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Molecular, functional and ultrastructural characterisation of plastids from six species of the parasitic flowering plant genus *Cuscuta*

T. A. W. van der Kooij, K. Krause, I. Dörr, K. Krupinska

Botanisches Institut, Universität Kiel, Olshausenstraße 40, D-24098 Kiel, Germany

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Abstract. Plastids of *Cuscuta reflexa* Roxb., *C. subinclusa* D. et H., *C. gronovii* Willd. and *C. campestris* Yunck. possess thylakoids and contain both chlorophyll *a* and *b* in a ratio similar to that of stem tissue of the systematically closely related but 'normal' green *Ipomoea tricolor*. In contrast, plastids of *C. odorata* R. et P. and *C. grandiflora* H.B.K. do not contain any chlorophyll or possess thylakoids. Light-driven electron transport, as measured by oxygen evolution and indicated by analysis of chlorophyll fluorescence, was present in all chlorophyll-containing species. The photosystem II efficiency was low and ranged from 0.511 to 0.687. The plastid *rbcL* gene could not be detected in *C. odorata*, but was present in all other tested species. Neither *rbcL* transcripts nor the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) could be detected in *C. odorata* and *C. grandiflora*. Low amounts of the large subunit of Rubisco were detected immunologically in all other *Cuscuta* species. Apparently, the genus *Cuscuta* comprises species with different degrees of plastid functionality, ranging from intact chloroplasts, via plastids with impaired protein production and gene expression to plastids with reduced plastome gene content.

Key words: *Cuscuta* – Photosynthesis – Plastid – Plastome – Thylakoid

Introduction

In the plant kingdom, there are several examples of plant evolution in the direction of parasitism, resulting in a partial loss of the unique autotrophic properties of plants and the dependence on a host for survival. This kind of plant evolution led in some cases to a loss of genes from the plastid genome, as described for *Epifagus virginiana* and *Orobanche hederae*, especially those genes related to the photosynthetic machinery (dePamphilis and Palmer 1990; Thalouarn et al. 1994).

Members of the large holoparasitic flowering plant genus *Cuscuta* have neither leaves, except for minute scale-like organs, nor roots and completely rely on their hosts to supply water, nutrients and carbohydrates (Dawson et al. 1994). However, many *Cuscuta* species do contain small amounts of chlorophyll, especially in the tips of the seedlings (Panda and Choudhury 1992; Dawson et al. 1994). Chlorophyll fluorescence, light-driven electron transport and incorporation of $^{14}CO_2$ have been reported for several *Cuscuta* species (Jandhanarao et al. 1984; Panda and Choudhury 1992; Dawson et al. 1994; Hibberd et al. 1998; Sherman et al. 1999). Further studies with *Cuscuta reflexa* revealed residual light- and CO_2-dependent photosynthetic activity and incorporation of ^{14}C-labelled carbonate into carbohydrates (Machado and Zetsche 1990). However, at the normal atmospheric CO_2 concentration the photosynthetic activity never exceeded the light compensation point (Hibberd et al. 1998; Sherman et al. 1999). The role of this residual photosynthetic activity is unclear, but might be important in view of the internal recycling of CO_2 produced during respiration in the light (Hibberd et al. 1998).

Sequence analysis of the plastome of *C. reflexa* revealed that many of the plastid genes of autotrophic higher plants are still present (Haberhausen et al. 1992). However, some genes are present in a slightly altered form, e.g. the *rbcL* gene (Haberhausen et al. 1992). Other plastid genes seem to have been lost in other *Cuscuta* species after the evolution towards a parasitic life, e.g. *ndh* genes (Haberhausen and Zetsche 1994). Sequence analysis of *Cuscuta* species other than *C. reflexa* are rather limited. Machado and Zetsche (1990) reported the presence of *rbcL*, *rbcS* and *psbA* in *C. europaea* although it seems to contain no chlorophyll and is hence unable to perform photosynthesis.

Abbreviations and symbols: LSU = large subunit of Rubisco; PFR = photon fluence rate; ΦPSII = efficiency of PSII; qN = non-photochemical quenching; qP = photochemical quenching

Correspondence to: K. Krupinska;
E-mail: kkrupinska@bot.uni-kiel.de; Fax: +49-431-8804238

The chlorophyll content of *Cuscuta* species is influenced by external factors such as nutrient supply, light intensities and the host plant species (Panda and Choudhury 1992). Some *Cuscuta* species have been reported to contain no chlorophylls at all (Machado and Zetsche 1990). Ultrastructural analysis of plastids from *C. reflexa* revealed that thylakoid structures are present, although in a reduced form in comparison to stem material of the normal green plant species *Ipomoea purpurea*, which is a member of the closely related family of the *Convolvulaceae*. The plastids of the chlorophyll-less species *C. europaea* possess no thylakoid-like structures (Machado and Zetsche 1990).

