Dual Targeting of Arabidopsis HOLOCARBOXYLASE SYNTHETASE1: A Small Upstream Open Reading Frame Regulates Translation Initiation and Protein Targeting1[W]

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Protein biotinylation is an original and very specific posttranslational modification, compartmented in plants, between mitochondria, plastids, and the cytosol. This reaction modifies and activates few carboxylases committed in key metabolisms and is catalyzed by holocarboxylase synthetase (HCS). The molecular bases of this complex compartmentalization and the relative function of each of the HCS genes, HCS1 and HCS2, identified in Arabidopsis (Arabidopsis thaliana) are mainly unknown. Here, we showed by reverse genetics that the HCS1 gene is essential for plant viability, whereas disruption of the HCS2 gene in Arabidopsis does not lead to any obvious phenotype when plants are grown under standard conditions. These findings strongly suggest that HCS1 is the only protein responsible for HCS activity in Arabidopsis cells, including the cytosolic, mitochondrial, and plastidial compartments. A closer study of HCS1 gene expression enabled us to propose an original mechanism to account for this multiplicity of localizations. Located in the HCS1 messenger RNA 5' -untranslated region, an upstream open reading frame regulates the translation initiation of HCS1 and the subsequent targeting of HCS1 protein. Moreover, an exquisitely precise alternative splicing of HCS1 messenger RNA can regulate the presence and absence of this upstream open reading frame. The existence of these complex and interdependent mechanisms creates a rich molecular platform where different parameters and factors could control HCS targeting and hence biotin metabolism.

The compartmentalization of most pathways of plant primary metabolism is generally covered in physiology textbooks. Cell fractionation and immunohistochemical studies have revealed the extensive compartmentalization of plant metabolism. This compartmentalization and its associated regulations are crucial for the integrated understanding of plant metabolism and its adaptation to both developmental and environmental changes. However, the intracellular locations of the majority of proteins are still not known (Lunn, 2007) and the intricacy of different metabolisms and their regulation in the cellular context is still to be unraveled. Biotin (vitamin H) is a cofactor for some carboxylases and decarboxylases dealing with crucial metabolic processes such as fatty acid and carbohydrate metabolism (Knowles, 1989). The biotinylation of these enzymes is a posttranslational modification allowing the transformation of inactive apoproteins into their active holo forms. The covalent attachment of biotin is catalyzed by biotin protein ligase, also called holocarboxylase synthetase (HCS). D-Biotin is attached to a specific Lys residue of newly synthesized apoenzyme via an amide linkage between the biotin carboxyl group and a unique ε-amino-group of a Lys residue (Samols et al., 1988). It occurs in two steps as follows:

\[
\begin{align*}
\text{d-biotin + ATP} & \rightarrow \text{d-biotinyl-5'-AMP + PPI} \\
\text{d-biotinyl-5'-AMP + apocarboxylase} & \rightarrow \text{holocarboxylase + AMP}
\end{align*}
\]

Biotin-dependent carboxylases are enzymes ubiquitously found in living cells. However, their number, structure, and subcellular localization are subject to variations according to organisms. In plants, four different biotin-dependent carboxylase activities have been evidenced: two structurally distinct isoforms of acetyl-CoA carboxylase (ACCase) in cytosol and plastids, geranoyl-CoA carboxylase in plastids, and methylcrotonyl-CoA carboxylase (MCCase) in mitochondria (Alban et al., 2000). Moreover, sequencing of the Arabidopsis (Arabidopsis thaliana) genome confirmed the occurrence of genes encoding for both structural forms of ACCase and for MCCase (Nikolaou et al., 2007).
produced by alternative splicing of HCS2. However, none of the putative HCS2 proteins, a family of alternatively spliced mRNAs (Denis et al., 2002), was soluble in E. coli and active in vitro when overproduced in Arabidopsis cultured cells, HCS activity was also essentially recovered in cytosol of fractionated protoplasts and, to a lesser extent, in the organelle subtraction (chloroplasts and/or mitochondria; Denis, 2002). This suggests that carboxylases are biotinylated in their compartment of residence.

The genetic and molecular bases of the compartmentalization of HCS activity are unclear. Two HCS genes have been evidenced in Arabidopsis (Tissot et al., 1997). Firstly, HCS1 cDNA has been isolated by functional complementation of an Escherichia coli mutant (Tissot et al., 1997). Subsequently, the systematic sequencing of the Arabidopsis genome enabled the identification of the HCS1 gene. Moreover, it confirmed the existence of a second HCS gene (HCS2) localized in the pericentromeric region of chromosome 1 (Arabidopsis Genome Initiative, 2000). HCS1 and HCS2 genes present very large similarities and probably result from the duplication of a common ancestor gene (Denis et al., 2002). The way both HCS genes share responsibility for in vivo HCS activity is unknown. A simple hypothesis would be that they present different substrate specificities or different subcellular localizations and cooperatively enable the biotinylation of the different carboxylase substrates. HCS1 has been shown to encode a plastidial isoform of HCS and could then account for the chloroplastic HCS activity (Tissot et al., 1998). However, HCS1 presented a broad specificity of substrates and was able to biotinylate efficiently in vitro all recombinant biotin-dependent apocarboxylases identified in Arabidopsis (Tissot et al., 1998; Denis, 2002; Denis et al., 2002). Interestingly, HCS2 expression produces a highly diverse family of alternatively spliced mRNAs (Denis et al., 2002). However, none of the putative HCS2 proteins, produced by alternative splicing of HCS2, was soluble and active in vitro when overproduced in E. coli, nor rescued an E. coli mutant affected in protein biotinylation (Denis, 2002). Also, western-blot analyses did not allow us to test the occurrence of these proteins in cellular extracts of Arabidopsis because HCS protein concentration in plant extracts was too low to be accurately detected (Denis et al., 2002). As a result, in vitro biochemical activity and substrate specificity alone did not give any definite evidence to partition the biotinylation of carboxylase proteins between HCS1 and HCS2. In this study, we report the isolation and characterization of hcs1 and hcs2 Arabidopsis mutants. From the result of our genetic and molecular analyses, we suggest that HCS1 is the only HCS gene responsible for the biotinylation of all carboxylases in plants. Closer scrutiny of HCS1 gene expression and splicing led us to revisit the specific compartmentalization of HCS1 proteins. We show that the HCS1 mRNA 5′-untranslated region (UTR) is subjected to alternative splicing that affects the translation initiation at two AUG codons, enabling dual localization of HCS1 protein in cytosol and chloroplasts.

