Transcription Factor RAP2.2 and Its Interacting Partner SINAT2: Stable Elements in the Carotenogenesis of Arabidopsis Leaves\textsuperscript{1[W]}

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The promoter of phytoene synthase, the first specific enzyme of carotenoid biosynthesis, shows two main regulatory regions: a G-box-containing region located near the TATA box, and a TATA box distal region containing the cis-acting element ATCTA, which mediates strong basal promoter activity. This second element was also present in the promoter of phytoene desaturase, the next step of the carotenoid pathway, suggesting a common regulatory mechanism. In this work, we demonstrate that AtRAP2.2, a member of the APETALA2 (AP2)/ethylene-responsive element-binding protein transcription factor family, binds to the ATCTA element. In Arabidopsis (Arabidopsis thaliana) leaves, ATRAP2.2 transcript and protein levels were tightly controlled as indicated by unchanged transcript and protein levels in T-DNA insertion mutants in the ATRAP2.2 promoter and 5' untranslated region and the lack of change in ATRAP2.2 protein levels in lines strongly overexpressing the ATRAP2.2 transcript. Homozygous loss-of-function mutants could not be obtained for the ATRAP2.2 5' untranslated region T-DNA insertion line indicating a lethal phenotype. In ATRAP2.2 overexpression lines, modest changes in phytoene synthase and phytoene desaturase transcripts were only observed in root-derived calli, which consequently showed a reduction in carotenoid content. The RING finger protein SEVEN IN ABSENTIA OF ARABIDOPSIS2 (SINAT2) was identified as an AtRAP2.2 interaction partner using a two-hybrid approach. The structure of SINAT2 and related proteins of Arabidopsis show developmental processes. The action of SINAT2 may explain the recalcitrance of ATRAP2.2 protein levels to change by altering AtRAP2.2 transcription.

Carotenoids fulfill important functions in photosynthesis, including harvesting of light energy and protection from damage by excess light energy (for a recent review, see Szabó et al., 2005). Accordingly, illumination of etiolated seedlings leads to the induction of carotenoid synthesis, coordinated with the synthesis of chlorophylls. Molecular analysis of this process revealed that the first committed enzyme of the carotenoid biosynthesis pathway, phytoene synthase (PSY), is strongly light induced both at the mRNA and protein levels in seedlings of mustard (Sinapis alba) and Arabidopsis (Arabidopsis thaliana; von Lintig et al., 1997; Welsch et al., 2000). In contrast, the mRNA and protein levels of enzymes acting upstream and downstream of PSY in the pathway, such as geranylgeranyl-diphosphate synthase and phytoene desaturase (PDS), respectively, remained relatively constant. A key role for PSY may not be restricted to deetiolation and green tissues. It has recently been shown that carotene desaturation and lycopene cyclization are not rate limiting in carotenoid synthesis in Golden Rice (Al-Babili et al., 2006) and that PSY activity plays the major role (Paine et al., 2005).

Because of its crucial regulatory role of PSY in the carotenoid pathway, the PSY promoter region was analyzed in more detail (Welsch et al., 2003). This revealed two main regions that are responsible for the regulation of transcriptional activities. A TATA box proximal region containing G-box-like elements is involved in light induction and discrimination between different light qualities. A TATA box distal region enables a high basal level of promoter activity. Using gel retardation assays, the pentameric sequence ATCTA, occurring in tandem in the TATA box distal region, was identified as the cis-acting element for a transcription factor mediating strong PSY promoter activity. Interestingly, this novel motif is also found in promoter regions of other carotenogenic genes (i.e. PDS from tomato [Lycopersicon esculentum] and photosynthesis-related genes, such as the chlorophyll a/b-binding protein [CAB] of mustard and Arabidopsis and plastocyanin of pea [Pisum sativum]). The formation of protein-DNA complexes with the same migration pattern was observed with all these promoter regions containing the ATCTA motif. In addition, the complex formed with the CAB-mustard promoter element could compete with the
PSY- Arabidopsis promoter element containing the ATCTA motif, indicating that the same transcription factor was involved.

These observations support the notion that the trans-acting factor involved might be able to regulate several genes involved in carotenogenesis, thereby also coordinating the expression of genes involved in photosynthesis. This, plus the proven importance of PSY in the carotenoid biosynthetic pathway, prompted us to identify cis-acting factors that govern the level of the basal PSY transcriptional activity using Arabidopsis as a model system.

RESULTS

South-Western Screening

Transcription factors that bind to cis-acting elements defined in the PSY promoter are unknown to date. To identify transcription factors binding to the previously identified upstream element (Welsch et al., 2003), we subjected a cDNA library of Arabidopsis to South-western screening. We used concatemers of the sequence 5’-CAATCTAAATATCTAAATATATAAA-3’ as a probe, defined previously as a specific competitor of the protein-DNA complexes in gel retardation assays (Welsch et al., 2003). A λZAPII cDNA library of Arabidopsis (Kieber et al., 1993) comprising cDNAs of 1 to 2 kb in size was screened in parallel under two conditions. For binding of the probe to the bacteriophage plaques, screening was performed under native conditions and after chaotropic denaturation/renaturation of the recombinant proteins (Vinson et al., 1988). The screening process revealed reproducible signals. In vivo excision and sequencing resulted in the identification of a sequence identical to the transcription factor RAP2.2 from Arabidopsis (AtRAP2.2), an uncharacterized member of the family of APETALA2 (AP2)/ethylene-responsive element-binding protein (EREBP) transcription factors (AGI no. At3g14230; accession no. NM_180252).

