AMPLIFICATION AND SEQUENCING OF SPACER REGION BETWEEN TWO tRNA GENES AND ITS FLANKING REGION IN THE CHLOROPLAST GENOME OF *Centella asiatica* L.

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DECLARATION

I hereby declare that this thesis entitled "**Amplification and sequencing of spacer region between two tRNA genes and its flanking region in the chloroplast genome of *Centella asiatica* L.**" is a bonofide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled **"Amplification and sequencing of spacer region between two tRNA genes and its flanking region in the chloroplast genome of *Centella asiatica* L."** is a record of research work done independently by Ms. Manju Elizabeth, P. (2004-11-31) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Dedicated to
Appachan, Ammachi and to My Teachers

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MANJU ELIZABETH, P.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

LIST OF PLATES

LIST OF ABBREVIATIONS

A	adenine
aadA	selection marker gene encoding aminoglycoside adenine transferase
BA	betaine aldehyde
BADH	betaine aldehyde dehydrogenase
BME	β- Mercaptoethanol
BLAST	Basic Local Alignment Search Tool
BLASTN	nucleotide-nucleotide BLAST
BLASTX	translated nucleotide-protein BLAST
bp	base pair
C	cytosine
cDNA	complementary DNA
cpDNA	chloroplast DNA
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	deoxy ribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
e PCR	electronic PCR
EPSPS	Enyl Pyruvyl Shikimate Phosphate Synthase
G	guanine
GFP	green fluorescent protein
IR	inverted repeat
kb	kilobase
kg	kilogram
LSC	long single copy
M	Molar
matK	maturase

mM	millimolar
m RNA	messenger RNA
NCBI	National Centre for Biotechnology Information
npt	neomycin phophotransferase
nt	nucleotide
PCR	Polymerase Chain Reaction
Pfu	*Pyrococcus furiosus*
ptDNA	plastid DNA
PVP	Poly Vinyl Pyrrolidone
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SSC	short single copy
T	thymine
TAE	Tris acetate EDTA
TE	Tris-EDTA
Tm	melting temperature
Tris	Tris (hydroxy methyl) aminomethane
Tris-HCl	Tris (hydroxy methyl) aminomethane hydrochloride
tRNA	transfer RNA
trnA	tRNA alanine
trnE	t RNA glutamic acid
trnF	t RNA phenyl alanine
trnfMet	t RNA formyl methionine
trnG	t RNA glucine
trnI	tRNa isoleucine
trnK	tRNA lysine

trnL	t RNA leucine
trnS	t RNA serine
trnT	t RNA threonine
trnQ	t RNA glutamine
µl	microlitre
µg	microgram

Introduction

1. INTRODUCTION

Centella asiatica L, commonly known as Indian Pennywort belonging to the family Apiaceae, native to India and South East Asia contains a blend of compounds including three triterpenes (asiatic acid, madecassic acid and asiaticoside) that have antioxidant properties and tissue regenerative property by stimulating collagen synthesis. Asiaticoside has a large demand world wide because it is widely used in many preparations. The triterpene asiaticoside content present in the Indian cultivars varies from 0.006-0.114 per cent. For the commercial extraction, the asiaticoside content should be at least three per cent. Hence, there is a need to improve the asiaticoside content in the plant.

Engineering of chloroplast genome is an evolving approach for crop improvement by circumventing the bottlenecks of nuclear transformation. Chloroplast genome is maternally inherited. Hence, the apprehensions of gene flow through pollen can be avoided through chloroplast transformation. In a typical plant cell a single chloroplast gene is represented at least 10,000 times. Hence, a high level of expression can be expected if the gene of interest is inserted into the plastome. In chloroplast transformation gene of interest can be targeted to the precise region of plastid genome. Target oriented integration is facilitated by a phenomenon called homologous recombination which is achieved by placing suitable flanking sequence on either side of the foreign piece of DNA in the vector. The gene of interest should be inserted into the plastome without disrupting the functions of the genes in the chloroplast. Insertion of foreign DNA into the non coding region such as spacer sequence between two genes is the best option. The major task towards chloroplast transformation is the development of a species specific vector having a region for homologous recombination. The plastid transformation technology has been

developed only in a few crop plants including tobacco, potato, tomato, *Arabidopsis*, rice, oilseed rape, *Lesquerella*, soybean, cotton, carrot and petunia.

The major challenges in chloroplast transformation include lack of homologous species-specific chloroplast transformation vectors containing suitable selectable markers, ability to regulate transgene expression in developing plastids. The chloroplast transformation vector consists of the gene of interest, sequence for homologous recombination, selectable marker and promoter sequence. The sequence for homologous recombination thus includes the spacer sequence where the gene is to be introduced and its flanking region for homologous recombination. To construct a vector for homologous recombination, knowledge about the sequence of the transforming plastid is necessary. Complete chloroplast genome of 79 organisms have been sequenced so far. The complete chloroplast genome of *C. asiatica* has not yet been sequenced. Hence it is needed to locate the spacer region between two genes along with its flanking region for insertion of the foreign gene and homologous recombination. This can be achieved either by directly sequencing the restriction digested fragment of chloroplast genome or by amplifying a fragment using heterologous primers.

The present study aims at sequencing the spacer region between two tRNA genes and its flanking region in the chloroplast genome of *C. asiatica* which may help in developing a vector for introducing the gene which can improve the asiaticoside content to commercially feasible level.

Review of Literature

2. REVIEW OF LITERATURE

Chloroplast transformation is an emerging alternative to nuclear transformation. Many limitations of nuclear transformation can be overcome by chloroplast genetic engineering. Expression of foreign genes in chloroplast is an attractive proposition because of precise targeting of defined fragment of DNA, maternal inheritance which limits the gene flow through pollen (Scott and Wilkinson, 1999), high levels of expression due to high gene copy number. The gene product is retained in the plastid and can be targeted to a specific compartment such as the internal space of the chloroplast. Chloroplasts have the ability to express multiple genes from a polycistronic mRNA, allowing the pyramiding of genes. Chloroplast transformation permits to insert several transgenes under the control of one promoter.

2.1CHLOROPLAST IN PLANTS

Plastids are plant-specific organelles which, have evolved from a prokaryotic, ancestor. The plastid genome has regulatory features reminiscent of prokaryotic genome, consistent with the endosymbiotic theory for evolution of plastids. However, more eukaryotic features such as splicing and RNA editing also occur (Sugiura, 1995). During plant cell differentiation, the undifferentiated proplastid found in meristematic cells of the shoot and the root can differentiate into a number of specialized plastid types. Chloroplast is the site of photosynthesis, where light energy in photons is converted into chemical energy, *via* redox reactions including inorganic carbon fixation at Calvin's cycle, finally yielding energy rich carbohydrate molecules (Lee *et al.*, 2006a).

2.2 CHLOROPLAST GENOME

Of the three compartments in the higher plant cell that contain their own DNA, the chloroplast harbors the least complex genetic material. Chiba (1951)

suggested that chloroplasts from the moss *Selaginella* and two flowering plant species contained DNA. Ris and Plaut (1962) demonstrated cytologically the presence of DNA in chloroplasts of *Chlamydomonas.* Bedbrook and Bogorad (1976) reported the first physical map for maize chloroplast. The first plant chloroplast genome completely sequenced was liverwort (*Marchantia polymorpha*) genome (Ohyama *et al.*, 1986). The rice (*Oryza sativa*) chloroplast genome sequence from Nipponnbare cultivar was published in 1989 (Hiratsuka *et al.*, 1989) and was reported to have a length of 134,525bp. Chloroplast DNA (cpDNA) is arranged into protein/DNA complexes that are similar to bacteria and are called cp-nucleoids or cp-nuclei. Proteins bound to DNA may determine the structure of the nucleoid and influence DNA function as a template for either transcription or replication (Kuroiwa, 1991). The gene order of tobacco chloroplast genome is found in most other angiosperms and also represents the ancestral gene order of vascular plants (Palmer, 1991). Therefore, tobacco DNA has often served as a reference for chloroplast genomes. The estimated coding capacity of the plastome, including open reading frames and known genes, is approximately 140 genes, most of which are components of the organellar transcriptional and translational apparatus or are involved in photosynthesis (Heinhorst and Cannon, 1993). The vast majority of plastid proteins are encoded in the nucleus and imported post-translationally into the plastid (Robinson *et al.*, 1998). Chloroplast contains 10 to 20 per cent of the total DNA in plant cell (Day, 2001).

With the development of recombinant DNA technologies, chloroplast DNA was selected as one of the first candidates for genome sequencing. Gene content and polycistronic transcription units of the chloroplast genome are also largely conserved in land plants (Lee *et al.*, 2004). Chloroplast genome of 79 organisms has been completely sequenced so far (*http://www.bch.umontreal.ca*).

2.3 CHLOROPLAST GENOME ORGANIZATION

Chloroplasts of higher plants are highly polyploid, with up to 50,000 identical copies of an approximately 150kb genome being present in a single cell and up to 900 genome copies in a single chloroplast (Bendich, 1987). The chloroplast maintains a highly conserved organization (Raubeson and Jansen, 2005) with most land plant genomes composed of a single circular chromosome with a quadrapite structure that includes two copies of an inverted repeat that separates the large and small single copy regions (Saski *et al.*, 2005). Although the genetic map of chloroplast genome is circular, alternative conformations, such as linear molecules and concatemers, also may exist *in vivo* (Oldenburg and Bendich, 2004). Ruhlman *et al.* (2006) sequenced the complete plastid genome of carrot. The complete plastid genome is 155,911bp long with 115 unique genes and 21 duplicated genes within the inverted repeat (IR) region. There are four ribosomal RNA (rRNA), 30 distinct transfer RNA (tRNA) genes, 18 intron containing genes, 12 direct repeats and two inverted repeats. This is the first sequenced plastid genome of the family Apiaceae and only the second published genome sequence of the species-rich euasterid II clade.

2.3.1 RNA genes in Chloroplast Genome

Among the chloroplast gene products identified to date are rRNAs (Whitfeld *et al.*, 1978) and some ribosomal proteins (Mets and Bogorad, 1971) as well as a virtually complete set of tRNAs (Driesel *et al.*, 1979) and elongation factors Tu and G (Tiboni *et al.*, 1978).

2.3.2 tRNA genes in Chloroplast Genome

Nuclear gene products include a number of plastid ribosomal proteins (Mets and Bogorad, 1971) as well as aminoacyl tRNA synthetase (Krauspe and Parthier, 1973). Chloroplast DNA codes for a set of tRNAs, probably all of the species

necessary for the protein synthesis of 70S ribosomes in chloroplast (Driesel *et al.*, 1979). There are greater sequence differences between the chloroplast and nuclear tRNA genes than between chloroplast and bacterial genes. This guess is based on heterologous hybridization data (Steinmetz and Weil, 1976) as well as hetero amino acylation studies (Guillemaut *et al.*, 1975). Chloroplast tRNAs show higher sequence homologies with bacterial tRNAs than eukaryotic cytoplasmic tRNAs (Sprinzl and Gauss, 1983). In contrast to prokaryotic tRNA genes, no plastid tRNA gene codes for its 3' CCA end, although in several cases the first C is present. This element is added post transcriptionally (Mache and Mache, 2001). The plastid $tRNA^{Glu}$ mediate the switch in RNA polymerase usage from nuclear encoded plastid RNA polymerase (NEP) to plastid encoded plastid RNA polymerase (PEP) during chloroplast development (Hanaoka *et al.*, 2005).

2.3.3 Non Coding Regions in Chloroplast Genome

Chloroplast DNA is an extremely valuable molecule for studying phylogenetic relationships between closely related species (Clegg *et al.*, 1991). Non coding regions evolve faster than do coding regions (Clegg and Zurawski, 1991). Most of the evolutionary hotspots that show high frequencies of indel mutations and base substitutions are concentrated on intergenic spacers that lack the polycistronic transcription units (Palmer, 1991). The *trnL* (UUA) intron and the intergenic spacer between the *trnL* (UUA) 3' and the *trnF* (GAA) gene seem to be well suited for inferring plant phylogenies between closely related taxa (Gielly and Taberlet, 1994). Some recent studies have revealed genetic variation between populations based on RFLPs of the total chloroplast genome (Schaal and Leverich, 1996) or on specific sequences, such as non coding spacers between several tRNAs of the chloroplast DNA (Demesure *et al.*, 1996).

Due to weak functional constraints, the spacer is evolving rapidly (Chiang *et al.*, 1998). The divergence levels of chloroplast genes are influenced not only by the location of the genes on the chloroplast genome but also by the functional

constraints of the genes (Kim and Lee, 2004a). According to Berger *et al.* (2005) a 437bp sequence present in tobacco chloroplast genome in the intergenic region of the genes *trnE* and *trnT* but completely absent in the tomato chloroplast DNA. Won and Renner (2005) studied the *trnT-trnF* spacer region in the large single copy region of the chloroplast genome of Gnetales. It consists of the *trnL* intron, a group I intron, and the *trnT-trnL* and *trnL-trnF* intergenic spacers. The *trnL* intron has a conserved secondary structure and contains elements that are homologous across land plants. The spacers are so variable in length and composition that homology cannot be found even among the three genera of Gnetales. Palindromic sequences that form hairpin structures were detected in the *trnL-trnF* spacer, but neither spacer contained promoter elements for the tRNA genes. The absence of promoters, presence of hairpin structures in the *trnL-trnF* spacer, and high sequence variation in both spacers together suggest that *trnT* and *trnF* are independently transcribed.. Small *et al.* (2005) used amplification of the non coding chloroplast DNA regions *trnS* (GCU)-*trnG* (UUC) spacer and *trnG* (UUC) intron for phylogenetic studies in lycophytes and monilophytes.

2.3.4 Inverted Repeat Regions in Chloroplast Genome

The intra molecular recombination between short repeats is the primary mechanism for generating a large inversion. The complete chloroplast DNA sequences in various land plants with large inversions show that short repeats are frequently situated closely to the end points of inversions (Ogihara *et al.*, 1988). Some large inversion end points are also associated with tRNA genes rather than recognizable specific short repeats (Shimada and Sugiura, 1989). The relative abundance of approximately oriented and located short dispersed repeats is often considered to be a major factor in determining chloroplast inversions (Palmer, 1991). Small inversions from four bp to 100bp may generate a large number of sequence differences (Kelchner and Wendel, 1996). A salient feature of the plastid genome in most of the higher plant species is duplication of a large region (about 25 kb) in an inverted orientation (Wakasugi *et al.*, 2001).

Large expansions and contractions of inverted repeat (IR) regions often create the length variation of chloroplast genomes in different groups of plants. The expansion or contractions of IR as observed in the IR or SSC borders are probably mediated by intra molecular recombination between two short direct repeat sequences that frequently occur within the genes located at the borders (Kim and Lee, 2004a). The small inversions were scattered over the chloroplast genome including the IR, SSC and LSC regions. Several small inversions were uncovered in chloroplast genomes even though they shared the same overall gene order. The majority of these small inversions were located within 100bp downstream of the 3' ends of genes. All had inverted repeat sequences, ranging from 11 to 24bp at their ends. Such small inversions from stem-loop hairpin structures that usually have function of stabilizing the corresponding mRNA molecules (Kim and Lee, 2004b). The carrot plastid genome contains a number of dispersed direct and inverted repeats scattered throughout coding and non coding regions (Ruhlman *et al.*, 2006).

2.3.5 Insertions and Deletions in Chloroplast Genome

Analysis of the complete sequences of tomato, potato, tobacco and *Atropa* chloroplast genomes revealed significant insertions and deletions within certain coding regions or regulatory sequences (eg. deletion of repeated sequences within 16S rRNA, ycf2 or ribosomal binding sites in ycf2). RNA, photosynthesis and ATP synthase genes are the least divergent and the most divergent genes are *clpP*, *cemA*, *ccsA* and *matK* (Daniell *et al.*, 2006).

2.4 COPY NUMBER OF PLASTIDS

The number of plastome copies per organelle and cell varies in a development and tissue specific manner. The plastome copies are greatly amplified in mesophyll cells of the developing leaf and can reach values of more

than 20,000 per cell (Lawrence and Possingham, 1986). In tobacco, the meristematic cells contain 10 to 14 proplastids, each containing one to two nucleoids per organelle, whereas leaf cells may contain 100 chloroplasts, with 10 to 14 nucleoids each, about 10,000 genome copies per cell. The number of plastids and pt DNA copies per cell is highly variable depending on the cell type (Bendich, 1987).

2.5 CHLOROPLAST DNA REPLICATION

Synthesis of nuclear and chloroplast DNA are not directly coupled. Nuclear DNA is replicated only once during the cell cycle, plastome replication does not seem to be limited to the synthesis phase (Lawrence and Possingham, 1986). The plastome is replicated by nuclear encoded proteins and its copy number seems to be highly regulated by the cell in a tissue specific and developmental manner (Heinhorst and Cannon, 1993). When the number of plastids per cell is low, plastid division is probably controlled by the cell cycle. When the cells contain a large number of plastids, they do not divide synchronously. Division would occur only when the plastid volume has reached a defined value. The number of nucleoids present in the plastid is probably another factor (Mache and Mache, 2001).

2.6 CHLOROPLAST GENOME INHERITANCE

Chloroplast genomes are inherited maternally in most angiosperms (Bricky, 1995). This is due to either exclusion of plastids by unequal cell divisions upon pollen grain mitosis or degradation of plastids and plastid DNA (ptDNA) during the male gametophyte development (Hagemann, 1992). Consequently, the sperm cell fertilizing the egg is free of plastids and pt DNA; hence the zygote receives its plastids exclusively from the pollen. Maternal inheritance of novel genes is highly desirable in situations where out crossing between crops is a concern (Daniell *et al.*, 1998). Studies using classic genetics as well as RFLP analysis

have demonstrated that rye, unlike most flowering plants, has biparental inheritance of both chloroplast and mitochondria (Mogensen and Rusche, 2000). Aksyonova *et al.* (2005) reported heteroplasmy and paternally oriented shift of the organellar DNA composition in barley-wheat hybrids during backcrosses with wheat parents.

2.7 ORGANELLAR GENES IN NUCLEUS

In rice it was reported that a total of sixteen chloroplast sequences (about 22 kb), ranging from 32bp to 6.8 kb in length were dispersed throughout the mitochondrial genome (Nakazono and Hirai, 1993). The precise molecular mechanism of DNA escape or transport out of the chloroplast into the nucleus currently remains unknown. According to Stegemann *et al.* (2003) besides an active transposition mechanism several possibilities of a more passive transfer can be envisaged such as occasional slippage of DNA molecules out of the chloroplast during organelle division, release of plastid DNA after chloroplast degradation or, even accidental uptake of organelle by the nucleus as observed microscopically for plant mitochondria (Yu and Russell, 1994). The Arabidopsis genome revealed 17 different tRNA and intron containing insertions of recently integrated chloroplast DNA in nuclear chromosomes indicating that recombination between nuclear DNA and escaped chloroplast DNA (The Arabidopsis Genome Initiative, 2000). Gene transfer from the chloroplast to the nucleus has occurred over evolutionary time. Functional gene establishment in the nucleus is rare, but DNA transfer without functionality is presumably more frequent. Huang *et al.* (2003) provided a quantitative estimate of one transposition event in about 16,000 pollen grains for the frequency of transfer of cp DNA to the nucleus in tobacco. Lister *et al.* (2003) found that DNA transfer from chloroplast to nucleus is much rarer in *Chlamydomonas* than in tobacco. Chromosome 10 of rice genome contains a 33 kb insertion of chloroplast DNA in addition to a 131 kb insertion representing nearly the entire plastid genome (The Rice Chromosome 10 sequencing Consortium, 2003).

2.8 CHLOROPLAST TRANSFORMATION

The first chloroplast transformation by biolistic methods was reported by Boynton *et al.* (1988) in *Chlamydomonas reinhardtii.* Engineering foreign genes (whose products are functional within organelles) through organelle genome instead of the nuclear genome could be energy wise economical for the cell, since synthesis and import of precursor proteins are highly energy consuming processes (Schindler and Soll, 1986). Transformation frequencies were maximal when the recipient cells were grown in 5'- fluorodeoxy uridine (FdUrd) before bombardment to reduce the chloroplast genome copy number (Wurtz *et al.*, 1977) and when sequence heterolog between the donor and recipient DNAs was minimized.

There are techniques available to target foreign gene products into chloroplasts (Cheung *et al.*, 1988) of the higher plants by way of the nuclear genome. Transformation of the plastid genome has several advantages over nuclear transformation. Stable plastid transformation was first reported for tobacco by Svab *et al.* (1990), who introduced spectinomycin resistance to tobacco. Although, plastid transformation has been accomplished *via* organogenesis in a number of dicotyledonous plant species including tobacco (Svab and Maliga, 1993), tomato (Ruf *et al*, 2001), *Arabidopsis* (Sikdar *et al.*, 1998), *Lesquerella* (Skarjinskaia *et al.*, 2003), oilseed rape (Hou *et al.*, 2003), petunia (Zubko *et al.*, 2004) potato (Nguyen *et al.*, 2005), and lettuce (Lelivelt *et al.*, 2005), this technology has proven to be highly efficient only in tobacco. Plastid transformation has been recently accomplished in several dicotyledonous crops, including soybean, carrot, and cotton, *via* somatic embryogenesis (Dufourmantel *et al.*, 2005). Lack of chloroplast genome sequence to provide 100 per cent homologous species specific chloroplast transformation vectors, containing suitable selectable markers and endogenous regulatory elements, is one of the major limitations to extend this concept to other useful crops (Daniell *et al.*,

2005a). According to Lee *et al.* (2006b) major obstacles to the extension of plastid transformation technology to crop plants *via* somatic regeneration include: (i) the difficulty of expressing transgenes in non-green plastids, in which gene expression and regulation systems are quite distinct from those of mature green chloroplasts, and (ii) inability to generate homoplastomic plants *via* subsequent rounds of regeneration, using leaves as explants.

2.8.1 Homologous Recombination

In *Chlamydomonas* an almost complete replacement of a continuous region of the plastid genome by the corresponding DNA fragment from the donor plasmid is the most common integration event and remarkably, a plastid donor/recipient homology (*C. reinhardii* by *C. smithii* plastid DNA and *vice versa*) decreased the integration frequency by 10 to 100 times (Newman *et al.*, 1990). Since gene integration into the plastome occurs *via* homologous recombination (Svab *et al.*, 1990) there are no position effects as normally experienced with random insertion of transgenes in nuclear transformation. Gene silencing does not occur in plastids and therefore transgene expression is stable in progeny of transplastomic plants. RecA mediated recombination system exists in chloroplasts (Cerutti *et al.*, 1995), a probable mechanism, which maintains the uniformity of the plastid DNA population, also drives the homologous integration events (Fejes *et al.*, 1990). Recombination between homeologous nucleotide sequences is influenced by degree of DNA homology that is monitored by the recombination/repair enzymes. A central component of the system that promotes homologous recombination in bacteria is the RecA protein and its functional homologs in eukaryotes (Camerini-otero and Hsieh, 1995). RecA protein responsible for facilitating recombination steps involving DNA pairing, strand transfer and branch migration. The presence and activity of a RecA homolog has also been observed in higher plant plastids (Cerutti and Jagendorf, 1993). The RecA mediated DNA strand exchange reaction *in vitro* readily bypasses short internal regions of heterology up to about 100bp (Morel *et al.*, 1994). The lack of

internal non homologous regions longer than 100bp that might have posed a barrier to branch migration and in contrast, the blocking effect of the extensive non homologous vector DNA, is the most plausible explanation of the high frequency of integration of both internal and peripheral donor plastid DNA markers observed. The most potent inhibitor of recombination between moderately diverged nucleotide sequences is the mismatch repair system (Modrich and Lahue, 1996). Even one per cent nucleotide sequence difference causes an order of magnitude reduction in the frequency of DNA recombination in various genetic systems (Elliott *et al.*, 1998). A similar observation was reported in the case of interspecific plastid transformation in *Chlamydomonas* (Newman *et al.*, 1990), although the involvement of a recombination hot spot can conceal the effect (Newman *et al.*, 1992). A characteristic feature of the plastid genome is its high recombinogenic activity, best demonstrated in the inverted repeat region as continuous flip-flop recombination and copy correction by gene conversion (Goulding *et al.*, 1996). When foreign genes are integrate into chloroplast DNA by homologous recombination, bacterial vector sequences are excluded and are not present in genetically modified chloroplast genomes (Day, 2001). Recent work has established that plastid transformation often occurs *via* a two step recombination mechanism: a first recombination event in one flanking sequence results in a co-integrate that is, a plastid genome with the entire transformation vector integrated which sooner or later resolved by a second cross over in the other flanking sequence block (Klaus *et al.*, 2004).

2.8.1.1 Length of Homologous Sequence for Recombination

The minimum length of homologous DNA required for efficient recombination varies from 23bp to 90bp in prokaryotes (Shen and Huang, 1986) to between 200bp to 400bp in eukaryotic nuclear DNA (Bollag *et al.*, 1989), although recombination between homologous sequences as short as 200bp has been observed in several system (Watt *et al.*, 1985). According to Staub and Maliga (1992) greater than 400bp of homologous sequence on each side of the

construct is generally used to obtain chloroplast transformants at a reasonable frequency. Longer flanks appear to be beneficial, but no correlation between size of the homologous region and transformation frequency has been established till date (Bock, 2001). According to Daniell *et al.* (2005b) approximately one kb of homologous flanking region is adequate to facilitate recombination.

2.8.2 Methods for Chloroplast Transformation

2.8.2.1 Biolistics

Biolistics is the most widely used and effective method of transforming chloroplasts due to its high efficiency rate, rapid regeneration of transformed tissue. The invention of the gene gun and biolistic technology provided the opportunity to introduce foreign DNA into living cells (Klein *et al.*, 1987). Tungsten or gold particles coated with plasmid DNA are shot through the chloroplast envelope by a helium powered gun.

2.8.2.2 PEG Mediated Transformation

Protoplasts take up DNA in the presence of PEG and changes in the plasma membrane allow DNA to penetrate and move into the cytoplasm. The foreign DNA is transported by some unknown means from the cytoplasm into the chloroplast, where it may be integrated into the genome (Golds *et al.*, 1993). *N. plumbaginifolia* protoplasts were directly transformed by PEG treatment with a cloned 16S rRNA gene isolated from a double antibiotic resistant *N. tabaccum* plastid mutant (O'Neill *et al.*, 1993).

2.8.2.3 Galistan Expansion femtosyringe

This is a novel approach involves the microinjection of DNA into chloroplast (Knoblauch *et al.*, 1999) and is not widely used. The heat-induced expansion of a liquid metal, galistan, within a glass syringe forces the transformation plasmid DNA through a capillary tip with a diameter of approximately 0.1 mm.

2.3.5 Vectors for Chloroplast Transformation

Stable chloroplast transformation depends on the integration of the foreign DNA into the chloroplast genome by homologous recombination; therefore the foreign gene that is being introduced must be flanked by sequences homologous to the chloroplast genome (Staub and Maliga, 1992). Chloroplast genes are transcribed by chloroplast-specific promoters and use chloroplast specific termination signals. Most chloroplast genes are transcribed as operons. This allows two open reading frames to be inserted into a vector in sequence under the same promoter. The selectable marker and the gene of interest are placed between the promoter and the terminator which are flanked by the 5' and 3' untranslated regions. Persistence of the unselected genome may have occurred due to integration into different plastid genomes followed by co replication with the selected genome population in plastid nucleoids (Sato *et al.*, 1993) or as part of multimeric plastid genomes that could undergo subsequent resolution to the monomer form (Lilly *et al.*, 2001).

The basic chloroplast specific expression cassette is comprised of a promoter, selectable marker, and 5'/3' regulatory sequences to enhance the efficiency of transcription and translation of the gene. The chloroplast specific promoters and regulator elements are amplified from the total cellular DNA of tobacco using specific primers designed on the basis of the sequence information available for the chloroplast genome of tobacco. The chloroplast specific expression cassette is cloned into a unique site in between the flanking sequences. Approximately one kb of homologous flanking region is adequate to facilitate recombination and site of insertion should be the intergenic spacer (Daniell *et al.*, 2005b).

