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Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect

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Abstract

Introduction of a constitutive antisense full-length chalcone synthase (CHS) cDNA gene in petunia can result in an inhibition of flower pigmentation. We have evaluated some of the factors which may be important for the effectiveness of an antisense CHS gene.

Antisense CHS genes encoding half-length or quarter-length RNA complementary to the 3′ half of CHS mRNA are able to affect flower pigmentation, while a gene encoding RNA complementary to the 5′ half of CHS mRNA did not show phenotypic effects in transgenic petunia plants. We demonstrate that the RNA encoded by the latter gene has a much lower average steady-state level in leaf tissue than the RNAs encoded by the other antisense gene constructs. We have compared the CaMV 35S and endogenous CHS promoter strengths and intrinsic stabilities of sense and antisense CHS RNAs. From the data we conclude that the constitutive antisense CHS genes are not likely to provide an excess of antisense RNA compared to the CHS mRNA derived from the endogenous genes.

Effective inhibition of flower pigmentation is also observed when the antisense CHS gene is under control of the homologous CHS promoter. The results indicate that the mechanism of antisense inhibition cannot solely operate via RNA duplex formation between sense and antisense RNA.

Introduction

Using antisense genes it has been demonstrated that target gene expression can effectively be inhibited in a number of different organisms varying from bacteria and slime molds to mammalian cells and plants [for reviews: see 7, 10, 15, 16]. It is generally accepted that antisense genes function by synthesis of antisense RNA which forms a duplex with the target mRNA. This mRNA is then either inaccessible for nuclear processing, rapidly degraded by RNases or blocked for translation. To evaluate the applicability of antisense technology in manipulation of valuable plant traits, flower pigmentation provides a nice model system since changes in its biosynthesis are readily scored by the eye.

Anthocyanins and flavonols are the most common pigments in flowers and are synthesized in the flavonoid biosynthesis pathway. In *Petunia*

hybrida this route has been characterized extensively at the genetic, biochemical and enzymatic level [for reviews: see 25, 27] and a number of relevant genes have been cloned and characterized [for listing: see 20]. In our laboratory we have cloned the petunia genes for chalcone synthase (CHS) [13], chalcone flavanone isomerase (CHI) [26] and dihydroflavonol reductase (DFR) [2]. The expression of these three gene families occurs in a coordinate fashion during flower development [26] and precedes the accumulation of flavonoids (flavonols and anthocyanins) in floral tissue. In a previous paper we reported on the effect of an antisense CHS gene on flower pigmentation in *P. hybrida* VR plants [17]. CHS performs a key reaction in flavonoid biosynthesis by forming the basic structure of flavonoids through condensation of one unit of 4-coumaroyl-CoA with three molecules of malonyl-CoA. Introduction in petunia of a full-length antisense CHS cDNA gene, under control of the CaMV 35S promoter, resulted in an effective reduction of CHS expression. The effect on flower pigmentation varies from completely colored flowers (no visible effect), reduced pigmentation or pigmentation patterns with colored rings or sectors to complete white flowers [17]. The different pigmentation patterns in flowers of independent transformants are ascribed to a qualitative position effect, revealing for the first time that the site of integration can confer ectopic patterns of expression within one plant organ. Furthermore, analysis of these transformants also shows that sequences surrounding the introduced gene may render its expression extremely susceptible to physiological changes, resulting in a variable flower phenotype on one plant (A.R. van der Krol *et al.*, Mol Gen Genet, in press).

In this study we evaluate a number of factors which may determine the efficiency of an antisense CHS gene. We have tested different subgenomic antisense CHS templates and different promoters driving the antisense gene transcription, for their ability to inhibit flower pigmentation in transgenic petunia. The data obtained from these studies suggest that antisense inactivation of CHS gene expression in floral tissue is not merely triggered by an RNA-RNA interaction. An alternative mechanism involving RNA-DNA interaction will be discussed.

Materials and methods

Transformation and growth of plants

Petunia hybrida VR plants were grown under standard greenhouse conditions unless stated otherwise. Transformants were obtained by the standard leaf disc transformation method [9]. Clones of transgenic plants were obtained either by regeneration of shoots from leaf discs under selective pressure (250 μg/ml kanamycin) or by taking cuttings from the primary transformant. Transformants are indicated by VIP number (see next section), followed by serial number (e.g. 167-1).

