ORIGINAL ARTICLE

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The *rbcL* genes of two *Cuscuta* species, *C. gronovii* and *C. subinclusa*, are transcribed by the nuclear-encoded plastid RNA polymerase (NEP)

Abstract Some species of the holoparasitic flowering plant genus *Cuscuta*, like *C. reflexa*, have retained a plastid genome that encodes photosynthesis-related gene products as well as the plastid-encoded RNA polymerase (PEP). In contrast, other species like *C. gronovii* and *C. subinclusa* have lost the *rpo* genes coding for the PEP subunits while photosynthetic genes have been retained. In order to ensure expression of the photosynthesis-related genes in the absence of PEP, a number of adaptations within the plastid genome were required that enable gene transcription mediated exclusively by the nuclear-encoded plastid RNA polymerase (NEP). In this study we analyzed promoter sequence conservation and transcription start sites of a typical PEP gene of non-parasitic plants, *rbcL*, which codes for the large subunit of ribulose bisphosphate carboxylase/oxygenase. We show that despite high sequence conservation of the coding region of *rbcL* among different *Cuscuta* species and tobacco, the 5′ non-coding regions of *C. gronovii* and *C. subinclusa* have suffered extensive deletions encompassing the PEP promoter that is present in *C. reflexa* and tobacco. Primer-extension analyses enabled the identification of transcripts initiated at NEP promoter motifs in *C. gronovii* and *C. subinclusa* that are not detectable in the 5′ non-coding region of *C. reflexa*.

Keywords *Cuscuta* · Holoparasitic plants · Plastid genome · Plastid promoters · Plastid *rbcL* gene · Plastid RNA polymerase

Abbreviations *atpB*: Gene coding for the β-subunit of the plastid ATPase · NEP: Nuclear-encoded plastid RNA polymerase · PEP: Plastid-encoded plastid RNA polymerase · *rbcL*: Gene coding for the large subunit of

Rubisco · Rubisco: Ribulose bisphosphate carboxylase/oxygenase

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Introduction

Transcription of plastid genes is mediated by a plastid-encoded RNA polymerase (PEP) in addition to one or two nuclear-encoded phage-type RNA polymerases (Hedtke et al. 1997; Chang et al. 1999; Hess and Börner 1999; Bligny et al. 2000). Each polymerase recognizes a distinct set of promoters. While PEP promoter sequences resemble the promoters of eubacterial genes (Igloi and Kössel 1992; Weihe and Börner 1999; Liere and Maliga 2001), NEP transcription starts at a YRTA-motif that shows great homology to bacteriophage and mitochondrial promoters (Hajdukiewicz et al. 1997; Kapoor et al. 1997; Liere and Maliga 1999; Weihe and Börner 1999). Whereas NEP is thought to be responsible mainly for the transcription of housekeeping genes, including the transcription of the subunits of the PEP enzyme, PEP is mainly responsible for the transcription of photosynthesis-related genes (Allison et al. 1996; Hajdukiewicz et al. 1997; Krause et al. 2000).

Previous studies have shown that in *C. reflexa*, despite the deletion of some plastid genes (Haberhausen et al. 1992; Haberhausen and Zetsche 1994), the plastid-encoded RNA polymerase (PEP) is still retained (Krause et al. 2003). Since *C. reflexa* accumulates chlorophyll and performs photosynthesis at low levels (Hibberd et al. 1998; van der Kooij et al. 2000) the existence of PEP is consistent with the notion that PEP is needed for the transcription of photosynthesis-related genes (Allison et al. 1996; deSantis-Maciossek et al. 1999). In contrast, in two other species (*C. gronovii* and *C. subinclusa*) the *rpo* genes are absent from the plastid genome although the capacity to perform photosynthesis at low levels has been retained (van der Kooij et al. 2000). A transfer of functional *rpo* genes to the nuclear genome and thus the import of the PEP subunits have been ruled

out by Southern Blot analysis and PCR (Krause et al. 2003) so that NEP has to be responsible for the expression of photosynthesis-related genes at levels sufficient to allow for photosynthesis. This is in clear contrast to observations that were made with PEP-deficient plastids of non-parasitic plants obtained either by mutagenesis of the plastid genome or by induction of plastid ribosome deficiency. All these PEP-deficient plants are characterized by achlorophyllous tissue that lacks photosynthetic capacity (Falk et al. 1993; Hess et al. 1993; Allison et al. 1996; deSantis-Maciossek et al. 1999; Krause et al. 2000).

