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Regulation and Manipulation of Flavonoid Gene Expression in Anthers of Petunia: The Molecular Basis of the *Po* Mutation

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Molecular mechanisms governing development of the male reproductive organs of flowers, the anthers, are largely unknown. In this article, we report on the investigation of the molecular basis of a mutation involving the expression of a gene encoding the flavonoid biosynthesis enzyme chalcone flavanone isomerase (CHI) in anthers of petunia. In petunia, the gene *Po* regulates the expression of CHI in anthers: *PoPo* petunia lines contain CHI enzyme activity in petals and anthers, whereas *popo* lines contain the CHI enzyme only in petals but not in anthers. As a result of the *Po* mutation, the substrate of CHI accumulates and therefore the pollen of a *popo* line are yellow or greenish. The genome of petunia contains two *chi* genes, *chi*A and *chi*B. In a restriction fragment length polymorphism analysis, a 100% linkage was observed between *Po* and *chi*A. This result suggested that *Po* is identical to *chi*A and that *Po* is not a regulatory gene of *chi*A. Introduction of a *chi*A gene isolated from a *PoPo* line into a *popo* line resulted in a complementation of the mutation that was directly visible because the pollen color shifted from yellow to white. This proved that *chi*A and *Po* are identical. Because *chi*A encodes a functional CHI enzyme in flower petals of a *popo* line, we propose that the *Po* mutation is a mutation in the regulatory region of *chi*A abolishing *chi*A promoter activity in anthers but not in corollas. This change in anther color is a fine illustration of how floral pigmentation can be manipulated in a predictable way and suggests the use of CHI as a visible marker.

INTRODUCTION

The flower is a complex structure composed of multiple organs, each with a distinctive and precisely defined role in the reproductive process of higher plants. In the male reproductive organs (anthers), gametophytes are produced in a developmental process that is not autonomously regulated but is programmed coordinately with the development of the flower as a whole and anthers in particular. Although anther and pollen development have been studied extensively at the cellular and subcellular levels, relatively little is known about molecular processes governing anther-specific and pollen-specific gene expression.

Flavonoids are plant secondary metabolites that are responsible for most of the pigmentation in flowers and fruits (for a review, see van Tunen and Mol, 1990) and serve as perfect markers to follow flower and anther development at the phenotypic level. To gain insight into regulatory processes of gene expression during development of the male flower organs, we investigated the expression of genes involved in the synthesis of flavonoids in anthers and pollen of petunia.

Flavonoid biosynthesis has been studied for many years and much knowledge has been gained at the biochemical, enzymatic, genetic, and molecular levels. The first flavonoid, naringenin chalcone, is formed in a condensation reaction by the enzyme chalcone synthase (CHS). Isomerization of naringenin chalcone is catalyzed by chalcone flavanone isomerase (CHI), leading to the synthesis of naringenin flavanone, the central intermediate of the flavonoid pathway. From this point, the pathway branches and the different flavonoid classes are formed (for reviews, see Heller and Forkmann, 1988; van Tunen and Mol, 1990). In petunia, genes encoding CHS, CHI, and dihydroflavonol 4-reductase (DFR) have been cloned and characterized (van Tunen et al., 1988, 1989, 1990a; Beld et al., 1989; Koes et al., 1989a, 1989b). It has been shown that members of the multigene families encoding CHS, CHI, and DFR are coordinately expressed during flower development. In contrast to the situation in corollas, anthers express flavonoid genes already in early stages of flower development (van Tunen et al., 1988; Beld et al., 1989). Immunochemical studies of tulip anthers showed that the CHS enzyme is localized in the tapetum cells (Kehrel and

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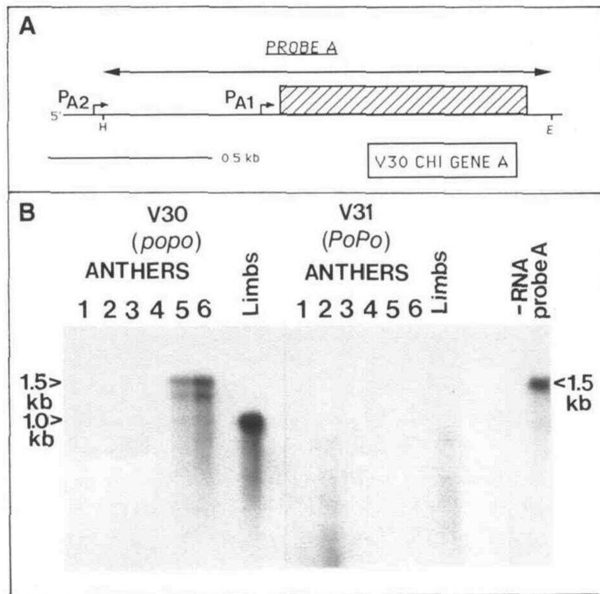

Figure 1. *chi*A Expression in Anthers of *Po*-Dominant (V31, *PoPo*) and *Po*-Recessive (V30, *popo*) Petunia Lines.

(A) Structure of V30 *chi*A. The two V30 *chi*A promoters (P_{A1} and P_{A2}) are shown. The hatched box represents the coding region. The V30 *chi*A in vitro synthesized RNA probe is indicated (probe A, horizontal arrow) and spans a 1.5-kb HindIII (H)/EcoRI (E) DNA fragment.

(B) RNase protection experiments. Total RNA was extracted from anthers isolated from flower buds of different developmental stages (lanes 1 through 6). As a control, samples from V30 or V31 limbs were included (Limbs). In addition, the probe control (probe A, no RNase treatment) and a sample without RNA added (−RNA) are shown.

