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Disciplines

Dentistry

Comments

At the time of publication, author Henry Daniell was affiliated with the University of Central Florida. Currently, (s)he is a faculty member at the School of Dental Medicine at the University of Pennsylvania.

Characterization of Heterologous Multigene Operons in Transgenic Chloroplasts. Transcription, Processing, and Translation[1]

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The first characterization of transcriptional, posttranscriptional, and translational processes of heterologous operons expressed via the tobacco (*Nicotiana tabacum*) chloroplast genome is reported here. Northern-blot analyses performed on chloroplast transgenic lines harboring seven different heterologous operons revealed that polycistronic mRNA was the predominant transcript produced. Despite the lack of processing of such polycistrons, large amounts of foreign protein accumulation was observed in these transgenic lines, indicating abundant translation of polycistrons. This is supported by polysome fractionation assays, which allowed detection of polycistronic RNA in lower fractions of the sucrose gradients. These results show that the chloroplast posttranscriptional machinery can indeed detect and translate multigenic sequences that are not of chloroplast origin. In contrast to native transcripts, processed and unprocessed heterologous polycistrons were stable, even in the absence of 3′ untranslated regions (UTRs). Unlike native 5′UTRs, heterologous secondary structures or 5′UTRs showed efficient translational enhancement independent of cellular control. Abundant read-through transcripts were observed in the presence of chloroplast 3′UTRs but they were efficiently processed at introns present within the native operon. Heterologous genes regulated by the psbA (the photosystem II polypeptide D1) promoter, 5′ and 3′UTRs have greater abundance of transcripts than the endogenous psbA gene because transgenes were integrated into the inverted repeat region. Addressing questions about polycistrons, and the sequences required for their processing and transcript stability, are essential in chloroplast metabolic engineering. Knowledge of such factors would enable engineering of foreign pathways independent of the chloroplast complex posttranscriptional regulatory machinery.

Plastid genes in higher plants are mainly organized as operons, of which more than 60 have been described in the tobacco (*Nicotiana tabacum*) chloroplast genome (Sugita and Sugiura, 1996). These may group genes of related or unrelated functions, the former being the most common (Barkan, 1988; Rochaix, 1996). Most of these genes are transcribed into polycistronic precursors that may be later processed and modified to render the transcripts competent for translation (Eibl et al., 1999; Barkan and Goldschmidt-Clermont, 2000; Monde et al., 2000b).

The processing mechanisms for translation regulation in chloroplast genes of higher plants are still largely unknown. The general consensus is that most native primary transcripts require processing to be functional (Barkan, 1988; Zerges, 2000; Meierhoff et al., 2003) and that posttranscriptional RNA processing of primary transcripts represents an important control of chloroplast gene expression (Hashimoto et al., 2003; Nickelsen, 2003). However, it is believed that more than one pathway may be involved in transcript processing (Danon, 1997; Choquet and Wollman, 2002).

For example, several studies have shown that the regulation of gene expression in the chloroplast relies more on RNA stability than on transcriptional regulation (Deng and Gruissem, 1987; Jiao et al., 2004). In chloroplast, such stability is mainly influenced by the presence of 5′ untranslated regions, or UTRs (Eibl et al., 1999; Zou et al., 2003), nucleus-encoded factors (Lezhneva and Meurer, 2004), and 3′UTRs (Adams and Stern, 1990; Chen and Stern, 1991), without which rapid degradation or low accumulation of primary transcripts has been observed. The role of plastid 3′UTRs differs from the role of its bacterial counterparts by being more involved in transcript stability and less involved in the effective termination of transcription (Stern and Gruissem, 1987).

Translation has also been a crucial step in the regulation of gene expression, as in many cases protein levels in the chloroplast did not correlate with steady-state transcript abundance (Monde et al., 2000b). Therefore, the transcription of native chloroplast operons and their posttranscriptional and translational patterns have been the target of several studies that showed that intercistronic processing enhanced translation of chloroplast operons, including the maize (*Zea mays*) *psb*B and *pet* clusters, which comprise genes from the PSII reaction center and the cytochrome b6/f

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complex, respectively (Barkan, 1988; Barkan et al., 1994). In addition, different species may experience various processing mechanisms for the same gene cluster. For example, species such as Arabidopsis (*Arabidopsis thaliana*; Meierhoff et al., 2003), tobacco (Monde et al., 2000a), and spinach (Westhoff and Herrmann, 1988) have a different mechanism than maize for the translation of *pet*D, which depends mainly upon the establishment of dicistrons and tricistrons of this gene. Alternative processing of the polycistron containing the *pet*D gene, which produces monocistronic *pet*D, causes the degradation of the transcript, inhibiting translation (Tanaka et al., 1987; Monde et al., 2000a, 2000b; Meierhoff et al., 2003). In contrast, in Chlamydomonas, nearly all chloroplast genes appear to be transcribed as monocistronic mRNAs, with translation being an essential regulatory step of gene expression (Rochaix et al., 1989; Zerges and Rochaix, 1994). Other mechanisms, such as editing, which can produce alternate start codons, have been linked to alternative processing and to a complete, different translation pattern (Hirose and Sugiura, 1997; del Campo et al., 2002). These examples provide evidence of different modifications of primary transcripts for efficient translation in chloroplasts.

Traditionally, plant genetic engineering had involved the introduction of single genes through nuclear transformation. In the past decade, the introduction of multiple genes has also been successful through this approach, allowing the incorporation of complete metabolic pathways (Nawrath et al., 1994; Ma et al., 1995; Ye et al., 2000). However, this approach required a long process of integration of individual transgenes followed by breeding to reconstruct the desired pathways. Additionally, transgene segregation from nuclear transformed plants may be possible in subsequent generations, which may result in loss of function of the introduced pathway. Furthermore, plant nuclear genes are typically transcribed monocistronically, which requires separate promoter sequences for each of the introduced genes. Expression of foreign genes may also be influenced by position effects and gene silencing, causing levels of gene expression to vary among independent transgenic lines (Daniell and Dhingra, 2002).

On the other hand, plant genetic engineering through chloroplast transformation presents several additional advantages over nuclear transformation, such as their ability to efficiently transcribe and translate operons (DeCosa et al., 2001; Lossl et al., 2003; Ruiz et al., 2003), as well as to confer hyperexpression capability (Daniell et al., 2004c). In addition, chloroplasts are able to accumulate foreign proteins that are toxic in the cytoplasm, such as cholera toxin β-subunit (Daniell et al., 2001), trehalose (Lee et al., 2003), and xylanase (Leelavathi et al., 2003), without any deleterious effects, due to the compartmentalization of transgene products (Bogorad, 2000). Concerns about position effect are also eliminated due to site-specific integration of transgenes via homologous recombination of chloroplast DNA flanking sequences (Daniell et al., 2002), and because chloroplasts are maternally inherited in most crops, the risk of outcrossing transgenes to related species through pollen is minimized (Daniell, 2002). Additionally, transformation of plastids in nongreen tissues, such as carrot (*Daucus carota*) roots, offer promising options for oral delivery of vaccine antigens (Kumar et al., 2004a).

As foreign genes are engineered into operons, the resulting transcript differs from the native operons by lacking native intergenic sequences. These sequences are removed during cloning or by PCR amplification of the coding sequences. The effect of such modifications in the transcription and translation of heterologous operons has not yet been investigated. Therefore, the purpose of this study is to examine the transcription, processing, and translation of several foreign operons engineered via the chloroplast genome. The results of this investigation provide sufficient evidence that suggests that engineered polycistrons in chloroplast transgenic lines are efficiently translated and that processing into monocistrons is not required to obtain overexpression of transgenes. Additionally, the role of 5′UTRs and 3′UTRs in posttranscriptional modifications, translation, and transcript stability are addressed. Addressing questions on polycistron translation as well as the sequences required for processing and transcript stability are essential for chloroplast metabolic engineering.

RESULTS

Multigene Engineering via the Tobacco Chloroplast Genome

Multigene engineering via the chloroplast genome has been achieved by using several different foreign genes, promoters, and 5′ and 3′ regulatory sequences (Daniell et al., 2004a; Kumar and Daniell, 2004). Chloroplast transgenic lines analyzed in this study were genetically engineered with multigene cassettes that contained the following basic features: the *aad*A (aminoglicoside 3′-adenylyltransferase) gene, which confers resistance to spectinomycin and helps in transgenic plant selection (Goldschmidt-Clermont, 1991), downstream from the constitutive chloroplast 16S ribosomal RNA gene promoter (*Prrn*). The heterologous gene or genes of interest were inserted downstream of the *aad*A gene and were flanked at the 3′ end by the *psb*A 3′UTR, which is involved in mRNA abundance and stability in the chloroplast (Deng and Gruissem, 1987; Stern and Gruissem, 1987). In some cases, the heterologous gene was also engineered to contain the *psb*A promoter and 5′ regulatory sequence (5′UTR) to enhance translation (Eibl et al., 1999; Fernandez-San Millan et al., 2003; Dhingra et al., 2004; Watson et al., 2004). The multigene cassettes were flanked at the 5′ and 3′ by sequences homologous to the tobacco chloroplast *trn*I (tRNA Ile) and *trn*A (tRNA Ala) genes, respectively, which allow site-

specific integration by homologous recombination into the inverted repeat region of the chloroplast genome (Daniell et al., 1998). More than 30 genes have been successfully integrated and expressed at this transcriptionally active spacer region (Daniell et al., 2004a, 2004b). In this study, the following foreign genes were inserted into the basic expression cassettes: human serum albumin (*hsa*), cholera toxin β-subunit (*ctx*B), *ctx*B-*gfp* (green fluorescent protein) fusion, *Bacillus thuringiensis* insecticidal protein (*cry*2Aa2) along with the associated chaperonin protein-coding open reading frames 1 and 2 (*orf*1 and *orf*2), and trehalose phosphate synthase (*tps*1). The transgenic lines engineered to express the CRY insecticidal protein contained the entire *cry*2Aa2 native operon.

