Molecular Heterogeneity of the Chalcone Synthase Intron in *Petunia*

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Abstract. A method was developed to characterize the genetic heterogeneity of the chalcone synthase gene intron within the *Petunia integrifolia* (Hook.) Schinz & Thell. species complex. The DNA from wild species collected from known locations was used to amplify the chalcone synthase gene intron through the polymerase chain reaction (PCR). The resulting PCR product was then characterized by Rsa I restriction, revealing a degree of heterogeneity that could be used to characterize the species genetically. Of the four different species that were characterized, two could be placed in the same genetic grouping. This study shows that the variation in the intron of the Chs A gene may be species-specific.

The genus *Petunia* was established by Jussieu (1803). The first monograph on this genus was written by Fries (1911), who separated the genus into two subgenera, *Pseudonicotianiana* and *Eupetunia*. Species within the *Pseudonicotianiana* subgenus had long and narrow corolla tubes, while the *Eupetunia* species had short, wide corolla tubes. The garden petunia, *Petunia ×hybrida* Hort. Vilm., is a complex hybrid of *P. integrifolia* (Hook.) Schinz & Thell. (*Eupetunia*) and *P. axillaris* Lindl. (*Pseudonicotianiana*) that originated about 1825 (Sink, 1984). Fries recognized three distinct *Eupetunia* species (*P. violacea* Lindl., *P. inflata* Fries, and *P. occidentalis* Fries). *Petunia violacea* had the largest flowers and pendant pedicles, while *P. occidentalis* had the smallest flowers and erect pedicles. *Petunia inflata* was intermediate between *P. violacea* and *P. occidentalis*. In addition, Fries recognized a diminutive subspecies of *P. violacea* (*P. violacea* subsp. *depauperata* Fries) with very small flowers and leaves.

There has been considerable confusion as to the taxonomic relationships between these *Eupetunia* species. Schinz and Thellung (1915) discovered that the plant previously described by Hooker (1831) as *Salpiglossis integrifolia* Hooker was actually a *Petunia* species (*P. integrifolia*). Subsequently, Smith and Downs (1966) recognized that *P. integrifolia* and *P. violacea* were identical. Because the description of *P. integrifolia* predated that of *P. violacea*, *P. integrifolia* was the correct name for the species.

Wijsman (1982) studied multiple ecotypes of *P. integrifolia* and discovered that both

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flower size and pedicel position were correlated with geographical distribution. The more western ecotypes of Brazil had smaller flowers and more erect pedicles. He concluded that *P. integrifolia*, *P. inflata*, and *P. occidentalis* were not distinct species, but were subspecies of a single, broadly defined species [i.e., *P. integrifolia* subsp. *integrifolia* (Fries) Wijsman, *P. integrifolia* subsp. *inflata* (Fries) Wijsman, and *P. integrifolia* subsp. *occidentalis* (Fries) Wijsman].

Not all taxonomists agree with Wijsman, and some have given ecotypes in the *P. integrifolia* complex independent species status: *P. scheideana* Smith & Downs (Smith and Downs, 1964), *P. littoralis* Smith & Downs (Smith and Downs, 1966), *P. bonjardinensis* Ando & Hash. (Ando and Hashimoto, 1993), *P. mantiiqueirensis* Ando & Hash. (Ando and Hashimoto, 1994), *P. guaraunaensis* Ando & Hash. (Ando and Hashimoto, 1995), *P. interior* Ando & Hash. (Ando and Hashimoto, 1996), *P. bajensis* Ando & Hash. and *P. riograndensis* Ando & Hash. (Ando and Hashimoto, 1998), and *P. occidentalis* Tsuk. (Tsukamoto et al., 1998). Wijsman believes that these ecotypes should have been described as subspecies of *P. integrifolia* (personal communication).

Several molecular marker studies of *Petunia* have been made using species-specific repeated DNA (Shepherd et al., 1990), rDNA (Kabbaj et al., 1995), and randomly amplified DNA (Cerny et al., 1996). However, in all of these studies, the species actually used is uncertain. For example, phylogenetic analysis using rDNA suggested that *P. integrifolia* was very distantly related to *P. violacea* (Kabbaj et al., 1995), but these are the same species! Furthermore, the complex taxonomy of *Petunia* has caused many cultivated species to be misidentified. For example, we have received from a botanical garden in Eu-

**Material and Methods**

**Plant material.** Two accessions, each from two different species, *P. integrifolia* ssp. *depauperata* ‘2’ (Longwood Gardens 77320), *P. integrifolia* ssp. *depauperata* ‘3’ (30°31’14” south, 53°27’09” west), *P. altiplana* ‘7’ (29°24’32” south, 50°26’57” west) and *P. altiplana* ‘8’ (28°12’37” south, 49°47’17” west), and a single accession of an unknown species *P. sp.* ‘Torres’ (29°21’26” south, 49°44’06” west) were analyzed. All but the first were collected from the wild, and each was from a genetically distinct population. We also analyzed two cultivated *Petunia* hybrids, *P. ×hybrida* ‘Magic White’ (Pan American Seed, West Chicago, Ill.) and *P. ×hybrida* ‘Prime Time Blue’ (Goldsmith Seed, Gilroy, Calif.), from genetically distinct breeding programs. Potted plants were grown under standard greenhouse conditions in Beltsville, Md., and fertilized weekly with a solution of 20N–8.7P–16.6K fertilizer at 200 mg L⁻¹ nitrogen.

