Transactions of the Illinois State Academy of Science
(1998), Volume 91, 1 and 2, pp.1-11

received 8/18/97
accepted 10/30/97

The Intron in Chloroplast Gene *rpl*16 is Missing From the Flowering Plant Families Geraniaceae, Goodeniaceae, and Plumbaginaceae.

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ABSTRACT

Previous studies have shown that in the vast majority of land plants examined to date, the chloroplast gene *rpl*16 is interrupted by an intron (of about 1 kilobase in size in most angiosperms), but that in *Limonium gmelinii* (Plumbaginaceae) and Geraniaceae sensu stricto the intron has been lost. In order to uncover other instances of intron loss, the complete *rpl*16 intron and flanking DNA regions from over 210 species, representing 86 families of angiosperms, were amplified using the polymerase chain reaction (PCR) technique. We report the widespread distribution of the *rpl*16 intron in angiosperm chloroplast DNAs, and confirm that the intron is missing from the chloroplast genomes of *Erodium chamaedryoides* and *Pelargonium ∗ hortorum* (the only two species of Geraniaceae included in our investigation), and from three of the four examined genera of Plumbaginaceae (*Armeria*, *Goniolimon*, and *Limonium*, but not *Plumbago*). Furthermore, we report that the intron is missing from *Goodenia ovata* and *Scaevola sericea*, the only representatives examined from the family Goodeniaceae. DNA sequencing of the *rpl*16 intron region in representatives of the seven genera lacking the intron confirms its absence and shows that the intron has been precisely removed from the gene along established exon/intron splice sites. Based upon available phylogenetic information and distribution of intron loss, we conclude that this rare genomic structural mutation has occurred independently at least three times during the evolution of flowering plants.

INTRODUCTION

The chloroplast gene *rpl*16, encoding the ribosomal protein L16, is interrupted by an intron in many, but not all, land plants. DNA sequencing has revealed that this intron is present in plants spanning diverse evolutionary lineages, such as in the liverwort *Marchantia polymorpha* (Ohyama et al., 1986), the gymnosperm black pine (*Pinus thunbergii*; Wakasugi et al., 1994), the dicot tobacco (*Nicotiana tabacum*; Shinozaki et al., 1986), and the monocots duckweed (*Spirodela oligorhiza*; Posno et al., 1986; Jordan et al., 1996) and corn (*Zea mays*; McLaughlin and Larrinua, 1987). Among these species, the intron varies considerably in length, from 536 base pairs (bp) in *Marchantia* to 1,411 bp in duckweed. In most angiosperms, the intron is about 1 kb (kilobase) in

size. Based on heterologous filter hybridization experiments using an *rpl*16 intron-specific probe from tobacco chloroplast DNA (cpDNA), the *rpl*16 gene was deemed to lack an intron in *Limonium gmelinii* (Plumbaginaceae; Downie and Palmer, 1992, 1994) and in all examined representatives of the family Geraniaceae (S. Downie, J. Logsdon, Jr., and J. Palmer, in Downie and Palmer, 1992).

Owing to the conservative nature of chloroplast genome evolution among photosynthetic angiosperms, particularly with regard to its gene and intron content, major structural rearrangements are relatively rare events (Palmer, 1991; Downie and Palmer, 1992). Because of their infrequent occurrence, these structural mutations usually can provide strong evidence of common ancestry (monophyly). Here we present the results of a PCR survey constructed to detect the presence or absence of the intron in chloroplast gene *rpl*16 across a broad representation of angiosperm species. Sequence analysis was used to confirm the absence of the intron when indicated by the PCR results. We report that the *rpl*16 intron is a highly stable component of angiosperm chloroplast genomes, being absent from very few taxa.