Transcription and Translation of the *cry*2Aa2 Operon

The chloroplast transgenic lines transformed with the transgene cassette containing the *aad*A gene and the complete *cry*2Aa2 operon (ORF1,2- Cry2Aa2 lines) were used to study the transcriptional and translational patterns of a heterologous operon in transgenic chloroplasts. This operon comprises the *orf*1, *orf*2, and *cry*2Aa2 genes under the transcriptional regulation of the *Prrn* promoter (Fig. 1A). Several transcripts were anticipated based on transcription initiation at the engineered promoter (P*rrn* promoter) and the native 16S ribosomal RNA promoter (native P*rrn*) in transgenic lines (Fig. 1A). Northern-blot analyses of three independent lines harboring the *cry*2Aa2 operon revealed that the predicted 4.9-kilonucleotide (knt) polycistron, which contained all four transgenes (*aad*A-*orf*1-*orf*2-*cry*2Aa2), was the most abundant transcript detected with the *cry*2Aa2 specific probe (Fig. 1B, c). Interestingly, the *cry* specific probe also revealed a shorter transcript of about 2.4 knt, which was about the same size as the *cry*2Aa2 gene (Fig. 1B, a), suggesting that this transcript could be the *cry*2aA2 monocistron. Densitometric analyses of the foreign mRNA transcripts revealed that the *cry*2Aa2 monocistron and the *aad*A-*orf*1-*orf*2-*cry*2Aa2 polycistron had similar abundances (Fig. 1C, a and c), indicating that processing in the intergenic region between *orf*2 and *cry*2Aa2 occurred in about 50% of the polycistrons transcribed from the P*rrn* promoter (Fig. 1, A and B, a and c). Another prominent 7.4-knt transcript was predicted, based on the calculation of the length of the coding sequence, initiating at the native 16S P*rrn* promoter (Fig. 1B, e). A low intensity, approximately 6.0-knt transcript detected (Fig. 1B, d) may be produced by read-through of the transcript starting at the *Prrn* promoter and terminating downstream of the engineered 3′ UTR. The low intensity, approximately 3.5-knt transcript (Fig. 1B, b) terminates at the same location as the 6.0-knt transcript, although it is smaller due to the processing between *orf*2 and *cry*2Aa2. Because this fragment only contained the *cry* gene and the sequences downstream from the 3′UTR, it could not be detected with the *aad*A probe (Fig. 1D). The read-through transcripts processed downstream of the 3′UTR represent an average of 27.3% ± 3% of those produced in these transgenic lines (Fig. 1B, b and d).

Northern-blot analyses with the *aad*A specific probe confirmed the results observed with the *cry*2Aa2 probe. The predicted 4.9-knt polycistron that harbors the *aad*A gene plus the complete *cry* operon was detected as expected (Fig. 1D, c). Although the predicted 2.5-knt tricistron (Fig. 1D, f) containing the *aad*A gene plus the *orf*1 and 2 was expected due to processing between the *orf*2 and the *cry*2Aa2 genes, a transcript of a similar intensity to that of the polycistron was observed instead (Fig. 1D, c and f). Densitometric analyses revealed a 1:1 ratio of the polycistron (*aad*A-*orf*1-*orf*2-*cry*2Aa2) versus the *aad*A-*orf*1-*orf*2 tricistron (Fig. 1E, c and f) due to processing in the intergenic region between *orf*2 and *cry*2Aa2 (Fig. 1A). These results showed that the two transcripts produced by the processing in the intergenic region between *cry*2Aa2 and *orf*2 resulted in transcripts with a similar abundance to the complete polycistron containing all four genes and the 3′UTR (Fig. 1C, a and c, and 1E, f and c, respectively). The fact that the tricistron containing the *aad*A, *orf*1, and *orf*2 genes did not contain a chloroplast 3′UTR but still was very stable suggests that polycistrons are stable in the chloroplast even in the absence of 3′UTRs. The results obtained by using the *orf*1-*orf*2 fragment (*orf*1,2) as a probe confirmed the detection of the *aad*A-*orf*1-*orf*2 tricistron (predicted 2.5 knt), indicating effective processing at the intergenic region between *orf*2 and *cry*2Aa2 (Fig. 1F, f). Other transcripts of larger size were also observed and corresponded to those obtained with the *cry*2Aa2 gene-specific probe (Fig. 1F, d and e).

Northern blots were also performed on the *cry*2Aa2 transgenic lines using the *psb*A 3′UTR probe (Fig. 7B). Results revealed a pattern similar to that obtained with the *cry*2Aa2 gene-specific probe, as well as the presence of the endogenous *psb*A transcript. In the case of the *cry*2Aa2 operon, transcripts were in much lower proportion to the native *psb*A operon. Because the native *psb*A and the heterologous *cry*2Aa2 operon are driven by different promoters, transcript abundance cannot be quantitatively compared. In contrast to the results obtained in Figure 1B, only the major transcripts (a and c) were detected.

Polysome fractionation assays of the *cry*2Aa2 operon support polycistron translation, as the larger transcripts corresponding in size to the complete operon (from the *prrn* promoter) were observed mainly in the lower fractions of the Suc gradients, when hybridized with the *cry*2Aa2 probe (Fig. 2A) and with the *orf*1,2 probe (Fig. 2B). Additionally, smaller transcripts corresponding in size to the *cry*2Aa2 gene processed from the rest of the operon also appear associated to polysomes, suggesting that processing may also occur and could be coupled to translation. Puromycin release controls confirm that the polycistronic transcripts found in the lower fractions were indeed associated to polyribosomes (Fig. 2C).

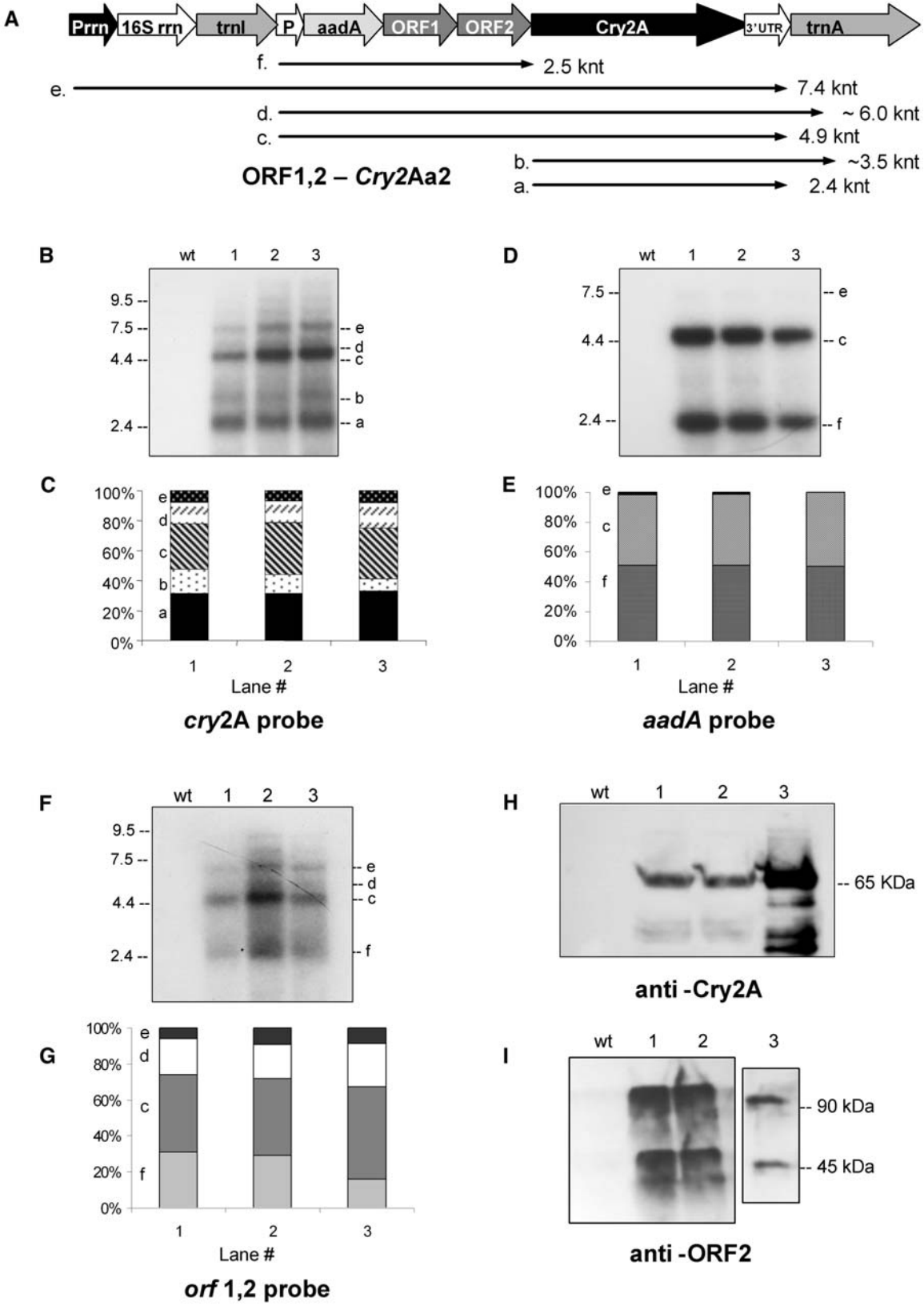

Figure 1. Transcriptional and translational analysis of the *Cry*2Aa2 operon. A, Schematic representation of the *orf*1-orf2-*cry*2Aa2 operon in transgenic lines, including the *aad*A gene and the upstream P*rrn* promoter (P); upstream native chloroplast 16S ribosomal RNA gene with its respective promoter (Prrn) and the *trn*I and *trn*A are shown. Arrows represent expected transcripts and their respective sizes. B, RNA hybridized with the *cry*2A probe, loaded as follows: wt, wild-type control; lanes 1 to 3,

Western-blot analyses revealed that the *Cry*2Aa2 (65 kD; Fig. 1H) and the *ORF*2 proteins (45 kD; Fig. 1) were highly expressed in the transgenic lines. The abundant expression of the *orf*2 confirmed that polycistrons were efficiently translated without the need for processing into monocistrons.

Transcription and Translation of the *hsa* Operon

Chloroplast transgenic lines transformed with three different multigene constructs, all containing the *hsa* gene, were used to study transcription, translation, and posttranscriptional modifications (Fig. 3A). The *Prrn* promoter drives the operon downstream in all three constructs. The first transgenic line (referred to as RBS-HSA) has an operon formed by the *aad*A gene, followed by the *hsa* gene, whereas the second transgenic line (5′UTR-HSA) harbored an expression cassette that contained the *aad*A gene under the transcriptional regulation of the P*rrn* promoter, as well as the *hsa* gene under the transcriptional regulation of the *psb*A promoter and the translational enhancement of the 5′ psbA UTR. This transgenic line was predicted to produce a monocistronic *hsa* transcript (Fig. 3A). Finally, the third transgenic line (ORF-HSA) contained a four-gene operon formed by the *aad*A and *hsa* genes, as well as the *orf*1 and *orf*2 sequences of the *cry*2Aa2 operon from the bacterium *B. thuringiensis* (Fig. 3A).