Wierman, 1985). Experiments in which *chs* and *chi* promoters were fused to the β-glucuronidase (GUS) reporter gene showed that in transgenic plants containing these constructs, *chs* and *chi* promoters are specifically active in the tapetum cells (Koes et al., 1990; van Tunen et al., 1990a). The flavonoids, however, are localized at the outer surface of the pollen grain (the exine), suggesting that flavonoid compounds have to be transported to the pollen grains (Kehrel and Wierman, 1985).

Many mutants blocked at specific steps in the flavonoid biosynthesis pathway are known in plants such as maize, snapdragon, and petunia (for a review, see van Tunen and Mol, 1990). These mutations include alterations in structural genes encoding the biosynthetic enzymes as well as aberrations in regulatory genes. In petunia, two mutations are known that influence anthocyanin synthesis in anthers. First, anthers of *An4* recessive lines lack anthocyanins (de Vlaming et al., 1984). The UDPglucose:flavonoid-3-*O*-glucosyltransferase enzyme from *an4an4* petunia plants shows a reduced thermostability, and the amount of the enzyme's activity is considerably reduced in those lines. This suggests that the gene *An4* or a closely linked gene is the structural gene for the UDPglucose:flavonoid-3-*O*-glucosyltransferase enzyme (Gerats et al., 1985). Second, the genetically defined gene *Po* is known to regulate CHI expression. The gene *Po* represents a single locus on chromosome V (de Vlaming et al., 1984; Wiering and de Vlaming, 1984). *Po*-dominant petunia plants express CHI enzyme activity in both the corolla and anthers, whereas in *Po*-recessive lines, expression is restricted to the corolla (Forkmann and Kuhn, 1979). Absence of CHI enzyme activity in floral tissue leads to the accumulation of the yellow chalcones, resulting in yellow or green (when mixed with anthocyanin) anthers. In a previous paper, we reported the absence of immunologically cross-reactive CHI protein in anthers of homozygous *popo* lines, which suggests that the *Po* mutation is not due to the presence of inactive forms of the CHI enzyme (van Tunen and Mol, 1987). The genome of the petunia line V30 contains two *chi* genes, designated *chi*A and *chi*B, which have both been isolated and characterized. Their structure and expression pattern have been investigated in detail (van Tunen et al., 1988, 1989, 1990a).

To investigate regulatory aspects of anther-specific gene expression, the molecular basis of the *Po* mutation in petunia was investigated. The *chi* genes from a *popo* and *PoPo* petunia line were isolated and compared with respect to structure, sequence, and expression pattern. Restriction fragment length polymorphism (RFLP) analysis was performed to investigate the linkage between the *Po* gene and both *chi* genes. To address the question of whether *Po* is a gene regulating *chi* gene expression or whether the *Po* mutation is an aberration within one of the *chi* structural genes, complementation experiments were performed using *chi* genes isolated from a *PoPo* petunia line.

RESULTS

chi Gene Expression in Anthers of *PoPo* and *popo* Petunia Lines

To investigate whether the absence of CHI protein in anthers of *popo* petunia lines is the result of an absence of *chi* mRNA, RNA gel blot analysis was performed using the isolated *chi* cDNA (van Tunen et al., 1988) as a probe. Surprisingly, this analysis revealed the presence of *chi* mRNA in anthers of both *PoPo* and *popo* lines (van Tunen et al., 1988, 1990b). To discriminate between *chi*A and *chi*B expression, RNase protection experiments were performed. The results are shown in Figures 1 and 2.

*chi*A expression was monitored using a V30 *chi*A in vitro synthesized RNA probe [probe A, which spans the entire coding region and an adjacent 0.5-kb 5′ region (see Figure 1A and van Tunen et al., 1988)]. As a control, *chi*A expression has been monitored in limbs of both a *PoPo*

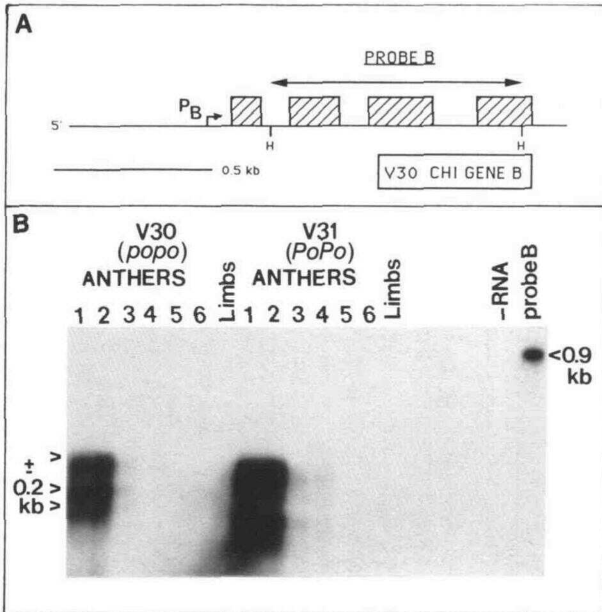

Figure 2. *chi*B Expression in Anthers of *Po*-Dominant (V31, *PoPo*) and *Po*-Recessive (V30, *popo*) Petunia Lines.

(A) Structure of V30 *chi*B. The V30 *chi*B promoter (P_B) is indicated. Hatched boxes represent the *chi*B exons. The V30 *chi*B in vitro synthesized RNA probe is indicated (probe B) and spans an internal HindIII (H)/HindIII DNA fragment.

