Construction of a Restriction Map and Gene Map of the Lettuce Chloroplast Small Single-Copy Region Using Southern Cross-Hybridization

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INTRODUCTION

Considerable effort has been put into the construction of detailed structural maps of chloroplast genes and genomes of plants (5). Such maps have allowed the detailed analyses of chloroplast genome evolution and of gene function. The conventional genome analysis methods involve the determination of restriction sites followed by the mapping of selected chloroplast genes using heterologous gene probes. The large size of the chloroplast genome necessitates the use of many individual gene probes for detailed mapping. Both of these procedures must be carried out prior to subcloning or sequencing a region of interest. Potter and Dressler (11) developed a Southern cross-hybridization procedure [devised by Hutchison (14)] to generate a restriction map of the phage λ genome, which allowed the simultaneous determination of restriction sites for up to ten different enzymes.

In this report, Southern cross-hybridization has been used to determine a restriction map of a large (26 kbp) cloned lettuce chloroplast small single-copy region (SSCR). Several chloroplast genomes such as tobacco (15), rice (4) and the liverwort Marchantia (9) have been fully sequenced and show that the major cluster of six ndh genes occurs in the SSCR, in the order: ndhH - ndhA - frxB - ndhG - ndhE - frxA - ndhD - ndhF. Southern cross-hybridization was also extended to determine a genetic map of the SSCR of the lettuce chloroplast genome by cross-hybridization to fragments of the tobacco chloroplast genome. The order of genes in the lettuce SSCR is unchanged relative to the tobacco SSCR.

MATERIALS AND METHODS

Cosmid Libraries

Lettuce (Lactuca sativa) and tobacco (Nicotiana tabacum) chloroplasts were purified over a Percoll® gradient (Sigma Chemical, St. Louis, MO, USA) according to Gruissem et al. (2). Chloroplast DNA was purified from RNA and protein over CsCl gradients. Partial HinDIII digests of chloroplast DNA were size-fractionated and fragments of average 25 kb were cloned in the broad host-range cosmid pVK102 (7). All restriction enzymes were obtained from Boehringer Mannheim (Mannheim, Germany). Recombinant cosmids were selected by differential resistance to tetracycline and kanamycin (Sigma Chemical). Cosmid DNA was prepared according to Birnboim and Doly (1) and purified on a single CsCl-ethidium bromide gradient followed by gel filtration on Sephacryl® S-1000 (Pharmacia Biotech, Milton Keynes, Bucks, UK) minicolumns to eliminate residual bacterial RNA (13).

Southern Cross-Hybridization

The procedure used for Southern cross-hybridization was essentially as...
described previously by Potter and Dressler (11). Aliquots of cosmid DNA (3–5 µg) were restricted and then fractionated on agarose gels (0.8%–1.5%; Sigma Chemical) using the entire width of the gel (10 cm). Two outer slots (3-mm wide) carrying DNA molecular weight markers (phage λ HindIII plus Marker VI from Boehringer Mannheim) flanked the (10 cm) sample slot. Unlabeled DNA fragments were Southern-blotted onto Hybond®-N nylon transfer membranes (Amersham International, Little Chalfont, Bucks, UK) and then fixed by baking at 85°C for 1 h. Labeled DNA fragments were similarly electrophoresed and Southern-blotted and were not covalently bonded. Instead, the unfixed membranes were used immediately for transfer of the labeled DNA to the fixed-DNA membranes by cross-hybridization. Hybridization membranes were washed twice in 2× standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 62°C for 15 min, then once in 0.1× SSC/0.1% SDS at 62°C for 10 min prior to rinsing in 2× SSC at room temperature and autoradiographed using Fuji RX film (Fuji Medical Systems, Stamford, CT) at -70°C for periods between 1 h and 3 days.

Labeling of Cosmid Restriction Fragments

Restricted 5′-dephosphorylated cosmid DNA (3 µg) was labeled at the 5′ terminus for 1–3 h at 37°C using 1 U of T4 polynucleotide kinase (Sigma Chemical) and [γ-32P]ATP (3000 Ci/mmol; Amersham International) under standard reaction conditions (13).

Analysis of Cross Hybridizations

Autoradiographs were overlaid on a light box, and intersecting cross-hybridization spots were analyzed manually. The position of dots was measured relative to the loading slot and related to the migration of the DNA fragments during electrophoresis.

