runner IAC-886	4*x*	AABB	Natural allotetraploid, commercial cultivar [b]
A. hypogaea subsp. *hypogaea* var. *fastigiata* L.	BR1	4*x*	AABB	Natural allotetraploid, commercial cultivar [b]
[*A. batizocoi* K9484 × *A. duranensis*, V14167] ^{4x}	BatDur1	4*x*	AAKK	Induced allotetraploid [c]
[*A. batizocoi* K9484 × *A. duranensis*, SeSn2848] ^{4x}	BatDur2	4*x*	AAKK	Induced allotetraploid [c]
[*A. batizocoi* K9484 × *A. stenosperma*, V10309] ^{4x}	BatSten	4*x*	AAKK	Induced allotetraploid [c]
[*A. gregoryi* V6389 × *A. stenosperma*, V10309] ^{4x}	GregSten	4*x*	AABB	Induced allotetraploid [d]
[*A. ipaënsis* K30076 × *A. duranensis*, V14167] ^{4x}	IpaDur1	4*x*	AABB	Induced allotetraploid [e]
[*A. ipaënsis* K30076 × *A. villosa*, V12812] ^{4x}	IpaVillo1	4*x*	AABB	Induced alotetraploid [d]

[a] USDA accessions.
[b] Allotetraploid derived from the natural hybridization between *A. duranensis* and *A. ipaënsis*, and tetraploidization of the diploid hybrid.
[c] Leal-Bertioli et al., 2015.
[d] Produced in this study.
[e] Fávero et al., 2006.

comparisons (data with normal distribution). When data did not follow a normal distribution, averages were compared with the nonparametric test of Kruskal–Wallis, with significance levels of 5%. The heat map was constructed using the percentage of increase or decrease of a character measurement for the induced allotetraploid compared with the midparent value. Changes ranged between a 210% increase and a 100% decrease. The libraries gplots and gtools of the R package were used for heat map production.

RESULTS

Induced allotetraploids—Six induced allotetraploids were used in this study: four previously reported: IpaDur1, [*A. ipaënsis* K30076 × *A. duranensis* V14167]^{4x} (where "4*x*" indicates the ploidy; Fávero et al., 2006), BatSten1, BatDur1, and BatDur2 ([*A. batizocoi* K9484 × *A. stenosperma* V10309]^{4x}; [*A. batizocoi* K9484 × *A. duranensis* V14167]^{4x}; [*A. batizocoi* K9484 × *A. duranensis* SeSn2848]^{4x} ; respectively; Leal-Bertioli et al., 2015) and two produced here: IpaVillo1 [*A. ipaënsis* K30076 × *A. villosa* V12812]^{4x} and GregSten1, [*A. gregoryi* V6389 × *A stenosperma* V10309]^{4x}. Like the ones previously reported, these two newly induced allotetraploids had high fertility and produced seeds in numbers comparable to production by *A. hypogaea* (data not shown).

Plant architecture—With few exceptions, the induced allotetraploids had greater central stem heights, canopy area, leaf area, and root and aerial dry biomass compared with those of the diploid parents and the midparent values. Even when the increase was not statistically significant, the value was numerically higher than that of the parent with the highest value (Figs. 1A–C, 2).

The peanut cultivars used here have compact, dense architecture, while the diploid wild species have a spreading growth habit. All induced allotetraploids had the same growth habit as their diploid parents, and most had central stems that were higher than the peanut cultivars (Fig. 3). They mostly had slightly larger canopy areas than their parents (Fig. 1A). The only exception was BatDur2, which was a frail plant that gradually increased in vigor over generations (not shown). With the exception of IpaDur1, the ratio of root to aerial biomass showed no significant differences between the induced allotetraploids and their diploid parents (Appendix S1, see online Supplemental Data with this article).

Leaf morphology: Size and density of stomata, trichomes, and epidermal cells—Most stomata are the paracytic type, which has two subsidiary cells along the aperture and both guard cells (according to Metcalfe and Chalk, 1979). Densities of both stomata and epidermal cells (StD and ECD) were higher on the adaxial surface than the abaxial in all genotypes. Conversely, trichome density (TD) was greater on abaxial surfaces. The stomatal index (StI) had the same value for both surfaces (Appendix S1). Wild diploids generally had smaller cells but at higher density (both epidermal and guard cells) and higher trichome density and stomatal index compared with induced allotetraploids and peanut cultivars. Induced allotetraploids either had significantly lower values or resembled the parent with the lowest value (Figs. 1D, 4; Appendix S1).

The overall tendencies in leaf cell morphology observed for the spontaneous allotetraploid *A. hypogaea* were also observed for the induced allotetraploids: in all cases, on both leaf surfaces, the allotetraploid had cells with larger dimensions—epidermal and guard cells—than the diploid parents (Fig. 1D–1F, Table 2; online Appendices S1, S2). The stomatal index on the abaxial surface presented little variation among genotypes with same ploidy level, but they significantly reduced upon tetraploidization (Appendix S1). Trichomes were uniseriate and filiform, according to Leelavathi and Ramayya (1983). The trichome density for different peanut cultivars was similar. Mostly, allotetraploids had trichome density values similar to the maternal parent; however, on the abaxial surface of GregSten1, IpaDur1, and IpaVillo1, the trichome density was significantly lower than either parent and more similar to cultivated peanut (Table 2; Appendix S1).