(B) RNase protection experiments. Total RNA was extracted from anthers isolated from flower buds of different developmental stages (lanes 1 through 6). As a control, samples from V30 or V31 limbs were included (Limbs). In addition, the probe control (probe B, no RNase treatment) and a sample without RNA added (−RNA) are shown.

(V31) and a *popo* (V30) recessive line (Figure 1B). Because *chi*A of the line V31 is not completely homologous to the V30 probe A (see below), the probe is digested into four fragments.

The generation of four protected fragments in young anthers of the line V31 (*PoPo*; Figure 1B, stage 2) shows that *chi*A is expressed in this tissue. In contrast, *chi*A mRNA is absent in (young) anthers of the line V30 (*popo*; Figure 1B, V30 stages 1 to 3).

A 1.5-kb *chi*A mRNA accumulates in mature anthers of the line V30 (*popo*) and more specifically in pollen grains (Figure 1B, V30 stages 5 and 6). This mRNA is the result of the use of the upstream P_{A2} *chi*A promoter (van Tunen et al., 1988, 1989). Only a low level of the expanded *chi*A transcript was detected in V31 mature anthers (see below). In an RNase protection assay (Figure 1B, V31 stages 4 to 6), however, this 1.5-kb V31 transcript was not detected, presumably because of the fact that the probe was completely degraded at the 5′ side and only partially protected at the 3′ part, thereby dividing the signal over four fragments. This suggests that the presence of this expanded *chi*A transcript is not related to the *Po* mutation because it is present in both *PoPo* (V31) and *popo* (V30) petunia lines. The function of the long *chi*A transcript is still obscure because its expression is not coordinate with that of *chs* or *dfr* genes and no CHI enzyme can be extracted from anthers of this developmental stage (van Tunen et al., 1988).

*chi*B expression was monitored using a 0.9-kb V30 *chi*B in vitro synthesized RNA probe, as shown in Figure 2A (probe B, spanning an internal HindIII fragment). The generation of protected fragments in both V30 (*popo*) and V31 (*PoPo*) anthers showed that *chi*B is expressed in anthers of both lines, as illustrated in Figure 2B. This explained the hybridization signal detected on an RNA gel blot in anthers of a *popo* line (see above). Taken together, these data indicate that anthers of *popo* and *PoPo* lines both express *chi*B mRNA, whereas the 1.0-kb *chi*A mRNA is only present in young anthers of *PoPo* lines. This shows that the *chi*B gene is not related to the *Po* mutation.

Linkage Analysis of *Po* with *chi* Genes

The mutant phenotype of *popo* petunia lines can be the result of a mutation in the *chi*A gene itself, preventing the synthesis of a functional *chi* mRNA. Alternatively, a regulatory gene that controls the expression of the *chi*A gene in anthers can be mutated. To discriminate between these possibilities, RFLP analysis was performed to investigate linkage between the *chi* structural genes and *Po*. Absence of linkage rules out the possibility that the *Po* mutation is a mutation in the structural part of one of the *chi* genes.

On the basis of several criteria, the lines V23 and R51 were selected for the linkage experiments. First, the line V23 is recessive for *Po* and the line R51 is dominant, enabling the visual scoring of the status of *Po* in a single back-cross of the F1 V23/R51 (VR) hybrid with V23. Second, the VR hybrid is heterozygous for at least one phenotypic marker on each chromosome (Wallroth et al., 1986), which allows chromosomal localization experiments in the back-cross with either parent. Finally, as can be seen in Figure 3, DNA gel blot analysis of HindIII-digested R51 and V23 DNA revealed the presence of a polymorphism for *chi*A, which is a prerequisite to perform the RFLP analyses. For these reasons, progeny of a (V23 × R51) × R51 and a (V23 × R51) × V23 cross were analyzed for segregation of the *Po* gene and *chi*A. Complete linkage was observed between *Po* and *chi*A, as shown in Figure 3 and Table 1.

Because R51 and V23 do not show an RFLP for *chi*B (not shown), a different cross was analyzed for the segregation of *chi*B alleles in relation to the status of *Po*. An RFLP analysis of (M65 × W62) × W62 using a *chi*B probe demonstrated that no linkage exists between *chi*B and *Po*

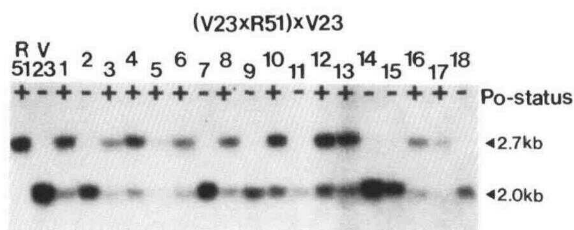

Figure 3. Linkage Analysis between *chi*A and *Po*.

DNA isolated from individual plants from a back-cross of the V23/R51 F1 hybrid with V23 (lanes 1 through 18) and the parental lines (V23 and R51) was digested with HindIII and subjected to DNA gel blot analysis using the V30 *chi* cDNA as a probe. The allelic status of *chi*A was compared with that of *Po*, which was determined visually [purple pollen (*Popo*) indicated with a plus sign and green pollen (*popo*) indicated with a minus sign].

(Table 1). Likewise, no linkage was observed between *chi*B and several other phenotypic markers segregating in the same cross (*An3* and *Rt*, data not shown). These results indicate that *chi*B is not located on chromosome IV (*An3*), V (*Po*), or VI (*Rt*).