Construction of the antisense CHS gene VIP 167, 168, 171, 172 and 176

The construction of the antisense CHS gene (VIP 102) containing the CaMV 35-S promoter, the full-length CHS cDNA and the nopaline synthase 3′ tail fragment, has been described before [17]. Using the VIP 102 DNA (see top of Fig. 1) we constructed two different antisense CHS genes, one containing the 3′ half and one containing the 5′ half of the CHS template. VIP 102 DNA was digested partially with *Bam* HI and the sticky ends were removed using Klenow polymerase I. Subsequently, the DNA was digested with *Sac* I, the sticky ends were removed and the DNA was re-circularized with ligase. After transformation to *Escherichia coli*, recombinant clones were identified containing the antisense CHS gene construct coding for the (antisense) 5′ half of the CHS template (VIP 167) or the antisense CHS gene construct coding for the (antisense) 3′ half of the CHS template (VIP 168). Since only the VIP 168 construct gave rise to phenotypic changes in flowerpigmentation we further divided this CHS template in two halves. The antisense

CHS gene VIP 171 was made from VIP 168 by digesting the DNA with *Bam* HI and *Stu* I. The sticky ends were removed and the DNA was re-circularized with ligase. VIP 172 was constructed similarly by digesting with *Stu* I and *Nco* I. DNA of recombinant clones was analyzed by restriction mapping and/or sequencing of the ligation site. Sequence analysis showed that the S1 nuclease reaction had removed an additional 8 basepairs form the *Sac* I site in VIP 167 and an additional 9 basepairs from the *Sac* I site in VIP 168. The RNAs encoded by VIP 167, 168, 171 and 172 are shown in Fig. 1. VIP 176 was constructed by replacing the *Eco* RI-*Bam* HI CaMV 35S promoter in VIP 102 by a 0.8 kb *Eco* RI-*Bam* HI CHS-A promoter fragment (I.M. van der Meer and A.R. Stuitje, in preparation). The different antisense CHS constructs (VIP 167, 168, 171, 172 and 176) were isolated as *Eco* RI-*Hin*d III fragment and ligated into the *Eco* RI-*Hin*d III site of the binary vector BIN 19 [4].

DNA/RNA isolation and Southern analysis

DNA and RNA was extracted from flowerbuds (stage 3–4 as defined in [13]). Flowerbuds stage 3–4 show an optimal expression of endogenous CHS genes. To average out small differences in developmental stage of the flower buds, nucleic acid was isolated from five flower buds taken from one plant. Total nucleic acid was dissolved in 0.5 ml H_2O and after the addition of 0.5 ml 4 M LiCl and 3 h incubation on ice the RNA was pelleted by centrifugation. The RNA pellet was dissolved in 300 μl H_2O, precipitated with 1 ml ethanol and redissolved in 50–100 μl H_2O. The genomic DNA in the 2 M LiCl solution was precipitated by adding 0.6 vol iso-propanol. The DNA pellet was dissolved in 400 μl H_2O and again precipitated with 2 vol ethanol. After centrifugation the pellet was dried and dissolved in 500 μl H_2O. Southern blot analysis was performed as described [13].

Northern blot analysis and RNase protection

Northern blotting and RNase protection experiments were performed according to van Tunen *et al.* [26]. Labeled single-stranded CHS sense or antisense RNA was synthesized *in vitro* using T7-polymerase (Promega Biotec), [^{32}P]UTP (Amersham) and the vector pTZ18U and pTZ19U (GeneScribe-Z TM, US Biochemical Corp.) in which an *Eco* RI-*Hin*d III full-length CHS gene A cDNA was cloned. CHI and DFR DNAs were labeled using [^{32}P]ATP (Amersham) and a nick translation reaction as described by Maniatis *et al.* [18].