Binding Assay with Recombinant AtRAP2.2

To confirm that AtRAP2.2 binds to the cis-acting element characterized in gel retardation experiments and used in South-western screening, gel retardation assays were performed with the recombinant protein. For this, AtRAP2.2 cDNA was subcloned into the expression vector pQE30, thereby providing the recombinant protein with an N-terminal 6×-His tag. Purification of the recombinant protein via metal affinity under native conditions was not possible and gel retardation assays using the bacterial lysate failed due to accumulation of the recombinant protein in inclusion bodies (data not shown). Therefore, a chaotropic denaturation/renaturation procedure was applied following the conditions used during the screening. The use of renatured bacterial lysate containing recombinant AtRAP2.2 and the −856 to −825 region of the PSY promoter as a radiolabeled probe revealed the formation of a protein-DNA complex that could be competed specifically (Fig. 1). Compared to the control complex formed with nuclear extracts from illuminated mustard seedlings (as in Welsch et al., 2003), the complex formed with recombinant AtRAP2.2 migrated slightly differently. This is probably due to the N-terminal 6×-His tag influencing electrophoretic mobility of the complex.

Effects of Changed AtRAP2.2 Transcript Amounts in Arabidopsis

Transgenic AtRAP2.2-overexpressing lines were produced in Arabidopsis (ecotype Wassilewskija) to study the regulation of carotenoid biosynthesis and possible effects on expression of other photosynthesis-related genes. AtRAP2.2 cDNA was expressed under the control of a strong promoter containing four tandem cauliflower mosaic virus (CaMV) enhancer elements (4CaMV-35S). The weaker nos promoter (nosP) was also used to create low expressing lines. From several transgenic lines, one homozygous line of each transformation was selected, grown under short-day conditions, and rosette leaves were harvested for further analyses. Real-time reverse transcription (RT)-PCR indicated that, relative to wild-type leaves, the

![Figure 1](Image 346x196 to 490x397)
AtRAP2.2 expression level was increased about 2-fold in the weak overexpressing line (nosP::AtRAP2.2; nosr-2) and almost 12-fold in the strong overexpressing line (4CaMV-35S::AtRAP2.2; cmr-5; Fig. 2B, left).

To investigate the effects caused by loss of AtRAP2.2 function, we took advantage of two T-DNA insertion lines, disrupted within the AtRAP2.2 gene (Fig. 2A). One line, rapDpro, carried a T-DNA in the TATA box proximal region, leaving an intact minimal promoter of only 130 bp. However, AtRAP2.2 transcript levels in homozygous rapDpro rosette leaves were relatively unchanged (Fig. 2B), indicating that the remaining intact promoter region provided sufficient activity to allow the accumulation of AtRAP2.2 transcript comparable to wild-type levels. The second T-DNA insertion line, rapDutr, contained the T-DNA within the 5′ untranslated region (UTR) of the AtRAP2.2 transcript (leaving 60 bp of the 5′ UTR). However, unlike rapDpro, homozygous progeny for rapDutr could not be identified, even after repeated analyses of several generations of T3 progeny, indicating that loss of AtRAP2.2 function is lethal for embryo/seed development. Analysis of AtRAP2.2 transcript levels in rosette leaves of heterozygous rapDutr progeny revealed almost no difference compared to wild type. Because the recessive loss-of-function rapDpro allele is lethal, it was not included in further analysis.

The two AtRAP2.2-overexpressing lines and two T-DNA insertion lines showed no apparent phenotypic differences compared to wild-type plants. Analysis of the carotenoid and chlorophyll content by HPLC revealed almost unchanged pigment content and patterns (Fig. 2F, left) and quantification of PSY and PDS transcript levels by real-time RT-PCR also revealed relatively unchanged mRNA levels compared to the wild type (Fig. 2, D and E, left). However, the two overexpressing lines did show strongly increased AtRAP2.2 transcript levels. To determine whether this increase in transcript level translated to an increase in AtRAP2.2 protein, antibodies directed against AtRAP2.2 were generated to conduct western-blot analyses.

Properties of AtRAP2.2

For antibody production, a glutathione S-transferase (GST) fusion protein of the N-terminal 166 amino acids of AtRAP2.2 was used as antigen because sufficient amounts of full-length AtRAP2.2 protein could not be produced in different bacterial (6×-His tag; GST) and yeast (Saccharomyces cerevisiae) expression systems. Western-blot analyses using total protein extracts from Arabidopsis wild-type rosette leaves yielded a signal with an apparent molecular mass of about 60 kD, whereas the calculated molecular mass of AtRAP2.2 is only 42.1 kD (Fig. 3). The same unexpected migration behavior was observed when AtRAP2.2 cDNA was translated in vitro in the presence of [35S]-Met, both in reticulocyte lysate (RL) and wheat (Triticum aestivum) germ (WG) lysate (lane 1 RL and 1 WG, respectively). Strongly denaturing conditions applied by using urea-containing loading buffer did not result in a change of electrophoretic mobility (lane 1 WGU). To confirm that the antibodies generated specifically recognize AtRAP2.2, western-blot analyses were performed with the in vitro-translated product. This revealed a signal identical to those detected in the autoradiograms of the corresponding in vitro translations. Taken together, this proves that the western-blot signals obtained with protein extracts from leaves correspond to the authentic AtRAP2.2. Furthermore, we partially purified AtRAP2.2 from wild-type Arabidopsis nuclear protein extracts by DNA affinity chromatography. For this, concatemers of the sequence used in the South-western screening described above were coupled to Sepharose. Chromatography and subsequent SDS-PAGE revealed one dominant band migrating at a molecular mass of 60 kD.

Because AtRAP2.2 shows higher apparent molecular mass than expected in SDS-PAGE, we investigated the structural nature of this migration behavior by examining the apparent molecular masses of different truncated forms of AtRAP2.2 produced as in vitro-translated [35S]-labeled proteins. Higher than expected masses were still observed when the AP2 domain was deleted (Fig. 3, lane 2 WG, RAPΔ109–192; calculated molecular mass 32.5 kD, apparent molecular mass 45 kD) and when only the N-terminal 250 amino acids were translated (lane 3 WG, RAP1–250; calculated molecular mass 28.7 kD; apparent molecular mass 37 kD). However, the expected molecular mass was obtained for the N-terminal 166-amino-acid translation product (lane 4 WG, RAP1–166, calculated and apparent molecular masses 18.8 kD). Therefore, the region between amino acids 192 and 250 is the source of the electrophoretic discrepancies observed. The primary and secondary structure of this region does not suggest a basis for the altered electrophoretic mobility of AtRAP2.2 and no distinguishing domains or motifs were identifiable within this domain.

