The *an11* locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals

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In petunia flowers, the loci *an1*, *an2*, and *an11* control the pigmentation of the flower by stimulating the transcription of anthocyanin biosynthetic genes. The *an1* and *an2* locus were recently cloned and encode a basic helix-loop-helix (bHLH) and MYB-domain transcriptional activator, respectively. Here, we report the isolation of the *an11* locus by transposon tagging. RNA gel blot experiments show that *an11* is expressed independently from *an1* and *an2* throughout plant development, as well as in tissues that do not express the anthocyanin pathway. It encodes a novel WD-repeat protein that is highly conserved even in species that do not produce anthocyanins such as yeast, nematodes, and mammals. The observation that the human *an11* homolog partially complements the *an11* petunia mutant in transient assays shows that sequence similarity reflects functional conservation. Overexpression of *an2* in *an11*⁻ petals restored the activity of a structural anthocyanin gene in transient assays, indicating that AN11 acts upstream of AN2. Cell fractionation experiments show that the bulk of the AN11 protein is localized in the cytoplasm. Taken together, this indicates that AN11 is a cytoplasmic component of a conserved signal transduction cascade that modulates AN2 function in petunia, thereby linking cellular signals with transcriptional activation.

[Key Words: signal transduction; anthocyanin; transcription control; MYB; WD-40; petunia]

Received January 29, 1997; revised version accepted April 9, 1997.

The flavonoid biosynthetic pathway in plants is branched and produces a variety of products with diverse biological functions. For instance, anthocyanin pigments in flowers are visual signals to pollinators, whereas flavonols protect the plant from UV light and are required for the development of pollen (for review, see Koes et al. 1994; Shirley 1996). Given these different functions, it is not surprising that the expression of the flavonoid biosynthetic genes, encoding enzymes of the pathway, is induced by a variety of factors. These factors include hormones and factors that determine tissue-specificity, as well as external signals such as light, temperature, and pathogens (for review, see Mol et al. 1996). The underlying regulatory mechanisms have been most intensively studied in relation to the biosynthesis of anthocyanin pigments, because they provide a convenient visible marker.

Some steps in the primary light signal transduction cascade have been elucidated. The tomato *aurea* mutant lacks active phytochrome and fails to synthesize anthocyanins. Anthocyanin synthesis can be restored by mi-

croinjection of different signaling compounds. On the basis of results with antagonists of animal G proteins (Neuhaus et al. 1993), it is suggested that phytochrome acts through heterotrimeric G proteins. Downstream from the G proteins, cyclic GMP is most likely the signaling molecule leading to activation of anthocyanin synthesis (Bowler et al. 1994a,b).

The transcription factors that are involved in the terminal steps of the signal-transduction cascades have been studied by genetic strategies in maize, snapdragon, and petunia (for reviews, see Dooner et al. 1991; Mol et al. 1996). Analysis of anthocyanin mutants in maize has shown that transcription of all the structural anthocyanin biosynthetic genes is controlled by two gene families (Fig. 1A). Both families comprise multiple genes that encode functionally similar proteins with distinct spatial expression patterns (Ludwig and Wessler 1990; Cone et al. 1993; Consonni et al. 1993). *c1* and *pl* encode MYB domain proteins (Paz-Ares et al. 1987; Cone et al. 1993), while the *r/b* gene family encodes proteins with a basic helix-loop-helix (bHLH) DNA-binding motif (Ludwig and Wessler 1990; Consonni et al. 1993). Recent studies indicate that the *c1/pl* and *r/b* regulators are highly conserved and interchangeable between plant species. Lloyd et al. (1992) showed that introduction of a maize *r* gene

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Figure 1. Genes involved in anthocyanin synthesis in maize and petunia and phenotypes of *an11*-mutants. (A) The anthocyanin biosynthesis pathway and its regulation in maize and petunia. Enzymes: (CHS) chalcone synthase; (CHI) chalcone flavanone isomerase; (F3H) flavanone 3-hydroxylase; (DFR) dihydroflavonol 4-reductase; (3GT) UDP-glucose flavonoid glycosyltransferase; (RT) anthocyanin rhamnosyltransferase; (5GT) UDP-glucose flavonoid 5-O-glycosyltransferase; (MT/MF) methyltransferase. Names of the regulatory loci controlling anthocyanin biosynthesis in maize (left) or in petunia (right) are given in italics. Changes in color following modifications to the basic flavonoid ring structure are indicated. (B) Flower pigmentation patterns specified by different *an11* alleles. The genotypes are specified above each flower.

in *Arabidopsis* and tobacco enhanced anthocyanin production. In snapdragon, *delila* regulates anthocyanin biosynthesis in the tube of the flower and was found to encode an *r/b* homolog (Goodrich et al. 1992). In petunia four loci, *an1*, *an2*, *an4*, and *an11*, control transcription of a subset of structural anthocyanin genes in the flower and mutations in these loci can be complemented by *c1* and an *r* gene from maize (Quattrocchio et al. 1993; see also Fig. 1A). In addition, *an1*, *an2*, and *an11* play a role in the control of intracellular pH in petal cells (Quattrocchio 1994). Recently, *an1* and *an2* were cloned by transposon tagging and shown to encode a bHLH protein (C. Spelt, J. Mol, and R. Koes, unpubl.) and an MYB-type protein homologous to *c1/pl*, respectively (Quattrocchio 1994).

Little is known about the mechanisms that control these MYB and bHLH regulators and the modulation of their activity by upstream signal transduction cascades. One good candidate for transcriptional regulation of *c1* is the maize *viviparous-1* (*vp1*) gene (McCarty et al. 1991). Mutations in the *vp1* gene have a pleiotropic effect on seed development, in that the gene is required for both seed dormancy and seed pigmentation. It was shown that *vp1* controls the anthocyanin pathway primarily through transcriptional regulation of the *c1* gene. The *r* gene apparently does not require *vp1* expression (Hattori et al. 1992).