Apparently, the different species of the genus *Cuscuta* present a range from low to no photosynthetic capacity. In order to gain a deeper insight into evolutionary plastome reduction and residual chloroplast functioning of *Cuscuta*, the pigment content, photosynthetic capacity and plastid ultrastructure of *C. reflexa*, *C. subinclusa*, *C. gronovii*, *C. campestris*, *C. grandiflora*, and *C. odorata* were examined. In addition, the presence of the plastidic *rbcL* gene and the occurrence of its product, the large subunit of Rubisco (LSU), were investigated.

Materials and methods

Plant material. All *Cuscuta* species used in this study were maintained on *Pelargonium zonale* in a greenhouse at the University of Kiel (Germany). Plants were exposed to day and night temperatures of 22 and 18 °C, respectively. The photoperiod was 14 h at a minimal light supply of 130 μmol photons m^{-2} s^{-1} (within the 400 to 700 nm range). *Ipomoea tricolor* Car. was grown in the botanical garden of the Botanical Institute of Cologne (Germany).

Pigment content, chlorophyll fluorescence properties and oxygen evolution. Stem tips of 5 cm length were ground in liquid nitrogen. Chlorophylls and carotenoids were extracted in 90% ethanol and the contents were determined photometrically according to Lichtenthaler (1987). Stem tips were detached and dark-adapted for 20 min on moist cotton cloth. Chlorophyll fluorescence measurements were performed at room temperature using a pulse amplitude fluorometer (PAM-101; Walz, Effeltrich, Germany). Irradiance was provided by a single halogen light source (KL1500T lamp unit; Schott, Mainz, Germany) and the photon fluence rate (PFR) was increased stepwise. Saturated pulses (3500 μmol m^{-2} s^{-1}, 1 s) were applied every 100 s. Photochemical quenching (qP), non-photochemical quenching (qN) and the efficiency of PSII [ΦPSII; in the dark-adapted state (the ratio of variable to maximal fluorescence; Fv/Fm) and during actinic light] were calculated according to Genty et al. (1989).

Stem tips (approx. five to seven stem tips of 5 cm each, equivalent to 150–300 mg FW) were detached and transferred to a Clark-type oxygen electrode (Bachofer, Reutlingen, Germany). Light response curves were made by increasing the PFR stepwise by applying neutral grey filters (Schott) with different transmission properties. Irradiance was increased once oxygen evolution/consumption remained constant for 10 min. Dark respiration was measured before and after illumination, in order to check for changes during the measuring procedure. The temperature inside the cuvette was kept constant at 20 °C.

Isolation and analysis of DNA. Plant stem material was ground in liquid nitrogen and resuspended in extraction buffer [4 M guanidine thiocyanate, 2% (w/v) laurosylsarcosinate, 25 mM Tris-HCl (pH 7.5), 10 mM EDTA and 100 mM *β*-mercaptoethanol]. After the removal of crude cell material by centrifugation at 17 500*g* and 4 °C for 3 min, the supernatant was extracted twice in 0.5 vol. phenol/chloroform/isoamylalcohol (25:24:1, by vol.), and once with 0.25 vol. chloroform/isoamylalcohol (24:1, v/v). The DNA was precipitated with 0.1 vol. of 3 M Na-acetate (pH 4.8) and 1 vol. of cold (4 °C) isopropanol followed by centrifugation (17 500*g*, 4 °C, 15 min). After air-drying, pellets were resuspended in 300 μl of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE-buffer) and incubated for 30 min at 37 °C with RNase A (Boehringer, Mannheim, Germany). The DNA was purified by subsequent phenol/chloroform extraction and ethanol precipitation (as described above). Finally, the DNA was resuspended in a small volume of TE buffer (pH 8.0).

Total DNA was digested with *Eco*RI and *Xho*I (Boehringer) and restriction fragments were separated on a 1% (w/v) agarose gel. The DNA fragments were transferred onto a Hybond-N+ membrane (Amersham) and hybridised according to the manufacturer's protocol. The *rbcL* probe of *Hordeum vulgare* was radiolabelled with ^{32}P using a random primed DNA labelling kit (Boehringer).