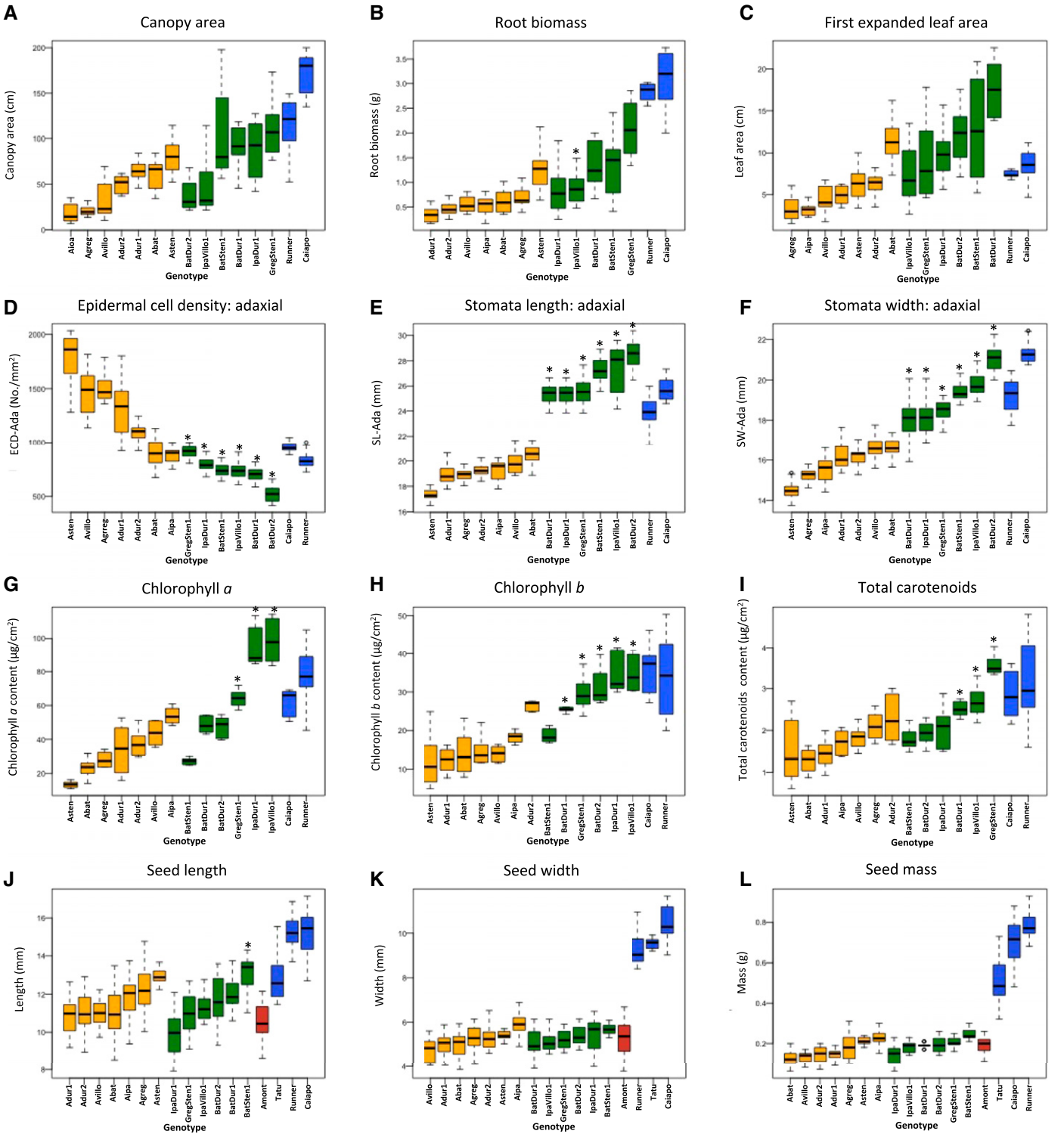


FIGURE 1 Box plot diagrams showing changes following tetraploidization on traits related to general plant characteristics (A–C), cell size on leaf adaxial surface (D–F), photosynthetic pigment concentrations per square centimeter of leaf area (G–I), and seed dimensions (J–L) of wild diploid species (yellow), induced allotetraploids (green), and peanut cultivars (blue). Seed dimensions plots of the wild allotetraploid *A. monticola* are in red. Boxes contain 50% of the data points. Bars across boxes represent the median. The top and bottom ends of the whiskers represent the highest and lowest values observed. Boxes with stars represent allotetraploids that have values significantly different from the midparent values at $P < 0.05$ (see online Appendix S1 for details). ECD-Ada = adaxial epidermal cell density; SL-Ada = adaxial stomata length; SW-Ada = adaxial stomata width.