A switch from PEP- to NEP-driven transcription in some *Cuscuta* species has so far only been observed for the gene encoding the 16S rRNA (Krause et al. 2003) that in non-parasitic plants already contains promoters for both PEP and NEP. Photosynthesis-related genes, like the plastid *rbcL* gene, in contrast, normally possess only a promoter that is recognized by PEP (Shinozaki and Sugiura 1982; Mullet et al. 1985; Orozco et al. 1990; Allison et al. 1996). Since transcripts of this gene were detected in a number of *Cuscuta* species, both with and without PEP (van der Kooij et al. 2000), this gene is a good candidate for the analysis of the consequences of PEP-deficiency on the promoter structures of photosynthesis genes.

Materials and methods

Plant material

Cuscuta reflexa, *C. gronovii* and *C. subinclusa* were kept in a greenhouse at the University of Kiel on *Pelargonium zonale* as the host plant, as described before (van der Kooij et al. 2000; Krause et al. 2003). *Nicotiana tabacum* L. cv. Xanthi, which was used as a non-parasitic control plant, was grown in a greenhouse.

PCR amplification and sequence analysis

The primers used for PCR amplification of fragments of the *rbcL* gene were designed based on the published sequence of the tobacco plastid DNA (Wakasugi et al. 1998; positions in parentheses):

- *atpB.P5-uni* (56495–56012) 5′-TGTAAAACGACG-GCCAGTCTGTGTCAATCACTTCC-3′;
- *atpB.P4-uni* (56747–56765) 5′-GTAAAACGACG-GCCAGTAGAACCAGAAGTAGTAGG-3′;
- *rbcL-for-uni* (57610–57629) 5′-GTAAAACGACGG-CCAGTAGACTAAAGCAAGTGTTG-3′;
- *rbcL.P1-rev* (57654–57636) 5′-AGGAAACAGCTA-TGACCGTACTCTTTAACACCAGC-3′;
- *rbcL.P5-rev* (57735–57754) 5′-AGGAAACAGCTA-TGACCGCTTCTTCAGGTGGAACT-3′;
- *rbcL.P3-uni* (58328–58342) 5′-GTAAAACGACGG-CCAGTACATGCGAAGAAATG-3′;

- *rbcL.P2-rev* (58342–58328) 5′-CAGGAAACAGCT-ATGACCATTTCTTCGCATGTACC-3′;
- *rbcL.P4-rev* (59056–59039) 5′-CAGGAAACAGCT-ATGACTTTCTCCTTATCCTTC-3′.

Different combinations of these primers were used to amplify overlapping fragments.

The PCR products were sequenced directly after purification from agarose gels as described by Krause et al. (2003). After alignment of the overlapping fragments, the resulting *rbcL* sequences from *C. gronovii* and *C. subinclusa* were deposited in the EMBL database under accession numbers AJ320207 (*C. gronovii*) and AJ320210 (*C. subinclusa*).

Primer extensions

Primer extensions were carried out essentially as described by Krause et al. (2003) with 10–50 μg of total RNA on a Li-Cor DNA sequencer (Model 4000 L; MWG Biotech) using the following IRD800-labeled primer (complementary nucleotides 57624–57595 in tobacco): 5′-ACTTGCTTTAGTCTCTGTTTGTGGT-GACAT-3′.