Results

The effects of constitutive expression of different antisense CHS transgenes on flower pigmentation in Petunia hybrida

In a previous paper we have shown that the expression of a full-length antisense CHS cDNA (VIP 104) may result in a severe reduction in flower pigmentation (sometimes with distinct ectopic patterns), caused by a specific reduction in CHS mRNA steady-state level [17]. Here we have analyzed some of the factors which may determine the effectiveness of an antisense CHS gene. First, we tested the effect of expressing subgenomic antisense CHS templates targeted either against the 5′ or 3′ end of the CHS mRNA. Using VIP 102 [17] we constructed antisense CHS genes containing half the CHS template (VIP 167, 704 bp, and VIP 168, 628 bp; see Materials and methods and Fig. 1). In these constructs transcription of the antisense CHS gene is driven by the CaMV 35S promoter and they contain a nopaline synthase 3′ tail fragment which provides a poly(A) addition signal. Of 13 transformants expressing the VIP 168 gene, nine plants show a wild-type flower pigmentation, while three transformants show an intermediate level of pigmentation and one transformant has virtually white flowers (Fig. 2A; VIP 168-6). The phenotypic effect of the VIP 168 transformants

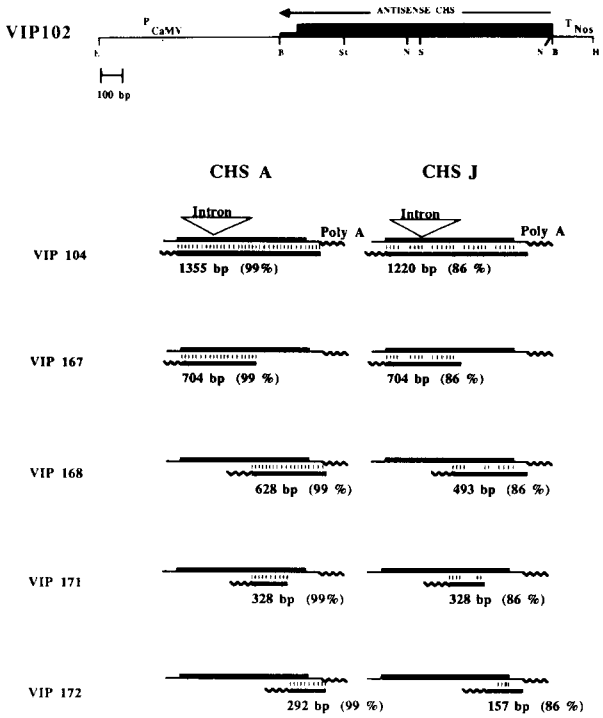

Fig. 1. VIP 102 and the RNA encoded by antisense CHS gene constructs VIP 167, 168, 171 and 172. The construction of the different antisense CHS genes using VIP 102 (top) is described in Materials and methods. The restriction endonuclease sites which were used for the construction of VIP 167, 168, 171 and 172 are indicated: E, *Eco* RI; B, *Bam* HI; St, *Stu* I; N, *Nco* I; S, *Sac* I; H, *Hin*d III. The RNA encoded by each construct and its complementarity to CHS-A or CHS-J mRNA is indicated. The number of basepairs (bp) refer to the number of nucleotides in the antisense RNA which are complementary to the target RNA. The percentage of the complementarity is given in parenthesis.