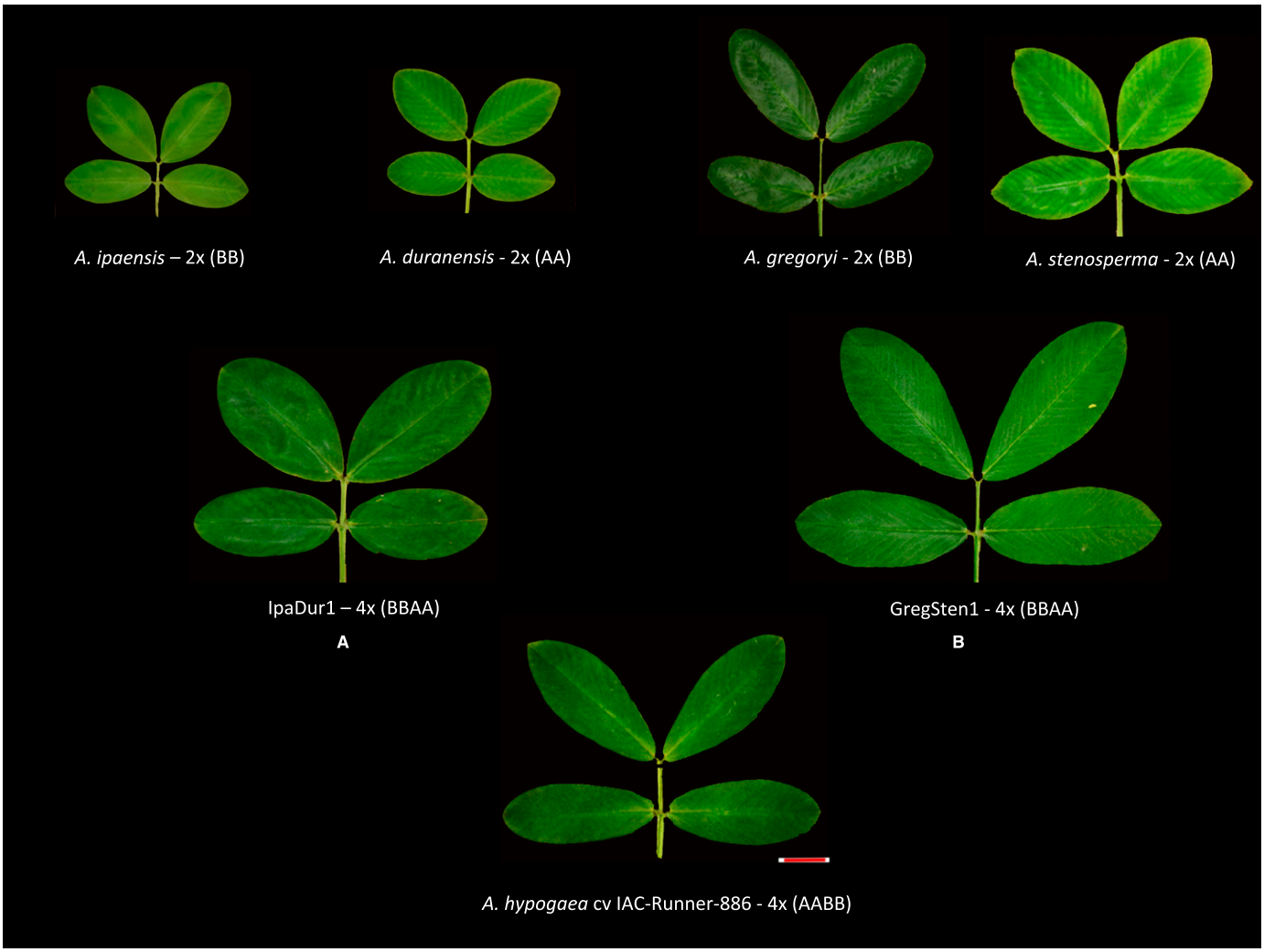


FIGURE 2 Examples of first expanded leaves of two sets of induced allotetraploids and their parents: (A) IpaDur1 (4*x*), *A. ipaënsis* K30076 (2*x*) and *A. duranensis* V14167 (2*x*); (B) GregSten1, *A. gregoryi* V6389 (2*x*) and *A. stenosperma* V10309 (2*x*) and cultivated peanut *A. hypogaea* cv. Runner IAC-886. Bar corresponds to 1 cm.

Leaf photosynthetic pigments—Photosynthetic pigment concentrations (μg/μL) were transformed to micrograms per square centimeter of leaf area. Significant differences were detected in all pigment concentrations between genotypes with the same ploidy and with different ploidy levels. Pigment concentrations for diploid genotypes ranged between 13.56 ± 1.74 and 53.93 ± 4.76 μg/cm^2 (Chla); 11.57 ± 5.83 and 26.37 ± 1.26 μg/cm^2 (Chlb) and 1.27 ± 0.25 and 2.29 ± 0.52 μg/cm^2 (X-car) (Fig. 1G–I; Appendix S1). On average, tetraploid genotypes had about double the pigment concentration of the diploids (Figs. 1G–1I, 3; Appendix S1).

All induced allotetraploids tested had numerically more Chla and Chlb and total X-car per leaf area than in their respective diploid parents, comparable to levels in cultivated peanut. However, these increases were only significant in the allotetraploids BatDur1, GregSten1, and IpaVillo1. Upon tetraploidization, Chla content increased, on average, 80.7%; Chlb, 110.9%; and X-car, 77.4% (Figs. 1G–I, 3).

Seed size, length, width, and mass—All diploid wild species used in this study have smaller and lighter seeds than those of cultivated peanut (Figs. 1J–L, 5). Previous observations showed that seeds of the allotetraploids BatDur1, BatDur2, and BatSten1 were very slightly larger and heavier than their diploid parents (Leal-Bertioli et al., 2015). However, compared with the midparent value, seed mass was the only trait without a statistically significant increase (Fig. 1J–L).

With the three other combinations (IpaDur1, IpaVillo1, and GregSten1), no significant differences in seed size or mass were found when compared with either parent or the midparent value (Appendix S1; Fig. 1J–1L). The wild allotetraploid *A. monticola* (also derived from *A. ipaënsis* and *A. duranensis*) and the cultivar IAC-Tatu (a Valencia type, with smaller seeds among peanut cultivars) were included in these measurements. The three *A. monticola* accessions evaluated are likely to be derived from the same wild population, deposited at the USDA (G. Seijo, IBONE, Argentina, personal communication). The values for all three accessions were statistically the same; therefore, the mean value for all three accessions is presented here. The results for *A. monticola* were similar to those of its diploid ancestral species. IAC-Tatu, as expected, had smaller seeds than the other two cultivars analyzed, but larger seeds than those of all