In this report, we present the molecular analysis of the anthocyanin regulatory gene *an11*. The *an11* gene encodes a novel protein containing five WD-repeats, a motif first noted in the G protein β subunit of the bovine

retinal transducin (Fong et al. 1986). Because the AN11 protein is localized in the cytoplasm, it may regulate gene expression of the anthocyanin biosynthetic pathway through post-translational modification of the transcription factors, most likely the MYB domain protein AN2. The presence of AN11 protein homologs in mammals and yeast suggests an evolutionary conserved function in all eukaryotes.

Results

Isolation of the *an11* gene

The unstable *an11-W137* allele was isolated in the petunia line W138 (Doodeman et al. 1984). Homozygotes for this allele have white flowers with red and occasionally pink spots, presumably caused by the somatic excision of a transposable element (Fig. 1B). Among progeny obtained by selfing of *an11* mutable plants, we identified three full revertant alleles, two partial revertant, and four stable recessive alleles that originated from independent sporogenic excision events (Fig. 1B). Furthermore, a novel unstable allele, *an11-G5543*, was recovered. *an11-G5543* flowers differ from *an11-W137* flowers in that they contain a large number of pink spots whereas red spots are seen only occasionally (Fig. 1B). A sporogenic excision event from *an11-G5543* yielded the partial revertant allele *an11-Z2297*.

All unstable alleles isolated in the W138 background that were analyzed so far contained insertions of the 300-bp transposable element *dTph1* (Gerats et al. 1990; Souer

in length and sequence. In many WD-repeats, including those of AN11, the amino-terminal part with the amino acids (G/S/A/V)H has diverged considerably from the consensus, whereas the carboxy-terminal part is usually much better conserved. Neer et al. (1994) stated that WD-repeat proteins, by definition, have at least one repeat that matches the consensus with zero or one mismatches, and at least one repeat with three or fewer mismatches. Consistent with these criteria, three of the five repeats (2, 3, and 5) of the AN11 protein have one or two mismatches with the consensus core repeat sequence, whereas the remaining two repeats (1 and 4) each have five mismatches. The length of the core between the GH and WD sequences in each WD-repeat of AN11 protein is 26–28 amino acids, which is also consistent with the consensus (Neer et al. 1994).

Spatiotemporal and genetic control of *an11* expression

To study the pattern of *an11* gene expression, we measured *an11* mRNA levels in various tissues relative to mRNA levels of *dfr* (an *an11* controlled structural anthocyanin gene encoding dihydroflavonol 4-reductase), *chs* (a structural gene encoding chalcone synthase that is coordinately expressed with *dfr*, but not controlled by *an11*) and *gapdh* (encoding the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase). Figure 4A shows that *an11* mRNA levels in the limb of developing petals closely parallel those of *dfr* and *chs*. The three mRNAs increase from a low level in young (stage 1) buds to a maximum in stage 4/5, when the petal starts to unfold. From this stage on, the *dfr* and *chs* mRNA quickly disappear, whereas the *an11* mRNA level decreases only slightly. In the flower tube, however, *an11*, *chs*, and *dfr* mRNA accumulation is not completely parallel, even though they reach a maximum at the same stage (5).

In anthers, *chs* and *dfr* genes are coordinately expressed at a slightly earlier stage of flower development than in the petals (Beld et al. 1989). Figure 4A shows that *chs* mRNA levels in anthers display a steep increase from stage 1 to 3, whereas *an11* mRNA levels are virtually constant during that period. Because the R27 line used for these experiments is an *an4*[−] mutant, no *dfr* mRNA is detected in the anthers (Quattrocchio et al. 1993). Ovaries express *an11* throughout development in a pattern very similar to that of *chs* and *dfr*. Strikingly, we also detected *an11* transcripts in sepals, leaves, stems, and roots. These tissues are normally not pigmented, consistent with the absence of *chs* and *dfr* expression.

To determine whether *an11* expression is controlled at the transcriptional level by the two other anthocyanin regulatory loci *an1* and *an2*, we measured *an11* transcript levels in *an1*[−] and *an2*[−] mutants (Fig. 4B, left). Comparison of the *an11* mRNA level in the line W138, harboring the unstable *an1*-W138 allele, with that in the isogenic wild-type R27, shows that the *an1* mutation has no significant effect on *an11* expression. Similarly, the *an11* mRNA level in the *an2*[−] mutant W115 is compa-

Figure 4. Spatiotemporal and genetic control of *an11* expression. (A) RNA gel blot analysis of total RNA from floral organs at different developmental stages (1–6) as indicated and from root (r), stem (s), and leaf (l). The filters were sequentially hybridized with *an11*, *dfr*, a structural gene regulated by *an11* and *chs*, a gene encoding for an anthocyanin biosynthesis enzyme and not regulated by *an11*, and *gapdh* to confirm the presence of RNA in all lanes. (B) Genetic control of *an11* expression in the flower corolla and leaves by the regulatory loci *an1* and *an2*. RNA was isolated from corollas from various petunia lines at three developmental stages. The petunia lines used and their genotype are indicated above the lanes.

able to that in the nonisogenic wild-type R27. As expected, the *dfr* mRNA levels are very low in the *an1* and *an2* mutants. In leaves, *an11* also appears to be expressed independently from the regulators *an1* and *an2* (Fig. 4B, right). No signal was detected in *an11*[−] leaves, indicating that the transcripts detected in vegetative tissues are indeed derived from the *an11* locus.

Taken together, these data show that the spatiotemporal expression of *an11* and that of the target genes (*dfr*) are not correlated; *an11* is expressed in pigmented as well as unpigmented tissues. Furthermore, it seems un-

likely that the other two regulators, *an1* and *an2*, control *dfr* transcription by controlling *an11* mRNA levels.