Unchanged Protein Amounts in Leaves of AtRAP2.2-Overexpressing Arabidopsis Lines

No apparent differences in AtRAP2.2 protein levels were detected (at the authentic electrophoretic mobility of 60 kD) in leaves of AtRAP2.2-overexpressing lines and the T-DNA insertion line rapDpro (Fig. 2). Whereas this was expected, with the latter considering its relatively unchanged transcript levels, it was surprising that the 12-fold increase in transcript levels in the AtRAP2.2-overexpressing line cmr-5 would not lead to an increase in protein levels. This finding provides an interpretation of Affymetrix ATH1 GeneChip expression analyses run using total RNA isolated from leaves from the two overexpressing lines and the Wassilewskija wild type (see Table I). When using a 2.5-fold difference in all transgenic-to-wild type chip-to-chip comparisons as a filter, only six and four genes showed significant changes in the nosr-2- and cmr-5-overexpressing lines, respectively. Out of these genes,
only two plastid-encoded genes occurred in both data-
sets, the PSI assembly protein, ycf4, and the ATPase I
subunit, atpF. Additional differently expressed genes
in the weak overexpressing line, nosr-2, belong to
processes involved in RNA and protein regulation.
Analysis of the datasets using less stringent parame-
ters increased the number of differently expressed
genes more than 10-fold in both datasets (see Supple-
mental Table S1, A and B), but the list of genes affected
in both nosr-2 and cmr-5 only increased by two genes
(see Supplemental Table S1C). These expression anal-
yses are consistent with the observation that in pho-
tosynthetically active tissues AtRAP2.2 protein levels
cannot be altered by increasing AtRAP2.2 transcript
levels and therefore no pronounced changes in the
transcriptome result from AtRAP2.2 overexpression.

Effects of AtRAP2.2-Overexpressing Arabidopsis Lines
on Root-Derived Calli

Numerous transgenic experiments in various plant
systems have demonstrated that photosynthetically
active tissues/cells are more recalcitrant to attempts to
engineer carotenoid flux and content than nongreen
tissues/cells (for a recent review, see Howitt and
Pogson, 2006). Therefore, we analyzed callus tissue
generated from roots of the AtRAP2.2-overexpressing lines (Fig. 2, right). Here, the weak overexpressing line, nosr-2, and the strong overexpressing line, cmr-5, showed a 2- and 17-fold increase in AtRAP2.2 transcript levels relative to wild-type levels. Western-blot analysis to investigate AtRAP2.2 protein amounts in callus failed because protein amounts were below the detection limit in all cases. However, if the increase in AtRAP2.2 transcript amounts correlated with an increase in protein amounts in this tissue, one would expect a detectable signal, at least with the high expressing line. We assumed that in root-derived calli—just as in leaves—AtRAP2.2 protein amounts were being kept at a low steady-state level and that there was no linear correlation between transcript and protein amounts. Consistent with this assumption, PSY expression levels and the carotenoid content of nosr-2 and cmr-5 were similar to wild type (Fig. 2, D and F, right). Only PDS expression was slightly increased relative to the wild type (24% and 62% increase in nosr-2 and cmr-5, respectively). This might represent effects caused by a small AtRAP2.2 increase.

In contrast to the overexpressing lines, root-derived calli generated from the T-DNA insertion line rapDpro carrying an insertion in the promoter region showed a significant decrease in AtRAP2.2 transcript levels to

Figure 3. Migration behavior of AtRAP2.2 in SDS-PAGE. A, Autoradiography of in vitro translation products of different AtRAP2.2 truncations (left). Western-blot analysis using anti-AtRAP2.2 antibodies and a silver stain of proteins obtained by DNA affinity chromatography is shown on the right. B, Schematic representation of the truncations used in A; the region responsible for the unusual migration behavior observed is marked between dashed lines. C, Amino acid sequence of AtRAP2.2. The AP2 domain is shown in bold; arrowheads indicate the amino acid positions used for the truncations in B. The calculated mass of AtRAP2.2 is 42.1 kD; however, an apparent molecular mass of about 60 kD is observed in western-blot analyses using 40 µg of leaf protein extracts and anti-AtRAP2.2 antisera (leaves wt). The [35S]Met translation products of the AtRAP2.2 cDNA showed the same electrophoretic mobility in RL (1 RL) and WG lysate (1 WG). The presence of urea in the loading buffer did not influence this behavior (1 WGU). Deletions lacking the AP2 domain (2 WG, RAPΔ109–192; calculated molecular mass, 32.5 kD; apparent, 45 kD) and truncation of the C-terminal 124 amino acids (3 WG, RAP1–250; calculated molecular mass, 28.7 kD; apparent, 37 kD) also showed anomalous electrophoretic mobility. The expected molecular mass was observed for the N-terminal 166 amino acids (4 WG, RAP1–166, calculated and apparent molecular mass, 18.8 kD). Western-blot analysis conducted with the in vitro translation product showed a signal identical to that observed in the autoradiography of the same sample (1 WG), whereas an in vitro translation performed with the empty vector pGEM4 showed no signal (K WG). Using concatemers of the sequence used for the screening of AtRAP2.2 and nuclear proteins of Arabidopsis leaves, DNA affinity chromatography was performed. Silver staining revealed selective enrichment of AtRAP2.2 in the eluate (DNAaffi).
AtRAP2.2-overexpressing lines