MATERIALS AND METHODS

Over two hundred and ten species from 86 angiosperm families, representing members of all six subclasses of dicots and all five subclasses of monocots (sensu Cronquist, 1981), were surveyed for the presence or absence of the *rpl*16 intron (Appendix 1). Included here, as controls, were tobacco (*Nicotiana tabacum*), previously reported to contain the intron (Shinozaki et al., 1986), and geranium (*Pelargonium ∗ hortorum*), previously reported not to contain the intron (S. Downie, J. Logsdon, Jr., and J. Palmer, in Downie and Palmer, 1992). Total genomic DNAs were isolated from fresh leaf or herbarium materials using the modified CTAB procedure of Doyle and Doyle (1987). Fresh leaves were obtained from the field or from plants cultivated in the University of Illinois greenhouse facilities. For some species, DNAs were supplied to us directly.

For each genomic DNA, the entire *rpl*16 intron (if present) and portions of its flanking exons and intergenic spacer regions were PCR-amplified using the pair of primers illustrated in Fig. 1. Primers were designed by comparing *rpl*16 exon 2 or *rps*3 sequences from tobacco, spinach, *Epifagus*, rice, corn, and *Marchantia* and choosing regions highly conserved among these taxa. In tobacco cpDNA, the *rpl*16 intron is 1,020 base pairs (bp) in size, the 3' end of the forward primer is 377 bp upstream from the exon 1/intron junction, and the 3' end of the reverse primer is 18 bp downstream from the intron/exon 2 junction (Shinozaki et al., 1986). The primers were synthesized by Operon Technologies, Inc. (Alameda, CA). Details of the amplification reactions, and the DNA purification and sequencing strategies used, were the same as outlined in Downie and Katz-Downie (1996) with the only exception being a reduction in the volume of each reaction (25 μl instead of 100 μl) in the PCR survey. The ensuing PCR fragments were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and sized against *Eco*RI/*Hin*dIII-digested lambda DNA standards. Each set of reactions was monitored by the inclusion of positive (tobacco and geranium cpDNAs, each with and without the intron, respectively) and negative (no template) controls. Successful PCR amplifications resulted in a single DNA band of about 1,400 bp when the intron was present (Fig. 1A), or about 400 bp when the intron was absent (Fig. 1B). To confirm the

suspected loss and precise excision of the *rpl*16 intron, the small-sized (i.e., 400 bp) PCR fragments were sequenced using the *rpl*16 exon 2 primer.

RESULTS AND DISCUSSION

Our survey revealed two major size categories of PCR products—one corresponding to the presence of the *rpl*16 intron (1,400 bp) and the other corresponding to its loss (400 bp). For those species possessing the intron, detectable size variation was evident in only ten species, differing from the tobacco PCR fragment by about 200 bp or less. As discussed in Doyle et al. (1995), most of the group II introns present in cpDNA (such as the *rpl*16 intron) are within a few hundred bp of the minimum size (ca. 500 bp) required for intron splicing. Thus, other than the loss of the entire intron itself, major deletions within the intron are not expected.

Our PCR experiments indicated that most of the 210 angiosperms examined contained an intron in chloroplast gene *rpl*16. Introns are highly conserved elements of land plant chloroplast genomes so it is not surprising that we detected the *rpl*16 intron in all but three of the 86 families surveyed. A previous investigation, encompassing 88 species (36 families) from subclass Asteridae and an additional 123 species from all other subclasses of monocots and dicots, revealed the near ubiquity of the *rpl*16 intron in angiosperm cpDNAs when assayed using an intron-specific probe (Downie and Palmer, 1992).