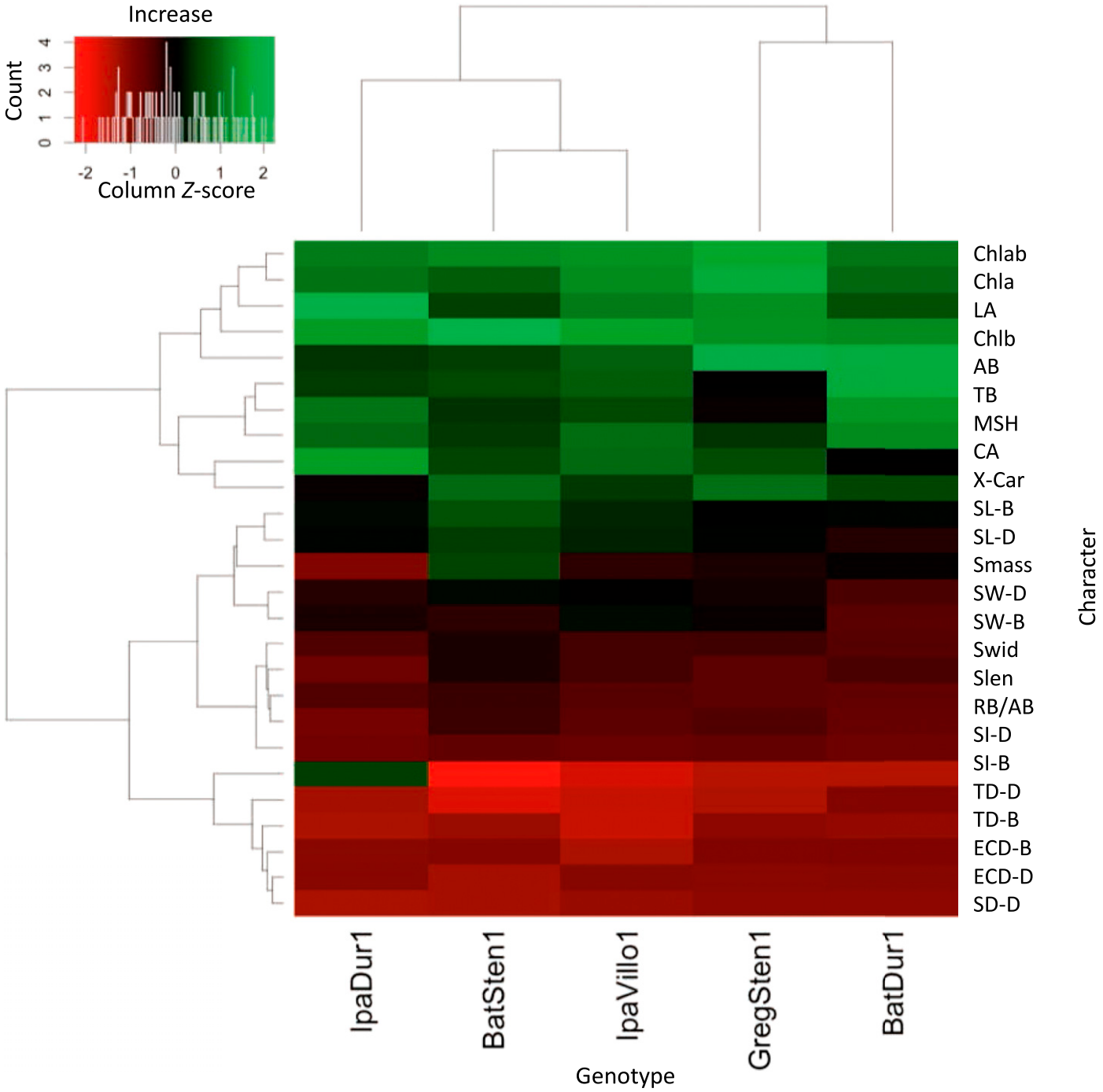


FIGURE 3 Heat map of character changes upon tetraploidization. Each cell contains the percentage of increase/decrease of the induced allotetraploid value compared with the midvalue between the corresponding diploid parents. Black = zero; green = maximum positive (210%); red = maximum negative (−100%). Chlab= foliar concentration of chlorophyll *a*+*b*; Chla = foliar concentration of chlorophyll *a*; LA = area of the first expanded leaf; Chlb = foliar concentration of chlorophyll *b*; AB = aerial biomass; TB = total biomass; RB = root biomass; MSH = main stem height; CA = canopy area; X-Car = foliar concentration of total carotenoids (carotene + xanthophylls); SL-B = abaxial stomata length; SL-D = adaxial stomata length; Smass = seed mass; SW-D = adaxial stomata width; SW-B = abaxial stomata width; Swid = seed width; Slen = seed length; RB/AB = ratio root biomass/aerial biomass; SI-D = adaxial stomatal index; SI-B = Abaxial stomatal index; TD-D = adaxial trichome density; TD-B = abaxial trichome density; SD-B = abaxial stomata density; ECD-B = abaxial epidermal cell density; ECD-D = adaxial epidermal cell density; SD-D = adaxial stomata density.

induced allotetraploids. Figure 5 shows typical seeds of the progenitors of cultivated peanut (*A. ipaënsis* and *A. duranensis*), the induced allotetraploid IpaDur1, the spontaneous wild allotetraploid *A. monticola*, and the cultivated spontaneous allotetraploid *A. hypogaea* subsp. *hypogaea* cv. Runner IAC-886. Note that only the cultivated peanut seed is visibly larger than the others.