Analysis of mutant *an11* alleles

As a first step toward defining the functional important domains in the AN11 protein, we analyzed the different alleles derived from *an11-W137* (Fig. 5A). Mapping and sequence analysis of the *an11-W137* allele showed that the *dTph1* element was inserted in the first WD-repeat, (321-bp downstream of the transcription start site, see Fig. 3A). The *dTph1* element was flanked by an 8-bp duplication of *an11* sequence, a structure similar to *dTph1* insertions in other genes (Gerats et al. 1990; Souer et al. 1996). Sequence analysis of four stable *an11*⁻ derivative alleles showed that *dTph1* had excised and generated a 5- or 8-bp footprint causing a frameshift. This frameshift results in the addition of 4 or 5 novel amino

acids after Pro-86 before a stop codon is reached. Conceptual translation of these transcripts produces proteins lacking the WD-repeats. The partial revertant alleles *an11-W2038* and *an11-W2053* both have a 3-bp insertion after Pro-86, which leads to an extra Asp. Three independent revertant alleles have been isolated, each having a 6-bp footprint. The footprints in *An11-T2141* and *An11-T152* are identical, conceptually leading to a Pro-Ile insertion between His-85 and Pro-86. The 6-bp footprint in *An11-T154* would cause the insertion of a Pro-Asp at the same position.

PCR and sequence analysis showed that in *an11-G5543*, 4 bp were deleted immediately upstream the *dTph1* element in *an11-W137*. Most likely, *an11-G5543* arose by an unsuccessful *dTph1* transposition event, in which the *dTph1* element was cleaved at the left end, but not at the right, followed by ligation. This 4-bp deletion apparently results in a slightly different spectrum

Figure 5. Analysis of *an11* alleles. (A) Footprint alleles generated after excision of the *dTph1* element from the *an11-W137*. Comparison of the DNA and deduced amino acid sequences of wild-type (*An11-R27*), *an11-W137*, stable mutant, partial revertant, and full revertant *An11* alleles. The 8-bp target site duplication upon *dTph1* insertion are underlined. (B) Expression of *an11* in various mutant, partial, and full revertant *An11* alleles. Total RNA was isolated from R27 (wild-type), two independent stable *an11* mutants (W134, X2197), a partial revertant (W2053), and two independent *An11* revertants. Blots were probed with *an11*, *dfr*, and *gapdh*. (C) Analysis of the transcription initiation of *chs*, *chi*, *dfr*, and *an11* in wild-type, partial revertant and mutant *an11* alleles. Nuclear run-on assays were performed utilizing nuclei of corollas in developmental stage 3/4. Nascent ³²P-labeled RNAs were hybridized to filters carrying single-stranded DNA clones of indicated genes. Empty M13mp10 vector was loaded as a control. Quantitation of the *dfr* and *an11* signals are shown in the histogram, which gives the relative transcription rates expressed as the *dfr* to *chs*, and the *an11* to *chs* signal ratios.

A WD-repeat protein controls flower pigmentation

of excision footprints, and a different ratio of full (red) and partial (pink) revertant spots. Sequencing of the sporogenic partial reversion allele *an11*-Z2297 derived from *an11*-G5543 showed that its sequence was identical to *an11*-W2038 and *an11*-W2053. RNA gel blot analysis of plants harboring mutant alleles (Fig. 5B) shows that flowers homozygous for the stable recessive alleles *an11*-W134 and *an11*-X2197, produce very little *an11* mRNA. The partial revertant *an11*-W2053 has an intermediate *an11* transcript level (threefold lower than in R27 wild type), whereas the full revertants *An11*-T152 and *An11*-T154 have *an11* mRNA levels comparable to that of the R27 wild-type plant. An identical pattern was obtained after hybridization with the *an11*-controlled gene, *dfr*.

To test whether the reduced levels of *an11* mRNA in the *an11*⁻ mutant are the result of reduced *an11* gene transcription or destabilization of the *an11* transcript, we analyzed nascent RNA by a nuclear run-on assay for *an11*, *dfr*, *chs*, and *chi* (encoding chalcone flavanone isomerase). *chs* and *chi* were used as an internal control to normalize the *an11* and *dfr* signals, because they have a similar temporal expression pattern in the petal limb but are not controlled by *an11*. Figure 5C shows that the transcription rate of the *dfr* gene was reduced in the partial *an11* revertant and *an11*⁻ line as expected, whereas the rate of transcription of the *an11* gene remained comparable to that in the wild-type R27. These results indicate that the mutations result in destabilization of the *an11* transcript. This is in agreement with the observation that an *an11* promoter- β -glucuronidase (*gus*) fusion is equally expressed in particle bombarded *An11*⁺ and *an11*-W134 petals (data not shown).

An11 protein is localized in the cytosol

To examine the subcellular localization of AN11 protein in the petunia corolla, we raised a polyclonal antiserum against the AN11 protein. The AN11 ORF was expressed in *Escherichia coli* with the pRSET expression vector. The IPTG-induced His tag/AN11 fusion polypeptide migrated at 40 kD and was purified by nickel affinity chromatography (Fig. 6A, lanes 2–6). Anti-AN11 serum reacted specifically with a protein doublet of 40 kD present in wild type (R27), but not in *an11*-W134 corolla limbs (Fig. 6A, lanes 7,8). The presence of two proteins cross-reacting with the anti-AN11 antiserum with little difference in molecular size could be caused by post-translational modification or proteolytic cleavage of the AN11 polypeptide.