On the basis of our PCR experiments, the intron was inferred to be absent in both examined species of Geraniaceae (*Erodium chamaedryoides* and *Pelargonium* ∗ *hortorum*), both examined species of Goodeniaceae (*Goodenia ovata* and *Scaevola sericea*), and four of the five examined species of Plumbaginaceae (*Armeria maritima*, *Goniolimon tataricum*, *Limonium gmelinii*, and *Limonium latifolium*, but not *Plumbago auriculata*; Table 1). The absence of the intron in *Limonium gmelinii* and *Pelargonium* cpDNAs corroborates the results of earlier blot-hybridization studies using an *rpl*16 intron-specific probe (Downie and Palmer, 1992, 1994). DNA sequencing of the *rpl*16 region in *Erodium*, *Pelargonium*, *Goodenia*, *Scaevola*, *Armeria*, *Goniolimon*, and *Limonium gmelinii* confirmed that the intron is indeed missing from these taxa. Furthermore, these sequence data showed that the *rpl*16 gene has undergone a precise deletion of the intron, with the two remaining exons juxtaposed into a single, uninterrupted gene. This excision coincides precisely with established splice sites for group II introns (Michel et al., 1989) and leads to the union of the two coding regions. This precise excision of an intron has been reported from other chloroplast genes (Hiratsuka et al., 1989; Downie et al., 1991; Freyer et al., 1995; Doyle et al., 1995; Wallace and Cota, 1996). The mechanism of intron loss is speculative, but might involve reverse transcription of the spliced RNA followed by reintegration into the chloroplast genome at precisely the same location (Hiratsuka et al., 1989; Downie et al., 1991).

Angiosperm families Geraniaceae, Goodeniaceae, and Plumbaginaceae are placed by Cronquist (1981) in three different subclasses (Rosidae, Asteridae, and Caryophyllidae, respectively; Appendix 1). These families are distantly related to one another in systems of classification based largely on morphology (e.g., Cronquist, 1981) and in phylogenies based on cpDNA *rbc*L gene sequences (e.g., Chase et al., 1993). Thus, based on these traditional and molecular data, we infer that the *rpl*16 intron has been lost at least three

times independently during the evolution of the flowering plants. Although shared structural mutations can provide strong evidence of common ancestry, it is apparent that similar rearrangements, such as the loss of the *rpl*16 intron, can occur in parallel.

Herein we present the phylogenetic significance of each of these three instances of intron loss. The family Geraniaceae, as defined by Cronquist (1981), consists of 11 genera and about 700 species. Using a blot-hybridization approach and an *rpl*16 intron-specific probe, Downie and Palmer (1992), citing unpublished results by S. Downie, J. Logsdon Jr., and J. Palmer, reported that this intron is missing from Geraniaceae representatives *Monsonia* (2 examined species), *Erodium* (2 species including *E. chamaedryoides*), *Geranium* (3 species), *Sarcocaulon* (3 species), and *Pelargonium* (34 species including *P.* * *hortorum*). These five genera comprise the tribe Geranieae (or Geraniaceae sensu stricto), a well marked natural group (Price and Palmer, 1993). The shared loss of the *rpl*16 intron in all examined members of this group supports its monophyly. The remaining six genera, encompassing about 25 species altogether, have sometimes been segregated into as many as four distinct families (Cronquist, 1981). On the basis of *rbc*L sequence comparisons, two of these segregate genera, *Wendtia* and *Viviania*, are clearly excluded from the Geraniaceae (Price and Palmer, 1993). Relationships of the remaining four genera (*Biebersteinia*, *Rhynchotheca*, *Dirachma*, and *Balbisia*) have yet to be examined using *rbc*L sequences. The distribution of the *rpl*16 intron in these six segregate taxa has not been determined.

The family Goodeniaceae is of tropical and subtropical distribution and consists of about 14 genera and 300 species, with the two largest genera being *Goodenia* and *Scaevola* (Cronquist, 1981). The one species examined from each of these genera lacks the intron. Additional representatives from the family should be investigated for intron loss.

The family Plumbaginaceae consists of about a dozen genera and some 400 species, and is widely distributed (Cronquist, 1981). Two subfamilies are traditionally recognized, Armerioideae and Plumbaginoideae, with *Plumbago* being the only examined representative of the latter. The presence of the *rpl*16 intron in *Plumbago auriculata* but not in *Armeria*, *Goniolimon* or *Limonium* cpDNAs identifies a major dichotomy in Plumbaginaceae; the distribution of the intron may coincide with traditional subfamilial circumscriptions. Like in the Geraniaceae and Goodeniaceae, the distribution of *rpl*16 intron in this family needs to be assessed more critically.