Zoubenko *et al* (1994) used pPRV plasmids vectors for targeted insertion of foreign genes into the tobacco ptDNA. The vectors are based on the pUC119 plasmid which replicates in *E. coli* but not in plastids. The spectinomycin resistance (*aadA*) gene and a multiple cloning site (MCS) are flanked by 1.8-kb and 1.2-kb pt DNA sequences. The pPRV vector series targets insertions between the divergently transcribed *trnV* gene and the *rps12/7* operon. The lack of read through transcription of appropriately oriented transgenes makes the vectors an ideal choice for the study of transgene promoter activity. Sikdar *et al.* (1998) developed an *Arabidopsis* plastid transformation vector pGS31A, a derivative of pBlue-script KS(+) phagemid vector. The vector carries a two kb *Hind*III – *Eco*R1 Arabidopsis pt DNA fragment containing the 5' end of the 16S rRNA gene, *trnV* and part of the *rps12/7* operon. During construction of the pGS31A plasmid, the *Hind*III site has been removed. The vector carries a selectable spectinomycin resistance (*aadA*) gene, which was excised as a *Sac*1/*Bsp*H1 fragment from plasmid pZS179 (Svab and Maliga, 1993) and cloned into the *Hinc*II site of the cloned pt DNA fragment. In vector pGS31A, the *aadA* gene coding region is transcribed from a synthetic promoter consisting of the promoter of the tobacco rRNA operon fused with a synthetic ribosome binding site (*Prrn*).

The *aadA* mRNA is stabilized by transcriptionally fusing sequences downstream of the coding region with the 3' untranslated region of the tobacco plastid *psbA* gene (*TpsbA*). Kanevski *et al.* (1999) cloned a 5.3 kb *Pvu*II/*Xho*1 DNA fragment of the tobacco plastid DNA (sites at nucleotides 55147 and 60484, Shinozaki *et al.*, 1986) in *Ec1136*II/*Xho*1 digested pBlue-script KS(+) phagemid (Stratagene) to obtain plasmid pIK28. Linker ligation was used to convert the Xba1 site at nucleotide 59234 (Shinozaki *et al.*, 1986) into a *Hind*III site (5'CAAGCTTG3') and *Acc*1 site at nucleotide position 59026 (Shinozaki *et al.*, 1986) into an *Xho*1 site (5'GCTCTAGAGC3') to obtain plasmid pIK76. Plastid transformation vectors are *E. coli* plasmid derivatives with cloned pt DNA sequences (one kb to two kb) that flank both sides of a selectable marker gene and cloning sites. The pt DNA sequences serve as targeting regions to direct integration into the plastid genome (Maliga, 2004). Lu *et al.* (2005) constructed a tobacco chloroplast multicistron expression vector pLM4 with *Prrn* promoter, 3' region of *psbA* gene, *aadA* gene, *man* gene, *gfp* gene and tobacco chloroplast high frequency homologous recombination ct DNA fragment (*psbA/psbC*, 3463bp). Lee *et al.*, (2006a) constructed a rice specific plastid transformation vector, pLD-RCtV-sGFP, targets insertion into the *trnI-trnA* in inverted repeat regions of the rice plastid genome.

2.8.3.1 Primer Designing

Primer design is the single largest variable in PCR applications and is the most important factor in determining the result of PCR reactions. Innis *et al.* (1990) suggested a set of rules for primer sequence design which include, length of primer (17-28 bases), G+C content (50-60%), the presence of G or C, or CG or GC at (3') end, and a preferred melting temperature between 55-80°C. It should not have three or more Cs or Gs at the 3'-ends, 3'-end complementarity and self-complementarity. Primer3 software (http://fokker.wi.mit.edu/primer3/.) designs primers for PCR reactions, according to the conditions specified by the user. Primer3 consider conditions like melting temperature, concentration of various

solutions in PCR reaction, primer bending and folding, and many other conditions when attempting to choose the optimal pair of primers for a reaction. The program produce primers based on the user specified conditions. The software was originally developed by Rozen and Skaletsky (2000) at the Whitehead Institute for Biomedical Research, USA.

Primer design has two essential phases: physical design and selectivity design. Physical design of primers involves the consideration of factors such as GC-content, primer length, annealing and melting temperatures, starting nucleotides and higher-order oligonucleotide structure. These factors are essential to ensure that a primer is able to bind to a template and initiate extension by the polymerase in an efficient, consistent manner. Primer selectivity refers to the ability of a primer to bind to a single location within the initial pool of DNA (Boutros and Okey, 2004).

Most of the primers were designed for amplifying spacers between tRNA genes which have been proved variable among species or populations (Demesure *et al*., 1996). Several universal primers for amplifying non coding spacers of the chloroplast genome have been reported (Taberlet *et al*., 1991; Lapegue *et al*., 1997). The PUNS (Primer-UniGene Selectivity) server simulates PCR reactions by running against BLASTN analysis on user entered primer pairs against both transcriptome and genome to assess primer specificity (Boutros and Okey, 2004). AutoDimer is a screening tool for primer dimer and hairpin structures (Vallone and Butler, 2004). Amplicon is a program for designing PCR primers on aligned groups of DNA sequences. The most important application for Amplicon is the design of group specific PCR primer sets that amplify a DNA region from a given taxonomic group but do not amplify orthologous regions from other taxonomic groups (Jarman, 2004). Primaclade is a web based application that accepts a multiple species nucleotide alignment file input and identifies a set of polymerase chain reaction primers that will bind across the alignment. Primaclade iteratively runs the primer3 application for each alignment sequences and collates the results.

Primaclade creates an HTML result page that recaps the original alignment, provides a consensus sequence and lists primers for each alignment area, with primers color coded to reflect the level of degeneracy in the primer (Gadberry *et al.*, 2005). Anderson *et al.* (2005) developed a method, implemented in the software DualPrime, that reduces the number of primers required to amplify the genes of two different genomes. The software identifies regions of high sequence similarity, and from these regions selects PCR primers shared between the genomes, such that either one or, preferentially, both primers in a given PCR can be used for amplification from both genomes. Miura *et al.* (2005) developed an algorithm which identifies the specificity determining subsequences of each primer and examine its uniqueness in the target genome.

MultiPLX is a new program for automatic grouping of PCR primers. It can use many different parameters to estimate the compatibility of primers, such as primer-primer interactions, primer-product interactions, difference in melting temperatures, difference in product length and the risk of generating alternative products from the template. A unique feature of the MultiPLX is the ability to perform automatic grouping of large number of primer pairs (Kaplinski, 2005). GENOMEMASKER is a software package which contains a repeat masking application and an e-PCR application for predicting primer binding sites and PCR products (Anderson *et al.*, 2006).

2.8.3.2 Polymerase Chain Reaction

Since its inception in 1986 (Mullis *et al.*, 1986), polymerase chain reaction (PCR) has become an essential part of molecular biology. Don *et al.* (1991) did touch down PCR to overcome the problems with melting temperature differences between primer pairs, in which the annealing temperature was lowered by 0.5^0 C per cycle during the first cycle from 50^0C to 54^0C. Virtual PCR is a web based service that predicts PCR products from different genomes (Lexa *et al.*, 2001). SPCR is a program that can help to choose a PCR primer pair giving the least

possible non targeted products (Cao *et al.*, 2005). Its algorithm is based on the hypothesis that the annealing of a primer to a template is an information transfer process. *In situ* PCR (isPCR) is designed for predicting possible PCR products that two primers could produce (http://www.soe.ucsc.edu/-kent/src/unzipped/isPcr).

2.8.4 Incorporation of Transforming DNA into the Chloroplast Genome

According to Bock (2001) primary chloroplast transformation events involve the transformation of only one or a few genome copies within a single plant cell resulting in cells that contain a mix of transformed and wild type chloroplast genomes. The cells are referred to as heteroplastomic and are genetically unstable. Heteroplasmy falls into two categories, interplastidic and intraplastidic. Interplastidic heteroplasmy is where a cell contains chloroplasts with wild type genomes and chloroplasts with transformed genomes. Intraplastidic heteroplasmy is where wild type and transformed genomes are located within the same chloroplast. Usually heteroplastomic cells are resolved spontaneously to a homoplastomic condition where the chloroplasts are all transgenic or all wild type. This is achieved by random genome segregation during chloroplast division and random chloroplast segregation during cell division. Homoplasmy can be achieved in chloroplast transformation studies by allowing for a sufficient number of cell divisions under high concentrations of the selection agent, usually spectinomycin. Plantlets go through a series of regeneration and selection steps with spectinomycin. Interplastidic heteroplasmy is more likely to disappear rapidly as chloroplasts containing only wild type genomes are sensitive to the selection agent and will not survive. Intraplastidic heteroplasmy is more difficult to eliminate as the spectinomycin resistance gene functions as a dominant selectable marker and only a few copies are sufficient to confer resistance. Homoplastomic transgenic shoots are typically obtained after two to four regeneration cycles under high selection pressure. Assays have been

developed to verify homoplasmy of transformed genomes in the shoots and include large scale seed assays (Svab and Maliga, 1993) and PCR based tests that amplify wild type genomes (Bock *et al.*, 1996).

The most common transformation event resulted in complete or nearly complete replacement of the resident chloroplast DNA sequence by the corresponding sequence in the donor plasmid without integration of the vector sequences. Positions of exchange events between the homologous recipient and donor sequences were biased and tended to occur near the insertion vector junction. The integrated donor sequences were present in both inverted repeat elements of all copies of the chloroplast genome in the transformed recipient cell, consistent with the presence of an active copy correction mechanism for maintaining the homogeneity of the inverted repeat (Palmer *et al.*, 1985). When the plasmid DNA was completely homologous to the corresponding region of the corresponding region of the recipient genome, higher frequencies of transformation were observed than when the donor DNA possessed several sequence heterologies dispersed throughout intergenic regions of the plasmid insert. Integration of donor DNA into one copy of the inverted repeat on the recipient sequences, which result in the formation of transient heteroplasmic genomes is followed by intra molecular copy correction that yields homoplasmic molecules with either the donor or recipient form of a given RFLP in both inverted repeat elements (Newman *et al.*, 1990).

2.8.5 Transgene Expression

Transgene expression is more stable in transplastomic plants than in nuclear transformants. Nuclear transformation in plants occurs by the random integration of transgenes into unpredictable locations in the genome by non homologous recombination and can result in varying levels of expression and, in some cases, gene silencing (Kooter *et al.*, 1999). Chloroplasts have the capacity to express

multiple genes from a polycistronic mRNA. This allows the pyramiding of genes to decrease the risk of promoting resistance in pest organisms (Gressel, 1999).

2.8.6 Selection of Transplastomes

The first system for high frequency plastid transformation in higher plants was established using a chimeric bacterial *aadA* gene encoding an aminoglycoside 3-adenyl transferase in combination with spectinomycin selection (Svab and Maliga, 1993). Spectinomycin is mainly used because it is a prokaryotic translational inhibitor and has little effect on plant cells. Spectinomycin is the most effective selectable marker used in chloroplast transformation. Khan and Maliga (1999) fused GFP with the *aadA* gene product (AAD) to be used as a bifunctional visual and selective (spectinomycin resistance) marker gene. Although *aadA* selection allows for efficient and reproducible plastid transformation in numerous plant species, the antibiotic resistance gene is not needed in the final transgenic product and its removal is desirable to eliminate the theoretical risk of antibiotic resistance gene flow. Methods used to remove the antibiotic resistance marker from transplastomic plants have included marker excision using the Cre/lox site specific recombination system (Hajdukiewicz *et al*, 2001) and generation of deletion derivatives after homologous recombination mediated excision (Iamtham and Day, 2000).

In nuclear transformation, insertion of the transgene into the nuclear genome and subsequent expression of the selectable marker provides protection of the entire cell. On the other hand, in plastid transformation, presumably only one plastid transformed, with only one or a few plastid genomes initially incorporating the transgene. Antibiotic selection using the *aadA* gene to confer spectinomycin resistance meets non lethal selection because cell responds only by bleaching and not by cell death (Maliga *et al*, 1993). The neomycin phosphotransferase (*npt II*) gene which confers kanamycin resistance may also be used for chloroplast transformation (Carrer *et al.*, 1993). Daniell *et al.* (2001) reported first genetic

engineering of the higher plant chloroplast genome without the use of antibiotic selection. The betaine aldehyde dehydrogenase (*BADH*) gene from spinach was used as a selectable marker. The enzyme is present only in chloroplasts of a few plant species adapted to dry and saline environment (Rathinasabapathi *et al.*, 1994). The lethal selection scheme kills the cells at an early stage of transformation before the integrated plastid transgene has had time to amplify sufficiently to express phenotypic resistance at the cellular level (Ye *et al.*, 2003). Chloroplast transformation efficiency was 25 fold higher in BA (betaine aldehyde) selection than spectinomycin. Green fluorescent protein (GFP) has been used to detect transient gene expression (Hibbered *et al.*, 1998) and stable transformation events (Skarjinskaia *et al.*, 2003). Kumar *et al.* (2004a) employed a 'Double Gene/Single Selection (DGSS)' plastid transformation vector that harbors two selectable marker genes (*aph A*-6 and *npt II*) to detoxify the same antibiotic by two enzymes, irrespective of the type of tissues or plastids. Cotton plastid transformation was achieved for the first time by combining this with an efficient regeneration system *via* somatic embryogenesis. The DGSS transformation vector is at least 8-fold (one event per 2.4 bombarded plates) more efficient than 'Single Gene/Single Selection (SGSS)' vector (*aph A-6*; 1 event per 20 bombarded plates). Chloroplast transgenic lines were fertile, flowered and set seeds similar to untransformed plants. Transgenes stably integrated into the cotton chloroplast genome were maternally inherited and were not transmitted *via* pollen when out crossed with untransformed female plants.

2.9 GENETIC MODIFICATION WORKS IN CHLOROPLAST GENOME

Boynton *et al.* (1988) showed that a non photosynthetic, acetate requiring mutant strain of *Chlamydomonas reinhardtii* with a 2.5 kb deletion in the chloroplast *Bam 10* restriction fragment region that removes the 3' half of the *atpB* gene and a portion of one inverted repeat can be transformed to photosynthetic competency following bombardment with microprojectiles coated with wild type *Bam* 10 DNA.

Smeda *et al.* (1993) reported high level of atrazine resistance by cultured variant potato cells due to a single amino acid substitution from serine to threonine at position 264 in the mature Q_B protein. *Bacillus thuringensis* (Bt) toxins are harmful to insects when ingested. Kota *et al.* (1999) observed hyper-expression of *Bt* toxins in transformed tobacco chloroplasts that resulted in a high insect mortality rate. The advantage of this application is that the toxins are located in green leaf tissue which is the most likely part of the plant to be consumed by insects. There is an added advantage that insecticidal proteins are not produced in fruit or pollen hence the proteins will not likely be consumed by humans or animals and the transgene will not be transmitted to other plants *via* pollen.

DeCosa *et al.* (2001) .over expressed the *Bt cry2Aa2* operon in tobacco chloroplast, and demonstrated *Bt* pro-toxin accumulation to a level of 45.3 per cent of the total soluble proteins in leaves. Kumar *et al.* (2004b) reported high-level expression of betaine aldehyde dehydrogenase (BADH) in cultured cells, roots, and leaves of carrot (*Daucus carota*) *via* plastid genetic engineering. Homoplasmic transgenic plants exhibiting high levels of salt tolerance (up to 400mM NaCl) were regenerated from bombarded cell cultures *via* somatic embryogenesis. Two bacterial enzymes that confer resistance to different forms of mercury known as mercuric ion reductase and organomercurial lyase were over expressed in the chloroplast of tobacco through chloroplast genetic engineering. When the chloroplast transgenic plants containing the operon with the mercuric ion reductase and organomercurial lyase were tested through a bioassay in which the extremely toxic organomercurial PMA was used , the transgenic plant were substantially more resistant than wild type tobacco plants growing under the same conditions (Daiell *et al.*, 2005). Plastid transgene expression to a particular tissue or developmental stage can be achieved by placing the transgene under the control

of a phage T7 RNA polymerase promoter which is normally not recognized by the plastid transcriptional apparatus. Plastid transgene expression can then be switched on by a nuclear encoded and plastid targeted T7 RNA polymerase (Mc Bride *et al.*, 1994). Hyper expression of the petunia EPSPS (which is highly sensitive to glyphosate) through chloroplast transformation conferred resistance to high levels of glyphosate and the transgene was maternally inherited (Daniell *et al.*, 1998). Expression of the nuclear T7 RNA polymerase gene can in turn, be controlled by tissue specific or developmental stage specific promoters or made dependent on chemical inducers of gene expression (Heifetz, 2000). Kuroda and Maliga (2001) reported that sequences downstream of the translation initiation codon are important determinants of translation efficiency in chloroplasts. The silent mutations in the *rbcL* segment downstream of the AUG of tobacco chloroplast genome reduced the NPT II accumulation 35- fold.

The human growth hormone (somatotropin) was successfully expressed from tobacco plastid genome and shown to get accumulated in greater than seven per cent of total soluble protein. The eukaryotic protein somatotropin was synthesized in chloroplast in its correct disulfide bonded form and proved to be biologically active in bioassays (Staub *et al.*, 2000). Helical structured antimicrobial peptides (AMPs) are expressed as protective agents against pathogens in many organisms. De Gray *et al.* (2001) expressed MSI-99, an analog of magainin 2 in transgenic chloroplast. This AMP confers protection against prokaryotic organisms because of high specificity for negatively charged phospholipids, which are mostly found in bacteria and less abundant in eukaryotic organisms.

Ruf *et al.* (2001) developed a plastid transformation system for tomato, which is the first report on the generation of fertile transplastomic plants in a food crop with an edible fruit. Chloroplast transformation with modified *accD* operon increases acetyl-*CoA* carboxylase and causes extension of leaf longevity and increase in seed yield in tobacco (Madoka *et al.*, 2002). Iamtham and Day (2003)

were transformed tobacco plants *via* chloroplast genetic engineering with more resistant forms of EPSPS, including *AroE* (*Bacillus*) and *CP4* (*Agrobacterium*). The TPS1 gene from yeast encodes the trehalose phosphate synthase, an enzyme that produces the osmoprotectant trehalose. Lee *et al.* (2003) reported hyper expression of the trehalose phosphate synthase and increased accumulation of trehalose in chloroplasts of transgenic plants. Production of trehalose in the chloroplasts of transgenic plants conferred membrane stability.

Sugiura and Sugiura (2004) integrated a foreign gene into the *trnR* - CCG coding region via homologous recombination and constructed stable plastid *trnR* - CCG knock out moss transformants which grew normally indicating that the *Physcomitrella patens trnR*-CCG gene is not essential for plastid transformation. Daniell *et al.* (2004) reported stable integration of transgenes into cotton chloroplast genome which were maternally inherited and not transmitted *via* pollen when out crossed with untransformed female plants. Sebastian *et al.* (2004) developed marker free chloroplast transformation in tobacco using the reconstitution of wild type pigmentation in combination with plastid transformation vectors, which prevented stable integration of the kanamycin selection marker. Lutz *et al.* (2004) reported an alternative approach to plastid transformation that relies on integration of foreign DNA by the phi C31 phage site specific integrase (INT) mediating recombination between bacterial and phage attachment sites (*attB* and *attP*, respectively).

Dufurmantel *et al.* (2005) generated fertile and homoplasmic soybean plastid transformants expressing the *Bacillus thuringiensis* insecticidal protoxin *Cry1Ab*. Transgenes were targeted in the transgenic region of *Glycine max* plastome, between the *rps12/7* and *trnV* genes and selection was carried out using the *aadA* gene encoding spectinomycin resistance. Glenz *et al.* (2006) generated transplastomic tobacco plants that accumulate the outer surface lipoprotein A (*OspA*) which is the basic constituent of the first generation monovalent human vaccine against Lyme disease. Dhingra and Daniell (2006) reported highly

efficient plastid transformation *via* somatic embryogenesis in *Arabidopsis*. Kanamoto *et al.* (2006) showed that lettuce leaf chloroplast can express transgene encoded GFP to approximately 36 per cent of the total soluble protein.

2.10 CENTELLA

Centella asiatica (L.) Urb., an Apiaceae member, is a clonally propagated herb native of Asia , southern and middle Africa , southeastern United States and Australia . The plant flourishes in damp, moist and shady habitats and grows by producing stolons that are characterized by long internodes and nodes, on which are borne reniform-cordate leaves and sessile flowers in simple umbels. It is used in Ayurvedic preparations either as whole plants or as leaves in the fresh or extract form is an important medicinal plant widely distributed in tropical and subtropical regions. Plants from India, Sri Lanka and Madagascar reportedly differ in presence of glycosides, such as isothankuniside, brahmoside, centelloside and madecassoside (Dutta and Basu, 1968). The plant is grown and consumed as a vegetable in many Asian countries and is finding acceptance in the western world (Peiris and Kays, 1996). Some researchers have termed *Mandukaparni* as an "Araliaceous hydrocotyloid". Although it is a member of the Apiaceae, it bears many similarities both botanically and in therapeutic action with species of the Araliaceae, such as *Panax* spp. (http://www.toddcladecott.com).

2.10.1 Medicinal Properties of *Centella asiatica* L.

A three week treatment of a triterpene fraction of *C. asiatica* in clients with postphlebitic syndrome significantly reduced the number of circulating endothelial cells, as compared to normal subjects (Montecchio *et al.*, 1991). *C. asiatica* has been found to have a GABAnergic activity (Chatterjee *et al.*, 1992).

Moharana and Moharana (1994) reported the use of *Centella asiatica* plant extracts in memory enhancing tonics and for the treatment of mental and stress-

related disorders. Herbert *et al.* (1994) studied the *in vitro* effect of an indigenously produced dry powder of *C. asiatica* on the acid-fastness and viability of *Mycobacterium tuberculosis*. The results indicate that it may not have any direct action on the acid-fastness or viability of *M. tuberculosis* H37Rv *in vitro*. A water-soluble fraction of *C. asiatica* was reported to inhibit hepatic enzymes responsible for barbiturate metabolism (Leung and Foster 1996). Titrated extract of *C. asiatica*, consisting of a mixture of three triterpenes (asiatic acid, madecassic acid and asiaticoside) stimulates collagen and glycosaminoglycan synthesis in rats surgically inserted with stainless steel wound chambers (Maquart *et al.*, 1999). Sharma and Sharma (2002) reported that *C. asiatica* has a considerable reputation in the Indian system of medicine. It is a rasayana (general tonic), brain tonic, improves memory and strengthens the central nervous system. According to Rao (2003), Ayurveda describes a group of plant drugs as "medhyarasayanas" (brain tonics). *C. asiatica* leaf extract treatment in rats during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization (Rao *et al.*, 2006).

2.10.1.1 Asiaticoside

Giardina *et al.* (1987) reported that modern drugs comprising the pharmacologically active triterpenoid fractions and glycosides such as asiaticoside and madecassoside are being currently used in the treatment of leprosy, lupus, eczema, skin lesions, psoriasis, wound healing, burns, ulcers of the duodenum, skin and cornea, tuberculosis and venous diseases. The usage of madecassol (asiaticoside) in tablet, ointment and powdered form was found to be efficacious in the treatment of chronic or subchronic systemic scleroderma with limited skin involvement, and in progressive and/or advanced focal scleroderma (Guseva *et al.*, 1998). Asiaticoside derivatives were found to inhibit or reduce H_2O_2 induced cell death and lower intracellular free radical concentration, protecting against the effects of beta-amyloid neurotoxicity (Jung *et al.*, 1999).

Kim *et al.* (2004) reported stimulation of asiaticoside accumulation in the whole plant cultures of *C. asiatica* by elicitors such as methyl jasmonate, $CdCl_2$, $CuCl_2$ and yeast extract. Findings of Guo *et al.* (2004) indicated that water extract of *C. asiatica* and its active constituent asiaticoside have an anti-inflammatory property that is brought about by inhibition of nitric oxide synthesis and thus facilitating ulcer healing in rats. Kim *et al.* (2005) cloned cDNA encoding oxidosqualene cyclase associated with asiaticoside biosynthesis from *C. asiatica*. According to Lee *et al.* (2006c), asiaticoside induces human collagen synthesis through TGF beta receptor I kinase independent Smad signaling.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Amplification and sequencing of spacer region between two tRNA genes and its flanking region in the chloroplast genome of *Centella asiatica* L." was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, during 2005 to 2006. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 ISOLATION OF SPACER SEQUENCE AND FLANKING REGIONS

3.1.1 Primer Designing

Primers were designed based on the chloroplast genome sequences of related crop species using Primer3 software (Rozen and Skaletsky, 2000). Certain parameters were fixed for primer designing. The primer length varied from 20nt-26nt with GC content greater than 50.0 percent. The 3' region of the primer ends with guanine or cytosine. The difference in melting temperature between forward and reverse primers was a maximum of five except in two primer pairs.

3.1.1.1 trnG-trnfMet Spacer

Primers (Af and Ar) were designed for the spacer region between tRNA glycine (*trnG*) and tRNA formylmethionine (*trnfMet*). Sequences of *Nicotiana tabacum* (GI: 76559634) and *Arabidopsis thaliana* (GI: 5881673) chloroplast genome were downloaded from the National Centre for Biotechnology Information (NCBI) Gen Bank in FASTA format. Homologous regions of the genes were identified using Clustal X 1.81 (Thompson *et al.*, 1997) multiple alignment program. The conserved regions were then selected for designing primers with Primer3 (Rozen and Skaletsky, 2000).

3.1.1.2 trnE-trnT Spacer

Primers (Bf, Br) were also designed for the spacer region between tRNA glutamic acid (*trnE*) and tRNA threonine (*trnT*) using the sequences of *N. tabacum* chloroplast genome (GI: 2924257). Primers (Cf, Cr) were also designed for the same spacer region using the chloroplast genome sequence of *Panax ginseng* (GI: 51235292), as the spacer sequence between genes *trnG* and *trnfMet* of *C. asiatica* showed similarity to this crop.

3.1.1.3 trnT-trnL Spacer

Primers (Df, Dr) were designed for spacer region between tRNA threonine (*trnT*) and tRNA leucine (*trnL*) using the chloroplast genome sequence of *P. ginseng* (GI: 51235292).

3.1.1.4 rps16-trnQ Spacer Region

Primers (Ef, Er) were designed for the spacer region between genes for ribosomal protein 16S (*rps16)* and tRNA glutamine (*trnQ)* using the sequences of *N. tabacum* chloroplast genome (GI: 2924257).

3.1.1.5 Right Flanking Region of rps16-trnQ Spacer

Primers were designed for the right flanking region of the spacer region between gene *rps16* and *trnQ* using the chloroplast genome sequence of *P. ginseng* (GI: 51235292). The forward primer (Hf) for the right flanking region was the reverse complement of the reverse primer (Er) for the spacer region between *rps16* and *trnQ* genes.

3.1.1.6 Left Flank of rps16-trnQ Spacer Region

Primers were designed for the left flanking region of the spacer region between genes *rps16* and *trnQ* using the chloroplast genome sequence of *P. ginseng* (GI: 51235292). Two primer pairs were designed for amplifying the left flanking region namely Ff, Fr and Gf, Gr. The primer pair Ff and Fr was designed from the sequence of *P. ginseng*. The forward primer (Gf) was designed from sequence of *P. ginseng*. The reverse primer (Gr) of the left flanking region was the reverse complement of the forward primer for the spacer region between gene *rps16* and *trnQ*. The primers for the flanking region were designed manually because Primer3 software found it difficult to give a primer pair with given conditions.

3.1.2 Primer Analysis

The primers were analyzed by BLAST N (Altschul *et al.*, 1997) to find the binding region of the primers in different organisms. Oligonucleotide properties calculation program (www.basic.northwestern.edu/biotools/oligocalc.html) was used to find the 3' complementarity, hairpin formation and self annealing of the primer pairs.

3.1.3 Synthesis of Primers

The primers were synthesized and supplied by Integrated DNA technologies (IDT), USA and Bioserve Biotechnologies India Pvt. Ltd., Hyderabad.

3.1.4 DNA Isolation

3.1.4.1 Plant Material

Total genomic DNA was isolated from pot grown healthy, pest and disease free *Centella asiatica* plants. Fresh green leaves were used for isolation.