RESULTS

HCS1 and HCS2 Are Neither Redundant Nor Cooperative to Mediate Carboxylase Biotinylation in Plants

Various collections of T-DNA and transposon insertion mutants were searched for disruption in HCS1 and HCS2 genes. We identified a line of Arabidopsis plants (SAIL_1277_E03; line hcs1-1) carrying an inverted tandem insert of T-DNA with a Basta resistance marker within the second exon of the HCS1 gene (At2g25710; Fig. 1A). Segregating plants originating from the mutant line were genotyped by PCR using primers specific of the HCS1 gene and of the T-DNA left border (Fig. 1B). This PCR analysis did not reveal any lines presenting a homozygous disruption of the HCS1 gene. Moreover, self-pollination of heterozygous plants for the T-DNA insertion produced, in the next generation, wild-type and heterozygous plants at a ratio close to 1:1. Among a total of 95 progeny plants examined, no plants homozygous for the T-DNA insertion were recovered. Basta resistance segregation indicated that no additional T-DNA was inserted elsewhere in the genome. Indeed, all wild-type plants were Basta sensitive and all heterozygous plants were Basta resistant. These segregation data suggest that transmission of the inserted allele might be impaired in either the female or male gametes (or both), consistent with a gametophytic defect. The examination of immature siliques of self-pollinated heterozygous plants confirmed this hypothesis. They were found to contain approximately 60% normal-sized seeds, 30% to 40% empty slots filled with apparently aborted ovules that had not been fertilized, and aborted embryos at a reduced frequency (<10%; Fig. 1, C and D). Given that homozygous mutant plants were never recovered, we assume that aborted seeds corresponded to missing homozygous mutants. Aborted ovules suggest a defect of female gametogenesis. This was examined by backcrossing of mutant plants. If the mutation is borne by the female parent (heterozygous mutant female flower crossed with wild-type pollen), the resulting siliques contained roughly one-half the number of seeds per siliqua as the wild-type siliques. Of the plants grown from 30 of these seeds, 25 of them were found to be of the wild-type genotype and the other five were of the heterozygous genotype, based on PCR genotyping and Basta selection. If the mutation was borne by the male parent (wild-type mutant female flower crossed with heterozygous pollen), the resulting siliques contained the same number of seeds per siliqua as the wild-type siliques. One-half of them had the
wild-type genotype and one-half had the heterozygous genotype (from 30 plants, analyzed by PCR and Basta resistance). As a result, genetic crossing data support the notion that **HCS1** disruption causes ovule lethality. In summary, our results reveal that insertion in the **HCS1** gene is lethal, resulting in decreased transmission through the female gametophyte and homozygous embryonic lethality. Examination of a second independent **hcs1** disruption allele (FLAG_486E08; line **hcs1-2** obtained from the Versailles collection) presenting a single T-DNA insertion within intron 9 confirmed that the ovule and embryo abortions were caused by the T-DNA insertions into **HCS1** gene (see Supplemental Fig. S1).

To obtain an Arabidopsis **hcs2** knockout mutant, we screened a T-DNA insertion population (Bouchez et al., 1993) by PCR analysis of DNA pools with specific sets of primers derived from the **HCS2** gene and the T-DNA and molecular hybridization. One candidate knockout line (line 21) was obtained, and the location of the T-DNA insertion was determined by DNA sequence analysis of the PCR product. As shown in Figure 2A, the T-DNA was found to be inserted in intron 9 of the **HCS2** gene, 5 bp upstream of the beginning of exon 10. The line 21 presented a single T-DNA insertion (shown by the segregation of the kanamycin resistance in the F2 progeny and Southern-blot analysis). Plants (T3 generation) were examined by PCR. Among this population, wild-type plants as well as homozygous and heterozygous siblings were identified (Fig. 2B). As a result, the homozygous disruption of **HCS2** is not lethal. Wild-type and homozygous plants for the insertion were then self-pollinated for further molecular and biochemical analyses. To assess the impact of T-DNA insertion on gene expression, real-time quantitative reverse transcription (RT)-PCR was performed on mRNA from aerial plant sections comprising rosette leaves, stems, inflorescence, and developing siliques (Fig. 2C). The **HCS2** mRNA level in the plants homozygous for the T-DNA insertion did not exceed the background noise of PCR, while wild-type levels of **HCS2** transcripts were found in heterozygous plants. Interestingly, expression of **HCS1** gene was not altered in the **hcs2** null mutant, indicating that abolition of **HCS2** gene expression was not compensated by an overexpression of the **HCS1** gene, at least at the transcriptional level. The phenotype of the homozygous **hcs2** knockout mutant was carefully inspected, compared with the wild type, under standard greenhouse growth conditions. No detectable difference was observed at any growth stage, including seed germination, plant morphology and growth, seed production, and fertility (data not shown). To determine **HCS2**’s part in biotinylation of biotin-dependent carboxylases, we first measured total HCS activity in soluble plant protein extracts using different Arabidopsis apocarboxylase substrates (recombinant proteins produced in **E. coli**). Surprisingly, protein extracts...

**Figure 1.** Characterization of a T-DNA insertion allele of Arabidopsis **HCS1** (SAIL_1277_E03; line **hcs1-1**). A, Structure of the **HCS1** gene carrying the inverted tandem insert of T-DNA. Black boxes and lines indicate exons and introns, respectively. The size of T-DNA is not drawn to scale. The locations of primer sequences used for PCR genotyping are marked with arrows. B, Characterization of the T-DNA insertion locus by PCR. Genomic DNA from a wild-type (Wt) and a heterozygous **HCS1/hcs1-1** mutant (Het) plant was amplified using the primer combinations indicated. Three PCR reactions per plant were performed. **Hcs1**.for and Hcs1.rev amplify a 0.46-kb product from the wild-type allele, LB3 and Hcs1.rev amplify a 0.29-kb product from the disrupted allele, and Hcs1.for and LB3 amplify a 0.41-kb product from the disrupted allele. Left and right flanking lanes (M) show DNA size markers. C, Light microscopy analysis of a wild-type immature silique showing uniform seed development and heterozygous **HCS1/hcs1-1** immature siliques containing aborted, white, and shrunken ovules (asterisks) and aborted, white or brown, and shriveled seeds (arrowheads). Scale bars = 500 μm. D, Seed production from wild-type (**HCS1/HCS1**) and heterozygous (**HCS1/hcs1-1**) Arabidopsis plants. Three types of seed/ovules were identified in the siliques and counted: normal seeds containing full-size embryos, abnormal seeds containing aborted embryos, and aborted white ovules.