RESULTS

Southern Cross-Hybridization Analysis of the Lettuce Chloroplast SSCR

A general scheme of the Southern cross-hybridization method used to map a defined DNA fragment is depicted in Figure 1. The method is based on the homology between DNA fragments produced by restriction of the same DNA fragment by two different enzymes “a” and “b”. Digests of the DNA were electrophoresed from a loading well extending the width of the gel. The restriction fragments shown in the
upper membrane “a” were not covalently bound to membrane and are labeled, whereas the restriction fragments shown in the lower membrane “b” were covalently bound to a membrane and are unlabeled. The two different transfer membranes are aligned at right angles to each other during the stringent hybridization procedure—thus each fragment of one digest intersects with each fragment of the other digest. Whenever a $^{32}$P-labeled fragment contacts a fragment containing homologous sequence, a “dot” of hybridization occurs at the point where the two fragments intersect, as the restriction fragments from “a” anneal to complementary sequences attached to membrane “b” (Figure 1). Since the DNA fragments result from different restriction digests, overlap between the $^{32}$P-labeled fragments and the nonlabeled fragments produces a two-dimensional (2-D) array of hybridization spots, from which a restriction map may be deduced (see Materials and Methods). Multiple, 2-D hybridization patterns for up to 10 restriction enzymes may be obtained from a single cross-hybridization experiment (11). In the Figure 1 illustration, the order of fragments 2 and 4 cannot be determined by this cross-hybridization alone since both hybridize only to fragment “a”. Rather, hybridization of digest “b” to labeled fragments produced by other appropriate restriction enzymes is also required to locate fragments 2 and 4 more precisely within fragment “a”. Several cross-hybridization experiments may be necessary to order the positions of small DNA fragments.

**Intraspecific Southern Cross-Hybridization**

To illustrate cross-hybridization by example, some representative hybridization blots are shown in Figure 2. Cosmid L32 contained a lettuce chloroplast genome fragment, which corresponds to the SSCR from HindIII fragment 22 to fragment 28 (5) (see Table 1). Figure 2 shows a cross-hybridization between unlabeled L32 digested by both HindIII and EcoRI (HE), and an end-labeled digest of L32 by HindIII (H) alone (Figure 2.1) or an end-labeled digest of L32 by HindIII, PstI and XhoI (HPX) (Figure 2.2). The HindIII restriction releases all chloroplast sequences from the cosmid vector. The combination of HindIII with a second enzyme allows the approximate position and number of restriction sites of the second enzyme to be determined within each HindIII fragment when hybridized to

![Figure 2. Autoradiograph of homologous cross-hybridization between lettuce chloroplast SSCR fragments.](image-url)
the HindIII digest. The complex 2-D hybridization array shows many well-separated hybridization dots that can be readily related to individual fragments in both the labeled and unlabeled gels. The array also illustrates the need for multiple cross-blots for localization of a minority of DNA fragments obtained through restriction by a frequent cutting enzyme such as EcoRI (Figure 2.1). Closely migrating DNA fragments may be difficult to resolve, while some signals are weak and others are strong. The reasons for the variation in signal intensity are not entirely clear; however, first, the extent of hybridization of large 5′ end-labeled DNA fragments were generally weaker than for smaller fragments due to the more limited transfer of the labeled region of the probe. Second, the hybridization and washing conditions were stringent to ensure elimination of incorrect hybridization. The stability of hybridization is dependent on DNA sequence, and the different intensity of individual signals may reflect this in part. Third, the regions of hybridization between probes and target vary with the size of overlap, and this variation could also affect signal intensity. The information obtained from the hybridization arrays shown in Figure 2 and several additional hybridization arrays using other combinations of enzymes (not shown) allowed a restriction map of the lettuce chloroplast SSCR to be constructed (see below and in Figure 4). The map is in agreement with a restriction map of the region determined by Jansen and Palmer (5). However, additional mapping data were determined for the enzyme EcoRI, which cuts the lettuce SSCR frequently. This detailed restriction mapping data would be laborious to obtain by conventional hybridization methods and demonstrates the advantage of cross-hybridization.

**Interspecific Cross-Hybridization**

Large chloroplast DNA fragments contain many genes and open reading frame (ORF) sequences that can be used as simultaneous interspecific gene mapping probes by Southern cross-hybridization. This is illustrated in Figure 3, where the lettuce cosmid L32 was cross-hybridized to the equivalent SSCR of tobacco [map positions 107041–134115 (15)] contained within cosmid W34. In Figure 3 it can be seen that the hybridization between HindIII/EcoRI-restricted L32 and HindIII/PstI/XhoI-digested W34 that interspecific hybridization of the SSCR was limited to DNA fragments encoding genes and ORFs, while fragments carrying non-coding DNA generally hybridized poorly. This data

Table 1. Restriction Fragments (F) and Electrophoresis Bands (B) Released by Restriction of Lettuce Cosmid L32 Listed from Largest to Smallest Band in kbp

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Restriction fragments (F) or electrophoresis bands (B) (kbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L32H (F)</td>
<td>a</td>
</tr>
<tr>
<td>23</td>
<td>9.0</td>
</tr>
<tr>
<td>L32HPX (P)</td>
<td>a</td>
</tr>
<tr>
<td>23</td>
<td>8.3</td>
</tr>
<tr>
<td>L32HE (B)</td>
<td>a</td>
</tr>
<tr>
<td>23</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Note that electrophoretic bands may represent more than one DNA fragment. Restriction enzymes were used singly or in combinations: HindIII (H); HindIII, PstI and XhoI (HPX); HindIII and EcoRI (HE).
allows a more detailed location of the conserved genes in the lettuce SSCR and illustrates the power of the cross-hybridization technique to identify homologous genes and ORF regions. Cross-hybridization between larger L32 and W34 fragments generated using different restriction digests (not shown) confirmed the same order of lettuce restriction fragments as deduced from L32 to L32 hybridizations.