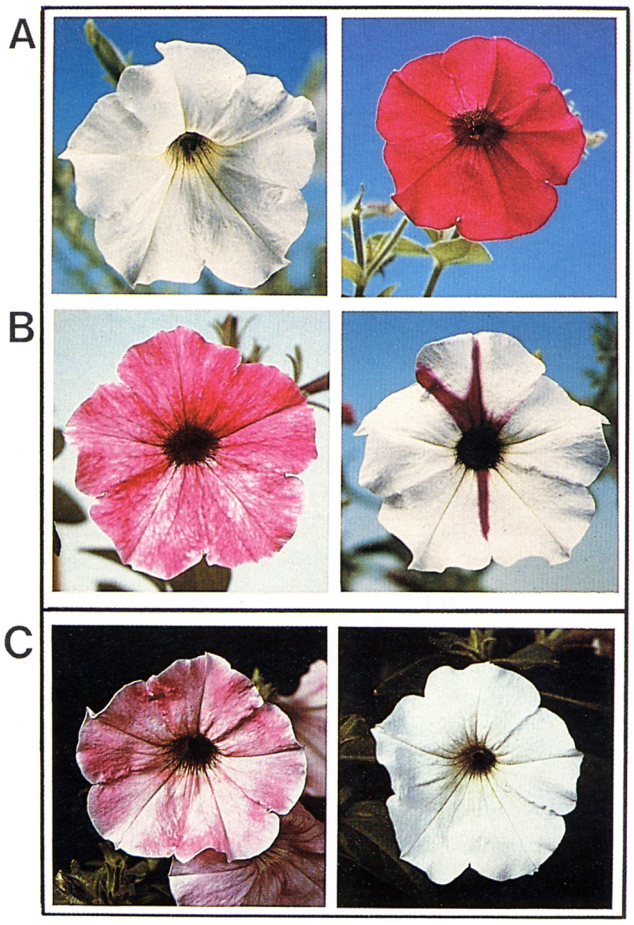

Fig. 2. Flower phenotype of transformants expressing different antisense CHS genes. *Petunia hybrida* VR plants were transformed with VIP 167, VIP 168, VIP 171, VIP 172 or VIP 176 (see Materials and methods). A reduced flower pigmentation was observed in some of the transformants containing either VIP 168 (four out of thirteen), VIP 171 (two plants sprayed with B9, out of ten), VIP 172 (one out of thirteen) and VIP 176 (two out of twenty). VIP 167 did not confer a phenotypic change on flower pigmentation. Shown in panel A: 168-6 (left) and 167-1 (right); panel B: 172-5 (left) and 171-9 (right, after spraying with B9); panel C: 176-3 (left) and 176-10 (right).

resembles that of the full-length antisense CHS gene (VIP 104); virtually complete inhibition of pigmentation in corolla tissue is obtained while pigmentation of the anthers remains unchanged. No distinct ectopic expression patterns were observed in flowers of the VIP 168 transformants.

In contrast, none of 20 transformants expressing the VIP 167 gene construct show an effect on flower pigmentation (Fig. 2A,; VIP 167-1). Since only the introduction of VIP 168 resulted in a reduced flower pigmentation, we further divided the antisense CHS template encoded by this gene in two halves. Two gene constructs containing either the 3′ (VIP 171; 328 bp) or the 5′ (VIP 172; 292 bp) half of the CHS DNA contained in VIP 168 were made (see Materials and methods and Fig. 1). Of these two antisense CHS gene constructs VIP 172 resulted in a visible effect on flower pigmentation in one out of 13 transformants (Fig. 2B; VIP 172-5). The plants carrying VIP 171 did not give a visible change in flower pigmentation when grown under normal

greenhouse conditions (10 plants analyzed). However, when two of these transformants were sprayed with B9 (an inhibitor of endogenous gibberellic acid synthesis and known to enhance the effect of antisense CHS genes; A.R. van der Krol *et al.*, Mol Gen Genet (in press), flowers with white sectors of varying size developed on the plants (Fig. 2B; VIP 171-9). Flowers on control VR plants sprayed with B9 did not show an altered flower pigmentation. These results indicate that the antisense RNA encoded by VIP 171 potentially still can cause an effective inhibition of flower pigmentation. Molecular analysis shows that CHS-A and CHS-J mRNA steady-state levels are equally affected by each of the antisense CHS gene constructs (A.R. van der Krol *et al.*, Mol Gen Genet, in press).

We have evaluated the intrinsic stability of the different antisense CHS RNAs encoded by each antisense CHS gene, by measuring antisense CHS RNA steady-state levels in leaf tissue. Total RNA from pooled leaf material (10 or more independent transformants) was isolated and analyzed by RNase protection experiments. Figure 3 shows that the antisense CHS RNA

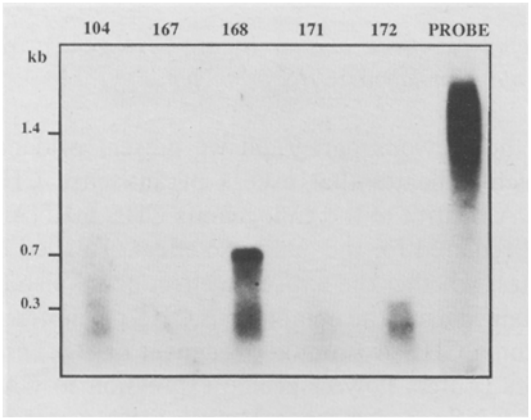

Fig. 3. Average steady-state level of the different antisense CHS RNAs in leaf tissue. Total RNA was isolated from pooled leaf material (10 transformants, see Materials and methods). To be able to directly compare the signals obtained for the different length antisense CHS RNAs, we used 10 μg of the VIP 104 (full-length), 20 μg of the VIP 168 and VIP 169 (half-length) and 40 μg of the VIP 171 and VIP 172 (quarter-length) leaf RNA in the RNase protection experiment, using a 32P-labeled full-length sense CHS RNA as probe. ^{32}P-labeled DNA fragments were used as size markers.

encoded by VIP 167 has an extremely low average steady-state level compared to the antisense CHS RNAs encoded by the other gene constructs. This may explain the absence of a phenotypic effect on flower pigmentation in the twenty plants carrying VIP 167.