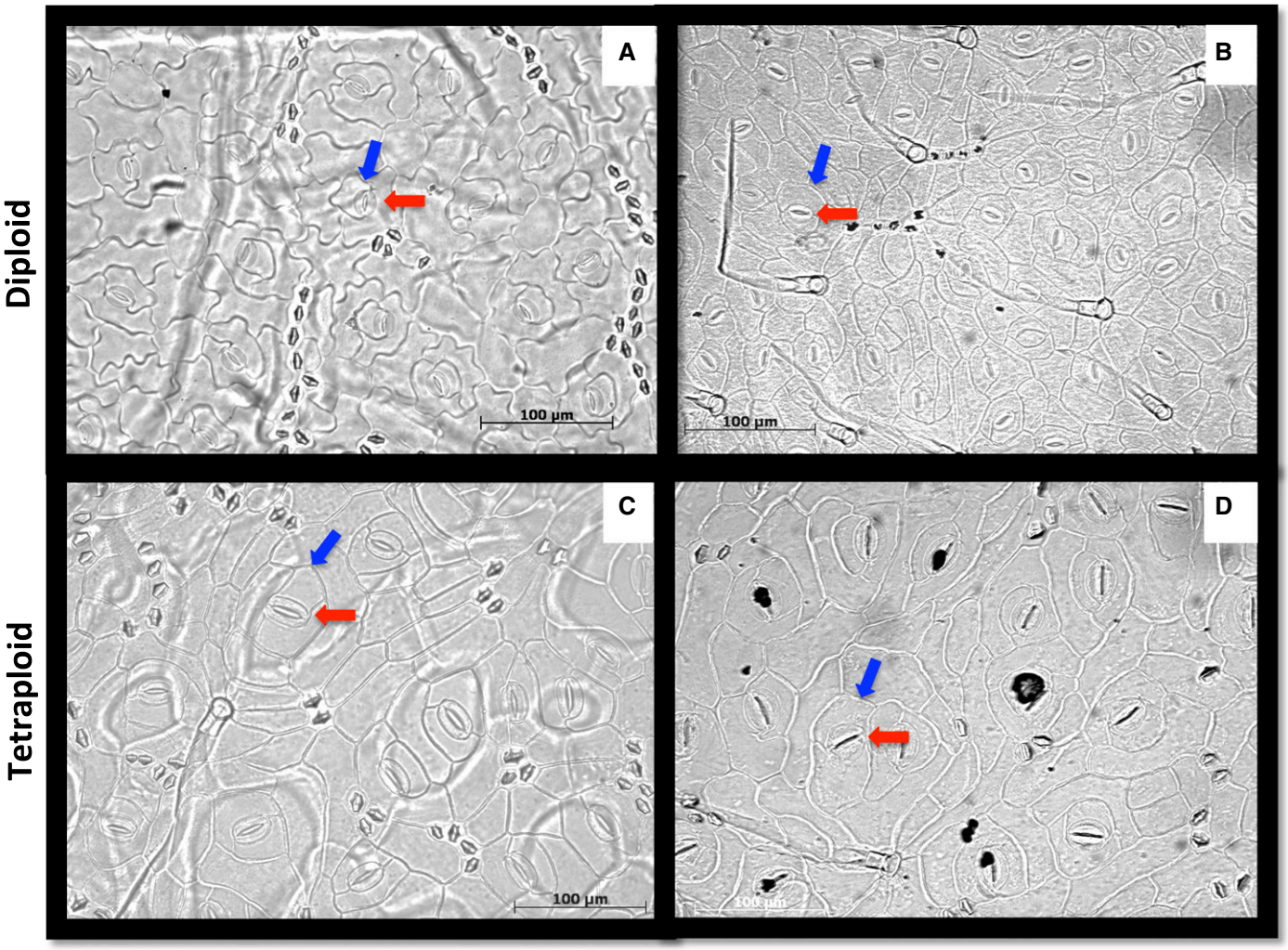


FIGURE 4 Leaflet surface after epidermal dissociation in DIC microscopy of (A) *A. batizocoi* K9484, (B) *A. stenosperma* V10309, (C) BatSten1, and (D) *A. hypogaea* cv. Runner IAC-886. Note that the stomata guard cells (red arrows) and surrounding epidermal cells (blue arrows) of tetraploid genotypes (C, D) are larger than in diploid genotypes (A, B).

DISCUSSION

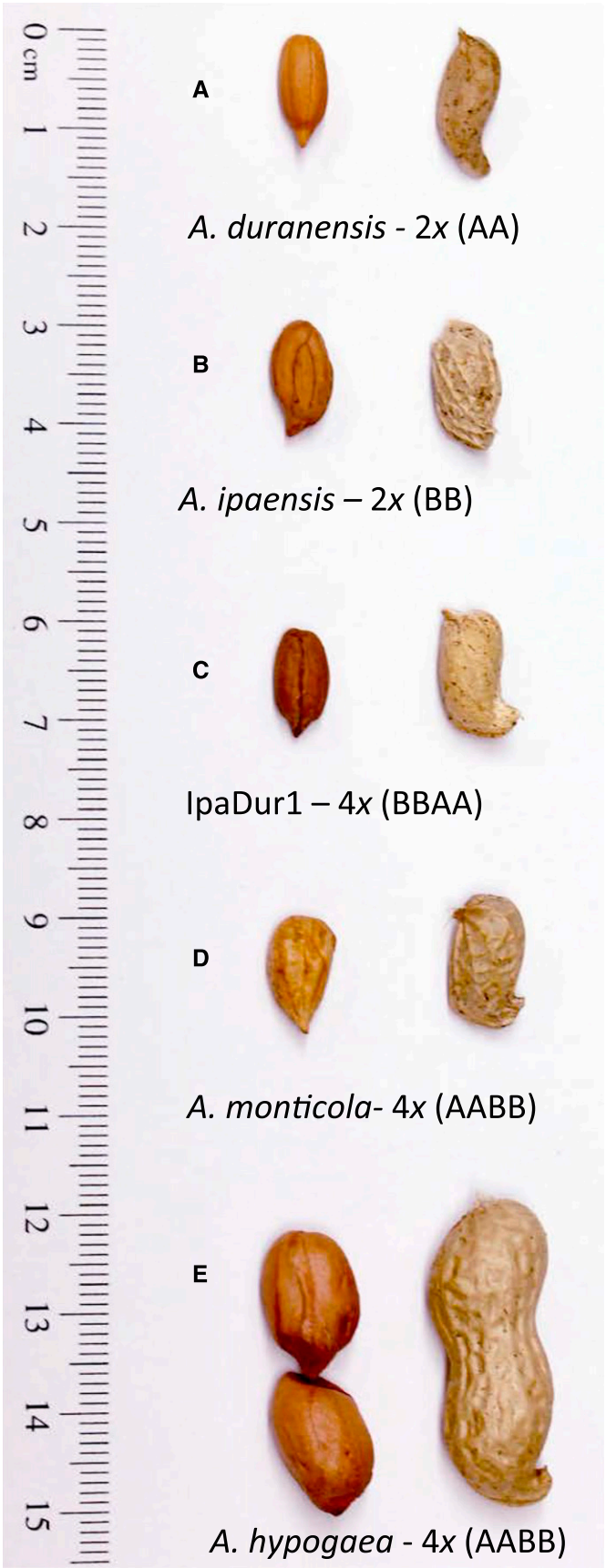
Whole-genome duplications have occurred in virtually all angiosperm lineages at some time and are believed to be a major driving force in their evolution (Cui et al., 2006). They generally result in polyploids that are new biological species, with increased heterosis and robustness, whose genomes have new possibilities for gene and genome evolution (e.g., Adams and Wendel, 2005). It has long been noted that a large proportion of domesticated plants are polyploids and that polyploidy may contribute to the plant's attractiveness for, or ability to respond to, domestication-and the ability to adapt to new environments (Soltis and Soltis, 2000; Jackson and Chen, 2010; Parisod et al., 2010; te Beest et al., 2011). However, there is some controversy, as some authors point out that polyploids are not over-represented among domesticated plant species because there are also high frequencies of polyploidy in wild plant species (Hilu, 1993), while others maintain that polyploid species are more likely to be domesticated than their wild diploid relatives (Renny-Byfield and Wendel, 2014; Salman-Minkov et al., 2016).