3.1.4.2 Isolation of Genomic DNA

Total genomic DNA was isolated using modified protocol of Murray and Thompson (1980). Fresh green leaves (2.0 gram) were taken and washed in distilled water and blot dried. The leaves were cut into small pieces and a pinch of PVP was added to it. Then the leaves were ground into fine powder using liquid nitrogen in an autoclaved mortar and pestle. The powder was then transferred quickly to a 30 ml oak ridge tube. To the tube 15 ml of pre warmed Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer (Appendix I) was added. The tube was incubated at 65^0 C for 30 minutes with occasional mixing. The mixture was cooled to room temperature and centrifuged at 5000rpm for five minutes. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and mixed well to get an emulsion by inverting the tube several times for 15 minutes. It was centrifuged at 10,000 rpm for 10 minutes and the aqueous phase was taken. To this equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 minutes. To the aqueous phase one tenth volume 3M sodium acetate (pH 5.2) and 2.5 volume chilled absolute alcohol was added and mixed carefully. It was kept at -20^0C for 30 minutes. The precipitated DNA was pelleted at 10,000 rpm for 10 minutes in a refrigerated centrifuge. Pellet was washed twice using 70 per cent ethanol and air dried. Pellet was dissolved in 0.5ml Tris EDTA (Ethylene Diamene Tetra Acetic acid) buffer (pH 8.0; Appendix I).

3.1.4.3 Quantification of DNA

UV Spectroscopy was used for assessing the concentration of DNA. The absorbance of diluted DNA samples was measured at 260 and 280 nm (SPECTRONIC GENESYS 5). Since an absorbance value of one at 260nm was corresponded to 50 µg/ml for DNA, the DNA concentration in the sample was calculated as follows

Amount of DNA (µg/ml) = A_{260} x 50 x dilution factor

A_{260} = Absorbance at 260 nm

DNA purity was determined by the ratio taken between A_{260}/A_{280} readings.

3.1.4.4 Agarose Gel Electrophoresis

The integrity of total DNA was determined by running 10µl aliquots of DNA on 0.8 per cent agarose gel. Electrophoresis was carried out at 40 V for four hours in 1X TAE buffer (Appendix II). The gel was stained with ethidium bromide and analyzed using gel documentation system (BIO-RAD).

3.1.4.5 PCR Amplification

The DNA was amplified using specific primers designed for the spacer regions and flanking regions of *rps16-trnQ* spacer region. 25µl reaction mixture was set in the Programmable Thermal Cycler (PTC-150, MJ Research, USA). *Pfu* DNA polymerase (recombinant) enzyme supplied by Fermentas was used since it exhibited proof reading activity that enables the polymerase to correct nucleotide incorporation errors. The reaction was set as follows.

Item	Volume (µl)
DNA sample (10ng/µl)	2.0
10x *Pfu* buffer with $MgSO_4$	2.5
$MgSO_4$	1 .0
dNTP mix (2mM each)	2.5
Forward primer (10pmol/µl)	1.0
Reverse primer (10pmol/µl)	1.0
Pfu DNA polymerase (2.5 U/µl)	0.75
Sterile water	14.25
Total	25.0

The PCR was carried out by denaturating the PCR mix at 94°C for five minutes followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing for 30 seconds and 72°C for four minute. Final extension at 72 °C for 10 minutes was given. Annealing temperature was changed based on the Tm of primers as shown below.

Primers	Annealing Temperature (^{0} C)
Af and Ar	50
Bf and Br	52
Cf and Cr	55
Df and Dr	55
Ef and Er	52
Ff and Fr	52
Ff and Gr	56
Gf and Fr	52
Gf and Gr	52
Hf and Hr	56

Control reactions were carried out without DNA to distinguish the target product from non target products and primer dimer formation. After PCR, gel electrophoresis was carried out on 1.6 per cent agarose gel in 1X TAE buffer at 40 V for six hours.

Sequencing was done at Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad. The protocol for sequencing reaction adopted by them was as follows:

After quality and quantity check of the DNA sample (direct PCR product) on agarose gel, purification was done by gel elution when many bands are present. Then sequencing PCR reaction was set up using Beckman Quick Master Mix, 100ng of DNA and 5 pmols of forward and reverse primer. Post processing of PCR products was done to remove dye terminators. Then sequencing reaction was carried out using Beckman Coulter CEQ 8000 machine.

3.2 SEQUENCE ANALYSIS

Similarity searches were carried out in the NCBI database using BLASTN (nucleotide-nucleotide) and BLASTX (translated nucleotide-protein) tools (Altschul *et al.*, 1997) to identify homologous sequences from other species.

Results

4. RESULTS

The results of the study entitled "Amplification and sequencing of spacer region between two tRNA genes and its flanking region in the chloroplast genome of *Centella asiatica* L." carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2005-2006 are presented below.

4.1 ISOLATION OF SPACER SEQUENCE AND FLANKIG REGIONS

4.1.1 Primer Designing

For the amplification and sequencing of spacer region between tRNA genes and its flanking region in the chloroplast genome of *Centella asiatica*, primers were designed using Primer3 software (Table 1).

Primers were designed for the spacer sequence between genes *trnG* and *trnfMet* based on the sequence of *Nicotiana tabaccum* and *Arabidopsis thaliana* chloroplast genome. The forward primer (Af) was 22 nucleotides (nt) long and the reverse primer (Ar) was 21nt long.

Based on the chloroplast genome sequence of *N. tabaccum* primers were designed to amplify the spacer between *trnE* and *trnT* genes. The forward primer (Bf) had a length of 21nt and the reverse primer (Br) had a length of 22nt. Chloroplast genome sequence of *P. ginseng* were used to design one more primer pair for the spacer region between genes *trnE* and *trnT*, because the BLASTN result of *trnG-trnfMet* spacer in *C. asiatica* showed similarity to this crop. Both forward (Cf) and reverse (Cr) primer had 22nt length.

Primers for the *trnT-trnL* spacer region were designed using the *P. ginseng* chloroplast genome sequence. The forward (Df) and reverse (Dr) primers were 22nt and 21nt long, respectively.

Table 1. Primers designed

Sl. No	Target region	Name of primer	Tm (^{0}C)	GC%	Primer length (nt)	Sequence of primer
1	*trnG-trnfMet* spacer	Af Ar	52.2 58.6	40.9 57.1	22 21	5'GGTAAAATTTCTCCTTGCCAA3' 5'GTTTGGTAGCTCGCAAGGCTC3'
2	*trnE-trnT* spacer	Bf Br	56.1 55.5	52.3 50.0	21 22	5'CTTGACCAACCGCCATCATAC3' 5'GAACCGATGACTTACGCCTTAC3'
3	*trnE-trnT* spacer	Cf Cr	55.0 55.0	50.0 50.0	22 22	5'CCTCCTTGAAAGAGAGATGTCC3' 5'GAACCGATGACTTACGCCTTAC3'
4	*trnT-trnL* spacer	Df Dr	55.0 54.0	50.0 52.3	22 21	5'GAGCTAAGCAGGCTCACATAAC3' 5'TGGGGATAGAGGGACTTGAAC3'
5	*rps16-trnQ* spacer	Ef Er	55.9 56.2	50.0 55.0	22 20	5'CTGTAGGTTGAGCCCCTTTTTC3' 5,GGGTTTTGGTCCCGCTATTC3'
6	Left flanking region of *rps16-trnQ* spacer	Ff Fr	57.0 55.0	50.0 50.0	24 22	5'AGCCACAAGGCTCTATCTCTATCC3' 5'CTCTCCCAATGAGCCGTCTATC3'
7	Left flanking region of *rps16-trnQ* spacer	Gf Gr	57.0 55.0	50.0 50.0	24 22	5'CAGATGGACTGGGTAGGGTATTAG3' 5'GAAAAAGGGGCTCAACCTACAG3'
8	Right flanking region of *rps16-trnQ* spacer	Hf Hr	54.0 61.0	55.0 53.8	20 26	5'GAATAGCGGGACCAAAACCC3' 5'CGCCCCGGATCATTAGGATAGAGGAATCC3'

For the amplification of spacer region between *rps16-trnQ* genes, primers were designed using the chloroplast genome sequence of *N. tabaccum.* The forward primer (Ef) was 22nt long and the reverse primer (Er) was 20nt long.

Primers for the flanking regions of the spacer region between *rps16* and *trnQ* genes were designed manually because, Primer3 software failed to produce primers with the given conditions. The forward primer (Hf) for the right flanking region of the spacer sequence between *rps16* and *trnQ* genes was the reverse complement of the reverse primer (Er) for the *rps16-trnQ* spacer region and had a length of 20nt. The reverse primer (Hr) for the right flanking region of *rps16-trnQ* spacer was designed based on the chloroplast genome sequence of *P. ginseng* was 26nt long.

Two primer pairs were designed for the amplification of the left flanking region of the *rps16-trnQ* spacer. One pair was designed based on the sequence of *P. ginseng* chloroplast genome. The forward primer (Ff) was 24nt long. The reverse primer (Fr) had a length of 22nt. Second pair was designed based on the chloroplast genome of *P. ginseng* and primer of *rps16-trnQ* spacer region. The forward primer (Gf) as designed using the sequence of *P. ginseng*, had a length of 24nt and the reverse primer (Gr) was the reverse complement of the forward primer (Ef) for the *rps16-trnQ* spacer region and had a length of 22nt.

4.1.2 Primer Analysis

Primers designed were analyzed using oligonucleotide properties calculation program (Table 2). To find out the binding sites of the primers in chloroplast genome of other species BLASTN analysis of all the primers was done (Table 3).

Table 2. Primers analyzed using oligonucleotide properties calculation program

Sl. No.	Primer	Hairpin formation	3' complementarity	Self annealing
1	Af	None	None	Yes
2	Ar	None	None	None
3	Bf	None	None	None
4	Br	None	None	None
5	Cf	None	None	None
6	Cr	None	None	None
7	Df	Yes	None	None
8	Dr	Yes	None	None
9	Ef	None	None	None
10	Er	None	None	None
11	Ff	Yes	None	None
12	Fr	None	None	None
13	Gf	None	None	None
14	Gr	None	None	None
15	Hf	None	None	None
16	Hr	Yes	None	None

Table 3. BLASTN analysis of primer sequences

Sl.No	Primer	Crop	Gene Identity Number (GI No.)	Primer sequence	Binding site (base position)
1	Af	*P. ginseng* *N. tabacum*	51235292 76559634	1-21	38730-38709 38079-38099
2	Ar	*P. ginseng* *N. tabacum*	51235292 76559634	1-22	38437-38458 38420-38400
3	Bf	*N. tabacum*	76559634	1-21	32341-32361
4	Br	*N. tabacum* *P. ginseng*	76559634 51235292	1-22	33240-33219 33461-33440
5	Cf	*Piper cenocladum*	112253730	1-22	32115-32136
6	Cr	*Piper cenocladum* *P. ginseng*	112253730 51235292	1-22	32939-32918 33461-33440
7	Df	*P. ginseng*	51235292	1-22	48658-48679
8	Dr	*P. ginseng*	51235292	1-21	50057-50037
9	Ef	*N. tabacum*	76559634	1-22	5162-5183
10	Er	*N. tabacum* *P. ginseng*	76559634 51235292	1-20	7465-7445 8209-8189
11	Ff	*P. ginseng*	51235292	1-24	4052-4075
12	Fr	*P. ginseng*	51235292	1-22	5447-5429
13	Gf	*P. ginseng*	51235292	1-24	3177-3140
14	Gr	*N. tabacum*	76559634	1-22	5183-5162
15	Hf	*N. tabacum* *P. ginseng*	76559634 51235292	1-20	7445-7464 8189-8208
16	Hr	*P. ginseng*	51235292	1-16	9267-9252

4.1.3 DNA Isolation

Modified protocol of Murray and Thompson (1980) was used for isolation of total genomic DNA from *C. asiatica* (Plate 1). On 0.8 per cent agarose gel the isolated DNA showed intact band (Plate 2). The quality of DNA was good with an A_{260}/A_{280} ratio 1.7 was measured using spectrophotometer. The yield of DNA was 893 $\mu g\ g^{-1}$ of the fresh leaf.

4.1.4 PCR

Polymerase chain reactions were carried out using the specific primers designed for spacer region between genes and flanking regions. Amplification was done on *C. asiatica* genomic DNA. The amplified product was electrophoresed on 1.6 per cent agarose gel.

The amplification of *C. asiatica* with primers Af and Ar for *trnG-trnfMet* spacer gave a band of about 270bp length. The amplified band was reproducible (Plate 3). PCR with primers (Bf and Br) for the spacer region between the *trnE-trnT* genes gave an intense band of size about 700bp (Plate 4). This band was gel eluted, purified and sequenced. The spacer region amplified with primers Cf and Cr produced many bands (Plate 5). The intense band was eluted, purified and sequenced. The intense band of about 1500bp size produced during the amplification of *trnT-trnL* spacer region with primers Df and Dr was gel eluted and purified (Plate 5).

The spacer region between *rps16* and *trnQ* amplified with primers Ef and Er gave a product size of nearly 1500bp (Plate 6). This band was gel eluted and purified. The purified sample was sequenced with both forward (Ef) and reverse (Er) primer.

A

B

C

Plate 1: ***Centella asiatica*** **L. (A) Plant (B) Flower (C) Seed**

Plate 3. *trnG-trnfMet* spacer region amplified

Lane 1. 500bp DNA marker

Lane 2. 100bp DNA marker

Lane 3. *trnG-trnfMet* spacer region amplified using primers Af and Ar

Lane 4. Control reaction for *trnG-trnfMet* spacer region without DNA

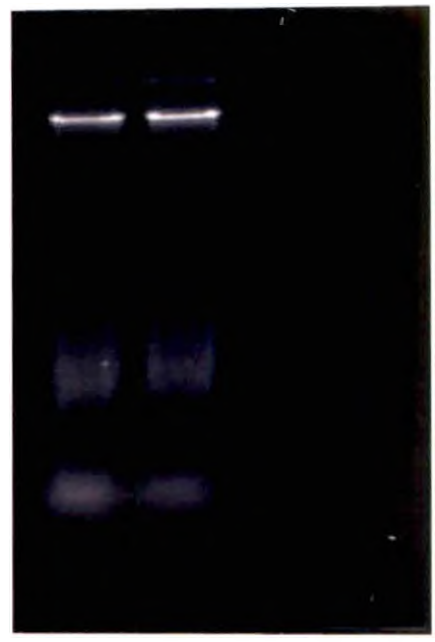

Plate 2: Genomic DNA of *C. asiatica*

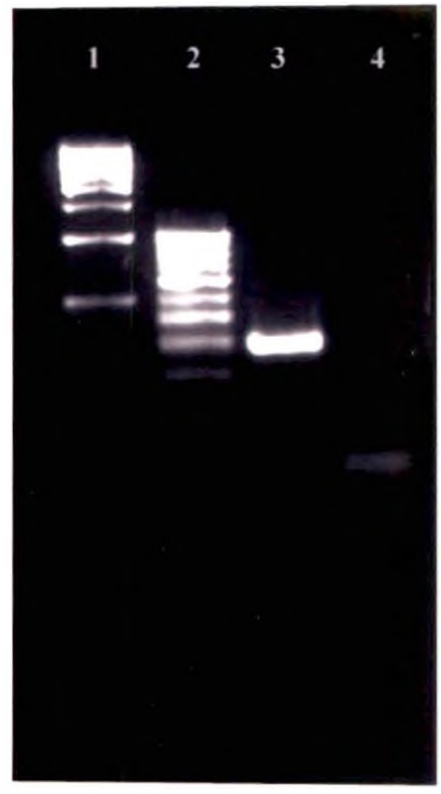

Plate 3: *trnG-trnfMet* spacer region amplified

Plate 4. *trnE-trnT* spacer region amplified

Lane 1. *trnE-trnT* spacer region amplified using primers Bf and Br

Lane 2. 100bp DNA marker

Lane 3. 500bp DNA marker

Plate 5. *trnE-trnT* and *trnT- trnL* spacer regions amplified

Lane 1. 500bp DNA marker

Lane 2. *trnE-trnT* spacer region amplified using primers Cf and Cr

Lane 3. Control reaction for *trnE-trnT* spacer region without DNA

Lane 4. Control reaction for *trnT-trnL* spacer region without DNA

Lane 5. *trnT-trnL* spacer region amplified using primers Df and Dr

Plate 4: *trnE-trnT* spacer region amplified

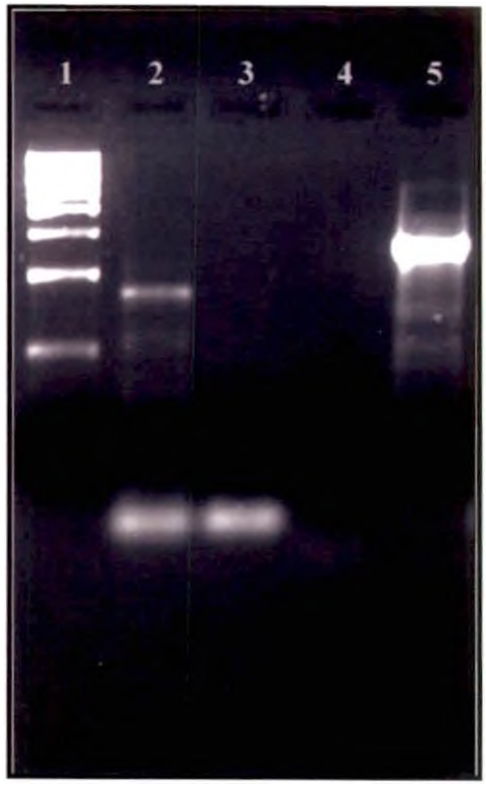

Plate 5: *trnE-trnT* and *trnT-trnL* spacer regions amplified

Plate 6. *rps16-trnQ* spacer region amplified

Lane 1. *rps16-trrnQ* spacer region amplified using primers Ef and Er

Lane 2. 100bp DNA marker

Lane 3. 500bp DNA marker

Plate 7. Left and right flanking regions of *rps16-trnQ* spacer region amplified

Lane 1. 500bp DNA marker

Lane 2. Left flanking region of *rps16-trnQ* spacer region amplified using primers Ff and Fr

Lane 3. Left flanking region of *rps16-trnQ* spacer region amplified using primers Ff and Gr

Lane 4. Left flanking region of *rps16-trnQ* spacer region amplified using primers Gf and Fr

Lane 5. Left flanking region of *rps16-trnQ* spacer region amplified using primers Gf and Gr

Lane 6. Right flanking region of *rps16-trnQ* spacer region amplified using primers Hf and Hr

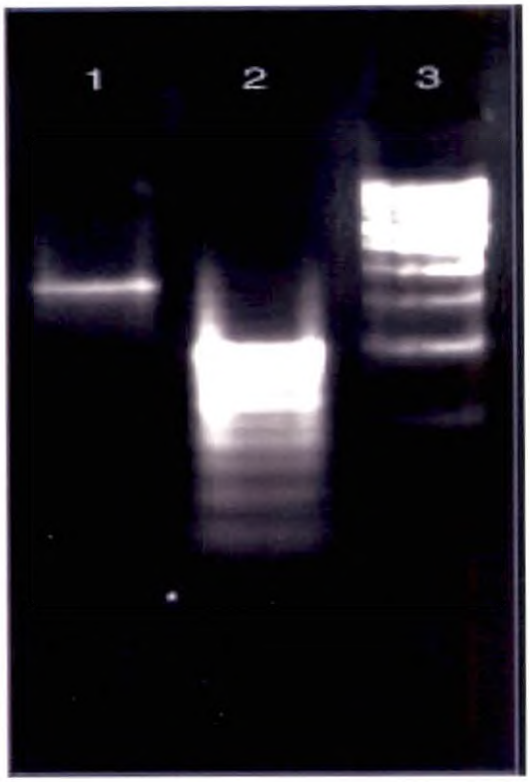

Plate 6: *rps16 -trnQ* spacer region amplified

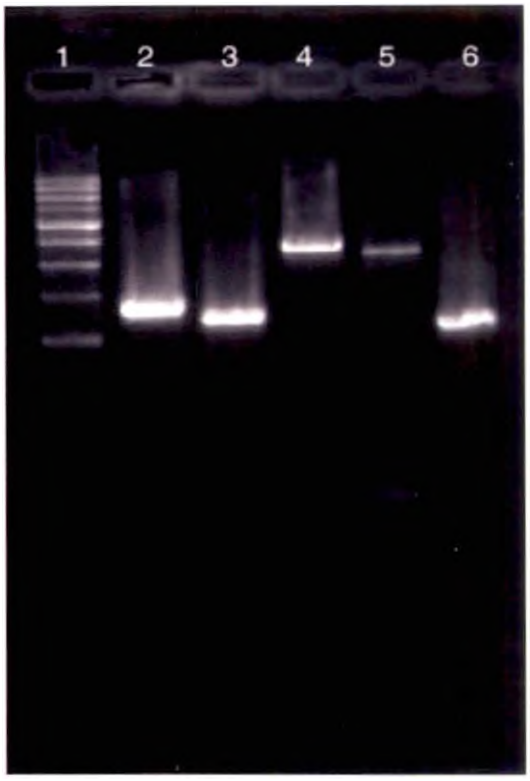

Plate 7: Left and right flanking regions of *rps16-trnQ* spacer region amplified

To amplify the left flanking region of *rps16-trnQ* spacer four primer combinations were tried (Ff and Fr, Ff and Gr, Gf and Fr, Gf and Gr). The flanking region amplified with primer combination Ff and Fr produced a band of about 1500bp (Plate 7). The band produced by amplification with primers Ff and Gr was about 1300bp (Plate 7). Amplification with primers Gf,Fr and Gf,Gr produced bands of 2500bp size (Plate 7). Amplified products using all primer combinations except Ff and Gr were sequenced.

Hf and Hr primer combination was used to amplify the right flanking region of the *rps16-trnQ* spacer in *C. asiatica*. The amplified product showed single band with a size of about 1500bp (Plate 7).

4.1.5 Sequencing of PCR products

The spacer region amplified between genes *trnG* and *trnfMet* were sequenced (CEN_PRIMER 1). The sequence had a length of 270bp (Table 4).

The intense band produced during PCR with primers Bf and Br for the spacer region between the *trnE-trnT* was gel eluted and purified. It was sequenced (RNE_V1) with primer Bf and was 633bp long (Table 4). The amplified product with Cf and Cr for *trnE-trnT* spacer was sequenced (SE_FWD) with primer Cf and had a length of 895bp (Table 4).

The region amplified with primers Df and Dr for *trnT-trnL* spacer region were gel eluted and purified. After gel elution and purification, sequencing PCR failed to produce amplification with the same primers. Hence the sequencing could not be done.

The amplified product with primers Ef and Er was sequenced. PCR product sequenced (RNQ_V3) with primer Ef gave 815bp long sequence (Table 5). The

Table 4. Sequence of spacer region between tRNA genes amplified using specific primers

Sl. No.	Spacer region	Sequence length (bp)	Sequence 5'-3'
1	*trnG-trnfMet*	270	>CEN_PRIMER1 sequence exported from CEN_PRIMER1.scf TAGCACAAATCCTGTCTCCGCAATATCTTTTTTTGTCAACCCTTTTTAGGTTTGACTCTG TTAACTAGTAATTAATTAACACTTTTATTGGGGGATGGGAGGGAAAAAGAAGGGGTGGAT AGAATCACTACATTATTACGGTCAACTATACCGAATCCTTTATGTTTTATTAATATGAAA TTTATCATATGAAAAAAATTACTCCATCTTAGGCGGATAGCGGGAATCGAACCCGCGTCT TCTCACTTGGCAAGGAGAAATTATAACCCA
2	*trnE-trnT*	633	>RNE_V1 sequence exported from RNE_V1.scf CGCACATCACGATGNTGTTTCGTCGAACACATGGGCTCCGTTTGATACACGTGGTGTACA TGGTCACTACTATAAATGTGGACCATAGGCATAATAACATGCAATGGACTAACTCAGCAG CACAGCCGACGGTGCGGACGAATTGCCATGTCTGTTAGGTTGACCCCCAGTATGCTTGCG TAGAGTACCAGAAGCGACTGGCTTATCACGGGCTCTGGCCACTCGATGAGCGTCCCTCCA ACGGGCGTTAAGCAGTACTAAAGTCCGACACTTTTTAATGTAAGTACGCACTGCCCCCAG ATCCCGGGTGGCGGATTCGTGTCGGCGTCAGTATAAAAAAAAAAAAAACAACTGAGGTACA TCCCAATGTAGATCGCCGCGCCTTTCCCAGTAGCCAGTACTTCTAAGAATCTCACGAGTC TTGCACGGATGGGCGATCGACCCGGGGGGAATATACCTGTTGCGATGGTACCGGNGGATA TTGTCGTCCTAAGTCTAGCTCGCGTCGCTGCATGATGTCTTCACCGTCTGGGGGGGATTC CTACCTCCCTGATATGCGACTGGTGTCGGCCTCGGCCGCGGTGGCCCGTCCGTACACGGT CTGGTCCTGCCCCCCCCCCCCCCCGACCACCCC

Table 4 continued

Sl. No.	Spacer region	Sequence length (bp)	Sequence 5'-3'
3	*trnE-trnT*	895	>SE_FWD sequence exported from SE_FWD CATATAACACTAAAGTAAGTGGTATAGCATCAGATGAACTAACTCAATCATCATCGATTT TTTATCAAACCAGAACTCCCCCCACACACGACTAAAGCGGGTAAGTAACTTACATTACCC CAAAATGGATTGAGACCCCCAACACTCTAAAATCACAATCTAACATCCCACATTCCAACT ACAAACCATACATAATCCCAATACCGAACAGATCTTATCTAAAGCCCCAGATGGCCCACT AAATAACATACATCGTAAGAAAAGAGGTTTACACGATTACCAACTCNAATTNCGTTGTTG CTTAAAACCCCCCAGATAAACCAATTATAAACTCAACACTGATCAATTACAACACGCTCG GANACACCCACTCAGGTGCTTCATTAAGCCATTTAACATTACACATTAAACGAACAACGG TAAATGTGGTTACACACAAATCAACAAGCGGAACAATTAAACCCAACCTACGTTAAGACC ACATAATAATAAATTGGGGGAGCAGTGATGAAACCAGCCCCAGCAATCCGAAACCCTCGN GTTAATAACCGGGATCGCAGTAGACCATAAATATATGCGAATGCCAATCTNGGTTTAATT TAGGCGTACGTTNACTCAGTTNATCTGCAAAACCACACCCTCAATCAAATACCCAAAACG GGGTTAGCCTTATCCGATACGGTTTATCATTACATTCAGCGGCCAAAAAAATGCCAATGC GAACGTTTCCCCCCGAACGGGGAAAACGTCCAAGCACCCAATCCACAATAGAATCACGGT TCCAACCCGTCAATGCCGCTTCGTCGGTACTAATGTTATTTTTTTTTTAAAACAATCCCG GAACGGTTCCCCCAACAACTCTTGATCTTAGATTTTATGCTGCCTTTCTTGGATT

Table 5. Sequence of spacer region between genes *rps16* and *trnQ* amplified using specific primers

Sl. No.	Spacer region	Sequence length (bp)	Sequence (5'→3')
1.	*rps16-trnQ*	815	>RNQ_V3 sequence exported from RNQ_V3.scf CAGGAACGTTTAAATAACTTTGATTCTTTATCGGATCATAAAAACCCACTTTCCGAAGAT CTCTTCCTTCTCTTCGGGATTGAACATCAATTGCAACGATTCGATAGACCGCTCATTGGG ATAGGTGTAAGTAAATGAACAAGACCCCCCCTAGAAACGTATAAGAAGTTTTCGCCTCGT ACGGCTCGAGAAAAAATGATTCGAGACTTGTCTACGTATAGAATTCGAATGAATGGCAAA AGGGTTGATAAAATCAATTAAAATGAAACTAGGATTTAACTCATTTTTCCTTCGTCCTTC CTGAAATAAATCATTTGTACTCATAACTCAAGTTGGATAATTTTCAAATAGCGCAAAAGA AAATCAAATCTTTAGGCAATTTATTTCATTTATTGAATGGTCTTTAACCCCCTTTTGTTT GTCTCGTTTAAAATTTAAATTGATTTGGATTTTTTATTCTGATCCAGTTAGTGAGACAAT TGAAACGGCGTTTTCTTGTTCTGAGATCCTCTTTTTGTTTTAAATCATTGGGTTTAGACA TTACTTCGGTGATTCTTAATCTTTCCAAAATGGCAGCAACATACCCCTTTTGTGATTTCT TTCTATTACTATTAAAGAATCATACGAACGGTTGATTCCCGNGTGATACACTTTGAATCG CAAAGGTTTTACCTATTCCAACAAATTTCCCTTTTGAATTGAAAACTCGCCCGAATCGAA TCCTTCGAATTCTATATTGATAATATACCTACGAAGTTGGTCCCAATTATTTTATTTATT TGAATATAACCCTAGAATCCTGGTACCTGAACGAA
2.	*rps16-trnQ*	934	>RNQ_V4 sequence exported from RNQ_V4.scf TCCCAGGACTTATACATGGTAGCAGAGTAAGGGTTGCTTTTGAAAATAACGTTCTCTTTA AAGGCCTTCTATTGGTATTGGATGGATAGAATAGAATGTGATTGTTGTTTATTTTTTTGA ATGATTCTTCTTGAAGGAAGTTAGTTACCTTAATTTGAAGAAGAATCTTACGTCTCATAT TTCGTTGAATTTCAACGAGACCCTTTGTTTTGAATTGCAATAAGAAAGAACCTATCTTTC CTATTTCTTATTCCCTATCCATTAACTAAAACGGGGTAGCCCAATTGCCAATTATTAGTA TCTCTGAATTCTAACCAAGAGGGGTCTATTACATTCAATCAATGGGATTAAGTCTTTGTA AGACTAAGTTAGGGTAAAATAAAAAATTAGGAGCAAGGGTTTAATTTGGACTTGATGAGT TGAATCTTTAGTATTCAAAGAAATGGGAGATGGGTCAATCTCTCCTATTGAGTCACTTGA AGATGTAGAGTCAAAAAGAATTCATATATTCGTGTTATTTCTGTTAGTTTGTTTTAAAAA AAATGTTGTATTCATACAATAAAAGATGGGGTCTTGCTAAAATTTCTTCATTCTTCAGAA AAATCTTTACCTTCTATGTATAGAAGAAAAAAGTATAGATTAATCTGTCTATGTACTGAC TGAACCAATGACTATTCATGATTTCGTATTTGAATCATTTGCATACTGGTTCCAATTTAG AGGAAGGGTATGGGTAAAACTTCGTTTTGAAACGANGTTGGTAGAAAGCAACGTGCGACT TGAAAGGACCCGAATCCGAGTGTGAATTCTTATTTTTTACATCCCCACTTTAAATATAGG AATGAAGGGTCCCTCTGGCCGACACCGTTCGGTTTCCCTAACCCCACATTGGTGGGTGTA AGTAAATAAACTAGTGACCTGAGTAAAGATTGAA

amplified region sequenced (RNQ_V4) with primer Er had a length of 934bp (Table 5).

