Cuscuta europaea plastid apparatus in various developmental stages
Localization of THF1 protein

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Abbreviations: Chl, chlorophyll; FLP, flu-like protein; Fv/Fm, maximal quantum yield of PSII; GluTR, Glutamyl-tRNA-reductase; LHCl, light-harvesting complex; RbcL, large rubisco subunit; THF1, thylakoid formation protein 1; ΦPSII, effective photochemical quantum yield of PSII

Introduction

Parasitic plants have a specialized absorptive organ, called haustorium, which is used to penetrate through the host tissues and connect to xylem or phloem. Cuscuta (dodder) is the only parasitic genus found in the morning-glory family Convolvulaceae. The genus Cuscuta has been considered to be a holoparasitic angiosperm, obtaining both water and organic nutrients from its host plants. Some species like C. gronovii D. Willd., C. campestris H.B.K. do not contain chlorophyll, the enzyme Rubisco and developed thylakoids.1 Using ultrastructural studies, Sherman et al.2 showed no differences between the structure of C. pentagona thylakoids in seedlings germinated and grown in the dark and seedlings grown in the light. Plastids had poorly differentiated endomembrane system with prolamellar bodies, which are prominent structures of etioplasts. It has been shown that C. reflexa Roxb. possesses a number of photosynthesis-related genes with significant homology to those found in higher plants.3,4 Immunoblots revealed plastid proteins associated with PSI and PSII, as well as cytochrome f and plastocyanin.5 Up to date literature provides information that C. europaea is more specialized for the parasitic lifestyle than other Cuscuta species, with no detectable chlorophylls, no capacity for CO2 assimilation and no thylakoids in plastids.6 Large deletions of photosynthesis-related genes has been documented as well.7,8 Several nucleus- and plastid-encoded Arabidopsis genes that can ultimately affecting thylakoid formation or chloroplast development have been identified. One of many, the thf1 gene, was found in various plant species.9–11 Based on sequence similarity

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and different functions that were initially attributed to the protein, various names of the same protein do exist—THF1, ToxA-binding protein, or Psb29. Previously, it was suggested that THF1 protein is involved in thylakoid membrane differentiation, in responses to fungal and bacterial attacks and as a G-protein (GPA1) interacting partner in sugar (D-glucose) signaling pathway. Huang et al. provided evidence that the GPA1 (GTP-bound form of GPA1). Zhang et al. have postulated that the THF1 interacts with the constitutively active form of GPA1 (GTP-bound form of GPA1). Zhang et al. proposed a model for G protein-mediated chloroplast development via FtsH proteases, which play a critical role during chloroplast development in Arabidopsis. Immuno-fluorescence microscopy revealed that THF1 localizes to the stroma (soluble THF1) and to the membranes of plastids in developing C. europaea haustorium, most abundantly in the digitate cells of the endophyte primordium. Zhang et al. have postulated that the THF1-mediated regulation of chloroplast development may be achieved via G protein-dependent and/or -independent pathways. In the G protein-independent pathway, the THF1 is at least in part, localized to the outer membrane of plastid and to stromules, which appear to associate with the plasma membrane. After the binding to GPA1, this activated form can transmit signals from the plasma membrane to nuclei via second messengers and ultimately control the expression of the FtsH genes needed for chloroplast development.

Our results proved that photosynthesis is not a main process in the plastids in C. europaea haustorium. Localization of THF1 protein in plasma membrane and plasmodesmata leading us to pronounce assumption, that this protein has probably other functions, for example in sugar sensing within the dodder haustorial cells. Furthermore, result from western blot showing high levels of THF1 in dodder and tobacco tissues which are actively involved in the development of parasitic connection. To clarify the precise role of THF1 in host-parasite interactions could be argued and needs further investigations.

