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Albinism does not correlate with biparental inheritance of plastid DNA in interspecific hybrids in *Cicer* species

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ABSTRACT

Cultivated chickpea (*Cicer arietinum*) was crossed with its wild relatives from the genus *Cicer* to transfer favorable genes from the wider gene pool into the cultivar. Post-hybridization barriers led to yellowing and subsequent senescence from as early as 5 days after fertilization, however, the ovules of hybrid embryos could be rescued *in vitro*. Hybrids were classified as green, partially green or albino. The hybrid status of regenerated plantlets *in vitro* was confirmed by amplification of nuclear DNA markers. To check whether chloroplast development correlated with plastid DNA inheritance in these crosses, primers were designed using conserved plastid gene sequences from wild and cultivated species. All three possible plastid inheritance patterns were observed: paternal, maternal and biparental. This is the first report of biparental inheritance of plastid DNA in *Cicer*. No correlation was observed between parental origin of the plastid genome and degree of albinism, indicating that chloroplast development in hybrid genotypes was mostly influenced by nuclear factors.

1. Introduction

The genus *Cicer* has 43 species, annual and perennial, of which chickpea (*Cicer arietinum* L.) is the only cultivated species. Increased genetic diversity is possible through hybridization of chickpea with its wild *Cicer* relatives. Eight wild *Cicer* species have the same annual growth habit and chromosome number ($2n = 16$) as domesticated chickpea and have been the primary focus of screening and introgression efforts. Only two of the eight species in the primary gene pool are readily hybridized with chickpea using conventional crossing techniques. Interspecific hybrids between Australian chickpea cultivars and all three *Cicer* species in the secondary gene pool (*C. pinnatifidum*, *C. judaicum* and *C. bijugum*) have been achieved [1]; however, complete to partial albino plants were observed following embryo rescue *in vitro*.

In interspecific hybrids, incompatibility between the plastome (plastid genome) and nuclear genome has long been recognized as a major factor inhibiting chloroplast development and chlorophyll or pigment formation [2–4]. Most of the hundreds of proteins in photosynthesis-related complexes, plastid ribosomes and plastid DNA metabolism are encoded by the nuclear genome [5], but key proteins are encoded in the plastome. In chickpea, the plastid genome has been sequenced, revealing 125,319 base pairs containing 108 genes, coding for 4 rRNAs, 29 tRNAs and 75 proteins [6]. This has opened the way for future molecular genetic studies on plastid development and plastome inheritance in this species. Differences in plastome sequences can provide the molecular basis for incompatibility leading to albinism [7], although there are many other opportunities for the complex process of plastid biogenesis in plants to go awry.

Understanding plastid inheritance might therefore provide important clues in overcoming crossability barriers and albinism faced by chickpea breeders using wide crosses. It is no longer thought that plastid DNA is always transmitted only from the female parent in angiosperms [8–10] and plastid DNA from either or both parents has been found in a number of albino hybrids in horticultural species [11–13]. The proportion of progeny containing biparental plastid DNA varied from species to species in these studies. Similar inheritance studies in legume crosses are rare, although one report in pea describes biparental plastid DNA inheritance in a

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Table 1
A summary of the *Cicer* interspecific hybrids and their parents used in this study.

Female parent	Male parent	Cross designation	Crosses	Embryos rescued
Sonali	*C. pinnatifidum* (ILWC33)	CP114[a]	105	31
Rupali	*C. judaicum* (ILWC46)	CP227	580	46
Rupali	*C. pinnatifidum* (ILWC29)	CP192; CP214	312	105
Kimberley Large	*C. pinnatifidum* (ILWC29)	CP210; CP213; CP190; CP197	452	91
Kimberley Large	*C. pinnatifidum* (ILWC248)	CP163	150	35

[a] CP is designation for chickpea.

hybrid associated with nuclear–cytoplasmic incompatibility [14]. Unlike most angiosperm groups, transmission of paternal plastid DNA is thought to be relatively frequent in at least some legume species, including *Lens* [15], *Vicia* [16] and *Medicago* [17]. Occasional paternal and biparental inheritance of organellar genomes complicates the genetic analysis of hybrids and makes it more difficult to solve potential problems due to nucleo-cytoplasmic incompatibility by controlling the direction of the cross. If nucleo-cytoplasmic incompatibilities are due to negative physical interactions between specific nuclear and cytoplasmic factors, then these can be avoided by performing the cross in a particular direction; this approach will not work if both parents are contributing the relevant cytoplasmic genomes. For these reasons, it is important to understand plastid inheritance patterns, particularly when chloroplast development is obviously affected in hybrid seedlings. There are no reports to date on plastid inheritance in chickpea. The research described in this paper was aimed at investigating the inheritance of plastid DNA in wide *Cicer* crosses and any potential correlation with chloroplast development in green and albino hybrids.