Restriction and Gene Map

Highly specific hybridization between the lettuce and tobacco restriction fragments allowed the gene order of the lettuce chloroplast small single-copy fragment to be deduced to a large measure. Figure 4 presents a restriction map, deduced from homologous L32 to L32 cross-blots (as shown in Figure 2), combined with a gene map, deduced from the heterologous L32 to W34 cross-blots (as shown in Figure 3). The map shows that the same linear arrangement of genes and ORFs occurs in both the tobacco and the lettuce SSCRs. The intensity of hybridization signals between tobacco and lettuce small single-copy gene regions is indicated by shading of the tobacco ORFs.

DISCUSSION

Southern cross-hybridization was used initially for restriction mapping of the phage λ genome (11) and Taq DNA (14). Subsequently, Southern cross-hybridization has been used with whole genome digests of Caenorhabditis elegans DNA to identify DNA mutations and restriction fragment polymorphism (12). Cross-hybridization between DNA and RNA blots has also been used to map alternative mRNA transcripts of genes and ORFs in maize (3) and liverwort (6) chloroplasts. Homologous DNA-DNA or DNA-RNA hybridizations were used in each of these reported Southern cross-hybridization experiments. In this report, the known and sequenced gene fragments of a reference tobacco chloroplast SSCR were used to generate a genetic map of an unmapped lettuce small single-copy fragment. This is the first description of the application of interspecific Southern cross-hybridization for genetic mapping of a complex, gene-rich DNA fragment. The order of genes in the ndh cluster of the SSCR is generally conserved in plants from different genera (4,8,9,15). Figure 4 showed that this gene order is also conserved in the lettuce SSCR. A representation of the intensity of hybridization between tobacco and lettuce regions is indicated by shading of the tobacco ORFs. This may not fully reflect the degree of sequence similarity of the annealing DNA fragments but may result in part from the general hybridization conditions used during the cross-hybridization procedure, which would favor strong representation of thermally stable sequences as well as regions of high sequence homology.

Mapping of Small Genomes

Chloroplast genomes are generally between 120–160 kbp and contain some 150–180 genes and ORFs (9,15). In conventional mapping studies (5), a small number of gene probes are used (usually less than 20) to provide “landmarks” from which a gene order is deduced. The small number of gene probes may not indicate all of the rearrangements present in the unmapped genome, such as fragment inversions or complex multiple rearrangements (10). The findings presented here suggest that an efficient use can be made of the high degree of DNA homology between chloroplast genes (16) to derive high-density gene maps by Southern cross-hybridization. The entire chloroplast genome of plants and algae could be located into small cosmid libraries comprising 8–20 overlapping clones. The pVK102 cosmid vector (7) used here has several advantages that aided the cross-hybridization analysis. Cosmid pVK102 lacks many commonly used restriction sites, thus allowing convenient restriction of the recombinant cosmids to release chloroplast fragments. The vector is also large (23 kb) and usually migrates behind all chloroplast fragments during electrophoresis, which permits their easy visualization. Cross-hybridization can simultaneously transfer labeled DNA

Figure 4. Combined restriction map (EcoRI, PstI, HindIII, XhoI) and gene map of the lettuce chloroplast SSCR. The cosmid L32 is represented by HindIII fragments numbers 22 to 28 as described by Jansen and Palmer (5). The restriction and gene map of complementary the tobacco SSCR in cosmid W34 [from map positions 107 041 to 134 115 (15)] is aligned for comparison. Cross-hybridizing fragments and regions between tobacco and lettuce are indicated by vertical lines and bars between the maps. Strong and weaker hybridizing lettuce and tobacco gene regions are indicated by stippling.
fragments to up to 10 hybridization membranes (11) and could allow cosmid clones covering the entire chloroplast genome to be examined in a single experiment. A reference chloroplast genome may be easily screened against an unknown chloroplast fragment to ensure that the possibility of complex genome rearrangements was examined. Although the method described takes advantage of the conservation of chloroplast DNA sequences between plant species, it could be applied to any unknown DNA sequence relative to a known, highly homologous sequence. Autoradiographs were interpreted manually during this work; however, automated 2-D gel scanning and data handling might also prove useful for map construction.

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REFERENCES


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