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ORIGINAL PAPER

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# Complete chloroplast genome sequences of *Solanum bulbocastanum*, *Solanum lycopersicum* and comparative analyses with other Solanaceae genomes

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**Abstract** Despite the agricultural importance of both potato and tomato, very little is known about their chloroplast genomes. Analysis of the complete sequences of tomato, potato, tobacco, and *Atropa* chloroplast genomes reveals significant insertions and deletions within certain coding regions or regulatory sequences (e.g., deletion of repeated sequences within 16S rRNA, *ycf*2 or ribosomal binding sites in *ycf*2). RNA, photosynthesis, and atp synthase genes are the least divergent and the most divergent genes are *clpP*, *cemA*, *ccsA*, and *matK*. Repeat analyses identified 33–45 direct and inverted repeats ≥30 bp with a sequence identity of at least 90%; all but five of the repeats shared by all four Solanaceae genomes are located in the same genes or intergenic regions, suggesting a functional role. A comprehensive genome-wide analysis of all coding sequences and intergenic spacer regions was done for the first time in chloroplast genomes. Only four spacer regions are fully conserved (100% sequence identity) among all genomes; deletions or insertions within some intergenic spacer regions result in less than 25% sequence identity, underscoring the importance of choosing appropriate intergenic spacers for plastid transformation and providing valuable new information for phylogenetic utility of the chloroplast intergenic spacer regions. Comparison of coding sequences with expressed sequence tags showed considerable amount of variation, resulting in amino acid changes; none of the C-to-U conversions observed in potato and tomato were conserved in tobacco and *Atropa*. It is possible that there has been a loss of conserved editing sites in potato and tomato.

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## Introduction

The chloroplast is a plant organelle that contains the entire enzymatic machinery in the stroma and electron carriers within the thylakoid membranes for photosynthesis. In addition to photosynthesis, several other biochemical pathways are present within chloroplasts, including biosynthesis of fatty acids, amino acids, pigments, and vitamins. The chloroplast genome generally has a highly conserved organization (Palmer 1991; Raubeson and Jansen 2005), with most land plant genomes composed of a single circular chromosome with a quadripartite structure that includes two copies of an inverted repeat (IR) that separate the large and small single copy regions (LSC and SSC). Our knowledge of the organization and evolution of chloroplast genomes has been expanding rapidly because of the large numbers of completely sequenced genomes published in the past decade. Currently, there are 47 completely sequenced plastid genomes (Raubeson and Jansen 2005; Jansen et al. 2005; http://www.megasun.bch.umontreal.ca/ogmp/projects/other/cp_list.html), and 29 of these are from various land plant lineages, with the best representation (21) from flowering plants. Comparative studies indicate that chloroplast genomes of land plants are highly conserved in both gene order and gene content. Several lineages of land plants have chloroplast DNAs (cpDNAs) with multiple rearrangements, including *Pinus* (Wakasugi et al. 1994) and the angiosperm families Campanulaceae (Cosner et al. 1997), Fabaceae (Palmer et al. 1988; Milligan et al. 1989; Kato et al. 2000), Geraniaceae (Palmer et al. 1987), and Lobeliaceae (Knox and Palmer 1998). In most of these studies, comparisons of gene content and order have been made among distantly related taxa because only one genome sequence was available from groups with rearranged genomes. Two exceptions are: grasses with genomic data available for four genera of crop plants (corn, wheat, sugar cane, and rice; Maier et al. 1995; Matsuoka et al. 2002; Tang et al. 2004) and legumes with genome sequences completed for three genera (alfalfa, soybean, and *Lotus*; Kato et al. 2000; Saski et al. 2005).

Chloroplast genetic engineering offers a number of unique advantages, including a high-level of transgene expression (DeCosa et al. 2001), multi-gene engineering in a single transformation event (DeCosa et al. 2001; Ruiz et al. 2003; Lossl et al. 2003; Quesada-Vargas et al. 2005), transgene containment via maternal inheritance (Daniell et al. 1998; Scott and Wilkenson 1999; Daniell 2002; Hagemann 2004) or cytoplasmic male sterility (Ruiz and Daniell 2005), lack of gene silencing (DeCosa et al. 2001; Lee et al. 2003; Dhingra et al. 2004), position effect (Daniell et al. 2002), pleiotropic effects (Lee et al. 2003; Daniell et al. 2001; Leelavathi and Reddy 2003) and lack of transformation vector sequences or selectable marker genes (Daniell et al. 2004a).

Plastid genetic engineering has also become a powerful tool for basic research in plastid biogenesis and function. This approach has helped unveil a wealth of information about plastid DNA replication origins, intron maturases, translation elements and proteolysis, import of proteins and several other processes (Daniell et al. 2004b). Although many successful examples of plastid engineering have set a solid foundation for various future applications, this technology has not been extended to many of the major crops. However, plastid transformation has been recently accomplished via somatic embryogenesis using partially sequenced chloroplast genomes in soybean (Dufourmantel et al. 2004), carrot (Kumar et al. 2004a), and cotton (Kumar et al. 2004b; Daniell et al. 2005). Transgenic carrot plants were able to withstand salt concentrations that only halophytes could tolerate (Kumar et al. 2004a).

The lack of complete chloroplast genome sequences is still one of the major limitations to extending this technology to useful crops; prior to 2004 only seven published *crop* chloroplast genomes were available and this number has increased to 23 during the past 2 years (Table 1). Chloroplast genome sequences are necessary for identification of spacer regions for integration of transgenes at optimal sites via homologous recombination, as well as endogenous regulatory sequences for optimal expression of transgenes (Daniell et al. 2005; Maier and Schmitz-Linneweber 2004). In higher plants, about 40–50% of each chloroplast genome contains non-coding spacer and regulatory regions (Saski et al. 2005; Lee et al. 2006; Jansen et al. 2006).

Once thought to be poisonous, tomato (*Solanum lycopersicum*) has become the second most commonly grown vegetable crop in the world behind potato. The total traded value of tomatoes in the United States is about US $13,493,496,000. The fresh-market export of US tomatoes was estimated to be 325,000 lbs while export was 2,095,000 lbs. Similarly, the volume of processed tomatoes exported in 2005 was about 1,295,500 lbs and imported about 3,080,000 lbs. Countries that export tomatoes to the United States include Canada, Chile, Mexico, Italy, and Israel (http://www.ers.usda.gov/Briefing/Tomatoes/trade.htm#tradetables). Traditional plant breeding has resulted in great progress in increasing yield, disease and pest resistance, environmental stress resistance, and quality and processing attributes. However, tomato plant breeding programs still strive to generate a better product. To assist in this goal, some plant breeding programs have been expanded to include biotechnological techniques. Tomato has long been recognized as an excellent genetic model for molecular biology studies. This has resulted in a flood of information including markers and genetic maps, identification of individual chromosomes, promoters and other nuclear genome sequences, and identification of genes and their function. However, there is not much information about the tomato chloroplast genome. Because of this, segments of the tobacco chloroplast genome were used as flanking sequences to facilitate integration of transgenes into the tomato chloroplast genome by homologous recombination, without knowing exact sequence identity (Ruf et al. 2001).

*Solanum tuberosum* (Irish or white potato) is the most economically significant crop in the US produce industry. With an annual farm value of US $2.5 billion and per capita use of 140 pounds in 2003, potato ranks first in value and consumption among all vegetables produced and consumed in the United States. Additionally, potato products such as french fries and potato chips generate billions more in revenue for the food-processing and food service industries. Currently exports account for 11% of US potato production in the form of fresh, seed, frozen and dehydrated potatoes (http://www.ers.usda.gov/Briefing/Potatoes). However, there is not much information on the potato chloroplast genome. When potato plastid genome was transformed, only the tobacco plastid genome flanking sequence was used to facilitate transgene integration by homologous recombination (Sidorov et al. 1999).