Northern-blot analyses of the RBS-HSA lines with the *hsa* and the *aad*A probes revealed that the most abundant transcript was a dicistron of a predicted 2.8 knt (*aad*A-*hsa*; Fig. 3, B and D, b), followed by two polycistronic transcripts: one transcribed from the native 16S *Prrn* promoter of an expected size of 5.3 knt (Fig. 3, B and D, h), and the 3.2-knt transcript transcribed (Fig. 3, B and D, d) from the engineered *Prrn* promoter, terminating downstream of the 3′UTR of the gene cassette. No monocistrons were detected in these RBS-HSA transgenic lines. Quantification of transcripts from the northern blots obtained with the *hsa* probe revealed that the polycistrons transcribed from the engineered P*rrn* promoter accounted for 65.5% ± 3% of the transcript detected in these lines (Fig. 3C). The polycistrons terminating downstream of the 3′UTR in the *trn*A region and the one transcribed from the native 16S P*rrn* were 24.9% ± 2% and 9.6% ± 1% of the total transcripts, respectively (Fig. 3C, d and h). Values for the northern analysis performed with the *aad*A probe were similar, with 61.8% ± 3% for the polycistron transcribed from the engineered P*rrn* promoter, 31.7% ± 3% and 6.5% ± 0.3% for the read-through transcript and the polycistron transcribed from the native promoter, respectively (Fig. 3E, b, d, and h). This analysis shows that there is abundant read-though transcription.

The ORF-HSA transgenic lines also showed a similar transcription pattern with respect to the RBS-HSA line when probed with the *hsa* or *aad*A probe. When hybridized with the *orf*1,2 probe, the same pattern to that obtained with the *aad*A probe was observed, and no processing was detected between *orf*2 and the *hsa* gene (Fig. 3F, lanes 7–9). The most abundant transcript was the polycistron containing all four genes (predicted size of 4.4 knt), which was transcribed from the engineered *Prrn* promoter, representing 68.1% ± 2%, 65.5% ± 1%, and 43.3% ± 4% of the total transcripts detected with the *hsa*, *aad*A, or *orf*1,2 probes, respectively (Fig. 3, B, D, and F, f). Additionally, the predicted 6.9-knt polycistron originating at the *Prrn* native promoter was also detected (Fig. 3D, k) and this represented 6.8% ± 0.4% of the polycistrons (Fig. 3E, k). An approximately 5.2-knt transcript (Fig. 3, B, D, and F, g) obtained from the engineered 16S P*rrn* and processed downstream of the 3′UTR was also observed. This transcript was about 27.7% ± 1% and 31.9% ± 2% of the polycistrons detected with the *aad*A or *hsa* probes (Fig. 3C, g, and 3E, g), respectively, and 44.2% ± 1% of those detected with the *orf*1,2 probe (Fig. 3G, g). Finally, transgenic lines engineered with the *aad*A-5′UTR-*hsa* construct produced transcripts about 200 nt longer than the transgenic lines transformed with the *aad*A-*hsa* construct (Fig. 3, B and D, c and e). This increase in transcript size is due to the presence of the *psb*A 5′UTR and promoter. Additionally, this transgenic line produced an abundant *hsa* monocistron (2.1 knt) that accounted for approximately 50% of the total transcript detected with the *hsa* probe (Fig. 3, B and C, a); this transcript was not detected with the *aad*A, nor with the *orf*1,2 probes (Fig. 3D, lanes 7–9). The polycistrons transcribed from the engineered P*rrn* and native promoter were 28.7% ± 3% and 9.4% ± 2% of the transcripts produced (Fig. 3E, g

Figure 1. (*Continued.*)
*cry*2Aa2 operon transgenic lines. Transcripts of the *cry*2Aa2 operon are indicated by lowercase letters and correspond to the transcripts depicted in A. C, Relative heterologous transcript abundance within each line hybridized with the *cry*2A probe. D, Transcript analysis showing RNA hybridization with the *aad*A probe, loaded as follows: wt, wild-type control; lanes 1 to 3, *cry*2Aa2 operon transgenic lines. Transcripts of the *cry*2Aa2 operon are of sizes as described for the *cry*2Aa2 probe; f is *aad*A/*orf*1/orf2 tricistron, 2.5 knt. E, Heterologous transcript quantification for samples hybridized with the *aad*A probe. F, RNA hybridization using the orf1,2 probe. Samples were loaded in the same order as in D and predicted transcript sizes correspond to those observed in D. G, Relative transcript abundance within each transgenic line obtained by hybridization with the *orf*1,2 probe. H, Western-blot analysis using the *Cry*2Aa2 antibody. wt, Wild-type control; lanes 1 and 2, *cry*2Aa2 operon transgenic lines; lane 3, positive control (*Cry*2Aa2 protein). The expected polypeptide of 65 kD is shown in both transgenic plants and the positive control. I, Western-blot analysis using the *ORF*2 antibody. wt, Wild-type control; lanes 1 and 2, cry2Aa2 operon transgenic lines; lane 3, positive control (*ORF*2 protein). The expected polypeptide of 45 kD is shown in both transgenic plants and the positive control.

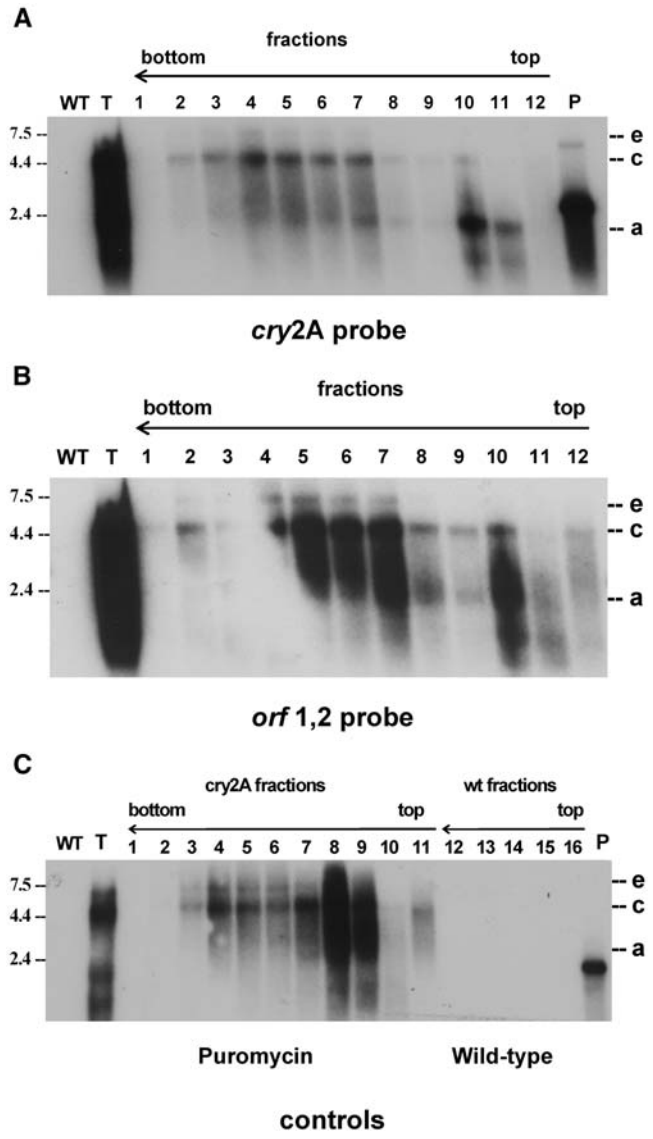

Figure 2. Polysome fractionation assays of the *cry*2Aa2 operon. A, RNA hybridized with the *cry*2A probe after fractionation through a Suc gradient. WT, Wild-type control; T, total RNA sample; lanes 1 to 12, RNA collected from the different fractions of the gradient. Lower fractions correspond to the bottom of the Suc gradient (polysomal fractions). P. *cry*2Aa2 probe. C, Transcript "c" (*aad*A-*orf*1-*orf*2-*cry*2A polycistron) described in Figure 1A. B, Same RNA blot after stripping and rehybridizing with *orf*1,2 probe. Lane P is omitted because no *orf*1,2 probe was loaded. C, Puromycin release and wild-type controls. Cry2Aa2 samples were treated with puromycin before loading onto Suc gradients, whereas an additional wild-type sample was loaded onto Suc gradients and used as a negative control. RNA was hybridized with the *aad*A probe. The gel was loaded as follows: WT, wild-type RNA; T, total RNA; 1 to 11, RNA collected from the different fractions of the Suc gradient and hybridized with the *aad*A probe. Lanes 12 to 16, Wild-type RNA from fractions 2, 4, 6, 8, and 10 collected from the Suc gradient. P, *aad*A probe. c, transcript "c" (*aad*A-*orf*1-*orf*2-*cry*2A polycistron) described in Figure 1A.

and k). Similar transcript abundance was detected in northern-blot analyses in which the *aad*A probe was used (Fig. 3E, g and k). Furthermore, read-through transcripts processed downstream of the transgene cassette, in the *trn*A native gene, were detected (Fig. 3, B and D, e and j). The combined abundance of these transcripts was 15.5% (Fig. 3C, e and j), whereas the overall polycistron abundance in this transgenic line was as much as the monocistronic transcript.

When RNA from the different transgenic *hsa* lines were hybridized with the *psb*A 3′UTR probe, a pattern similar to that obtained with the *hsa* gene-specific probe was observed (Fig. 7A). Because the native *psb*A transcript was also detected, the abundance of both native and heterologous operons could be observed. However, endogenous versus heterologous transcript abundance could only be compared among transcripts that were regulated by the same *psb*A promoter (Fig. 7A, lanes 4–6a). The results showed that the 5′UTR-HSA monocistronic transcript was approximately 1.6 times as abundant as that of the native *psb*A. This may be due to the effect of gene dosage, as the transgene is integrated into the inverted repeat region, whereas the *psb*A gene is located in the large single copy region.

Western-blot analyses of the different constructs showed expression of the HSA monomer (66 kD) and dimer (132 kD) in the transgenic lines harboring the 5′UTR-*hsa* and the *orf*1,2-*hsa* constructs (Fig. 3H). Transgenic lines expressing the monocistrons showed expression levels similar to the ORF-HSA transgenic line, in which only polycistrons were translated. The abundant translation of ORF2 protein (45 kD) from the *aad*A-*orf*1-*orf*2-*hsa* transgenic lines (Fig. 3I), which only transcribed tricistrons and polycistrons, support the view that polycistrons are highly stable in the chloroplast and can be efficiently translated without further processing. This was also observed in polysome fractionation assays, in which larger polycistronic transcripts of the ORF-HSA lines were detected in the lower fractions of the gradient (data not shown). Expression of the *hsa* gene in the transgenic line ORF-HSA at levels similar to the ones produced by the *psb*A-5′UTR-*hsa* transgenic lines suggest a similar translation efficiency for heterologous polycistrons and monocistrons in the chloroplast (Fig. 3H).