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<th>Genotype</th>
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<td>100</td>
<td>0</td>
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<tr>
<td><strong>HCS1/hcs1-1</strong> (Het)</td>
<td>17</td>
<td>59±13</td>
<td>8±6</td>
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from hcs2 mutant plants presented wild-type levels of HCS activities, in vitro, irrespectively of the apocarboxylase substrate used in the reaction (Fig. 2D). Western-blot analyses of biotinylated proteins using streptavidin coupled to peroxidase showed that the absence of HCS2 expression does not affect the biotinylation of biotin-dependent carboxylases in planta (Fig. 2F). In the same protein extract, biotin-dependent carboxylase (MCCase and ACCase) activities were found to be similar in both mutant and wild-type lines (Fig. 2E). This confirms that in planta protein biotinylation was not affected by HCS2 disruption.

Altogether, our results support the idea that the HCS2 gene has no or limited implication in biotin-dependent carboxylase biotinylation in planta, and that the HCS1 gene is responsible for most, if not all, plant HCS activity and as such is essential for plant viability. Therefore, HCS1 and HCS2 are not functionally redundant.
and they do not share the activity of biotinylating different carboxylases in planta. How could HCS1 be responsible for the different HCS activities evidenced in different compartments of the plant cell?

The 5′-UTR of HCS1 mRNA Is Subjected to Alternative Splicing

Functional complementation of an E. coli mutant enabled us to identify one mRNA variant of HCS1 (Tissot et al., 1997). It encodes an in vitro active form of HCS and was thought to be the only mRNA transcribed from the HCS1 gene. However, 5′-RACE experiments evidenced two populations of HCS1 mRNA (Denis et al., 2002), HCS1.s (for spliced) and HCS1.un (for unspliced), generated by alternative splicing of the same HCS1 pre-mRNA (Fig. 3). HCS1.un sequence was identical to the previously identified cDNA sequence. However, in the 5′-UTR of HCS1.s mRNA, 101 nucleotides are spliced out. A closer analysis of HCS1.un cDNA sequence enabled us to evidence a small open reading frame (ORF) of 24 nucleotides length (including the stop codon) located four bases upstream of the first ATG codon (Fig. 3). In HCS1.s mRNA, this sequence is spliced out, the stop codon of the 24-nucleotide small ORF coinciding with the 3′ acceptor signal.

Such elements, referred to as upstream ORF (uORF), are often found in the 5′-UTR of eukaryotic mRNA, yeast and fungi (McCarthy, 1998; Vilela and McCarthy, 2003), plants (Hanfrey et al., 2003), and mammals (Vattem and Wek, 2004). uORF can regulate the downstream ORF encoding the major gene product (Morris and Geballe, 2000; Meijer and Thomas, 2002). HCS1 mRNA offers two in-frame AUG (AUG1 and AUG2), possibly representing initiation codons (Tissot et al., 1997). The amino acid sequence encoded by the region located between AUG1 and AUG2 corresponds to the cleavable transit peptide, targeting the longest form of HCS1 into chloroplasts (Tissot et al., 1998). Initiation at AUG1 or AUG2 could then produce HCS proteins with different subcellular localizations. The double occurrence of an alternatively spliced uORF (now referred to as uORF24) and two possible AUG start codons in the 5′-end of the HCS1 main ORF offers a good environment for a possible translational control. This interesting observation led us to investigate the influence of uORF24 on translation initiation at AUG1 and AUG2.

uORF24 Controls in Vitro Translation of the Downstream HCS1 Main ORF

To determine the influence of the uORF on translation initiation, we studied translation of both HCS1 mRNA species by in vitro transcription-translation of full-length cDNA variants, isolated by RT-PCR, and subcloned in pPCRscript (Fig. 4A). In vitro transcription-translation of HCS1.un cDNA produced a major peptide of 37 kD corresponding to the translation beginning at AUG2. As a result, translation at AUG1 is impeded when it is preceded by the full-length HCS1 5′-UTR. The HCS1.s mRNA translation profile was quantitatively and qualitatively different. The amount of HCS1 protein produced in vitro at AUG1 is several-fold less than at AUG2, reflecting the translational control exerted by the upstream ORF.
protein synthesized is increased when HCS1.s is translated as compared to HCS1.un. Therefore, the 5'-UTR sequence, spliced out in HCS1.s and retained in HCS1.un, has an inhibitory effect on HCS1 translation. Moreover, even if the translation of HCS1.s produced the same 37-kD polypeptide as HCS1.un, it was not the major translated product. A more abundant 41-kD protein was also synthesized, from the initiation of translation at AUG 1. These results suggested that the 5'-UTR of HCS1 mRNA can influence the choice between AUG 1 and AUG 2. Can these results be explained by the presence (in HCS1.un) or absence (in HCS1.s) of uORF24? To understand uORF24 influence on HCS1 translation initiation, we performed site-directed mutagenesis. uORF24 initiation codon (ATG0) was changed to ATC codon in HCS1.un cDNA and the mutant analyzed by in vitro transcription and translation. The mutated HCS1.un cDNA presented the same pattern as HCS1.s and can produce a 41-kD protein in large abundance. This suggests that the modification of translation efficiency and AUG choice associated with the splicing of 5'-UTR of HCS1 is directly dependent on the presence or absence of uORF24 itself and not on some structural changes possibly produced by the splicing removal of 101 nucleotides. In the presence of the uORF24 within the 5'-UTR of HCS1 mRNA (HCS1.un), translation initiates mainly at AUG 2. In its absence, either due to elimination by alternative splicing or translation from the AUG 1 site, the translated product is a 41-kD protein.