Isolation of RNA and Northern blotting. The RNA was isolated using TRIzol (Gibco BRL) according to Krause et al. (1998). Total RNA (15 μg) was separated on a 1.2% agarose/6.7% formaldehyde gel and transferred to Hybond-N+ (Amersham) by capillary blotting. Transcripts were detected by hybridisation with a radiolabelled ^{32}P probe specific for the *rbcL* gene of *H. vulgare*.

Protein extraction and Western blotting. Stem tips were ground in liquid nitrogen. Total proteins were extracted in buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS and 5% (v/v) *β*-mercaptoethanol]. Proteins were separated by SDS-PAGE and blotted onto polyvinylidenedifluoride (PVDF) membrane (Roti-PVDF; Roth, Karlsruhe, Germany) according to Humbeck et al. (1996). Membranes were incubated with an antiserum specific for LSU from *Pisum sativum*. Anti-rabbit IgG peroxidase conjugate was used as the secondary antibody and antibody-protein conjugates were visualised by enhanced chemoluminescence.

Electron microscopy. Stem segments of 2–3 mm length, taken approx. 10 cm from the tip, were fixed and embedded according to Dörr (1997). In the case of *Cuscuta* species with thick stems, e.g. *C. reflexa*, stems were split into half for better fixation. The material was fixed in 5% (v/v) glutaraldehyde/4% (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 0 °C and postfixed overnight at 0 °C in 2% (w/v) osmium tetroxide in sodium cacodylate buffer. The material was prestained in aqueous [0.5% (w/v)] uranyl acetate and finally embedded in Spurr's epoxy resin. Ultra-thin longitudinal sections of the cortex were post-stained in aqueous uranyl acetate and subsequently incubated in concentrated lead citrate. A transmission electron microscope (CM10; Philips, Eindhoven, The Netherlands) was used to investigate the plastid ultrastructure.

Results

Chlorophylls *a* and *b* were detectable in young stem tissue in four out of six tested *Cuscuta* species (Table 1). The total chlorophyll contents were much lower in comparison to stem tissue of the normal green plant species *Ipomoea tricolor* whereas the chlorophyll *a* to *b* ratios were comparable to that of *I. tricolor* (Table 1). All *Cuscuta* species, including the chlorophyll-less species, contained high amounts of carotenoids (Table 1). Especially, high amounts of carotenoids were measured in *C. gronovii* and *C. campestris*.

Table 1. The chlorophyll and carotenoid contents of *Ipomoea tricolor* and six different *Cuscuta* species. Pigment content was determined using 5-cm-long stem ends (in the case of *I. tricolor* with the leaves removed). Data represent the mean of four measurements (±SD)

Species	Chlorophylls [μg (g FW)$^{-1}$]				Carotenoids [μg (g FW)$^{-1}$]
	a	*b*	Total	*a*/*b*	Total
Ipomoea tricolor	245 ± 20	78 ± 4	323 ± 22	3.1 ± 0.2	91 ± 14
Cuscuta reflexa	88 ± 18	24 ± 6	112 ± 24	3.7 ± 0.1	42 ± 7
C. subinclusa	41 ± 7	11 ± 5	52 ± 11	3.9 ± 1.0	41 ± 8
C. gronovii	49 ± 0.2	9 ± 1	58 ± 1	5.5 ± 0.3	115 ± 8
C. campestris	51 ± 7	12 ± 3	63 ± 9	4.3 ± 0.4	177 ± 19
C. odorata	nd	nd	nd	–	28 ± 5
C. grandiflora	nd	nd	nd	–	48 ± 13

nd = not detectable

All chlorophyll-containing *Cuscuta* species showed thylakoids in plastids of the stem cortex, as revealed by electron-microscopic studies (Fig. 1). Grana formation could clearly be observed in the cortex plastids of *C. reflexa* and *C. subinclusa*. Grana formation was less developed in plastids of *C. gronovii* and *C. campestris*. The plastids in the stem cortex of *C. grandiflora* and *C. odorata* had an amoeboid proplastid like shape, contained no visible thylakoid structures but were filled with irregular tubular or vesicular structures.

All the chlorophyll-containing *Cuscuta* species exhibited more or less normal chlorophyll fluorescence kinetics, with decreasing qP and increasing qN at increasing irradiance. The maximal ΦPSII ranged from 0.501 for *C. campestris* to 0.687 for *C. reflexa* and was therefore lower than the maximal ΦPSII of 0.711 of stem tissue of *I. tricolor* (Table 2). The levels of qP and qN differed between the different *Cuscuta* species at the same PFR (120 μmol m^{-2} s^{-1}), indicating differences in the light-driven electron transport rates and formation of a transmembrane pH gradient (Table 2). The value for ΦPSII decreased with increasing irradiance in a similar way as in *I. tricolor* (Fig. 2A). The theoretical light-driven electron transport rate, as calculated by multiplying ΦPSII by the corresponding PFR, reached its maximum at the rather low PFR of 200 μmol m^{-2} s^{-1} in most *Cuscuta* species (Fig. 2B). An exception was *C. reflexa*, which exhibited a light response curve almost identical to that of stem material from *I. tricolor*.