The accumulation of human serum albumin in transgenic lines *aad*A-*orf*1-*orf*2-*hsa* was monitored under different photoperiods and developmental stages by performing ELISA analyses. These experiments were conducted to determine whether *hsa* expression under the *cry* 5′UTR, which is a heterologous 5′UTR, is light dependent or developmentally regulated. The data obtained from the analysis of cell extracts from young, mature, and old leaves exposed to periods of 0, 4, 8, and 16 hours of light revealed no significant differences among age of leaf or among different periods of illumination. Therefore, HSA accumulation in this transgenic line regulated by a heterologous 5′ UTR is independent of light regulation and is free of cellular control (Fig. 4).

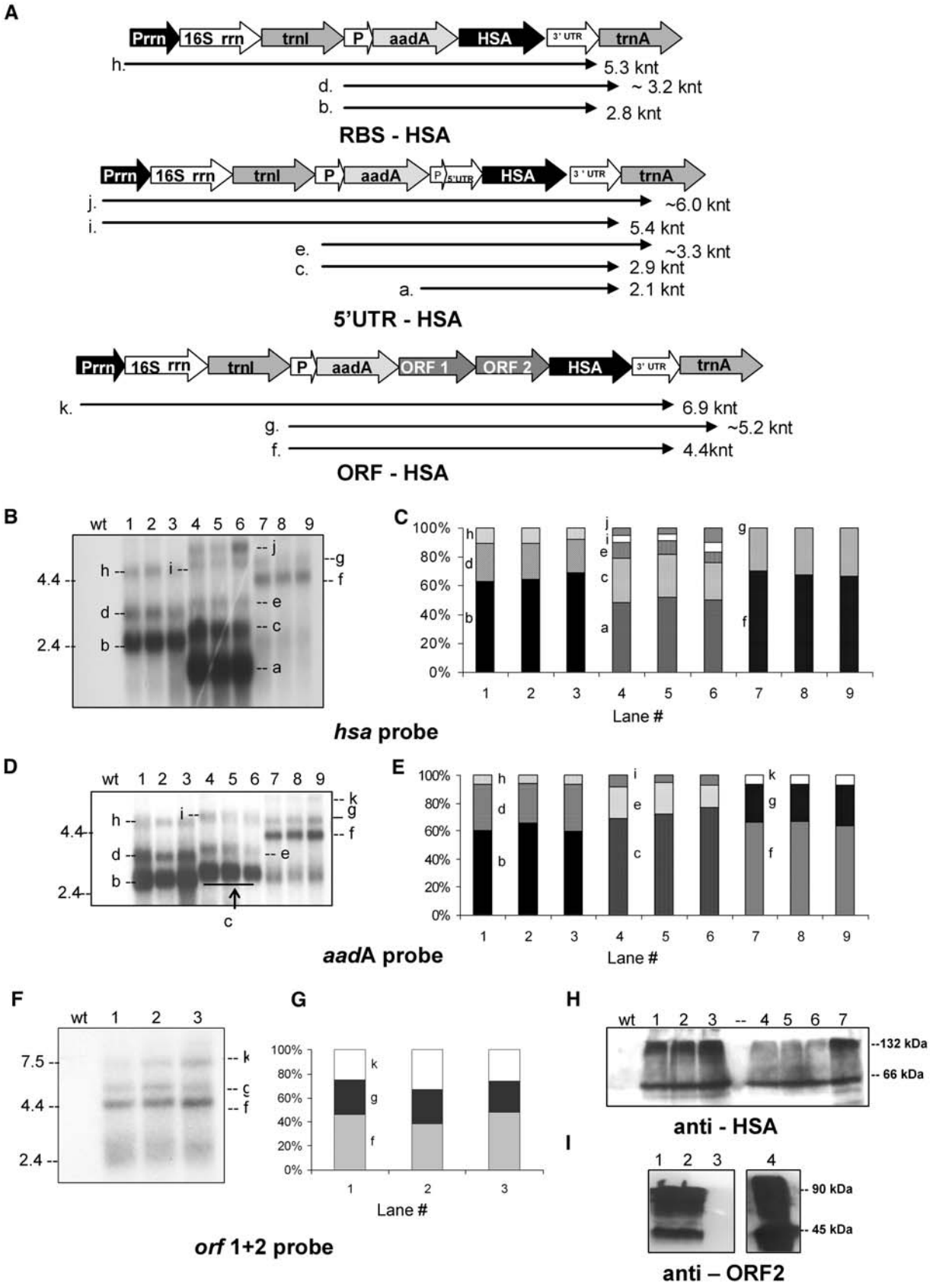

Figure 3. Transcriptional and translational analysis of the *hsa* operons. A, Schematic representation of the *hsa* operons (rbs-*hsa*, 5′UTR-*hsa*, *orf*1-*orf*2-*hsa*) in transgenic lines, including the *aad*A gene and upstream P*rrn* promoter (P); upstream native

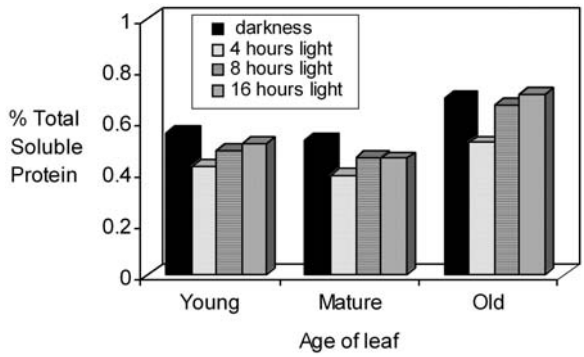

Figure 4. ELISA analysis of the *orf*1-*orf*2-*hsa* transgenic line. Total soluble protein content of young, mature, and old leaf extracts of the *orf*1-*orf*2-*hsa* transgenic lines determined by ELISA analyses. Transgenic plants were subjected to the following light conditions: 4, 8, and 16 hours of light, as well as total darkness.

Transcription and Translation of the *tps*1 Operon

The *tps*1 gene coding for trehalose phosphate synthase was engineered into a two-gene operon, formed by the *aad*A and the *tps*1 genes, and transcribed from the engineered *Prrn* promoter (RBS-TPS1 lines; Fig. 5A). Northern-blot analyses with either the *tps*1-specific or *aad*A-specific probes detected the expected 2.7-knt dicistron (*aad*A-*tps*1) as the most prominent transcript (Fig. 5, B and D, a). Densitometric analyses of the northern blots showed that the *aad*A-*tps*1 dicistron accounted for 43.3% ± 3% of the total transcripts detected by the *tps*1 probe, and 59.8% ± 5% of the total transcript when the *aad*A probe was used (Fig. 5C, a, and 5E, a). A predicted 5.2-knt polycistron observed in the northern blots with either the *tps*1 and *aad*A probes (Fig. 5B, c, and 5D, c) is transcribed from the native 16S *Prrn* (Fig. 5A, c). Additionally, the *tps*1 probe detected less abundant polycistrons of about 3.5 knt (Fig. 5, B and D, b) and approximately 6.5 knt (Fig. 5B, d) transcribed from the engineered *Prrn* promoter and the native 16S P*rrn* promoter, respectively, terminating downstream of the 3′UTR. The 3.5-knt polycistron was also detected by the *aad*A probe. Transcripts that ended in the *trn*A intron region (downstream of the engineered 3′UTR) were also detected in the *cry* (Fig. 1B, b, and 1D, e) and *hsa* transgenic lines (Fig. 3B, d, e, and g; 3D, d, e, and g; and 3F, g), indicating that this region may contain different processing sequences. The transcripts processed at the *trn*A location account for about 37% of the total transcripts detected in the transgenic lines (Fig. 5C, b and d, and 5E, b). Transcripts longer than the 6.5-knt polycistrons may terminate at undetermined locations and these were not quantified densitometrically. No monocistron was detected in the northern blots with the *tps*1 probe nor with the *aad*A probe, indicating that the polycistron is not being processed in these transgenic lines, whereas the larger transcripts detected are likely to be read-through.

Northern-blot analyses performed using the *psb*A 3′UTR probe (Fig. 7C, lanes 1–3) revealed a pattern consistent to that obtained using the gene-specific *tps*1 probe (see Fig. 5B). In addition, the native *psb*A transcript was also detected and was similar in abundance to the *aad*A/*tps*1 dicistron (Fig. 7C, a). However, transcript abundance cannot be quantitatively compared because they are regulated by different promoters. Larger, less abundant transcripts (Fig. 7C, b–c) were also detected with the gene-specific *tps*1 probe (see Fig. 5B) and may correspond to read-through transcripts.

Western-blot analyses performed to detect the trehalose phosphate synthase revealed efficient translation of polycistrons, as shown by the abundant accumulation of a 65-kD polypeptide corresponding to this protein (Fig. 5F). Because no monocistrons for *tps*1 or *aad*A were detected in the northern-blot analyses, hyperexpression of TPS1 should thus be the result of efficient translation of polycistrons in transgenic chloroplasts.

Transcription and Translation of the *ctb* Operon

RNA from chloroplast transgenic lines transformed with the *aad*A-*ctxb* (referred to as RBS-CTB lines) or 5′UTR-*ctxb*-*gfp* fusion constructs (5′UTR-CTB-GFP lines) were also analyzed by northern blots. The RBS-CTB lines showed dicistrons and polycistrons, whereas the 5′UTR-CTB-GFP transgenic lines showed monocistrons along with several polycistrons. Pre-

Figure 3. (*Continued.*)
chloroplast 16S ribosomal RNA gene and promoter (Prrn) as well as *trn*I/*trn*A genes are shown. Arrows represent expected transcripts and their respective sizes. B, RNA hybridization with the *hsa* probe. wt, Wild type; lanes 1 to 3, rbs-*hsa* transgenic lines; lanes 4 to 6, 5′UTR-*hsa* transgenic lines; lanes 7 to 9, *orf*1,2-*hsa* transgenic lines. Lowercase letters correspond to the transcripts predicted in A. C, Relative abundance of the transcripts obtained with the *hsa* probe. D, mRNA transcripts hybridized with the *aad*A probe and loaded in the same order as in B. Transcripts a to i corresponded to the same transcripts observed in B; k corresponds to the 16*rrn*/*hsa* polycistron (6.9 knt). E, Quantification of relative heterologous transcript abundance obtained with the *aad*A probe. F, mRNA transcripts of wild-type (wt) and *orf*1,2-*hsa* transgenic lines (lanes 1–3) hybridized with the *orf*1,2 probe. G, Relative abundance obtained for the transcripts detected with the *orf*1,2 probe. H, Western-blot analysis using the *HSA* antibody. wt, Wild-type control. Lanes 1 and 2, RBS-*hsa* transgenic lines; lanes 3 and 4, 5′UTR-*hsa* transgenic lines; lanes 5 and 6, *orf*1,2-*hsa* transgenic lines; lane 7, positive control (HSA protein). Lane marked with (--) was left blank. All samples presented 66-kD and 132-kD peptides, corresponding to the size of the HSA protein and its dimeric form, respectively. I, Western-blot analysis using the ORF2 antibody. Lanes 1 and 2, *orf*1,2-*hsa* transgenic lines; lane 3, wild-type control; lane 4, positive control (ORF protein). Shown are 45-kD ORF2 and 90-kD dimer.