The subcellular localization of the AN11 protein was determined by fractionating petal extracts into a cytosolic, microsomal membrane and nuclear fraction and immunological detection of the protein (Fig. 6B). Incubation with anti-AN11 antibodies showed that the 40-kD AN11 protein was found predominantly in the cytosolic fraction of wild-type R27 corolla limbs, not in the microsomal membrane fraction or in the nucleus. To assess the purity of the protein fractions, we subsequently used the same blot to probe with anti-F3H antiserum [detects the flavonoid biosynthetic enzyme flavanone 3-hydrox-

Figure 6. Subcellular localization of AN11 protein. (A) Expression of AN11 fusion protein in *E. coli*. SDS-polyacrylamide gel electrophoresis showing protein extracts from *E. coli* containing (lanes 4,5) or lacking (lanes 2,3) the *an11* cDNA. Bacteria were either induced (lanes 3,5) or uninduced (lanes 2,4) with IPTG, and AN11 was affinity purified by use of the histidine tag (lane 6). Lane 1 contains molecular mass standards. Numbers on the left are given in kD. Anti-AN11 antibodies recognize AN11 protein in wild-type R27 petunia corolla, not present in the *an11*⁻ mutant W134. Total protein extracts from wild-type (lane 7) and *an11*⁻ mutant (lane 8) corollas were separated on SDS-PAGE and transferred to nitrocellulose. Filters were incubated with anti-AN11 antiserum and AN11 protein was detected by an enhanced chemiluminescence detection method. (B) Subcellular localization of AN11. Proteins isolated from the cytosolic (lanes 1,2), microsomal membrane (lanes 3,4) and nuclear fraction (lanes 5,6) of wild-type R27 (lanes 1,3,5) or *an11*⁻ mutant W134 (lanes 2,4,6) petunia flower corolla were separated on an SDS-polyacrylamide gel and AN11 protein was detected by immunoblotting with AN11-specific antiserum. The same filter was subsequently incubated with antiserum raised against F3H (a cytosolic localized enzyme of the anthocyanin biosynthesis pathway) and FBP1 (a nuclear localized MADS box protein).

ylase [Britsch et al. 1991]] and anti-FBP1 antiserum [detects a nuclear localized MADS box protein [Cañas et al. 1994]]. Anti-F3H antiserum recognized a 38-kD protein predominantly present in the cytosolic fraction and also in the microsomal membranes. The anti-FBP1 antibodies recognize the 25-kD FBP1 protein in the nucleus of both wild-type R27 and W134 *an11*⁻ mutant corolla limbs, whereas no protein is detected in the cytosolic protein fraction. The presence of FBP1 protein in the microsomal membrane fraction suggests that this fraction is slightly contaminated with nuclei. These data show that the cytosolic and nuclear fractions have very little cross-contamination. We conclude that the AN11 protein is predominantly present in the cytosol.

AN11 is evolutionary conserved among eukaryotes

For a number of WD-repeat proteins, conserved homologs with similar function have been identified in different species. According to the sequence criteria of Neer et al (1994), none of the WD-repeat proteins in the GenBank and EMBL databases can be considered as an *an11* ortholog. Therefore, we tested whether *an11* homologs exist in other eukaryotic species by Southern blot analysis. Figure 7 shows that *an11* hybridizes to single *Eco*RI or *Hind*III fragments in petunia, maize, *Chlamydomonas*, yeast, *Caenorhabditis elegans*, and human. It would

seem, therefore, that these species contain a single *an11* homologous gene. Three hybridizing fragments were detected in *Arabidopsis*, whereas in tobacco a number of strong and weaker hybridizing bands were observed, indicating the presence of multiple *an11* homologs.

In a search of the entire yeast genomic sequence we found only one gene, which we designated *Scan11*, with high homology to *an11*. The restriction sites surrounding *Scan11* match the sizes of the fragment seen on Southern blots of yeast DNA. This indicates that *Scan11* is the closest *an11* homolog in the yeast genome. Subsequent searches of the National Center of Biotechnology Information (NCBI) database revealed sequences in the genomes of *Arabidopsis*, *C. elegans*, and human with even higher homology to *an11*. In *Arabidopsis*, two expressed sequence tags (ESTs) and one genomic open reading frame (ORF) were identified, consistent with the number of hybridizing fragments on the Southern blot (Fig. 7). The genomic ORF, designated *Atan11-b*, is located on chromosome 3, upstream of the *gapdhA* gene and lacks introns. The cDNA, *Atan11-a*, corresponding to one of the ESTs was fully sequenced. A human cDNA with high homology to *an11* appears in various EST libraries prepared from heart, placenta, liver, spleen, fetal lung, and brain tissue, suggesting that the gene is expressed in a wide variety of tissues. A full-length cDNA, *Hsan11*, was amplified from a cDNA library with primers derived from the ESTs and fully sequenced. A *C. elegans* *an11* homolog, *Cean11*, had been sequenced by the genome consortium and has at least four introns. Because of a lack of homology between the 5' end of the coding sequence of *Cean11* and the other *an11* homologs and the possible presence of an intron in this region of the *Cean11*, we have been unable to locate the translational start of *Cean11*.

Except for *SCAN11*, all AN11 homologous proteins have similar sizes. They all contain five WD-repeats at corresponding positions (Fig. 8A) and the sequence similarity extends across the WD-repeats and the carboxyl terminus. The yeast *SCAN11* protein is almost 200 amino acids larger, mainly caused by the considerably larger spacing between the WD-repeats, and an amino-terminal extension. In pairwise comparisons, the AN11 proteins have 26% (yeast) to 59% (*Arabidopsis*) overall sequence identity with the petunia AN11. A phylogenetic tree of the AN11 homologous amino acid sequences shows that the mammalian and plant sequences are more related to each other, whereas the yeast *SCAN11* appears to represent a separate branch.

To test whether the observed sequence similarities between *an11* homologs reflect functional similarity, we examined whether they could complement the *an11*⁻ mutation in petunia. Therefore, the coding sequences of the petunia, *Arabidopsis*, human, and yeast *an11* homologs were fused to the strong and constitutive 35S promoter from cauliflower mosaic virus (CaMV). To measure the activity of these constructs, we used a luciferase (*luc*) reporter gene fused to the *dfr* promoter. The effector (35S-*an11* homolog fusion) and reporter gene were reintroduced into petunia *an11*-W134 petal cells

Figure 7. The presence of *an11* homologs in different eukaryotic species. Total genomic DNA from different eukaryotes was digested with *Eco*RI (E), *Hind*III (H), *Pst*I (P), or *Sal*I (S), electrophoresed and blotted to Hybond N+. Genomic DNA isolated from yeast, *C. elegans*, and human was digested with *Eco*RI. Blots were probed with the petunia *an11* cDNA and washed with 0.5× SSC at 60°C.