Light-dependent oxygen evolution was observed for *C. reflexa*, *C. subinclusa*, *C. gronovii* and *C. campestris* (Fig. 3). For all the *Cuscuta* species the rate of oxygen evolution was lower than that observed for stem material of *I. tricolor*. Oxygen evolution of none of the *Cuscuta* species exceeded the light compensation point, whereas that of *I. tricolor* stem material did at PFRs of 300–400 μmol m^{-2} s^{-1}. Oxygen evolution was maximal at the rather low irradiance of 200 μmol m^{-2} s^{-1}, except for *C. reflexa* reaching maximal rates at higher PFRs (Fig. 3).

The total DNA used for a genomic Southern hybridisation with the *rbcL* gene was normalised to equal amounts of plastid DNA. This was done by using the 16S rDNA, which is present in all species, as a probe (data not shown). The plastidic *rbcL* gene was detected in all *Cuscuta* species except *C. odorata* (Fig. 4). The heterologous *rbcL* probe of *Hordeum vulgare* hybridised with restriction fragments of equal length for *C. grandiflora*, *C. campestris* and *C. gronovii*, which indicates extensive sequence homology between these species. In contrast, the size of restriction fragments in *C. reflexa* and *C. subinclusa* differed between each other and with respect to the other species (Fig. 4).

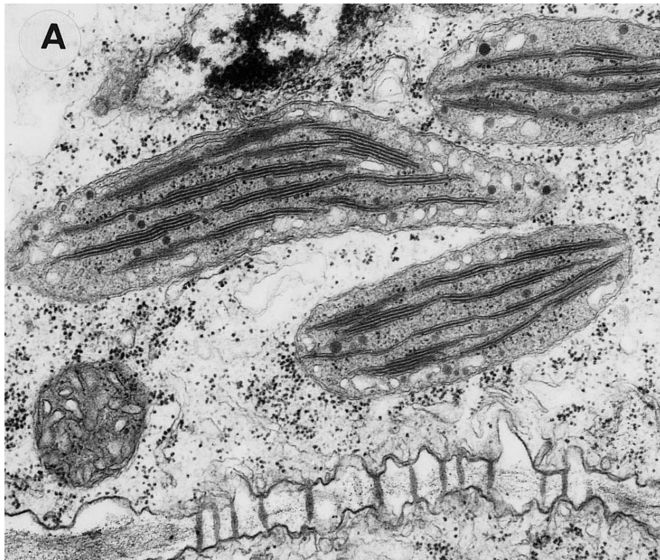
Northern analysis, with the same *rbcL* probe as used for the Southern analysis, revealed the presence of *rbcL* transcripts in most *Cuscuta* species (Fig. 4). In comparison to *I. tricolor*, the intensity of the hybridisation signal was much lower in the *Cuscuta* species. No *rbcL* transcripts could be detected for either *C. odorata* or *C. grandiflora* (Fig. 4).

Western blotting revealed that LSU, the product of the *rbcL* gene, is present in *C. reflexa*, *C. subinclusa*, *C. gronovii* and *C. campestris* (Fig. 4). In comparison to *I. tricolor* the LSU signal intensity of the *Cuscuta* species was much lower; LSU was not detected in protein extracts of *C. odorata* and *C. grandiflora* (Fig. 4).

Discussion

The presence of chlorophylls in the holoparasitic plant genus *Cuscuta* has been known for a long time (Abbott 1942). However, some species within this genus have no detectable amounts of chlorophyll, e.g. *C. europaea* (Machado and Zetsche 1990), *C. odorata* and *C. grandiflora* (this study). Chlorophyll contents of different *Cuscuta* species are highly influenced by many factors such as the host species, nutrient supply and the age of the tissue (Panda and Choudhury 1992). This might be an explanation for the large differences in chlorophyll content reported by others so far. Machado and Zetsche (1990) reported a very low chlorophyll content for *C. reflexa* with less than 2.5% of the chlorophyll contents of comparable tissue of *Ipomoea purpurea*, whereas in our studies the chlorophyll content of *C. reflexa* was 25% of that determined for *I. tricolor*. This difference might be due to using exclusively young tissue of *C. reflexa* in this study.