**DNA isolation.** Plants were placed in the dark 24 h before DNA extraction in order to reduce their polysaccharide content. DNA was extracted by grinding young fully expanded leaves (100 mg fresh weight) in liquid nitrogen and isolated using the Nucleon PhytoPure Plant DNA Extraction Kit (Vector Laboratories, Burlingame, Calif.) as recommended by the manufacturer.

The DNA was further purified from polysaccharides by elution from DEAE-cellulose (Marechal-Drouard et al., 1995). The DEAE-cellulose (1.5 g) was resuspended in 20 mL of elution buffer (2 M NaCl, 1 mM EDTA, and 10 mM TRIS, pH 7.5), washed several times in the same buffer, and then in wash buffer (400 mM NaCl, 1 mM EDTA, and 10 mM TRIS, pH 7.5), and finally resuspended in 5 mL of wash buffer. The DNA pellets from four 100-mg extractions were combined, dissolved in 100 µL of TE (100 mM TRIS, pH 8.0, and 1 mM EDTA), and thoroughly mixed with 100 µL of DEAE-cellulose. The suspension was centrifuged at 3000 g, for 30 s, the supernatant discarded, and DEAE-bound DNA washed several times with wash buffer. The DNA was released from the DEAE by suspension in 500 µL of elution buffer. The eluted DNA was precipitated with an equal volume of isopropanol, washed with 70% ethanol, and resuspended in 50 µL of TE.

**PCR amplification.** The DNA sequences of the chalcone synthase genes (Chs) from both *P. ×hybrida* ‘V30’ (Koes et al., 1989) and *P. ×hybrida* ‘Roter Traum’ (Niesbach-Kløsgen et al., 1987) have been reported. The sequence from *P. ×hybrida* ‘Roter Traum’ was selected for creating primers to amplify the Chs intron. The forward primer sequence (5’-GAGAAATTCAAGCGNATGTG-3’),
designated CHS-1, was selected from the region immediately before the intron. The reverse primer sequence (5′-AACCTGCTGTCATCAGT-3′), designated CHS-4, was selected from a transcribed region of the gene 312 bp downstream from the intron. The sequences complementary to CHS-4 are very highly conserved between unrelated species in different genera (Niesbach-Klösgen et al., 1987).

The PCR reactions were performed in a Perkin Elmer DNA Thermal Cycler Version 2.3 (Perkin-Elmer Corp., Norwalk, Conn.). The reaction mix (100 µL) contained 1 µL genomic DNA (0.1 mg·mL⁻¹), 1 µL AmpliTaq Gold DNA polymerase (PE Applied Biosystems, San Jose, Calif.), 10 µL of 10X buffer (500 mM KCl and 150 mM Tris, pH 8.0), 8 µL mixed dNTPs (each at 10 mM), 25 µL 10 mM MgCl₂, 5 µL of 20 µM CHS-1 primer, 5 µL of 20 µM CHS-4 primer, and 45 µL of water. Each reaction mixture was overlaid with 25 µL of mineral oil and was preheated at 95 °C for 12 min. Following a 2-min incubation at 92 °C and 40 cycles of 92 °C (30 s), 60 °C (2 min), and 72 °C (1 min), the reaction was terminated with a 72 °C 10-min incubation and held at 5 °C.

Restriction analysis. Analysis of the published sequence of the CHS intron in P. ×hybrida ‘V30’ (Koes et al., 1989) revealed that only the PCR products and restriction fragments were resolved by gel electrophoresis (50-V constant voltage) in 4% AmpliSize Agarose (Bio-Rad, Hercules, Calif.) in TAE. Gels were stained in 0.5 µg·mL⁻¹ ethidium bromide for 45 min. The AlphaEase image analysis system (AlphaInnotech Corp., San Leandro, Calif.) was used to digitally record the resulting images and to determine molecular weights.

From the published DNA sequence of the CHS intron in P. ×hybrida ‘V30’ (Koes et al., 1989), fragments <100 bp were expected. However, the molecular weight of these small fragments could not be accurately determined and were not included in the analysis.

Results and Discussion

Chalcone synthase is the key enzyme of the anthocyanin biosynthetic pathway, and cDNAs of this gene have been isolated and sequenced from many species in isolated families. Comparison of the coding region of the CHS gene in these species revealed a 66% nucleotide similarity (Niesbach-Klösgen et al., 1987). In P. ×hybrida ‘V30’, chalcone synthase is part of a multigene family with eight complete (CHS A, B, D, F, G, H, J, and L) and four incomplete (CHS C, E, I, and K) copies of the CHS gene per haploid genome (Koes et al., 1989). The CHS A gene is the only CHS gene transcribed to a significant extent in flower tissue. The CHS A, B, D, and F genes are located on chromosome V, whereas the CHS G and L genes are located tightly linked together on chromosome II. Each complete CHS gene consists of two exons separated by an intron of variable size and sequence (Koes et al., 1989). The lengths of the introns in CHS A, B, D, F, G, H, J, and L are, respectively, 1346, 3776, 694, 563, 2438, 406, 728, and 123 bp. Homologous genes for each of the complete CHS genes in P. ×hybrida ‘V30’ have been found in species identified as P. axillaris, P. parodii Smith & Downs, P. inflata, and P. violacea (Koes et al., 1987). The incomplete CHS genes do not contain an intron.