Despite evolution in parallel, the loss of the *rpl*16 intron can be used as a molecular character in each of the families in which it has occurred. As sampling is expanded and the evolutionary relationships within each of these three families better understood, the utility of these intron loss characters in demarcating monophyletic groups can be more readily ascertained.

ACKNOWLEDGMENTS

The authors thank Deborah S. Katz-Downie and Jonathan W. Luttrell for laboratory assistance; Jim Kramer of the University of Illinois Plant Sciences Lab for permitting us to sample the greenhouse collections; Jeffrey Palmer for supplying some of the DNAs; and Deborah S. Katz-Downie and two anonymous reviewers for comments on the

manuscript. This work was supported, in part, by grants from the UIUC Campus Research Board, the National Science Foundation (DEB-9407712), and the Federal Work Study Program.

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Table 1. Species without an intron in chloroplast gene *rpl*16. Asterisks denote those species whose intron absence has been confirmed by DNA sequencing.

Geraniaceae[a]
Erodium chamaedryoides L'Her.*
Pelargonium ∗ hortorum L. H. Bailey*
Goodeniaceae
Goodenia ovata Smith*
Scaevola sericea Vahl*
Plumbaginaceae
Armeria maritima Willd. *
Goniolimon tataricum (L.) Boiss. *
Limonium gmelinii Kuntze*
Limonium latifolium Kuntze

[a]S. Downie, J. Logsdon Jr., and J. Palmer report in Downie and Palmer (1992), on the basis of filter hybridizations using an *rpl*16-intron specific probe, that cpDNAs from Geraniaceae genera *Monsonia* (2 species), *Erodium* (2 species), *Geranium* (3 species), *Sarcocaulon* (3 species), and *Pelargonium* (34 species) do not have the *rpl*16 intron.

Appendix 1. Angiosperms surveyed for the presence or absence of the chloroplast DNA *rpl*16 intron. System of classification follows that of Cronquist (1981); subclass names are boldfaced. Voucher information and sources of plant material are available upon request.

MAGNOLIOPSIDA (DICOTYLEDONS)

Asteridae

Acanthaceae
- *Graptophyllum pictum* Griff.

Apocynaceae
- *Amsonia tabernaemontana* Walter
- *Apocynum sibiricum* Jacq.
- *Aspidosperma myristicifolium* Markgr.
- *Nerium oleander* L.
- *Vinca minor* L.

Asclepiadaceae
- *Asclepias curassavica* L.

Asteraceae
- *Barnadesia caryophylla* (Vell.) S. F. Blake
- *Lactuca sativa* L.

Bignoniaceae
- *Campsis radicans* (L.) Seemann.
- *Paulownia tomentosa* (Thunb.) Steudel

Boraginaceae
- *Borago officinalis* L.
- *Mertensia virginica* (L.) Pers.
- *Myosotis sylvatica* Hoffm.

Calyceraceae
- *Boopis* sp.
- *Calycera sympaganthera* Kuntze
- *Gamocarpha poeppigii* DC.

Campanulaceae
- *Campanula garganica* Ten.
- *Lobelia mildbraedii* Engl., + 4 other species

Caprifoliaceae
- *Kolkwitzia amabilis* Graebn.
- *Sambucus canadensis* L.
- *Symphoricarpos albus* (L.) S. F. Blake

Convolvulaceae
- *Calystegia sepium* (L.) R. Br.
- *Convolvulus arvensis* L.

Dipsacaceae
- *Dipsacus sylvestris* Huds.

Gentianaceae
- *Gentiana saponaria* L.
- *Obolaria virginica* L.

Goodeniaceae
- *Goodenia ovata* Smith
- *Scaevola sericea* Vahl

Hydrophyllaceae
- *Hydrophyllum virginianum* L.

Lamiaceae
- *Teucrium canadense* L.

Loganiaceae
- *Gelsemium sempervirens* (L.) Aiton f.

