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RESEARCH ARTICLE

LEAF STOMATAL CHARACTERISTICS AND KEY AGRONOMIC TRAITS OF INTERSECTIONAL PEANUT F_1 HYBRIDS BETWEEN HUAYU 665 AND *Arachis paraguariensis*

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ABSTRACT

The cultivated peanut is a globally important crop, valued for its oil, food, and feed uses, but has a narrow genetic base. High stress resistance, good-quality and high-yield factors residing in wild species constitute valuable resources for genetic improvement of the peanut cultigen. Some wild species are used as groundcovers, while others utilized as potted plants. Previous studies have focused on compatible wild relatives, but there is a lack of research on the use of incompatible *Arachis* species. This study aimed to investigate the hybrids produced from a cross between the peanut cultivar Huayu 665 and the incompatible species *Arachis paraguariensis*, enhancing our understanding of distant hybridization. Ture F_1 intersectional hybrids were identified by transposon element marker pairs. Leaf stomata were observed, and main agronomic traits were investigated. The F_1 hybrids exhibited significantly fewer large stomata (14.78 per mm²) and longer stomata (26.52 µm) on the abaxial leaf epidermis compared to the female parent, Huayu 665, which had 20.56 per mm² and 16.86 µm, respectively. Compared to Huayu 665, the F_1 hybrids exhibited a longer first pair of lateral branches, a wider range of seed set, and more branches, but produced fewer pods per plant. A hybrid with a plant type similar to the female parent was identified. The authenticity of the hybrids was confirmed through molecular, anatomical, and morphological analyses. The hybrid resembling the cultigen may accelerate the utilization of incompatible wild species in peanut breeding. However, its chromosome composition is yet to be determined. To avoid missing true hybrid identification in peanut remote crosses, use of molecular markers distributed across different chromosomes of the wild species was proposed.

Keywords: groundnut, incompatible, intersectional hybrid, stomata, transposon element marker

INTRODUCTION

The cultivated peanut (*Arachis hypogaea* L.), a globally significant cash crop, plays a vital role in rural economies worldwide (Smartt, 1994). Its seeds are an essential source of protein and oil, while its leaves and vines serve as valuable fodder for livestock (Smartt, 1994). However, the genetic base of the cultivated peanut is alarmingly narrow due to six evolutionary bottlenecks (Mallikarjuna and Varshney, 2014) This issue has been further compounded by the repeated use of backbone parental lines in breeding programs, which leads to diminished adaptability to environmental stresses.

In contrast, wild *Arachis* species, particularly those genetically incompatible with cultivated peanuts, represent a treasure trove of genetic diversity. These wild relatives exhibit high levels of resistance—or even immunity—to a wide range of biotic and abiotic stresses that often threaten the cultivated peanut (Williams, 2022; Wang and Zhang, 2013) Moreover, wild species harbour desirable agronomic traits, including higher yield potential and increased oil or protein content compared to their cultivated counterpart (Cherry, 1976; Jiang *et al*., 2010; Nigam *et al*., 1991). This diversity makes them a highly valuable genetic resource for peanut breeding. However, the use of wild incompatible *Arachis* species in

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breeding has been limited due to biological and technical barriers.

Recent efforts in peanut breeding have primarily focused on exploiting compatible wild species to expand the genetic base of cultivated peanut (Stalker *et al*., 2016; Cason *et al*., 2023). Yet, incompatible wild species remain underutilized, despite their promise in significantly enhance genetic diversity and resilience. Hybridization between peanut cultivars and incompatible wild species has historically been hindered by challenges such as delayed fertilization and aborted embryos. *In vitro* embryo rescue methods have been employed to overcome these obstacles, using ovules, embryos, and pegs as explants to generate hybrid seedlings or seeds. However, these processes are resource-intensive and time -consuming, and often face hybrid lethality and sterility problems.

To address these gaps, based on our experience with *in vitro* peanut peg culture, we proposed and confirmed the hypothesis of hormonal imbalance in peanut incompatible crosses (Li *et al*., 2023), and developed an innovative *In Situ Embryo Rescue* (ISER) technique, which facilitates the generation of hybrids between cultivated peanut and incompatible wild *Arachis* species (Wang *et al*., 2020; Jiang *et al*., 2024). This technique simplifies the process of obtaining hybrids and allows for characterization of a large number of intersectional hybrids.