In this article we present the complete sequence of the chloroplast genomes of tomato and potato. One goal of this paper is to compare the genome organization of potato and tomato with the other two completely sequenced Solanaceae chloroplast genomes (tobacco and *Atropa*). In addition to examining gene content and gene

**Table 1** Alphabetical list of 23 complete plastid genome sequences of crop plants as of January 25, 2006 (see http://www.megasun.bch.umontreal.ca/ogmp/projects/other/cp_list.html and http://www.ncbi.nlm.nih.gov:80/genomes/static/euk_o.html for access to genomic sequences)

| Species | Reference | Accession number | Year completed | Genome size (bp) |
|---|---|---|---|---|
| *Citrus sinensis* | Bausher et al. (2006) | NA | 2006 | 160,614 |
| *Cucumis sativus* | Plader et al., unpublished/ Kim et al. (2006) | NC_007144/ DQ119058 | 2005/ 2006 | 155,293/ 155,527 |
| *Eucalyptus globules* | Steane (2005) | AY780259 | 2005 | 160,286 |
| *Glycine max* | Saski et al. (2005) | DQ317523 | 2005 | 152,218 |
| *Gossypium hirsutum* | Lee et al. (2006) | DQ345959 | 2006 | 160.301 |
| *Helianthus annuus* | Timme et al. (2006) | DQ383815 | 2006 | 151,104 |
| *Lactuca sativa* | Kanamoto et al., unpublished/ Timme et al. (2006) | NC_007578/ DQ383816 | 2005/ 2006 | 152,765/ 152,772 |
| *Medicago truncatula* | Lin et al., unpublished | AC093544 | 2001 | 124,033 |
| *Nicotiana tabacum* | Shinozaki et al. (1986) | Z00044 | 1986 | 155,939 |
| *Oryza nivara* | Masood et al. (2004) | NC_005973 | 2004 | 134,494 |
| *Oryza sativa* | Hiratsuka et al. (1989)/ Tang et al. (2004) | NC_001320/ AY522329, AY522331 | 1989/2004, 2004 | 134,525/134,496, 134,551 |
| *Panax schinseng* | Kim and Lee (2004) | NC_006290 | 2004 | 156,318 |
| *Pinus thunbergii* | Wakasugi et al. (1994) | NC_001631 | 1994 | 119,707 |
| *Populus trichocarpa* | http://www.genome.ornl.gov/ poplar_chloroplast/ | NA | 2003 | 157,033 |
| *Saccharum* hybrid | Calsa et al., unpublished | NC_005878 | 2004 | 141,182 |
| *Saccharum officinarum* | Asano et al. (2004) | NC_006084 | 2004 | 141,182 |
| *Solanum bulbocastanum* | Daniell et al., this article | DQ347958 | 2006 | 155,371 |
| *Solanum lycopersicum* | Daniell et al., this article | DQ347959 | 2006 | 155,461 |
| *Solanum tuberosum* | Chung et al., unpublished | DQ231562 | 2005 | 155,312 |
| *Spinacia oleracea* | Schmitz-Linneweber et al. (2001) | NC_002202 | 2000 | 150,725 |
| *Triticum aestivum* | Ogihara et al. (2000) | AB042240 | 2001 | 134,545 |
| *Vitis vinifera* | Jansen et al. (2006) | DQ424856 | 2006 | 160,928 |
| *Zea mays* | Maier et al. (1995) | NC_001666 | 1995 | 140,384 |

*NA* not available

order, we determine the distribution and location of repeated sequences among members of the Solanaceae. A second goal is to compare levels of DNA sequence divergence between chloroplast coding and noncoding regions. Intergenic spacer regions have been examined to identify ideal insertion sites for transgene integration and they are commonly used by plant systematists for resolving phylogenetic relationships among closely related species (Kelchner 2002; Shaw et al. 2005). A final goal of this paper is to examine the extent of RNA editing in Solanaceae chloroplast genomes by comparing the DNA sequences with available expressed sequence tags (EST) sequences. RNA editing is known to play an important role in several lineages of plants (Wolf et al. 2004; Kugita et al. 2003), but most of our knowledge about the frequency of this process in crop plants comes from studies in maize (Maier et al. 1995) and tobacco (Hirose et al. 1999).

## Materials and methods

### DNA sources

The bacterial artificial chromosome (BAC) libraries of potato and tomato were constructed by ligating size fractionated partial *Hin*dIII digests of total cellular high molecular weight DNA with the pINDIGOBAC vector. The average insert size of the potato and tomato libraries is 177 and 155 kb, respectively. BAC related resources for these public libraries can be obtained from the Clemson University Genomics Institute BAC/EST Resource Center (http://www.genome.clemson.edu).

BAC clones containing the chloroplast genome inserts were isolated by screening the library with a soybean chloroplast probe. The first 96 positive clones from screening were pulled from the library, arrayed in a 96-well microtiter plate, copied, and archived. Selected clones were then subjected to *Hin*dIII fingerprinting and *Not*I digests. End-sequences were determined and localized on the chloroplast genome of *Arabidopsis thaliana* to deduce the relative positions of the clones, then clones that covered the entire chloroplast genomes of potato and tomato were chosen for sequencing.

### DNA sequencing and genome assembly

The nucleotide sequences of the BAC clones were determined by the bridging shotgun method. The purified BAC DNA was subjected to hydroshearing, end repair, and then size-fractionated by agarose gel electrophoresis. Fractions of approximately 3.0–5.0 kb were eluted and ligated into the vector pBLUESCRIPT IIKS+. The libraries were plated and arrayed into 40 96-well microtiter plates, respectively, for the sequencing reactions.

Sequencing was performed using the Dye-terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, USA). Sequence data from the forward and reverse priming sites of the shotgun clones were accumulated. Sequence data equivalent to eight times the size of the genome was assembled using Phred-Phrap programs (Ewing and Green 1998).

## Gene annotation

Annotation of the potato and tomato chloroplast genomes was performed using DOGMA (Dual Organellar GenoMe Annotator; Wyman et al. 2004; http://www.evogen.jgi-psf.org/dogma). This program uses a FASTA-formatted input file of the complete genomic sequences and identifies putative protein-coding genes by performing BLASTX searches against a custom database of previously published chloroplast genomes. The user must select putative start and stop codons for each protein coding gene and intron and exon boundaries for intron-containing genes. Both tRNAs and rRNAs are identified by BLASTN searches against the same database of chloroplast genomes.

## Molecular evolutionary comparisons

### *Comparisons of gene content and gene order*

Gene content comparisons were performed with Multipipmaker (Schwartz et al. 2003). Comparisons included four genomes: tobacco (NC_001879), potato (DQ 347958), tomato (DQ 347959), and *Atropa* (NC_004561) using tobacco as the reference genome. Gene orders were examined by pair-wise comparisons between the tobacco, potato, tomato, and *Atropa* genomes using PipMaker (Elnitski et al. 2002).

### *Examination of repeat structure*

The repeat structure of the chloroplast genomes was examined in two stages. First, REPuter (Kurtz et al. 2001) was used to identify the number and location of direct and inverted (palindromic) repeats in the species of Solanaceae using a minimum repeat size of 30 bp and a Hamming distance of 3 (i.e., a sequence identity of ≥90%). Second, the repeats identified for tobacco were blasted against the complete chloroplast genomes of all four Solanaceae genomes. Blast hits of size 30 bp and longer with a sequence identity of ≥90% were identified to determine the shared repeats among the four genomes examined.

### *Comparisons of DNA sequence divergence*

An aligned data set of all of the shared genes among the four Solanaceae chloroplast genomes was constructed by extracting these sequences from the annotated genomes either using DOGMA (Wyman et al. 2004) or the Chloroplast Genome Database (Cui et al. 2006; http://www.cbio.psu.edu/chloroplast/index.html). The sequences were aligned using ClustalX (Higgins et al. 1996) followed by manual adjustments using Seq Ap.

Molecular evolutionary analyses were then performed on the aligned data matrix using MEGA2 (Molecular Evolutionary Genetics Analysis; Kumar et al. 2001). Estimates of sequence divergence were based on the Kimura 2-parameter distance correction (Kimura 1980).

### *Comparison of intergenic spacer regions*

Intergenic regions from four Solanaceae chloroplast genomes were compared using MultiPipMaker (Schwartz et al. 2003; http://www.pipmaker.bx.psu.edu/pipmaker/tools.html). MultiPipMaker offers a suite of software tools to analyze relationships among more than two sequences. In the current study, we used a program known as ‘all_bz’ that iteratively compares a pair of nucleotide sequences at a time until all possible pairs from all species have been compared. However, this program processes only one set of intergenic regions at a time. For genome-wide comparisons of corresponding intergenic regions from all species, we developed two programs written in PERL. The first program iteratively creates a set of input files containing corresponding intergenic regions from each species and compares them using ‘all_bz’ program, until all the intergenic regions in the chloroplast genome are processed. The second program parses the output from the above comparisons, calculates percent identity by using the number of identities over the length of the longer sequence and generates results in tab-delimited tabular format.

### *Variation between coding sequences and cDNAs*

Each of the gene sequences from the potato chloroplast genome was used to perform a BLAST search of expressed sequence tags (ESTs) from Genbank. The retrieved EST sequences from potato, tomato, and tobacco were then aligned with the corresponding gene for each species separately, using Clustal X. In the case of *Atropa*, no sequences were retrieved from the Genbank even though its chloroplast sequence has been completed and studies of RNA editing have been previously performed (Schmitz-Linneweber et al. 2002). To maintain consistency in this study, only EST sequences were used and no other genomic sequences were considered. The aligned sequences were then screened and nucleotide and amino acid changes were detected using the Megalign software. The following criteria were used for comparisons of the DNA and EST sequences: (1) when more than one EST sequence was retrieved using BLAST, a change was recorded only if all sequences had the same change (substitution); (2) changes were recorded based on the base substitutions, that is, if there was an indel that affected the DNA sequence, it was not considered; and (3) if a retrieved EST sequence was too different (more than three consecutive nucleotide substitutions in a given sequence), it was not used for the analysis. In most cases, EST sequences were not of the same length as that of the corresponding gene, so the length of the analyzed sequence was recorded. Once a variable site was detected, the sequence was translated using the Megalign program using the plastid/bacterial genetic code and differences in the amino acid sequence were recorded.