In summary, we have shown that *chi*A and *Po* are strongly linked (84 plants tested), whereas *chi*B is not linked with *Po* (50 plants tested). This places *chi*A on chromosome V at or near the *Po* locus and indicates that *chi*A and *Po* might be identical.

Complementation of the *Po* Mutation

To definitely exclude the possibility that *Po* is a regulatory gene controlling *chi*A expression, introduction of a wild-type *chi*A gene into a *popo* petunia line should complement the *Po* mutation. To perform this experiment, *chi* genes from a *Po*-dominant petunia line were isolated. Therefore, a V31 genomic library consisting of approximately 400,000 recombinant λ phages was constructed and screened with the V30 *chi*A-derived cDNA probe (van Tunen et al., 1988). Two positive clones were obtained, one being homologous with the probe (harboring V31 *chi*A) and one melting at higher stringencies (harboring V31 *chi*B). Restriction maps were constructed for both λ clones, and DNA gel blot analysis revealed that the V31 genome, like that of V30, contains two *chi* genes (not shown).

A 6.5-kb SstI V31 *chi*A fragment containing the entire *chi*A coding region, 3.5-kb 5′ promoter region, and a 2.0-kb tail was ligated into Bin19 (Bevan, 1984). The resulting construct was introduced into the *Po*-recessive line W115 and transgenic plants were regenerated. From the data presented in Figure 4A and Table 2, it can be concluded that anthers from eight of the 10 transgenic plants contained pollen grains that were white instead of yellow. Because the line W115 contains an additional block in the pathway (recessive for the gene *An4*), complemented transgenic plants accumulate colorless flavonoids instead of the pigmented anthocyanins and, therefore, the pollen grains are white. The complementation of the mutation correlated with the absence of naringenin chalcone (Figure 4B, lane 6) and the presence of CHI enzyme activity (Figure 4C, lane 8; Table 2) and *chi* mRNA in anthers (Figure 4D, lanes 4 and 5). Although the remaining two transgenic plants (32 and 33) contained the introduced *chi* gene in an intact form (as determined by DNA gel blotting; data not shown), no shift in pollen color was observed. Probably these plants lack expression of the introduced *chi* gene.

Complementation of the *Po* mutation could also be accomplished by introduction of a chimeric gene consisting of the 35S cauliflower mosaic virus (CaMV) promoter fused to a full-size *chi* cDNA. A transgenic W115 plant containing one copy of this gene construct also has white pollen grains. Analysis of plants of a back-cross of this transgenic plant showed a 100% correlation of the complemented phenotype (white pollen grains) with kanamycin resistance: 18 of 30 plants were resistant to kanamycin and showed complementation of the mutation, whereas all the others

Table 1. Linkage Analysis between *Po* Gene and *chi*A and *chi*B

	(V23 × R51) × V23		(V23 × R51) × R51		(M65 × W62) × W62	
Allele	*Popo*	*popo*	*PoPo*	*popo*	*Popo*	*popo*
*chi*A[a]						
R51/R51	–	–	18	0		
R51/V23	18	0	0	19		
V23/V23	0	29	–	–		
*chi*B[b]						
M65/W62					11	18
W62/W62					9	12

[a] Eighty-four plants tested.
[b] Fifty plants tested.