Figure 4. The nature of the 5'-UTR of HCS1 mRNA influences AUG choice in vitro and controls the targeting of HCS1 protein in vivo. A. In vitro transcription-translation experiments. Polypeptides radioactively labeled with [35S]Met were subjected to SDS-PAGE and analyzed by phosphorimaging as described in “Materials and Methods.” Fluorography of translation products obtained with 1 μg of pPCRscript-HCS1.s (lane 1), 1 μg of pPCRscript-HCS1.un (lane 2), and 1 μg of pPCRscript-HCS1.ATG0m (lane 3). The apparent molecular masses of the polypeptides produced are indicated on the right. B and C. Transient expression of GFP-fusion proteins in Arabidopsis protoplasts. B. GFP alone (35S-GFP) and chimera between GFP and the transit peptides of the small subunit of Rubisco (ATS1A-GFP) or dihydropterin pyrophosphokinase/dihydropteroate synthase (HPPK DHPS-GFP) were used as controls for the targeting of the reporter protein to the cytosol, chloroplasts, and mitochondria, respectively. C. Various plasmid constructs engineered with HCS1 cDNA sequences fused upstream and in frame with GFP sequence were introduced into Arabidopsis protoplasts as described in “Materials and Methods.” Schematic representations of cDNA constructs used are shown on the right of corresponding images. TP , Transit peptide. Images are optical photomicrographs (bright field), GFP fluorescence (GFP; green pseudocolor), and chlorophyll fluorescence (chlorophyll; red pseudocolor). Scale bars = 10 μm.
ing of the HCS1 pre-mRNA (HCS1.s) or by mutation of AUG₀ codon (HCS1.ATG₀,m), translation initiates predominantly at AUG₂, yielding the longer HCS1 form.

**In Vivo uORF24 Controls the Targeting of HCS1 Protein**

To follow the consequences of HCS1 5′-UTR splicing in vivo, we designed reporter plasmids bearing spliced or unspliced HCS1 5′-UTR tagged on the sequence coding the first 142 residues of HCS1 main ORF in frame with GFP coding sequence. These constructs, along with different control constructs, were transiently expressed into Arabidopsis protoplasts, and the resulting GFP fluorescence (reporting HCS1 expression and localization) was analyzed (Fig. 4, B and C). As expected, the expression in protoplasts of the control construct 35S:HCS1.ATG₁-GFP (translation starting at AUG₁) marked the plastids with GFP fluorescence (colocalized with the red autofluorescence of chlorophyll). This result was in good agreement with a previous immunocytological study showing that tobacco plants stably transformed with the Arabidopsis HCS1 cDNA coding sequence driven by the cauliflower mosaic virus 35S promoter accumulated recombinant HCS1 protein within chloroplasts (Tissot et al., 1998). On the other hand, the control construct 35S:HCS1.ATG₂-GFP (translation starting at AUG₂) expression led to a diffuse cytosolic GFP fluorescence. Arabidopsis protoplasts transfected with 35S:HCS1.un-GFP construct exhibited GFP fluorescence spread throughout the cytosol. This suggests that, in vivo, an unspliced 5′-UTR favors the utilization of AUG₂, which, by eluding the transit peptide, leads to a cytosolic localization. In contrast, the expression of 35S:HCS1.s-GFP construct resulted in a pattern of GFP fluorescence corresponding to the plastid pattern observed after transfection of protoplasts with p35S:HCS1.ATG₁-GFP control plasmid. This suggests that splicing of HCS1 5′-UTR abrogates in vivo the inhibition of AUG₁ initiation and enables the synthesis of a longer HCS1 precursor form and its plastidial targeting. Finally, mutagenesis of ATG₀ codon in HCS1.un-GFP (35S:HCS1.ATG₀,m-GFP) restored the plastidial fluorescence localization associated with the expression of HCS1.s-GFP. This confirms that uORF24 itself (and not a global structure of HCS1 5′-UTR) controls the initiation of translation in vivo and the subsequent targeting of HCS1 protein.

Both in vivo and in vitro, our data demonstrate that alternative splicing of the 5′-UTR of HCS1 mRNA controls the dual targeting of HCS1 protein through alternative use of distinct initiation codons and that uORF24 is essential for the AUG choice. The presence of uORF24 within the 5′-UTR of HCS1 cDNA (HCS1.un) precludes AUG₀ utilization and favors the synthesis of a short protein form initiated at AUG₂, which consequently localizes in the cytosol. In the absence of uORF24 (HCS1.s or HCS1.ATG₀,m), the translation initiation begins at AUG₁, allowing the production of a HCS1 protein headed by a transit peptide.

**HCS1.un and HCS1.s Are Expressed in Every Organ of Arabidopsis, But the Former That Encodes a Cytosolic HCS1 Protein Is Predominant**

The model previously presented was drawn from the results of RACE and RT-PCR experiments, and the subsequent molecular and functional analysis of the two resulting cDNA products. To test the significance and relevance of this model, we sought to check the presence of HCS1.un and HCS1.s transcripts in planta. We also investigated the cellular distribution of HCS1 protein by means of subcellular fractionation studies.

By means of quantitative RT-PCR measurements, we evaluated HCS1.un and HCS1.s transcript relative abundance in various plant organs (Fig. 5). HCS1.un and HCS1.s mRNA were detected in all analyzed organs (roots, stem, leaves, green siliques, flowers, and mature seeds). This evidenced the ubiquitous existence of an alternative splicing of HCS1 and validated the significance of our model. Moreover, in all plant organs tested, the relative amount of HCS1.un mRNA was much greater (about 50- to 200-fold higher) than HCS1.s mRNA. This last observation was in good agreement with previous findings showing that HCS activity is mostly present (up to 90% of total activity) in cytosol of plant cells (Tissot et al., 1997; Denis, 2002). HCS1.un transcript encoding a cytosolic HCS1 form. In addition, we showed by in vitro translation experiments that HCS1.s is more efficiently translated than HCS1.un. The maintenance of a large pool of unspliced HCS1 mRNA could then, at the same time, assure that a large majority of HCS1 protein is targeted to the cytosol and enable, upon its splicing, a rapid and efficient synthesis of plastidial HCS1 protein.

As a complementary approach, we investigated the subcellular distribution of HCS1 in Arabidopsis by western blot. Importantly, under our assay conditions, affinity-purified HCS1 antibody used in this study was specific to HCS1 proteins and did not cross react with recombinant HCS2 (Fig. 6A). Intact chloroplasts and mitochondria from Arabidopsis leaves were purified on Percoll density gradients, thus providing organelles devoid of contamination from the other compartments (Fig. 6B). Also, a cytosolic-enriched fraction was prepared. Soluble proteins from purified chloroplasts, mitochondria, and the cytosolic-enriched fractions were then analyzed. To overcome the difficulty in immunodetection of the endogenous protein due to its low abundance, we used a sensitive chemifluorescent system for detection. As expected, antibodies raised against recombinant HCS1 protein identified HCS1 mainly in the cytosol (Fig. 6A). The apparent molecular mass of the detected polypeptide was identical to that of recombinant HCS1-ATG₂, in good agreement with an initiation of translation at AUG₂ encoded Met residue (Tissot et al., 1998) was also detected in chloroplasts and in mito-
chondria, albeit to a lower proportion. This labeling pattern correlated well with HCS specific activity measured in the same fractions. Taken together, these results suggest that the model deduced from our molecular analysis is relevant and most probably presents an important physiological significance in planta.