A

B

C

Effector	LUC/GUS	Fold. Act.
none	0.15±0.05	
35S- <i>an11</i>	12.2±6.2	81±41
35S- <i>Atan11-a</i>	1.13±0.67	7.5±4.5
35S- <i>Hsan11</i>	0.82±0.11	5.4±0.7
35S- <i>Scan11</i>	0.12±0.08	1.0±0.5

Figure 8. Comparison of AN11 from plants, mammals and yeast. (A) Alignment of the petunia AN11 sequence with homologs from *Saccharomyces cerevisiae* (SCAN11), *Arabidopsis thaliana* (ATAN11-a and ATAN11-b), *Homo sapiens* (HSAN11), and *Caenorhabditis elegans* (CEAN11). Identical amino acids present in three or more sequences (black boxes). WD-40 domains (underlined). Dots represent gaps for optimal alignment. The sources of the sequences are: AN11, this work; ATAN11-a, this work; ATAN11-b, *Arabidopsis* Genome Consortium; CEAN11, *C. elegans* Genome Consortium cosmid F53C11; SCAN11, Yeast Genome Consortium. (B) Phylogenetic tree of the AN11 homologs. All sequences were truncated to the same length as the CEAN11. The standard errors indicate the validity of the branch points as determined by the algorithm within the UPGMA program utility of GeneWorks (Intel-Genetics). This tree shows potential evolution or functional relationship among the sequences described in A. (C) Complementation of *an11*⁻ mutants flowers by AN11 homologs. *An11*, *Atan11-a*, *Hsan11*, and *Scan11* cDNAs were fused to the 35S promoter and introduced in *an11*-W134 corolla limbs by particle bombardment. Activity was measured with a *dfi-luc* reporter gene and standardised using a *chs-gus* fusion gene to assay transformation efficiency. The values represent the mean of four independent bombardments. Errors shown are the standard error of the mean.

by particle bombardment. Figure 8C shows that cobombardment of the petunia *an11* gene with *dfr-luc* in the *an11*⁻ mutant corolla resulted in strong activation of the *dfr-luc* reporter gene. The level of activation is more than 80-fold over the basal expression. The *Arabidopsis* and human AN11 activated the *dfr-luc* reporter, albeit at a 10-fold lower efficiency, whereas the yeast AN11 homolog was inactive. These results indicate that the human and *Arabidopsis an11* homologous sequences are functional *an11* homologs. The yeast *Scan11* might be too diverged to be functional in plants or not translated sufficiently in plants.

dfr promoter activity is restored in an *an11*⁻ mutant by overexpression of the MYB-type transcription factor AN2

The cytosolic localization and the nature of the AN11 protein make it unlikely that AN11 is a DNA-binding protein, but that it is more likely to exert its action through the putative transcription factors AN1 and/or AN2, resulting in the activation of the anthocyanin biosynthesis pathway. If this is true, one would predict that expression of the AN1 or AN2 protein in the *an11*⁻ mutant corolla would overcome the absence of AN11. To test this hypothesis, *an1* and *an2* cDNAs fused to the CaMV-35S promoter were coexpressed with the *dfr-luc* reporter gene in the *an11*⁻ mutant W134. Table 1 shows that overexpression of *an2* in the *an11*⁻ mutant induced *dfr-luc* activity 12-fold, similar to the activation by the AN11 protein. The AN1 protein was unable to activate the *dfr* promoter (data not shown). The reverse experiment, introduction of 35S-*an11* in *an2*⁻ petals, did not reactivate the *dfr-luc* reporter. These results are consistent with previously published results (Quattrocchio et al. 1993), showing that *dfr* promoter activity in *an11*⁻ petals could be restored (though with low efficiency) by the maize *an2* homolog *c1*, but not by *lc*, encoding the maize bHLH protein. Taken together, these data indicate that AN11 acts upstream of AN2.

Discussion

Over the last few years, a number of regulatory anthocyanin genes have been identified in maize, petunia, and snapdragon. So far, they all encode transcription factors with an MYB or a bHLH domain (for review, see Mol et

al. 1996). Here we show that the regulatory anthocyanin gene of petunia *an11* encodes an evolutionary conserved WD-repeat protein that is primarily localized in the cytosol. In transient expression assays, overexpression of the MYB domain protein AN2 in *an11*⁻ petals can restore the promoter activity of the *dfr* gene, indicating that AN11 acts upstream of AN2. We propose that AN11 is involved in a signal transduction cascade leading to activation of AN2.

an11 encodes a WD-repeat protein that is conserved in nature

WD-repeat proteins represent a family of regulatory proteins that have been implicated in a variety of processes like cell-cycle control, signal transduction, RNA splicing, and vesicular trafficking (Neer et al. 1994). WD-repeat proteins, in general, seem to have a regulatory function; no member of this class of proteins has yet been shown to have catalytic activity (Neer et al. 1994). The WD-repeat structure may facilitate interaction with other proteins, consistent with the observation that many of these proteins are found in multi-protein complexes as shown for the β -subunit of the heterotrimeric G protein (for review, see Neer and Clapham 1988), protein phosphatase A2 (Kamibayashi et al. 1992) and yeast TUP1 (Komachi et al. 1994). Such protein-protein interaction could result in a protein-induced conformational change, leading to activation of the complex. Some WD-40 proteins, like RACK1, are made up entirely of WD-repeats (Ron et al. 1994). Others, including AN11, contain amino-terminal and carboxy-terminal extensions of various lengths, which may contribute to the regulatory function, as shown for the G- β subunit and the phospholipase A2 activating protein (Clark et al. 1991; Garritsen et al. 1993).