## Results

### Size, gene content and organization of the tomato and potato chloroplast genomes

The complete sizes of the tomato and potato chloroplast genomes are 155,461 and 155,371 bp (Fig. 1), respectively. The genomes include a pair of inverted repeats of 25,611 bp (tomato) and 25,588 bp (potato), separated by a small single copy region of 18,363 bp (tomato) and 18,381 bp (potato) and a large single copy region of 85,876 bp (tomato) and 85,814 bp (potato). The difference in size of the two genomes is due partly to a slight expansion of the IR in tomato resulting in a partial duplication *rps19*, a phenomenon that is quite common in chloroplast genomes (Goulding et al. 1996).

The potato and tomato chloroplast genomes contain 113 unique genes, and 20 of these are duplicated in the IR, giving a total of 133 genes (Fig. 1). There are 30 distinct tRNAs, and seven of these are duplicated in the IR. Seventeen genes contain one or two introns, and five of these are in tRNAs. The genomes consist of 58.3% (tomato), 59.6% (potato) coding regions that includes 50.7% (tomato), 52.0% (potato) protein coding genes and 7.6% (tomato and potato) RNA genes and 41.7% (tomato), 40.4% (potato) noncoding regions, containing both intergenic spacers and introns. The overall GC and AT content of the potato and tomato chloroplast genomes are 37.86% (tomato), 37.88% (potato) and 62.14% (tomato), 62.12% (potato), respectively.

### Gene content and gene order

Gene content of the four sequenced species of Solanaceae (potato [DQ347958] & tomato [DQ347959] published here; tobacco [NC_001879] and *Atropa* [NC_004561]) is identical. Similarly, the gene order is identical among all four sequenced Solanaceae genomes. However, there are significant additions or deletions of nucleotides within certain coding sequences. For example, ACACGGGAAAC sequence is uniquely present within the 16S rRNA gene of potato, tomato, and *Atropa* but absent in tobacco or any other sequenced chloroplast genome (Fig. 2). Several deletions also occur within the coding sequence of *ycf2* in *Atropa*, tomato, potato, and tobacco (Fig. 3). It should be noted that deleted nucleotides within the 16S rRNA and *ycf2* are repeated sequences. In tomato *ycf2* has three ribosome binding sites (GGAGG), whereas there is only one in all other Solanaceae members sequenced so far (Fig. 3).

### Repeat structure

REPuter found 33–45 direct and inverted repeats 30 bp or longer with a sequence identity of at least 90% among the four chloroplast genomes examined (Fig. 4; see Supplemental Table 1 for a list of all repeats in all four genomes). The majority of the repeats in all four genomes are between 30 and 40 bp in length. The longest repeats other than the inverted repeats are found in tomato and consist of four 57 bp repeats not found in any of the other three genomes. Both tobacco and potato share a 50 and 56 bp repeat, whereas *Atropa* does not have a single repeat in the 50+ bp size range (excluding the IR).

BlastN comparisons of the tobacco repeats (excluding the inverted repeat) against the chloroplast genomes of *Atropa*, potato, and tomato identified 42 repeats that show a sequence identity ≥90% with sequences ≥30 bp and a bit score greater than 40 (Table 2, Fig. 1). Thirty-seven of the 42 repeats are found in all four Solanaceae chloroplast genomes and all of these are located in the same genes or intergenic regions.

### Intergenic spacer regions

All intergenic spacer regions except those less than 11 bp across the four Solanaceae chloroplast genomes were compared (Fig. 5a, Table 3; see Supplemental Table 2 for a list of sequence identities for all intergenic spacers). Only four spacer regions (*rps11 - rpl 36*, *rps 7 - rps 12* 3′ end, *trnI*-GAU - *trnA*-UGC, *ycf 2 - ycf 15*) have 100% sequence identity among all genomes (~2.5% of the spacer regions) and three of these regions are in the inverted repeat. Between tomato and potato 21 intergenic spacer regions have 100% sequence identity, whereas only eight regions have 100% sequence identity between tomato and *Atropa*, tobacco and potato, *Atropa* and potato, nine regions between tobacco and tomato and ten regions between tobacco and *Atropa*. The number of intergenic spacer regions with 100% sequence identity reflects the close phylogenetic relationship among the four Solanaceae genomes (Bohs and Olmstead 1997; Olmstead et al. 1999). It is noteworthy that one of the intergenic spacer regions that has 100% sequence identity between *Atropa* and potato (*trnI*-CAU - *ycf 2*) has only 66–69% sequence identity among the other Solanaceae species examined. Similarly, *ycf4 - cemA* has only 27% identity between tobacco and *Atropa*, potato and tomato, whereas it has greater than 90% identity between other Solanaceae species examined. There are several deletions or insertions in the intergenic spacer regions between *trnQ - rps16*, *trnE - trnT*, *trnK - rps16*, *trnT - ycf 15*, *trnS - trnG*, *ycf2 - trnI*, *ycf 4 - cemA*, *ycf15 - trnL*.

### Sequence divergence

We classified the chloroplast genes into 11 functional groups for comparisons of sequence divergence among

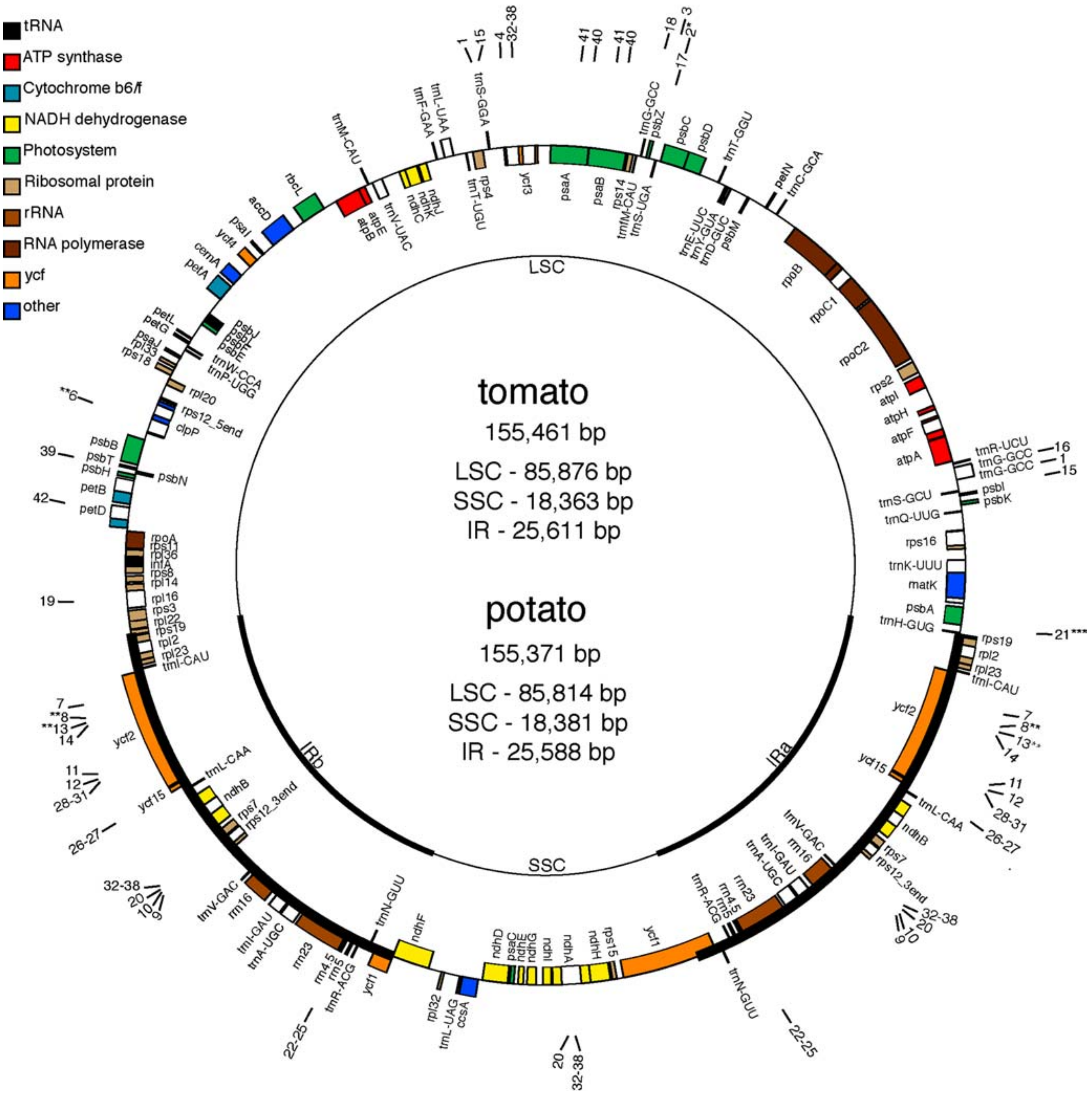
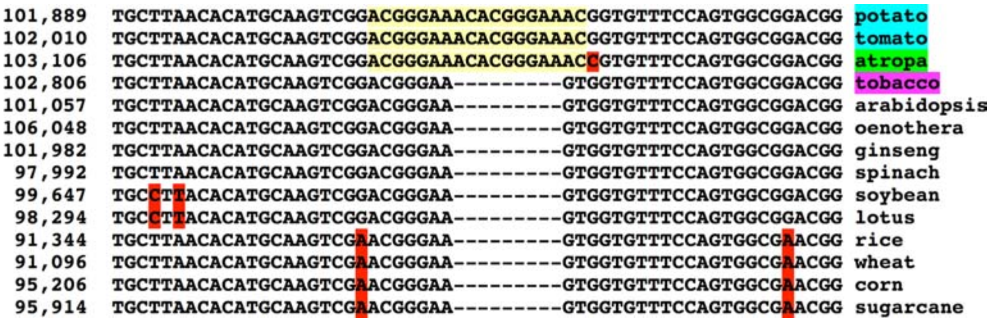