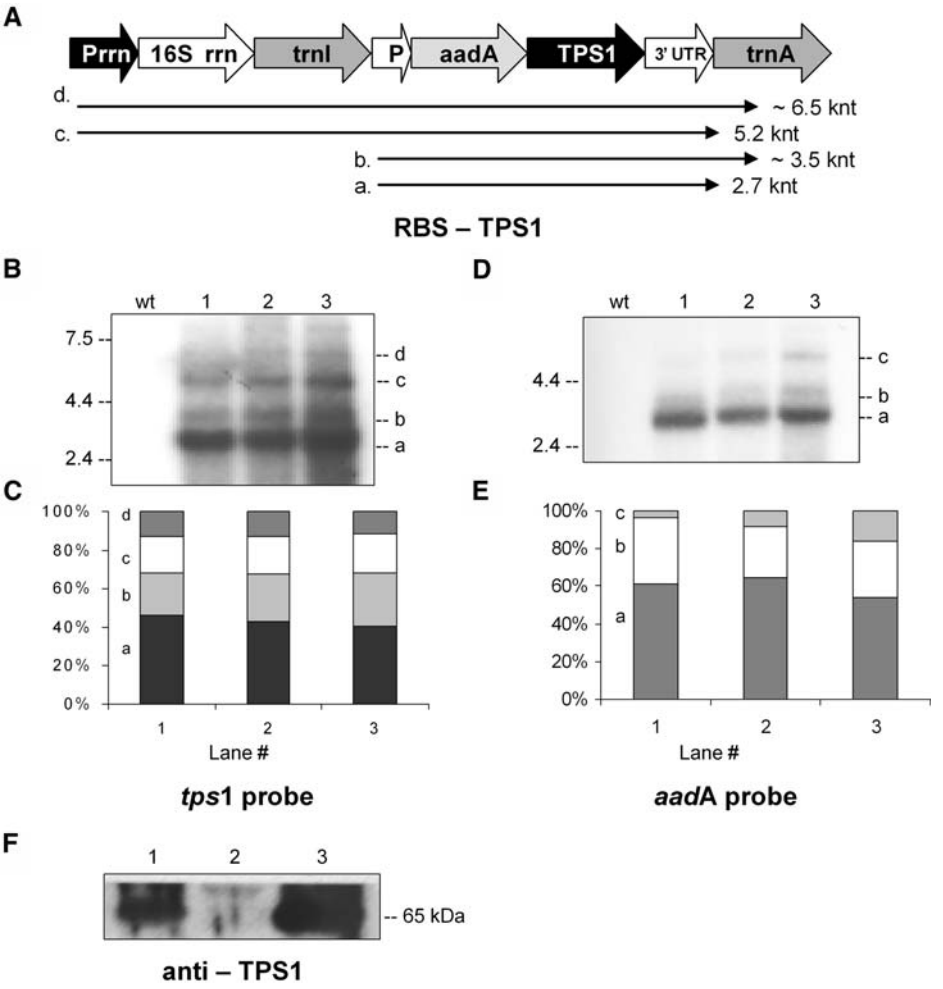

Figure 5. Transcriptional and translational analysis of the *tps*1 operon. A, Schematic representation of the *tps*1 operon in transgenic lines, including the *aad*A gene and upstream P*rrn* promoter (P). Upstream native chloroplast 16S ribosomal RNA gene and promoter (Prrn) as well as *trn*I/*trn*A genes are shown. Arrows represent expected transcripts and their respective sizes. B, Northern-blot analysis obtained by hybridization with the *tps*1 probe, loaded as follows: wt, wild-type control; lanes 1 to 3, *tps*1 transgenic lines. Transcripts of the *tps*1 operon correspond to those depicted in A, indicated with lowercase letters. C, Relative transcript abundance per transgenic line, obtained with the *tps*1 probe. D, RNA transcripts hybridized with the *aad*A probe, loaded as follows: wt, wild-type control; lanes 1 to 3, *tps*1 transgenic lines. Transcript bands obtained for the *tps*1 operon are of sizes as described for *tps*1 probe (B). D, Relative abundance of transcripts in each sample after hybridization with the *aad*A probe. E, Western-blot analysis using the TPS1 antibody. Lane 1, Positive control (TPS1 protein); lane 2, wild-type control; lane 3, *tps*1 transgenic line. A polypeptide of 65 kD was observed in the transgenic clone, corresponding to the expected size of the TPS1 protein, as observed in the positive control.

dicted dicistrons of 1.3 knt (Fig. 6B, a, and 6D, a) and 2.3 knt (Fig. 6B, d, and 6D, d) transcribed from the engineered *Prrn* promoter were detected with either the *ctxb* or *aad*A probe. Additionally, polycistrons transcribed from the native 16S *Prrn* were observed in both transgenic lines. In the RBS-CTB transgenic lines, the *aad*A-*ctxb* polycistron was of a predicted size of 3.8 knt (Fig. 6B, f, and 6D, f), while in the 5′UTR-CTB-GFP transgenic line, *aad*A-5′-UTR-*ctxb-gfp* polycistron was 4.8 knt (Fig. 6B, g, and 6D, g); both polycistrons code for four genes (16 rRNA gene, *trn*I gene plus the two heterologous genes). Polycistronic transcripts of higher molecular weight appear to terminate downstream from the engineered 3′UTR (Fig. 6, B and D, i and h), as well as the transcripts of approximately 2.2 knt (Fig. 6, A and B, c) and approximately 3.5 knt (Fig. 6, A and B, e) obtained from the engineered *Prrn* promoter and processed downstream of the 3′UTR in the gene construct. The *cxtb-gfp* monocistron of 1.4 knt (Fig. 6B, b) was detected with the *ctxb* probe but not with the *aad*A probe; besides this transcript, no other monocistron was detected in these analyses. Its average relative abundance was 42.1% ± 3% of the total heterologous transcripts in the 5′UTR-CTB-GFP transgenic lines (Fig. 6C, b), while the total combined abundance of the polycistrons averaged 56% (Fig. 6C, d and g), with the polycistron transcribed from the engineered P*rrn* accounting for 22.9% ± 1% of the total transcripts (Fig. 6C, d). For the RBS-CTB transgenic line, 100% of the transcripts were polycistrons, of which the most abundant transcript was the *aad*A-*ctx*B dicistron (about 45% of the total transcripts), followed by approximately 30% of the polycistron transcribed from the engineered P*rrn* and processed downstream at the *trn*A gene (Fig. 6D, a and C, and 6E, a and c).

Additional northern-blot analyses performed with the *psb*A 3′UTR probe (Fig. 7D) revealed a transcript pattern consistent to that obtained with the *ctx*B gene-specific probe (see Fig. 6B). Furthermore, the native *psb*A transcript was also detected. Because the size of the *aad*A/*ctx*b dicistron (1.25 knt) is similar to that of the endogenous psbA (1.3 knt), they could not be distinguished from each other. However, this may account for the increase in transcript abundance observed in relation to the native psbA transcript (Fig. 7D, lanes wt and 1–3a*). Due to similar reasons, the increase in transcript abundance of the native *psb*A