The left flanking region of *rps16-trnQ* spacer amplified with three primer combinations was sequenced. The PCR product with primers Ff and Fr was sequenced. Amplified region sequenced (A_FWD) with forward primer Ff had a length of 639bp (Table 9) and that sequenced (A_REV) with primer Fr was 558bp long (Table 9). The left flanking region amplified using Gf and Fr primer combination sequenced (C_FWD) with Gf had a length of 513bp (Table 9) and that sequenced (C_REV) with primer Fr was 576bp long (Table 9). Amplified region using primers Gf and Gr was sequenced with forward and reverse primer and found to have a length of 283bp (D_FWD) and 259bp (D_REV), respectively (Table 9).

The right flanking region of *rps16-trnQ* spacer amplified with Hf and Hr was sequenced with both primers (Table 8). The PCR product sequenced with primer Hf had a length of 594bp (E_ME5F) and that with primer Hr had a length of 988bp (E_REV).

4.1.6 Similarity search

Similarity search was carried out for the amplified sequence using BLASTN (nucleotide-nucleotide) and BLASTX (nucleotide-protein) program of NCBI.

4. 1. 6. 1 trnG-trnfMet spacer region

The *tnrG-trnfMet* spacer sequence from *C. asiatica* showed maximum similarity to *P. ginseng* (GI: 51235292, AY582139.1) chloroplast genome on BLASTN similarity search with an expectation value (E value) of 9e-41 and a bit score of 174 (Fig. 1). The region of similarity includes part of sequences of both genes and spacer region between them (Table 6). BLASTX result of *trnG-trnfMet*

Table 6. BLASTN similarity for the spacer regions amplified using specific primers

Sl. No	Sequence description and base positions showed similarity	Target spacer	Crops showed similarity and base positions	Gene Identity Number (GI No.)	Accession No.
1.	CENPRIMER 1 (6-262)	*trnG-trnfMet*	*P. ginseng* chloroplast genome (38687-38442)	51235292	AY582139.1
			N. tabacum chloroplast DNA (38084-38378)	76559634	Z00044.2
2.	RNE_V1 (16-34)	*trnE-trnT*	*Oryza sativa* genomic DNA chromosome 4 (33253984-33254002)	5830790	AP008210.1
	SE_FWD (313-336)		*Medicago truncatula* mth2-5j2 (102479-102502)	38194258	AC125475.1
	(256-274)		*Scenedesmus obliquus strain* UTEX 393 chloroplast genome (75583-75565) (158309-158327)	88696597	DQ396875.1
3	RNQV3 (5-776)	*rps16-trnQ*	*Centella trifolia* 30S ribosomal protein gene intron (984-212)	6692895	AF110604.1
			Centella asiatica 30S ribosomal protein gene intron (984-212)	6692894	AF110603.1
	RNQV4 (1-784)		*P. ginseng* chloroplast genome (8169-6316)	51235292	AY582139.1

Table 7. BLASTX similarity for the spacer regions amplified using specific primers

Sl. No	Sequence description and base positions showed similarity	Target spacer region	Crops showed similarity and base positions	Gene Identity Number (GI No.)	Accession No.
1.	CEN_PRIMER 1 (242-111)	*trnG-trnfMet*	*Oryza sativa* japonica cultivar hypothetical protein (65-109)	57900033	BAD88075
2.	RNE_V1 (185-57)	*trnE-trnT*	*Medicago truncatula* retrotransposon gag protein (592-635)	928742247	ABE82543.1
	SE_FWD (660-812)		*Picea glauca* heat shock like protein (10-60)	1350548	AAB015721
3	RNQ_V3 (118-2)	*rps16-trnQ*	*Lactuca sativa* ribosomal protein S16 (13-51)	81176232	YP398311.1
	RNQ_V4 (546-403)		*Arabidopsis thaliana* putative protein (14-59)	7406448	CABB5550.1
	(98-6)		*Arabidopsis thaliana* disease resistant putative protein (242-272)	21618001	AAM67051

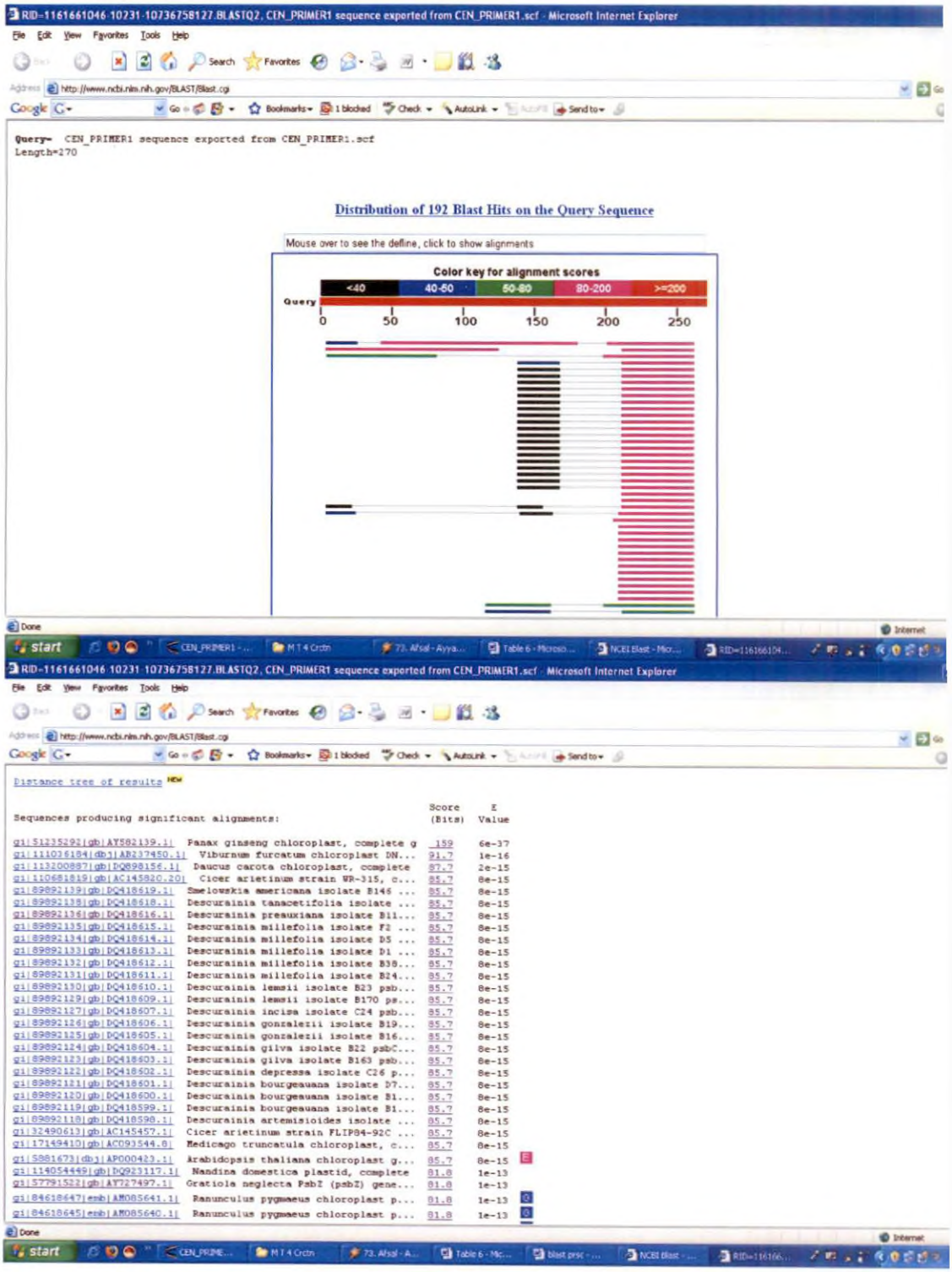

Fig. 1. BLASTN result for *trnG-trnfMet* spacer region (CEN_PRIMER1) sequenced v primer Af

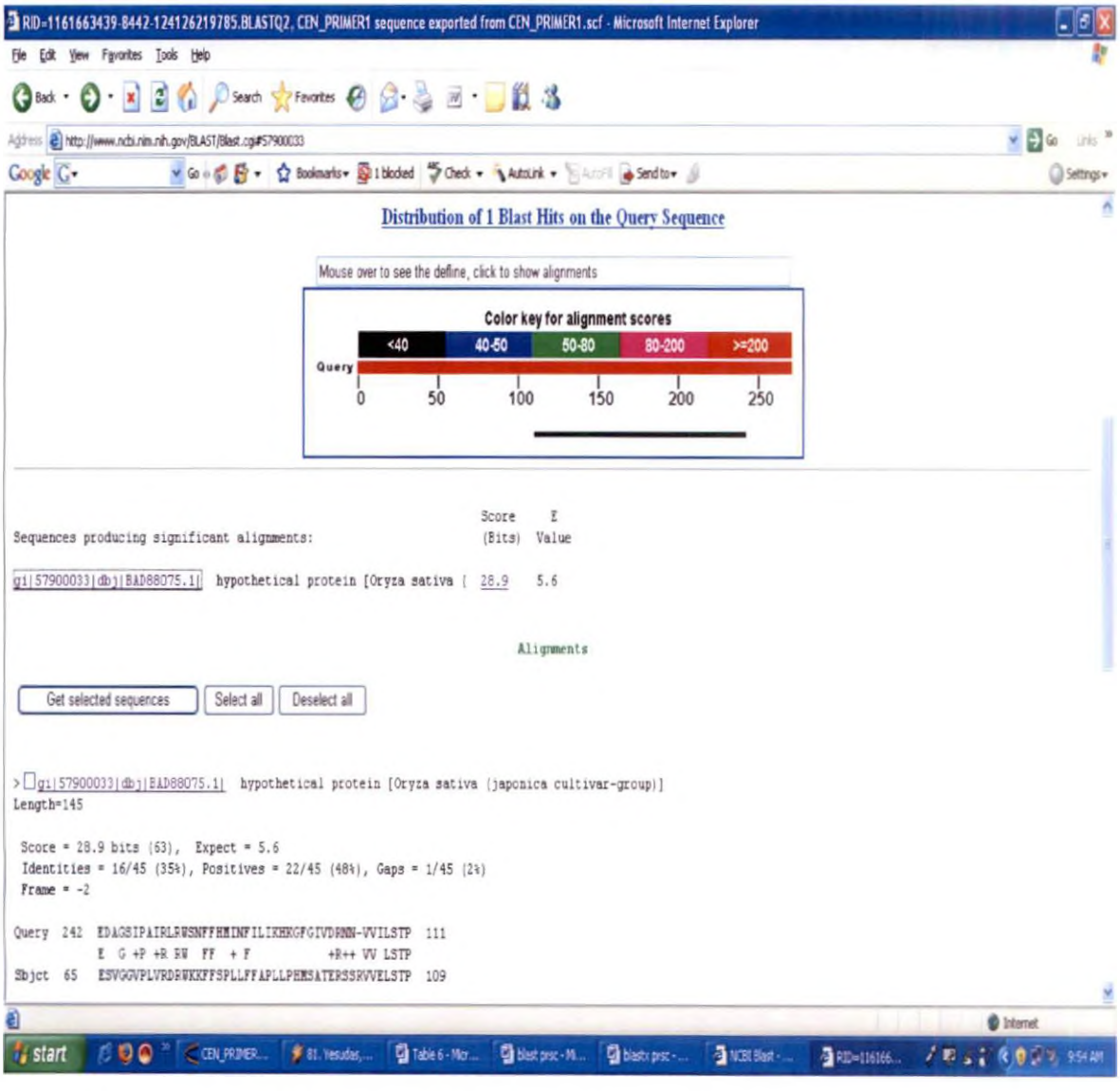

Fig. 2. BLASTX result for *trnG-trnfMet* spacer region (CEN_PRIMER 1) sequenced with primer Af

spacer sequence of *C. asiatica* showed similarity to a hypothetical protein in *Oryza sativa* japonica cultivar-group (GI: 57900033, BAD88075.1) with an E value of 5.6 and score bit of 28.9 (Fig 2). Only 131bp out of 270bp showed similarity (Table 7).

4. 1. 6. 2 trnE-trnT spacer

The spacer region amplified using primers Bf and Br were sequenced with Bf and had a length of 633bp. Similarity search of this sequence using BLAST N showed maximum similarity to *Oryza sativa* (GI: 58530790, AP008210.1) genomic DNA chromosome 4 with an E value of 3.9 and a bit score of 38.2 (Fig. 3). Only similarity with short sequence of 18bp was found (Table 6). BLAST X result of it showed similarity to retrotransposon gag protein in *Medicago truncatula* (GI: 92874247, ABE82543.1) with an expect value of 9.4 and score bit of 29.6 (Fig. 4). A similarity for only 128bp was found (Table7).

BLAST N similarity search of the sequence SE_FWD showed similarity to *Medicago truncatula* mth2-5j2 (GI: 38194258, AC125475.11) *UbiA* prenyl trnasferase mRNA with an E value of 1.4 and bit score of 40.1. It also showed similarity to *Scenedesmus obliquus* strain UTEX 393 (GI: 88696597, DQ396875.1) chloroplast genome with an E value of 5.5 and bit score of 38.2 (Fig. 5). The region of similarity was to small subunit of ribosomal RNA present in the inverted repeat region (Table 6). BLASTX analysis of the SE_FWD showed maximum similarity to heat shock like protein of *Picea glauca* (GI: 1350548, AAB01572.1) with an E value of 5.7 and bit score of 31.2 (Fig. 6). About 152bp showed similarity (Table 7).

4. 1. 6. 3 trnT-trnL spacer

Sequencing PCR reaction failed to amplify the sample. Hence no sequence result could be obtained.

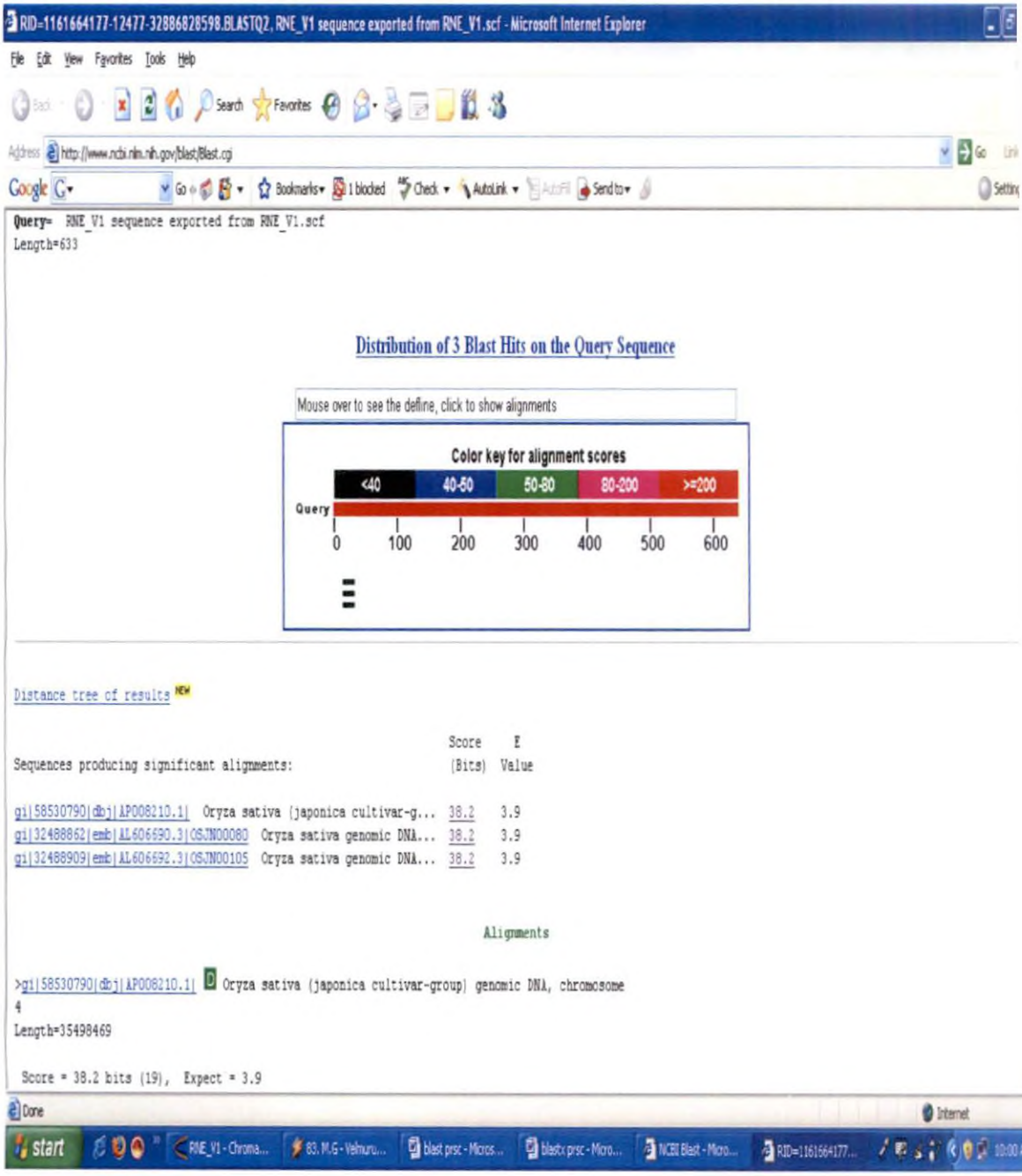

Fig. 3. BLASTN result for *trnE-trnT* spacer region (RNE_V1) sequenced with primer Bf

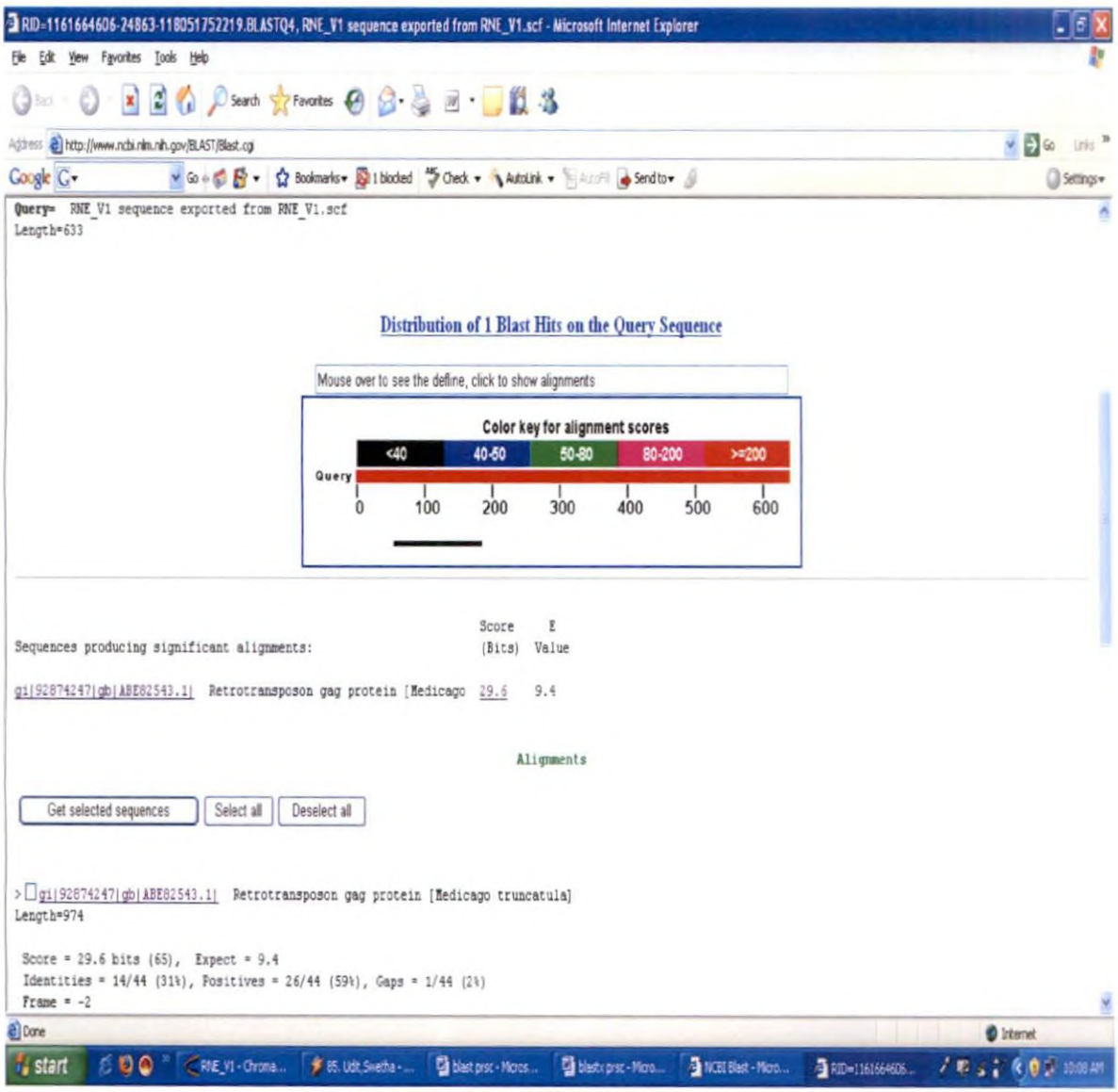

Fig. 4. BLASTX result for *trnE-trnT* spacer region (RNE_V1) sequenced with primer Bf

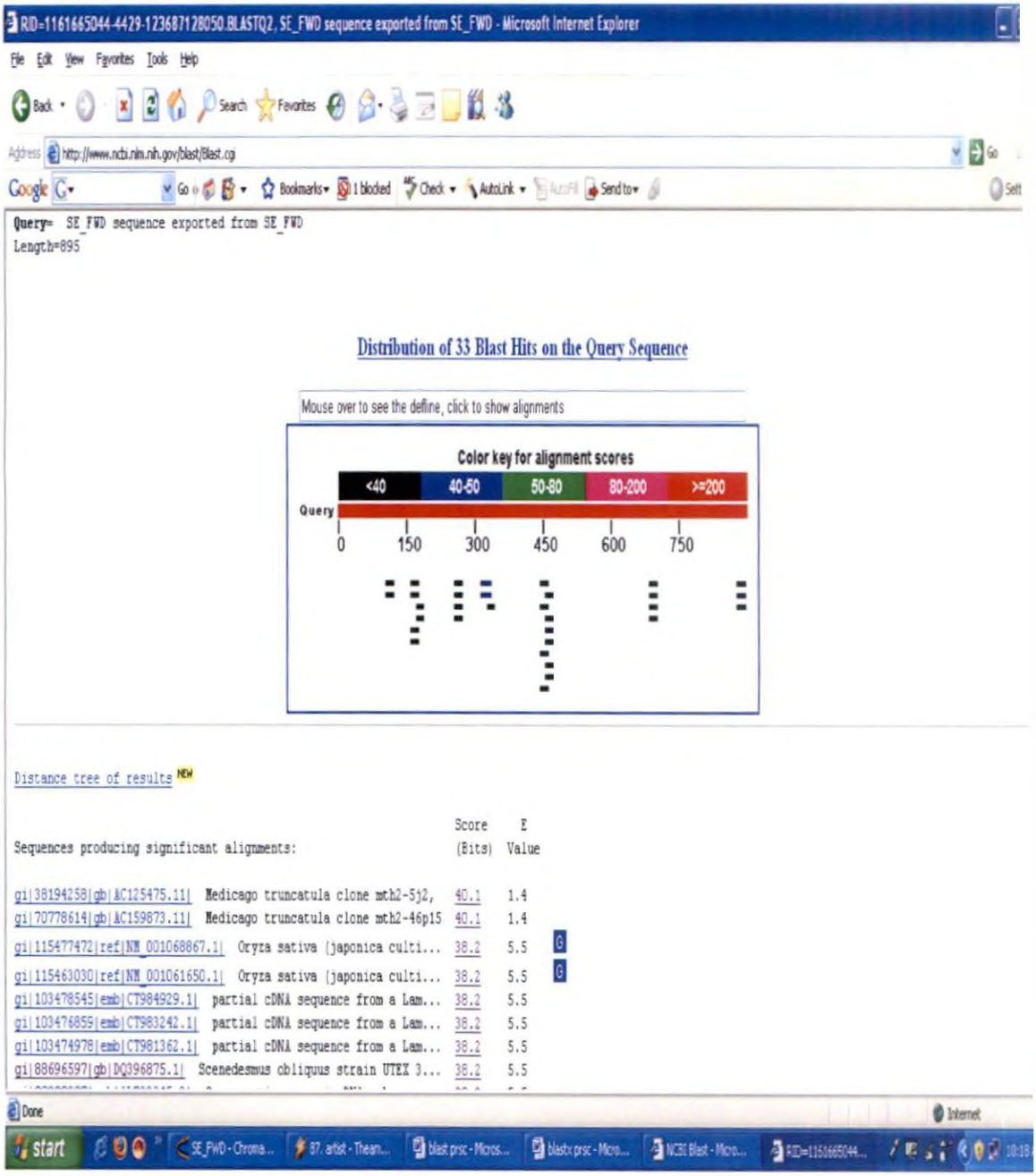

Fig. 5. BLASTN result for *trnE-trnT* spacer region (SE_FWD) sequenced with primer C

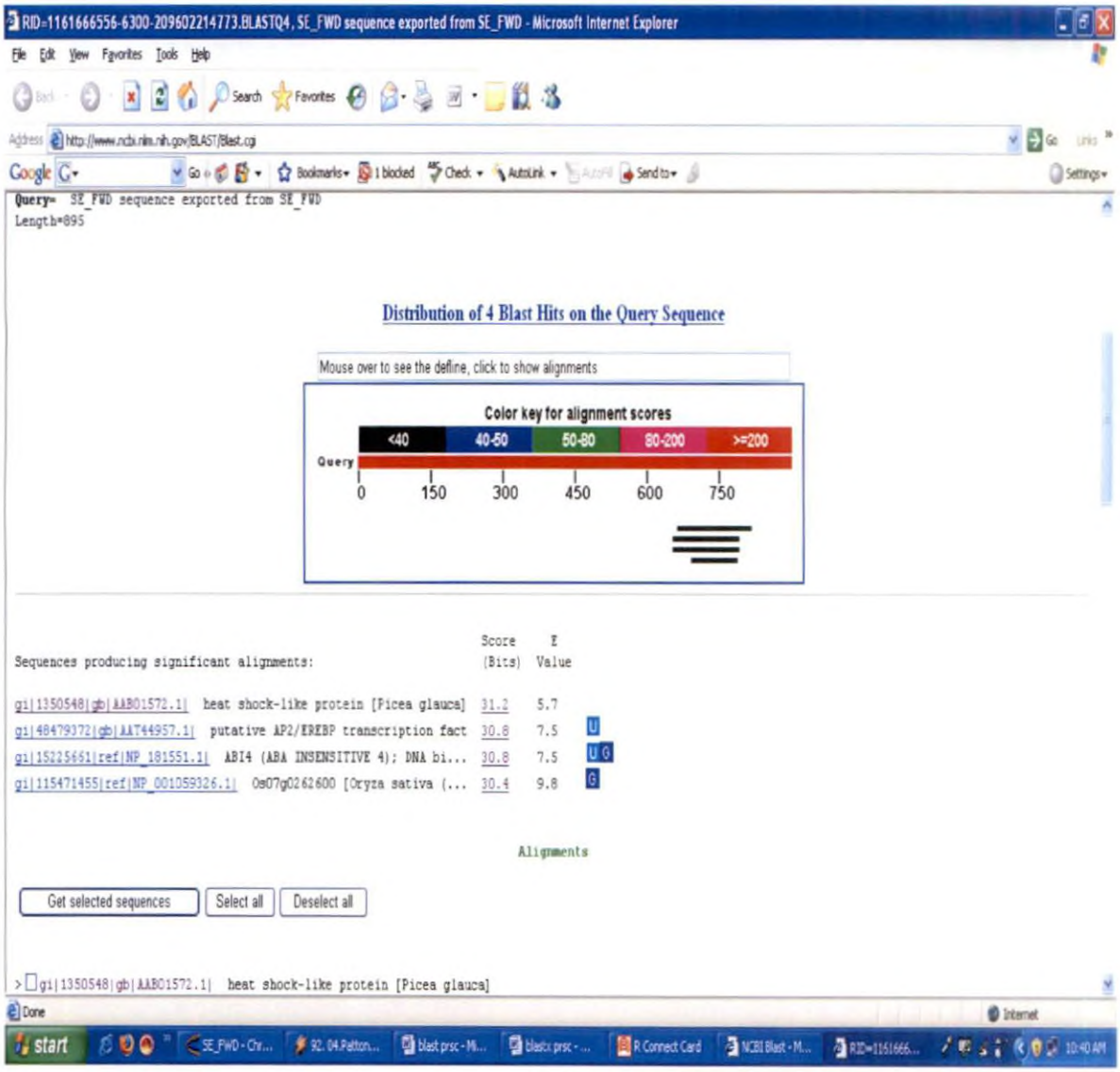

Fig. 6. BLASTX result for *trnE-trnT* spacer region (SE_FWD) sequenced with primer Cf

4.1.6.4 rps16-trnQ spacer

BLASTN analysis of RNQ_V3 showed maximum similarity to 30S ribosomal protein S16 (*rps16*) gene intron and partial coding sequence of *Centella trifolia* (GI: 6692895, AF110604.1) and *C. asiatica* (GI: 6692894, AF110603.1) with an E value of zero and a bit score of 1358 and 1342, respectively (Fig. 7). The region showed similarity was 771bp long (Table 6). BLASTX result of RNQ._V3 gave similarity with *Lactuca sativa* (GI: 81176232, YP398311.1) ribosomal protein S16 with an E value of 3e-13 and bit score of 75.1 (Fig 8). About 116bp long sequence showed similarity (Table 7).