Comparison of CaMV 35S and endogenous CHS promoter strengths

It is generally believed that, as in prokaryotes, antisense gene effects in eukaryotes are caused by the formation of duplex RNA structures. However, repeated attempts to detect RNase A-resistant double-stranded RNA in white floral tissue of antisense transgenic plants have failed. Moreover, antisense inhibition would only occur if a large excess of antisense over sense RNA is present in the cell. Surprisingly, no large amounts of antisense RNA were detected in floral tissue of antisense CHS transformants. In fact, only a very weak signal is obtained on northern blots of floral RNA after prolonged exposure of the film [17]. Even in very young flower buds (stage 1–2) shortly before the onset of endogenous CHS expression [14] only very low levels of CHS antisense RNA are detected (not shown). These data suggest that for the efficient inhibition of CHS gene expression, a large excess of antisense CHS RNA is not an absolute requirement. This notion was confirmed by comparing the relative strength of the CaMV 35S and endogeneous CHS promoters in floral tissue of transgenic petunias. We have analyzed transgenic plants expressing a (sense) CHS cDNA (VIP 103) under the same CaMV 35S promoter used in the antisense CHS genes described above. Since in these CHS 'sense' transformants the endogenous and CHS transgene encode similar mRNAs (presumably with a similar turnover), we assume that in this case the relative steady-state levels reflect relative promoter strengths. Since there is a small sequence divergence between the sense CHS-A cDNA gene construct (which derives from petunia V30) and the sequence of endogenous CHS-A gene(s) in the petunia VR hybrid [14], RNase protection

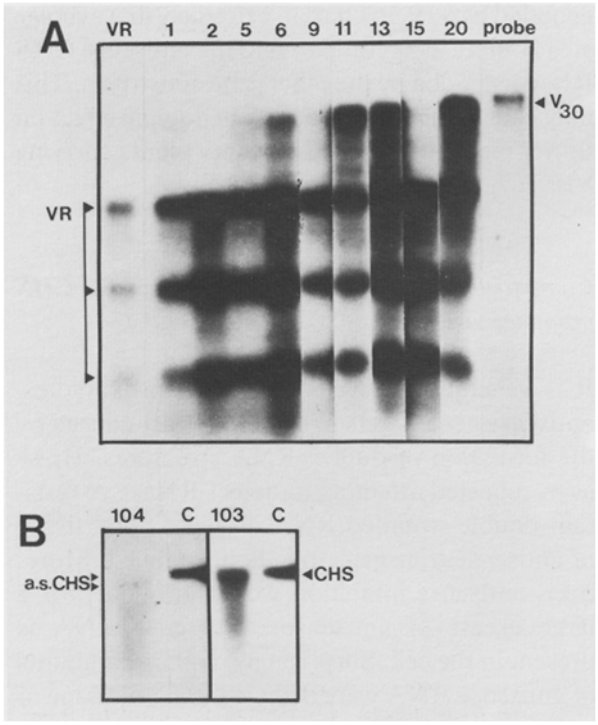

Fig. 4. Comparison of the CaMV 35S and endogenous CHS promoter strengths in floral tissue of VIP 103 transformants. A. Total RNA was isolated from flowerbuds (stage 1–2) of VR control plants and CHS sense transformant 103-1, 2, 5, 6, 9, 11, 13, 15 and 20 (see Materials and methods). In an RNase protection experiment, using 32P-labeled antisense V30-CHS RNA as probe, the VIP 103 transcript is completely protected (indicated by V30, 1.4 kb), while endogenous VR CHS-A transcripts result in three subfragments (indicated by VR, approximately 0.8 kb, 0.4 kb and 0.2 kb). B. Total RNA was isolated from pooled leaf material of ten VIP 104 and ten VIP 103 transformants. The RNA was fractionated on agarose-formaldehyde gel blotted onto Highbond-N filter (Amersham). VIP 104 RNA was hybridized with 32P-labeled sense CHS RNA, while VIP 103 RNA was hybridized with 32P-labeled antisense CHS RNA (see Materials and methods). Control lanes C1 and C2 show the hybridization signals to 100 pg CHS cDNA.