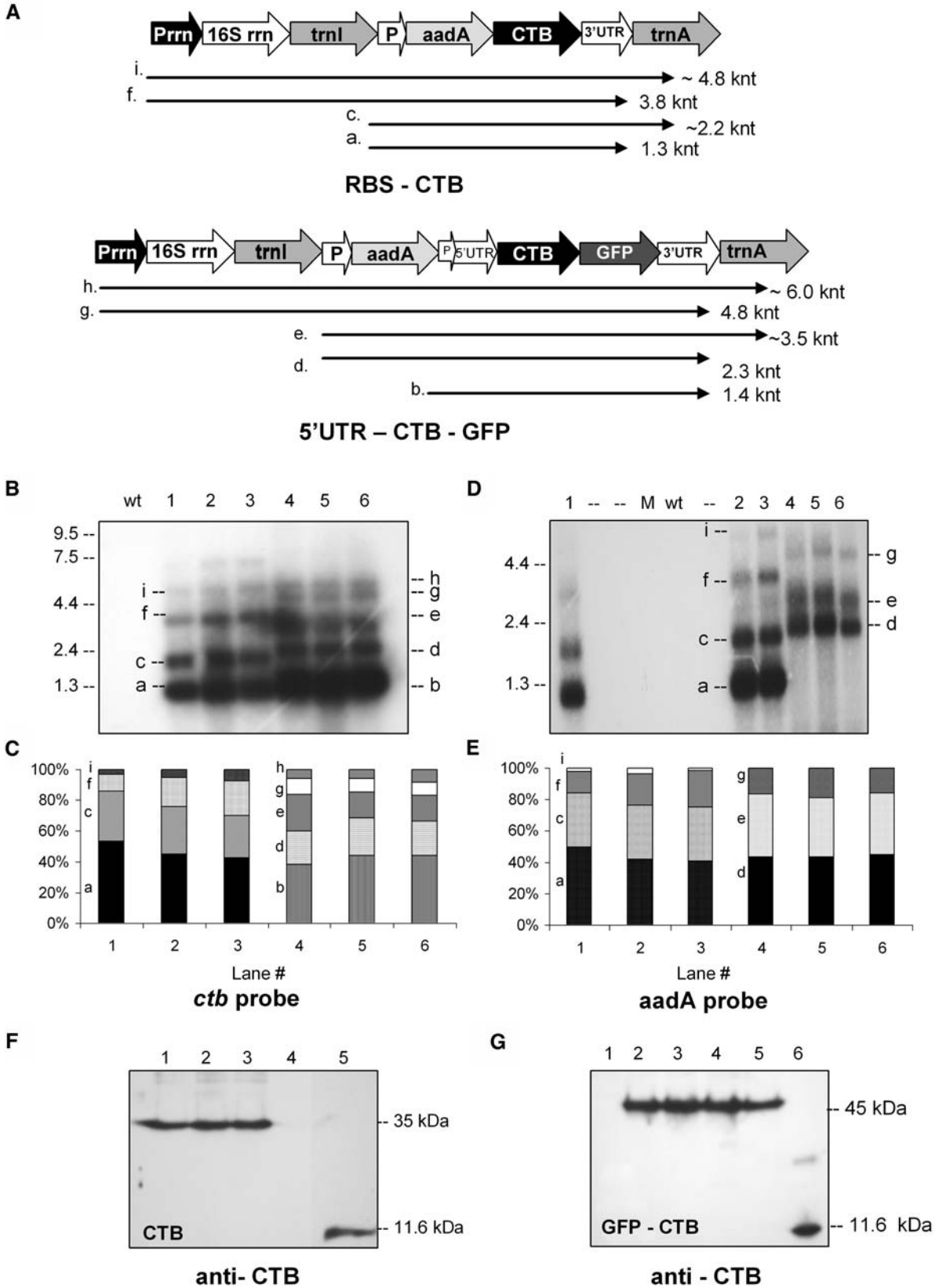

Figure 6. Transcriptional and translational analysis of the CTB operons. A, Schematic representation of the 5′UTR-*ctb*-*gfp* and RBS-*ctb* operon in transgenic lines, including the *aad*A gene and the upstream P*rrn* promoter (P); upstream native chloroplast 16S ribosomal RNA gene with its respective promoter (Prrn) and the *trn*I and *trn*A are also shown. Arrows represent expected

transcript observed on the 5′UTR-CTB-GFP transgenic lines (Fig. 7D, lanes 4–6b*) could be due to the presence of the *ctx*b-*gfp* monocistron (1.4 knt). Although the native *psb*A and the *ctx*b-*gfp* genes are regulated by the same *psb*A promoter, transcript abundance could be quantitatively compared. However, because both transcripts are similar in size, comparison between these native and heterologous transcripts was not possible.

The western-blot analyses showed that transgenic lines expressing either CTB or CTB-GFP fusion produced large amounts of either protein (Fig. 6, F and G). CTB protein was detected as a higher molecular weight polypeptide (trimer of 35 kD) than the *Escherichia coli* expressed CTB (Fig. 6F). High protein level was also detected for the *gfp-ctx*B fusion protein (Fig. 6G), which was detected in the monomeric form (45 kD). Interestingly, expression levels in both transgenic lines were similar, even though in the *aad*A-5′UTR-*ctxb-gfp* transgenic line the more abundant transcript was the monocistron. This again suggests that polycistrons are translated as effectively as monocistrons.

DISCUSSION

The chloroplast genome has been engineered with single genes to confer useful agronomic traits including herbicide resistance (Daniell et al., 1998), insect resistance (McBride et al., 1995; Kota et al., 1999; De Cosa et al., 2001), disease resistance (DeGray et al., 2001), drought tolerance (Lee et al., 2003), salt tolerance (Kumar et al., 2004a), and phytoremediation (Ruiz et al., 2003). Recent success in transforming the chloroplast genome of several major crops, including cotton (*Gossypium hirsutum*; Kumar et al., 2004b) and soybean (*Glycine max*; Dufourmantel et al., 2004), has opened this field for commercial development. Because most of the desired traits require multigene engineering, it is important to understand transcription, posttranscriptional changes, and translation of heterologous polycistrons within plastids.

Transcript analyses performed in this study repeatedly confirmed that different transgenic lines harboring multigenic operons generated polycistrons as the most abundant transcript form, along with monocistronic mRNA. This observation is further supported by the polysome fractionation assays performed on the *cry*2Aa2 samples, in which larger transcripts were collected from the fractions associated to polyribosomes. Smaller transcripts were observed mainly in the upper fractions of the gradient, suggesting that polycistrons may be preferentially translated without processing. Similar results were obtained after stripping the membranes and reprobing with the *orf*1,2 probe. Polycistronic polysomal RNA has been previously reported in native chloroplast operons, as well as multiple open reading frames simultaneously translated from polycistions (Barkan, 1988); however, in such a case, polycistronic transcripts were less abundant than monocistrons. In the case of the cry2Aa2 operon, ribosome-associated polycistrons were in much higher abundance than the monocistronic transcripts, suggesting that the heterologous operon is preferentially translated as a polycistronic unit. Similar results were observed with chloroplast transgenic lines harboring the *aad*A-*orf*1-*orf*2-*hsa* operon (data not shown). These observations contrast with the general consensus for native chloroplast translation mechanisms (Barkan, 1988; Barkan et al., 1994; Zerges, 2000; Meierhoff et al., 2003), thus showing that multigene operons engineered into the chloroplast genome do not necessarily require processing of polycistrons to monocistrons or dicistrons for efficient translation.

Processing was observed in the native *cry*2Aa2 operon, between *orf*2 and the *cry*2Aa2 genes on the transgenic lines. However, this event did not occur between *orf*1 and *orf*2 of this operon or at intergenic sequences of the other engineered operons studied. The fact that processing occurred only in the *cry*2Aa2 5′UTR suggests that this intergenic sequence might contain unique information required for processing. By using computer simulation, it was observed that the heterologous bacterial intergenic transcript sequences may form secondary structures. Evidence for the protection of chloroplast RNA by 5′UTRs, as well as the role of 5′UTR secondary structures in RNA stability (Zou et al., 2003), has been previously discussed (Drager et al., 1998). Additionally, previous reports have shown that intergenic sequences forming stable secondary structures that mask the ribosome-binding site may affect the translation of the downstream gene (Barkan et al., 1994; Hirose and Sugiura 1997; del Campo et al., 2002). These observations offer the possibility of further studies involving the role of intergenic secondary structures of native and heterologous operons in posttranscriptional processes.

Figure 6. (*Continued.*)
transcripts and their respective sizes. B, Northern-blot analysis showing RNA hybridized with the CTB probe. Samples were loaded as follows: wt, wild-type control; lanes 1 to3, 5′UTR-*ctb-gfp* transgenic lines; lanes 4 to 6, *rbs-ctb* transgenic lines. The transcripts and respective sizes correspond to those indicated in A with lowercase letters. C, Relative transcript abundance, within each line, of the transcripts shown in B. D, RNA hybridization using the *aad*A probe, and loaded according to the following: M, molecular weight marker; wt, wild-type control; lanes 1 to 3, RBS-*ctb* transgenic lines; lanes 4 to 6, 5′UTR-*ctb-gfp* transgenic lines. Lanes marked with (--) were left blank. The transcripts observed correspond to the same as in B. E, Relative transcript abundance, per line, for the transcripts shown in C. F, Western-blot analysis of the RBS-CTB transgenic lines using anti-CTB antibody. Lanes 1 to 3, transgenic clones; lane 4, wild-type control; lane 5, positive control (CTB protein). CTB from transgenic lines is in trimeric form. E, Western-blot analysis of the 5′UTR-*ctb-gfp* transgenic lines using the CTB antibody. Lane 1, Wild-type control; lanes 2 to 5, transgenic lines; lane 6, positive control (CTB protein).

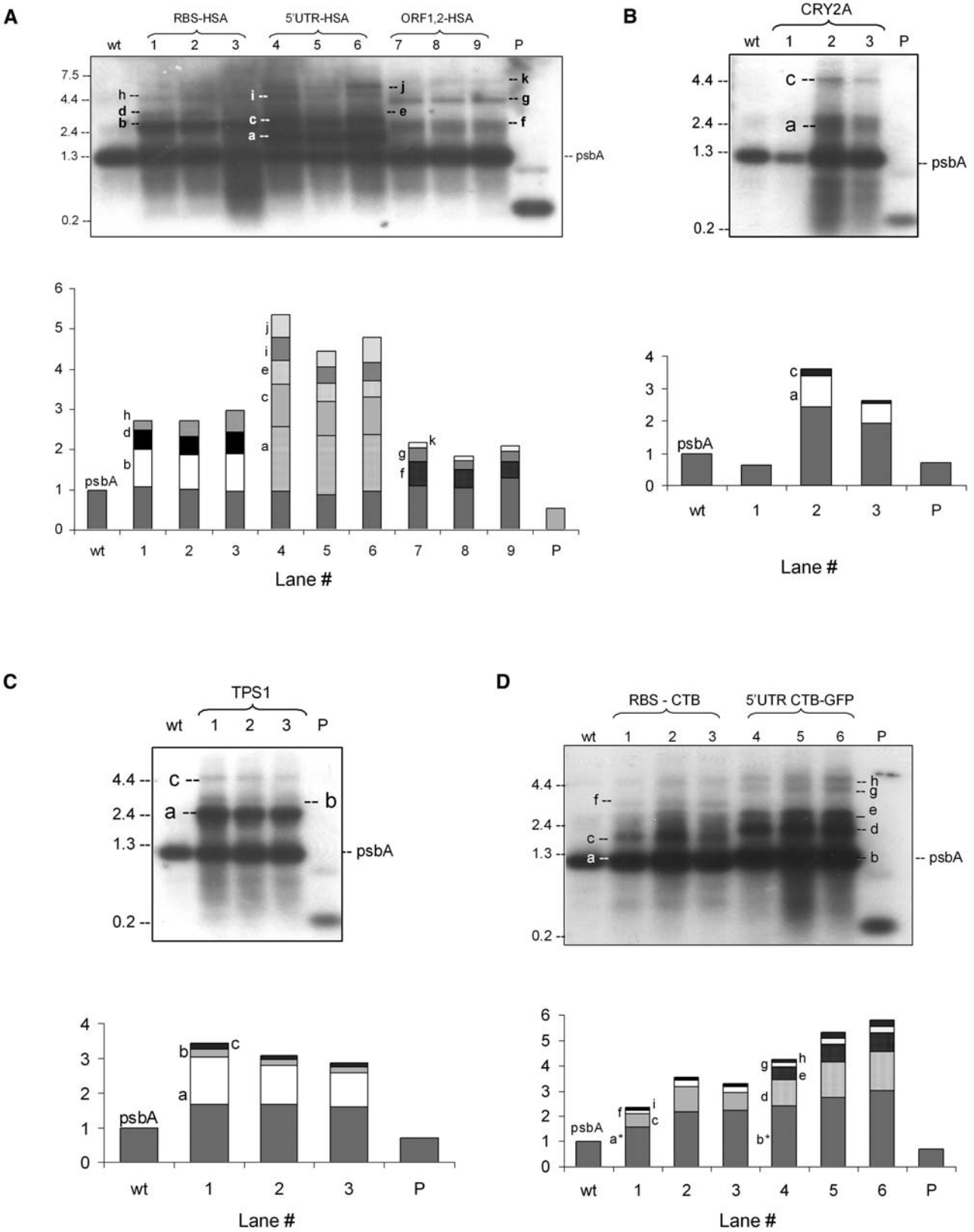

Figure 7. Transcription of heterologous operons using the *psb*A 3′UTR probe. A, Northern-blot analysis and corresponding quantification of transcripts obtained from different HSA transgenic lines described in Figure 3, as well as of the native *psb*A transcripts. The RNA gels were loaded as follows: wt, wild-type; lanes 1 to 3, RBS-HSA transgenic lines; lanes 4 to 6, 5′UTR-HSA transgenic lines; lanes 7 to 9, ORF-1,2-HSA transgenic lines; P, *psb*A 3′UTR probe. Lowercase letters correspond to the same