Menyanthaceae
- *Menyanthes trifoliata* L.
- *Nymphoides thunbergiana* Kuntze
- *Villarsia exaltata* F. Muell., + 9 other species

Myoporaceae
- *Myoporum acuminatum* R. Br.

Oleaceae
- *Forsythia ovata* Nakai
- *Fraxinus americana* L.
- *Syringa vulgaris* L.

Pedaliaceae
- *Proboscidea louisiana* (Miller) Thell.

Plantaginaceae
- *Plantago lanceolata* L.

Rubiaceae
- *Galium boreale* L.
- *Rubia tinctorum* L.

Scrophulariaceae
- *Digitalis purpurea* L.

Solanaceae
- *Nicotiana tabacum* L.

Verbenaceae
- *Callicarpa americana* L.

Caryophyllidae

Aizoaceae
- *Monilaria moniliformis* (Thunb.) H. D. Ihlenf. & S. Jörg.
- *Tetragonia tetragonioides* (Pall.) Kuntze

Amaranthaceae
- *Alternanthera dentata* (Moench) Scheygrond
- *Amaranthus tricolor* L., + 2 other species
- *Celosia argentea* L.

Basellaceae
- *Anredera cordifolia* (Ten.) Steenis

Cactaceae
- *Pereskia grandifolia* Haw.

Caryophyllaceae
- *Agrostemma githago* L.
- *Corrigiola littoralis* L.
- *Lychnis coronaria* (L.) Desr.
- *Lychnis chalcedonica* L.

Chenopodiaceae
- *Archiatriplex nanpinensis* Chu
- *Beta vulgaris* L.
- *Camphorosma monspeliaca* L.
- *Ceratoides lanata* (Pursh) J. T. Howell
- *Chenopodium album* L.
- *Grayia spinosa* (Hook.) Moq.
- *Hablitzia thamnoides* Marsch.
- *Sarcobatus vermiculatus* (Hook.) Torr.

Spinacia oleracea L.
Didiereaceae
Alluaudia montagnacii Rauh
Didierea madagascariensis Baillon
Molluginaceae
Mollugo verticilliata L.
Nyctaginaceae
Bougainvillea glabra Choisy
Mirabilis nyctaginea (Michx.) MacMill.
Phytolaccaceae
Petiveria alliacea L.
Phytolacca americana L.
Stegnosperma halimifolium Benth.
Plumbaginaceae
Armeria maritima Willd.
Goniolimon tataricum (L.) Boiss.
Limonium gmelinii Kuntze
Limonium latifolium Kuntze
Plumbago auriculata Lam.
Polygonaceae
Polygonum caespitosum Blume
Portulacaceae
Calandrinia ciliata (Ruiz & Pav.) DC.
Claytonia perfoliata Donn
Portulaca oleracea L.
Portulaca umbraticola Kunth.

Dilleniidae

Begoniaceae
*Begonia * semperflorens-cultorum*
Bixaceae
Bixa orellana L.
Brassicaceae
Brassica rapa L.
Caricaceae
Carica papaya L. 'Solo'
Cucurbitaceae
Cucurbita moschata (Duchesne) Poir.
Datiscaceae
Datisca glomerata Baill.
Droseraceae
Drosera sp.
Flacourtiaceae
Flacourtia inermis Roxb.
Oncoba spinosa Forssk.
Fouquieriaceae
Fouquieria splendens Engelm.
Malvaceae
Gossypium hirsutum L.
Nepenthaceae
Nepenthes sp.
Paeoniaceae
Paeonia lactiflora Pall.
Passifloraceae
Passiflora incarnata L.
Primulaceae
Anagallis arvensis L.
Salicaceae
Populus deltoides Marshall
Salix amygdaloides Andersson
Sarraceniaceae
Sarracenia sp.
Violaceae
Viola dissecta Ledeb. var. *chaerophylloides* (Regel) Makino.

Hamamelidae

Ulmaceae
Ulmus americana L.