This study focuses on hybrids derived from a cross between the cultivated peanut cultivar Huayu 665 and the wild incompatible species *A. paraguariensis*. Specifically, we aim to characterize their leaf stomatal traits and key agronomic features to enrich our understanding of these distant hybrids and their potential contributions to peanut breeding.

MATERIALS AND METHODS

Plant materials

The maternal parent, Huayu 665, is a high-oleic peanut variety released by the Shandong Peanut Research Institute (SPRI). The paternal parent, *A. paraguariensis* PI 338297, is a wild incompatible peanut species of Section Erectoides. Their F_1 hybrids were recovered through ISER (Wang *et al*., 2020). All the peanut materials were sown in double rows (one seed per hill) on an elevated seedbed (bottom width: 85 cm, height:12 cm) with an inter-row and an inter-plant spacing of 30 cm and 17.5 cm, under polyethylene film mulch, at SPRI Laixi Experimental Station, on May 1, 2021. Standard agronomic practices were followed as described by Wan (2003).

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted using a plant genomic DNA extraction kit (Tiangen, Beijing) from fresh leaves of parents and hybrids. PCR mixture (20 μl) was composed of 10 μl of 2 × *Taq* PCR Master Mix (Tiangen, Beijing), 1μl of DNA template, and 1 μl of each forward and reverse primer (10 μmol/L) (Table 1). PCR program was an initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 5 min. PCR products were separated and visualized on a 1% agarose gel.

Table 1: Transposable element (TE) marker primer sequence information

Primer name	Sequence (5'-3')
AhTE0416-F	GAGTTACATCGGTATTGAA-TATGTGA
AhTE0416-R	CTCCGTGATTGAGGCGTTA
AhTE0489-F	TGACTACAATGTTTGGTCATTTTG
AhTE0489-R	GAACCACCCATGATTTGTGA

Source: http://marker.kazusa.or.jp/Peanut/marker/list/Transposable%20Element

Observation on foliar stomatal characteristics

On August 27, 2021, the third fully expanded leaf on the main stems of the parents and their true F_1 hybrids was sampled for stomatal observation. Care was taken to collect leave samples at the same maturity level. Immediately after the leaves were removed from the plants, the upper and lower epidermis of the leaves were brushed evenly with a thin layer of flexible collodion (Hubei

Kotan Pharmacology, Hubei, China) using a small brush. Once the adhesive dried, the film was carefully torn off with forceps, placed on a slide, and covered with a coverslip. The stomatal characteristics were observed under an IX73P+DP80 inverted fluorescence microscope (Olympus, Japan).

Three leaflets were randomly selected from the same position of the compound leaves from the parents and the hybrids. For each leaflet, three fields of view were randomly selected for either the upper or lower surface. The field size was 260×340 μm^2, and the primary and secondary veins were avoided. Two representative stomata were randomly selected for each field of view to calculate the length of the longitudinal diameter and the width of the transverse diameter. The total number of stomata in each field of view was also counted. Each leaflet was a replicate, and the average of the measurements or counts from the three fields of view was calculated to represent the value for each leaflet.

Investigation of key agronomic traits

Five true F_1 hybrids and five plants of the female parent, Huayu 665, were randomly selected to evaluate agronomic traits. The angle between the main stem and the cotyledonary branch was measured using a digital angle ruler (Shenzhen Dupuy Electronic, Guangdong, China) on September 10, 2021. At harvest, additional agronomic traits were assessed following the methods outlined by Yu (2008).

Statistical analysis

Stomatal characteristics were analyzed using a two-factor completely randomized design (fixed model) with factors including three genotypes (hybrids, male parent, and female parent) and two positions (upper and lower leaflet surfaces). Each leaflet was treated as a replicate, with three replicates per genotype. Tukey's method was used for multiple comparison. Key agronomic traits were evaluated using Fisher's small-sample non-parametric randomization test. All statistical analyses were performed using the DPS 14.50 software package (Tang and Zhang, 2013).