Chlorophyll *a* to *b* ratios of the *Cuscuta* species ranging between 3.7 and 5.5 do not indicate drastic alterations in light-harvesting complex composition in comparison to 'normal' green plants. The measured oxygen evolution clearly shows that the observed light-dependent ΦPSII and photon capture are indeed used in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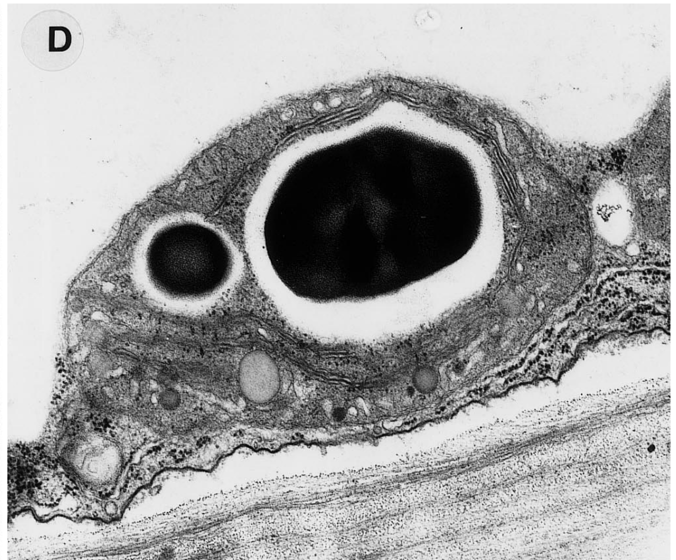
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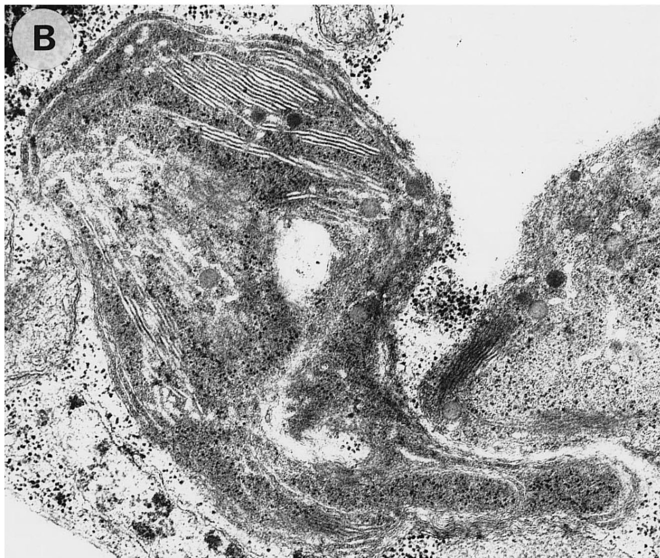
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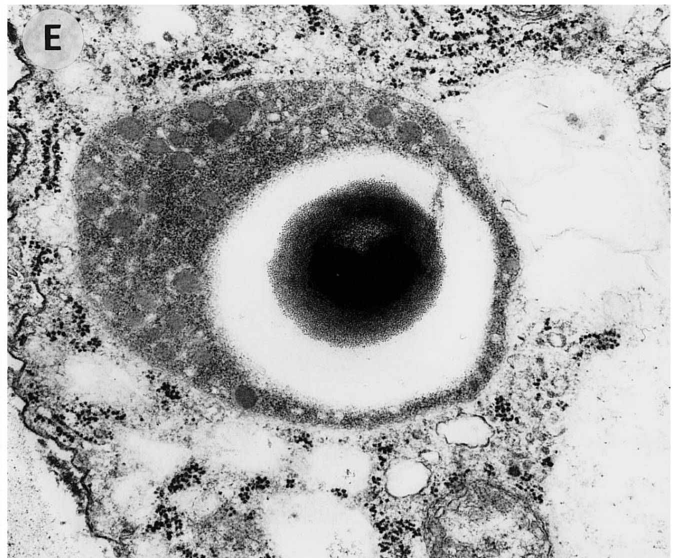
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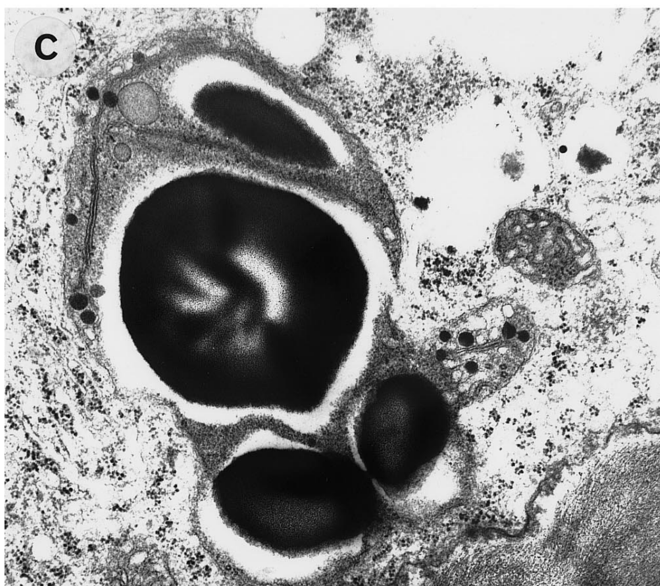
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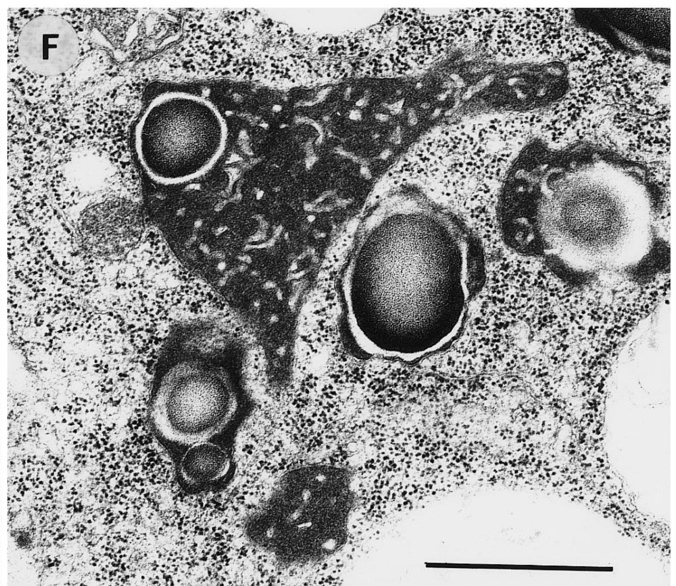
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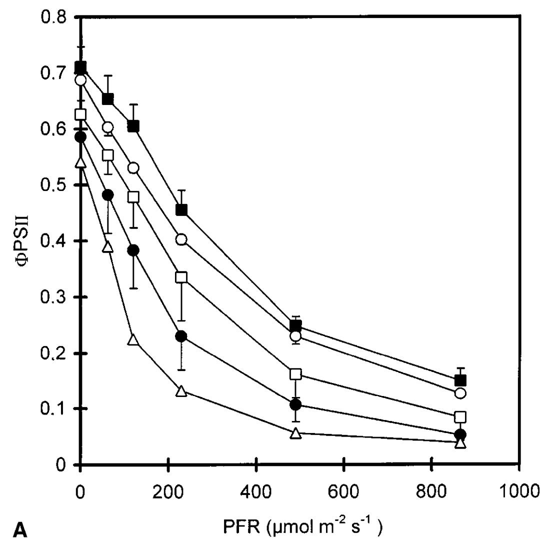
Fig. 1A–F. Ultrastructure of plastids in cortical cells of different *Cuscuta* species. *Cuscuta reflexa* (**A**) plastids with a shape typical of chloroplasts of higher plants, containing stroma and grana thylakoids. *Cuscuta subinclusa* (**B**), showing amoeboid-like plastids with thylakoids rarely arranged in grana as was observed for *C. gronovii* (**C**) and *C. campestris* (**D**), the latter two showing an even more reduced thylakoid system and plastids that are to a large extent filled with starch grains. *Cuscuta grandiflora* (**E**) and *C. odorata* (**F**) show plastids without typical thylakoid structures. The internal membranous structures appear tubular or vesicular. The plastids, in particular those of *C. odorata*, are extremely variable in shape and filled with starch grains. Bar = 1 μm