Apart from the relatively loose WD-repeat consensus sequence, no significant sequence homology has been found between AN11 and any other identified WD-repeat protein, indicating that AN11 is a new member of this rapidly expanding family of proteins. Based on three criteria, WD-repeat proteins are grouped into subfamilies that presumably define functional homologs (Neer et al. 1994). First, the proteins must have the same number of WD-repeats. Second, the WD-repeats, at equivalent positions in other subfamily members, should be more similar to each other than to any other repeat. Third, the homology must stretch beyond the repeating units into the amino- and carboxy-terminal extensions. So far, five subfamilies have been identified that meet these requirements: (1) the family of G- β subunits, (2) the nuclear neurogenic Groucho-related proteins, (3) protein phosphatase regulatory subunits, and (4) a group defined by the protein kinase C-binding protein RACK1 (Neer et al. 1994). Recently, a fifth subfamily has been proposed consisting of HIRA and HIR1 repressors of transcription of the histone core genes H2A and H2B (Lamour et al. 1995). The AN11 protein defines a sixth subfamily of functionally related WD-repeat proteins, present in yeast, animals and plants (Figs. 7,8). These proteins sat-

Table 1. Complementation of the *an11*-W134 mutant by AN2

Effector	<i>an11</i> -W134		<i>an2</i> -W115	
	LUC/GUS ^a	fold. act.	LUC/GUS ^a	fold act.
None	0.44 ± 0.44		0.85 ± 0.27	
35S- <i>an2</i>	5.31 ± 2.63	12 ± 5.9	29.0 ± 15.3	34 ± 18
35S- <i>an11</i>	4.88 ± 2.42	11 ± 5.5	1.27 ± 1.21	1.5 ± 1.4

^aRatio of *dfr-luc* activity/*chs-gus* in arbitrary units; mean ± S.E. (n = 4).

isfy the three sequence criteria that identify functional homologs. Functional homology is substantiated by complementation of the petunia *an11* mutation with the human and *Arabidopsis an11* homologs.

Given that AN11 homologs presumably coevolved with yet unknown partners, it is remarkable that proteins from distant species are apparently still capable of recognizing the petunia AN11 partner. Therefore, it is not surprising that the most diverged AN11 homolog, SCAN11 from yeast, does not complement the *an11*⁻ mutation, as it may simply fail to complex with the petunia AN11 partner.

Role of AN11 in the regulation of anthocyanin biosynthesis

Mutations at the *an11* locus affect two seemingly unrelated processes: transcriptional inactivation of the anthocyanin-specific genes resulting in a loss of pigmentation, and the control of intracellular pH in petal cells (Quattrocchio et al. 1993; 1994). Strikingly, the *an11* gene is expressed in a wide variety of tissues including tissues that are not anthocyanin pigmented. Thus, *an11* is unlikely to determine the pattern of pigmentation. Because no obvious phenotypic alterations are seen in leaves, stem, and roots, the function of *an11* in these tissues, if any, is unclear. It seems unlikely that *an11* function is redundant in these tissues as genomic DNA gel blots indicate that *an11* is a single copy gene.

Mutations at the *an1* and *an2* locus have similar effects, although *an2*⁻ mutations only affect pigmentation of the corolla. The products of these genes share homology with MYB and bHLH proteins indicating that they are putative transcription activators. Because AN11 is predominantly localized to the cytoplasm, it is unlikely to be part of a transcription complex. This, together with the observation that *dfr* promoter activity in *an11*⁻ petals could be restored by overexpressing the AN2 protein, suggests that AN11 acts upstream of the putative transcription factor AN2.

We can envisage a number of scenarios to explain the restoration of *dfr* promoter activity. The simplest explanation, the transcriptional regulation of *an2* by AN11, can be excluded, because the levels of *an2* mRNA in wild-type and *an11*⁻ flowers are similar (Quattrocchio 1994). Thus, AN11 is more likely to be involved in the post-translational control of AN2, either by modifying the AN2 activity or its cellular localization. For instance, an increased level of AN2 may simply compensate for a lower DNA-binding affinity or transcription activation potential. It has been shown for plant and animal MYB-domain proteins that phosphorylation reduces DNA-binding activity (Lüscher et al. 1990; Moyano et al. 1996) and in addition a WD-repeat protein has been identified as the regulatory subunit of a protein phosphatase (Mayer et al. 1991). Alternatively, AN11 could have a role in nuclear transport of AN2. The hydrophilic nature and the cytosolic localization of AN11 argue against the notion that AN11 functions as a receptor for AN2. It is possible, however, that AN11 maintains AN2 in, or con-

verts it into, an import-competent form. It is noteworthy in this respect that a WD-repeat protein, PAS7, has been shown to be involved in the import of thiolase into peroxisomes (Marzioch et al. 1994). Post-translational regulation of AN2 by AN11 could explain the substantial time lag between the appearance of *an2* mRNA and the induction of the *dfr* structural gene during petal development (F. Quattrocchio, J. Wing, K. van der Woude, J. Mol, and R. Koes, in prep.). The timing of *dfr* induction is, however, closely correlated with the appearance of *an11* mRNA (Fig. 4).

Conservation of AN11 during evolution

The different flavonoid end products serve a wide variety of biological functions and their distribution through the plant kingdom indicates that they appeared sequentially during evolution, thus the appearance of the structural genes and their linking to regulatory networks also occurred stepwise (Koes et al. 1994). On the basis of sequence homologies, it was argued that structural flavonoid genes originated from genes encoding enzymes of primary metabolism (Stafford 1991). There is very little useful information, however, to deduce how and when the regulatory genes appeared.

The best information on the origin of regulatory flavonoid genes that has been obtained so far comes from expression analysis of the *chs* gene(s). Only in legumes, *chs* genes are expressed in the root tip where they are involved in the synthesis of flavonoids that serve as signaling molecules for nodulating Rhizobia. In a non-legume host, tobacco, the bean *chs* promoter is active in the root tip even though the endogenous *chs* genes are not (Schmid et al. 1990). This indicates that the regulatory networks are more widespread and evolutionary older than the biological function of the flavonoids. One interpretation is that newly appeared or pre-existing structural genes are simply linked to pre-existing regulators (with, e.g., a useful expression pattern) to establish new expression patterns and biological functions for flavonoid biosynthetic genes. Our observation that the *an11* gene is highly conserved in nature, even in organisms that can not synthesize anthocyanins, supports this notion. Before the anthocyanin pathway appeared, *an11* presumably controlled an old set of target genes. It is unclear if these old target genes are still functional in a higher plant, and in which process they are involved. In this light, it will be interesting to elucidate the biological functions of the *an11* homologs in species other than higher plants, for instance, by gene replacement of *Scan11* in yeast.