To get complete spacer region, the amplified product was sequenced with reverse primer Er. It (RNQ_V4) showed similarity to *P. ginseng* chloroplast genome with an E value of 1e-41 and bit score of 176 (Fig. 9). This region which showed similarity was corresponded to *rps16* gene, spacer and starting region of *trnQ* gene in *P. ginseng* chloroplast genome (Table 6). BLASTX analysis of RNQ_V4 showed similarity to a putative protein in *A. thaliana* (GI: 7406448, CABB5550.1) with an E value of 6.1 and bit score of 31.2. There was also similarity to a disease resistance putative protein in *A. thaliana* (GI: 21618001, AAM67051.1) with an E value of 7.9 and bit score of 30.8 (Fig. 10). Different regions of RNQ_V4 showed similarity to the two sequences (Table 7).

4.1.6.5 Right flank of rps16-trnQ spacer

BLASTN analysis of E_ME5F showed maximum similarity to *P. ginseng* chloroplast genome with an E value of zero and a bit score of 753 (Fig. 11). This region correspond *trnQ* gene, spacer region after *trnQ*, *psbK* gene and spacer after *psbK* in *P. ginseng* (Table 10). BLASTX analysis of E_ME5Fshowed similarity to photosystem II protein I of *Nuphar advena* (GI: 69217353, AAZ04035.1) with an E value of 7e-13 (Fig. 12). The sequence showed similarity to 57bp of the

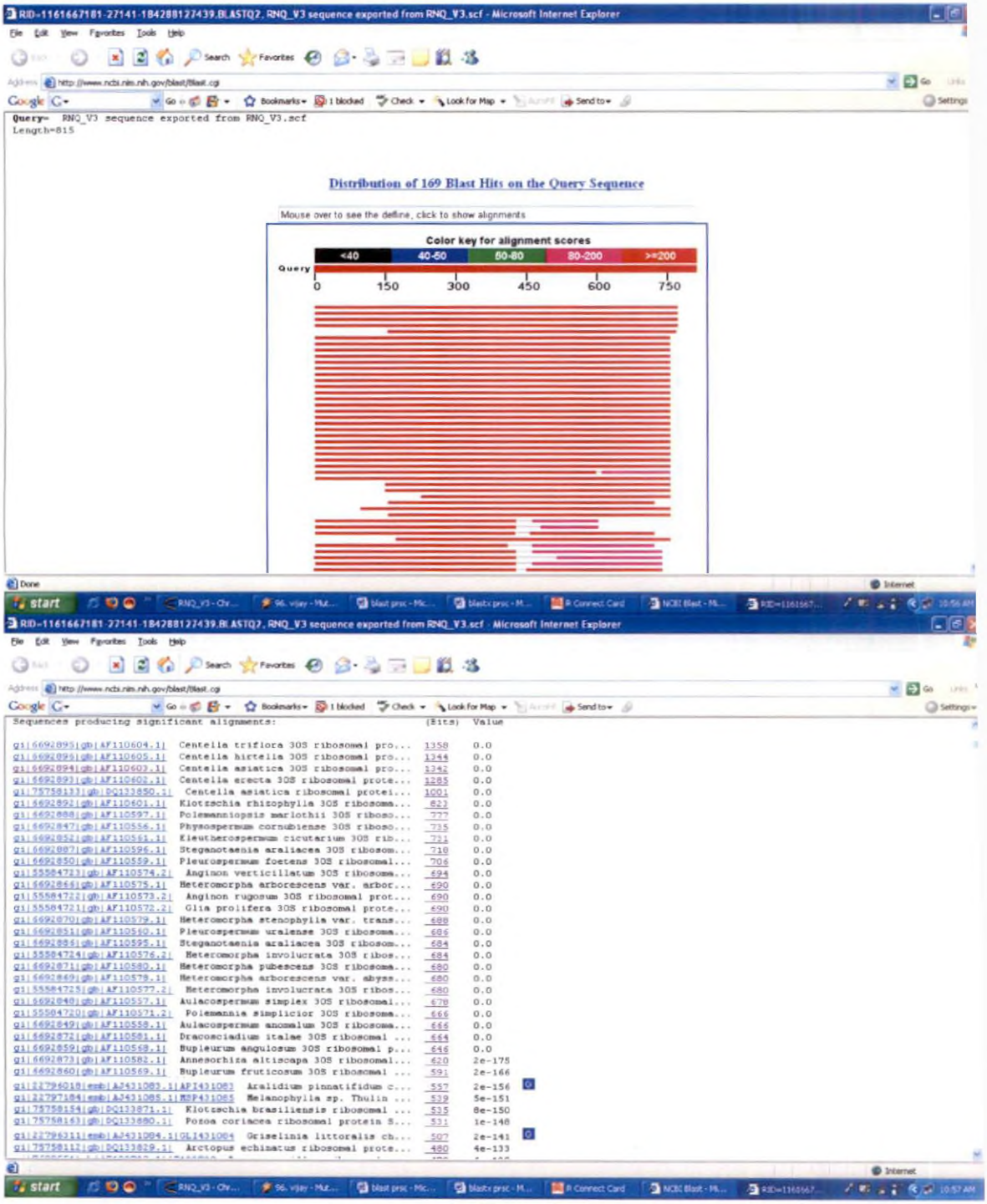

Fig. 7. BLASTN result for *rps16-trnQ* spacer region (RNQ_V3) sequenced with primer Ef

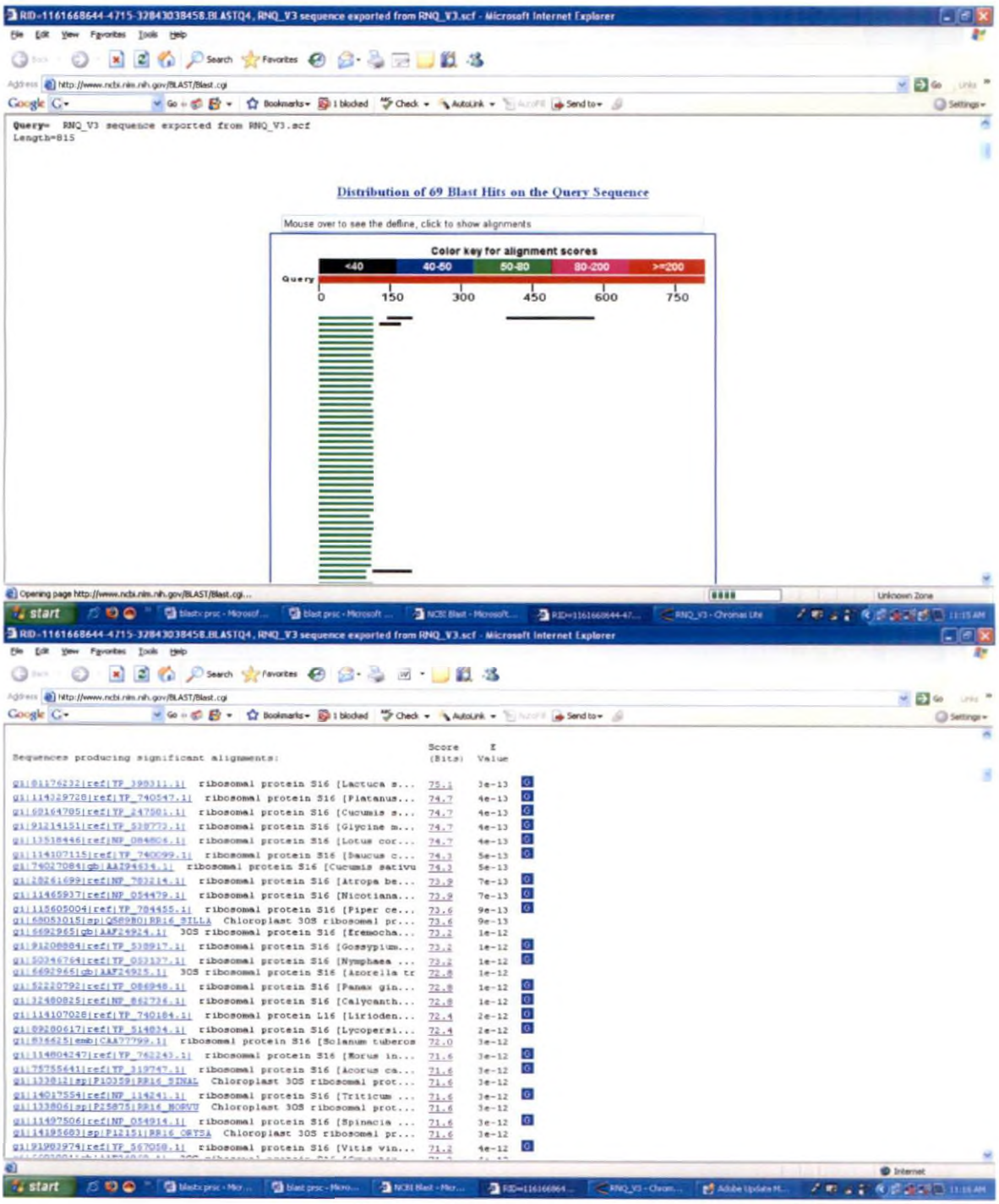

Fig. 8. BLASTX result for *rps16-trnQ* spacer region (RNQ_V3) sequenced with primer Ef

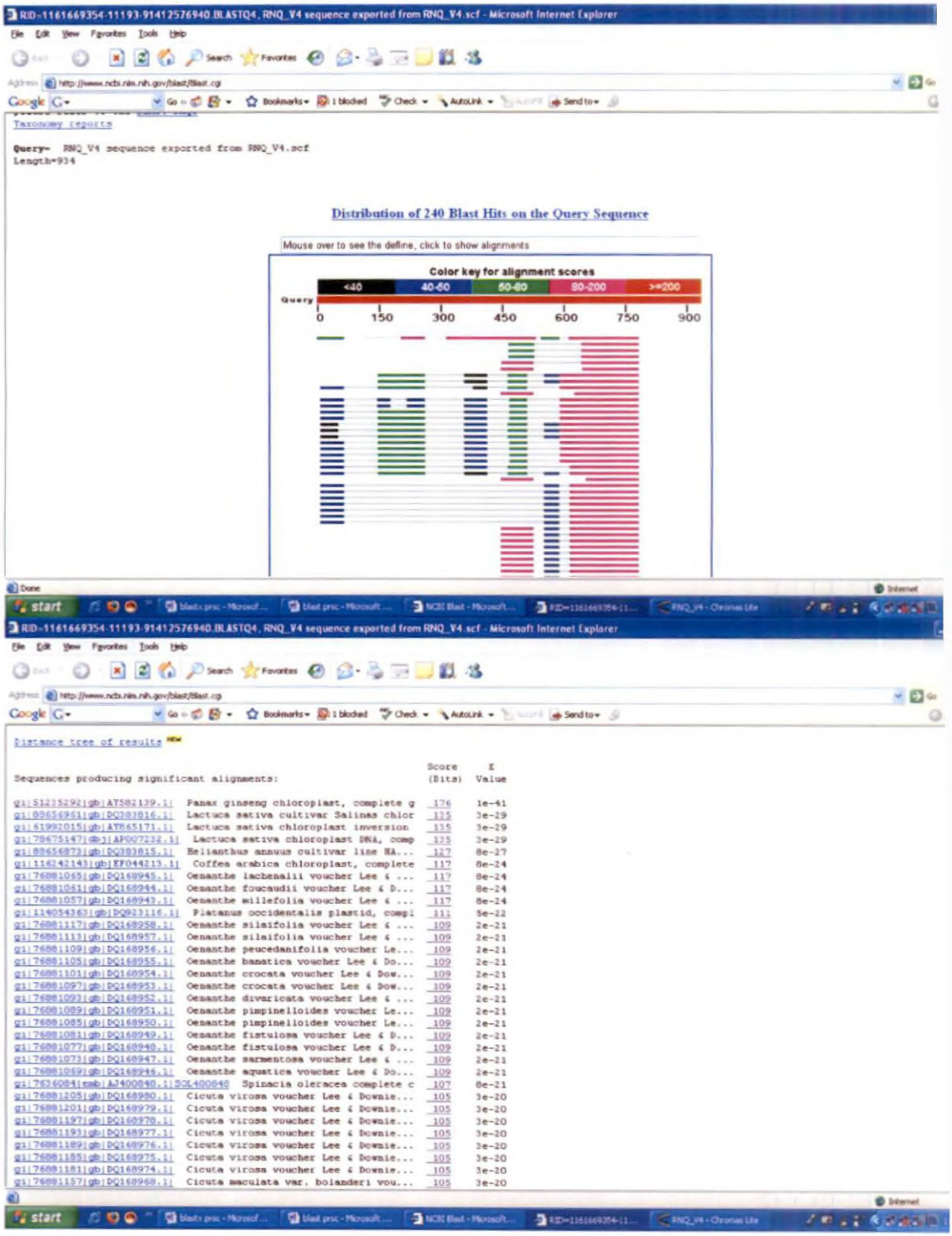

Fig. 9. BLASTN result for *rps16-trnQ* spacer region (RNQ_V4) sequenced with primer

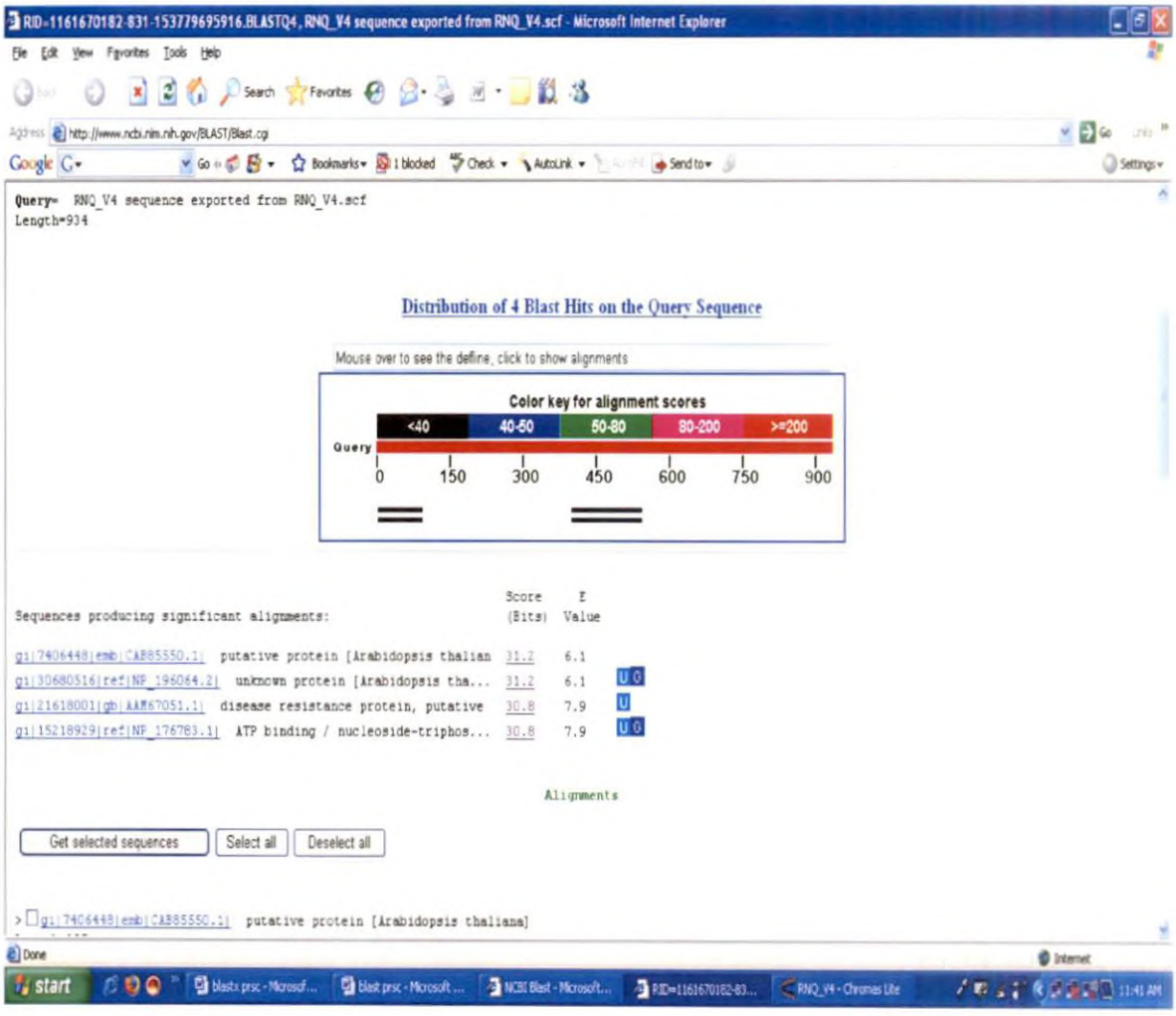

Fig. 10. BLASTX result for *rps16-trnQ* spacer region (RNQ_V4) sequenced with primer Er

protein (Table 11). BLASTN analysis of E_REV showed similarity to the region 9218-8819 of *P. ginseng* chloroplast genome (Fig. 13). This region corresponded to spacer after *psbK* gene and gene sequence of *psbI* (Table 10). BLASTX similarity search of this sequence showed maximum similarity to photosystem II protein I of *Liriodendron tulipifera* (GI: 114107030, YP740186.1) (Fig. 14). The region showed similarity was 56bp long (Table 11).

4.1.6.6 Left flank of rps16-trnQ spacer

BLASTN analysis of A_FWD showed similarity to *P. ginseng* chloroplast genome with an E value of 3e-105 and a bit score of 387 (Fig. 15). This region corresponded to spacer after *matK* (maturase) gene, *trnK* (tRNA lysine) gene and spacer after *trnK* (Table 10). There was no significant similarity found when BLASTX analysis of A_FWD done (Fig.16; Table 11).

Similarity search using BLASTN on A_REV showed similarity to *P. ginseng* chloroplast genome with an E value of 3e-84 (Fig. 17). This region corresponded to spacer after *trnK* gene and *rps16* gene (Table 10). BLASTX analysis of A_REV showed similarity to *Lactuca sativa* (GI: 88656965, ABD47215.1) ribosomal protein S16 with an E value of 3e-23 and bit score of 107 (Fig. 18; Table 11).

BLASTN similarity search of C_FWD showed similarity to *C. erecta* (GI: 2281236, U58599.1) *matK* gene with an E value of zero and bit score of 655 (Fig. 19, Table 10). BLASTX analysis of C_FWD showed similarity to *Micropleura renifolia* (GI: 2281265, AAB64070.1) maturase protein with an E value of 7e-60 and bit score of 228. There was also similarity with *C. erecta* (GI: 2281237, AAB64056.1) with an E value of 2e-58 and bit score of 224 (Fig. 20; Table 11).

Similarity search of C_REV using BLASTN showed maximum similarity to *P. ginseng* chloroplast genome with an E value of 1e-30 and bit score of 139 (Fig.

21). It corresponded to spacer after *trnK* and *rps16* gene (Table 10). BLASTX analysis of C_REV showed similarity to ribosomal protein 16 of *Helianthus annus* (GI; 94502473, YP5880999.1) with an E value of 2e-04 and bit score 33.9 (Fig. 22)

BLASTN analysis of D_FWD showed similarity to *Rhododendron occidentale* (GI: 39932768, AY494184.1) *trnK* gene with an E value of 3e-07 and bit score of 60.8 (Fig. 23, Table 10). BLASTX analysis of D_FWD showed similarity to *Actinotus helianthi* (GI: 14276781, AAK58392.1) maturase protein (Fig 24; Table 11)

Since the sequence result of D_REV contained sequence for mixed bases BLASTN and BLASTX analysis of it was not done.

Table 8. Sequence of right flanking region of *rps16 - trnQ* spacer region amplified using specific primers

Sl. No.	Target region	Sequence length (bp)	Sequence (5'→3')
1.	Right flanking region of *rps16-trnQ* spacer	594	>E_ME5F sequence exported from E_ME5F.scf GGATGACGACTCGTGTGCCACGCCCCATTTAGATTTCTATTCAATCACTACTAAAGAATA ATATTAGTATCGGGTCTTGGTCAATTCCAAGCCAAATATCTATATAAAATCTTTATAGAA TAAATTAGATTCCTGTTAGGATTTTTACACGTGTAGATATAGAATTCAACTGAATTCATT GATCATTACATAGAATTCAATTAAGATATTTTATGAAAGTATGATTTCTTCTATTCTCCT TCGAGAATTGGAGCATTTTTGATTGGATGAGTTCAAAGAAAAAGAAGGATTTTTTGTCTA CCTTACTTTACTTCATTTTTCTTTATATCAATAACTCAATCAAAATGCAATTATCTCCTA AGAACAAAATGTCTGTTATGCTTAATATCTTTAGTTTGATCTGTATCTGTCTTAATTCTG CCCTTTATTTGAGTAGTCTTTTCTTCGCCAAATTGCCCCGAGGCCTATTCTTTTTTGAAT CCAATCCGTAGATCTTATGCCAGTCATACCTTTGTTCTTTTTTCTCTTAGCCTTTGTTTG GCAAGCTGCTTGTAAAGTTTTCCGATGAGATTTTTTAATTACTTATTCCTAGAA
2.	Right flanking region of *rps16-trnQ* spacer	988	>UMA-4-10-06 sequence exported from E_REV.ab1 AAGGCWAGGAGAGACAAGATATCACTACTGTATAAACGAAAAGTTTGAGAGTAAGCATTA CACAATGTAAARCYSKTTTTTGGAAAAAAAAAACGAGAAAAGAGAATAGAACCTCCATCT TTATTTTAGACCAACTTTTTTGGCACCAAGAAATGAAGTGCTTTCTAGAAACAGAAAAGA ATTTCAGAAATTCAAATTCCGTTGTAATAAGAGTCTTTCAGTACCAAAAATTTCTTTTAT ACCTTCAATTAGATATTGTGGGGACCCAAACCTAACCCTATTAGGGTCTTTTACTCGAAA AGGTCAGAATGGGGAATACGGGGGTGAGCCAGATTTGAATTTATCGGGCTATCCAAGACT TTCAATGTTTAAATCGAAGGTTGATACTCCAAGACTTATTTGATCTTATTAATTGGTAGA ATTTTTTTCCCGAATAAAATAATAAATTATGAATTTTCYWGGAKGGGATTAAAAATCCCA TCSAAAACTTACMGCMSCYTGCCMAACAAAGGSKWARRAGAAAAAMRRYCCMWGGKRTGA CKYGGCRMARAWTCTMWSATKGGRTMYWAAAARAAWAGGSCCKSGGGSMWWTKGGGSRAR RRAAARSWYWYTYWAAWAARGGGGSGRATTMSMCRRSYYCCSKYCYAMMMMWRRRWTTWK YSMAACRRSCCYTYGKTSYSGGGRAAAARRMCWTTYKRRKGGKTTTTGSWTARAGAAAWT KGSRKTKWKKWGGKGGRSMMAAMCCCYYTTTTTTTTKGSRCSCKCYMYMAMAWARCYCCA YTCYSRWARGGRWKKWSAWAWSSMCMCKCYYCKMAYYGWRWWAKATTTYTGWSAMRMSRA KATWTTCKGARAMWCWCAYAMRCMSCGCMMSMAMAWRTRTYSASRWKTAMRRRARTKTWK TWWTCTCTCTTTTKSCSCRYMCKSYSWCATKCSTMKTGTGTTTTATRMMATRMGMGSSKG WGRGTGKKKRKKKGSCSYSMRCCGMATC

54

Table 9. Sequence of left flanking region of *rps16 - trnQ* spacer region amplified using specific primers