In the case of *A. hypogaea*, we consider that evidence strongly suggests that polyploidy conferred some advantage, or attraction for domestication. Diploid species have much greater genetic diversity than *A. hypogaea* and were cultivated from an earlier date and over a larger geographic area. In spite this, it was *A. hypogaea* that was domesticated and transformed by artificial selection into one of the world's most important crops: completely distinct in plant architecture, seed size and pod characteristics from all wild species (Krapovickas and Gregory, 2007; Freitas et al., 2007).

TABLE 2. Summary of evaluations of leaf cells of *Arachis* genotypes.

Characteristic	Leaf surface	Ploidy level	Type
Stomatal width	ABA > ADA	2*x* < 4*x*	W < Ah < IA
Stomatal length	ABA > ADA	2*x* < 4*x*	W < IA < Ah
Stomatal density	ABA < ADA	2*x* > 4*x*	W > Ah > IA
Epidermal cell density	ABA < ADA	2*x* > 4*x*	W > Ah > IA
Stomatal index	ABA = ADA	2*x* > 4*x*	Ah > W > IA
Trichome density	ABA > ADA	2*x* > 4*x*	W > (Ah = IA)

Notes: ABA = abaxial surface of the leaf; ADA = adaxial surface of the leaf; W = wild diploid species; IA = induced allotetraploid; Ah = cultivated *Arachis hypogaea.*



To investigate the changes that accompanied polyploidy in *Arachis*, we used six induced allotetraploids, their parental diploids, and the spontaneous allotetraploid *A. hypogaea*. We considered that the induced allotetraploid that is derived from the same two wild species as peanut (IpaDur1) was likely to give the best proxy for a primitive peanut. However, since the exact genetics of peanut's ancestors is not completely known, another five induced allotetraploids were also analyzed to enable inferences about general consequences and trends of tetraploidization in *Arachis*.

First, the induced allotetraploids generally display increased vigor and biomass when compared with the respective diploid parents. Increases were observed in leaf size, canopy, and root biomass (Appendix S1, Fig. 1A–C). Second, induced allotetraploids have larger leaf cells. Tetraploidization had a large impact on stomata and epidermal cell dimensions—induced allotetraploids presented large transgressive segregation, often surpassing the values of cultivated peanut. These changes are consistent with previous trends observed in *Arachis* (Singsit and Ozias-Akins, 1992; Leal-Bertioli et al., 2012) and other plants (e.g., *Triticum*, Jellings and Leech, 1982; *Medicago*, Molin et al., 1982; *Pisum*, Cavallini et al., 1993; and *Musa*, Vandenhout et al., 1995) and are likely to be linked to the changes in transpiration responses to water deficit following polyploidy previously observed (Leal-Bertioli et al., 2012). The allotetraploid's longer response of continuing transpiration under water deficit is likely to result in greater productivity under certain conditions.

Third, leaf photosynthetic pigments increase upon tetraploidization. Except for BatSten1, all allotetraploids had higher concentrations of Chla, Chlb, and X-car. Increases of up to 207.98%, 174.62%, and 142.33%, respectively, were found (Fig. 3; Appendix S2). These increases likely resulted in a higher photosynthetic capacity and, for peanut, remained during domestication. These observations are consistent with increased photosynthetic capacity following polyploidy in other species, including the soybean relative *G. dolichocarpa* (Coate et al., 2012; Ilut et al., 2012) and *Arabidopsis*, *Brassica*, and wheat allopolyploids (Ni et al., 2009). Since photosynthesis plays a fundamental role in plant fitness (Warner and Edwards, 1993), these results suggest that the induced allotetraploids could have greater fitness in some environments.

Fourth and last, in spite of the large changes in plant morphology observed, changes in seed dimensions in response to tetraploidy were not significant. For the seed measurements, we added three accessions of the wild species *A. monticola* as it is the only other natural tetraploid from the section *Arachis* and has the same parental species as peanut (Krapovikas and Gregory, 2007; Vyas et al., 2007). It probably arose from the same tetraploidization event as peanut (Seijo et al., 2004; Moretzsohn et al., 2013) and is either a wild descendant of the progenitor of peanut or a feral species that escaped at early stages of domestication. *Arachis monticola* seed dimensions and mass are comparable to those of the wild parents and to the induced allotetraploid IpaDur1. Furthermore, for all the newly produced allotetraploids, seeds had similar mass and dimension to their parents. Although our previous study did show that seeds of the *A. batizocoi*-derived allotetraploids were very slightly larger than their diploid parents (Leal-Bertioli et al., 2015), the

FIGURE 5 Typical pods of wild species (A) *A. duranensis*, (B) *A. ipaënsis*, and (D) *A. monticola*; (C) the induced allotetraploid IpaDur1; and (E) cultivated peanut *A. hypogaea* cv. Runner IAC-886. (image credit: Dr. JinHee Shin)

difference here was not statistically significant. We believe that many will find this result surprising, but it is not without precedent. Tetraploidization does cause seed size increase for some plant genera, such as *Catharanthus* (Hosseini et al., 2013) and *Dactylis* (Bretagnolle et al., 1995), but it has also been reported to induce small seeds in other genera, such as *Brassica* (Howard, 1942) and *Nasturtium* (Howard, 1947).

In summary, the *Arachis* allotetraploids show some general phenotypic trends that are common, regardless of the exact combination of diploid parents. For these trends, nucleotypic effect (higher DNA quantity, irrespective of that DNA's information content) is likely to be more important than new allelic combinations, as also suggested by Bennett (1987). These same trends were almost certainly displayed by the ancestor of cultivated peanut following polyploidy—newly formed allotetraploids are more robust, have increased photosynthetic capacity, and different transpiration responses to diploid species. These characteristics, or others, may have proved attractive for domestication. However, our findings suggest that the characteristic that is probably most cited as being likely to make domestication more attractive, increased seed size (Simpson et al., 2001) was unlikely to have been important for the initial domestication of peanut.

ACKNOWLEDGEMENTS

The authors thank Leandro Mesquita, Karinne Dantas, and Igor Bacon for greenhouse and technical assistance; Dr. JinHee Shin for photograph (Figure 5); and Drs. Jose Valls, Jeff Doyle, and Guillermo Seijo for constructive discussions. This work was funded by the Generation Challenge Program Tropical Legumes 1 and MARS Inc. They also thank the authors' institutions for financial and logistical support and the reviewers for their very helpful comments.