Results

Amplification and sequence analysis of the *rbcL* gene

The 5′-region of the *rbcL* gene containing the promoter sequence as well as the first 700 nucleotides of the coding region were amplified in separate reactions with primer pairs derived from the tobacco plastid DNA sequence. While the PCR with the primers specific for the coding region revealed no size differences of the amplified fragments in *C. reflexa*, *C. gronovii*, *C. subinclusa* or tobacco, amplification of the intergenic region between *atpB* and *rbcL* yielded differently sized PCR products (Fig. 1a,b)

Sequence analysis demonstrated that the coding regions of the *rbcL* gene in the three *Cuscuta* species were highly homologous to each other and to the *rbcL* gene of tobacco (Fig. 1c). Comparison of the *rbcL* 5′-non-coding sequences of the three *Cuscuta* species and tobacco revealed that *C. gronovii* and *C. subinclusa* have suffered several large deletions in this area. The characteristic promoter sequence motif for the plastid-encoded RNA polymerase (PEP) is strongly altered in *C. gronovii* and completely absent from the *C. subinclusa* sequence. In contrast, *C. reflexa* has suffered only a few changes in the region containing the PEP promoter sequence (Fig. 1c).

Expression of the *rbcL* gene

The detection of the mature *rbcL* mRNA and the accumulation of the corresponding protein (van der
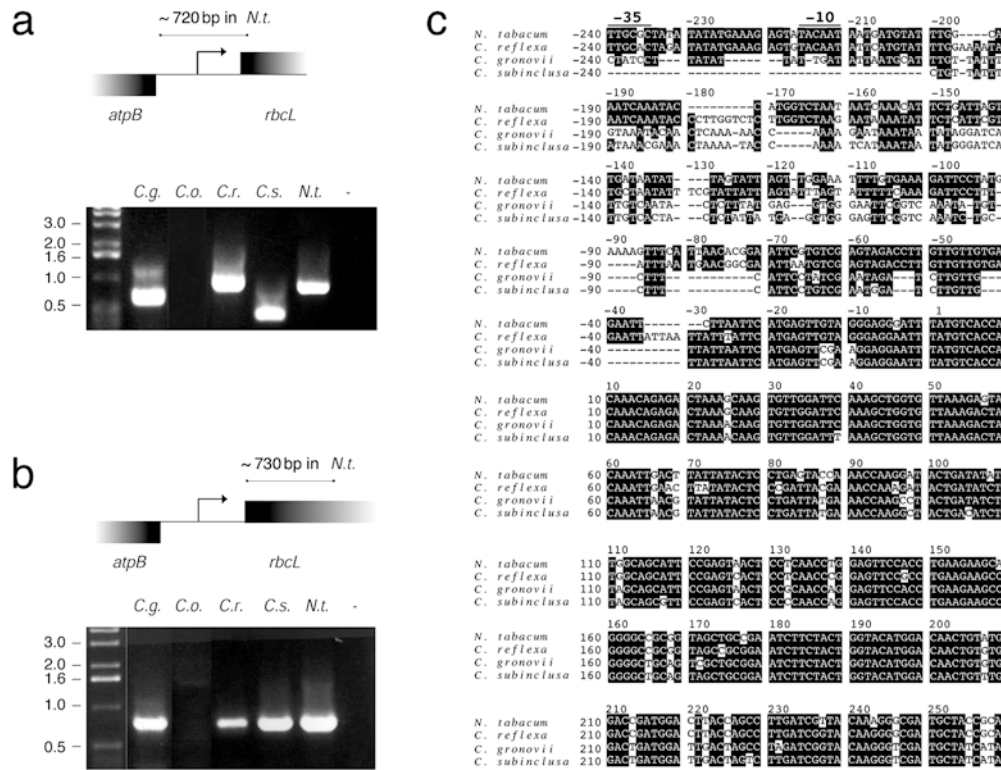
Fig. 1a–c Conservation of the *rbcL* gene from different *Cuscuta* species. **a,b** PCR amplification of fragments from the intergenic region between *atpB* and *rbcL* (**a**) and the first 700 bp of the *rbcL* coding region (**b**). Ethidium bromide-stained gels containing the PCR fragments of *C. gronovii* (*C.g.*), *C. reflexa* (*C.r.*) and *C. subinclusa* (*C.s.*) are shown below a schematic depiction of the amplified region. The sizes of bands from the molecular weight standard (*left lane*) are given on the *left*. *C. odorata* (*C. o.*) lacking the *rbcL* gene (van der Kooij et al. 2000) and tobacco (*N.t.*) were included as a negative and positive controls, respectively. The genes (*grey boxes*) are not drawn to scale. *Arrows* indicate the direction of transcription of the *rbcL* gene. **c** Sequence comparison of the 5′-region of the *rbcL* gene and part the coding region. The numbering of the nucleotides refers to the tobacco sequence and starts with nucleotide number 1 at the start codon. Nucleotides identical to the *rbcL* sequence of *N. tabacum* are given in *white on a black background*. A *hyphen* (-) indicates that a nucleotide is missing with respect to the tobacco sequence. The *black bars* mark the position of the σ70-type promoter ('−10' and '−35'-boxes) of the tobacco *rbcL* gene. GenBank accession numbers are NC_001879 (*N. tabacum*), X61698 (*C. reflexa*), AJ320207 (*C. gronovii*) and AJ320210 (*C. subinclusa*)