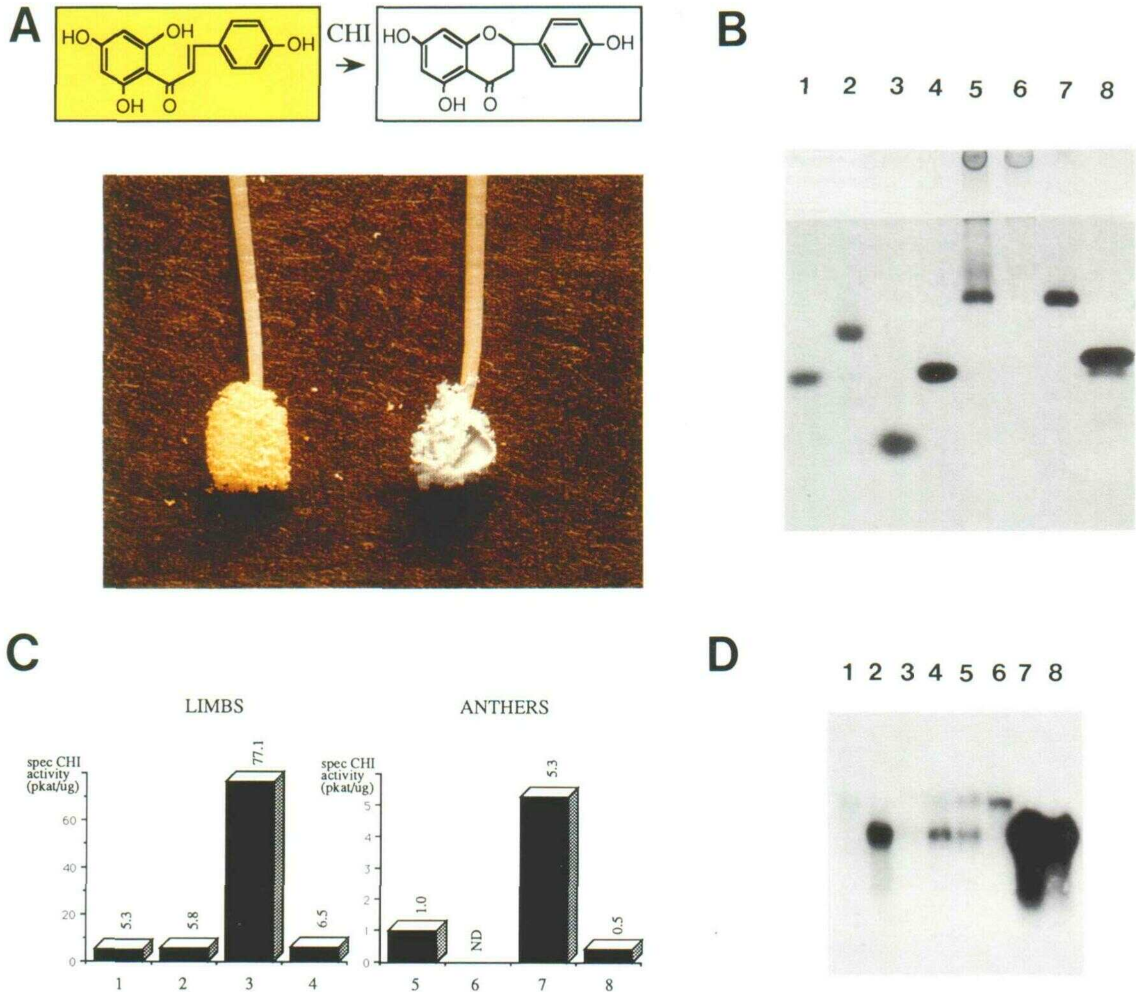

Figure 4. Complementation of the *Po* Mutation.

(A) Anther of an untransformed W115 plant (yellow anther at left) and an anther of a W115 transformant containing the *chi*A gene isolated from the *PoPo* line V31 (white anther at right). At the top, the reaction catalyzed by the CHI enzyme is shown; the yellow 2′,4,4′,6′-tetrahydroxychalcone (left) is converted into the corresponding colorless flavanone (right).

(B) TLC of pollen extracts. As a marker, 5-μL samples of various purified flavonoids (1 μg/μL) were also chromatographed. Lane 1, 5,7,4′-trihydroxyflavanone (naringenin); lane 2, 5,7,3′,4′-tetrahydroxyflavanone (eriodictyol); lane 3, 5,7-dihydroxyflavanone (pinocembrin); lane 4, 2′,4′,4-trihydroxychalcone (yellow); lane 5, pollen extract of an untransformed W115 (*popo*) plant; lane 6, pollen extract from a transformant containing the *PoPo chi*A from V31; lane 7, 2′,4′,6′,4-tetrahydroxychalcone (yellow); lane 8, 4,4′-dihydroxy-2′-methoxy-chalcone (yellow).

(C) CHI enzyme activity in limbs or anthers of untransformed V31 (*PoPo*, lanes 1 and 5), untransformed W115 (*popo*, lanes 2 and 6), transgenic W115 containing a 35S CaMV-*chi* cDNA chimeric gene (lanes 3 and 7), and a transgenic W115 containing a *chi*A gene isolated from V31 (lanes 4 and 8). ND, not detectable.

(D) RNA gel blot analysis showing the presence of *chi* RNA in pollen of developmental stage 5/6 of untransformed V31 (*PoPo*, lane 1), immature anthers of developmental stage 2/3 of untransformed V31 (lane 2), W115 (*popo*, lane 3) plants, transgenic W115 plants showing complementation of the *Po* mutation (lanes 4 and 5), transformed W115 plants without complementation of the *Po* mutation (lane 6), and petals of developmental stage 3 of W115 (lane 7) or V31 (lane 8) untransformed plants. Under the hybridization conditions used (2 × SSC, 65°C), the *chi*A cDNA probe cross-hybridizes only slightly with the *chi*B transcript found in immature anthers (see also van Tunen et al., 1988). Therefore, the hybridization signals detected on this blot represent only the 1.0 kb or 1.5 kb *chi*A transcripts.

Table 2. Complementation of the *Po* Mutation in Transgenic W115 (*popo*) Plants Containing the V31 (*PoPo*) *chi*A Gene

Transformant No.	Pollen Color	Specific CHI Enzyme Activity in Anthers (pkat/μg of protein)
W115 control	Yellow	ND[a]
V31 control	White	1.05
1	White	0.56
2	White	0.38
31	White	0.25
32	Yellow	ND
33	Yellow	ND
52	White	0.26
53	White	0.24
54	White	0.57
80	White	0.46
101	White	0.16

[a] ND, not detectable.

were both kanamycin sensitive and had yellow pollen. In plants harboring the 35S CaMV-*chi* construct, the presence of CHI enzyme activity is not only restricted to floral tissues alone but high levels of CHI activity were also detected in pistil, leaf, and stem, as shown in Table 3. In corollas and tubes, higher levels (up to 10-fold) of CHI enzyme activity were measured (Table 3).

Taken together, these data clearly show that the *Po* mutation can be overcome by the introduction of a *chi*A gene isolated from a *Po*-dominant petunia line or a 35S CaMV-*chi* cDNA construct. We conclude, therefore, that the *Po* mutation is a mutation in the *chi*A gene itself.

Comparison of *chi* Genes from *PoPo* and *popo* Petunia Lines

The tight linkage between *chi*A and *Po* and the complementation of the *Po* mutation by the *Po*-dominant *chi*A gene (see above) proves that both are identical. Because the *chi*A gene from a *popo* line gives rise to a functionally active CHI enzyme in the corolla (van Tunen et al., 1988), the *Po* mutation most likely involves a mutation in the promoter region of *chi*A preventing its expression in anthers (see also Figure 1A). This rules out the possibility that *Po* is a regulatory gene controlling *chi*A expression. To identify sequences potentially involved in the regulation of genes in the anthers, the DNA sequences of *chi*A genes from a *Po*-dominant line and a *Po*-recessive line were compared.