experiments can distinguish between transcripts from the introduced CHS-A (VIP 103) and endogenous CHS-A gene transcripts. The results of such RNase protection experiments are shown in Fig. 4A. Protection by the VR CHS-A transcripts results in three main subfragments, while the ^{32}P-labeled probe is completely protected by the VIP 103 transcript. The experiment shows that even for the strongest expressor analyzed (103-20), the steady-state level of CHS mRNA transcribed from the transgene in flowers does not exceed that of the endogenous CHS genes.

To compare the intrinsic stabilities of antisense and sense CHS RNA, we measured their steady-state levels in leaf tissue. Leaf tissue from ten independent VIP 103 or ten VIP 104 transformants was pooled, RNA was extracted, fractionated on agarose-formaldehyde gel and blotted onto a Highbond-N filter (see Materials and methods). The blot was probed either for CHS sense RNA or CHS antisense RNA, using strand-specific probes. Fig. 4B shows that antisense CHS RNA has a much lower average steady-state level in leaf tissue, compared to that of sense CHS RNA. Hybridization to control DNA resulted in an equal signal with the sense and antisense CHS probe (see Fig. 4B, lanes C), from which we conclude that the signals for CHS in leaf RNA can be compared directly. Taken together, the data from Fig. 4A and 4B suggest that in VIP 104 transgenic petunias the level of expression of the antisense CHS gene remains below that of endogenous CHS gene expression in floral tissue.

Phenotypic effects of an antisense CHS gene driven by the homologous CHS promoter

In the previous paragraph we present evidence which indicates that excess of antisense CHS RNA relative to the endogenous CHS mRNA is not required for the antisense effect. This led us to test whether the antisense effect could also be obtained using the homologous CHS promoter. A petunia CHS-A promoter fragment of 880 base-pairs confers flower-specific expression to CAT and GUS reporter genes. However, RNA steady-state levels from these transgenes in petunia flower tissue is only a few percent of that of the endogenous gene (A.R. van der Meer and A.R. Stuitje, in preparation). An antisense CHS gene construct containing this CHS promoter fragment, the full-length CHS cDNA and the nopaline synthase 3′ tail fragment, was introduced into petunia VR plants using the standard transfor-

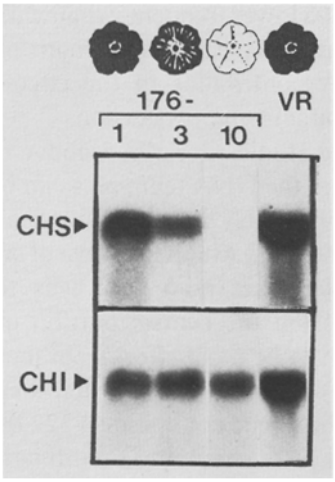

Fig. 5. CHS and CHI mRNA steady-state levels in floral tissue of transformants 176-1, 176-3 and 176-10. RNA was isolated from flower buds (stage 4) of transformants 176-1, 3, 10 and from VR control plants. The RNA was fractionated on a formaldehyde-agarose gel, blotted onto Highbond-N filter (Amersham) and hybridized either with ^{32}P-labeled antisense CHS RNA (top) or antisense CHI RNA (bottom). The flower phenotype of the transformants is indicated.