**DISCUSSION**

HCS, catalyzing the covalent attachment of biotin, is ubiquitously represented in living organisms. In Arabidopsis, two HCS genes have been identified. However, our results establish that of the two HCS genes present in the Arabidopsis genome, only HCS1 is essential and hence plays a major role in biotin-dependent carboxylase biotinylation. Moreover, we characterized an alternative splicing of the 5’-UTR of the HCS1 mRNA. Interestingly, splicing of the 5’-UTR removes a small ORF (uORF24) situated upstream from the first AUG start codon. We showed that uORF24 could affect the initiation of HCS1 translation and the choice between two in-frame AUG codons, targeting HCS1 either to the cytosol or to the chloroplasts. Altogether, these data enabled us to propose a model explaining the compartmentalization of HCS activity in planta with HCS1 gene alone by an alternative splicing of its 5’-UTR.

**Two Unequal Copies of HCS Genes in Arabidopsis**

The two HCS genes identified in Arabidopsis, HCS1 and HCS2, share 71% identity and probably result from an ancient event of duplication (Denis et al., 2002). However, the observation of their correspond-

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**Figure 5.** Relative abundance of HCS1.s and HCS1.un mRNA species in Arabidopsis organs. A, Schematic representation of left plus, left minus, and right oligonucleotide positions on HCS1 cDNA variants. Left plus oligonucleotide enabled the quantification of HCS1.un mRNA. Left minus oligonucleotide enabled the quantification of HCS1.s mRNA. B, Real-time RT-PCR experiment on poly(A+) RNA from various Arabidopsis organs (roots [R], stems [St], leaves [L], flowers [F], siliques [Si], and seeds [S]) using HCS1.s- and HCS1.un-specific primers. Data (relative transcripts abundance normalized to the expression level of HCS1.un in seeds) are means of three independent experiments performed with three cDNA dilutions ± SD. ACTIN1 was used as an internal control to normalize for variation in the amount of cDNA template. Note the log scale in y axis.

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**Figure 6.** Evidence for multiple subcellular localizations of HCS1 in Arabidopsis. A, Soluble proteins (100 µg per lane) from a total leaf extract (T), purified mitochondria (Mi), purified chloroplasts (Cp), and a cytosolic-enriched fraction (Cy) were analyzed by western-blot analysis with affinity-purified polyclonal antibodies raised against recombinant HCS1 (Tissot et al., 1998). The same fractions were assayed for HCS specific activity. Pure recombinant HCS1-ATG1 (HCS1 41-kD precursor form, 10 ng), HCS1-ATG2 (HCS1 37-kD mature form, 10 ng), and HCS2 (37-kD gene product, 25 ng) proteins were run on the same gel as controls. B, Specific activities of cytosolic (pyrophosphate:Fru-6-P 1-phosphotransferase; PFP), chloroplast stroma (NADP-dependent glyceraldehyde-3-P dehydrogenase; GraPDH), and mitochondrial matrix (fumarase) markers were measured (Tissot et al., 1997) to analyze cross-contaminations between subfractions. Data are means ± SD of three replicates.
Defection is probably enough to explain the lethality observed upon HCS1 disruption. HCS1 is able to biotinylate in vitro apocarboxylase substrates from various subcellular compartments (Tissot et al., 1998; Denis et al., 2002). In Arabidopsis, MCC-α, the biotinyl subunit of MCCase, is localized in the mitochondrion; ACC1, the homomeric ACCase isoform, is localized in the cytosol; and BCCP1 and BCCP2, the biotinyl subunits of heteromeric ACCase isofoms, are localized in the plastid (Alban et al., 2000). Our data offer a mechanism by which HCS1 can be targeted to cytosol and plastids, compartments where it could biotinylate its different substrates and where HCS activity had been detected (Tissot et al., 1997; Denis, 2002). Finally, the question arises whether HCS1 could also be targeted to mitochondria. Indeed, HCS activity also occurs in mitochondria. Indeed, HCS activity also occurs in mitochondria. Furthermore, HCS1 does not present a canonical transit peptide sequence, indicative of a possible joint

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<td>5'-CCAGTTTGTTCCAGTTGTCCTGAATAAC-3'</td>
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<td>Hcs2.Q3</td>
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<td>60°C</td>
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<tr>
<td>Left minus</td>
<td>5'-GAAAGGACTGCTCCATATAACGCTT-3'</td>
<td>60°C</td>
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<tr>
<td>Left plus</td>
<td>5'-GAAGGACTGCTCCATATAACGCTT-3'</td>
<td>60°C</td>
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<tr>
<td>Right</td>
<td>5'-AGCTCTCTGCTGTTGAGGACTTATGC-3'</td>
<td>60°C</td>
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<tr>
<td>Actin.5</td>
<td>5'-GTCTTTGCTGTTGAGGACTTATGC-3'</td>
<td>55°C</td>
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<tr>
<td>Actin.3</td>
<td>5'-GGATTAGCTTATCGGCC-3'</td>
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<td>Hcs1.BAM</td>
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*The annealing temperature used for the real-time PCR is indicated.*
plastid and mitochondrial targeting. Examples of dual targeting of a single translation product in chloroplasts and mitochondria through the utilization of a single ambiguous presequence have been described (Peeters and Small, 2001; Karmiely and Pines, 2005). Western-blot analyses strongly suggest that in addition to the cytosol and chloroplasts, HCS1 is also targeted to mitochondria in Arabidopsis. However, despite a thorough survey of protoplasts expressing the HCS1-GFP constructs, we were unable to detect fluorescence in mitochondria. This may reflect a lack of sensitivity in the GFP assay; in case of an uneven dual distribution of a protein, its most abundant localization can impede the detection of the minor one (Duchene et al., 2005). It is also possible that the GFP promoter fusion missed essential cis-elements that aid its targeting to mitochondria but are not necessary for plastidic targeting (Christensen et al., 2005; Kabeya and Sato, 2005).