RESULTS AND DISCUSSION

Identification of true hybrids

Screening of TE marker primers with DNA templates of both parents, Huayu 665, wild species *A. paraguariensis* PI 338297, resulted in two TE marker primer pairs, AhTE0416 and AhTE0489 (Table 1), with reproducible, discernable and male parent-specific bands. Both primer pairs were therefore used in hybrid identification for cross-validation. All the 13 seeds tested were identified as true hybrids (Figure 1).

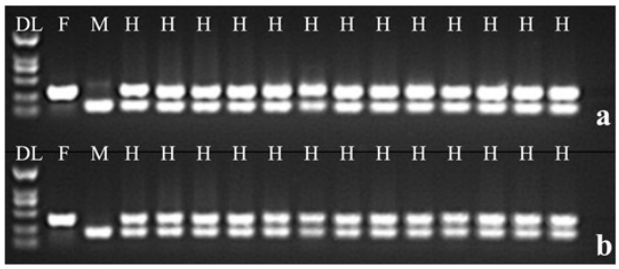

Figure 1: Identification of true F_1 hybrids using AhTE primer pairs AhTE0416 (a) and AhTE0489 (b)

DL: D2000 DNA Marker (Tiangen, Beijing); F: female parent, Huayu 665; M: male parent, *A. paraguariensis* PI 338297; H: true hybrid.

Foliar stomatal features of parents and true hybrids

Leaf stomata length and width

Regarding foliar stomata length, significant differences were detected at the 0.01 and 0.05 levels among genotypes and between the adaxial and abaxial epidermis, respectively, with no significant interaction between the two factors (*Supplementary Table 1*). The stomata length of the hybrids averaged 23.93 μm, much higher than that of Huayu 665 (16.81 μm) or the wild species (15.88 μm) (Table 2). The adaxial leaf surface had an epidermal stomata length (17.59 μm) lower than the abaxial leaf surface (20.16 μm) (Table 3). Stomata lengths by peanut materials and by epidermis positions were shown in Table 3. The abaxial leaf surface stomata length of the hybrids was significantly higher than that of the female parent (26.52 μm vs 16.86 μm) (Table 4).

Table 2: Comparison of stomatal characteristics among peanut genotypes

Identity	Stomata length (µm)	Stomata width (µm)	Stomata density (No. of stomata per mm^2)	Large stomata density (No. of large stomata per mm^2)
Hybrid	23.93^{A}	5.01^{a}	334.34^{B}	14.61^{B}
Female parent	16.81^{B}	3.29^{b}	424.84^{B}	19.39^{AB}
Male parent	15.88^{B}	3.69^{b}	676.22^{A}	23.11^{A}

Note: Values in the same column with different upper- or lower-case letters indicate significant differences at the 0.01 or 0.05 level.

Table 3: Comparison of stomatal characteristics between upper and lower leaf surface

Position	Stomata length (µm)	Stomata width (µm)	Stomata density (No. of stomata per mm^2)	Large stomata density (No. of large stomata per mm^2)
Adaxial surface	17.59^{b}	3.82	626.36^{A}	19.07
Abaxial surface	20.16^{a}	4.18	330.57^{B}	19.00

Note: Values in the same column with different upper- or lower-case letters indicate significant differences at the 0.01 or 0.05 level.

Table 4: Comparison of stomatal characteristics of the upper and lower epidermis of leaves of different peanut genotypes

Identity	Stomata length (µm)		Stomata width (µm)		Stomata density (No. of stomata per mm^2)		Large stomata density (No. of large stomata per mm^2)	
	Adaxial surface	Abaxial surface	Adaxial surface	Abaxial surface	Adaxial surface	Abaxial surface	Adaxial surface	Abaxial surface
Hybrid	21.35^{A}	26.52^{A}	4.98	5.05	369.53^{B}	299.15	14.44^{B}	14.78^{b}
Female parent	16.76^{AB}	16.86^{B}	3.30	3.28	509.05^{B}	340.62	18.22^{AB}	20.56^{a}
Male parent	14.65^{B}	17.10^{B}	3.18	4.21	1000.50^{A}	351.94	24.56^{A}	21.67^{a}

Note: Values in the same column with different upper- or lower-case letters indicate significant differences at the 0.01 or 0.05 level.