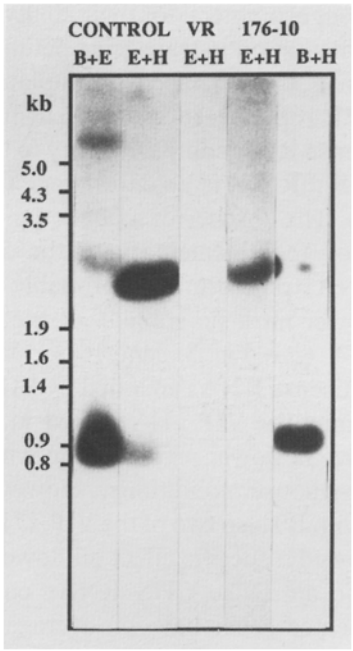

Fig. 6. Southern blot analysis of genomic DNA of antisense CHS transformant 176-10. Genomic DNA was isolated from transformant 176-10, digested with *Eco* RI plus *Bam* HI or *Eco* RI plus *Hin*d III. Genomic DNA of untransformed petunia VR was digested with *Eco* RI plus *Hin*d III. The DNA was fractionated on an agarose gel, blotted onto Highbond-N filter and hybridized with a ^{32}P-labeled petunia V30 CHS-A promoter fragment (see Materials and methods). The blot was washed in 0.1 × SSC at 65 °C. Under these conditions the V30 CHS promoter DNA does not hybridize to VR DNA. Copy number was estimated by comparing the hybridization signal with that of a single-copy reconstruction (lanes Control: 10 pg VIP 176 DNA, digested *Bam* HI plus *Eco* RI or *Eco* RI plus *Hin*d III).

mation procedures (see Materials and methods). Of twenty plants regenerated, two transformants showed an effect on flower pigmentation. One transformant exhibits a reduced level of pigmentation (176-3) while the other one gave virtually white flowers (176-10; see Fig. 2C). Northern blot analysis of floral RNA from these transformants shows that the reduced pigmentation is again accompanied with a reduction in CHS mRNA steady-state level while the CHI mRNA steady-state level is unaffected in these plants (see Fig. 5). We analyzed genomic DNA of one of the transformants to make sure that no rearrangements of the introduced DNA had occurred. Genomic DNA of 176-10 was digested with different enzymes, blotted and hybridized with the labeled 880 bp CHS promoter fragment. Under stringent washing conditions (0.1 × SSC, 65 °C) no hybridization to DNA from untransformed petunia VR plants is observed (Fig. 6, lane VR, E + H). Lanes 176-10 E + H and E + B show that transformant 176-10 contains one insert (compare single copy reconstructions with lanes 176-10 E + H and E + B), while no aberrations in the introduced gene construct could be detected.

Discussion

We have previously shown that flower pigmentation can be blocked by introduction of a constitutively expressed antisense CHS gene in petunia and tobacco plants [17]. In this paper we evaluate a number of factors which may determine the efficiency of an antisense CHS gene to inhibit endogenous gene expression. We have tested different antisense CHS templates in combination

with different promoters, for their ability to inhibit flower pigmentation in transgenic petunia. Of the two half-size CHS antisense templates tested, only the VIP 168 gene, encoding a 628 nucleotides long antisense RNA complementary to the 3′ half of the CHS mRNA, is able to reduce CHS gene expression. The absence of a phenotypic effect in the VIP 167 (complementary to the 5′ half of CHS mRNA) transformant is probably caused by a much lower intrinsic stability of the antisense CHS RNA (see Fig. 3, lane 167). Of the two shorter antisense RNAs encoded by VIP 171 and VIP 172, only the VIP 171 resulted in a phenotypic change in flower pigmentation when grown under greenhouse conditions. However, upon spraying with B9 also two of the VIP 171 transformants showed a specific effect on flower pigmentation. The antisense CHS RNAs encoded by these truncated genes have on average an about equal steady-state level in leaf tissue (Fig. 3, lanes 171 and 172). We note that in RNase protection experiments for each of the antisense RNAs multiple bands are obtained, while the ^{32}P-labeled probe derives from the same CHS cDNA used to construct the antisense CHS genes. These different length RNAs may be caused by utilization of multiple poly(A) addition signals in the antisense template. However, on northern blots the antisense RNAs show only one or two main bands which are of the expected size (for VIP 104 see [17]). Alternatively, the multiple fragments obtained in RNase protection experiments could be explained by a modification of the antisense RNA which affects protection of the ^{32}P-labeled probe. Such modification of RNA has been reported for *Xenopus* eggs, where it is associated with an RNA-duplex unwinding activity [1]. However, in leaf tissue of the antisense CHS transformants no duplex RNA can be formed since the target gene is not expressed here. The discrepancy between the antisense CHS RNA signals observed on northern blots and in RNase protection experiments will be subject to further investigation. The effects obtained with the quarter-length antisense CHS RNAs (VIP 171 and 172) are comparable to those obtained with the full-length antisense CHS RNA (VIP 102), but occur at a lower frequency than with VIP 102. This indicates that sequence length of the antisense RNA contributes to the effectiveness of inhibiting target gene expression.