Results

Plant material cultivation. Seeds of Cuscuta started to germinate approximately 10 d after inoculation to the soil around the base of 8-week-old tobacco plants, but germination was very asynchronous. After 20 d of cultivation, dodder vines attacked their host plant. Cuscuta set up flowers approximately 90 d after the host plant-parasite connection establishment.

Pigment analysis. Early developmental stages and flowers of C. europaea accumulated chlorophylls. Seven-day-old seedlings show the highest level of these photosynthetic pigments (Fig. 1A, column 2). We detected only trace amounts of the Chls (a+b) in 30-d-old dodder stems compared with young seedlings and flowers. The decrease was statistically significant (Fig. 1A). Dodder flowers and 30-d-old vines accumulate high amounts of carotenoids (Fig. 1B, columns 1, 3). The lowest level of carotenoids was identified in young dodder seedlings (Fig. 1B, column 2). The high levels of Chls (a+b) and carotenoids were observed in tobacco leaves (Fig. 1A and B, column 4). Significantly lower content of Chls (a+b) and carotenoids were observed in tobacco stems (Fig. 1A and B, column 5).

Chlorophyll a fluorescence. Maximum quantum yield of PSII (F/ΦPSII), which is proportional to the quantum yield of O2 evolution, was relatively high and did not differ significantly between Cuscuta stem and flower. Effective photochemical quantum yield of PSII (ΦPSII), which measures the proportion of the light absorbed by chlorophylls associated with PSII, did not show any significant changes between Cuscuta stem and flower. However, the values of F/ΦPSII were lower in comparison to the host plant, as documented by the 2-D chlorophyll fluorescence imaging system (Fig. 2).

Plastid ultrastructure. Detailed ultrastructural study revealed differences in the plastid apparatus between young and older developmental stages of the dodder stem. In growing tips of the young dodder seedlings, chloroplasts with poorly differentiated
endomembrane system are shown (Fig. 3A). Chloro-amyloplasts in the cortical cells of young dodder stems contained both thylakoids and large starch grains (Fig. 3B). Thirty-day-old stems represented advanced ontogenesis stage and their cells contained only amyloplasts with enlarged and sometimes numerous starch grains (Fig. 3C and D).

Western blot analysis. The highest levels of THF1 protein were detected in Cuscuta stems attached to the host, Cuscuta haustorium and tobacco stems attacked by the parasite (Fig. 4A, columns 2, 4 and 5). In dodder seedlings and tobacco stem unattacked by Cuscuta we observed notable decrease in THF1 protein accumulation (Fig. 4A, columns 1 and 3). Presence of RbcL and D1 proteins were proved in dodder seedlings, tobacco leaves, tobacco stems and stems attacked by dodder, although the levels of these proteins were lower in dodder seedlings (Fig. 4A, columns 1, 3, 5 and 6). LHC complex polypeptides were observed in all examined samples, the highest accumulation was in tobacco tissues. FLP protein was detected only in tobacco. We found the accumulation of the GluTR in dodder young seedlings and stems attached to the host (Fig. 4A, columns 1 and 2), but it was not detected in haustorium (Fig. 4A, column 4).

Using western blot analysis we detected the THF1 protein accumulation in plastid membrane and stromal fractions isolated from chloroplasts of 7-d-old seedlings (Fig. 4B, columns 2 and 3). In plasma membrane fractions originated from dodder haustorium, we confirmed lower levels of THF1 protein (Fig. 4B, column 1).

THF1 protein in situ immulocalization. Microscopical analysis and THF1 protein immulocalization were performed on C. europaea stems attached to the host plant (N. benthamiana) at the stage of dodder haustorium penetration into the phloem area of the host plant (Fig. 5). Based on immunofluorescence microscopy results we found high accumulation of THF1 protein in endophytic tissue of the haustorium and in cortical cells of the parasite. Tissues of the host plant accumulated low levels of THF1 protein (Fig. 5B and C).