Sl. No.	Target region	Sequence length (bp)	Sequence (5'→3')
1.	Left flanking region of *rps16-trnQ* spacer	639	>UMA-4-10-06 sequence exported from A_FWD.ab1 CAATWWAWYWYYTGACCAACTTGAATTGATTCTTTTTGTTCCGTTTCAAAAAATCAAACA AAGGTTTGTACCGAAAAGGGAAAACGAAATATTCTCAAAACCCTTCGTTGATACGACAAG CTATTTTTTCCATTCATTCCCTTTCAGGATCAGTCGTGGTCTGCCAAACTTTACCGATGG TATGGACGAATCCTTCGCTTCATCCAAATGTGTAAAAGATCCTAGCCGCACTTAAAAGCC GAGTACTCTACCGTTGAGTTAGCAACCCGAATAAAAAAGTATGTAGATACAATCAAAAAA AAAATTAGACAAGATAATTAAACCATTGAATTAAGAAAGAAAAATCGAAAAAAAAAAAAA ACAAATTTTGAAATTGATTTGGAACATCAAAATGAATCTTTGAAAAAAAAATMCAAAAAA TTCTTTCGATAAAAAAAARATARATAAATCCWCAAATCTTKRGACRCCCCCCYTTTCYTWY CAWTTTTYCWTTTTAMCMMAAAACCCCTTTKGWWYYAAATCRAAWMCAMCRRACACCCCK CTTTTGAATGATGAASARSAAAAAWCMAACCCKKGGMRMMMAAAARAAAACSMYCCCYCT TWTCWMGARGAAWTWWWRTTGCKCMAWACRSTTCCCTMC
2.	Left flanking region of *rps16-trnQ* spacer	558	>UMA-4-10-06 sequence exported from A_REV.ab1 GGRYWWTKMYTTGGATGTTCATCCCGAAGAGAGGAAGAGATCTTCGGAAAGTGGGTTTTT ATGATCCGATAAAGAAACAGGMTTATTTAAACGTTCCTGCTATTCTATATTTCCTTGAAA AAGGGGCTCAGCCTACAGGAACTGTTCGGGATATTTTAAAGAAAGCGGAGGTTTTTAAGT AACTTTGCCCTAATCAAAAATCAAAGGAAACTCAATTTCAATTAAGGAAAGAAAAAAGAG GGGTAGTGATTCGTATATAATTTTGTATAACTTTTCTATGTTTTTTTATTTCCCCCTTTT GTAGCTCTCTTCATAGCTATTTTTCATTGTTTCCATAGAATGTCATGTTCTATAAGGGCA AAACCTAGTTGGGTGATCCTAGAAATAGAAAATTCTGGTATTTCTTTCAACTGAGCGATT TCGTTAGAATTTTCCTAACAAGGAATAAATCAAATTTGTTTATTCTACTTATTATTGTYA YTGAYTYMATAAMTCYAAATCTGAYATWWWTTTCCCTTCAAAWTTWTTTAAAAACCKWTC MAACCTTTYTYYMTCMAT
3	Left flanking region of *rps16-trnQ* spacer	513	>UMA-4-10-06 sequence exported from C_FWD.ab1 AWWYCTTWMMYTAAATTAAATGTGAATTTTGTCCTCTAAAAAAGGAAATATTGAATGAAT TGATCGTAAAGAGCGGCTTATTATCCCTTTCCTTTCTAGGGAAGAATTAAAGGACGAAAA GGGAATTTCCACAATGACTGTAACCCCCTCTGATATCAATTGAGAATACAAATTCTTGTT ACGCCCCCAAAAGATATTTTGGTTAAAATCATTAGTAGAAAGAATCAAATGATTCCGTTG ATACTGATACATTCGAGCAATTAAACGTTTCACAATTAGTAAGCTGGATTTATTGTCATA ACCTGCATTTTCTAACAAAATTGATCTATTTAAACCATGATCATGCGCAAGTGCATAAAT ATACTCCTGAAAGATAAGTGGATATAAGAAGTAGTATTGTTGAGATCTATTTAGCTCTAA ATATCTTTGGAATTCTTCCATTTGAAATTCGAATTTAAACTAAAGGTGGAGGATTTGGGG GGTTATCCAATGATCCMYACYMGCKATYCWWTC

Table 9 continued

Sl. No.	Target region	Sequence length (bp)	Sequence (5'→3')
1.	Left flanking region of *rps16-trnQ* spacer	576	>UMA-4-10-06 sequence exported from C_REV.ab1 TGRKKKTKSMYKSRKGMWTMRYCWSSCRAGAGAAGGAAGAGAKCTTCGGARRGKGGGTTT TTAKGATCCGATAAAGAACAAGTTATTKRAACGTTCSTGCTATTCYAAKTKGGAAMCGRG GCYCGGSTACAGGAACTGTTCGGGATATTTTAAAGAAAGCGGAGGTTTTTAGTAACTTTG CCCAAATCAAAAATCAAAGGAAACTCAATTTCAATTAAGGAAAGAAAAAAGAGGGGTAGT GATTCGTATATAATTTTGYAKSASTSSTSKATWTTTWYWTATTSCCCCCYYKSGWAWCTC TCTTCATKGYTATTTCWCAMTGTTSYMKTAGAATGSSMTYTTSTWTGASGGRAAASYRTA GKYGGWSMMSYCTARACATASAAAKTTSRGGYWTTWCWWWYCYMGSCSGCCMTYTRTARY CWTCYYCTMTYKAWGMCCCCMCCKGCCTSGTTGGTWGGATSYCSAYTCKYYYCYAYMAAM WGWSTTTGRKTGCYWGKTCSCCACCWCTTSTYCCAKTTTWKCCKCKYWTTTTWAYYYCCR RGSMKGGWSWRWSCMMAAGGMTTRAAWWMWCGGARW
2.	Left flanking region of *rps16-trnQ* spacer	283	>UMA-4-10-06 sequence exported from D_FWD.ab1 GSSMMCYMAMYAATTAATTGGATTTTCTCTCTAAAAACGGAATATTGAATGAATTGATCG TWAACTGATTCTRKCCSRSCCTTTCTAGGGAAGATATAATGCCTAWWGGGRTTTCCRCRR TGACTGYAACCCCCTCSWMKKKSKRKYGSGARKRSRAATTYYKGTTAYKTWMMMAARAGA YATYTKGGTTAAAWTCATMAGTAGSAAGYAKKKWKTGRKSCCGTTGARARTGATKCATTC GAGCSGGGGGSGGCGCYCCSSCRMCRGGSCGGYTTSASYCCCC
3	Left flanking region of *rps16-trnQ* spacer	259	>UMA-4-10-06 sequence exported from D_REV.ab1 TGGRYAKWRASGAWTWWKAAGAAARAGGASGTTTTKAAKTAASTTTGSAKARKSRKRRAT CAAAGGATATGGCTCCCCCARGRSGTMRGGGGARYKAKKARACTCCTTTRSGGGSMTGGA RRAYATRCMMCKMSGSWRKYWRRKYRWRRMKSKWMYYWKWWTTTTMWYARGAKGYYRYGW KWAKAARGRYYAYMAKAATTKTWKAKASASRASCCMACAAATTTAAWAAAAASAGGGGGR RKKKGAAAAATTTWYCAAA

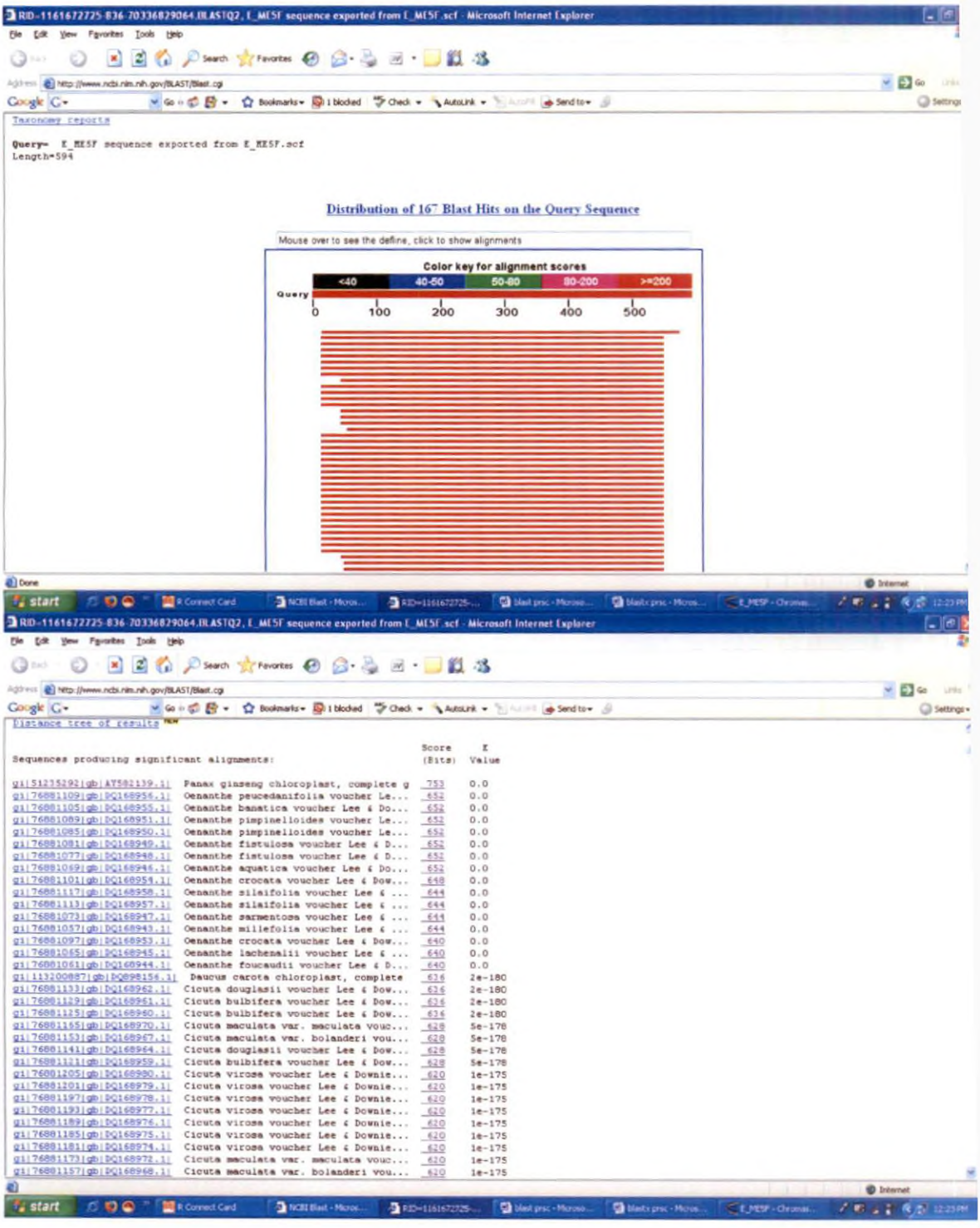

Fig. 11. BLASTN result for right flanking region (E_ME5F) of *rps16-trnQ* spacer region sequenced with primer Hf

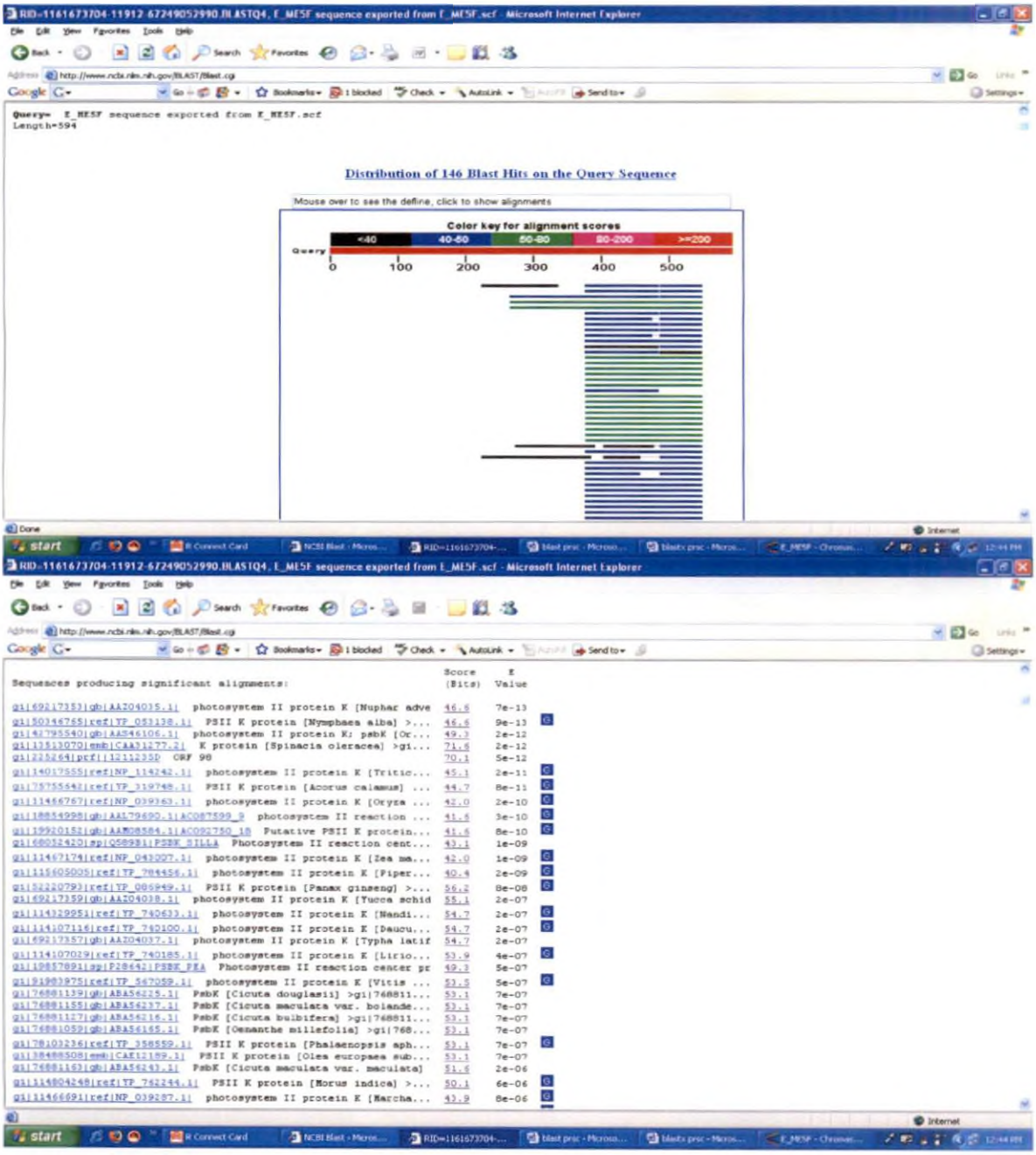

Fig. 12. BLASTX result for right flanking region (E_ME5F) of *rps16-trnQ* spacer region sequenced with primer Hf

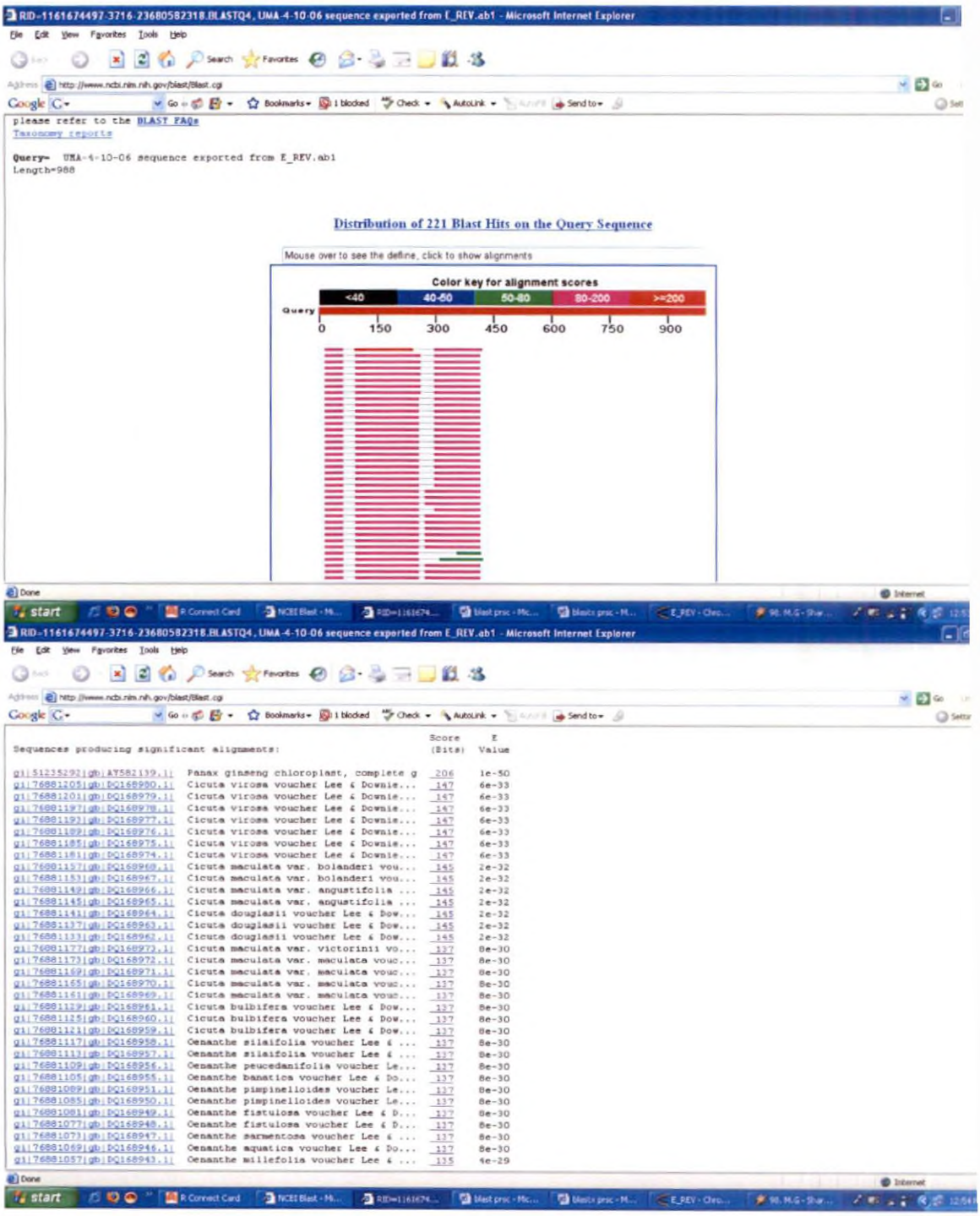

Fig. 13. BLASTN result for right flanking region (E_REV) of *rps16-trnQ* spacer region sequenced with Hr

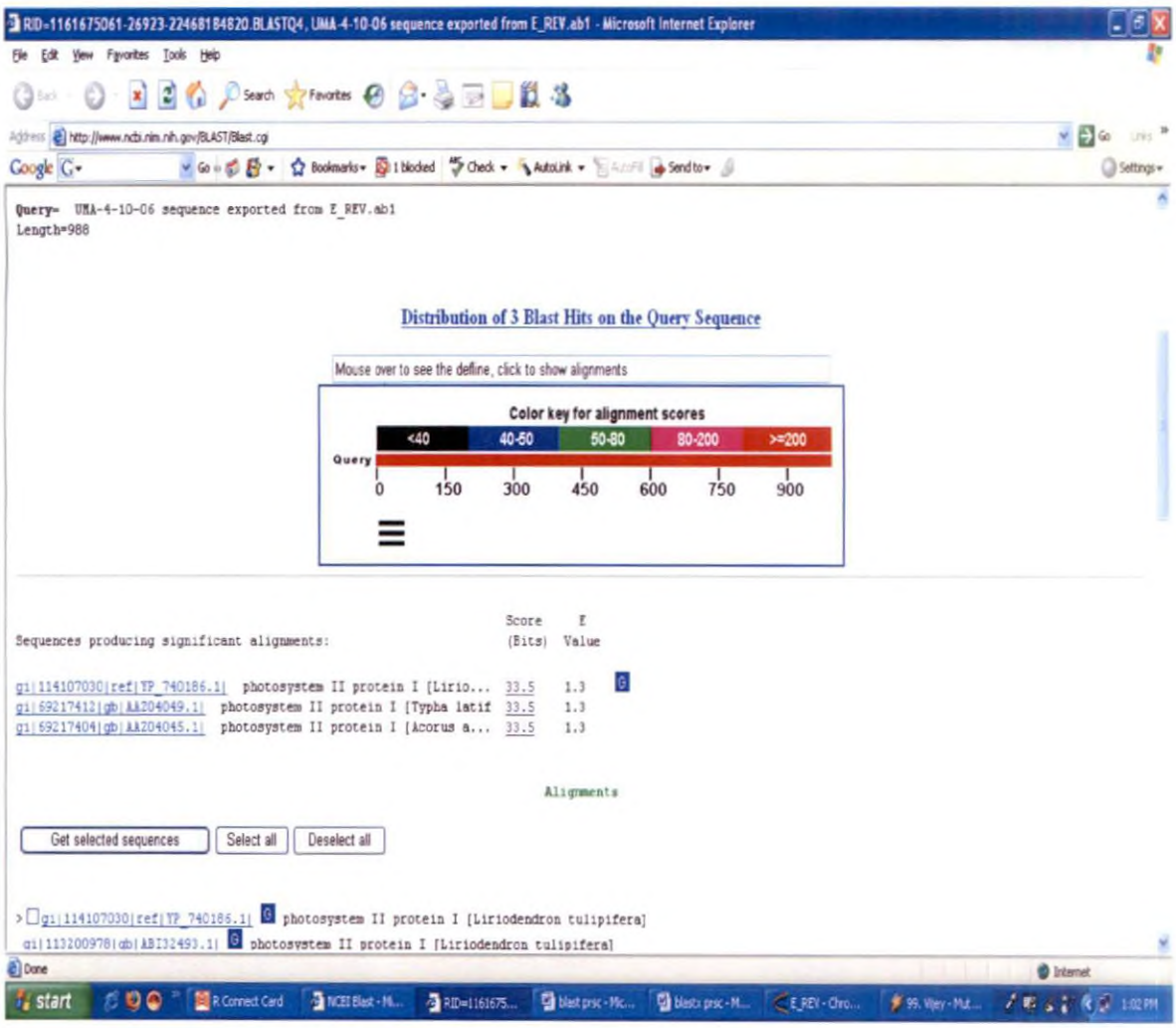

Fig. 14. BLASTX result for right flanking region (E_REV) of *rps16-trnQ* spacer region sequenced with Hr

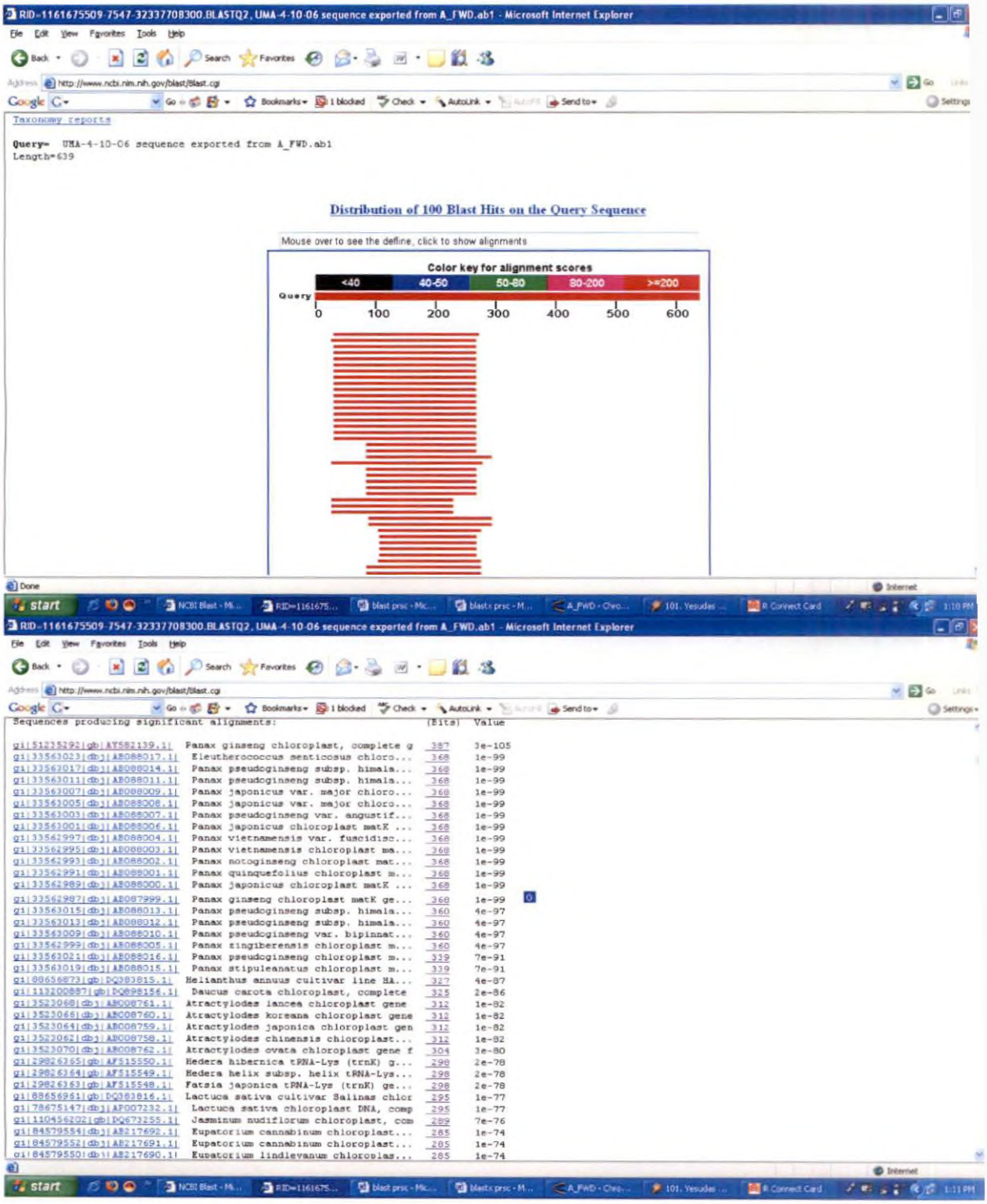

Fig. 15. BLASTN result for left flanking region (A_FWD) of *rps16-trnQ* spacer region sequenced with primer Ff

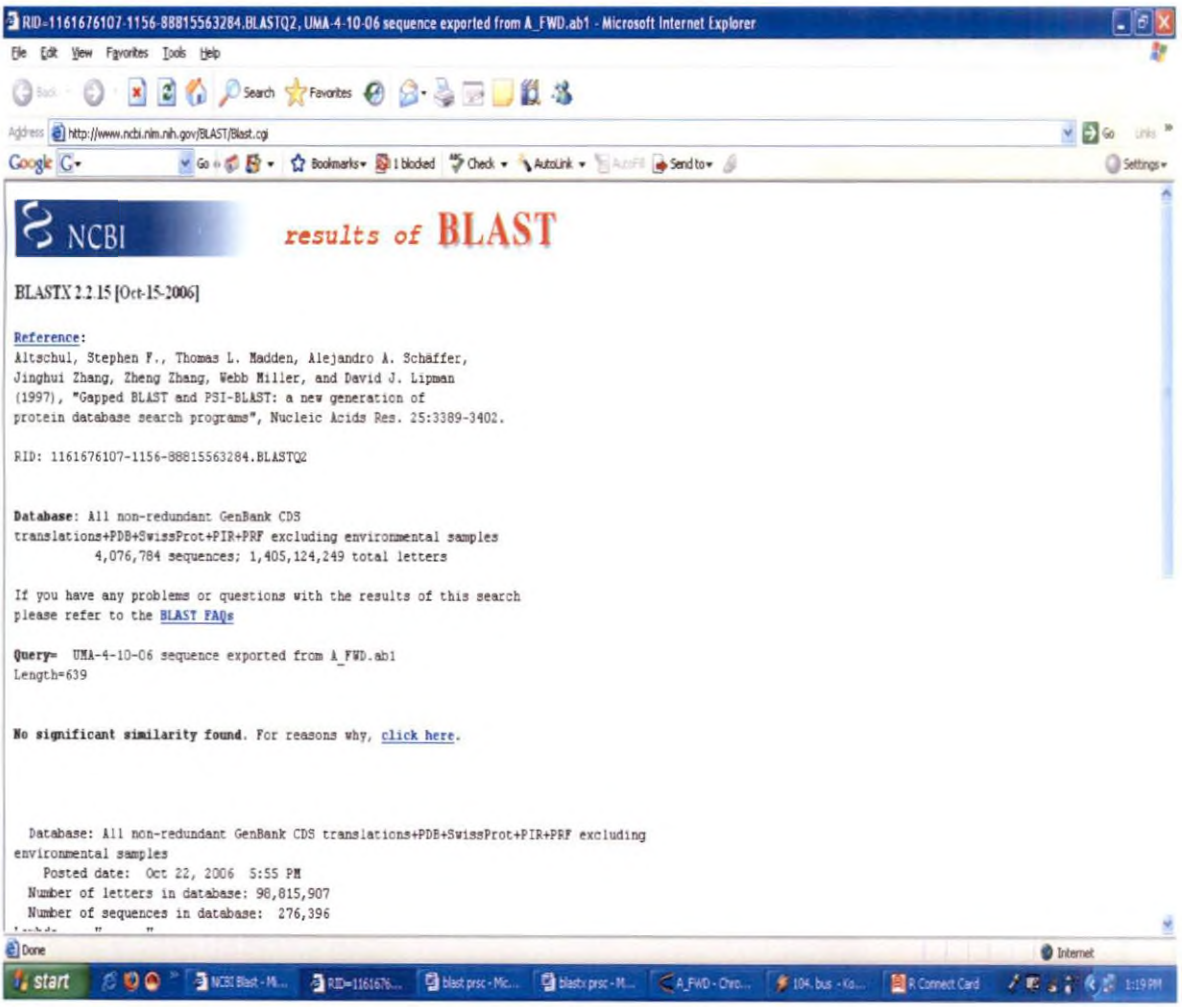

Fig. 16. BLASTX result for left flanking region (A_FWD) of *rps16-trnQ* spacer region sequenced with primer Ff