From the studies described above no specific region within the DNA templates can be assigned which is absolutely required for the antisense effect. In a study in which the effect of an antisense nopaline synthase (nos) gene was tested, nos sequences from the central portion of the gene were shown to be most effective in mediating the antisense effect [23, 24]. On the other hand, an antisense rbcS gene containing a 322 bp fragment of the rbcS gene, spanning the initiation codon, resulted in a significant reduction of rbcS synthesis in transgenic tobacco plants [22]. As long as it is not fully understood how the antisense transcripts exert their function *in vivo*, each antisense gene must be empirically tested to determine which portion is most effective in inhibiting target gene expression.

The absence of a detectable level of duplex RNA, the highly reduced levels of CHS mRNA and the barely detectable antisense CHS RNA steady-state levels in floral tissue are in agreement with numerous other reports on the action of antisense genes [6, 11, 19, 21, 22]. These observations may be explained by a rapid turnover of the antisense-sense RNA duplex. Alternatively, these results may reflect somekind of mechanism which interferes with the transcription process itself, thus preventing the accumulation of (sense) RNA or duplex RNA. If the mechanism of antisense inhibition in plants relies on the formation of an RNA duplex, it can be reasoned that effective antisense inhibition would require a high level of antisense over sense gene transcription. However, our studies in which we compare transcription initiated from the CaMV 35S promoter to transcription from endogenous CHS gene(s), indicate that the viral promoter is not particularly strong compared to the endogenous CHS promoter(s). Also, when we used the homologous CHS promoter driving antisense gene transcription (VIP 176), in two out of twenty transformants we observed an effect on flower pigmentation, accompanied with reduction in CHS mRNA

steady-state level (see Figs. 2 and 5). The same promoter fragment when used to drive chloramphenicol acetyl transferase or glucuronidase gene transcription, gives rise to a flower specific mRNA steady-state level which is only a few percent of the wild-type CHS mRNA steady-state level (A.R. van der Meer and A.R. Stuitje, in preparation). Therefore it is not likely that transcription from this CHS promoter fragment leads to an excess of antisense CHS RNA compared to endogenous CHS mRNA. Furthermore, Southern analysis shows that only a single copy of the antisense gene has integrated in the genome of transformant 176-10 (Fig. 6). Thus, the effect on flower pigmentation cannot be explained by a titration of a (hypothetical) CHS-specific trans-acting factor. The results presented above imply that inhibition of gene expression by antisense genes in plants is not only the result of a straightforward RNA-RNA interaction.

Alternatively, the antisense effect may (also) be the result of the interaction of antisense RNA with the DNA, affecting the transcription process itself. An effect on target gene transcription by an antisense gene has been reported by Yokoyama and Imamoto [28]. They reported the effect of an antisense *myc* gene in mammalian (HL-60) cell cultures. *In vitro* run-on experiments on nuclei isolated from cells transformed with an antisense *myc* gene showed a severe reduction of *myc* gene transcription compared to controls. It should be noted that in this case reduction of *myc* gene expression may also be related to the complex differential transcription of this gene, which even involves transcription of both strands in a short region upstream of exon 1 [3]. In another case, for the *Dictyostelium* antisense discoidin 1 gene, no effect in run-on experiments was observed with the target gene, while discoidin 1 mRNA steady-state levels were reduced by about 90% [6].

Direct proof of RNA-RNA interaction *in planta* comes from experiments where the antisense RNA is not targeted against nuclear encoded genes but against cytoplasmic viral RNAs. Antisense viral coat protein RNA, when constitutively expressed in plants is able to limit viral infection to some extent [5, 8]. Other proof comes from duplex RNA that has been detected in nuclei of *Dictyostelium* or mammalian cells, transformed with an antisense discoidin gene [12] or an antisense *myc* gene [28] respectively. Although these latter reports seem to support the involvement of RNA-RNA interactions in the mechanism of antisense inhibition of gene expression, in our case one might favor a mechanism in which an RNA-DNA interaction interferes with the transcription process. In floral tissue the flavonoid genes are switched on in cells that are no longer dividing thus an RNA hybridizing to DNA would not readily be removed by the DNA replication process. When this hypothetical RNA-DNA hybrid is not rapidly removed, one could envision an inhibitory effect on the transcription of the gene. This model explains the absence or extremely low levels of duplex RNA, while at the same time target mRNA steady-state levels are specifically reduced.

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