As can be seen in Figure 5, comparison of the V30 and V31 *chi*A genes showed that both of their coding sequences are highly conserved and lack introns; only 19 base pair substitutions have occurred, from which 10 are at the third base. Furthermore, most amino acid substitutions are in the N-terminal part of the *chi* genes, a region in which a high variability was observed when *chi* sequences from different plant species were compared (Dr. E.R. Blyden, personal communication). Finally, no substitutions have occurred in the region of the gene likely to encode the active site of the CHI enzyme (predicted to be around the cysteine residue at position 647, Figure 5) (Dr. E.R. Blyden, personal communication).

Comparison of the regulatory regions of *chi* genes from the *Po*-dominant (V31) line and *Po*-recessive (V30) line revealed that the V31 5′ promoter region and 3′-untranslated tail show a high overall homology to the corresponding V30 regions. However, significant differences were also observed. A number of additions in the V30 promoter region relative to that of V31 were observed, the largest being around positions −67, −194 (deletion of half of the direct repeat of V30 *chi*A), −310, −377, and −400. Only one deletion located around position −289 was found in the V30 promoter region. Finally, the V30 perfect inverted repeat around position −330, capable of forming a relatively stable hairpin loop, was not found in the V31 promoter region.

In summary, it can be concluded that although the promoter regions of V30 (*popo*) and V31 (*PoPo*) are highly homologous, deletions and additions have occurred in the V30 *chi*A promoter that might have led to the inactivation of this promoter in anthers.

DISCUSSION

In anthers of petunia, expression of CHI is regulated by the gene *Po*. In *Po*-recessive petunia lines, the presence

Table 3. CaMV-Driven CHI Enzyme Activity in *PoPo* and *popo* Petunia Plants Transformed with a CaMV-*chi* Chimeric Gene (WA1, Transformed W115; VRA3, Transformed VR)

	Specific CHI Enzyme Activity (pkat/μg of protein)			
Tissue[a]	WA115 Control (*popo*)	WA1	VR Control (*Popo*)	VRA3
Limb	6.5	77.1	8.3	90.4
Tube	1.4	17.9	6.6	20.6
Anthers	0	5.3	1.9	4.6
Pistil	2.2	16.9	4.2	32.5
Leaf	0.3	6.3	0.4	26.9
Stem	0.5	21.7	6.1	34.8

[a] All flower tissues are from buds of developmental stage 3 (W115 buds, 3.0 cm to 4.5 cm; VR buds, 2 cm to 3 cm).

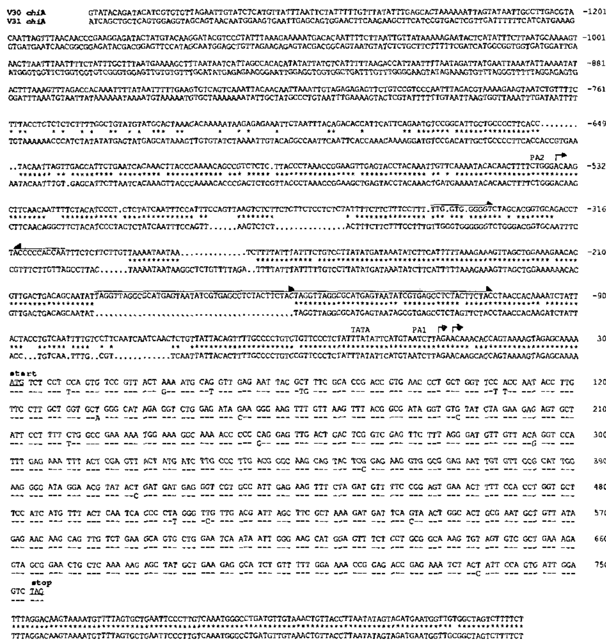

Figure 5. Sequence Comparison of *chi*A Genes of *PoPo* (V31) or *popo* (V30) Petunia Lines.

The coding sequence of V30 *chi*A is depicted in a triplet code (top row); nucleotides from V31 that differ from V30 are indicated (bottom row). The 5′ promoter regions of V30 and V31 are arranged in such a way as to produce the best fit. Identical bases in the 5′ and 3′ regions are indicated with an asterisk (*). The stop and start codons (underlined), TATA box, transcription starts (bent arrows), a direct repeat (horizontal arrows around position −100), and an inverted repeat (horizontal arrows around position −330) from the V30 *chi*A are indicated. Numbering is such that the V30 P_{A1} transcription start is labeled as +1.

of the CHI enzyme is strongly reduced in the anthers, whereas normal CHI enzyme levels are observed in corollas of the same line. As a consequence of the *Po* mutation, the yellow substrate of CHI accumulates in anthers and can be easily seen. In this paper, we investigated the molecular basis of this mutation as a way to investigate molecular aspects of anther-specific gene regulation.

Because no mutations in the coding region of *chi* genes of a *popo* petunia line are observed (van Tunen et al., 1989), we might explain the *Po* mutation in several ways: *Po* is either a regulatory gene controlling *chi*A and/or *chi*B expression or *Po* is identical to *chi*A or *chi*B and a mutation in *chi* regulatory sequences prevents *chi* expression in anthers.