Kooij et al. 2000) in the three *Cuscuta* species implies that the gene is actively transcribed and functional in the plastids. In non-parasitic plants, all transcripts of this gene are initiated at a PEP promoter. As PEP is absent in *C. gronovii* and *C. subinclusa* (Krause et al. 2003), it was important to determine the transcription start sites and thus gain further information on the promoters that are involved in directing *rbcL* gene transcription. For this purpose we mapped the 5′-ends of *rbcL* transcripts by primer-extension reactions. In the case of *C. reflexa* the 5′-end of the *rbcL* transcript mapped to position −200 relative to the start of the coding region of the *rbcL* mRNA. The sequence preceding this 5′-end resembles the sequence of the published transcription start site of

tobacco at −180 (Shinozaki and Sugiura 1982; Mullet et al. 1985; Allison et al. 1996). The signal corresponding to position −59 of tobacco *rbcL* represents the processed transcript (Mullet et al. 1985). In the other two *Cuscuta* species, 5′-ends were mapped to completely different positions. In *C. subinclusa* and *C. gronovii*, signals were obtained at positions −116 and −108, respectively, relative to the translation start site of the mRNA (Fig. 2). Both 5′-ends are preceded by sequences that contain a YRTA-box as well as a GAA-motif. These sequence motifs are known to be associated with NEP-based transcription (Weihe and Börner 1999) and were found at a similar distance from the start site of transcription in many other genes (Fig. 3).

Discussion

In non-parasitic higher plants the plastid-encoded RNA polymerase (PEP) is thought to be mainly responsible for the transcription of genes associated with photosynthesis (Allison et al. 1996; deSantis-Maciossek et al. 1999), while the nuclear-encoded RNA polymerase(s) (NEP) are mainly responsible for transcription of housekeeping genes (Maliga 1998; Hess and Börner 1999). In most holoparasitic plants the genes encoding PEP are missing, and transcription of plastid genes depends exclusively on NEP (Morden et al. 1991; Wolfe et al. 1992; Thalouarn et al. 1994; Lusson et al. 1998). *C. gronovii* and *C. subinclusa*, though being green and capable of performing photosynthesis at low levels (van der Kooij et al. 2000), lack PEP likewise as white parasitic plants like *Cuscuta odorata* (van der Kooij et al.