Differences in stomata width among genotypes were significant at the 0.05 level, while differences between the upper and lower epidermis and genotype-position interactions were not significant (*Supplementary Table 1*, Tables 2 and 4). The stomata width of the hybrids (5.01 µm) was significantly higher than that of either parent (3.69 µm for the wild species, 3.29 µm for Huayu 665) (Table 2).

Leaf stomata density and large leaf stomata density

In terms of stomata density, the effects of genotype, stomata position, and their interaction were all significant at the 0.01 level (*Supplementary Table 1*). The male parent had a significantly higher stomata density (676.22 per mm^2) than either the female parent (424.84 per mm^2) or the hybrids (334.34 stomata per mm^2) (Table 2). These differences were most evident in the upper epidermis, where the stomata density (626.36 per mm^2) was significantly higher than that of the lower epidermis (330.57 per mm^2) (Tables 3 and 4).

Large leaf stomata density significantly differed among the parents and the hybrids at the 0.01 level ($P=0.0017<0.01$), but not between the upper and lower epidermis or for genotype by epidermal factor (position) interaction (*Supplementary Table 1*). The F_1 hybrids exhibited the lowest density of large stomata on the leaf surface, with a value of 14.61 per mm², significantly lower than that of the male parent (23.11 per mm²) (Table 2). This trend was particularly evident on the abaxial leaf epidermis, where the large stomata density of the hybrids was 14.78 per mm², compared to 20.56 per mm² in Huayu 665 and 21.67 per mm² in the wild peanut (Table 3).

Agronomic traits

Supplementary Table 2 shows that compared to the female parent, the hybrids had longer first pairs of lateral branches, a greater range

of seed-set, and more branches. However, they had fewer pods, full pods, and two-seeded pods per plant.

As previously noted by C. T. Wang (unpublished data), segregation in plant type may occur in F_1 hybrids between the cultivated peanut and wild incompatible species. In this study, one hybrid was similar to the female parent in plant type, while others were clearly different (Figure 2).

Figure 2: F_1 hybrids displaying plant types distinct from the female parent, Huayu 665 (a), as illustrated in (b), or resembling the female parent, as shown in (c).
bar size = 10 cm

In the present study, TE molecular markers were employed to confirm the authenticity of F_1 hybrids in peanut wide crosses. Stomatal characteristics and key agronomic traits were analyzed, revealing distinct differences between the hybrids and their parents. While some hybrids exhibited morphological traits and seed-set characteristics resembling the female parent, this phenomenon, consistent with our prior unpublished findings, warrants further investigation. We postulated that trait segregation in the F_1 generation was due to genomic incompatibility between the distantly related incompatible wild relative and the cultivated peanut. Zhou *et al.* (1988) hypothesized that fragment hybridization occurs in distant plant hybrids, based on their surveys of such hybrids, and proposed the molecular breeding theory involving the introduction of exogenous DNA. Inconsistent trait expression in F_1 hybrids may stem from the varied extent of rejection/acceptance of alien genome by the genome of the cultivated peanut and differential genome recombination. Notably, in a recent unpublished study, we observed the loss of some wild species chromosome-specific molecular markers in hybrids between the cultivated peanut and certain incompatible wild species (Jiang *et al*., 2024). This raises concerns about the reliability of hybrid identification using only 1–2 molecular markers, as it could lead to true hybrids being misclassified as false hybrids. We recommend using a larger number of markers distributed across wild species' chromosomes alongside morphological evaluation for accurate hybrid identification. From a breeding perspective, maternally inclined hybrids with superior agronomic traits may offer rapid utility, although their chromosomal constitution remains to be elucidated.

To date, no formal reports have addressed trait segregation in F_1 generations of peanut wide crosses, leaving a significant gap in understanding this phenomenon. Chromosomal elimination has been hypothesized as a potential underlying cause, with molecular markers providing preliminary supportive clues. To gain a more comprehensive understanding, cytogenetic

techniques such as chromosome counting and fluorescent *in situ* hybridization (FISH) will be essential, as they can provide direct and definitive insights into chromosomal behavior, chromosomal numerical changes and structural variations in hybrids. Investigating this further will be a critical focus of our future work, aiming to elucidate the mechanisms behind trait segregation and facilitate the effective utilization of wild species in peanut breeding programs.