Leaves of weak AtRAP2.2-overexpressing line nosr-2 versus wild-type leaves

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>Fold-Change</th>
<th>Probe Set No.</th>
<th>AGI No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleosidein/PDI-related protein, putative receptor kinase</td>
<td>-2.6</td>
<td>253519_at</td>
<td>At4g31240</td>
</tr>
<tr>
<td>Transducin/WVD40 repeat family protein, putative splicing factor</td>
<td>-2.5</td>
<td>263261_at</td>
<td>At1g0580</td>
</tr>
<tr>
<td>Cullin 3B, ubiquitin-protein ligase</td>
<td>-2.5</td>
<td>260416_at</td>
<td>At1g69670</td>
</tr>
<tr>
<td>Zinc (RING) finger family protein</td>
<td>-2.5</td>
<td>259800_at</td>
<td>At1g27175</td>
</tr>
<tr>
<td>PSI assembly protein, ycf4</td>
<td>3.2</td>
<td>245018_at</td>
<td>AtCG00520</td>
</tr>
<tr>
<td>ATPase 1 subunit, atpF</td>
<td>3.1</td>
<td>245025_at</td>
<td>AtCG00130</td>
</tr>
</tbody>
</table>

Leaves of strong AtRAP2.2-overexpressing line cmr-5 versus wild-type leaves

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>Fold-Change</th>
<th>Probe Set No.</th>
<th>AGI No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exocyst subunit EXO70 family protein</td>
<td>2.4</td>
<td>247693_at</td>
<td>At5g59730</td>
</tr>
<tr>
<td>PSI assembly protein, ycf4</td>
<td>3.6</td>
<td>245018_at</td>
<td>AtCG00520</td>
</tr>
<tr>
<td>ATPase 1 subunit, atpF</td>
<td>3.1</td>
<td>245025_at</td>
<td>AtCG00130</td>
</tr>
<tr>
<td>Ribosomal protein L14, rpl14</td>
<td>2.5</td>
<td>244982_at</td>
<td>AtCG00780</td>
</tr>
</tbody>
</table>

Genes affected in leaves of the weak AtRAP2.2-overexpressing line nosr-2 and the strong AtRAP2.2-overexpressing line cmr-5, respectively, are shown as fold induction/repression relative to wild-type leaves, each as the mean of two biological replicates. For analysis, data were filtered for flags present in three of four samples, followed by filtration for 2.5-fold difference between transgenic and wild-type lines. For data analysis with less stringent parameters, see Supplemental Table S1.

about 35% of wild type (Fig. 2B, right). This difference relative to leaves (where transcript levels remained unchanged) may lie in the fact that AtRAP2.2 mRNA levels in wild-type calli are 5-fold higher than in leaves. Therefore, the remaining promoter activity in leaves is sufficient to reach wild-type levels in leaves of AtRAP2.2, but obviously too weak to provide the higher levels found in wild-type calli. Conclusions about AtRAP2.2 protein levels from western-blot analysis were not possible because they were below the detection limit.

Interestingly, the decrease in AtRAP2.2 transcript levels in rapΔpro was accompanied by significant reduction of both PSY and PDS transcripts to 50% of the wild-type level and was correlated with a 30% decrease in carotenoid content relative to wild-type calli (Fig. 2, D–F, right).

Interaction Partners of AtRAP2.2

The results presented so far led to the conclusion that AtRAP2.2—at least in leaves—is regulated mainly at the posttranslational level: Protein levels were unresponsive to changes in mRNA abundance. This might indicate that AtRAP2.2 protein is subjected to specific protein degradation, a process that involves protein–protein interaction.

To obtain information on possible AtRAP2.2 interaction partners, protein overlay assays using nuclear extracts from light-grown mustard seedlings were performed. For this, in vitro-translated [35S]-labeled AtRAP2.2 and a radiolabeled C-terminal truncation, RAP1-250, were used. Both translation products recognized at least two proteins, both with a molecular mass of about 36 kD (Fig. 4).

In subsequent yeast two-hybrid screens, the entire AtRAP2.2 cDNA was fused to the GAL4 activation domain of pGBT9 to yield pGBT9-RAP. Yeast H7c cells transformed with this construct alone were able to grow on synthetic dropout medium lacking Trp and His, which indicated autoactivation of the HIS3 reporter gene. This is frequently observed with transcription factors and limits the use of the two-hybrid screen. Attempts to repress this autoactivation by the addition of the His biosynthesis suppressor 3-aminol,2,4-triazol were unsuccessful. Therefore, we set out to eliminate the responsible region by using truncated forms of AtRAP2.2 in an autoactivation growth test (Fig. 5). This revealed that AtRAP2.2 contains at least two regions that can independently act as autoactivation domains. One region is located in the C-terminal third between positions 252 and 374, because yeast transformed with pGBT9-RAP252-374 was able to grow on selective medium. The second region is located in the central third of the amino acid sequence between positions 166 and 250 because the N-terminal 250 amino acids of AtRAP2.2 (in pGBT-RAP1-250) show autoactivation, whereas the N-terminal 166 amino acids (in pGBT-RAP1-166) do not.

Because the RAP1-166-GAL4BD fusion protein was devoid of autoactivation activity, the construct pGBT9-RAP1-166 was used as the bait vector in the two-hybrid screen. An Arabidopsis library with cDNAs connected to the GAL4 activation domain in pGAD424 was used as the prey library; 3 × 10⁶ yeast transformants were screened. After eliminating false-positive clones by confirming the interaction with RAP1-166 by reconstitution, by using the second reporter gene lacZ, and by testing possible autoactivation of the prey cDNAs alone, five positive clones were obtained. Restriction endonuclease digestion and DNA sequencing showed that they contained identical cDNAs corresponding to a 5′-proximal truncated cDNA of SEVEN IN ABSENTIA IN ARABIDOPSIS2 (SINAT2; AGI no. At3g58040; accession no. AY087768.1). The result of these two-hybrid assays showed that AtRAP2.2
may interact with this particular gene product. The protein overlay assay described above demonstrated interaction between AtRAP2.2 and a protein of 35 kD, which corresponds to the molecular mass of SINAT2. Interestingly, SINAT2 contains a RING zinc finger motif involved in proteasomal-mediated degradation of proteins as part of many E3 ubiquitin ligases.

