S40-008

In vitro analysis of *cis*-elements and *trans*-factors for the RNA editing of tobacco chloroplast *psbE* and *petB* mRNAs

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Keywords: chloroplast, RNA editing, in vitro, psbE, petB

Introduction

RNA editing is one of the posttranscriptional processes in higher plant chloroplasts (Bock, 2000). Editing is found in many cases in internal protein-coding regions, resulting in amino acid substitutions. Therefore, it is important to understand editing events to use the chloroplast as a factory for protein production. The number of editing sites so far observed is 31 in tobacco and these editing events are always C-to-U conversions (Hirose *et al*., 1999). The recognition of these sites, approx. one C residue to be edited out of 1000 Cs in the whole transcripts of tobacco chloroplasts, should be extremely accurate and efficient. Therefore, a major question for RNA editing is how editing sites are specifically recognized. Using transgenic approaches in tobacco chloroplasts, the only available transformation system among higher plant organelles (Svab and Maliga, 1993), *cis*-acting elements important for the recognition of editing sites have been identified in *psbL* and *ndhB* mRNAs (Chaudhuri and Maliga, 1996; Bock *et al*., 1997).

To analyze the biochemical mechanism of RNA editing reactions in chloroplasts, we have developed an *in vitro* RNA editing system from tobacco green chloroplasts (Hirose and Sugiura, 2001). This *in vitro* system with mRNA substrates labeled specifically at editing sites allowed us to detect accurate editing with high sensitivity. Using this system, mutational analysis of substrate mRNAs derived from tobacco chloroplast *psbL* and *ndhB* mRNAs confirmed the participation of *cis*-acting elements that had previously been identified *in vivo*. Competition analysis revealed the existence of site-specific *trans*-acting factors interacting with the corresponding upstream *cis*-elements. A chloroplast protein of 25 kDa was found to be specifically associated with the *cis*-element involved in *psbL* mRNA editing (Hirose and Sugiura, 2001). Here, we report our preliminary results on *cis* and *trans* elements for the editing of additional mRNAs (details will be published elsewhere).

Materials and methods

Active extracts were prepared from intact chloroplasts isolated from 5~10 cm tobacco leaves as described by Hirose and Sugiura (2001). Pre-mRNA portions in which the C residue to be edited was specifically labeled with ^{32}P were used as *in vitro* substrates. Editing activity was assayed by detecting ^{32}P-labeled U mononucleotides on cellulose TLC after digestion of substrates with nuclease P1.

Results and discussion

Using the *in vitro* system, we analyzed the editing of chloroplast mRNAs from tobacco chloroplast *psbE* and *petB* genes. Both the mRNAs were found to be edited accurately *in vitro* by the chloroplast extract. To define *cis*-acting elements which requires *trans*-acting factor binding, competition assays by mutated RNAs were carried out. In the case of *psbE* mRNA, competitor RNAs mutated in a sequence between -15/-6 could not inhibit editing, therefore the *cis*-acting element is likely to be located in this region. In the case of *petB* mRNA, a sequence in a similar position is suggested as its *cis*-element.

To examine whether distinct *trans*-acting factors interact with each *cis*-acting element, cross-competition experiments were carried out. Depletable *trans*-acting factors were shown to be site-specific because heterologous competitor RNAs could not inhibit editing. The above factors are likely to be proteins, since the interaction of an RNA-binding protein (p25) was shown for the cis-element of *psbL* mRNA (Hirose and Sugiura, 2001). Therefore, to detect protein species binding to the proposed *cis*-acting elements, UV cross-linking experiments were carried out. Using the *psbE* mRNA substrate, several protein bands of 56 to 58 kDa and 28 to 33 kDa in sizes were detected. On the other hand, other RNA-binding proteins were found to be crosslinked with the *petB* mRNA substrate, suggesting that *trans*-acting factors are site-specific.

Although binding of *trans*-acting factors should be highly site-specific, it is possible that other C residues followed by sequences similar to *cis*-elements for editing are targeted by such a *trans*-acting factor. Therefore, we searched sequences similar to the *cis*-element for *psbE* mRNA editing from the entire sequence of tobacco chloroplast genome (Shinozaki *et al*., 1986; Wakasugi *et al*., 1998). Twenty-three sequences which match at least 8 nt out of the 10 nt *cis*-element of *psbE* mRNAs were found. Since an editing site was reported to be defined by its distance from the upstream element (Hermann and Bock, 1999), we further selected sequences in which C residues are located at 5 nt downstream position. Only two sequences fit this condition; one is in *rbcL*, the other is in *psbD* coding regions (Figure 1). To analyze whether these Cs are edited, total RNA was isolated from tobacco chloroplasts, amplified by RT-PCR and subjected to direct sequencing. Neither C was found to be edited, suggesting that the sequence-specificity of the *cis*-acting element is rigid so that one specific C residue, the editing site of *psbE* mRNA, is recognized precisely from all chloroplast transcripts.

psbE **66931-ACUGGCCGUUuugauC-66916**

psbD **35041-AUUGGACGUUgaaccC-35056**

rbcL **58371-AUUGGGCGUUccgauC-58386**

Figure 1. Alignment of sequences similar to the *cis*-acting element of *psbE* mRNA editing. The sequence upstream of the editing site in *psbE* mRNA is shown on the top. Upper-case letters indicate sequences corresponding to the *cis*-element and common residues in this region are underlined. Uppercase C residues at the right end are candidates for editing sites.

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