Subcellular localization by confocal microscopy revealed plastid-related but also plastid-unrelated (extra-plastidial) distribution of the THF1 protein. In host tobacco plants the protein was found mainly in the stroma of plastids in cells of the stem, however in parasite cells, in addition of plastid-localized protein, the plasma membrane expressed strong labeling (Fig. 6). In general, a higher accumulation of this protein was detected in dodder tissues. Chloro-amyloplasts with one starch grain in cortical cells of the host stem, as well as amyloplasts with 2 to 3 small starch grains in parasite cortex and haustorial cells were evenly distributed at the cell periphery (Fig. 6). Amyloplasts in digitate haustorial cells comprised 4 to 5 large starch grains which depressed THF1-positive stroma to margins and to the center of plastids (Fig. 6G–I). In cortex and haustorial cells of the C. europaea stems the THF1 protein was distributed in stroma and outer membrane of plastids and in the plasma membrane (Fig. 6G–I). Especially in digitate haustorial cells of the dodder, the protein was detected also in plasmodesmata (Fig. 6J–L).

Discussion

The current literature provides some information about in vitro and in vivo cultivation of parasitic plants. It is generally known, that before inoculation of dodder seeds on the cultivation medium or in the soil, it is necessary to treat dodder seeds with concentrated H2SO4. After this step, the germination is running...
Choisy with concentrated H\textsubscript{2}SO\textsubscript{4} for 45 min and after washing presence of chloroplasts in growth tips and chloro-amyloplasts in tron microscopy observations. Ultrastructural analysis has shown only in 30-d-old dodder stems. These facts are supported by elec-

rophylls and increase in carotenoids concentration were observed levels of chlorophyll \( \text{a} \) and \( \text{b} \) accumulate relatively high developmental stages of \( C. \) reflexa possesses a number of photosynthesis-related genes with significant homology to those found in higher plants. Machado and Zetsche\textsuperscript{5} reported a very low chlorophyll content for \( C. \) reflexa [112 ± 24 [μg (g FW)\textsuperscript{-1}]]. \( C. \) odorata and \( C. \) grandiflora had an amoeboid-like shape of plastid and did not contain visible thylakoid structures. Western blotting revealed that LSU, the product of \( rbcL \) gene, was not detected in protein extracts of \( C. \) odorata and \( C. \) grandiflora.\textsuperscript{3} It has been stated that \( C. \) europaea appears to be the best adapted to a parasitic lifestyle with characteristics of photosynthetic plants, while older plants have lost this ability and are not photosynthetic.

Development of thylakoid membranes depends upon the transport of membrane vesicles from the chloroplast inner envelope and subsequent fusion of vesicles within the interior of the plastid.\textsuperscript{31} Several nuclear- and plastid-encoded \( Arabidopsis \) genes have been identified as they can ultimately affect thylakoid for-

mation or chloroplast development. One of them, the \( \text{thf}1 \) gene, has been previously identified in various plant species. The \( Arabidopsis \) \( \text{thf}1 \) gene product controls specifically an important step required for leaf development, the normal organization of vesicles into mature thylakoid stacks. Disruption of the \( \text{thf}1 \) gene via T-DNA insertion results in variegated leaf patterns. Non-green sectors of variegated leaves lacking \( \text{thf}1 \) expression contain plastids that accumulate membrane vesicles on the interior, but lack organized thylakoid structures.\textsuperscript{12} It has been reported, that THF1 protein localizes to the outer plastid membrane, stroma, thylakoids and stromules.\textsuperscript{11,15,38,39} Our results have shown faster and more effectively. Lee\textsuperscript{26} scarified seeds of \( C. \) japonica Choisy with concentrated H\textsubscript{2}SO\textsubscript{4} for 45 min and after washing with distilled water, seeds were placed on moist paper. Seeds of \( C. \) europaea were not able to germinate after this procedure and thus, we shortened the time period for H\textsubscript{2}SO\textsubscript{4} treatment to 15 min, which yielded effective germination.