Fig. 2 Mapping of 5′-ends of *rbcL* transcripts. The 5′-ends of the *rbcL* transcripts were detected by primer-extension analysis and are indicated by *black arrowheads* on the *right* of each panel. For comparison, sequences were generated using the same primers that were used for the primer-extension reactions. For each species, the sequence ladders are shown on the *left* next to the lane with the primer extensions, respectively. The loading order (AGCT) is indicated above. Promoter consensus motifs (PEP −10/−35-boxes and NEP YRT- and GAA-boxes) that were found in the regions immediately upstream of the primer extension signals are shown on the *left* of each panel
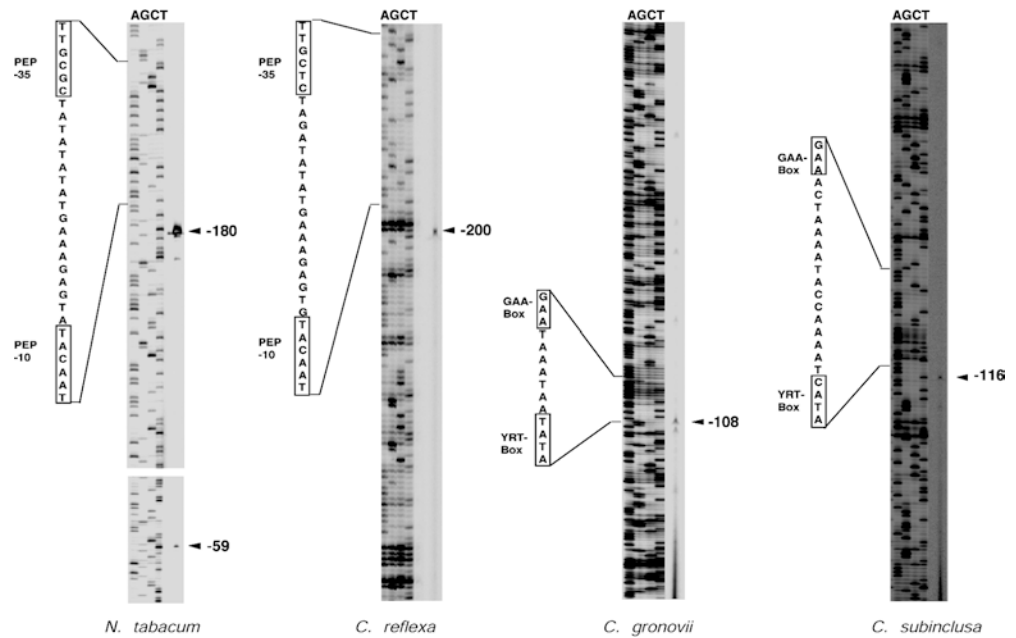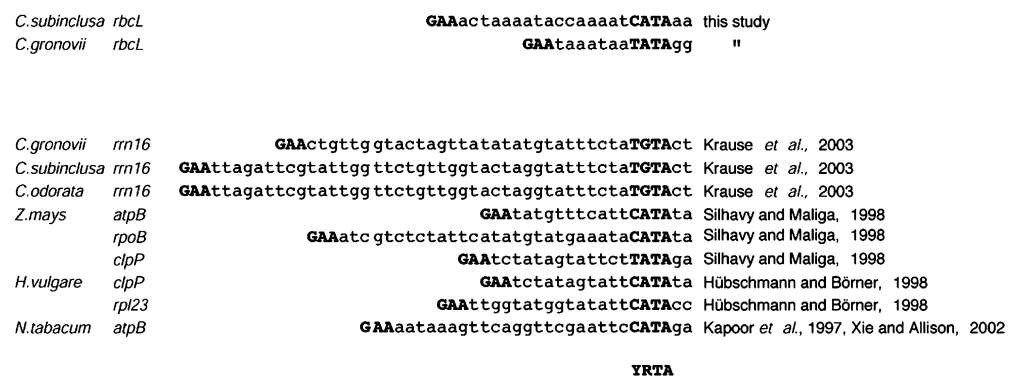

N. tabacum   C. reflexa   C. gronovii   C. subinclusa

Fig. 3 Alignment of NEP promoter sequences. Sequences upstream of the 5′-ends of the *rbcL* transcripts of *C. subinclusa* and *C. gronovii* were compared with published sequences of the NEP promoters of different genes from various species. The conserved consensus motifs (YRTA-box and GAA-box, Weihe and Börner 1999) are indicated by *bold upper case letters*

C.subinclusa	*rbcL*	**GAA**actaaaataccaaaat**CATA**aa	this study
C.gronovii	*rbcL*	**GAA**taaataa**TATA**gg	"
C.gronovii	*rrn16*	**GAA**ctgttg gtactagttatatatgtatttcta**TGTA**ct	Krause *et al.,* 2003
C.subinclusa	*rrn16*	**GAA**ttagattcgtattgg ttctgttggtactaggtatttcta**TGTA**ct	Krause *et al.,* 2003
C.odorata	*rrn16*	**GAA**ttagattcgtattgg ttctgttggtactaggtatttcta**TGTA**ct	Krause *et al.,* 2003
Z.mays	*atpB*	**GAA**tatgtttcatt**CATA**ta	Silhavy and Maliga, 1998
	rpoB	**GAA**atc gtctctattcatatgtatgaaata**CATA**ta	Silhavy and Maliga, 1998
	clpP	**GAA**tctatagtattct**TATA**ga	Silhavy and Maliga, 1998
H.vulgare	*clpP*	**GAA**tctatagtatt**CATA**ta	Hübschmann and Börner, 1998
	rpl23	**GAA**ttggtatggtatatt**CATA**cc	Hübschmann and Börner, 1998
N.tabacum	*atpB*	**GAA**aataaagttcaggttcgaattc**CATA**ga	Kapoor *et al.,* 1997, Xie and Allison, 2002