2. Materials and methods

2.1. Germplasm used for wide hybridization

Chickpea and its wild relatives were grown under controlled conditions in a glasshouse (20 °C day/15 °C night). Chickpea cultivars, accessions of wild *Cicer* and their progenies are detailed in Table 1. Kimberley Large (KL) was commercially released in 2004 in Australia as a premium quality large seeded kabuli type chickpea. Rupali (R) and Sonali (S) were commercially released in the same year as chilling tolerant desi type cultivars adapted to winter cropping where early podding at low temperatures provides an advantage in regions likely to experience a short season with terminal drought. These three cultivars were selected as desirable parents in the interspecific breeding program in Western Australia. Floral buds of chickpea were hybridized early in the morning before anthesis, emasculated, and the stigma was hand pollinated with fresh pollen from the wild species. Immature pods, 14–22 days after cross-pollination, were removed from mother plants before they aborted. Embryos were rescued *in ovulo* and hybrid plantlets were regenerated according to the procedures described by [1]. Tissue cultures were maintained in the growth room with 16 h day/8 h night photoperiod at 25 °C constant temperature.

2.2. DNA isolation and PCR amplification

Total genomic DNA was extracted from 50 mg of fresh *in vitro* leaves frozen and ground in liquid nitrogen (Retsch MM301 Mixer Mill) following a rapid and high-throughput method of Edwards et al. [18]. Three replicate samples were prepared for each genotype. A nuclear sequence (external transcribed spacer (ETS) associated with 18S rRNA) and a chloroplast gene (*matK*) were chosen as genetic markers. Multiple sequence alignments performed using CLUSTAL W (1.81) revealed regions in these sequences that are polymorphic between *Cicer arietinum* and its wild relatives (Table 2). As shown in Fig. 1,species-specific primers were designed within these polymorphic regions in order to amplify these genetic markers (Fig. 1, Table 2). 18S rRNA ETS and *matK* regions from nuclear and plastid genomes, respectively, were amplified by PCR. PCR amplifications were carried out in a total volume of 20 μl containing 50 ng of template DNA, 0.1 μM of each primer, 0.5 μM of each dNTP, in 1× reaction buffer and 0.5 unit of *Taq* DNA polymerase. Amplification was carried out using Eppendorf Mastercycle ep gradient S with initial denaturation at 94 °C for 2 min and 35 cycles at 94 °C for 30 s, primer annealing (58–62 °C) for 30 s and primer extension at 72 °C for 2 min and a final amplification step at 72 °C for 5 min. PCR amplifications were performed on each of the three replicate samples for each genotype. A blank as a negative control was also run in parallel. As the polymorphic regions were discrete, the primer annealing temperatures were determined for each primer pair to discriminate between genotypes. DNA was separated by electrophoresis in 1% agarose gel at 120 V, stained in 0.5% ethidium bromide for 30 min and viewed on a gel documentation system (BioRad).

3. Results

3.1. Hybridization and confirmation of hybrid status

Hybridization efficiency ranged from 67% to 94% in the four different chickpea × *C. pinnatifidum* cross combinations. Hybridization efficiency was significantly lower at 30% in the *C. judaicum*

Table 2
List of gene-specific primers used for the plastid inheritance studies.

Species	Primer length	Primer sequence	Tm (°C)
ETS & 18S RNA gene partial sequence (nuclear gene)			
Cicer arietinum	A/F (19b)	5′GCTTTTGCACTGAACGGTT3′	62.8
	A/R (19b)	5′CACCTCCCCAGACATACGA3′	63.6
Cicer bijugum, *C. pinnatifidum*, *C. judaicum*	B/F (19b)	5′GCTTTTGCATCGAACGGTC3′	65.6
(identical at primer picking spots)	B/R (23b)	5′GGCACCTCCCTAGACATACATAC3′	62.4
matK (maturase) gene complete sequence (plastid gene)			
Cicer arietinum	A/F (22b)	5′ATCCCATTTTTCCTACAATCTG3′	60.6
	A/R (24b)	5′GAATATCCAAATACCAAATACGAT3′	58.6
Cicer bijugum, *C. pinnatifidum*, *C. judaicum*	B/F (25b)	5′GAAATTCCATTTTTCCTACAATTTA3′	60.0
(identical at primer picking spots)	B/R (22b)	5′GAATATCCAAATACCAAATCCG3′	60.8