Research on peanut stomata has been reported, with a focus on physiology, ploidy studies, drought tolerance, and resistance to foliar diseases. Li *et al*. (2014) found that calcium deficiency significantly reduced stomata density on the abaxial surface of upper peanut leaves, while it had no significant effect on the stomata density of lower leaves. Singst and Ozias-Akins (1992) detected a positive correlation between ploidy level and the number of chloroplasts in guard cells and pollen grain size when identifying *in vitro*-regenerated interspecific *Arachis* hybrids. Shen and Zhu (1985) noted discrepancies in foliar stomata density and size of peanut genotypes of different ploidy. Qiu *et al.* (1983) found that stomata density was higher in the upper epidermis of leaves than in the lower epidermis, in agreement with the findings of the present study. They concluded that drought-tolerant varieties had a lower density of stomata, and that the size of the stomata was not related to drought tolerance (Qiu *et al.* 1983). Jyosthna *et al.* (2004) observed that the late leaf spot-resistant cultivar FDRS-10 had lower foliar stomata density and smaller stomata size compared to the susceptible variety TMV-2. Further investigation is needed to determine if there is a relationship between stress resistance and stomatal characteristics in peanut intersectional hybrid derivatives.

CONCLUSIONS

This study conducted molecular marker analysis, stomatal observations, and key agronomic trait evaluations on the interspecific hybrid F_1 generation derived from the cultivated peanut variety Huayu 665 and the incompatible wild species *A. paraguariensis*. The results confirmed the authenticity of the hybrids and, for the first time, reported the phenomenon of trait segregation in the F_1 generation of distant peanut hybrids. Chromosomal elimination is proposed as a potential underlying cause. For hybrid identification in peanut remote crosses, the use of molecular markers distributed across different chromosomes of the wild species was proposed. F_1 hybrids with an upright growth habit and superior productivity hold the potential for rapid utilization in breeding programs.

AUTHOR CONTRIBUTION

CTW and HJS conceptualized the study. CJJ, HWW, CYX, GDY, JY carried out the experiments and analysis. CJJ, HWH, and CTW wrote the manuscript with input from all authors. All authors discussed the results and commented on the manuscript.

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Supplementary Table 1: **Analysis of variance of foliar stomatal features**

Source of variation	DF	No. of large leaf stomata			Stomata length			Stomata width			Stomata density		
		MS	*F*-statistic	*P*-value	MS	*F*-statistic	*P*-value	MS	*F*-statistic	*P*-value	MS	*F*-statistic	*P*-value
Genotype (G)	2	108.9321	11.407	0.0017	116.5841	32.625	0	4.8646	4.902	0.0278	188265.2097	17.3710	0.0003
Position (P)	1	0.0247	0.003	0.9603	29.7221	8.318	0.0137	0.5898	0.594	0.4557	393722.1556	36.3290	0.0001
G × P	2	10.4136	1.09	0.3672	9.6564	2.702	0.1074	0.5007	0.504	0.6161	143609.7216	13.2510	0.0009
Error	12	9.5494			3.5734			0.9925			10837.6355		
Total	17												

Note: DF=Degree of freedom, MS=Mean sum of squares. Position denotes upper (adaxial) and lower (abaxial) leaf surface.

Supplementary Table 2: **Main single plant agronomic characters of the intersectional hybrids, Huayu 665 ×*A. paraguariensis* PI 338297 and female parent, Huayu 665**

Identity	Main stem height (cm)	Length of cotyledonary branches (cm)	Stem thickness (cm)	Range of seed-set (cm)	Number of branches per plant	Number of effective branches per plant	Number of pods per plant	Number of well-filled pods per plant	Number of poorly-filled pods per plant	Number of one-seeded pods per plant	Number of two-seeded pods per plant	Angle between main stem and cotyledonary branch
Hybrid	37.00	65.40^{A}	0.44	13.10^{a}	10.20^{a}	10.40	10.40^{b}	10.40^{b}	0	6.00	4.40^{B}	51.68
Female parent	32.40	40.40^{B}	0.48	5.70^{b}	9.40^{b}	9.40	25.00^{a}	23.60^{a}	1.40	4.80	20.20^{A}	43.36

Note: The values within each column marked with different uppercase or lowercase letters were significant at 0.01 or 0.05 levels.