Niesbach-Klösgen et al. (1987) did not report the number of CHS genes in P. ×hybrida ‘Roter Traum’ or the size of their introns. Only a single DNA sequence was published. From this sequence, primers were designed to amplify the intron plus an additional 312 bp of the gene. Because of the small difference between the CHS A, B, D, F, G, and J gene sequences in P. ×hybrida ‘V30’, we expected the primers to amplify more than one of the CHS genes (Fig. 1). However, in all but two accesses a single PCR product was obtained (Table 1; Fig. 2).

The PCR products in P. ×hybrida ‘Prime Time Blue’ and ‘Magic White’ were the same length (1275 bp) and did not match the length of any of the P. ×hybrida ‘V30’ CHS genes (Table 1). Rsa I digestion of these products resulted in three fragments >100 bp in length at 615, 390, and 280 bp. These fragments did not correspond in length to any of the published sequences of the CHS genes (Table 1). We are confident that our primers amplified the CHS A gene because the sequence of the 234 nucleotides from the 3′ end of the PCR product differed from the reported CHS A gene sequence by only a single nucleotide (data not shown).

The lengths of the PCR product (1275 bp) and Rsa I restriction fragments (615, 390, and 280 bp) of P. ×hybrida ‘Prime Time Blue’ and

| Species                  | hybrida | integerrifolia | altiplana 7 | altiplana 8 | 'Torres' |
|--------------------------|---------|----------------|-------------|-------------|---------|
| hybrida                  | 1275    | 1220           | 1220/1185   | 1320        | 1678    | 4105   | 1016   | 894   | 2770   | 1060   |
| altiplana 7              | 615     | 825            | 825/785     | 710         | 718     | 1316   | 677    | 794   | 1249   | 357    |
| altiplana 8              | 390     | 165            | 165         | 280         | 357     | 705    | 185    | 697   | 278    |
| 'Torres'                 | 280     | 190            | 273         | 528         | 154     | 273    | 100    |       |        |        |
| CHS A                    |         |                |             |             | 116     | 351    | 273    | 101   |
| CHS B                    |         |                |             |             | 108     | 291    | 180    | 139   | 104    |

Table 1. The lengths (bp) of the Rsa I restriction fragments of the PCR products containing the intron of chalcone synthase. Only fragments over 100 bp are reported. The PCR primers were not expected to amplify the intron in CHS H or L. The first line is the length of the unrestricted PCR product. In P. altiplana 7 and 8, there were two unrestricted products. In P. altiplana 8, the 1220 bp PCR product produced a 825 bp fragment, while the 1185 bp PCR product produced a 785 bp fragment. The specific CHS gene fragments were calculated from the sequences reported in the literature for P. ×hybrida ‘V30’ (Koes et al., 1989). The accession number of the CHS genes were: CHS A, X14591; CHS D, X14596; CHS E, X14597; CHS F, X14598; CHS G, X14599; and CHS B, X14592.
‘Magic White’ were identical and differed from that of either P. altiplana or P. integrifolia ssp. depauperata (Fig. 2; Table 1). Both P. integrifolia ssp. depauperata accessions produced a single PCR product of the same length (1220 bp) and two Rsa I restriction fragments of the same length (825 and 165 bp). Both P. altiplana accessions produced two PCR products, one of which was of the same length (1220 bp) as the P. integrifolia product. In P. altiplana ‘7’, the other PCR product contained 225 bp and was not digested by Rsa I, whereas in P. altiplana ‘8’, it contained 1185 bp and was digested by Rsa I, producing a 785 bp fragment.

The Rsa I restriction data suggest that P. altiplana and P. integrifolia ssp. depauperata are genetically more similar to each other than to P. x hybrida. This was not unexpected because Petunia altiplana was separated from P. integrifolia ssp. depauperata based upon its procumbent leaves and ability to form adventitious roots on its shoots (Ando and Hashimoto, 1993). Both Smith and Downs (1966) and Wijsman (1982) collected P. altiplana but did not consider it a separate species distinct from P. integrifolia ssp. depauperata.

The unknown species P. sp. ‘Torres’ is morphologically distinct from either P. x hybrida, P. altiplana, or P. integrifolia ssp. depauperata, with round, glabrous, and succulent leaves. The Rsa I banding pattern of P. sp. ‘Torres’ was clearly different from those of either P. x hybrida or P. integrifolia/P. altiplana.

This study shows that variation exists in the intron of the Chs A gene, and that this variation may be species-specific. Of the four different species that were characterized, two could be placed in the same genetic grouping.

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