**Fig. 1** Gene map of tomato and potato chloroplast genomes. The *thick lines* indicate the extent of the inverted repeats (IRa and IRb), which separate the genome into small (*SSC*) and large (*LSC*) single copy regions. Genes on the outside of the map are transcribed in the clockwise direction and genes on the inside of the map are transcribed in the counterclockwise direction. *Numbers* around the map indicate the location of repeated sequences found in Solanaceae genomes (see Table 2 for details). *Lines* with *asterisks* indicate the five groups of repeats that are shared by all four Solanaceae genomes: *tobacco and tomato, **tobacco and *Atropa*, ***tobacco

coding regions (Table 4; Fig. 5b). Sequence divergence, which represents the proportion of nucleotide sites that differ, were estimated for all genes using the Kimura 2-parameter method (Kimura 1980). Overall, sequence divergence corresponds to the phylogenetic relationships among the four species of Solanaceae examined (Bohs and Olmstead 1997; Olmstead et al. 1999; Spooner et al. 1993). For example, the two most closely related species, potato and tomato, have the lowest divergence values for all classes of genes. Comparisons of sequence divergence among functional groups indicates that the RNA, photosynthesis, and atp synthase genes are the least divergent and that the most divergent genes are *cemA*, *clpP*, *matK*, and *ccsA*. Our comparisons of the levels of sequence



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101,889  TGCTTAACACATGCAAGTCGGACGGGAAACACGGGAAACGGTGTTTCCAGTGGCGGACGG potato
102,010  TGCTTAACACATGCAAGTCGGACGGGAAACACGGGAAACGGTGTTTCCAGTGGCGGACGG tomato
103,106  TGCTTAACACATGCAAGTCGGACGGGAAACACGGGAAACCGTGTTTCCAGTGGCGGACGG atropa
102,806  TGCTTAACACATGCAAGTCGGACGGGAA---------GTGGTGTTTCCAGTGGCGGACGG tobacco
101,057  TGCTTAACACATGCAAGTCGGACGGGAA---------GTGGTGTTTCCAGTGGCGGACGG arabidopsis
106,048  TGCTTAACACATGCAAGTCGGACGGGAA---------GTGGTGTTTCCAGTGGCGGACGG oenothera
101,982  TGCTTAACACATGCAAGTCGGACGGGAA---------GTGGTGTTTCCAGTGGCGGACGG ginseng
 97,992  TGCTTAACACATGCAAGTCGGACGGGAA---------GTGGTGTTTCCAGTGGCGGACGG spinach
 99,647  TGCCTTACACATGCAAGTCGGACGGGAA---------GTGGTGTTTCCAGTGGCGGACGG soybean
 98,294  TGCCTTACACATGCAAGTCGGACGGGAA---------GTGGTGTTTCCAGTGGCGGACGG lotus
 91,344  TGCTTAACACATGCAAGTCGAACGGGAA---------GTGGTGTTTCCAGTGGCGAACGG rice
 91,096  TGCTTAACACATGCAAGTCGAACGGGAA---------GTGGTGTTTCCAGTGGCGAACGG wheat
 95,206  TGCTTAACACATGCAAGTCGAACGGGAA---------GTGGTGTTTCCAGTGGCGAACGG corn
 95,914  TGCTTAACACATGCAAGTCGAACGGGAA---------GTGGTGTTTCCAGTGGCGAACGG sugarcane
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**Fig. 2** Alignment of a portion of the 5′ end of the 16S ribosomal RNA showing a 9 bp insertion in *Atropa*, potato, and tomato. Nucleotides shown in *red* indicate base substitutions, *yellow* indicate the repeated sequence. Nucleotides shown are for the 16S rRNA gene, from nucleotides 46 to 96 or 105

divergence between noncoding and coding regions (Fig. 5a, b) indicate that the noncoding regions are more divergent than coding regions.

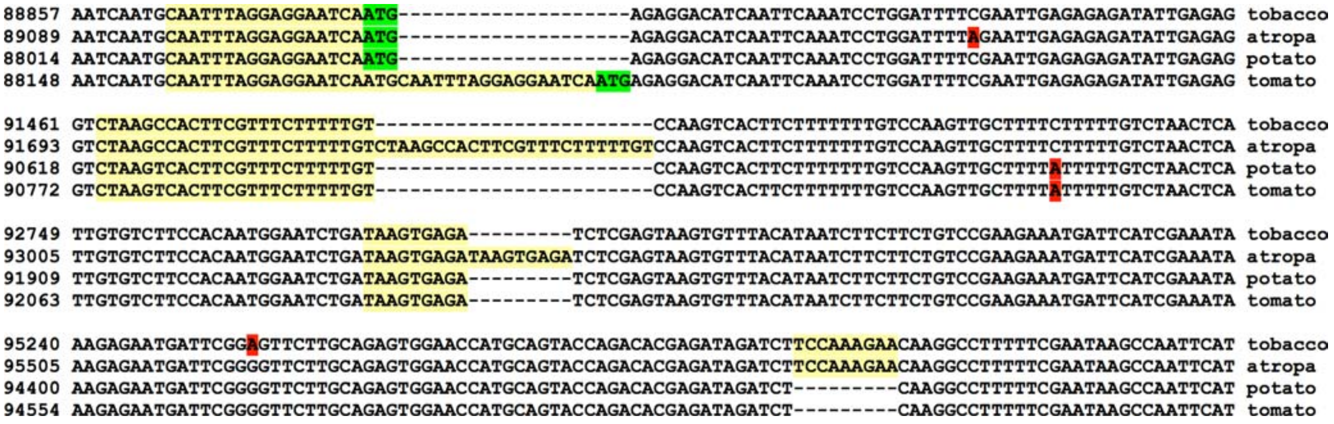
### RNA variable sites in tomato and potato chloroplast transcripts

Based on the alignment of EST sequences retrieved from Genbank, 53 nucleotide substitution differences were observed in the tomato sequence (Table 5) and 47 were observed in potato (Table 6). However, with the exception of *rpl23*, all nucleotide substitutions occurred in different positions among both species. Of these substitutions, 11 were synonymous and 42 were nonsynonymous in tomato, whereas potato had 19 synonymous and 24 nonsynonymous substitutions. Potato had nine C-to-U conversions, five of which resulted in amino acid changes (Table 6). In tomato, seven C-to-U conversions were observed, all of which resulted in an amino acid change (Table 5). Although most genes in both species experienced one and three nucleotide substitutions, four genes had more than five variable sites. These were *rpl36* and *rpoC2* in tomato, with 7 and 10 nucleotide substitutions, respectively (Table 5), and *rpl16* and *ycf1* in potato, with 5 and 7 substitutions, respectively (Table 6). In addition, an amino acid alteration was observed in the tomato *ycf*1 gene that results in a stop codon at position 604. There is a complete copy of *ycf1* and the truncated copy is at the IR/SSC boundary. It is the truncated copy that has the stop codon due to RNA editing. Thus there is still a full, functional copy of *ycf1*. Although there is evidence that *ycf1* is a necessary chloroplast gene, it is missing from all grass genomes (Maier et al. 1995).