Transgenic lines harboring the engineered *aad*A-*orf*1-*orf*2-*hsa* operon showed no difference in HSA accumulation in response to light or dark conditions, in contrast to those transformed with the *hsa* gene and native *psb*A 5′UTR. This suggests that the translation enhancement observed is not light-dependent. Thus, the heterologous *cry*2aA2 operon UTR region is independent of nuclear and chloroplast control, unlike the *psb*A regulatory sequences (Fernandez San Millan, et al., 2003; Zerges, 2004). Such heterologous UTRs have played a major role in transgene expression in nongreen tissues, such as carrot roots (Kumar et al., 2004a), or in nongreen cultured cells (Kumar et al., 2004a, 2004b), to facilitate transformation of recalcitrant crops.

Data shown here supports the idea that engineered operons in the chloroplast, which do not carry any intergenic sequences capable of forming stable secondary structures, can be translated very efficiently and do not require processing into monocistrons in order to be translated. The processing observed in the *cry*2Aa2 transgenic lines may be due to endonucleolytic cleavage of a region in the intergenic sequence, but it does not indicate that this processing has to occur for translation to take place. An interesting observation is that the *aad*A-*orf*1-*orf*2 tricistron produced by the processing event does not contain a 3′ UTR region, yet this transcript is as abundant as the polycistrons, which contain the 3′UTR and are efficiently translated. This shows that polycistrons may be stable in the chloroplast, even in the absence of the 3′UTR.

In chloroplasts, all of the genes in the 16S *rrn* operon, including the *trn*A, *trn*I, as well as 23S, 4.5S, and 5S *rrn* genes (which are downstream of the integrated transgenes), are transcribed from the native P*rrn* promoter. Therefore, disruption of these polycistrons by the insertion of the foreign operon due to effective termination at the 3′UTR would mean that the *trn*A and other downstream genes would not be transcribed, affecting chloroplast protein synthesis. However, this was not the case; all the transgenic lines grew similar to the wild-type plants, indicating that the read-through transcripts formed by the insertion of foreign operons were sufficient for optimal ribosome synthesis in chloroplasts. Read-through transcripts processed at the *trn*A region accounted for about 26% to 39% of the total heterologous transcripts in all transgenic lines tested, whereas in HSA-expressing transgenic lines, this percentage was between 15% and 32%. Introns within the *trn*A gene undergo splicing and other posttranscriptional modifications in order to produce the functional *trn*A (Barkan., 2004). Therefore, such processing may modify polycistronic transcripts that read through from the 3′UTR *psb*A engineered in these chloroplast vectors. Additionally, larger polycistrons were also detected, although these were not quantified.

The transcript profile for the transgenic lines 5′UTR-*hsa* and 5′UTR-*ctx*b-*gfp* (the only two transgenic lines in this study that transcribed monocistrons) was very similar. The monocistronic transcripts accounted for about 42% to 50% of the total heterologous transcripts examined. The total polycistronic levels in these two transgenic lines, including read-through transcripts, were between 50% and 57%. In all the transgenic lines that did not transcribe monocistrons, the most abundant transcript was transcribed from the engineered P*rrn* promoter, terminating at the 3′UTR, which accounted for 43% to 59% of the total transcripts detected.

Data generated by analyzing the different transcripts with the *psb*A 3′UTR probe not only supported the previous results observed with the gene-specific probes, but also allowed comparison with the native *psb*A transcripts. In two transgenic lines (5′UTR-HSA and 5′UTR-CTB-GFP), the *psb*A 5′UTR was used upstream of the genes of interest. In such cases, endogenous versus heterologous transcript abundance could be quantitatively compared, unless the 5′*utr-ctx*b-*gfp* transcript was similar in size to the native *psb*A gene. Comparison of the native *psb*A and 5′*utr-hsa* transcripts showed a greater abundance (1.6 times) of the heterologous transcript. This could be attributed to gene dosage, as the heterologous operons are integrated into the inverted repeat region, whereas the native *psb*A gene is located in the single-copy region. In addition, transcript abundance was variable among the remaining heterologous operons regulated by the 16S *prrn* promoter. Variability could be attributed to

Figure 7. (*Continued.*)
transcripts predicted in Figure 3A. Transcript abundance was normalized against the wild-type *psb*A, to which a value of 1 was assigned. B, Northern-blot analysis and corresponding transcript quantification of the *cry*2Aa2 operon. Gel loading was as follows: wt, wild-type RNA; lanes 1 to 3, Cry2Aa2 transgenic lines; P, *psb*A 3′UTR probe. Lowercase letters correspond to transcripts predicted in Figure 1A. Native *psb*A transcript is indicated. Transcript abundance was normalized against the wild-type *psb*A, to which a value of 1 was assigned. The low transcript abundance of lane 1 is due to partial RNA degradation in the sample. C, RNA blot and transcript quantification of the transgenic TPS1 lines. The RNA gel was loaded as follows: wt, wild type; lanes 1 to 3, TPS1 transgenic lines; P, *psb*A 3′UTR probe. Lowercase letters correspond to transcript sizes shown in Figure 5A. Native *psb*A transcript is indicated. Transcript abundance was normalized against the wild-type *psb*A, showing a value of 1. D, Northern-blot analysis of the RBS-CTB and 5′UTR-CTB-GFP transgenic lines. Samples were loaded as follows: wt, wild type; lanes 1 to 3, RBS-CTB transgenic lines; lanes 4 to 6, 5′UTR-CTB-GFP transgenic lines; P, *psb*A 3′UTR probe. Lowercase letters correspond to transcripts shown in Figure 6A. Transcripts a* and b* are similar in size to the native *psb*A and therefore they cannot be distinguished from the native transcript. Because such transcripts were shown to be very abundant in Figure 6B, and because of increase in transcript abundance in comparison to the wild-type *psb*A transcript, it is assumed that such transcripts are present. Transcript abundance was normalized against the wild-type *psb*A, to which a value of 1 was assigned.

differences in mRNA stability, as well as in the level of posttranscriptional processing of the primary transcripts (Barkan and Goldschmidt-Clermont, 2000; Monde et al., 2000b; del Campo et al., 2002).

Although less abundant transcripts have been addressed in this work, they should be characterized further. With the current analyses, alternately processed and other minor transcripts have been explained by their sizes, with predicted sizes based on the coding sequences. More extensive analyses should be performed to further confirm the nature of these minor transcripts, including alternate splicing. The major and more abundant transcripts, however, are very consistent and strongly support the hypothesis that heterologous operons can be transcribed and translated without need for further processing.

The ability to engineer foreign genes without promoters or other regulatory sequences has several advantages. Also, repeated sequences may cause deletion of the transgene (Iamtham and Day, 2000). Observations reported here show evidence for transcription and processing of heterologous operons. While endogenous polycistrons require processing for effective translation, this is not required for expression of foreign operons. Native polycistrons require chloroplast specific 3′UTRs for stability, which is not always required for heterologous polycistrons. UTRs in native transcripts are regulated by nuclear factors, whereas heterologous transcripts have not been shown to be dependent of such regulations. Specific nuclear-encoded factors recognize sequences in native transcripts for the processing of primary mRNA (Barkan, 2004). This is not the case in foreign operons where heterologous sequences can be recognized and processed by the chloroplast posttranscriptional machinery. Finally, in both native and foreign operons there are abundant read-through transcripts that allow the expression of genes downstream of 3′UTRs. Addressing questions of the translation of polycistrons and sequences required for transcript processing and stability is essential for chloroplast metabolic engineering. Knowledge of such factors would enable engineering pathways that will not be under the complex posttranscriptional regulatory machinery of the chloroplast.

One of the primary advantages of using heterologous sequences for increasing gene expression is the lack of cellular control over these sequences, allowing the enhancement of transgene expression in green and nongreen tissues. Recently, the use of the g10 5′UTR facilitated the transformation of nongreen plastids of carrot (Kumar et al., 2004a). Additionally, the use of a gene cassette containing the selectable marker genes under the regulation of heterologous UTRs increased transformation efficiency and facilitated cotton plastid transformation (Kumar et al., 2004b). Recent accomplishments in the transformation of agronomically important species through somatic embryogenesis using species-specific chloroplasts vectors also broadens the possibility of extending this technology to crops that have been, until now, recalcitrant to chloroplast transformation (Dufourmantel et al., 2004; Kumar et al., 2004a, 2004b; Daniell et al., 2005).

In this study, we report the translation of polycistronic transcripts without processing, the expression of multigene operons independently of cellular control, and the stability of heterologous polycistrons lacking a 3′UTR. These results suggest that it is possible to effectively express multiple genes via the chloroplast genome without significant intervention of chloroplast regulation. The findings of this study should facilitate multigene engineering via the plastid genome in both green and nongreen plastids.

The results reported here are initial attempts to understand multigene engineering in transgenic plastids. Further studies on determination of 5′and 3′ ends of the transcripts by primer extension and RNase protection assays, as well as the possible role of intergenic sequences, should help to understand processing of heterologous transcripts. Such studies, however, are beyond the scope of this article.

MATERIALS AND METHODS

Chloroplast Transformation, Selection, and Characterization of Transgenic Plants

The chloroplast transformation, selection, and characterization of the transgenic lines used in this study have been previously reported (Daniell et al., 2001; De Cosa et al., 2001; Lee et al., 2003; Fernandez-San Millan et al., 2003) with the exception of the *ctb-gfp* transgenic lines. Sterile tobacco (*Nicotiana tabacum*) leaves were bombarded using the Bio-Rad PDS-1000/He biolistic device (Hercules, CA) as described previously (Daniell, 1997; Daniell et al., 2004a, 2004b).