The HCS2 gene does not seem to bear any fundamental function in carboxylase biotinylation in plants, reporting on HCS1 gene, which is essential for plant viability, the whole responsibility of biotin-dependent carboxylase biotinylation within the cell. Is there a function for HCS2? The event of whole duplication of the Arabidopsis genome gives many examples of pairs of duplicate genes, and their relative functions and evolution have been documented. Many duplicated genes are retained and their redundancy might facilitate genetic robustness against null mutations (Gu et al., 2003). Unequal genetic redundancies, where the absence of a mutant phenotype in loss-of-function mutants of one gene contrasts with a strong phenotype in loss-of-function mutants of its homolog, have also been recently discussed (Briggs et al., 2006). Examples have been described in which genes do not act redundantly in vivo because of a difference of expression level or pattern. Such a scenario has been observed, for instance, for the proteasome subunit RPN1A and RPN1B: RPN1A loss-of-function results in embryo lethality, whereas rpm1b mutants are fully viable and resemble the wild type (Brikkhin et al., 2005). The nondispensable allele RPN1A is expressed at much higher levels than RPN1B. This does not seem to be the case for HCS1 and HCS2 duplicated pair. Although HCS2 is located in the pericentromeric region of chromosome 1, it is constitutively and ubiquitously expressed in Arabidopsis plants (Denis et al., 2002). However, none of the mRNA produced by HCS2 gene expression was shown to encode an in vitro active HCS protein. Thus, one cannot exclude that HCS2 could be an inactive pseudogene in Arabidopsis. This possibility would be consistent with the observations that the HCS2 gene, unlike the HCS1 gene, is characterized by a high level of nucleotide polymorphism inside ectotype Wassilewskija, indicating relatively weak functional constraint on the HCS2 gene (Denis et al., 2002). This is also consistent with global sequence analyses showing that pericentromeres are remarkably dynamic, undergoing rapid changes in structure and sequence content (HALL et al., 2006). Another possibility is that HCS2 could have a function as a noncoding RNA. Recent evidence points to a widespread role of these molecules in eukaryotic cells (MATTICK and MAKUNIN, 2006; COSTA, 2007). Interestingly, in mice, Makorin1-p1, an expressed pseudogene, was shown to regulate the mRNA stability of its protein-coding homologous gene, Makorin1 (HIROSUTUNE et al., 2003). However, we showed that in the hcs2 mutant, the HCS1 mRNA level was unchanged. As a result, HCS2 does not seem to have any direct function in HCS1 mRNA stability. Finally, besides its classical role in carboxylase biotinylation, evidence is emerging that HCS in mammalian cell nuclei participates in the epigenetic control of chromatin structure and gene expression through biotinylation of histones (NARANG et al., 2004; ZEMPLENI, 2005). To date, these challenging fields of research remain unexplored in plants. Thus, one cannot exclude such functions for HCS2 gene in Arabidopsis. This question clearly deserves further study.

A Control of HCS1 Translation Initiation by uORF24

We have identified a uORF within 5′-UTR of HCS1 mRNA and showed that it could influence AUG choice and HCS1 main ORF translation initiation. The uORFs are common features of eukaryotic genes, occurring in 10% to 25% of 5′ leader sequences (CROWE et al., 2006; NEASFSEY and GALAGAN, 2007). They have been generally found to decrease translational efficiency of the downstream coding sequence (MEIJER and THOMAS, 2003; vILELA and MCCARTHY, 2003). Indeed, in accordance with the scanning model of translation initiation (Kozak, 2002), uORFs interfere with translation of a downstream main ORF. They have been shown to negatively impact translational efficiency through a variety of means, including ribosome blocking by the encoded peptide, ribosome stalling at the uORF termination codon, and failure to reinitiate at the genic translation start site after disengaging from the uORF (GABA et al., 2001). The reinitiation mechanism describes the ability of 40S subunits to continue to scan and initiate at a downstream main AUG codon after translating a small independent uORF. Our data show that the presence of uORF24 not only decreases the translation of downstream HCS1 but also influences AUG choice. This makes it possible that uORF24 acts by inducing a reinitiation of translation after disengaging of the ribosome at its termination codon. In the presence of uORF24 within 5′-UTR (HCS1.un mRNA), ribosomes would initiate translation at the AUG1 codon (more favorable to initiation than AUG2; Pedersen and Nielsen, 1997). After translation of uORF24, the 40S subunit may hold on the mRNA, resume scanning, and reinitiate at a downstream AUG codon. However, reinitiation at AUG1 would probably not be possible because it is too close (four nucleotides) from uORF24 termination (see Fig. 3) and would not give the 40S ribosomal subunit its required distance to reacquire Met-tRNAi and initiation factor eIF2 (Kozak, 1999, 2002). Possibly, after translation of uORF24, ribosomes could recover a suf-
sibility of the NMT1 phosphate. Thiamine pyrophosphate binding on the splicing by a riboswitch that bound thiamine pyrophosphate was regulated at the level of pre-mRNA ORF (Cheah et al., 2007). Our results and model, in light of previous studies which adopt alternate structures depending on the presence of metabolites, offer exciting prospects for the study of metabolic-related regulations and rapid variations of the cellular metabolism under developmental or stress conditions.

CONCLUSION

Control of gene expression by sequence elements in the mRNA 5’ leader is still not well understood, and new mechanisms of control continue to be uncovered. Our work describes a novel situation in which the presence or removal, by alternative splicing, of a uORF governs start codon selection in the main ORF, which in turn controls organelle versus cytosolic localization of a multi-targeted enzymatic gene product. This provides a possibility for fine molecular regulation and, beyond the specific issue of HCS protein, unveils the general complexity of plant metabolism compartmentalization.

MATERIALS AND METHODS

Media, Bacterial Strains, and Chemicals

d-[8,9-3H]Biotin (33 Ci mmol-1), [α-32P]dCTP (1,000 Ci mmol-1), and [35S]L-Met (1,75 Ci mmol-1) were purchased from GE Healthcare. Isopropyl-thio-β-galactoside was obtained from Bioprobe Systems. All other biochemicals were obtained from Sigma-Aldrich. The oligonucleotide primers utilized in this study are listed in Table I.

Temperature-sensitive Escherichia coli birA215 mutant (strain BM4050) was generously provided by Dr. A.M. Campbell. Mutations in the birA gene affect the biotin ligase function of the BirA protein. The mutant had been lysogenized with the helper phage (ADE3) harboring a copy of the T7 RNA polymerase gene, using the ADE3 lysogenization kit from Novagen (Tissot et al., 1998). The resulting E. coli birA215 (DE3) was transformed with pET vectors encoding biotin-dependent carboxylases under the control of the T7 promoter. The recombinant carboxylases were then essentially expressed as their apo forms.