## Discussion

### Implications for integration of transgenes

Several intergenic spacer regions have been used to integrate foreign genes into the tomato and potato plastid genomes. These spacer regions are located between the following genes: *trnfM* and *trnG*, *rbcL* and *accD*, *trnV* and 3′-*rps 12*, and *16S rRNA* and *orf 70B* (Ruf et al. 2001; Sidorov et al. 1999; Nguyen et al. 2005). Unfortunately, none of these regions have 100% sequence identity to the tobacco flanking sequence used in plastid transformation vectors. Potato plastid transformants were generated at 10–30 times lower frequencies than tobacco (Nguyen et al. 2005) and the intergenic spacer region between *rbcL* and *accD* shows only 94% identity. Similarly, the *trnfM* and *trnG* intergenic spacer region used for tomato plastid transformation has only 82% sequence identity, resulting in inefficient transgene integration. There are major deletions in the tomato chloroplast genome in this intergenic spacer region when compared to tobacco, which was used for plastid



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88857 AATCAATGCAATTTAGGAGGAATCAATG--------------------AGAGGACATCAATTCAAATCCTGGATTTTCGAATTGAGAGAGATATTGAGAG tobacco
89089 AATCAATGCAATTTAGGAGGAATCAATG--------------------AGAGGACATCAATTCAAATCCTGGATTTTAGAATTGAGAGAGATATTGAGAG atropa
88014 AATCAATGCAATTTAGGAGGAATCAATG--------------------AGAGGACATCAATTCAAATCCTGGATTTTCGAATTGAGAGAGATATTGAGAG potato
88148 AATCAATGCAATTTAGGAGGAATCAATGCAATTTAGGAGGAATCAATGAGAGGACATCAATTCAAATCCTGGATTTTCGAATTGAGAGAGATATTGAGAG tomato

91461 GTCTAAGCCACTTCGTTTCTTTTTGT------------------------CCAAGTCACTTCTTTTTTTGTCCAAGTTGCTTTTCTTTTTGTCTAACTCA tobacco
91693 GTCTAAGCCACTTCGTTTCTTTTTGTCTAAGCCACTTCGTTTCTTTTTGTCCAAGTCACTTCTTTTTTTGTCCAAGTTGCTTTTCTTTTTGTCTAACTCA atropa
90618 GTCTAAGTCACTTCGTTTCTTTTTGT------------------------CCAAGTCACTTCTTTTTTTGTCCAAGTTGCTTTTATTTTTGTCTAACTCA potato
90772 GTCTAAGTCACTTCGTTTCTTTTTGT------------------------CCAAGTCACTTCTTTTTTTGTCCAAGTTGCTTTTATTTTTGTCTAACTCA tomato

92749 TTGTGTCTTCCACAATGGAATCTGATAAGTGAGA---------TCTCGAGTAAGTGTTTACATAATCTTCTTCTGTCCGAAGAAATGATTCATCGAAATA tobacco
93005 TTGTGTCTTCCACAATGGAATCTGATAAGTGAGATAAGTGAGATCTCGAGTAAGTGTTTACATAATCTTCTTCTGTCCGAAGAAATGATTCATCGAAATA atropa
91909 TTGTGTCTTCCACAATGGAATCTGATAAGTGAGA---------TCTCGAGTAAGTGTTTACATAATCTTCTTCTGTCCGAAGAAATGATTCATCGAAATA potato
92063 TTGTGTCTTCCACAATGGAATCTGATAAGTGAGA---------TCTCGAGTAAGTGTTTACATAATCTTCTTCTGTCCGAAGAAATGATTCATCGAAATA tomato