Magnoliidae

Aristolochiaceae
Aristolochia durior Hill
Asarum canadense L.
Berberidaceae
Podophyllum peltatum L.
Magnoliaceae
Liriodendron tulipifera L.
Ranunculaceae
Aquilegia canadensis L.
Caltha palustris L.
Saururaceae
Saururus cernuus L.
Winteraceae
Drimys winteri J. R. & G. Forster

Rosidae

Apiaceae
Coriandrum sativum L.
Daucus carota L., + numerous other genera
Balsaminaceae
Impatiens balsamina L.
Cornaceae
Aucuba japonica Thunb.
Cornus florida L.
Euphorbiaceae
Acalypha hispida Burm. f.
Euphorbia milii Des Moul.
Fabaceae
Acacia farnesiana L., + 17 other species
Caesalpinia pulcherrima (L.) Sw.
Glycine max (L.) Merr.
Lablab purpureus L.
Lysiloma sp.
Medicago sativa L., + 9 other species
Phaseolus vulgaris L.
Pisum sativum L.
Pithecellobium saman (Jacq.) Benth.
Trifolium repens L.
Geraniaceae
Erodium chamaedryoides L'Her.
*Pelargonium * hortorum* L.H. Bailey
Grossulariaceae
Escallonia rubra (Ruiz & Pav.) Pers.
Hydrangeaceae
Hydrangea sp.
Linaceae
Linum usitatissimum L.

Malpighiaceae
Banisteriopsis caapi (Griseb.) Morton
Onagracaceae
Fuchsia hybrida Voss
Oenothera macrocarpa Nutt.
Oxalidaceae
Oxalis stricta L.
Pittosporaceae
Pittosporum tobira (Thunb.) Ait.
Punicaceae
Punica granatum L.
Rosaceae
Agrimonia gryposepala Wallr.
Alchemilla vulgaris L.
Amelanchier canadensis (L.) Medikus
Duchesnea indica (Andrews) Focke
Fragaria virginiana Duchesne
Geum aleppicum Jacq.
Gillenia trifoliata Moench
Physocarpus opulifolius (L.) Maxim.
Potentilla anserina L.
Prunus serotina Ehrh.
Rosa sp.
Rubus odoratus L.
Sanguisorba canadensis L.

LILIOPSIDA (MONOCOTYLEDONS)
Alismatidae
Alismataceae
Sagittaria latifolia Willd.
Arecidae
Araceae
Spathiphyllum floribundum N. E. Br.
Symplocarpus foetidus (L.) Nutt.
Commelinidae
Commelinaceae
Commelina tuberosa L.
Poaceae
Avena sativa L.
Secale cereale L.
Triticum aestivum L.
Zea mays L.
Typhaceae
Typha latifolia L.
Liliidae
Iridaceae
Iris sp.
Liliaceae
Allium cepa L.
Erythronium albidum Nutt.
Muscari botryoides (L.) Miller
Narcissus pseudonarcissus L.
Tulipa sp.
Pontederiaceae
Eichhornia crassipes (Martius) Solms-Laub.
Zingiberidae
Bromeliaceae
Tillandsia usneoides (L.) L.

Figure. 1. Structural organization of the *rpl*16 gene and flanking DNA regions. Coding regions are indicated by shaded boxes; the *rpl*16 intron is indicated by an open box. Scale units are in kilobase pairs (kb). (A) In tobacco and the vast majority of angiosperms chloroplast DNAs examined, the *rpl*16 gene is interrupted by an intron of about 1.0 kb. The two arrows indicate the placements and relative positions of the forward and reverse PCR primers used to assess the presence or absence of the intron. When the intron is present, the resultant PCR product is approximately 1,400 bp in size. (B) When the intron is absent, such as in geranium cpDNA, the resultant PCR product is reduced by the size of the intron and is about 400 bp in size. Forward and reverse primer sequences, written 5' to 3', are TTTCCTTTCGAAAAGCAATG and TCTTCCTCTATGTTGTTTACG, respectively.