The genus \( Cuscuta \) is considered to be a holoparasitic angio-
sperm, obtaining both water and organic nutrients from its host plants. Previously, it has been shown that pre-parasitic seedlings of \( C. \) campestris, \( C. \) subinclusa and \( C. \) gronovii contain chlo-
rophylls, enzyme Rubisco and thylakoids, especially in their growth tips.\textsuperscript{1,2} According to Haberhausen and Zetsche,\textsuperscript{6} \( C. \) reflexa possesses a number of photosynthesis-related genes with significant homology to those found in higher plants. Machado and Zetsche\textsuperscript{6} reported a very low chlorophyll content for \( C. \) reflexa [112 ± 24 [μg (g FW)\textsuperscript{-1}]]. \( C. \) odorata and \( C. \) grandiflora had an amoeboid-like shape of plastid and did not contain visible thylakoid structures. Western blotting revealed that LSU, the product of \( rbcL \) gene, was not detected in protein extracts of \( C. \) odorata and \( C. \) grandiflora.\textsuperscript{3} It has been stated that \( C. \) europaea appears to be the best adapted to a parasitic lifestyle with a number of deletions in photosynthesis-related genes, without development of thylakoid membranes and with no detectable levels of chlorophylls.\textsuperscript{8-10,40} However, we found out that young developmental stages of \( C. \) europaea accumulate relatively high levels of chlorophyll \( a \) and \( b \). Significantly lower content of chlo-
rophylls and increase in carotenoids concentration were observed only in 30-d-old dodder stems. These facts are supported by electron microscopy observations. Ultrastructural analysis has shown presence of chloroplasts in growth tips and chloro-amyloplasts in cortical cells of young dodder stems, while no chloroplasts were present in cells of older dodder stems. At points of contact with the host, the coiled dodder stems produces haustoria that penetrate host tissues and form vascular connections.\textsuperscript{37} Haustorial cells contained only amyloplasts with 4 to 5 starch grains. Our results shown low photochemical activity of PSII (Fig. 2) as well as low content of examined photosynthesis-related proteins (Fig. 4A, column 4). GluTR, the key regulatory enzyme in biosynthe-
sis of 5-aminolevulinic acid, universal precursor of tetrapyrroles, is tightly controlled at transcriptional and posttranslational levels.\textsuperscript{29} In young dodder seedlings (7-d-old) we observed relatively high GluTR accumulation. These results are in correlation with measuring of Chls (\( a+b \)) concentrations. FLP protein, which is thought to bind to GluTR and represses its activity to prevent overproduction of Pchlide,\textsuperscript{29} was not detected in dodder seedlings, stems, haustoria. Photosystem I (PSI) is a membrane-bound mult-
isubunit protein complex located in the chloroplast thylakoids. It utilizes light energy to oxidize plastocyanin or cytochrome \( c \) and to reduce ferredoxin or flavodoxin. In higher plants, green and red algae, the outer light-harvesting system associated with PSI is made up of LHCa proteins that are encoded by the \( cab \) genes and known collectively as LHCl (light-harvesting com-
plex I). Some studies have explored the structural basis by which LHCl act as the outer light-harvesting system of PSI.\textsuperscript{29} LHCl polypeptide complex was observed in all examined \( Cuscuta \) and \( tobaccco \) samples, but the highest accumulation was detected in tobacco. Accumulation of D1 protein, which represents a reaction center protein of photosystem II (PSII), was observed in 7-d-
old dodder seedlings and in tobacco (Fig. 4A) and never in older dodder stems and in haustoria. Results of Zhang et al.\textsuperscript{14} strongly suggest that THF1 and G proteins are the new regulators for FtsH protease and THF1 probably functions as a regulator for FtsH expression. The FtsH, a well-characterized family of mem-
brane bound proteases, are required for damaged D1 protein degradation, which is a core protein of the PSII reaction center. The low activity of FtsH proteases leads to the accumulation of damaged D1 protein, and to the inhibition of photosynthesis. We can conclude that 7-d-old seedlings of \( C. \) europaea have some characteristics of photosynthetic plants, while older plants have lost this ability and are not photosynthetic.