Materials and methods

Plant material

The *an11*-W137 allele arose spontaneously in the progeny of the unstable *an1* petunia line W138 (Doodeman et al. 1984). The lines W138 (harboring the unstable *an1*-W138 allele) and W137 (harboring the unstable *an11*-W137 allele) resulted from *dTph1* insertions in the background of the wild-type line R27. Full revertants (*An11*⁺-T152, *An11*⁺-T154 and *An11*⁺-T152),

partial revertants (*an11*-W2038 and *an11*-W2053) and stable null alleles (*an11*-W134, *an11*-T150, *an11*-T155, and *an11*-X2197) were isolated from selfed progeny of independent *an11*-W137 homozygous plants. The unstable *an11*-G5543 allele was identified in progeny of a cross between *an11*-W137 and *an11*-W134 homozygotes on the basis of a novel spotting pattern in the flower, and made homozygous by further selfings. Excision of the transposon from *an11*-G5543 resulted in the partial revertant allele *an11*-Z2297. The line W115 contains stable recessive alleles at the *an2* and *an4* locus. All plants were kept in a normal greenhouse.

Isolation and DNA sequencing of *an11* cDNA and genomic clones

Genomic DNA from young petunia leaves was isolated by a standard procedure (Dellaporta et al. 1983) with modifications (Souer et al. 1995). DNA prepared from the *an11* mutable W137 line was used to isolate mutant specific *dTph1*-flanking sequences as described (Souer et al. 1995). Briefly, 2 µg of DNA from an *an11*-W137 homozygous plant (plant T2141-10), a pool of siblings homozygous for the *An11*-T2141 revertant allele and a more distantly related family (V2060) of *an11*-W137 homozygotes was digested with *RsaI*, *HaeIII*, or *HincII*/*EcoRV* and recircularized by overnight ligation. The first round of amplification was performed with the *dTph1*-specific primers out5 (5'-dCCCTTATTAGAATTCTTGGCTCCGC-3') and out6 (5'-dCCTTTGCACCAAGGAGCTCCGCC-3') with amplification conditions: 45 sec at 94°C, 45 sec at 65°C, and 1.5 min at 72°C. One-half microliter of amplification product was used for nested PCR with the *dTph1*-specific primer out1 as described (Souer et al. 1995). Inverse PCR products from the *an11*-W137 homozygous plant T2141-10 were digested with *EcoRI* and ligated in M13mp18 vector. Duplicate filters were lifted and hybridized with ³²P-labeled inverse PCR product obtained from the V2060 family (+ probe) the *An11*-T2141 revertants (- probe). Differentially hybridizing plaques were picked, classified in cross-hybridization groups, and their inserts hybridized to dot blots containing inverse PCR products isolated from a number of plants segregating for *An11*-T2141 and *an11*-W137. Two identical clones R15 and R24 were identified that are part of the *an11* locus.

A corolla V26 cDNA library (100,000 PFU) was hybridized with the R24 insert, resulting in fourteen positive plaques. The longest insert was purified, subcloned in pBluescript KS, and sequenced. The 5'-flanking region of the *an11* gene was isolated by inverse PCR. One microgram of V26 genomic DNA was digested with *Bst*YI and ligated overnight in 300 µl at 16°C. For the first round of amplification, 200 ng of ligated DNA was used and the primers: *an11*-fw (5'-dGCTCTAGAATTAAACAACCGGTC-3') and *an11*-race (5'-dAAGGTACCCACGGCAATGCGGCG-3'). PCR conditions were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C by use of Vent polymerase. Part of this reaction (0.5 µl) was reamplified with the *an11*-fw and *an11*-race2 primer (5'-dGCGGATCCATGGAGTAAATTGGGTAAG-3'). The PCR products were digested with *Bam*HI and *Xba*I and cloned in pBluescript-KS.

Transposon insertion and excision alleles were amplified by PCR with the primers *an11*-fw and *an11*-rev (5'-dC-GAATTCTTTAACTTCCCATAAACG-3'), digested with *EcoRI* and *Xba*I, cloned into pBluescript KS and sequenced.

By use of EST databases available at the NCBI, *Arabidopsis* and human *an11* homologs were identified. The *Arabidopsis an11* homolog *Atan11-a* was identified by the EST sequences R90717 and H35958. The cDNAs, obtained from Dr. T. Newman, were sequenced completely on both strands. A number of

ESTs corresponding to a human *an11* homolog were identified. Based on the ESTs W07303 and N78624 primers were designed (5'-CCATAGATCTCAGGCTCG-3' and 5'-TAGGTACCAACACTACTCTGAG-3') and the cDNA amplified from a liver cDNA library in λgt11 (Clontech). The products were cloned and sequenced.

Nested deletions of cDNA and genomic clones were sequenced by the dideoxy chain termination method with fluorescent M13 primers, employing an Applied Biosystems DNA sequencer model 370A.

RNA analysis

RNA isolation and blot analysis were performed according to van Tunen et al. (1988). Transcription run-on assays were performed as described previously by van Blokland et al. (1994).

Expression of AN11 protein in Escherichia coli and antibody production

The *an11* cDNA fragment between an *NcoI* site at the in frame ATG codon, Met-28, and a *HindIII* site following the stop codon was cloned into pRSET B (InVitrogen). This construct was transformed into BL21 (DE3) and cultures were grown at 37°C in Luria broth with 50 µg/ml carbenicillin to an *A*₆₀₀ nm of 0.5. Then isopropylthio-β-galactoside was added to a final concentration of 1 mM, and cells were grown for an additional 3 hr. Induced cells were harvested by centrifugation and resuspended in binding buffer (100 mM NaH₂PO₄, 6 M urea, 10 mM Tris-HCl) at pH 8.0. After 1 hr at room temperature, the lysate was centrifuged at 10,000g for 15 min to remove debris. Soluble protein was loaded on a Ni-NTA resin column (Qiagen) and washed extensively with binding buffer at pH 8.0. The column was then washed with binding buffer at pH 6.3 and proteins were eluted with binding buffer at pH 4.5. The presence of the 40-kD AN11 protein in the eluate fraction was confirmed by SDS-PAGE.