Identification of SINAT2 as Interaction Partner of AtRAP2.2

To verify the interaction of AtRAP2.2 with SINAT2, pull-down assays were carried out. The C terminus of SINAT2 was subcloned in the vector pGEX4T2 enabling the expression of an N-terminal GST-fusion protein (GST-SINAT2-C). After purification, pull-down assays were performed with [35S]-labeled AtRAP2.2. As shown in Figure 6, GST-SINAT2-C interacted with AtRAP2.2, whereas GST alone did not. To repeat pull-down assays with the entire SINAT2 amino acid sequence, the full-length cDNA of SINAT2 was cloned by RT-PCR using total RNA isolated from Arabidopsis leaves, subcloned into pGEX4T2, and expressed as N-terminal GST fusion protein as above (GST-SINAT2). The pull-down assay performed with GST-SINAT2 and [35S]AtRAP2.2 confirmed the results obtained with the N-terminally truncated SINAT2, as shown in Figure 6.

This interaction of AtRAP2.2 and SINAT2 may explain the observed discrepancies between increased transcript amounts and unchanged protein amounts in leaves of AtRAP2.2-overexpressing lines described above. SINAT2 might target AtRAP2.2 for proteasomal degradation, ensuring constant steady-state AtRAP2.2 protein amounts, independent of transcript levels. Therefore, variations of SINAT2 transcript amounts might lead to more pronounced phenotypic effects than variations of AtRAP2.2 transcript amounts.

Analysis of SINAT2 T-DNA Insertion Lines

To investigate the effects of decreased SINAT2 transcript amounts, a T-DNA insertion line was characterized that carries T-DNA within the second exon of the SINAT2 gene (sinat2Δ; Fig. 7). If a stable transcript were produced in this line, it would contain a premature translational stop after amino acid 134 that would result in the deletion of both RING finger domains, rendering protein-protein interaction impossible. Homozygous sinat2Δ progeny showed no apparent phenotypic differences compared to wild type. As shown in Figure 7, neither rosette leaves nor root-derived calli from the homozygous SINAT2 insertion line showed differences in carotenoid content or composition. Accordingly, transcript levels of PSY, PDS, and also AtRAP2.2 remained almost unchanged in sinat2Δ compared to wild type. AtRAP2.2 protein amount was examined by western-blot analysis in leaves and was found to be unchanged as well. This unexpected result...
may be due to the existence of four SINAT2 homologous proteins in Arabidopsis that may functionally substitute in this regard for the loss of function of SINAT2.

**DISCUSSION**

The cis-acting element ATCTA on the PSY promoter of Arabidopsis is responsible for high-level basal expression of the gene. Regulation of PSY promoter activity via this upstream element is independent of the G-box-like elements involved in light response (Welsch et al., 2003). Using the promoter region containing the ATCTA motif as a probe in South-western screening procedures, we isolated the transcription factor AtRAP2.2, a member of the large family of AP2/EREBP transcription factors (Riechmann and Meyerowitz, 1998) and verified its binding specificity by gel retardation assays. The ATCTA motif occurs in both the PSY and PDS promoters and is recognized by the same transcription factor, probably AtRAP2.2 (Welsch et al., 2003).

**AtRAP2.2 Is an Essential Gene Whose mRNA Is Recalcitrant to Down-Regulation**

Plant lines carrying a T-DNA insertion in the AtRAP2.2 promoter region and in the 5'-UTR region were used to investigate the phenotypic effects of altering AtRAP2.2 expression. Surprisingly, down-regulation of AtRAP2.2 mRNA could not be achieved in leaves. rapΔpro, which carries a T-DNA insertion approximately 70 bp upstream of the putative TATA box, apparently maintains sufficient promoter activity to drive AtRAP2.2 expression to wild-type levels. Homozygous rapΔpro progeny were viable and with AtRAP2.2 expression and visible phenotype indistinguishable from wild type. These data suggest AtRAP2.2 is an essential gene and that negative impact on AtRAP2.2 mRNA levels due to moderate down-regulation of transcription are avoided by feedback regulation of mRNA levels. The situation differs somewhat in nongreen tissues, such as root-derived calli where wild-type AtRAP2.2 mRNA levels are 5 times higher than in leaves. In this tissue, the minimal promoter activity of the homozygous rapΔpro mutant is not sufficient to attain the elevated level in wild type. This yielded the expected effects, namely, down-regulation of PSY and PDS expression (both of which carry the AtRAP2.2-binding motif in their promoters) and consequently decreased carotenoid levels.

**AtRAP2.2 Interacts with SINAT2, a RING Finger Protein**

In contrast to attempts to down-regulate AtRAP2.2 expression, AtRAP2.2 mRNA levels could be readily increased in overexpression experiments. However, despite greater than 10-fold increases in AtRAP2.2 mRNA levels in transgenics, AtRAP2.2 protein levels were unchanged. This might be due to an mRNA-based regulatory mechanism (e.g. miRNA; Aukerman and Sakai, 2003; Chen, 2004) because a computational approach taken by Wang and coworkers (2004) identified AtRAP2.2 as a target for a yet-uncharacterized miRNA (Wang et al., 2004). However, using the truncated RAP1–166 as bait, we identified the RING finger protein SINAT2 as an interaction partner of AtRAP2.2 and these data point to proteasome-mediated protein degradation as the underlying regulatory principle controlling AtRAP2.2 protein levels.

SINAT2 contains two zinc finger domains of different types (see Supplemental Fig. S1). One shows a RING-type profile and is located between amino acids 60 to 96; the second has a SEVEN IN ABSENTIA HOMOLOG (SIAH)-type profile and is located between amino acids 113 and 173. The SIAH-type domain...
defines a zinc finger type found in the *Drosophila* SEVEN IN ABSENTIA (SINA) protein and its mammalian orthologs. The SINA gene encodes a nuclear protein that is required for the correct development of R7 photoreceptor cells in the *Drosophila* eye (Carthew and Rubin, 1990). The architecture found in SINAT2—a RING-type zinc finger followed by a SIAH-type zinc finger—is found in a small group of five related proteins in Arabidopsis (SINAT1 to SINAT5; Xie et al., 2002).