light-driven electron transport in the chlorophyll-containing *Cuscuta* species, as reported for *C. santapui*, *C. pentagona* and *C. reflexa* (Janardhanarao et al. 1984; Panda and Choudhury 1992; Hibberd et al. 1998; Sherman et al. 1999). The light dependence of qN illustrates the formation of a transthylakoid membrane proton gradient. These observations together suggest that the photosynthetic machinery in the chlorophyll-containing *Cuscuta* species is rather intact. However, both qP and qN differ among the various species, illustrating differences in the use of light-driven electron transport for the capture of CO_2.

The differences in photosynthetic performance and chlorophyll content among the various species correlate with the differences in complexity of the thylakoid structures, which are in fact lower and less developed in the more weakly performing species. The description by Machado and Zetsche (1990) of the plastids of *C. europaea* containing rather complex vesicular structures in the stroma nicely corresponds with our observations for both *C. odorata* and *C. grandiflora*. Apparently, a strong correlation exists between chlorophyll content and the thylakoid organisation among the different *Cuscuta* species, as was also observed within the species *C. pentagona* where greenish tissue and tissue that lacked any green appearance had chloroplasts with different thylakoid structures (Sherman et al. 1999).

Since all the chlorophyll-containing *Cuscuta* species also contain LSU, the capture of CO_2 in the light is possible with the use of the light-driven electron transport as has been reported recently for *C. reflexa* and *C. pentagona* (Hibberd et al. 1998; Sherman et al. 1999). For other species the presence of this residual photosynthetic activity had been under debate (Janardhanarao et al. 1984; Dawson et al. 1994). This might be due to the fact that, firstly, the actual photosynthetic activity is very low and hardly detectable, secondly, CO_2 exchange rates with the atmosphere are extremely low and, thirdly, the photosynthetic activity depends on the part of the plant that is studied. In this study the youngest tissue was used and all species were growing under the same conditions using the same host species, which is a prerequisite for reliable comparison of the different species. In general, chlorophyll content and levels of both *rbcL* transcripts and LSU protein were much lower in all tested chlorophyll-containing *Cuscuta* species in comparison to *I. tricolor* stem material, which explains



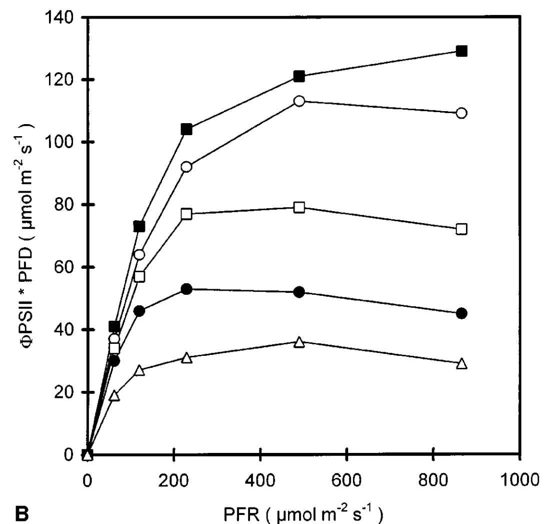


Fig. 2A,B. The PSII efficiency (ΦPSII, **A**) and the electron transport rate (**B**) of *Ipomoea tricolor* and different *Cuscuta* species at varying PFRs. The electron transport rate at different PFRs was calculated by multiplying ΦPSII by PFR. ■, *Ipomoea tricolor*; ○, *C. reflexa*; □, *C. subinclusa*; ●, *C. gronovii*; △, *C. campestris*. Data represent the mean of three measurements (± SD)

Table 2. Maximal PSII efficiency (Fv/Fm), qP and qN in stem material of *Ipomoea tricolor* and different *Cuscuta* species. Parameters qP and qN were determined at an actinic PFR of 120 μmol m^{-2} s^{-1}. Data represent the mean of three measurements (±SD)

Species	Fv/Fm	qP	qN
Ipomoea tricolor	0.711 ± 0.036	0.876 ± 0.049	0.400 ± 0.040
Cuscuta reflexa	0.687 ± 0.036	0.882 ± 0.022	0.312 ± 0.055
C. subinclusa	0.626 ± 0.026	0.820 ± 0.057	0.166 ± 0.059
C. gronovii	0.586 ± 0.055	0.727 ± 0.034	0.229 ± 0.062
C. campestris	0.541 ± 0.061	0.625 ± 0.070	0.379 ± 0.102