95240 AAGAGAATGATTCGGAGTTCTTGCAGAGTGGAACCATGCAGTACCAGACACGAGATAGATCTTCCAAAGAACAAGGCCTTTTTCGAATAAGCCAATTCAT tobacco
95505 AAGAGAATGATTCGGGGTTCTTGCAGAGTGGAACCATGCAGTACCAGACACGAGATAGATCTTCCAAAGAACAAGGCCTTTTTCGAATAAGCCAATTCAT atropa
94400 AAGAGAATGATTCGGGGTTCTTGCAGAGTGGAACCATGCAGTACCAGACACGAGATAGATCT---------CAAGGCCTTTTTCGAATAAGCCAATTCAT potato
94554 AAGAGAATGATTCGGGGTTCTTGCAGAGTGGAACCATGCAGTACCAGACACGAGATAGATCT---------CAAGGCCTTTTTCGAATAAGCCAATTCAT tomato
```

**Fig. 3** Alignment of four regions of the *ycf2* gene among the four Solanaceae chloroplast genomes showing insertion and deletion events. Nucleotides shown in *red* indicate base substitutions, *yellow* indicate the repeated sequence, and *green* indicate the start codon

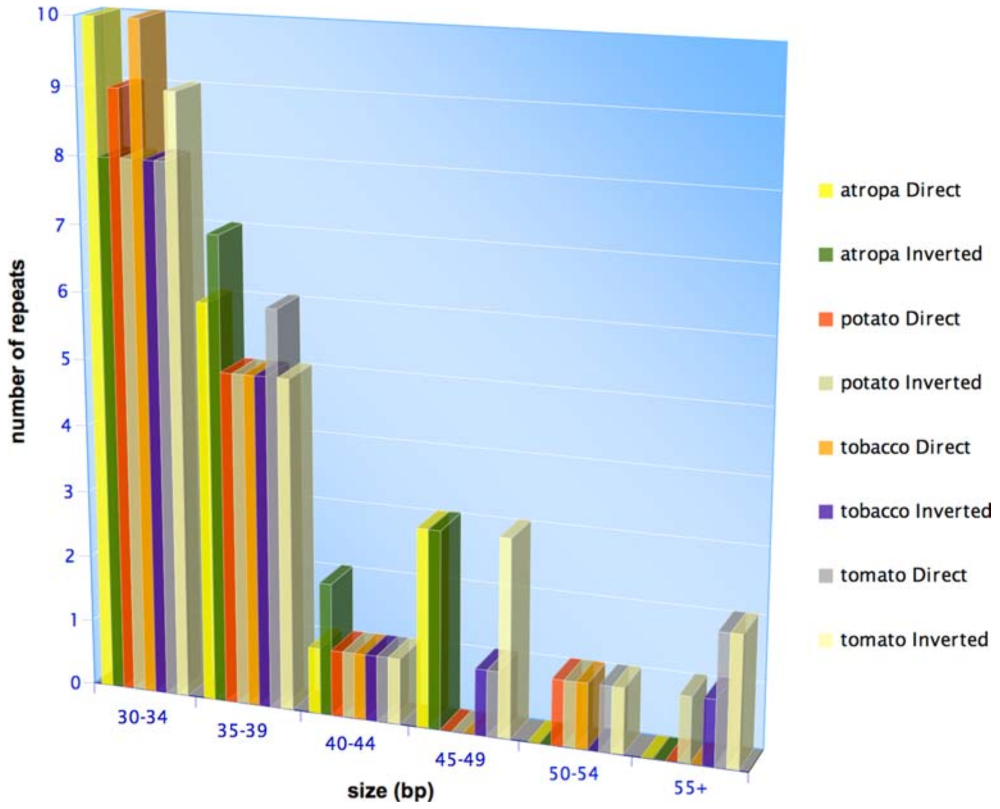

**Fig. 4** Histogram showing the number and type (direct or inverted) of repeated sequences ≥30 bp long with a sequence identity ≥90% in the four Solanaceae chloroplast genomes using REPuter (Kurtz et al. 2001)

transformation (Ruf et al. 2001). These studies point out the importance of choosing appropriate intergenic spacers for plastid transformation. The use of one of the regions between tobacco and tomato or potato with 100% sequence identity (Table 3) might have enhanced recombination efficiency and thereby increased the efficiency of plastid transformation. Alternatively, if species-specific vectors are used, then one could use any of the intergenic spacer regions for transgene integration.

In addition to providing insight into genome organization and evolution, availability of complete DNA sequence of chloroplast genomes should facilitate plastid genetic engineering. Thus far, transgenes have been stably integrated and expressed via the tobacco chloroplast genome to confer several useful agronomic traits, including insect resistance (DeCosa et al. 2001; McBride et al. 1995; Kota et al. 1999), herbicide resistance (Daniell et al. 1998; Iamtham and Day 2000), disease resistance (DeGray et al. 2001), drought tolerance (Lee et al. 2003), salt tolerance (Kumar et al. 2004a), phytoremediation (Ruiz et al. 2003), and cytoplasmic male sterility (Ruiz and Daniell 2005). The chloroplast has been used as a bioreactor to produce vaccine antigens (Daniell et al. 2001; Molina et al. 2004; Tregoning et al. 2003; Watson et al. 2004; Koya et al. 2005), human therapeutic proteins (Daniell et al. 2004a; Staub et al. 2000; Fernandez-San Millan et al. 2003; Grevich and Daniell 2005), industrial enzymes (Leelavathi et al. 2003), and biomaterials (Lossl et al. 2003; Guda et al. 2000; Vitanen et al. 2004). Although many successful examples of plastid engineering in tobacco have set a solid foundation for various future applications, this technology has not been extended to many of the major crops. Complete chloroplast genome sequences should provide valuable information on spacer regions for integration of transgenes at optimal sites via homologous recombination, as well as endogenous regulatory sequences for optimal expression of transgenes and should help in extending this technology to other useful crops.

## Evolutionary implications

Our comparisons of chloroplast genome organization between tomato and potato parallel earlier mapping studies of the nuclear genome of these important crop plants. Gene order of tomato and potato chloroplast genomes is identical, and this conservation extends to more distantly related genera (tobacco and *Atropa*) of Solanaceae. This is in contrast to the syntenic differences in the nuclear chromosomes of tomato and potato, which can be explained by three paracentric and two pericentric inversions (Bonierbale et al. 1988; Tanksley et al. 1992).

The analysis of repeated sequences in Solanaceae chloroplast genomes revealed 42 groups of repeats shared among various members of the family (Table 2, Fig. 1). Both direct and inverted repeats were identified. The origin of the repeats in the Solanaceae is not known, although replication slippage could be responsible for generating direct repeats. This mechanism has been

**Table 2** Tobacco repeats blasted against all four Solanaceae chloroplast genomes

| Repeat number | Size (bp) | Number of hits | Type of repeat | Location | Genomes |
|---|---|---|---|---|---|
| 1 | 30 | 2 | P | IGS(1 bp) - *trnS*-GCC | NAPT |
| 2 | 30 | 1 | F | IGS - (*psbC* - *trnS*-UGA)$^{N}$, Intron – (*clpP* exon 2 – *clpP* exon 3)$^{T}$ | NT |
| 3 | 30 | 1 | P | IGS(1 bp) - *trnS*-UGA | NAPT |
| 4 | 30 | 1 | P | Intron - (*ycf3* exon 2 - *ycf3* exon 3) | NAPT |
| 5 | 30 | 2 | P | *trnS*-GCU - IGS(1 bp), *trnS*-GGA - IGS(1 bp) | NAPT |
| 6 | 30 | 1 | F | Intron - (*clpP* exon 2 - *clpP* exon 3) | NA |
| 7 | 30 | 2 | F | *ycf2* | NAPT |
| 8 | 30 | 2 | P | *ycf2* | NA |
| 9 | 30 | 2 | F | IGS - (*rps12* 3′end - *trnV*-GAC) | NAPT |
| 10 | 30 | 2 | P | IGS - (*trnV*-GAC – *rps12* 3′end) | NAPT |
| 11 | 30 | 2 | F | *ycf2* | NAPT |
| 12 | 30 | 2 | F | *ycf2* | NAPT |
| 13 | 30 | 2 | F | *ycf2* | NA |
| 14 | 30 | 2 | F | *ycf2* | NAPT |
| 15 | 31 | 2 | F | IGS(2 bp) - *trnS*-GCU, IGS(1 bp) - *trnS*-GGA | NAPT |
| 16 | 31 | 1 | F | *trnG*-GCC - IGS(4 bp) | NAPT |
| 17 | 31 | 1 | F | IGS(2 bp) - *trnS*-UGA | NAPT |
| 18 | 31 | 1 | F | *trnG*-GCC - IGS(3 bp) | NAPT |
| 19 | 31 | 1 | F | Intron - (*rpl16* exon 1 - *rpl16* exon 2) | NAPT |
| 20 | 31 | 3 | F | IGS - (*rps12* 3′end - *trnV*-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 21 | 32 | 2 | P | IGS - (*trnH*-GUG - *psbA*) | N |
| 22 | 34 | 4 | P | IGS - (*rrn4.5* - *rrn5*) | NAPT |
| 23 | 34 | 4 | F | IGS - (*rrn4.5* - *rrn5*) | NAPT |
| 24 | 34 | 4 | F | IGS - (*rrn4.5* - *rrn5*) | NAPT |
| 25 | 34 | 4 | P | IGS - (*rrn4.5* - *rrn5*) | NAPT |
| 26 | 35 | 4 | P | IGS - (*ycf15* - *trnL*-CAA) | NAPT[a] |
| 27 | 35 | 4 | P | IGS - (*ycf15* - *trnL*-CAA) | NAPT[b] |
| 28 | 37 | 4 | F | *ycf2* | NAPT |
| 29 | 37 | 4 | P | *ycf2* | NAPT |
| 30 | 37 | 4 | P | *ycf2* | NAPT |
| 31 | 37 | 4 | P | *ycf2* | NAPT |
| 32 | 39 | 4 | F | Intron - (*ycf3* exon 2 - *ycf3* exon 3), IGS - (*rps12* 3′end - *trnV*-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 33 | 39 | 4 | F | Intron - (*ycf3* exon 2 - *ycf3* exon 3), IGS - (*rps12* 3′end - *trnV*-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 34 | 39 | 4 | F | Intron - (*ycf3* exon 2 - *ycf3* exon 3), IGS - (*rps12* 3′end - *trnV*-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 35 | 39 | 4 | P | Intron - (*ycf3* exon 2 - *ycf3* exon 3), IGS - (*rps12* 3′end - *trnV*-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 36 | 41 | 4 | F | Intron - (*ycf3* exon 2 - *ycf3* exon 3), IGS - (rps12 3′end - trnV-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 37 | 41 | 4 | P | Intron - (*ycf3* exon 2 - *ycf3* exon 3), IGS - (rps12 3′end - trnV-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 38 | 41 | 4 | P | Intron - (*ycf3* exon 2 - *ycf3* exon 3), IGS - (*rps12* 3′end - *trnV*-GAC) x2, Intron - (*ndhA* exon 1 - *ndhA* exon 2) | NAPT |
| 39 | 48 | 2 | P | IGS(47 bp) - *psbN*(1 bp) | NAP[c]T |
| 40 | 50 | 2 | F | *psaB*, *psaA* | NAPT |
| 41 | 50 | 2 | F | *psaB*, *psaA* | NAPT |
| 42 | 56 | 2 | P | Intron - (*petD* exon 1 - *petD* exon 2) | NAPT[d] |

Table includes blast hits at least 30 bp in size, a sequence identity ≥90%, and a bit-score of great than 40. Type of repeat is either (F)orward or (P)alandromic. Abbreviation for genomes are: $N$ = *Nicotiana* (tobacco) (Shinozaki et al. 1986), $A$ = *Atropa* (Schmitz-Linneweber et al. 2002), $P$ = potato, $T$ = tomato; *IGS* = intergenic spacer. See Fig. 1 for location of repeats on the gene map and supplementary data provided in the on-line version