The RING finger domain is related to the zinc finger domain family that represents one of the most abundant domains in the Arabidopsis proteome. In contrast to zinc finger domain-containing proteins, which are a functionally diverse group, the RING finger domain is generally considered to be involved in protein-protein interactions (Laitly et al., 2001; Kosarev et al., 2002). Many RING finger proteins are involved in ubiquitylation of substrate proteins to initiate their proteasome-mediated degradation. During this process, RING finger domain-containing proteins are constituents of E3 ligases that are ubiquitylated by an E2 protein (ubiquitin-conjugating protein). Consecutively, transfer of the ubiquitin moiety to its substrate target proteins is mediated, a process that requires protein-protein interaction. RING domains are assumed to contribute to the scaffold by providing optimal positioning of E2 and the substrate for the transfer of ubiquitin (Zheng et al., 2002).

The regions involved in the interaction between AtRAP2.2 and SINAT2 were deduced from yeast two-hybrid screening and pull-down assays. Due to the autoactivating properties of the C-terminal half of AtRAP2.2, yeast two-hybrid screening was performed using the N-terminal 166 amino acids of the protein, which removed the C-terminal half of SINAT2. Interestingly, the C-terminal half of SINAT2 does not contain any of the two zinc finger domains, but the corresponding domain has been identified as the substrate-binding domain in SIAH and is thus involved in the recognition and binding of a variety of different protein substrates (Reed and Ely, 2002). Because most of the AP2 domain was included in the N-terminal part of AtRAP2.2 used for the screening and pull-down assays, it appears that SINAT2 and the other four SINAT2-homologous proteins present in Arabidopsis regulate additional AP2 domain-containing proteins via their C-terminal substrate-binding domain.
Several lines of evidence support the involvement of SINAT2 in protein degradation processes. SINAT5, a close homolog of SINAT2 in Arabidopsis, is involved in ubiquitin-mediated regulation of auxin-regulated developmental processes, as suggested by its in vitro ubiquitin protein ligase activity (Xie et al., 2002). Furthermore, both the Drosophila protein SINA and homologous mammalian proteins (SIAHS) are involved in the ubiquitin-mediated degradation of proteins with important functions in developmental processes (Li et al., 1997; Tang et al., 1997; Dong et al., 1999; Boulton et al., 2000). Therefore, it appears likely that SINAT2 is also an E3 ligase constituent acting on AtRAP2.2. Thus, the observation that AtRAP2.2 protein levels in leaves of AtRAP2.2-overexpressing lines are unchanged is best explained by a regulated protein degradation process involving SINAT2 that maintains a constant steady-state level of the protein despite alterations in mRNA levels. However, in leaves of SINAT2 T-DNA knockout lines, the AtRAP2.2 protein amounts remain unchanged. Interestingly, the identity of the four other members of the Arabidopsis SINAT protein family to SINAT2 is very high (homology to SINAT1, 94.2%; SINAT3, 72.7%; SINAT5, 72.4%; SINAT4, 71.6%; see Supplemental Fig. S1). It is therefore conceivable that one or more of the members of this group are able to functionally compensate for SINAT2 in the SINAT2 line.

At the protein level, it is not surprising that the whole-genome microarray expression analyses conducted in parallel showed little variability. Expression of only six genes was changed in leaves of the weak AtRAP2.2-overexpressing line nosr-2 when applying a minimal filter for expression changes of at least 2.5-fold in all pair-wise analyses of wild type. Furthermore, in comparison with the strong AtRAP2.2-overexpressing line cmr-5, no common nuclear-encoded genes were affected. Therefore, the slight changes in expression levels observed in leaves are most probably due to minor variations and do not represent an effect of the overexpression of AtRAP2.2.

AtRAP2.2: General Considerations

The AP2/EREBP family of transcription factors, to which AtRAP2.2 belongs, is classified according to the existence of one or two DNA-binding AP2 domains, initially characterized in AP2 (Weigel, 1995). Apart from the AP2 domains, homology is very low among almost all AP2/EREBP proteins. Recently, three groups presented data from other AP2 domain-containing transcription factors from tomato, tobacco, and barley (Hordeum vulgare), respectively, with 25% to 38% identity to AtRAP2.2 at the protein level. For JERF1 from tomato, Zhang and coworkers demonstrated that this transcription factor binds to the GCC box and the DRE sequence in vitro, both of which are involved in stress responses (Zhang et al., 2004). Overexpression of JERF1 in tobacco led to increased expression of GCC box-containing genes in leaves under nonstressed growth conditions, consequently leading to enhanced salt stress tolerance. Lee and coworkers identified NtCEF1 from tobacco, which shows 68.5% identity to JERF1, and demonstrated its binding to the GCC box and the C/DRE element in vitro by gel retardation assays (Lee et al., 2005). However, only GCC box-containing genes were affected in vivo. Overexpression of NtCEF1 in Arabidopsis led to enhanced resistance to a bacterial pathogen. Jung and coworkers reported that overexpression of HvRAF from barley in Arabidopsis also activated stress-responsive genes under nonstressed conditions and conferred enhanced pathogen resistance (Jung et al., 2007).

The question arises as to whether AtRAP2.2 represents the Arabidopsis equivalent of NtCEF1, JERF1, or HvRAF. Because of the generally low degree of homology among the members of the AP2/EREBP family, despite a common AP2 domain, functional equivalency based on homology is often difficult. Given this constraint, the 38% identity that AtRAP2.2 shares with JERF1 and NtCEF1 is relatively high. However, the main argument against AtRAP2.2 being functionally equivalent is the difference in the binding motif. The stress-related family members mentioned above bind to motifs containing the GCC box, which is commonly involved in stress-mediated responses, whereas AtRAP2.2 recognizes the motif ATCTA. Interestingly, the binding specificity for several AP2 domain-containing transcription factors appears to be very complex in vivo. This was concluded from a systematic approach conducted for the tomato transcription factor Pt4 for which binding of the GCC box was shown in vitro (Chakravarthy et al., 2003). Analysis of transcripts regulated by the overexpression of Pt4 in Arabidopsis revealed that most of the promoters of Pt4-regulated genes did not contain a GCC box, leading to the hypothesis that Pt4 might be able to recognize both GCC as well as non-GCC box-containing motifs. Data from other AP2/EREBP-overexpressing plants also indicate possible regulation of non-GCC box-containing genes (Wu et al., 2002; Tournier et al., 2003).