Plant Materials and Growth Conditions

A putative Arabidopsis (Arabidopsis thaliana) T-DNA insertion line for HCS1 (SAIL_1227_E03; line hcs1-1) of the ecotype Columbia identified in the Syngenta Arabidopsis Insertion Library (Sessions et al., 2002) was obtained from the Arabidopsis Biological Resource Center. The Arabidopsis hcs2 (EAC52 line) mutant of the ecotype Wassilewskija was obtained by screening of the T-DNA insertion line collection from the Station Génétique et d’Amélioration des Plantes (INRA, Versailles, France). Plants were grown in soil under greenhouse conditions (23°C) with a 16-h photoperiod and a light intensity of 200 μmol photons m-2 s-1 until harvested for analysis. For screening, seeds were surface sterilized and germinated on half-strength Arabidopsis medium solidified with agar (0.7%, w/v; Estelle and Somerville, 1987). After a cold treatment of 48 h at 4°C in the dark, the plates were transferred to a growth chamber and incubated at 24°C under a 16-h-light/8-h-dark regime. Selection of T-DNA-containing seeds was performed by germination on half-strength Arabidopsis medium supplemented with 50 mg L-1 kanamycin (hcs1-2 and hcs2 mutants) or by spraying seedlings (hcs1-1 mutant) with 240 μg mL-1 glufosinate ammonium (BASTA). Arabidopsis (ecotype Columbia) cell suspension cultures were grown under continuous white light (40 μE m-2 s-1) at 23°C with rotary agitation at 125 rpm in Gamborg’s B5 medium supplemented with 1 μM 2-naphthalene acetic acid and 1.5% (w/v) Suc.

Isolation of T-DNA Insertion Line of HCS2

DNA pools of the Arabidopsis T-DNA insertion lines from the Versailles collection were screened for T-DNA insertion in the HCS1 locus (At1g37150). Forward (hcs2.met) and reverse (hcs2.stop) primers from the coding sequence

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of the HCS2 locus were designed for PCR screening of the DNA pools by the combination of T-DNA left (Tag5) and right (Tag3) border-specific primers. PCR products were analyzed by southern hybridization of duplicate membranes to both the HCS2 gene probe generated from the entire HCS2 gene and the T-DNA probe. A positive PCR product was identified from megapool-16 and further amplified positively in Superpool-48 and in primary pool 189A by HCS2 locus primer hcs2.stop and T-DNA primer Tag5. T-DNA insertion in the HCS2 gene was confirmed by sequencing the resulting positive PCR fragment. The 48 lines from the primary pool 189A were further screened, and line 21 (EAG32) was identified for T-DNA insertion in the HCS2 gene. Homozygous mutant plants were isolated from line 21 by PCR analysis using two sets of primers (exon7/hcs2.stop gene primers and Tag5[T-DNA primer]/hcs2.stop) combined with kanamycin selection (resistance is conferred by the T-DNA). Homozygous mutant plants were confirmed by Southern-blot analysis using the HCS2 gene probe generated by PCR amplification of the entire HCS2 gene.

Plant DNA Isolation and Blotting

DNA was isolated from plant tissues by a standard method (Dellaporta et al., 1983). Blotting and hybridization were performed as described previously (Tissot et al., 1997).

Identification of HCS1 Transcription Start Site

The transcription start site of HCS1 was determined by 5′-RACE using Arabidopsis leaf cDNAs (Denis et al., 2002). Two sequential PCRs were performed with two pairs of nested adaptor primers and gene-specific primer in exon 4 of hcs1 (Hcs1.E4). The resulting PCR products were cloned into pfPCRscript (Stratagene) and sequenced.

Cloning of HCS1 cDNA Splicing Variants

Poly(A) mRNAs from Arabidopsis rosette leaves were prepared using the Straight A’s mRNA isolation system according to the manufacturer’s instructions (Novagen), followed by a treatment with RNase-free DNase I. First-strand cDNA was synthesized from 250 ng of DNA-free mRNA in a final volume of 20 μL using oligo(dt)20 primers (Thermoscript RT-PCR system; Life Technologies). PCR reaction was then conducted using 1 μL aliquots of the RT reaction and HCS1-specific primers in the presence of 1 unit of Pwo polymerase (Roche). HCS1-specific oligonucleotides (RACE/KPN/STOP1.SAC) were chosen according to hcs1 cDNA and 5′-RACE sequences to amplify both full-length splicing variants. They introduced a KpnI restriction site and a SacI restriction site. After digestion with KpnI and SacI enzymes, RT-PCR products were cloned into pfPCRscript digested by the same enzymes, yielding pfPCRscript-HCS1.s and pfPCRscript-HCS1.un constructs, respectively.

Real-Time PCR

Relative quantification experiments were done by real-time PCR using Rotor Gene system (Corbett Research) and SYBR Green Jump Start Taq DNA polymerase, pfPCRscript constructs as templates, and the specific flanking primers listed in Table I. The PCR products were digested with Xhol and BamHI and inserted between the Xhol and BamHI sites of pGIP to give plasmids p35S:HCIS1.un-GFP and p35S:HCIS1.s-GFP. p35S:HCIS1.ATGm-mGFP was obtained by site-directed mutagenesis of p35S:HCIS1.s-GFP plasmid. Control 35S:HCIS1-GFP fusion constructs lacking the 5′-UTR region of HCS1 cDNAs and starting at the ATG ([p35S:HCIS1.ATG-mGFP] or the ATG ([p35S:HCIS1.ATG-GFP]) codon were also performed, using specific primers (Table I). GFP chimeras bearing the transit peptide sequences of the small subunit of Rubisco from Arabidopsis (55 residues, AT51A gene, GenBank accession no. X13661) and dihydropterin pyrophosphokinase/dihydropteroate synthase from pea (Pisum sativum; 28 residues; Rébéille et al., 1997) were used as controls for the targeting of GFP to plastids and mitochondria, respectively. Transient transformation of Arabidopsis protoplasts prepared from a 4-d-old cell suspension culture was achieved using 40 μg of plasmid construct by the polyethylene glycol method, essentially as described (Abel and Theologis, 1994). Transformed cells were incubated at 23°C for 36 h and examined by epifluorescence microscopy using a Zeiss Axioplan2 fluorescence microscope, and images were captured with a digital CCD camera (Hamamatsu). The filter sets used were Zeiss filter set 13, 480/13-0000 (exciter BP 470/20, beam splitter FT 493, emitter BP 505–530) and Zeiss filter set 15, 480/15-0000 (exciter BP 546/12, beam splitter FT 580, emitter LP 590), for GFP and chlorophyll fluorescence, respectively.