A 100% linkage has been shown by RFLP analysis between *chi*A and *Po* (Figure 3 and Table 1), indicating that *Po* and *chi*A are identical and that a mutation in *chi*A is responsible for the absence of CHI enzyme in anthers of *popo* petunia plants. However, this mutation is not within the *chi*A coding region because the *chi*A gene from a *popo* line encodes a functional CHI enzyme in the petals (van Tunen et al., 1988).

The complementation of the *Po* mutation by introduction of a *PoPo chi*A gene proves that *Po* is identical to *chi*A. This is in line with RNase protection experiments that showed that the absence of *chi*A expression in anthers of *popo* correlates with the absence of the *chi* mRNA (Figure 1). Analysis of transgenic *PoPo* petunia plants containing a chimeric gene consisting of a V30 (*popo*) *chi*A promoter fragment and the GUS reporter gene (Jefferson et al., 1987) showed that *chi*A-driven GUS activity was present at high levels in limb, tube, leaf, sepal, stem, ovarium, and seed but not in anthers (van Tunen et al., 1990b). This result also suggested that the *Po* mutation acts in *cis* on the expression of *chi*A and that the V30 (*popo*) *chi*A promoter cannot be activated in *trans* by factors present in anthers of a *Po*-dominant line. Therefore, we conclude that the *Po* mutation involves a mutation in regulatory regions of *chi*A that prevents the expression of *chi*A in anthers.

The presence of multiple additions and deletions in the *chi*A promoter of V30 (*popo*) relative to that of V31 (*PoPo*, Figure 5) might be responsible for the absence of *chi*A transcripts in anthers of this line. To define the differences in wild-type and mutant promoter regions more precisely, we are performing promoter deletion and addition experiments.

A number of mutations in flavonoid gene expression in different plants have been characterized at the molecular level. These mutations include aberrations in the coding sequence that prevent the synthesis of a functional enzyme [*bz-1* encoding 3-*O*-glycosyltransferase in maize and *nivea* encoding chalcone synthase in snapdragon (Dooner et al., 1985; Martin et al., 1987)] or aberrations in the promoter sequences preventing the transcription of the genes *nivea* and *pallida* [encoding CHS and DFR, respectively, in snapdragon (Martin et al., 1985, 1987; Sommer et al., 1988)]. This results in lower levels of enzyme activity, causing a reduction of flower pigmentation. Besides an even reduction of flower pigmentation, mutations in the *nivea* or *pallida* promoter also can give rise to new color patterns in which the accumulation of anthocyanins is restricted to certain floral organs (lobes), whereas other floral organs (tubes) are colorless. The generation of color patterns appears to be a property of sequence alterations within the promoter (Coen et al., 1986). Sequence alterations are also found in the *Po* mutant where the mutation resides within the *chi*A promoter region. These changes may explain the altered CHI enzyme expression within only one organ (anther), whereas another organ (petal) is unaffected.

The experiments in which the *Po* mutation was complemented by the introduction of a *PoPo* V31 *chi* gene or a chimeric 35S CaMV-*chi* gene suggest the use of CHI as a phenotypic marker in a *popo* petunia background. There-

fore, promoters active in anthers and, more specifically, in the tapetum cells [where flavonoids are produced and the *chs*A and *chs*J and the *chi*B promoters are active (Koes et al., 1990; van Tunen et al., 1990a)] of such mutants can be fused with the full-size *chi* cDNA. If the promoter retains its specificity, anthers of transgenic (*popo*) plants should stain white. In many plant species, a mutation similar to the *Po* mutation occurs, giving rise to the formation of yellow anthers (e.g., Tulipa; Ebel and Hahlbrock, 1982). Likewise, corollas of *Callistephus chinensis* and carnation are devoid of CHI enzyme activity, resulting in the production of yellow flowers (Kuhn et al., 1978; Forkmann and Danglmayr, 1980). CHI can also be used as a phenotypic marker in flowers of those plant species. The major advantage of CHI as a marker system is that CHI expression can be monitored at the phenotypic, enzymic, protein, RNA, and DNA levels, enabling the investigation of promoter activity in great depth.

The complementation experiment (Figure 4) proved that *chi*A and *Po* are identical. From this, we can conclude that *chi*B is not related to the *Po* mutation. Because *popo* lines are devoid of CHI enzyme activity in anthers but express *chi*B in this tissue, this gene does not seem to encode a functional CHI. Therefore, the question arises of what is the function, if any, of this *chi*B mRNA. At present, it is not known whether this *chi*B mRNA is translated to CHI protein. It is also possible that *chi*B does not encode the actual CHI itself but rather an enzyme catalyzing a CHI-related reaction. One candidate may be an aurone synthase catalyzing the conversion of chalcones into aurones (van Tunen and Mol, 1990). Experiments to investigate this hypothesis are in progress.

METHODS

Plant Material

Petunia hybrida varieties were grown under normal greenhouse conditions. The lines R51 (*an4an4 PoPo*), M65 (*an4an4 PoPo*), V23 (*An4An4 popo*), V30 (*An4An4 popo*), V31 (*an4an4 PoPo*), W62 (*An4An4 popo*), and W115 (*an4an4 popo*) and the V23/R51 F1 hybrid (*An4an4 Popo*) were used for RNA extractions and cloning of *chi* genes (V30 and V31), RFLP analysis (V23, R51, W62, and M65), or used for transformation experiments (W115 and VR).