Regulatory factors, such as AtRAP2.2, may have the potential to improve complex multigene traits through genetically modified organism approaches or marker-assisted breeding for variation at the AtRAP2.2 locus. For AtRAP2.2, this might allow one to favorably alter the nutritional composition of crop plants by influencing entire biosynthetic pathways or improving agronomic properties. This is the case when those genes are master regulators of traits, such as the maize (Zea mays) LC/C1 transcription factors capable of increasing flavonol content in tomato (Bovy et al., 2002) or the Sub1A transcription factor conferring submergence tolerance in rice (Oryza sativa; Xu et al., 2006). The fact that AtRAP2.2 bound to at least PSY and PDS upstream regulatory sequences raised expectations for...
its potential biotechnological value to modify provita-
imin A biosynthesis. However, our data indicate that AtRAP2.2 and SINAT2 are just two constituents of a significantly more complex regulatory network in-
volved in carotenogenesis.

MATERIALS AND METHODS

South-Western Screening
A pZAPII cDNA-library of Arabidopsis (Arabidopsis thaliana; Kiefer et al., 1993) was screened via South-western screening. As a radiolabeled probe, the primers 5′-caatctaaatatcaataaaa-3′ and 5′-ttatatgtagattagttg-3′ were used to produce concatenated oligonucleotides according to Sambrook et al. (1989), resulting in concatemers of the sequence 5′-caatctaaatatcaataata-aa-3′. Nitrocellulose filters were prepared in two sets; one set was used for the incubation with the radiolabeled probe directly, whereas the second copy was subjected to denaturation using 6 M GuHCl followed by renaturation (Vineon et al., 1988; Sambrook et al., 1989). Approximately 3 × 10⁴ pfu per filter set were screened. Both filter sets were incubated for 12 h at 4°C with hybridization buffer, consisting of binding buffer (25 mM HEPES/KOH, pH 7.9; 7.5 mM MgCl₂; 20 mM KCl; 0.07 mM EDTA) containing 1 mM dithiothreitol (DTT), 0.25% (w/v) fat-free milk powder, and 10 mM l-cysteine. The filters were washed 3 times for 5 min with 60 mL hybridization buffer. After autoradiography. Clones showing a positive signal were isolated and identified by their absorption spectra using a photodiode array detector (PDA 2996; Waters). Normalization of the samples to the internal standard and quantification of carotenoid amounts were performed as described previously (Schaub et al., 2005).

Carotenoid Extraction and Quantification
The lipophilic compounds of 5 mg lyophilized leaf or 100 mg root callus material, respectively, were extracted three times by adding 2 mL of acetone and sonicating. One hundred microliters of α-tocopherolacetate (2 mg mL⁻¹ in acetone; Sigma) were added as an internal standard. After centrifugation (5 min, 6,000g), the acetone phases were pooled and dried by rotary evaporation; pigments were resuspended in 2 mL petroleum ether:dithyl ether (2:1 [v/v]); 1 mL of water was added and the samples were centrifuged (5 min, 6,000g). The organic phase was dried and the pigments were dissolved in 30 μL chloroform. Ten microliters of each sample were subjected to quanti-
tative analysis using HPLC with a C30 reversed-phase column (YMC Europe GmbH) and a gradient system as described (Hsu et al., 2003). Carotenoids were identified by their absorption spectra using a photodiode array detector (PDA 2996; Waters). Normalization of the samples to the internal standard and quantification of carotenoid amounts were performed as described previously (Schaub et al., 2005).

RNA Extraction, Microarray Experiments, and Data Analysis
Total RNA was isolated using Concert reagent (Invitrogen). RNA cleanup and on-column DNAseI digestion was performed using the Qiagen RNasy mini kit. After RNA quality control by formaldehyde agarose gel analysis, biotinylated target RNA (cRNA) was prepared from 15 μg of total RNA using the Affymetrix GeneChip® one-cycle target labeling kit (Airism). Two biological replicates for each line were hybridized to the Affymetrix Arabi-
dopsis ATH1 GeneChip. GeneChip Suite 5.0 (Affymetrix) was used for data normalization using default settings. The target intensity for all probe sets of each array was scaled to 500. GeneChip data files were imported into GeneSpring 7.2 (Agilent Technologies) for further analyses.

Two normalization steps were applied to each sample. First, per-chip normalization was performed using the medians for each gene was applied. For analysis, data filtration based on flags present in at least three of four samples used for comparison (wild-
type and overexpressing line, two biological replicates each) was applied. For a less stringent analysis (see Supplemental Table S1), data filtration was based on flags present and marginal in at least one of four samples used for comparison; statistical analysis and filtration on 2.5-fold difference was performed as described. The intersection of both gene lists was determined using the Venn diagram function of GeneSpring 7.2. Statistical significance was deter-
mained by ANOVA analysis using a P value of 0.05 as the cutoff. Lists of genes that were either induced or suppressed more than 2.5-fold between wild-type versus transgenic lines were created by filtration-on-fold function within the presented list. For a less stringent analysis (see Supplemental Table S1), data filtration was based on flags present and marginal in at least one of four samples used for comparison; statistical analysis and filtration on 2.5-fold difference was performed as described. The intersection of both gene lists was obtained using the Venn diagram function of GeneSpring 7.2. Affymetrix GeneChip data were deposited at the ArrayExpress (http://www.ebi.ac.uk/arrayexpress) database in compliance with Minimum Informa-

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overnight at 4°C labeled in vitro-translated AtRAP2.2 in prehybridization buffer and incubated at temperature in prehybridization buffer, air dried, and exposed to a PhosphorImager screen (Fuji Film) to visualize radiolabeled proteins. Membranes were blocked with prehybridization buffer (3% [w/v] bovine serum albumin, 100 mM NaCl, 1 mM EDTA) and 1% [v/v] Tween 20 overnight at 4°C. The solution was then replaced with 5 × 10^6 cpn [35S] labeled in vitro-translated AtRAP2.2 in prehybridization buffer and incubated overnight at 4°C. Membranes were rinsed three times for 5 min at room temperature in prehybridization buffer, air dried, and exposed to a PhosphorImager screen (Fuji Film) to visualize radiolabeled proteins.