Mutagenesis of HCS1 5′-UTR

Plasmids pPCRscript-HCIS1.ATG,m and p35S:HCIS1.ATG,m-GFP were obtained with the QuikChange mutagenesis protocol (Stratagene) and plasmids pPCRscript-HCIS1.un and p35S:HCIS1.un-GFP, respectively. Oligonucleotides were designed to replace the initiation codon (ATG1) of the uORF of HCS1 5′-UTR region by an ATC codon to modify the restriction enzyme digestion profile for identification of mutants. Positive mutants were sequenced to ensure that no other mutations were present.

Cloning, Expression, and Purification of Recombinant apo-BCCP1, apo-BCCP2, apo-MCC220, and apo-ACC138

Recombinant Arabidopsis apo-BCCP1 (the biotinylation carrier subunit of chloroplastic ACCase; At5g16390) devoid of the plastid transit peptide and apo-MCC220 (the C-terminal 220 amino acids of methylcrotonyl-CoA carboxylase α-subunit, comprising the biotinylation domain; At1g03090) were produced as described previously (Tissot et al., 1998; Denis et al., 2002).

The pET-28b+–BCCP2 and pET-28b+–ACC138 plasmids encoding the Arabidopsis biotinyl proteins BCCP2 (a second isoform of the biotin carboxyl carrier subunit of plastid ACCase; At5g15530) devoid of the plastid transit peptide and ACC138 (the 138 amino acids biotinylation domain of cytosolic ACCase isoform 1, comprising amino acids 609–746 of the protein sequence; At1g36160) were constructed by means of PCR using Arabidopsis leaf cDNAs.
Primers used for amplifications were synthesized in accordance with the published cDNA or gene sequences and to the comparison of biotinylation domains of various known biotin-dependent carboxylases (Table I; Samols et al., 1988; Cronan, 1990; Yanai et al., 1995; Thelen et al., 2001). They introduced a NdeI restriction site in 5′ and SacI restriction site in 3′. After digestion with NdeI and SacI, the PCR fragments were subcloned into pET28b+ vector (Novagen) digested with the same enzymes. The E. coli birA215 (DES) strain transformed with plasmid constructs was grown and induced at 37°C in Luria-Bertani medium supplemented with kanamycin (50 µg mL−1). When A600 reached 0.6, 0.5 mM isopropylthio-β-D-galactoside was added and growth was continued at 28°C for 3 to 4 h. Cells were collected by centrifugation and resuspended in buffer A containing Tris-HCl 25 mM, pH 7.5, 0.3 M NaCl, and 10 mM imidazole. Cells were then disrupted by sonication with a Vibra-Cell disruptor (100 pulses every 3 s on power setting 5). The soluble protein extracts were separated from the cell debris by centrifugation at 40,000g for 15 min. Finally, recombinant proteins produced were purified by metal-chelate column chromatography (Ni-NTA agarose, Qiagen) according to the manufacturer’s instructions.

Activity Measurements

Samples of Arabidopsis plants were harvested, ground in liquid nitrogen with a mortar and pestle, and homogenized in 1 volume of chilled buffer B composed of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM diithiothreitol, 5 mM z-aminocaproic acid, 1 mM benzamidine-HCl, and 1 mM phenylmethylsulfonyl fluoride. After 15 min centrifugation (40,000g), the supernatants were subjected to ammonium sulfate precipitation with crystalline ammonium sulfate to 80% saturation. The resulting precipitates were collected by centrifugation and resuspended in a minimum volume of buffer B. The suspensions were desalted onto PD10 columns (Pharmacia) and used for further analysis. HCS activity in the protein extracts was measured according to the protocol described previously (Tissot et al., 1996) using purified BCCP1, BCCP2, ACC138, and MCC220 as the apocarboxylase substrates. Biotin-dependent carboxylase activities were measured as the incorporation of radioactivity from NaH14CO3 into acid-stable products (Baldet et al., 1993). Acetyl-CoA and methylcrotonyl-CoA were used as acceptor substrates.

Western-Blot Analyses

Total soluble proteins from Arabidopsis leaves were extracted as described above proteins were resolved by SDS-PAGE and electroblotted to nitrocellulose membrane. Biotin-containing polypeptides were detected with a system analogous to western blotting using streptavidin conjugated with horseradish peroxidase (Baldet et al., 1992). The presence of streptavidin-biotin complexes was analyzed by chemiluminescence. For the purpose of HCS1 immunodepletion, chloroplasts and mitochondria from Arabidopsis leaves were isolated and purified with Percoll gradients as described previously (Pinon et al., 2005). Soluble proteins were obtained from the purified organelles after osmotic lysis and centrifugation (Pinon et al., 2005). A cytosolic-enriched fraction was prepared after ammonium sulfate precipitation with crystalline ammonium sulfate to 80% saturation. The resulting precipitates were collected by centrifugation and resuspended in a minimum volume of buffer B. The suspensions were desalted onto PD10 columns (Pharmacia) and used for further analysis. HCS activity in the protein extracts was measured according to the protocol described previously (Tissot et al., 1996) using purified BCCP1, BCCP2, ACC138, and MCC220 as the apocarboxylase substrates. Biotin-dependent carboxylase activities were measured as the incorporation of radioactivity from NaH14CO3 into acid-stable products (Baldet et al., 1993). Acetyl-CoA and methylcrotonyl-CoA were used as acceptor substrates.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis of a second independent T-DNA insertion allele of Arabidopsis HCS1 (FLAG-486E08; hcs1-2 line).

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center (The Ohio State University, Columbus) and the Arabidopsis Resource Centre for Genomics (INRA, Versailles, France) for providing seeds of the T-DNA-inserted hcs1 Arabidopsis mutants. We thank David Bouchez and Fabienne Granier from the Station Génétique et d'Amélioration des Plantes (INRA, Versailles, France) for providing access to the collection of T-DNA insertion mutants and enabling us to isolate the T-DNA inserted hcs2 Arabidopsis mutant. We also thank Laetitia Brunel for technical assistance, François Parcy for his help with genetic crossing studies, and Michel Matringe and Stéphane Ravel for critical reading and helpful comments on the manuscript.

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LITERATURE CITED

Dual Targeting of Arabidopsis HOLOCARBOXYLASE SYNTHETASE1