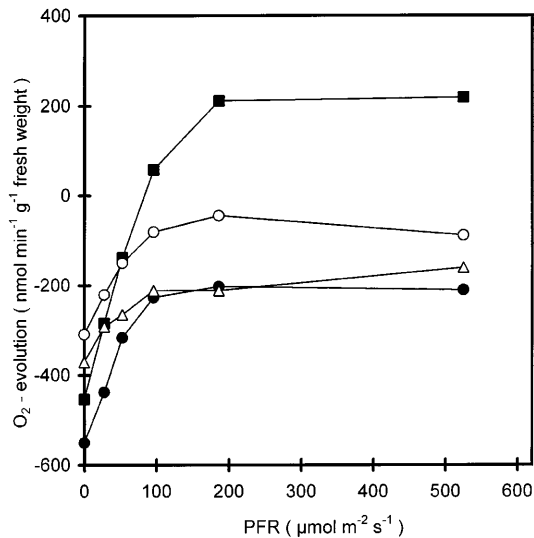


Fig. 3. The rate of oxygen evolution/consumption of stem material of *Ipomoea tricolor* and different *Cuscuta* species at different PFRs. Data represent single measurements including at least five stem ends. ■, *Ipomoea tricolor*; ○, *C. reflexa*; ●, *C. gronovii*; △, *C. campestris*

the low rates of photosynthesis and the light saturation of oxygen evolution at a rather low PFR of 200 µmol m^{-2} s^{-1}. On one hand, this low photosynthetic capacity may be expected for a holoparasitic plant genus with no need for photosynthesis at all. On the other hand, it is hard to understand the reason for maintaining such a high energetic cost and complex photosynthetic machinery considering that its carbon gain is far to low to allow its survival without a host.

The presence of the *rbcL* gene in *C. reflexa* and *C. europaea* has been reported previously (Machado and Zetsche 1990). Our results show that further *Cuscuta* species do possess this gene. An exception is *C. odorata*, which lacks an intact *rbcL* gene. Apparently, during evolution *C. odorata* has lost part of its plastome, including the *rbcL* gene, as reported for other parasitic flowering plant species (dePamphilis and Palmer 1990; Thalouarn et al. 1994).

In contrast to *C. odorata*, *C. grandiflora* possesses the *rbcL* gene while it lacks the corresponding transcript and the LSU protein. This situation is comparable to that observed for *C. europaea* (Machado and Zetsche 1990). The evolutionary degeneration of the capacity to perform photosynthesis in *C. grandiflora* and *C. europaea* is caused by impaired gene expression at the level of transcription. Whether this holds true for other plastid genes or is restricted to those involved in photosynthesis is still a question to be answered. The impairment of plastid gene expression correlates with a disturbed plastid ultrastructure and plastid differentiation in *C. odorata* and *C. grandiflora*.

In conclusion, the present results clearly show that within the holoparasitic plant genus *Cuscuta* a complete range of various stages of evolutionary degeneration of photosynthetic capacity exists: intact photosynthetic machinery with low photosynthetic activity in young tissue of *C. reflexa* and C. *subinclusa*, disturbances at the level of plastid ultrastructure in *C. gronovii* and *C. campestris*, disturbances at the level of plastome transcription in *C. grandiflora* and deletion of a plastid gene in *C. odorata*.

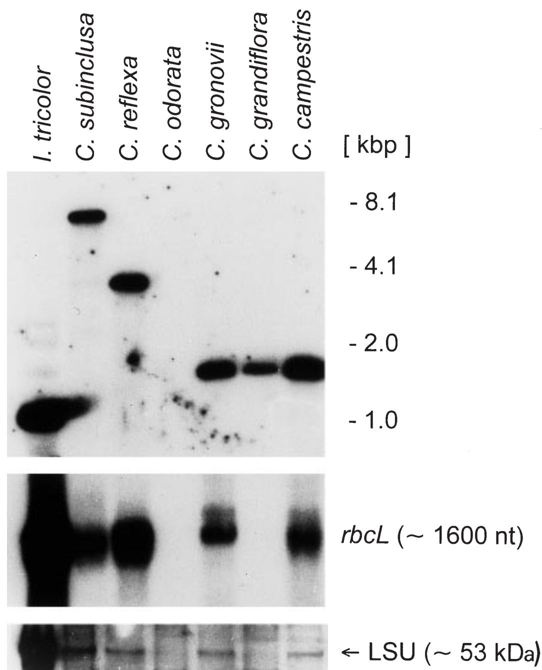


Fig. 4. Detection of the *rbcL* gene by Southern blot analysis (*upper panel*), the corresponding mRNA by Northern blot analysis (*middle panel*) and the protein product, LSU, by Western blot analysis (*lower panel*) in stem material of *I. tricolor* and different *Cuscuta* species. Total DNA was digested with *Xho*I and *Eco*RI. The *rbcL* probe of *Hordeum vulgare* served as a probe for Southern and Northern hybridisation. The large subunit of Rubisco (*LSU*) was detected immunologically with an antibody raised against LSU of *Pisum sativum*

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