LITERATURE CITED

Acquaah, G. 2012. Principles of plant genetics and breeding. Wiley-Blackwell, Chichester, UK.

Adams, K. L., and J. F. Wendel. 2005. Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology* 8: 135–141.

Bennett, M. D. 1987. Variation in genomic form in plants and its ecological implications. *New Phytologist* 106: 177–200.

Berlyn, G., and J. Miksche. 1976. Botanical microtechnique and cytochemistry. Iowa State University Press, Iowa, Ames, USA.

Bertioli, D. J., S. B. Cannon, L. Froenicke, G. Huang, A. D. Farmer, E. K. Cannon, X. Liu, et al. 2016a. The genome sequences of *Arachis duranensis* and *Arachis ipaensis*, the diploid ancestors of cultivated peanut. *Nature Genetics*.

Bertioli, D. J., S. C. M. Leal-Bertioli, and H. T. Stalker. 2016b. The peanut genome: The history of the consortium and the structure of the genome of cultivated peanut and its diploid ancestors. *In* H. T. Stalker and R. F. Wilson [eds.], Peanuts: Genetics, processing, and utilization. Elsevier, New York, New York, USA.

Bertioli, D. J., G. Seijo, F. O. Freitas, J. F. Valls, S. C. M. Leal-Bertioli, and M. C. Moretzsohn. 2011. An overview of peanut and its wild relatives. *Plant Genetic Resources* 9: 134–149.

Bretagnolle, F., J. D. Thompson, and R. Lumaret. 1995. The influence of seed size variation on seed germination and seedling vigour in diploid and tetraploid *Dactlys glomerata* L. *Annals of Botany* 76: 607–615.

Burow, M., C. Simpson, J. Starr, and A. Paterson. 2001. Transmission genetics of chromatin from a synthetic amphidiploid to cultivated peanut (*Arachis hypogaea* L.). Broadening the gene pool of a monophyletic polyploid species. *Genetics* 159: 823–837.

Cavallini, A., L. Natali, G. Cionini, and D. Gennai. 1993. Nuclear DNA variability within *Pisum sativum* (Leguminosae): Nucleotypic effects on plant growth. *Heredity* 70: 561–565.

Coate, J. E., A. K. Luciano, V. Seralathan, K. J. Minchew, T. G. Owens, and J. J. Doyle. 2012. Anatomical, biochemical, and photosynthetic responses to recent allopolyploidy in *Glycine dolichocarpa* (Fabaceae). *American Journal of Botany* 99: 55–67.

Cui, L., P. K. Wall, J. H. Leebens-Mack, B. G. Lindsay, D. E. Soltis, J. J. Doyle, P. S. Soltis, et al. 2006. Widespread genome duplications throughout the history of flowering plants. *Genome Research* 16: 738–749.

Cutter, E. G. 1986. Anatomia vegetal. Roca Publisher, São Paulo, S.P., Brazil.

Dillehay, T. D., J. Rossen, T. C. Andres, and D. E. Williams. 2007. Preceramic adoption of peanut, squash, and cotton in northern Peru. *Science* 316: 1890–1893.

Fávero, A. P., R. F. Santos, C. E. Simpson, J. F. M. Valls, and N. A. Vello. 2015. Successful crosses between fungal-resistant wild species of *Arachis* (section *Arachis*) and *Arachis hypogaea. Genetics and Molecular Biology* 38: 353–365.

Fávero, A. P., C. E. Simpson, F. M. J. Valls, and N. A. Velo. 2006. Study of evolution of cultivated peanut trough crossability studies among *Arachis ipaënsis, A. duranensis* and *A. hypogaea. Crop Science* 46: 1546–1552.

Freitas, F. O., M. C. Moretzsohn, and J. F. M. Valls. 2007. Genetic variability of Brazilian Indian landraces of *Arachis hypogaea* L. *Genetics and Molecular Research* 6: 675–684.

Gepts, P. 2003. Ten thousand years of crop evolution. *In* M. Chrispeels and D. Sadava [eds.], Plants, genes, and crop biotechnology, 328–359. Jones and Bartlett, Sudbury, Massachusetts, USA.

Halward, T. M., H. T. Stalker, E. A. Larue, and G. Kochert. 1991. Genetic variation detectable with molecular markers among unadapted germplasm resources of cultivated peanut and related wild species. *Genome* 34: 1013–1020.

Hilu, K. W. 1993. Polyploidy and the evolution of domesticated plants. *American Journal of Botany* 80: 1494–1499.

Hosseini, H., M. Chehrazi, M. M. Sorestani, and D. Ahmadi. 2013. Polyploidy and comparison of diploid and autotetraploid seedling of Madagascar periwinkle (*Catharanthus roseus* cv. alba). *International Research Journal of Applied and Basic Sciences* 4: 402–406.

Howard, H. W. 1942. The effect of polyploidy and hybridity on seed size in cross between *Brassica chinensis*, *B. carinata*, amphidiploid *B. chinensis-carinata* and autotetraploid *B. chinensis. Journal of Genetics* 43: 105–119.

Howard, H. W. 1947. Seed size in crosses between diploid and autotetraploid *Nasturtium officinale* and allotetraploid *N. uniseriatum. Journal of Genetics* 48: 111–118.

Ilut, D. C., J. E. Coate, A. K. Luciano, T. G. Owens, G. D. May, A. Farmer, and J. J. Doyle. 2012. A comparative transcriptomic study of an allotetraploid and its diploid progenitors illustrates the unique advantages and challenges of RNA-seq in plant species. *American Journal of Botany* 99: 383–396.

Jackson, S., and Z. J. Chen. 2010. Genomic and expression plasticity of polyploidy. *Current Opinion in Plant Biology* 13: 153–159.

Jellings, A. J., and R. M. Leech. 1982. The importance of quantitative anatomy in the interpretation of whole leaf biochemistry in species of *Triticum*, *Hordeum* and *Avena. New Phytologist* 92: 39–48.

Kochert, G., H. T. Stalker, M. Gimenes, L. Galgaro, C. R. Lopes, and K. Moore. 1996. RFLP and cytogenetic evidence on the origin and evolution of allotetraploid domesticated peanut, *Arachis hypogaea* (Leguminosae). *American Journal of Botany* 83: 1282–1291.