[a] Blast hit is 4 bp shorter in tomato

[b] Blast hit is 4 bp shorter in tomato

[c] Blast hit is 17 bp shorter in potato

[d] Blast hit is 2 bp shorter in tomato

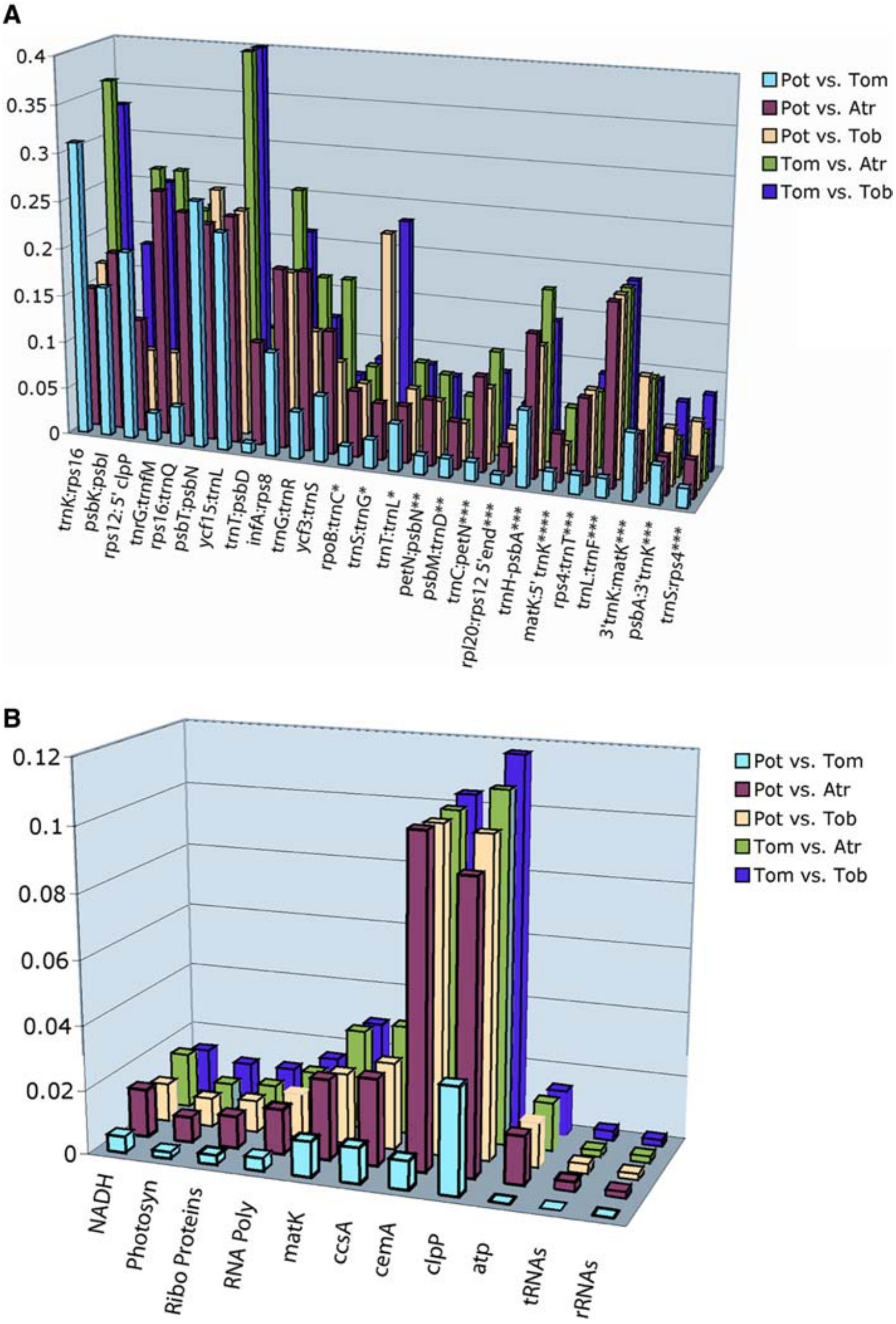

**Fig. 5** Histogram showing sequence divergence in pairwise comparisons among four Solanaceae chloroplast genomes for intergenic spacers (**a**) and coding regions (**b**). *Pot* potato, *Tom* tomato, *Atr Atropa*, and *Tob* tobacco. **a** Comparisons of 21 of the most variable intergenic regions. *, **, and *** indicate the tier 1, tier 2, and tier 3 regions reported in Shaw et al. (2005). The values plotted in this histogram come from Supplemental Table 2, which showed percent sequence identities for all intergenic spacers. The plotted values were converted from percent identity to sequence divergence on a scale from 0 to 1 and included on the *Y*-axis. **b** Sequence divergence of coding regions for the 11 different functional groups (Table 3)

suggested for chloroplast DNA (Palmer 1991) and evidence for replication slippage has been reported in the *Oenothera* chloroplast genome (Sears et al. 1996).

The fact that 37 of these 42 repeats are found in all four genomes examined suggests a high level of conservation of repeat structure. Furthermore, examination of the location of these repeats in the four genomes suggests that all of them occur in the same location, either in genes, introns or within intergenic spacers. This high level of conservation of both sequence identity and location suggests that these elements may play a functional role in the genome.

Except for the large inverted repeat, repeated sequences have generally been considered to be relatively uncommon in chloroplast genomes (Palmer 1991). One extraordinary exception is *Chlamydomonas*, which was estimated to have a genome comprised of more than 20% dispersed repeats (Maul et al. 2002). Dispersed repeats have also been identified in several families of flowering plants, including *Trachelium* (Cosner et al. 1997) (Campanulaceae), *Trifolium* (Milligan et al. 1989) (Fabaceae), wheat (Bowman and Dyer 1986; Howe 1985) (Poaceae), and *Oenothera* (Hupfer et al. 2000; Sears et al. 1996; Vomstein and Hachtel 1988) (Onagraceae). All of these

**Table 3** Intergenic spacer regions that are 100% identical in *Atropa*, tobacco, potato, and tomato or 100% identical to at least one other member of the Solanaceae

| Intergenic ID | Tob vs Atr | Tob vs Pot | Tob vs Tom | Atr vs Pot | Tom vs Pot | Tom vs Atr |
|---|---|---|---|---|---|---|
| *rps11*:*rpl36* | 100 | 100 | 100 | 100 | 100 | 100 |
| *rps12*_3'end:*rps7* | 100 | 100 | 100 | 100 | 100 | 100 |
| *trnA*-UGC:*trnI*-GAU | 100 | 100 | 100 | 100 | 100 | 100 |
| *ycf15*:*ycf2* | 100 | 100 | 100 | 100 | 100 | 100 |
| *trnV*-GAC:*rrn16* | 100 | 98 | 98 | 98 | 100 | 98 |
| *rrn4.5*:*rrn5* | 100 | 100 | 97 | 100 | 97 | 97 |
| *psbJ*:*psbL* | 96 | 96 | 96 | 100 | 100 | 100 |
| *trnA*-UGC:*rrn23* | 96 | 100 | 100 | 96 | 100 | 96 |
| *trnfM*-CAU:*rps14* | 100 | 97 | 97 | 97 | 100 | 97 |
| *trnN*-GUU:*ycf1* | 100 | 96 | 100 | 96 | 96 | 100 |
| *ycf1*:*trnN*-GUU | 100 | 96 | 100 | 96 | 96 | 100 |
| *rrn23*:*trnA*-UGC | 96 | 100 | 100 | 95 | 100 | 96 |
| *psbN*:*psbH* | 95 | 95 | 95 | 100 | 100 | 100 |
| *rpl23*:*trnI*-CAU | 97 | 97 | 97 | 97 | 100 | 97 |
| *rrn4.5*:*rrn23* | 100 | 95 | 95 | 95 | 100 | 95 |
| *rps8*:*rpl14* | 94 | 95 | 95 | 95 | 100 | 95 |
| *trnL*-UAG:*ccsA* | 95 | 94 | 94 | 95 | 100 | 95 |
| *trnD*-GUC:*trnY*-GUA | 94 | 94 | 94 | 94 | 100 | 94 |
| *ndhJ*:*ndhK* | 92 | 93 | 93 | 95 | 100 | 95 |
| *ndhD*:*psaC* | 93 | 93 | 93 | 94 | 100 | 94 |
| *rpoA*:*rps11* | 89 | 100 | 100 | 89 | 100 | 89 |
| *psbH*:*petB* | 95 | 92 | 92 | 92 | 100 | 92 |
| *rpoC2*:*rpoC1* | 95 | 92 | 92 | 91 | 100 | 93 |
| *rps14*:*psaB* | 95 | 91 | 91 | 91 | 100 | 92 |
| *trnI*-CAU:*ycf2* | 69 | 69 | 81 | 100 | 66 | 66 |

Names of genomes compared are abbreviated: *Pot* potato, *Tom* tomato, *Atr Atropa*, and *Tob* tobacco

**Table 4** Comparisons of sequence divergence of Solanaceae chloroplast genes among the 11 different functional groups

| Gene group | Length (bp) | Number of genes | Pot vs Tom | Pot vs Atr | Pot vs Tob | Tom vs Atr | Tom vs Tob | Atr vs Tob |
|---|---|---|---|---|---|---|---|---|
| NADH | 12,102 | 11 | 0.005 (0.001) | 0.015 (0.001) | 0.012 (0.001) | 0.017 (0.001) | 0.014 (0.001) | 0.013 (0.001) |
| Photosynthesis | 14,081 | 26 | 0.002 (0.000) | 0.008 (0.001) | 0.009 (0.001) | 0.009 (0.001) | 0.011 (0.001) | 0.008 (0.001) |
| Ribosomal protein | 10,207 | 22 | 0.003 (0.001) | 0.010 (0.001) | 0.010 (0.001) | 0.010 (0.001) | 0.011 (0.001) | 0.009 (0.001) |
| RNA polymerase | 10,473 | 4 | 0.004 (0.001) | 0.014 (0.001) | 0.014 (0.001) | 0.016 (0.001) | 0.016 (0.001) | 0.012 (0.001) |
| matK maturase | 1,530 | 1 | 0.011 | 0.025 | 0.022 | 0.031 | 0.029 | 0.017 |
| ccsA-cytochrome synthesis | 942 | 1 | 0.011 | 0.027 | 0.027 | 0.034 | 0.034 | 0.023 |
| cemA-envelope membrane protein | 690 | 1 | 0.009 | 0.102 | 0.101 | 0.102 | 0.104 | 0.010 |
| clpP-protease | 621 | 1 | 0.033 | 0.090 | 0.099 | 0.109 | 0.117 | 0.026 |
| atp synthase genes | 4,968 | 6 | 0.000 (0.000) | 0.015 (0.003) | 0.014 (0.003) | 0.015 (0.003) | 0.014 (0.003) | 0.015 (0.003) |
| TRNAs | 2,751 | 27 | 0.000 (0.000) | 0.003 (0.001) | 0.003 (0.001) | 0.002 (0.001) | 0.003 (0.001) | 0.003 (0.001) |
| RRNAs | 9,064 | 4 | 0.000 (0.000) | 0.002 (0.000) | 0.002 (0.001) | 0.002 (0.000) | 0.002 (0.001) | 0.002 (0.000) |

Standard errors are in parentheses. Pairwise distances were calculated using the Kimura 2-parameter model (Kimura 1980). Names of genomes compared are abbreviated: *Pot* potato, *Tom* tomato, *Atr Atropa*, and *Tob* tobacco

genomes have gene order changes, suggesting that the repeats may have played a role in these changes. The chloroplast genomes of Solanaceae are not rearranged yet they still have a substantial number of repeats. A similar comparison of repeat structure among three legume chloroplast genomes (Saski et al. 2005) also identified a substantial number of repeat elements. Thus, it is becoming evident that chloroplast genomes contain a substantial number of repeated sequences other than the inverted repeat. Additional studies are needed to assess the possible functional role of these repeat elements.

Intergenic spacer regions are the most widely used chloroplast markers for phylogenetic investigations at lower taxonomic levels in plants (Kelchner 2002; Raubeson and Jansen 2005; Shaw et al. 2005). Plant phylogeneticists have utilized these markers because IGS regions are considered more variable and therefore should provide more characters. Several early studies support this contention; however, other studies questioned the systematic utility of chloroplast intergenic spacer regions (see references in Kelchner 2002). Our first genome-wide comparisons of the levels of sequence conservation in the intergenic spacer regions of four Solanaceae chloroplast genomes (Table 3, Fig. 5a, and Supplemental Table 2) demonstrate a wide range of sequence divergence in different regions. Furthermore, comparisons of coding (Fig. 5b) and noncoding (Fig. 5a) regions generally support the contention that intergenic spacer regions are more variable and could