![Figure 4. (A) Western blot analysis of the THF1, GluTR, FLP, D1, LHCl complex and RbcL in \( C. \) europaea: (1) 7-d-old seedlings, (2) 30-d-old \( Cuscuta \) stems attached to the host, (4) haustorium and in \( N. \) benthamiana, (3) stems, (5) stems attacked by \( Cuscuta \), and (6) leaves. Twenty-five μg proteins have been loaded. (B) Western blot analysis of the THF1 protein in \( C. \) europaea, (1) plasma membrane fraction isolated from dodder haustorium. Purity of fraction was tested using anti-LHI antibody, (2) plastid membrane fraction isolated from chloroplasts of 7-d-old dodder seedlings, (3) plastid soluble (stromal) fraction isolated from chloroplasts of 7-d-old dodder seedlings. Purity of fractions were tested using anti-D1 and anti-RbcL antibodies. Fifteen microgram proteins have been loaded.](image-url)
presence of THF1 protein in both, parasite and host plant tissues (Fig. 4A). Confocal microscopy revealed localization of THF1 protein to outer plastid membrane and stroma of haustorial cells (Fig. 6G–L), what agree with previous observations of other authors,11,13 but in addition to this, we observed localization of this protein also in plasma membrane and plasmodesmata (Fig. 6J–L). The western blot of haustorial plasma membrane fractions verified the presence of THF1 protein in the plasma membrane (Fig. 4B, column 1). Huang et al.15 predicted that THF1 protein plays a role as a G-protein (GPA1) interacting partner in sugar (D-glucose) signaling pathway. This interaction requires proximity of the plastid or its stromule with the plasma membrane. Stromules may also play a role in stress response.39 Wangdi et al.18 considered that THF1 have a role in COR signaling pathway in bacterial speck disease development.

In conclusion, localization of THF1 protein in plasma membrane and plasmodesmata leading us to pronounce assumption, that this protein has probably other functions, for example in sugar sensing within the dodder haustorial cells. Furthermore, result from western blot showing high levels of THF1 in dodder and tobacco tissues, which are actively involved in the development of parasitic connection. To clarify the precise role of THF1 in host-parasitic interactions could be argued and needs further investigations.

Materials and Methods

Seeds sterilization and growth conditions. In vivo cultivated stems of parasitic dodder (Cuscuta europaea) and tobacco (Nicotiana benthamiana) as a host plant were used in our study. Seeds of C. europaea originate from the locality Ivanka pri Dunaji (2009, Slovak Republic), the N. benthamiana seeds were obtained from Gene Bank in Gatersleben, Germany. Dodder seeds were scarified by soaking in concentrated H2SO4 for 15 min, washed with distilled water and planted in soil around the base of 8-week-old tobacco plants. The plant material in soil (tobacco plants with dodder) was cultivated in the growth chamber (16/8 h photoperiod) at 23 ± 2°C, 40 μmolm−2s−1 PAR.

Protein isolation and western blot analysis. Samples (100 mg) were ground in liquid nitrogen and suspended in protein extraction buffer [28 mM dithiothreitol, 175 mM sucrose, 28 mM Na2CO3, 10 mM EDTA, 5% (w/v) SDS, chemicals from Sigma-Aldrich] with antiprotease pill (Roche). After 30 min incubation at 70°C and 15 min centrifugation (12,100 × g), supernatant was used for determination of protein concentration using Bichinonic Acid Kit for Protein Determination (Sigma-Aldrich). Protein samples (25 μg) were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Millipore) using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). For protein immunodetection, specific primary antibodies were used. Antibodies against THF1, GluTR, FLP, D1, LHCI and RbcL were purchased from Agrisera. Secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (Bio-Rad) was used. Signal was revealed using chemiluminiscent kit Immobilon Western (Millipore).