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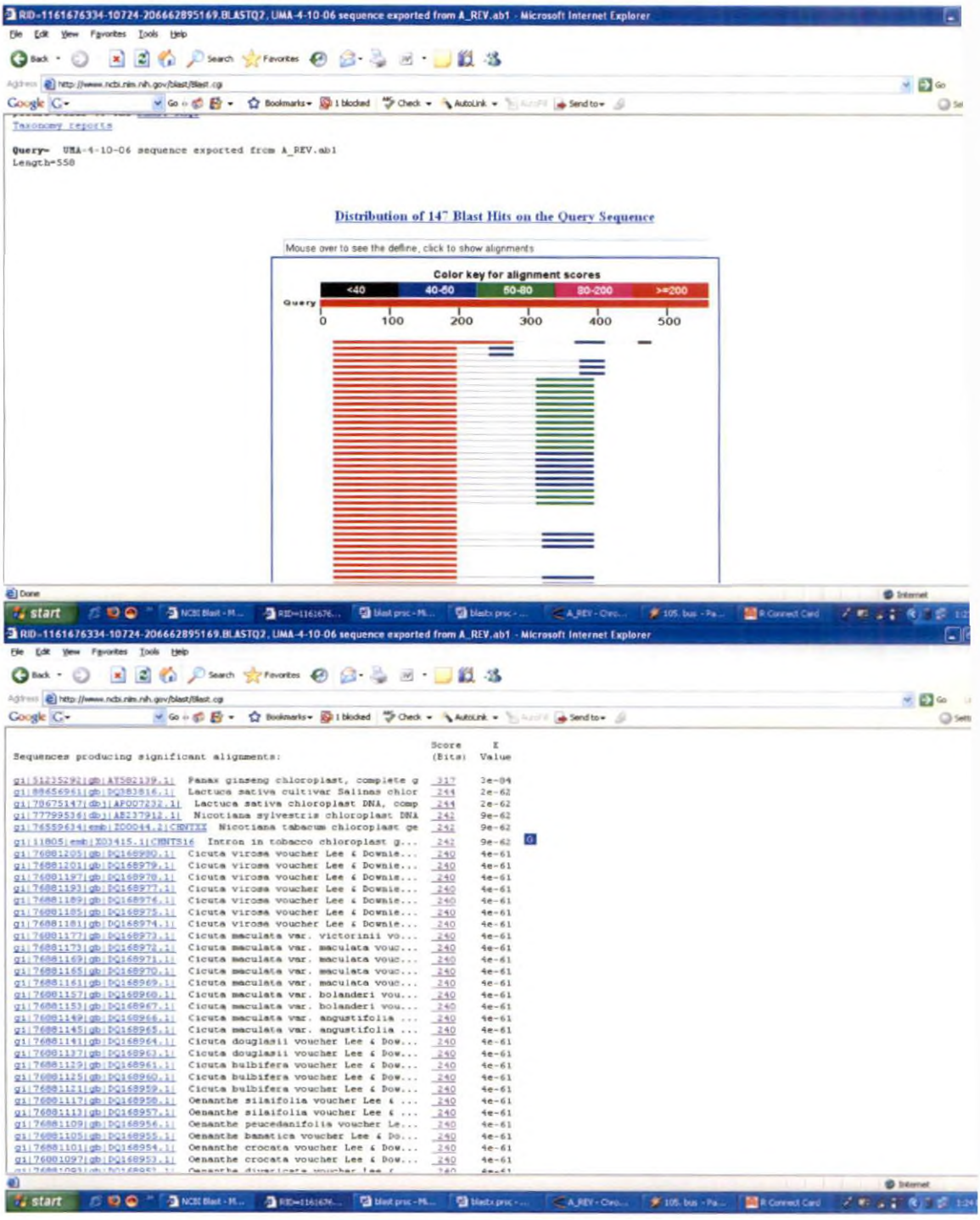

Fig. 17. BLASTN result for left flank of *rps16-trnQ* spacer region (A_REV) sequenced with primer Fr

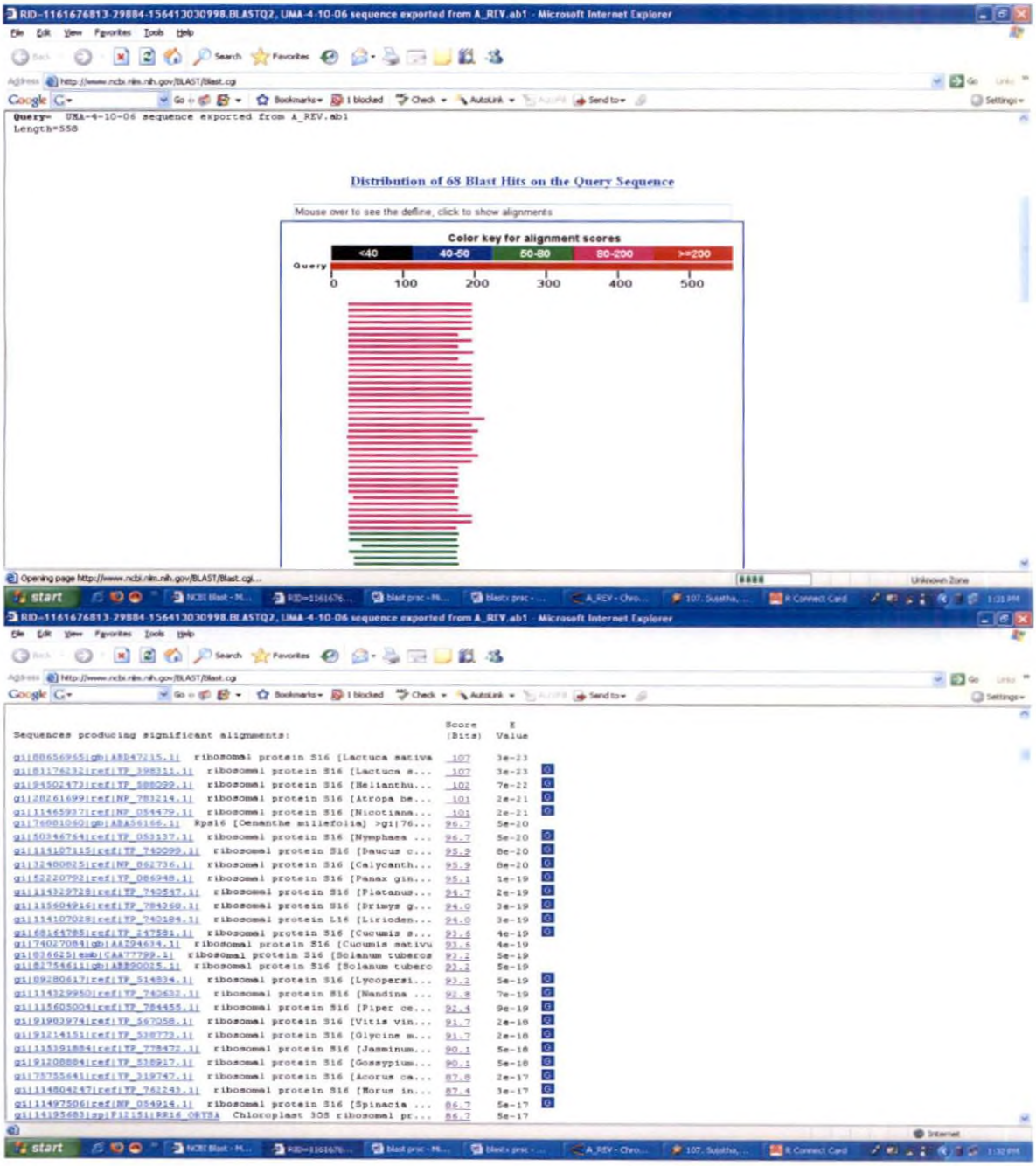

Fig. 18. BLASTX result for left flank of *rps16-trnQ* spacer region (A_REV) sequenced with primer Fr

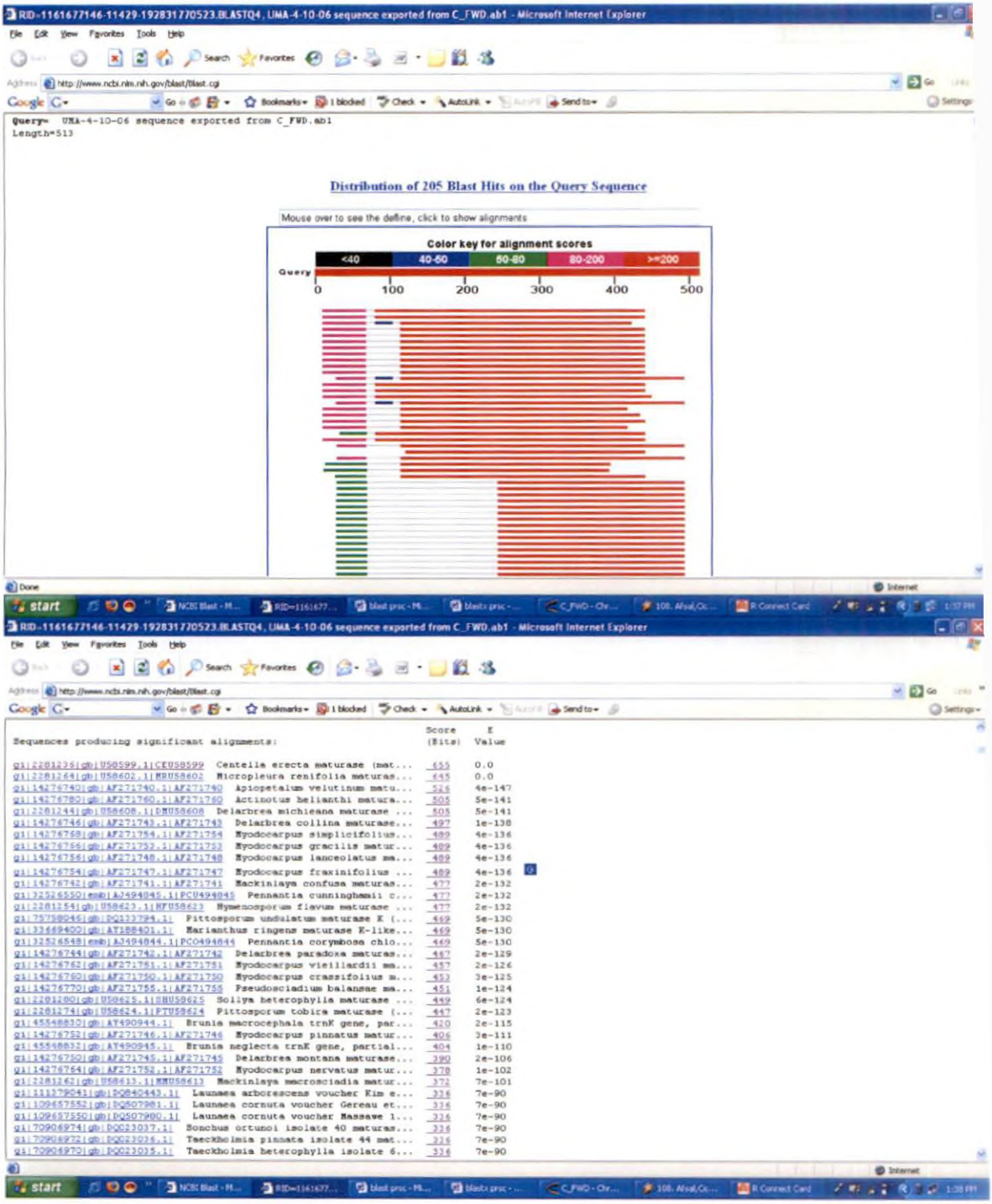

Fig. 19. BLASTN result for left flanking region (C_FWD) of *rps16-trnQ* spacer region sequenced with primer Gf

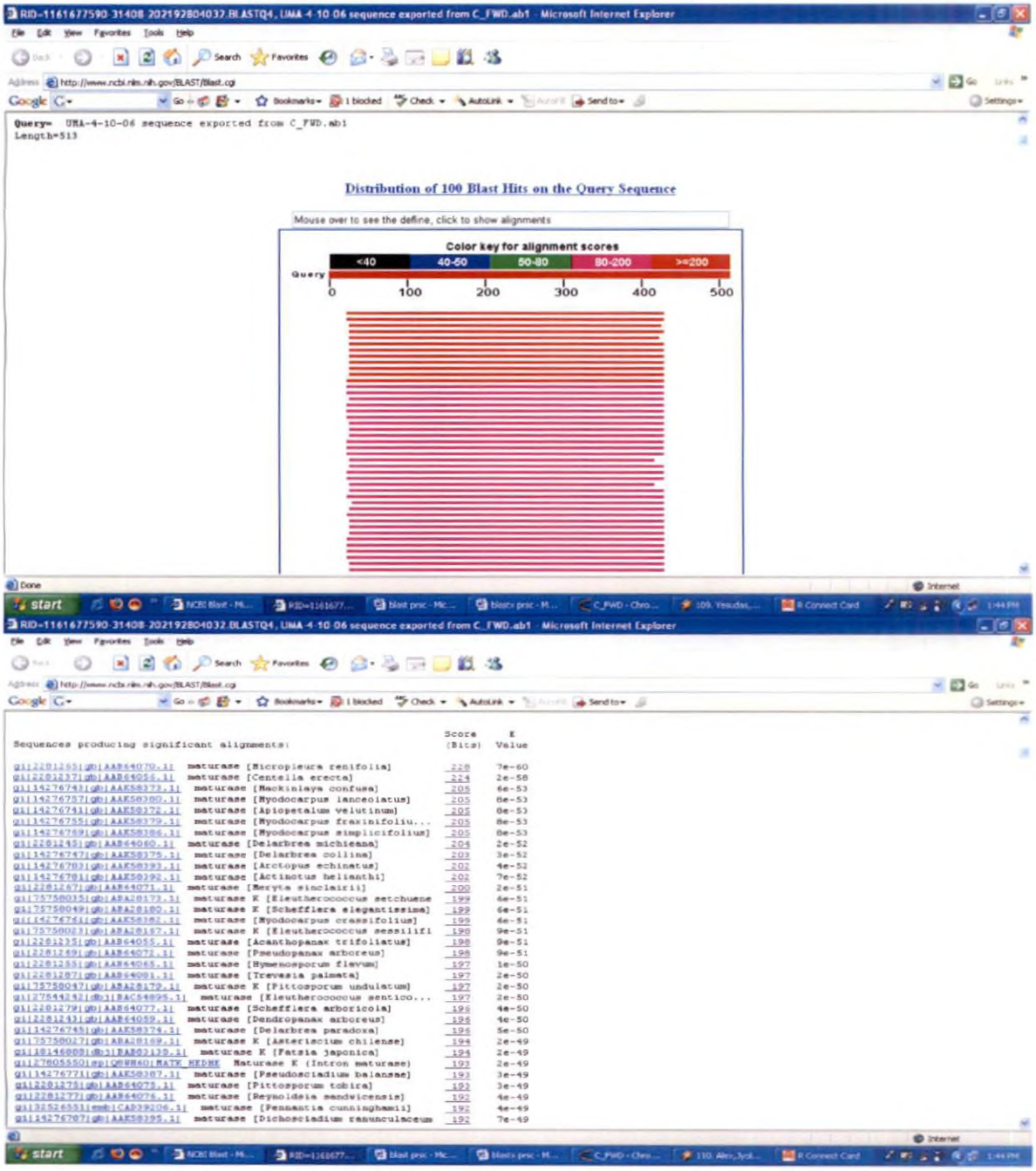

Fig. 20. BLASTX result for left flanking region (C_FWD) of *rps16-trnQ* spacer region sequenced with primer Gf

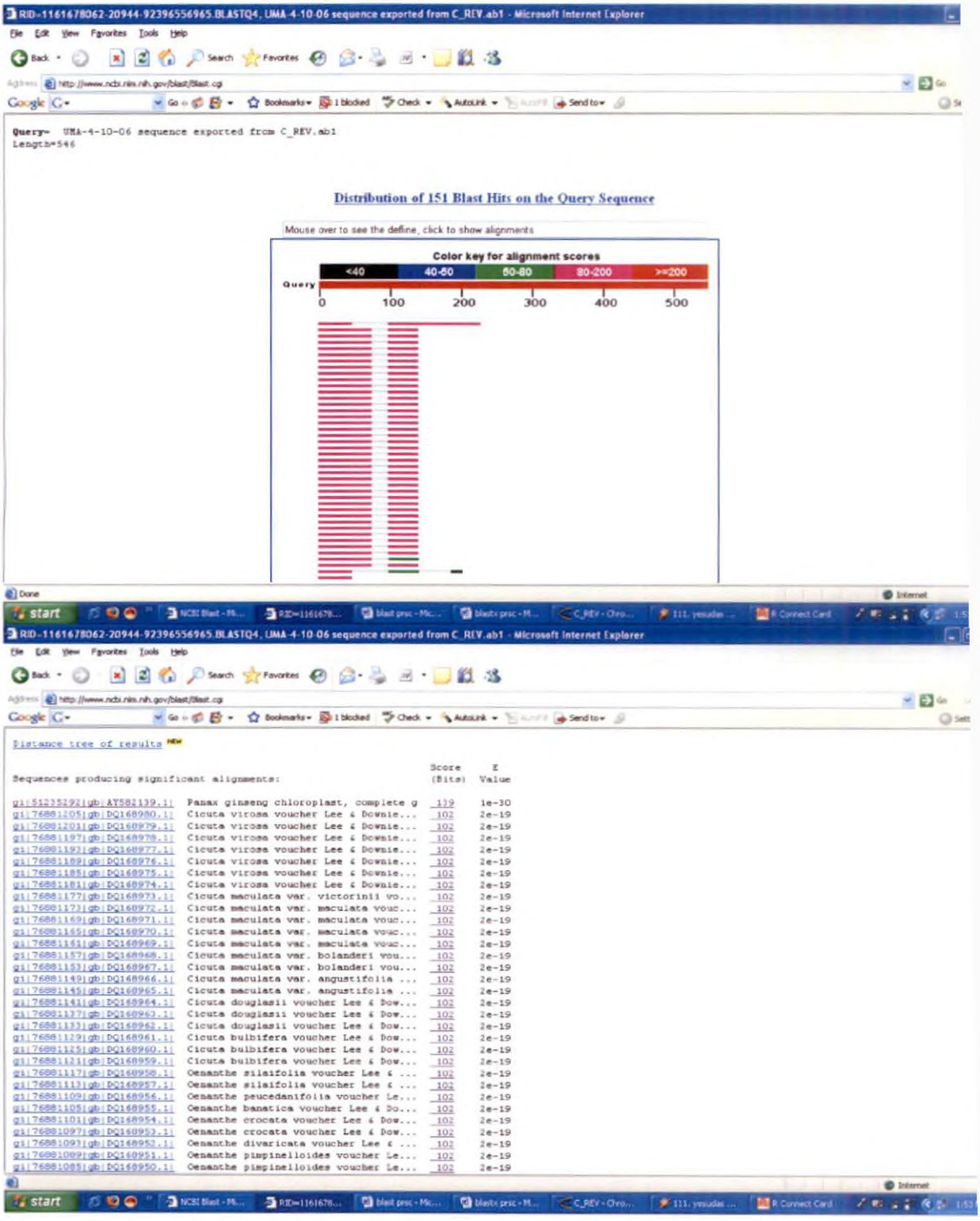

Fig. 21. BLASTN result for left flanking region (C_REV) of *rps16-trnQ* spacer region sequenced with primer Fr

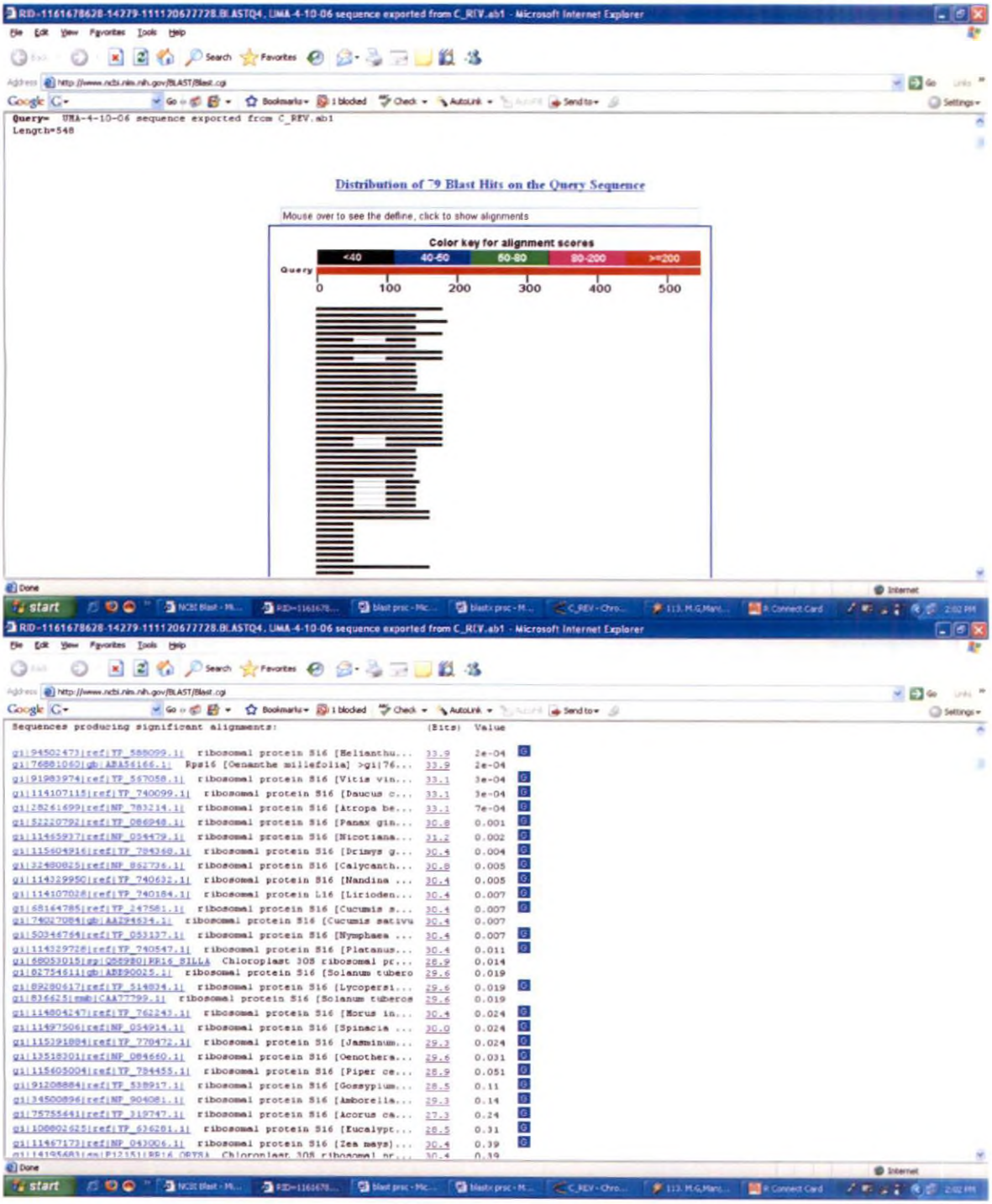

Fig. 22. BLASTX result for left flanking region (C_REV) of *rps16-trnQ* spacer region sequenced with primer Fr

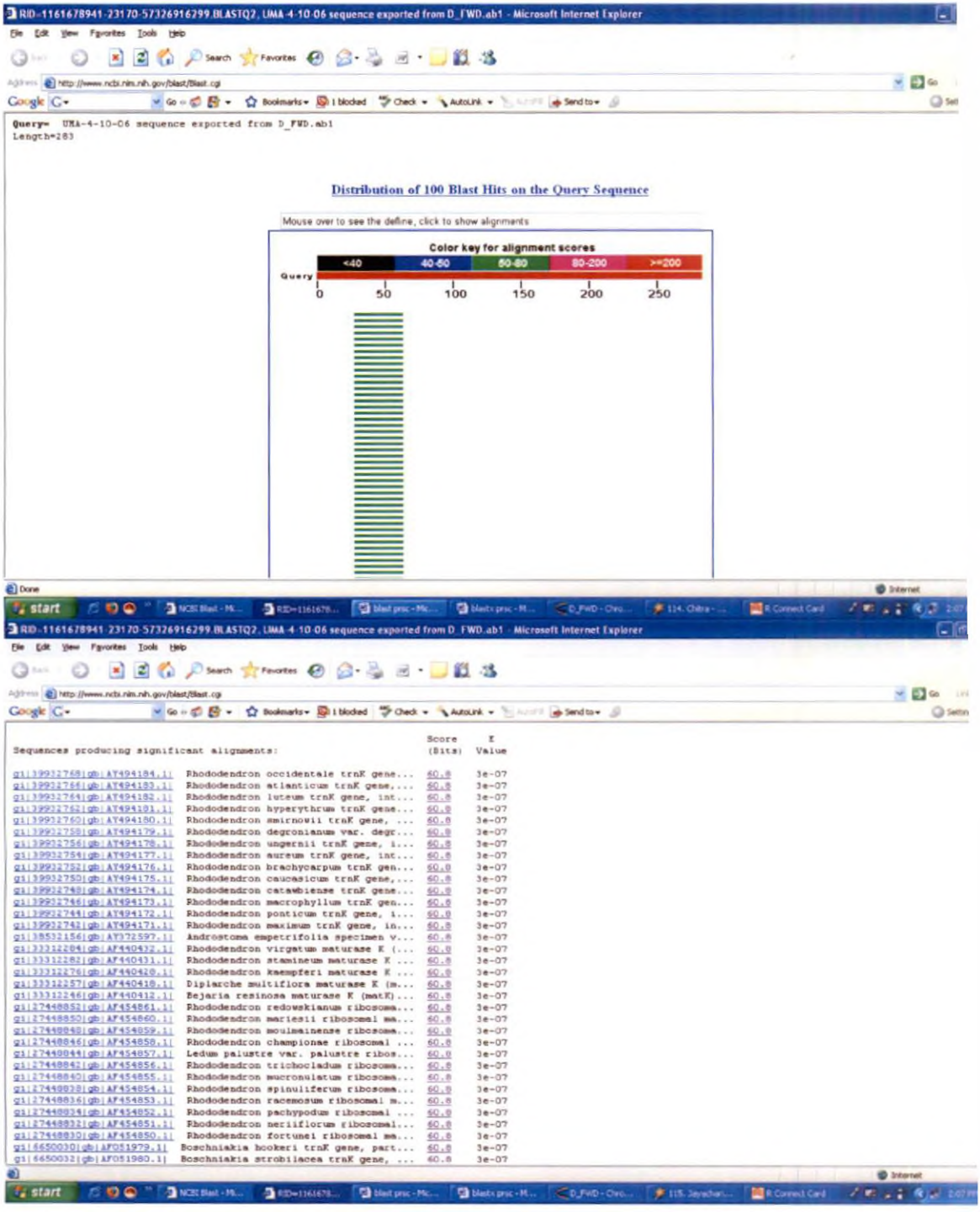

Fig. 23. BLASTN result for left flanking region (D_FWD) of *rps16-trnQ* spacer regio sequenced with primer Gf