**Table 5** Differences observed by comparison of tomato chloroplast genome sequences with EST sequences obtained by BLAST search in Genbank

| Gene | Gene size (bp) | Sequence analyzed[a] | Number of variable sites | Variation type | Position(s)[b] | Amino acid change |
|---|---|---|---|---|---|---|
| *atpA* | 1,526 | 1–837 | 2 | C-A | 87 | T-T |
| | | | | G-A | 653 | G-E |
| *atpB* | 1,497 | 769–1497 | 2 | C-A | 954 | D-E |
| | | | | A-G | 1062 | R-R |
| *atpF* | 555 | 322–555 | 1 | G-A | 408 | A-A |
| *atpH* | 246 | 29–246 | 1 | A-C | 141 | G-G |
| *ndhG* | 531 | 229–531 | 4 | A-G | 362 | Y-C |
| | | | | G-C | 393 | Q-H |
| | | | | T-C | 455 | F-S |
| | | | | T-G | 494 | V-G |
| *ndhH* | 1,182 | 692–1015 | 2 | G-C | 927 | R-R |
| | | | | T-G | 928 | F-V |
| *psaB* | 2,205 | 1778–2198 | 2 | T-C | 2138 | F-S |
| | | | | G-A | 2146 | G-S |
| *psaJ* | 135 | 1–135 | 1 | C-U | 22 | L-F |
| *infA* | 105 | 1–105 | 1 | C-U | 46 | Y-H |
| *PsbC* | 1423 | 756–1423 | 4 | T-C | 1310 | F-L |
| | | | | A-C | 1323 | H-P |
| | | | | T-A | 1324 | |
| | | | | A-U | 1418 | N-Y |
| *rbcL* | 1,436 | 469–1436 | 1 | A-G | 494 | Y-C |
| *rpl14* | 369 | 1–339 | 2 | G-A | 31 | A-T |
| | | | | T-C | 254 | V-A |
| *rpl22* | 472 | 1–268 | 1 | A-C | 180 | A-A |
| *rpl23* | 282 | 1–282 | 2 | C-U | 71 | S-F |
| | | | | C-U | 89 | S-L |
| *rpl36* | 114 | 1–114 | 7 | T-G | 20 | V-G |
| | | | | T-G | 24 | R-R |
| | | | | T-C | 31 | C-R |
| | | | | T-G | 54 | R-R |
| | | | | T-A | 77 | I-N |
| | | | | T-G | 81 | C-W |
| | | | | T-G | 82 | S-A |
| *rpoA* | 1,014 | 1–594 | 3 | C-U | 65 | T-I |
| | | | | C-U | 200 | S-F |
| | | | | A-C | 594 | I-I |
| *rpoC2* | 4,179 | 2392–3283 | 10 | G-U | 2409 | Q-H |
| | | | | G-A | 2432 | R-Q |
| | | | | G-A | 2518 | V-I |
| | | | | G-C | 2606 | R-P |
| | | | | G-U | 2629 | V-L |
| | | | | C-A | 2652 | I-I |
| | | | | T-A | 2728 | I-I |
| | | | | G-A | 2785 | S-T |
| | | | | G-A | 2817 | G-R |
| | | | | T-G | 3192 | K-K |
| | | | | | | C-W |
| *rps7F* | 468 | 109–468 | 1 | C-G | 137 | A-G |
| *rps12* | 258 | 1–258 | 1 | C-U | 107 | S-L |
| *rps18* | 306 | 163–306 | 1 | T-G | 223 | L-V |
| *ycf1* | 1,140 | 10–628 | 2 | A-U | 603 | N-K |
| | | | | T-A | 604 | K-stop |
| *ycf1R* | 3,599 | 500–1094 | 1 | A-G | 751 | K-E |
| *ycf2* | 6,837 | 981–1726 | 1 | G-A | 1704 | K-K |

[a] Sequence based on the gene sequence, considering the first base of the initiation codon as 1

[b] Variable position is given in reference to the first base of the initiation codon of the gene sequence

provide more phylogenetically informative characters for phylogenetic studies at lower taxonomic levels. Shaw et al. (2005) recently compared the phylogenetic utility of 21 noncoding chloroplast DNA regions. In their study, they ranked these 21 regions into three tiers based on their phylogenetic utility with tier one being the most useful by calculating the number of potentially informative characters. Although our genome-wide comparisons are based on sequence divergence, our results agree with the relative ranking of these regions in the Solanaceae (Fig. 5a; number of asterisks by gene names indicate Shaw et al.'s tiers). However, our comparisons have identified several intergenic regions that have higher sequence divergence than the most variable tier 1 regions identified by Shaw et al. (2005). Thus, our genome-wide comparisons provide valuable new information for the plant systematics community about the potential phylogenetic utility of the chloroplast intergenic spacer regions.

**Table 6** Differences observed by comparison of potato chloroplast genome sequences with EST sequences obtained by BLAST search in Genbank

| Gene | Gene size | Sequence analyzed[a] | Number of variable sites | Variation type | Nucleotide position(s)[b] | Amino acid change |
|---|---|---|---|---|---|---|
| *atpA* | 1,525 | 435–1050 | 3 | C-U | 436 | P-S |
| | | | | G-A | 651 | G-G |
| | | | | C-U | 711 | Y-Y |
| *AtpB* | 1,497 | 564–1260 | 4 | A-C | 1158 | E-D |
| | | | | G-A | 1246 | E-R |
| | | | | A-G | 1247 | |
| | | | | G-A | 1248 | |
| *atpH* | 247 | 1–247 | 3 | G-U | 16 | A-S |
| | | | | T-C | 18 | |
| | | | | G-A | 76 | V-I |
| *petB* | 648 | 20–648 | 2 | G-U | 405 | G-G |
| | | | | C-U | 611 | P-L |
| *psaC* | 247 | 1–177 | 3 | T-C | 147 | V-V |
| | | | | T-C | 151 | C-R |
| | | | | G-A | 156 | K-K |
| *psbA* | 1,062 | 1–699 | 1 | C-U | 489 | I-I |
| *psbB* | 1527 | 856–1425 | 3 | C-G | 856 | R-G |
| | | | | C-U | 1389 | F-F |
| | | | | T-C | 1390 | F-L |
| *clpP* | 598 | 1–383 | 1 | G-A | 190 | V-I |
| *psbD* | 1,062 | 321–534 | 1 | T-G | 532 | A-A |
| *rbcL* | 1,436 | 886–1302 | 2 | G-U | 1255 | A-S |
| | | | | G-A | 1300 | G-R |
| *rpl16* | 405 | 10–405 | 5 | C-A | 65 | S-Y |
| | | | | A-U | 219 | P-P |
| | | | | C-U | 226 | L-L |
| | | | | C-G | 234 | P-P |
| | | | | A-C | 243 | T-T |
| *rpl23* | 282 | 1–282 | 2 | C-U | 71 | S-F |
| | | | | C-U | 89 | S-L |
| *rpl36* | 114 | 1–114 | 2 | C-U | 31 | R-C |
| | | | | G-U | 73 | L-V |
| *rpoA* | 1,014 | 298–798 | 4 | G-A | 420 | T-T |
| | | | | G-U | 597 | L-L |
| | | | | T-C | 780 | L-L |
| | | | | C-A | 789 | N-K |
| *rps19* | 93 | 1–93 | 1 | T-C | 69 | N-N |
| *ycf1R* | 5,669 | 647–1275 | 7 | T-G | 1080 | F-L |
| | | | | A-C | 1195 | K-Q |
| | | | | A-U | 1225 | T-S |
| | | | | T-G | 1246 | F-V |
| | | | | A-G | 1269 | G-G |
| | | | | C-A | 1273 | Q-T |
| | | | | A-C | 1274 | |

[a] Sequence based on the gene sequence, considering the first base of the initiation codon as 1

[b] Variable position is given in reference to the first base of the initiation codon of the gene sequence

Our comparisons of DNA and EST sequences identified a substantial number of differences. Many of these differences are not likely due to RNA editing because previous studies of both *Atropa* (Schmitz-Linneweber et al. 2002) and tobacco (Hirose et al. 1999) have indicated that these types of events are exclusively C-to-U edits. Our analyses of both potato and tomato sequences (Tables 5, 6) showed a lower number of C-to-U changes than previously observed for these species (Hirose et al. 1999; Schmitz-Linneweber et al. 2002). In addition, none of the C-to-U conversions observed in potato and tomato were conserved with respect to the previous observations in tobacco and *Atropa*. It is more likely that the differences observed between the DNA and EST sequences are due to polymorphisms within these species, or even errors in the EST sequences. However, if future studies in the Solanaceae confirm that these differences are real and due to RNA editing, then it is possible that there has been a loss of conserved editing sites in potato and tomato. Evolutionary loss of RNA editing sites has been previously observed and could possibly be due to a decrease in the effect of RNA-editing enzymes (Wolf et al. 2004). Additionally, a considerable number of variable sites other than C-to-U conversions were observed in tomato and potato, suggesting that these chloroplast genomes may be accumulating considerable amounts of nucleotide substitutions, and some of the genes accumulate more variable sites than others. This has been previously observed in several chloroplast genes, such as *petL* and *ndh* genes, which have a high frequency of RNA editing (Fiebig et al. 2004). This suggests that, even though the chloroplast genome is relatively highly conserved among species, much of its variability could also be accounted for at the transcript level.

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