YRTA

2000) and *Epifagus virginiana* (Morden et al. 1991; Wolfe et al. 1992). Among the *Cuscuta* species analyzed so far (van der Kooij et al. 2000), only *C. reflexa* is known to possess a PEP. In order to elucidate how plastid genes associated with photosynthesis are transcribed in the absence of PEP, the transcription initiation sites and promoter sequences of *rbcL* transcripts from *C. gronovii* and *C. subinclusa* were investigated.

The *rbcL* gene belongs to the group of genes that is exclusively transcribed by PEP in non-parasitic plants (Mullet et al. 1985; Allison et al. 1996; Hajdukiewicz et al. 1997). With the exception of *C. odorata*, all *Cuscuta* species analyzed so far possess and express the *rbcL* gene (van der Kooij et al. 2000), though some of them lack PEP (Krause et al. 2003). As shown in this study the loss of PEP is accompanied by alterations in the promoters of the *rbcL* genes. The *rbcL* transcripts in *C. gronovii* and *C. subinclusa* are initiated from NEP promoter sequences that are neither present in *C. reflexa* nor in the non-coding region of *rbcL* from non-parasitic

plants. The transcripts detected by primer-extension reactions are likely to be true primary transcripts since in contrast to the processed *rbcl* transcripts of tobacco they could not be ligated to an RNA linker (data not shown). The method of RNA-linker ligation is an alternative to capping experiments (Vera and Sugiura 1992), which can be used to distinguish between original 5′-ends and those that result from processing (Miyagi et al. 1998). The newly evolved NEP promoter sequences in the *Cuscuta* species lacking PEP consist of a GAA-motif followed by a YRTA-motif (Hübschmann and Börner 1998; Liere and Maliga 1999; Weihe and Börner 1999). Similar alterations in the promoter region have been observed also in the *rrn* promoter sequence of different *Cuscuta* species. Though non-parasitic plants possess both NEP and PEP promoter elements in the *rrn* promoter, a new NEP promoter at a different position from that found in non-parasitic plants is used for transcription of the operon in *Cuscuta* species lacking PEP (Krause et al. 2003).

In the achlorophyllous *Lathraea clandestina*, *rbcL* transcripts were found to have an unusually long 5′-noncoding region (Lusson et al. 1998). In spite of high sequence conservation, the *rbcL* PEP promoter appears not to be used. Instead, based on sequence comparisons, an NEP promoter was proposed that could take over transcription of the *rbcL* gene (Lusson et al. 1998). However, no attempts to positively identify the transcription start site of *rbcL* in this species have been reported so far.

In contrast to achlorophyllous *Lathraea clandestina*, both *C. gronovii* and *C. subinclusa* are capable of synthesizing chlorophylls and performing photosynthesis, albeit at low levels. Obviously NEP-mediated expression of the *rbcL* gene, and most likely further genes associated with photosynthesis, is sufficient to allow for photosynthetic carbon fixation at a low level. In contrast to *C. gronovii* and *C. subinclusa*, tobacco *rpo* mutants are not capable of performing photosynthesis. One explanation for this could be that the mutants show severe disturbances in processing of primary transcripts (Krause et al. 2000; Legen et al. 2002) as a secondary effect of the loss of PEP, which could negatively influence translation efficiency. It is hypothesized that parasitic plants may cope with the loss of PEP not only by evolution of new promoter elements, but, possibly, by an adaptation of posttranscriptional mechanisms enabling the stabilization and correct processing of NEP-derived transcripts required for building up a photosynthetic apparatus.

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