**Protein Overlay Assay**
PcGem4 constructs were used for coupled transcription and translation using the TNT SP6 coupled RL and WG extract system (Promega) in the presence of [35S]Met and [35S]Cys (GE Healthcare) according to the manufacturer's instructions. The alkaline phosphatase system was used to visualize radiolabeled proteins.

**Protein Extraction and Western-Blot Analysis**
Total RNA was extracted as described above. First-strand cDNA synthesis was performed using the TaqMan RT reagents (Applied Biosystems) according to the manufacturer’s protocol. Primers and TaqMan MGB probes were designed from cDNA sequences of Arabidopsis, using Primer Express software (Applied Biosystems). The following primers and probes were used: AtRAP2.2 forward, 5′-gggtgttgcctcctgatgattt-3′; reverse, 5′-gggcagttcctgatgattt-3′; probe, 5′-htgctgctcctgatgattt-3′; PSY forward, 5′-ggggtgcctcctgatgattt-3′; reverse, 5′-cgcagggctgatcctgatcctgat-3′; probe, 5′-htgctgctcctgatgattt-3′; PDS forward, 5′-tggtgctcctcctcctgatgattt-3′; reverse, 5′-cgcagggctgatcctgatcctgat-3′; probe, 5′-htgctgctcctgatgattt-3′.

Specific mRNA levels were quantified by real-time RT-PCR (ABI Prism 7000; Applied Biosystems) using 18S rRNA levels for normalization. For 18S rRNA quantification, the eukaryotic 18S rRNA endogenous control kit (Applied Biosystems) was used. Reporter (5′ end) dyes for the TaqMan MGB probes were 6FAM, except for 18S rRNA, where VIC was used. The relative quantity of the transcripts was calculated by using the comparative threshold cycle method (Livak, 1997). Data were normalized first to the corresponding 18S rRNA levels and then expressed as relative to the wild-type transcript levels.

**Yeast Two-Hybrid Assay**
Constructs to test for autoactivation were produced by subcloning the AtRAP2.2 cDNA into the vector pGBT9, truncation were constructed by using appropriate restriction sites and/or by amplification and subcloning PCR fragments using mutagenized primers. Yeast (Saccharomyces cerevisiae) two-hybrid screening was performed using the MATCHMAKER GAL4 two-hybrid system (CLONTECH). An Arabidopsis (ecotype Columbia-0) MATCH-MAKER cDNA library present in the vector pGAD424 and the bait vector pGBT9-RAP1-166 were sequentially transformed into the yeast reporter strain H7c and cultured on synthetic dropout agar lacking Leu, Try, and His. Yeast transformants that appeared on selection medium within 2 d were transferred on fresh selection plates and allowed to grow for two more days. From these transformants that appeared on selection medium within 2 d were transferred, 61 yeast clones that grew on selection medium were tested for β-galactosidase activity by the colony-lift filter assay with 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside. Plasmids were rescued from 20 His-/lacZ yeast transformants and identical cDNAs were identified by restriction analysis. Interaction was tested by cotransformation of the selected pGAD424-cDNA plasmids with pGBT9-RAP1-166 into H7c followed by both His- and lacZ assays. Analyzing pGAD424-cDNA plasmids were cotransformed with the empty pGBT9 vector into the H7c yeast strain to check for possible autoactivation of the gene product.

**Pull-Down Assays**
Recombinant GST, GST-SINAT2-C, and GST-SINAT2 bound onto glutathione-Sepharose beads (GE Healthcare) were produced according to Frangioni and Neel (1993), using 0.5% (w/v) N-laurylsarcosyl and 1% (v/v) Triton X-100. To equal amounts of GST or GST fusion protein, respectively, 5 μL of in vitro-translated [35S]-labeled AtRAP2.2 were added. Fifty microliters of 5X hybridization buffer (100 mM Tris/HisCl pH 8.0; 500 mM NaCl, 5 mM DTT, 5 mM EDTA) and 200 μL water were added and incubated with light agitation for 20 min at room temperature. After centrifugation at 5000 × g for 2 min, the pellet was washed five times with wash buffer (150 mM NaCl, 10 mM Tris/HisCl pH 8.0; 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride; 1 mM DTT; 1 μg mL⁻¹ leupeptin, 1 μg mL⁻¹ antipain, 10% [v/v] glycerol). The pellet was resuspended in 25 μL SDS sample buffer, boiled for 5 min, subjected to SDS-PAGE, and transferred onto nitrocellulose membranes. A PhosphorImager screen was used to visualize radiolabeled proteins.

**Protein Extraction and Western-Blot Analysis**
Total RNA was extracted as described above. First-strand cDNA synthesis was performed using the Reverse Transcription Primer (RT-PCR) kit (AMV; Promega) in the presence of [35S]Met and [35S]Cys (GE Healthcare) according to the supplier’s protocol to generate [35S]-labeled proteins. Nuclear extracts from illuminated mustard (Sinapis alba) seedlings were prepared according to Frangioni and Neel (1993). GST fusion protein was separated by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were blocked with prehybridization buffer (3% [w/v] bovine serum albumin, 100 mM NaCl, 1 mM EDTA) and 1% [v/v] Tween 20 overnight at 4°C. The solution was then replaced with 5 × 10^6 cpn [35S]-labeled in vitro-translated AtRAP2.2 in prehybridization buffer and incubated overnight at 4°C. Membranes were rinsed three times for 5 min at room temperature in prehybridization buffer, air dried, and exposed to a PhosphorImager screen (Fuji Film) to visualize radiolabeled proteins.

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

**Supplemental Figure S1.** SIAH proteins from Arabidopsis.

**Supplemental Table S1.** SINA-homologous proteins from Arabidopsis.

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**LITERATURE CITED**


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Transcription Factor RAP2.2 and Interaction Partner SINAT2