Preparation of plasma membrane, plastid membrane and stromal fractions. The plasma membrane fraction from 6 g of dodder haustorium (4 mo-long picking) was enriched by partitioning microsomes in an aqueous dextran/polyethylene glycol two-phase system. The purified plasma membrane fraction was treated with 40 mM CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] for 1 h at 4°C and centrifuged at 50,000 g for 30 min at 4°C.32 Purity of plasma membrane fraction was tested using anti-LHC antibody (Agrisera). Plastids were isolated and fractionated as described previously by Inaba et al.19 Essentially, chloroplasts were isolated from 10 g of 7-d-old seedlings (2 mo-long picking). Isolated intact chloroplasts were separated into membrane and soluble fractions by lysis in 0.1 M Na2CO3, pH 11.5, followed by centrifugation at 200,000 g for 20 min. The pellets (membrane fraction) were directly dissolved in SDS-PAGE sample buffer. The soluble proteins were recovered by precipitation with trichloroacetic acid and dissolved into SDS-PAGE sample buffer. Purity of membrane fraction was tested using anti-RbcL antibody and purity of soluble (stroma) fraction was tested using anti-D1 antibody (Agrisera). Samples (15 μg) were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.
Figure 6. For figure legend, see opposite page.
transferred to nitrocellulose membrane using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). THF1 protein was detected by western blot analysis using rabbit polyclonal anti-THF1 antibody (Agrisera). Secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (Bio-Rad) was used. Signal was revealed using chemiluminiscent kit Immobilon Western (Millipore). Protein concentrations were determined according to Bichinonic Acid Kit for Protein Determination (Sigma-Aldrich).

**Pigment analysis.** Chlorophyll (a+b) and carotenoids were extracted with 80% (v/v) chilled acetone and spectrophotometrically (Jenway 6400) quantified: Chl a at 663.2 nm, Chl b at 646.8 nm, carotenoids at 470 nm. Concentration was calculated according to Lichtenthaler.34

**Measurements of chlorophyll a fluorescence.** Prior to measurements, the parasitic plants with their hosts were dark-adapted for 30 min. Chlorophyll a fluorescence was measured by fluorocam FC1000-LC (Photon Systems Instruments). Minimal fluorescence ($F_0$) and maximal fluorescence ($F_{m}$) were measured using a saturation pulse (4000 μmolm$^{-2}$s$^{-1}$ PAR, 800- ms duration, $\lambda = 620$ nm) and maximal quantum yield of PSII ($F_m/F_0$) was calculated as ($F_m - F_0$)/$F_m$. Then an actinic light of 100 μmolm$^{-2}$s$^{-1}$ PAR ($\lambda = 620$ nm) was switched on for induction of photosynthesis and steady-state fluorescence was measured ($F_s$). Saturation pulses were applied in 60 sec intervals, for estimation of maximum chlorophyll fluorescence in the light-adapted state ($F_{m}'$). Effective photochemical quantum yields of photosystem II ($F_{psii}'$) were calculated according to Roháček35 and to Maxwell and Johnson36 as ($F_{m} - F_s$)/$F_{m}'$.35,36 Then an actinic light of 100 μmolm$^{-2}$s$^{-1}$ PAR ($\lambda = 620$ nm) was switched on for induction of photosynthesis and steady-state fluorescence was measured ($F_{s}'$).