Krapovickas, A., and W. C. Gregory. 2007. Taxonomy of the genus *Arachis* (Leguminosae). *Bonplandia* 16 (supplement): 1–205.

Leal-Bertioli, S. C. M., D. J. Bertioli, P. M. Guimarães, T. D. Pereira, I. Galhardo Silva, J. P. Silva, A. C. M. Brasileiro, et al. 2012. The effect of tetraploidization of wild *Arachis* on leaf morphology and other drought-related traits. *Environmental and Experimental Botany* 84: 17–24.

Leal-Bertioli, S. C. M., S. P. Santos, K. M. Dantas, P. W. Inglis, S. Nielen Araujo, A. C. G. Araújo, J. P. Silva, et al. 2015. *Arachis batizocoi*: A study of its relationship to cultivated peanut (*A. hypogaea*) and its potential for introgression of wild genes into the peanut crop using induced allotetraploids. *Annals of Botany* 115: 237–249.

Leelavathi, P., and N. Ramayya. 1983. Structure, distribution and classification of plant trichomes in relation to taxonomy III. Papilionoideae. *Proceedings of the Indian Academy of Sciences, Plant Science* 92: 421–441.

Lichtenthaler, H. K. 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *In* L. Packer and R. Douce [eds.], Methods in enzymology, vol. 148: 350–382. Academic Press, Waltham, Massachusetts, USA.

Mallikarjuna, N., S. Senthilvel, and D. Hoisington. 2011. Development of new sources of tetraploid *Arachis* to broaden the genetic base of cultivated groundnut (*Arachis hypogaea* L.). *Genetic Resources and Crop Evolution* 58: 889–907.

Metcalfe, C. R., and L. Chalk. 1979. Anatomy of the dicotyledons, 2nd ed. Vol. I, Systematic anatomy of leaf and stem, with a brief history of the subject. Clarendon Press, Oxford, UK.

Molin, W. T., S. P. Meyers, G. R. Baer, and L. E. Schrader. 1982. Ploidy effects in isogenic populations of alfalfa II. Photosynthesis, chloroplast number, ribulose-1,5-bisphosphate carboxylase, chlorophyll, and DNA in protoplasts. *Plant Physiology* 70: 1710–1714.

Moretzsohn, M. C., E. G. Gouvea, P. W. Inglis, S. C. M. Leal-Bertioli, J. F. M. Valls, and D. J. Bertioli. 2013. A study of the relationships of cultivated peanut (*Arachis hypogaea*) and its most closely related wild species using intron sequences and microsatellite markers. *Annals of Botany* 111: 113–126.

Ni, Z., E. D. Kim, M. Ha, E. Lackey, J. Liu, Y. Zhang, Q. Sun, and Z. J. Chen. 2009. Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. *Nature* 457: 327–331.

Parisod, C., R. Holderegger, and C. Brochmann. 2010. Evolutionary consequences of autopolyploidy. *New Phytologist* 186: 5–17.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website http://www.R-project.org/.

Ramsey, J., and D. W. Schemske. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology, Evolution, and Systematics* 29: 467–501.

Renny-Byfield, S., and J. Wendel. 2014. Doubling down on genomes: Polyploidy and crop plants. *American Journal of Botany* 101: 1711–1725.

Salman-Minkov, A., N. Sabath, and I. Mayrose. 2016. Whole-genome duplication as a key factor in crop domestication. *Nature Plants* 2: 16115.

Seijo, J. G., G. I. Lavia, A. Fernández, A. Krapovickas, D. Ducasse, and E. A. Moscone. 2004. Physical mapping of the 5S and 18S-25S rRNA genes by FISH as evidence that *Arachis duranensis* and *A. ipaënsis* are the wild diploid progenitors of *A. hypogaea* (Leguminosae). *American Journal of Botany* 91: 1294–1303.

Simpson, C. E., A. Krapovickas, and J. F. M. Valls. 2001. History of *Arachis* including evidence of *A. hypogaea* L. progenitors. *Peanut Science* 28: 78–80.

Simpson, C. E., S. C. Nelson, L. J. Starr, K. E. Woodard, and O. D. Smith. 1993. Registration of TxAG-6 and TxAG-7 peanut germplasm lines. *Crop Science* 33: 1418.

Singsit, C., and P. Ozias-Akins. 1992. Rapid estimation of ploidy levels in in vitro-regenerated interspecific *Arachis* hybrids and fertile triploids. *Euphytica* 64: 183–188.

Soltis, P. S., and D. E. Soltis. 2000. The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences, USA* 97: 7051–7057.

Stalker, H. T., and R. E. Lynch. 2002. Registration of four insect-resistant peanut germplasm lines. *Crop Science* 42: 313–314.

Tal, M. 1980. Physiology of polyploids. *In* W.H. Lewis [ed.], Polyploidy: Biological relevance, 61–75. Plenum, New York, New York, USA.

te Beest, M., J. J. Le Roux, D. M. Richardson, A. K. Brysting, J. Suda, M. Kubešová, and P. Pyšek. 2011. The more the better? The role of polyploidy in facilitating plant invasions. *Annals of Botany* 109: 19–45.

Vale, F. X. R., E. Fernandes Filho, and J. R. Liberato. 2001. Quant—Quantificação de doenças: versão 1.0.1 [computer program]. Federal University of Viçosa [UFV], Viçosa, Brazil [distributed by the authors].

Vandenhout, H., R. Ortiz, D. Vuylsteke, R. Swennen, and K. V. Bai. 1995. Effect of ploidy on stomatal and other quantitative traits in plantain and banana hybrids. *Euphytica* 83: 117–122.

Vyas, P., M. S. Bisht, S. Miyazawa, S. Yano, K. Noguchi, I. Terashima, and S. Funayama-Noguchi. 2007. Effects of polyploidy on photosynthetic properties and anatomy in leaves of *Phlox drummondii*. *Functional Plant Biology* 34: 673–682.

Warner, D. A., and G. E. Edwards. 1993. Effects of polyploidy on photosynthesis. *Photosynthesis Research* 35: 135–147.