CHI Enzyme Measurements and TLC

CHI enzyme measurements were performed as described previously (Mol et al., 1983) using tetrahydroxychalcone as a substrate. Ten millimolar KCN was present in the enzyme assay to inhibit the aspecific (peroxidase) breakdown of the chalcone. CHI enzyme extracts were prepared from floral organs dissected from 5 to 10 flower buds. After correction for spontaneous isomerization, the specific CHI enzyme activity (picokatals per microgram of protein) was calculated. Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard.

TLC was performed with HPTLC-Kieselgel 60 (13727, 10 × 10 cm, Merck, Darmstadt, Federal Republic of Germany) by using chloroform/methanol/formic acid (90:9:1, v/v/v) as solvent. Homogenized pollen was extracted with methanol (15 mg/mL) and the methanol phase was concentrated by using a Speedvac (Savant Instruments Inc., Farmingdale, NY). For chromatography, 5 μL (corresponding with 0.3 mg fresh weight) of pollen extracts and 5 μL (1 μg/μL) of flavonoids (naringenin from Sigma; pinocembrin and eriodictyol from Roth GmbH, Karlsruhe, Federal Republic of Germany; 2′,4′,4-trihydroxychalcone, 2′,4′,6′,4-tetrahydroxychalcone, and 4,4′-dihydroxy-2′-methoxychalcone from Dr. Donald Phillips, University of California, Davis) were applied in methanolic solutions and detected under UV light at a wavelength of 366 nm.

RNA/DNA Extractions and RNase Protection Analysis

All plasmid DNA isolations were performed as described earlier (Maniatis et al., 1982). Total RNA was extracted from various V30 or V31 flower tissues of different developmental stages: stage 1, flower buds up to 1.5 cm; stage 2, flower buds 1.5 cm to 3.0 cm; stage 3, flower buds 3.0 cm to 4.5 cm; stage 4, flower buds 4.5 cm to 6.0 cm; stage 5, open flowers/closed anthers; stage 6, open flowers/open anthers (as described by van Tunen et al., 1988, 1989b). After isolation, 10 μg of RNA was subjected to RNase protection experiments using in vitro synthesized V30 *chi*A or *chi*B probes (as described previously; see also van Tunen et al., 1988) and RNase T1 and A. Protected fragments were run on a 2% denaturing agarose gel.

Cloning of V31 *chi* Genes and Sequence Analysis

Total DNA was isolated from top leaves of young nonflowering petunia V31 plants. Cesium chloride-purified DNA was partially digested with Sau3A and size fractionated by glycerol gradient centrifugation. DNA fragments were cloned into λGEM 11 BamHI-digested arms (Promega Biotec). Approximately 400,000 clones were screened by hybridization with a ^{32}P nick-translated *chi* cDNA probe (van Tunen et al., 1988).

Physical maps of the isolated λ clones were made by analysis of partial restriction enzyme digests specifically labeled at the cos R-site (Rackwitz et al., 1984). λGEM 11 inserts were subcloned in M13mp18 and M13mp19 vectors using standard cloning procedures. Sets of overlapping clones were generated using the Erase-a-Base™ kit (Promega Biotec) and sequenced using the dideoxy chain-termination method of Sanger et al. (1977). Sequence comparisons were performed using the Apple DNA Inspector IIe program.

RFLP Analysis

RFLP analysis was performed as described by Koes et al. (1987). Different crosses segregating for the gene *Po* and *chi* genes were analyzed for linkage. In crosses where the status of the *Po* gene (*PoPo* or *Popo*) could not be determined directly, back-crosses

with the *Po*-recessive parent (at least 20 plants) were made and scored for the status of the *Po* gene by eye. DNA from individual plants was isolated by a miniprep procedure (Dellaporta et al., 1983).

After digestion and size separation on a 0.8% agarose gel, the DNA was blotted on a GeneScreen *Plus* membrane as described by the manufacturer (Du Pont-New England Nuclear). The blots were hybridized with a ^{32}P nick-translated V30 *chi*A cDNA probe (van Tunen et al., 1988) and subsequently washed under conditions specified in the figures.

Gene Constructions and Plant Transformation

A construct was made in which the 35S CaMV promoter was fused in front of a full-size *chi* cDNA. First, the chloramphenicol acetyltransferase coding sequence from the plasmid p35S chloramphenicol acetyltransferase (obtained from Jurgen Deneke, Plant Genetic Systems, Gent, Belgium) was removed by digestion with XbaI and NcoI. After S1 nuclease treatment, a blunt-end, full-size *chi* cDNA fragment was ligated into the vector. A clone with the *chi* cDNA insert in the right orientation was selected, digested with BglI and HindIII, and cloned into pBin19 (Bevan, 1984) digested with BamHI and HindIII. The resulting clone contained a 1-kb 35S CaMV promoter fused to a *chi* cDNA, retaining 5 bp of its leader, 751 bp of coding sequence, and 29 bp of the 3'-untranslated region and a 700-bp octopine synthase tail. After mobilization of the construct to the *Agrobacterium tumefaciens* strain LBA 4404 (Bevan, 1984), plasmid DNA from the resulting cell culture was checked by restriction enzyme analysis for the occurrence of recombination events.

Transgenic W115 and VR plants were obtained by the standard leaf disc transformation method (Horsch et al., 1985). Leaf discs were prepared from top leaves of young nonflowering plants. All transgenic plants raised were checked for transformation on the basis of resistance for kanamycin (leaf discs were taken and grown in kanamycin-containing MS plates supplemented with hormones) and DNA gel blot analyses. On the average, the transgenic plants contained one to five copies of the construct per haploid genome (data not shown).

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