**THF1 protein in situ immunolocalization.** Steedman’s embedding for THF1 visualization in dodder plants attached to host was performed according to Vitha et al.37 with some modifications: sections (10 μm-thick) were incubated for 1 h at room temperature in the primary antibody (1:200 dilution in PBS buffer: 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.3, chemicals from Sigma-Aldrich), washed 2 x 10 min in stabilizing buffer (SB buffer: 50 mM PIPES, 5 mM KH$_2$PO$_4$, 1.5 mM Na$_2$HPO$_4$, 5 mM MgSO$_4$, 5 mM EGTA, pH 6.9, chemicals from Sigma-Aldrich) and incubated overnight at RT, in the dark in secondary antibody (1:100 dilution in PBS). Antibody against THF1 protein was purchased from Agrisera. Secondary antibody Goat Anti-Rabbit IgG (whole molecule) F(ab')fragment-FITC was purchased from Sigma-Aldrich. Sections were examined under the fluorescence microscope (Axioskop 2 plus, Zeiss), equipped with 485/20 nm exciter filter, 510 nm beamsplitter and 515 nm LP barrier filter (filter Zeiss set 16, 25) and using confocal microscope (CLSM Fluoview FV1000, Olympus) with the excitation laser line 488 nm and BA505-550 barrier emission filter.

**Electron microscopy.** Samples were fixed in 5% (v/v) glutaraldehyde in 0.06% (w/v) cacodylate buffer (pH 6.8) for 4 h, washed with 0.06% (w/v) cacodylate buffer (pH 6.8) 6 x 10 min and postfixed in 1% (v/v) osmium tetroxide in the same buffer overnight. Fixed specimens were dehydrated in ethanol and propyleneoxide series and embedded in Spurr medium (BIOTECH). Ultrathin sections were cut on ultramicrotome Reichert-Jung Ultracut E and observed with JEOL 2000 FX electron microscope (Jeol Ltd.).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

1. Panda MM, Choudhury NK. Effect of irradiance and nutrients on chlorophyll and carotenoid content and Hill reaction activity in Cuscuta reflexa. Photosynthesica 1992; 26:585-92.

2. Dawson JH, Muselman LJ, Wolowinlak P, Dörö I. Biology and control of Cuscuta. Rev Weed Sci 1994; 6:265-317.

3. van der Kooi JAW, Krause K, Dörö I, Krupinska K. Molecular, functional and ultrastructural characterization of plastids from six species of the parasitic flowering plant genus Cuscuta. Planta 2000; 210:701-7; PMID:10805440; http://dx.doi.org/10.1007/s000250506707.

4. Lee KB, Park JB, Lee S. Morphology and anatomy of mature embryos and seedlings in parasitic angiosperm Cuscuta japonica. J Plant Biol 2005; 43:22-7; http://dx.doi.org/10.1007/BF03031032.

5. Sherman TD, Pettigrew WT, Vaughan KC. Structural and immunological characterization of the Cuscuta pentagona L. chloroplast. Plant Cell Physiol 1999; 40:592-603; http://dx.doi.org/10.1093/oxfordjournals.pcp.a029582.

6. Haberhausen G, Zerse K. Functional loss of all ndh genes in an otherwise relatively unaltered plastid genome of the holoparasitic flowering plant Cuscuta reflexa. Plant Mol Biol 1994; 24:217-22; PMID:8111019; http://dx.doi.org/10.1007/BF00040588.

7. Funk HT, Berg S, Krupinska K, Maier UG, Krause K. Complete DNA sequences of the plastid genomes of two parasitic flowering plant species, Cuscuta reflexa and Cuscuta gemmifera. BMC Plant Biol 2007; 7:45; http://www.biomedcentral.com/1471-2229/7/45; PMID:17714582; http://dx.doi.org/10.1186/1471-2229-7-45.

8. Machado MA, Zerse K. A structural, functional and molecular analysis of plastids of the holoparasites Cuscuta reflexa and Cuscuta europaea. Plant 1999; 181:91-6; http://dx.doi.org/10.1016/S0022-2836(99)00347-0.

9. Freyer R, Neckermann K, Maier RM, Kössel H. Structural and functional analysis of plastid genomes from parasitic plants: loss of an intron within the genus Cuscuta. Curr Genet 1995; 27:580-6; PMID:7539345; http://dx.doi.org/10.1007/BF00034451.