Chloroplast Expression Vector Carrying the *hsa* Gene

The pLDA-sdHSA vector was constructed by inserting the *hsa* gene (1.8 kb) into *Eco*RI/*Not*I sites of the multiple cloning site of the chloroplast transformation vector (pLD-ctv). This construct contained the *hsa* gene and a ribosome binding site sequence (ggagg) upstream of the gene. For the pLDA-5′UTR-*hsa* vector, the promoter and 5′UTR (205 bp) from *psb*A gene were amplified by PCR from tobacco chloroplast DNA and then sequenced. The subsequent in-frame cloning of the promoter/5′UTR upstream and *hsa* gene into pLD-ctv vector by *Eco*RI/*Not*I digestion produced the functional gene cassette.

Chloroplast Expression Vector Carrying the *ctx*B Gene

A ribosome binding site (GGAGG) was engineered five bases upstream of the start codon of the *ctx*B gene. The PCR product was then cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and subsequently cloned into the chloroplast transformation vector (pLD-ctv) after the sequencing of the open reading frame. The pLD vector carrying the *ctx*B gene was used for successive transformation of tobacco chloroplast genome according to the published protocol.

Chloroplast Expression Vector Carrying the *tps*1 Gene

The yeast *tps*1 gene was inserted into the *Xba*I site of the universal chloroplast expression (pCt) vector between the *aad*A selection marker gene for spectinomycin resistance and the *psb*A terminator to form the final pCt-*tps*1 vector.

Chloroplast Expression Vector Carrying the *Cry*2Aa2 Operon

The *cry*2Aa2 operon from the HD-1 strain (Delattre et al., 1999) was inserted into the universal chloroplast expression vector, pLD ctv, to form the final shuttle vector pLD-BD Cry2Aa2 operon (De Cosa et al., 2001). This vector contains the 16S ribosomal RNA (rRNA) promoter (P*rrn*) upstream of the *aad*A gene for spectinomycin resistance, the three genes of the *cry*2Aa2 operon, and the *psb*A terminator from the 3′ region of the chloroplast PSII gene.

Plant Transformation

Tobacco leaves were transformed by particle bombardment (Bio-Rad PDS-1000He device), using 0.6 μm gold microcarriers coated with the pCt-TPS1 chloroplast expression vector and delivered at 1,100 psi with a target distance of 9 cm (Daniell, 1997). The bombarded leaves were selected on RMOP medium containing 500 μg/ml spectinomycin to regenerate the transformants, as previously described (Kumar and Daniell, 2004; Daniell et al., 2004a).

Northern-Blot Analysis

Total plant RNA from untransformed tobacco (var. Petit Havana) and from three clones of T_1 chloroplast transgenic tobacco plants, was isolated by using the RNeasy Mini kit (Qiagen, Valencia, CA) and protocol. Northern-blot analyses were performed essentially as follows. Total RNA (1 μg) per plant sample was resolved in a 1.2% (w/v) agarose/formaldehyde gel at 55 V for 2.5 h. The RNA was transferred overnight to a nitrocellulose membrane by capillarity. The next day, the membrane was rinsed twice in 2× SSC (0.3 M NaCl and 0.03 M sodium citrate), dried on Whatman paper, and then cross-linked in the GS Gene Linker (Bio-Rad) at setting C3 (150 njouls).

The probes used for northern-blot analyses were obtained as follows: the *aad*A probe was obtained by *Bst*EII/*Xba*I restriction digestion of plasmid pUC19-16S/*aad*A; the *ctx*B and *tps*1 probes were obtained by *Xba*I restriction digestion of plasmids pSBL-CTB and pSBL-TPS1, respectively. The *Cry*2Aa2 probe was obtained by *Xba*I digestion of plasmid pSBL-ctv-*Cry*IIA. The *hsa* probe was obtained by *Eco*RV/*Not*I digestion of plasmid pCR2.1 ATG-*HSA*. Finally, the *orf*1,2 probe was obtained by *Eco*RI digestion of plasmid pCR2.1 ORF1,2 and the *psb*A 3′UTR probe was obtained by *pst*I/*Xba*I digestion of plasmid pLD-ctv.

Probes were radio labeled with ^{32}P dCTP by using Ready Mix and Quant G-50 micro columns for purification (Amersham, Arlington Heights, IL). Prehybridization and hybridization were performed using the Quick-Hyb solution (Stratagene, La Jolla, CA). The membrane was then washed twice for 15 min at room temperature in 2× SSC with 0.1% (w/v) SDS, followed by two additional washes at 60°C (to increase the stringency) for 15 min with 0.1× SSC with 0.1% (w/v) SDS. Radiolabeled blots were exposed to x-ray films and then developed in the Mini-Medical Series x-ray film processor (AFP Imaging, Elmsford, NY). When required, membranes were stripped by applying boiling 0.1% SSC and 0.1% SDS to the membrane, washing for 15 minutes, and repeating before rehybridizing with a different probe.

Relative transcript levels within each lane were measured by spot densitometry (Alphaimager 3300, Alpha Innotech, San Leandro, CA) on radiograms from the different northern-blot analyses, except those obtained using the *psb*A 3′UTR probe. The former are shown as percentage of abundance within each line and therefore comparison among lines cannot be made. For the blots obtained by hybridization with the *psb*A 3′UTR probe, transcript abundance was quantified by using the wild-type native *psb*A transcript, to which a value of 1 was assigned. All other transcripts show values greater or smaller than 1, depending on abundance in relation to the wild-type *psb*A transcript, and are shown as additive values in each line. Average transcript abundance was calculated among corresponding clones and the standard deviation was determined.

Polysomal Fractionation Assays

Approximately 0.3 g of leaf material from transgenic tobacco harboring the *cry*2Aa2 operon were thoroughly ground in liquid nitrogen and resuspended in polysome extraction buffer (A. Barkan, personal communication). The ground tissue was treated according to the protocol described by Barkan (1988), with some modifications. The samples were treated with 0.5% sodium deoxycholate and loaded onto 15% to 55% Suc gradients and centrifuged at 45,000 rpm for 65 minutes (Beckmann rotor SW52Ti). Fractions were collected from the bottom of the tube onto microcentrifuge tubes containing 50 μl 5% SDS and 0.2 M EDTA, up to a volume of about 500 μl for each fraction. Polysomal RNA was extracted with phenol:chloroform:isoamyl-alcohol (25:24:1), followed by ethanol precipitation. The resulting pellets were resuspended in RNase-free TE buffer, pH 8.0, and stored at −80°C or loaded onto denaturing 1.2% agarose-formaldehyde gels (5 μl from each fraction). Northern-blot analyses were then performed as described above. Blots were hybridized with *cry*2Aa2 and *orf*1,2 probes.

To provide further evidence that the RNA obtained from the bottom fractions of the Suc gradient corresponded to polysome-associated RNA, a puromycin-release control was included. Before treatment with sodium deoxycholate, samples were treated with 150 μl of 2 M KCl and 170 μl of puromycin (3 mg/ml stock) and incubated 10 min at 37°C. Sodium deoxycholate was then added to 0.5%, and the samples were incubated 5 min on ice before loading onto the Suc gradient. RNA extraction and northern-blot analyses were performed as described above. The blots were hybridized with the *aad*A and *orf*1,2 probes.

Western-Blot Analyses

Protein samples were obtained from 100 mg of leaf material from the same wild-type and transgenic lines used in the northern analyses by grinding the tissue to a fine powder in liquid nitrogen. Subsequent homogenization in 200 μl plant protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl, 0.05% (w/v) Tween-20, 0.1% (w/v) SDS, 14 mM β-mercaptoethanol, 400 mM Suc, and 2 mM phenylmethylsulfonyl fluoride) was performed, followed by a centrifugation step at 15.7*g* for 1 min to remove solids. The *Bacillus thuringiensis Cry*2Aa2 protein was extracted from 100 mg of transgenic leaf material by adding 200 μl of 50 mM NaOH to solubilize the *Cry* protein from the crystals formed in the transgenic plants, and centrifuged at 10,000*g* for 1 min to remove cell debris. Total protein concentrations for the samples were determined by Bradford assay (Bio-Rad Protein Assay) with bovine serum albumin as the protein standard.

Approximately 60 μg of total soluble protein was loaded onto 12% (v/v) SDS-polyacrylamide gels and separated by electrophoresis. The separated proteins were then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked for 1h with PTM buffer (1× PBS [phosphate buffer solution], 0.05% [v/v] Tween-20, and 3% [w/v] nonfat dry milk). The membranes were probed with primary and secondary antibodies as follows: for 2 h with primary antibody, then rinsed with water twice, and probed with secondary antibody for 1.5 h. Finally, the membranes were washed three times for 15 min with PT buffer (1× PBS, 0.05% [v/v] Tween-20) and one time with 1× PBS for 10 min, followed by incubation in Lumi-phos WB (Pierce, Rockford, IL) reagent for the alkaline phosphatase reaction or SuperSignal (Pierce) reagent for horseradish peroxidase reaction.

Film exposure took place for 1, 3, 5, or 10 min, depending on the strength of the signal of each blot. The antibodies used and their respective dilutions were the following: anti-*cry*2A (Envirologix, Portland, ME), dilution 1:3,000; anti-*ORF*2 (Moar et al., 1989), dilution 1:1,000; anti-*HSA* (Sigma, St. Louis, MO), dilution 1:3,000; anti-CTB (Sigma), dilution 1:2,500; anti-*PA* (Dr. Stephan Leppla, NIH), dilution 1:30,000. Secondary antibodies were used as follows: alkaline phosphatase conjugated anti-rabbit antibody (Sigma) was used to probe against every primary antibody with the exception of anti-PA, which was probed with horseradish peroxidase conjugated anti-mouse antibody; dilutions of 1:5,000 anti-rabbit antibody were used for anti-*HSA*, anti-*ORF*2 and anti-*Cry*2A, for anti-*CTB* the dilution was 1:4,000. Anti-mouse antibody was used in a 1:5,000 dilution.

ELISA Quantification

The Human Albumin Quantitation kit (Bethyl Laboratories) was used for ELISA quantification. Leaf material (100 mg) of the *aad*A-*orf*1-*orf*2-*hsa* transgenic line was ground in liquid nitrogen and resuspended in 700 μl of 50 mM NaOH. The leaf extracts were then diluted to fit in the linear range of the provided HSA standard. Absorbance was read at 450 nm. The DC protein assay (Bio-Rad) was used to determine total soluble protein concentration following the manufacturer's protocol.

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