For polyclonal antibody generation, AN11 protein was electrophoresed through a 12% polyacrylamide SDS gel, and electrotransferred to nitrocellulose (Schleicher & Schuell). The filter was stained with amido black for 1 min and destained with 10% acetic acid, 25% methanol. The AN11 band was cut out, ground to a fine powder, and resuspended in a 0.9% NaCl solution. A Swiss female mouse was immunized with approximately 75 µg of protein. Two booster injections were given with the same amount of protein every 3 weeks. After an additional 7 days, the serum was collected.

Immunoblot analysis

Total protein extracts were prepared, and protein concentration determined, as described previously (de Vetten et al. 1992). For subcellular protein fraction, 20 grams of frozen corolla tissue of wild-type R27 and *an11*-W134 was ground in liquid N₂ and the powder was resuspended in 40 ml of ice-cold buffer (10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 250 mM sucrose). The suspension was passed through a Miracloth filter and was centrifuged for 10 min at 5,000g. The pellets of nuclei were further purified on a Percoll gradient as described by van Blokland et al. (1994). The cytosolic fraction was obtained by centrifuging the postnuclei extract for 30 min at 100,000g. The pellet obtained after the 100,000g centrifugation was designated the microsomal membrane fraction. Identical amounts of cytosolic, microsomal membrane, and nuclear protein (50 µg) were electrophoresed on a 12.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Filters were blocked for 2 hr in 1% BSA in TBST buffer (10 mM Tris-

HCl at pH8.0, 150 mM NaCl, 0.05% Tween 20, 0.1% BSA) and incubated with 1:2000 dilution of mouse anti-AN11 antiserum for 1 hr. After three washes with TBST, the filters were incubated with a 1:5000 of goat anti-mouse horseradish peroxidase-conjugated IgG. After washing the filters with TBST, immunodetection was performed with an enhanced chemiluminescence western blotting detection reagent (Amersham).

Plasmid constructions used for transient expression experiments

The plasmid pANU6 was a gift of Dr. T. Teeri (University of Helsinki, Finland), having the CaMV 35S promoter fused upstream of the luciferase gene and the nopaline synthase polyadenylation signal. The *dfr-luc* vector was constructed by replacing the CaMV promoter with the 1800-bp petunia *dfr* promoter (Huits et al. 1994). The *chsA*-GUS construct used was as described by Koes et al. (1990). The 35S-*an11* plasmid was derived from PIP114, which includes the CaMV 35S promoter and the 3' sequence of the nopaline synthase gene. The petunia *an11* cDNA was amplified with the primers *an11*-ATG (5'-dGAG-GATCCATGGAAAATTCAAGTCAAGTC-3') and *an11*-trail (5'-dAAACAAGAAGCAGCAGGATCC-3') and cloned into the *Bam*HI site of PIP114, resulting in plasmid 35S-*an11*. The *Arabidopsis* cDNA of *Atan11-a* was amplified from the λ ZipLox plasmid 172B4 by use of the T7 promoter primer and *AtAn11Kpn* (5'-dTAGGTACCCCTAATACGATAGAAAATG-3'), digested with *Kpn*I and ligated in PIP114 to yield 35S-*Atan11*.

The yeast *an11* homolog, *Scan11* was amplified from yeast genomic DNA with the primers Yan01 (5'-dATGAATTCAAATGGATCCCTTTTAC-3') and Yan02 (5'-dTGTAAAGCTTCTCAAAGGACGCGGACG-3') digested with *Eco*RI and *Hind*III and cloned into PIP114, resulting in 35S-*Scan11*. The human *an11* homolog, *Hsan11*, was amplified from a human liver λ gt11 library with the primers HAN11B5 (5'-CCATAGATCTCAGGCTCG-3') and Human Trail (5'-dTAGGTACCAACTACACTCTCAG-3') and cloned in the *Sma*I site of PIP114 to yield 35S-*Hsan11*. For all these PCR amplifications Vent polymerase was used and all constructs were partially sequenced.

Particle bombardment and gene expression assays

Particle bombardments were performed essentially as previously described (Quattrocchio et al. 1993). After one day incubation at 25°C, protein extracts were made by grinding the corolla in 400 μ l 50 mM NaPi at pH 7.0, 2 mM EDTA, 20 mM DTT and 4% PVP. Luciferase activity was measured with the Luciferase Assay System (Promega Corp., WI), and GUS activity was measured as described (Koes et al. 1990). Data are expressed as the ratio of arbitrary light units to arbitrary units of fluorescence.

Acknowledgments

We thank Dr. E. Grotewold for critical comments on the manuscript. We thank Drs. L. Britsch and G. Angenent for the kind gift of anti-F3H and anti-FBP1 antiserum, Drs. H. van Luenen, M. Haring, and A. Schinkel for providing DNA samples, Drs. T. Teeri and T. Newman for providing plasmids, and M. de Haas and Dr. P. Borst for providing a liver cDNA library. Thanks are also due to P. Hoogeveen and M. Meesters for their care of the plants. N.d.V. is supported by a grant from the EU-BIOTECH Research program (grant BIO2 CT93 0101), F.Q. is supported by the Netherlands Organization for Chemical Research (SON).

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Note added in proof

The sequence data described in this paper have been submitted to GenBank under accession nos. U94748 (*an11*), U94747 (*Hsan11*), and U94746 (*Atan11-a*).

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The an11 locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals.

N de Vetten, F Quattrocchio, J Mol, et al.

Genes Dev. 1997, **11**:

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