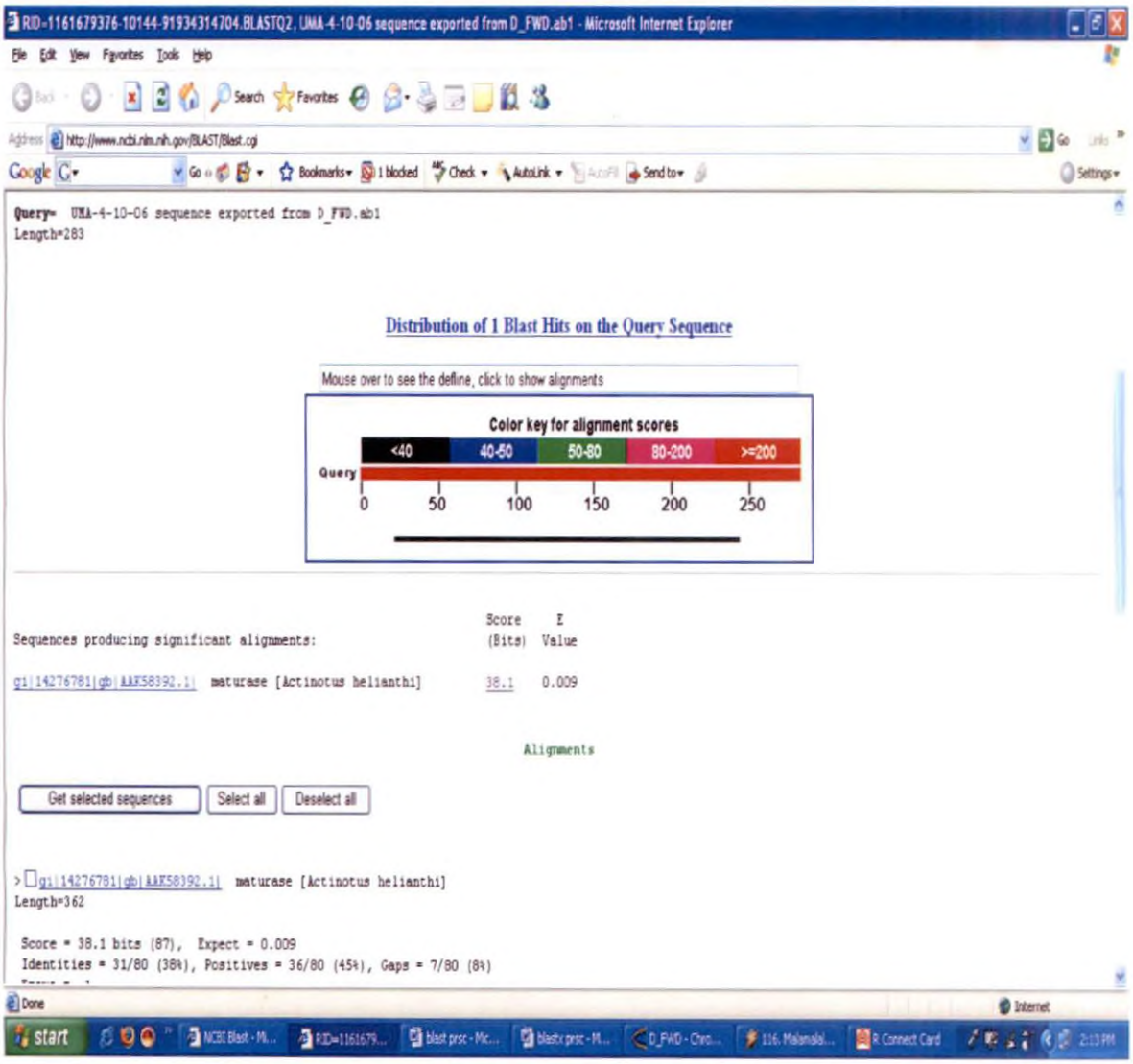

Fig. 24. BLASTX result for left flanking region (D_FWD) of *rps16-trnQ* spacer region sequenced with primer Gf

Table 10. BLASTN similarity for the flanking regions of *rps16-trnQ* spacer region amplified using specific primers

Sl. No	Sequence description	Region in *C. asiatica*	Crops showed similarity.	Gene identity number (GI No.)	Accession No
1.	E_FWD (17-575)	Right flanking region of *rps16-trnQ* spacer	*P. ginseng* chloroplast genome (8225-8777)	51235292	AY582139.1
	E_REV (19-421)		*P. ginseng* chloroplast genome (9218-8819)	51235292	AY582139.1
2.	A_FWD (33-273)	Left flanking region of *rps16-trnQ* spacer	*P. ginseng* chloroplast genome (4113-4353)	51235292	AY582139.1
	A_REV (21-477)		*P. ginseng* chloroplast genome (5406-4935)	51235292	AY582139.1
3	C_FWD (12-441)	Left flanking region of *rps16-trnQ* spacer	*Centella erecta*, *matK* gene (437-1)	2281236	US8599.1
	C_REV (1-228)		*P. ginseng* chloroplast genome (5397-5168)	51235292	AY582139.1
4	D_FWD (29-64)	Left flanking region of *rps16-trnQ* spacer	*Rhododendron occidentale trnK* gene intron, *matK* gene(450-414)	39932768	AY494184.1

Table 11. BLASTX similarity for the flanking regions of *rps16-trnQ* spacer region amplified using specific primers

Sl. No	Sequence decription	Region in *C. asiatica*	Crops showed similarity.	Gene Identity Number (GI No.)	Accession No.
1.	E_ME5F (378-550)	*rps16-trnQ* right flanking region	*Nuphar advena* photosystem II protein K (1-57)	69217353	AAZ04035.1
	E_REV (64-8)		*Liriodendron tulipifera* photosystem II protein I (14-32)	114107030	YP740186.1
2.	A_FWD	*rps16-trnQ* left flanking region	No significant similarity found		
	A_REV (27-197)		*Lactuca sativa* ribosomal protein S16 (24-80)	88656965	ABD47215.1
3	C_FWD (429-25)	*rps16-trnQ* left flanking region	*Micropleura renifolia* maturase protein (5-141)	2281265	AAB64070.1
			C. erecta maturase (5-141)	2281237	AAB64056.1
	C_REV (1-181)		*Helianthus annus* ribosomal protein 16 (24-84)	94502473	YP588099.1
4	D_FWD (244-23)	*rps16-trnQ* left flanking region	*Actinotus helianthi* maturase protein (62-140)	14276781	AAK58392.1

Discussion

5. DISCUSSION

Centella asiatica L. is an outstanding medicinal herb that is widely used in Indian system of medicine. Its vernacular name is 'Brahmi' which means 'bringing knowledge to supreme reality'. In India it is chiefly valued as a revitalizing herb that strengthens nervous function and memory. The glycosides found in Indian plants include brahmoside, brahminoside, and minor amounts of asiaticoside, which vary in their relative contents depending on the ecotypes. Asiaticoside content of *Centella* in India varies from 0.006 to 0.114 per cent. Current world demand of 95 per cent pure asiaticoside is 3000 kg. For the preparation of 95 per cent pure asiaticoside, raw materials with 2 to 3 per cent asiaticoside are needed by the industry. There is a scarcity of raw materials with high content of asiaticoside, in India. Hence, evolving plants with higher content of asiaticoside, suitable for the demand of Indian industry is very much essential. Genetic transformation is a useful option in this regard, for hastening the crop improvement programe by enhancing the productivity of secondary metabolites.

Genetic engineering of chloroplast is an evolving approach for crop improvement by avoiding some of the limitations of nuclear transformation. Chloroplast genetic engineering offers several advantages including a high level of transgene expression, multigene engineering in a single transformation event, transgene containment *via* maternal inheritance or cytoplasmic male sterility, lack of gene silencing, position effect due to site specific transgene integration, and lack of pleotropic effects due to subcellular compartmentalization of transgene product.

The present study aimed at isolation and sequencing of the spacer region between two tRNA genes and its flanking regions in the chloroplast genome of *C. asiatica*, which is needed for the development of a species specific chloroplast transformation vector for this crop. According to Daniell *et al.* (2005b), the basic chloroplast specific expression cassette is comprised of a promoter, selectable

marker, and 5'/3' regulatory sequences to enhance the efficiency of transcription and translation of the gene. They recommended approximately one kb of homologous flanking region to facilitate recombination and the site of insertion should be the intergenic spacer region with out interrupting any genes. According to Bock and Hagemann (2000) chloroplast transformation generally results from homologous recombination with a fragment of transforming DNA replacing the corresponding chloroplast DNA. Hence, it is important to know the sequence of chloroplast DNA region to which the gene of interest should be specifically targeted.

The availability of complete sequences of chloroplast genomes enhances their use for genetic engineering. In chloroplast transformation, finding appropriate intergenic spacer regions is very important for efficient integration of transgenes. In tomato and potato researchers have used *trnfM-trnG*, *rbcL-accD*, *trnV-3'-rps12* and 16S rRNA *orf* 70B intergenic spacer regions of tobacco to integrate transgenes. None of these regions have 100 per cent sequence identity. The intergenic spacer region between *rbcL* and *accD* of potato and tobacco shows only 94 per cent sequence identity and potato chloroplast transformants generated only at 10 to 30 per cent lower frequencies than tobacco (Nguyen *et al.*, 2005). The *trnfM-trnG* intergenic spacer region used for tomato chloroplast transformation has only 82 per cent identity with tobacco, resulting in inefficient transgene integration. There are major deletions in the chloroplast genome in intergenic spacer region, compared to tobacco, which was used for transformation. Therefore the development of species specific vectors for transgene integration would enable the use of any of the intergenic spacer regions within the respective chloroplast genome. Moreover, genome organization is different among some species. The *rbcL* and *accD* genes are adjacent in genomes, including citrus. However, they are not adjacent in the soybean chloroplast genome because an inversion has altered the gene order (Saski *et al.*, 2005). These examples emphasize the importance of choosing appropriate spacer regions for chloroplast transformation. Berger *et al.* (2005) reported several

unique restriction sites in the intergenic spacer regions of tomato suggesting that potential utility of these sequences in species specific vector construction for chloroplast transformation

Knowledge about the chloroplast genome sequence is needed for the isolation of a homologous flanking region. The complete chloroplast genome sequence of *C. asiatica* is not available so far. Different approaches are there to isolate a region from unknown. Restriction digestion of the chloroplast genome with specific restriction enzymes and sequencing of the digested fragment is a method (Zubenko *et al.*, 1994). Polymerase chain reaction based approach (Hamilton, 1999) was selected for this study. In this approach, heterologous primers were used for amplification. Primers for different spacer regions were designed based on the chloroplast genome sequences of *N. tabacum*, *A. thaliana* and *P. ginseng* using Primer3 software. According to Palmer (1991) the gene order of tobacco was found in most other angiosperms and also represents the ancestral gene order of vascular plants. Therefore, tobacco DNA has often served as a reference for chloroplast genomes. According to Calsa *et al.* (2004) related species display homologous DNA sequences and high level of conservation concerning both gene content and gene order. Hence, heterologous primers can amplify the specific sequence in *Centella*. The genome content and the relative position of 114 genes in chloroplast genome of *P. ginseng* are identical with the chloroplast DNA of *N. tabacum* (Kim and Lee, 2004a).

Polymerase chain reaction was carried out for the amplification of the spacer region using the specific primers designed. The reaction was carried out in 25 µl reaction volume containing buffer 1X, $MgSO_4$ 1mM,dNTP mix 200µM, forward and reverse primer 10pmols each, DNA 10pmols *Pfu* DNA polymerase 1.9U and sterile distilled water to make up the volume. The genomic DNA of *C. asiatica* was used as template for amplification. Daniell *et al.* (2005b) used total cellular DNA of tobacco to amplify promoters and regulator elements using specific primers. They used polymerase enzyme with proof reading activity to

amplify the flanking sequence required for homologous recombination in tobacco. They had done the PCR for the amplification of region for homologous recombination in tobacco using a reaction mixture containing 100-200ng DNA, buffer 1X, dNTPs 200-30µM, primers 15-20pmols, enzyme 1-2.5U in 50µl final reaction volume.

For the amplification of spacer region and its flanking regions from *C. asiatica* the thermal cyclic reaction used was the same, except annealing temperature which was changed with the Tm of primers. The reaction was denaturation at 94^0 C for five minute, followed by 35 cycles of denaturation at 94^0 C for 60 seconds, annealing for 30 seconds and extension at 72^0 C for four minute. Final extension at 72 °C for 10 minutes was given. Daniell *et al.* (2005b) carried out PCR in tobacco by denaturing at 94^0C for five minutes followed by 30 cycles of denaturation at 94^0C for 30seconds, annealing at 55^0 -60^0 C for 30seconds, and extension at 72^0 C for 2-4 minutes as per the expected size of PCR product followed by an eight to ten minute extension at 72^0C.

Primers (Af,Ar) for the *trnG-trnfMet* spacer were designed based on the sequence of *N. tabacum* and *A. thaliana* chloroplast genome. The amplified product was 270bp long and showed maximum similarity to the *trnG-trnfMet* spacer region of *P. ginseng* and to *N. tabacum* with 90 per cent identity. Hence, the spacer region might have conserved in *C. asiatica*. Small *et al.* (2005) amplified the non coding chloroplast DNA regions *trnS-trnG* spacer and *trnG* intron for phylogenetic studies in lycophytes and monilophytes.

Since the *trnG-trnfMet* spacer region was small, primers were designed for larger spacer region between tRNA genes based on the sequence of *N. tabacum* chloroplast genome. The spacer region selected was in between genes *trnE* and *trnT*. PCR was carried out using the primers Bf and Br. Intense band was eluted, purified and sequenced with primer Bf. This sequence had a length of 633bp and showed maximum similarity to chromosome 4 of *Oryza sativa* genomic DNA on

BLASTN similarity search. Nakazono and Hirai (1993) reported the presence of chloroplast sequence in mitochondrial genome of rice. Lister *et al.* (2003) reported DNA transfer from chloroplast to nucleus in tobacco. BLASTX analysis of the sequence showed similatirty to retrotransposon gag protein of *Medicago truncatula.*

Since the primers Bf and Br failed to produce expected similarity, primers (Cf, Cr) for the *trnE-trnT* spacer were again designed based on the sequence of *P. ginseng* chloroplast genome, as the *trnG-trnfMet* spacer of *C. asiatica* showed similarity to this crop. This crop belongs to the order Apiales, the same order of *C. asiatica.* The amplified product produced many bands. The intense band produced during amplification was sequenced after gel elution and purification. It had a length of 895bp and showed similarity to *Medicago truncatula* mitochondrial genome. Since genomic DNA of *C. asiatica* was used for the amplification, the primers might have got the site for amplification in nuclear genome or mitochondrial genome. Nakazono and Hirai (1993) reported the presence of chloroplast sequences in the mitochondrial genome of rice. BLASTX analysis showed similarity to heat shock like protein of *Picea glauca.* According to Berger *et al.* (2005) a 437bp sequence present in the intergenic region of the genes *trnE* and *trnT* in tobacco chloroplast genome was completely absent in the tomato chloroplast genome. Even though tobacco and tomato belongs to same family solanaceae, sequences at *trnE-trnT* spacer region are missing. Some sequences in the *trnE-trnT* spacer region of *P. ginseng* chloroplast genome might have been absent in *C. asiatica* chloroplast genome, although they belongs to same order Apiales.

Primers (Df, Dr) for another spacer region (*trnT-trnL*) was designed using *P. ginseng* chloroplast genome sequence. Amplification with primers Df and Dr also produced many bands. Inverted repeat region of *P. ginseng* sequence had sites for *trnL* along with a site in the long single copy region. There were two sites for *trnT* also. That may be the reason for production of many bands. Intense

band was eluted and purified. But it failed to produce amplification with the same primer during sequencing PCR. During purification the amplified product might have been lost the primer binding site or the integrity of the product might have lost. According to Berger *et al.* (2005) the intergenic spacers are less conserved and evolutionary divergence occurrs mainly in putative non coding regions.

As larger spacer region between tRNA genes isolated failed to show similarity with chloroplast genome, primers (Ef, Er) were designed for the spacer region between genes *rps16* and *trnQ* based on the sequence of *N. tabacum* chloroplast genome. Single band was produced on amplification. The PCR product sequenced with primer Ef was 815bp long and showed similarity to *rps16* gene intron of *C. trifolia* and *C. asiatica*. The amplified product when sequenced (RNQ_V4) with reverse primer Er showed similarity to *P. ginseng* chloroplast genome. The region of similarity corresponded to *rps16* gene, spacer and starting region of *trnQ* gene in *P. ginseng*. The gene order and sequence of this region in *C. asiatica* and *P. ginseng* might have been the same. Sugiura and Sugiura (2004) used PCR based approach to isolate a fragment containing *rbcL*, *trnR* and *accD* from plastid genome of *Physcomitrella patens*.

Subsequent step was to isolate the flanking regions to get adequate sequence length to facilitate homologous recombination. The overall A-T content of *Panax schingeng* chloroplast genome was 62 per cent. The A-T content in the non coding regions (65 per cent) was higher than in coding regions (59 per cent). The A-T content in IR regions amounted to 57 per cent, whereas LSC and SSC regions are 64 per cent and 68 per cent, respectively (Kim and Lee, 2004a). According to Innis *et al.* (1990) the primer sequence should contain 50.0 to 60.0 per cent G-C content. High percentage of A-T content in the non coding region of chloroplast genome made it difficult to design primer in the present instance. Primers were designed for both the flanking regions of *rps16-trnQ* spacer manually, as Primer3 software failed to produce primers for the given sequence as per the given conditions.

Manually designed primers were analyzed using the oligonucleotide properties calculation program to find out the hairpin formation, 3' complementarity and self annealing property. After analysis only good primers were selected. To find the binding specificity of the primers BLASTN analysis was also done.

Primers were designed for the right flanking region of the spacer region between gene *rps16* and *trnQ* using the chloroplast genome sequence of *P. ginseng* (GI: 51235292). The forward primer (Hf) for the right flank was the reverse complement of the reverse primer (Er) of the *rps16-trnQ* spacer. Two primer pairs designed for amplifying the left flank region *viz.*, Ff, Fr and Gf, Gr. The primer pair Ff and Fr was designed from the sequence of *P. ginseng*. The forward primer (Gf) was designed from sequence of *P. ginseng*. The reverse primer (Gr) of the left flank was the reverse complement of the forward primer of *rps16-trnQ* spacer.

The right flanking region of *rps16-trnQ* spacer was amplified with Hf and Hr and sequenced with both primers. Sequencing with Hf gave 594bp long sequence and showed similarity to region *trnQ* gene, spacer region after *trnQ*, *psbK* gene and spacer after *psbK* in *P. ginseng* chloroplast genome. Sequencing with Hr gave 988bp long sequence and showed maximum similarity to spacer region after *psbK* gene and gene sequence of *psbI* gene in chloroplast genome of *P. ginseng*.

The left flanking region of *rps16-trnQ* spacer amplified with three primer combinations was sequenced. Sequencing of PCR product with forward primer Ff gave a 639bp long sequence (A_FWD) and showed similarity to spacer region after *matK* (maturase) gene, *trnK* (tRNA lysine) gene and spacer after *trnK* of *P. ginseng* chloroplast genome. Sequencing with Fr was 558bp long (A_REV) and showed similarity to spacer after *trnK* gene and *rps16* gene of *P. ginseng*

chloroplast genome. The sequence C_FWD had a length of 513bp and showed similarity to *matK* gene of *C. erecta*. The sequence C_REV had 576bp long sequence and had similarity to spacer after *trnK* and *rps16* gene of *P. ginseng* chloroplast genome.

Amplified region with primers Gf and Gr was sequenced with forward and reverse primer and found to have a length of 283bp (D_FWD) and 259bp (D_REV), respectively. The sequence D_FWD showed similarity to *Rhododendron occidentale trnK* gene. The sequence of D_REV contained sequences for mixed base. Hence similarity search was not done. The sequencing machine was so sensitive to detect the minutest peak in case of non specific amplification. It gave degenerated code in the sequence. The letters such as W, Y, K, M, R and S were the machine's default settings for purines and pyramidines for ambiguous sequences. Zou (2001) used a similar approach by synthesizing primers amplify flanking regions from tobacco chloroplast genome to develop a vector.

As compared to primers designed for the spacer region between tRNA genes, primers for spacer region between *rps16* and *trnQ* genes were able to produce specific amplification. This is because tRNA genes are also present in the nuclear and mitochondrial genome, but genes specific for chloroplast are present only in chloroplast genome. The gene contents and the polycistronic transcription units of chloroplast genome are largely conserved among most vascular plant species (Kanno and Hirai, 1993).

Lack of complete genome sequences to provide a 100 per cent species specific chloroplast transformation vector, containing suitable selectable markers and regulatory elements, is one of the major limitations to extend the concept of chloroplast transformation to other useful crops (Daniell *et al.*, 2005a).

This work was an initial step towards chloroplast transformation of *C. asiatica*. The spacer region between *trnG* and *trnfMet* and *rps16* and *trnQ* genes and flanking regions of *rps16-trnQ* spacer region were successfully isolated and sequenced. These sequences showed sufficient similarity with the chloroplast genome of *P. ginseng*. The isolated sequence from the chloroplast genome of *C. asiatica* can be utilized for developing a chloroplast transformation vector for homologous recombination along with a gene which can improve the asiaticoside content of the crop. Thus a species specific vector for *Centella asiatica* can be generated to produce transplastomes in this crop which will have higher asiaticoside content.

Summary

6. SUMMARY

The study entitled "Amplification and sequencing of spacer region between two tRNA genes and its flanking region in the chloroplast genome of *Centella asiatica* L." was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2005 to 2006. The objective of the study was to design primers and sequence the spacer region between two tRNA genes and its flanking regions from the *C. asiatica* chloroplast genome, useful in developing species specific vector for chloroplast transformation. The salient findings of the study are summarized below.

PCR based approach was used in this study. Heterologous primers were designed to isolate the spacer sequence from the chloroplast genome of *C. asiatica* based on the chloroplast genome sequences of *A. thaliana*, *N. tabacum* and *P. ginseng* using Primer3 software. The length of the designed primers ranged from 20nt to 26nt and Tm from 52.0^{0}C to 61.0^0 C. Genomic DNA of *C. asiatica* was isolated using the modified protocol of Murray and Thompson (1980) and was used as the template for amplification reaction. The polymerase enzyme used for amplification was *Pfu* DNA polymerase (recombinant) enzyme which had the proof reading activity. The thermal cyclic reaction given for all the regions were same. Annealing temperature was changed based on the Tm of primers.

The spacer region between *trnG-trnfMet, trnE-trnT, trnT-trnL* and *rps16-trnQ* genes were isolated. The entire isolated regions were sequenced, except *trnT-trnL* spacer region. All sequenced regions were subjected to BLASTN and BLASTX similarity search. The spacer region between *trnG-trnfMet* isolated (270bp) showed maximum similarity to the same region in *P. ginseng* (GI: 51235292, AY582139.1) chloroplast genome. The sequences isolated for *trnE-trnT* spacer region failed to produce exact similarity to this region in chloroplast genome of other crops. The spacer region isolated (1749bp) between genes *rps16*

and *trnQ* showed maximum similarity to *rps16*gene and its intron in *C. asiatica* (GI: 6692894, AF110603.1) chloroplast genome with an E value of zero and to *rps16* gene, spacer region after *rps16* gene and starting region of *trnQ* gene in *P. ginseng* (GI: 51235292, AY582139.1) chloroplast genome in BLASTN similarity search.

Primers for the flanking regions of *rps16-trnQ* spacer were designed manually based on the sequence of *P. ginseng* chloroplast genome and primers of this spacer region. The right flanking region was isolated with primers Hf and Hr (1582bp) showed maximum similarity to *trnQ* gene, spacer region after *trnQ*, *psbK* gene, spacer region after *psbK* and *psbI* gene in *P. ginseng* (GI: 51235292, AY582139.1) chloroplast genome.

The left flanking region was isolated by three primer combinations. Sequencing result with two of the primer combination showed similarity to *P. ginseng* chloroplast genome. The sequence amplified (1227bp) with primer combination Ff and Fr showed similarity with *rps16* gene, spacer region after *trnK* gene, *trnK* gene and spacer region after *matK* gene of *P. ginseng* chloroplast genome. The sequence amplified (1089bp) with primers Gf and Fr showed similarity to *rps16* gene, spacer region after *trnK* of *P. ginseng* and to *matK* gene of *C. erecta* (GI: 2281236, US8599.1).

The isolated sequences can be used for developing a species specific vector for the chloroplast transformation in *C. asiatica* to improve the asiaticoside content of this crop.

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* Original not seen

Appendices

APPENDIX I

CTAB DNA Extraction buffer

NaCl	1.4M
Tris HCl (pH 8.0)	100.0mM
EDTA (pH 8.0)	20.0mM
CTAB	2.0% w/v
BME	0.1% w/v

(BME should be added only prior to use)

TE buffer

Tri Buffer (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

APPENDIX II

50X TAE buffer (pH 8.0)

Tris base	242.0 g
EDTA (0.5M)	100 ml
Glacial acetic acid	57.1 ml

(Make up the volume to 1000ml)

AMPLIFICATION AND SEQUENCING OF SPACER REGION BETWEEN TWO tRNA GENES AND ITS FLANKING REGION IN THE CHLOROPLAST GENOME OF *Centella asiatica* L.

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Abstract of the
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ABSTRACT

The study entitled "Amplification and sequencing of spacer region between two tRNA genes and its flanking region in the chloroplast genome of *Centella asiatica* L." was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2005-2006 with the objective of isolating a spacer region and its flanking regions from the chloroplast genome of *Centella asiatica* to develop a species specific vector for the chloroplast transformation.

Heterologous primers were designed based on the chloroplast genome sequences of *Arabidopsis thaliana*, *Nicotiana tabacum* and *Panax ginseng* using Pimer3 software for the spacer regions *trnG-trnfMet*, *trnE-trnT*, *trnT-trnL* and *rps16-trnQ* and were amplified on genomic DNA of *Centella asiatica*. The entire isolated regions were sequenced except *trnT-trnL* spacer region. All sequenced regions were subjected to BLASTN and BLASTX similarity search. The *trnG-trnfMet* spacer region (270bp) showed maximum similarity to the same region in *Panax ginseng* (GI: 51235292, AY582139.1) chloroplast genome. The spacer region between genes *rps16* and *trnQ* (1749bp) showed maximum similarity to *rps16* gene and its intron in *Centella asiatica* (GI: 6692894, AF110503.1) chloroplast genome and to *rps16* gene, spacer region after *rps16* and starting region of *trnQ* gene in *Panax ginseng* (GI: 51235292, AY582139.1) chloroplast genome.

Primers for flanking regions of *rps16-trnQ* spacer were designed manually based on the primers of this spacer and the chloroplast genome of *Panax ginseng*. The right flanking region amplified (1582bp) with primers Hf and Hr showed maximum similarity to *trnQ* gene, spacer region after *trnQ*, *psbK* gene, spacer region after *psbK* and *psbI* gene in *Panax ginseng* chloroplast genome. The left flanking region of *rps16-trnQ* spacer sequence amplified (1227bp) with primer combination Ff and Fr showed similarity to *rps16* gene, spacer region after *trnK*

gene, *trnK* gene and spacer region after *matK* gene of *Panax ginseng* chloroplast genome. The sequence amplified (1089bp) with primers Gf and Fr showed similarity to *rps16* gene, spacer region after *trnK* of *Panax ginseng* and to *matK* gene of *Centella erecta* (GI: 2281236, US8599.1). The spacer region between *rps16* and *trnQ* gene and its flanking region isolated can be used in developing a *Centella asiatica*L. specific vector for chloroplast transformation.