Single-site manipulation of tomato chromosomes \textit{in vitro} and \textit{in vivo} using Cre-\textit{lox} site-specific recombination

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\textbf{Abstract}

With the aim of developing new techniques for physical and functional genome analysis, we have introduced the Cre-\textit{lox} site-specific recombination system into the cultivated tomato \textit{(Lycopersicon esculentum)}. Local transposition of a Ds(\textit{lox}) transposable element from a T-DNA(\textit{lox}) on the long arm of chromosome 6 was used to position pairs of \textit{lox} sites on different closely linked loci. \textit{In vitro} Cre-\textit{lox} recombination between chromosomal \textit{lox} sites and synthetic \textit{lox} oligonucleotides cleaved the 750 Mb tomato genome with 34 bp specificity to release unique 65 kb and 130 kb fragments of chromosome 6. Parallel \textit{in vitro} experiments on \textit{Saccharomyces cerevisiae} chromosomes show the efficiency of cleavage to be 50\% per chromosomal \textit{lox} site at maximum. By expressing the Cre recombinase in tomato under control of a constitutive CaMV 35S promoter, efficient and specific somatic and germinal \textit{in planta} inversion of the 130 kb fragment is demonstrated. The combined use of \textit{in vitro} and \textit{in vivo} recombination on genetically mapped \textit{lox} sites will provide new possibilities for long range restriction mapping and \textit{in vivo} manipulation of selected tomato genome segments.

\textbf{Introduction}

Techniques for high-resolution molecular linkage mapping and pulsed-field gel electrophoresis (PFGE) of megabase DNA have opened the way to integration of genetic linkage maps and DNA restriction maps of large eukaryotic genomes. However, the short average size of fragments generated by conventional restriction enzymes usually confines the span of integrated maps to small genome segments, and leaves the upper range of PFGE resolution power (6–9 Mb) largely unexploited. In addition, genetic functions encoded within genomic restriction fragments generally remain unknown until mutagenized.

It would be useful to devise a method that can cleave a genome only at selected pairs of genetic markers \textit{in vitro}, and that can be used to specifically remove, invert or duplicate the selected genetic interval in a living cell. Physical information about large genetic distances could be obtained from the size of a single mega-restriction fragment, without the need for contig building. Precise \textit{in vivo} deletion of a specific fragment could map existing gene markers in segmental haploids, while new genetic functions may be uncovered in homozygous deficiency or duplication stocks.

The required technology for such a method can be provided by site-specific recombination systems like Cre-\textit{lox}, FLP/FRT and R/RS. These systems consist of a specific target DNA sequence (\textit{lox} (34 bp), \textit{FRT} (34 bp), \textit{RS} (31 bp)), and a recombinase (Cre, FLP, R) that is necessary and sufficient to induce crossovers between two target sites (reviewed in [22]). High specificity and a simple two-element design make this class of recombination systems attractive for use in heterologous hosts. Site-specific chromosomal deletions, inversions, duplications and translocations have been induced efficiently with all three systems in a variety of different higher eukaryotes, including plants and mammals (reviewed in [17, 27, 33]). In addition, 34 bp specific cleavage of the 15 Mb \textit{Schizosaccharomyces pombe} genome by intermolecular \textit{in vitro} recombina-
tion between artificial chromosomal \textit{lox} sites and \textit{lox} oligonucleotides has been demonstrated [28].

We are interested in applying the in vitro and in vivo systems of Cre-\textit{lox} site-specific recombination for long-range restriction mapping and genome manipulation in the cultivated tomato (\textit{Lycopersicon esculentum}). Although genetic mapping of this important model and crop species has been intense [37], current techniques for genomic restriction mapping are unlikely to bridge distant located markers on the genetic map of its relatively large (750 Mb) genome. Functional analysis of the tomato genome has long been restricted to mutagenesis by chemical and physical means, and has more recently been supplemented with heterologous transposons (\textit{Ac/Ds}) from maize [16, 41]. Introduction and genetic mapping of \textit{lox} sites in the tomato genome will provide new possibilities to select specific genetic intervals for PFGE restriction mapping using the 34 bp cleavage specificity of the in vitro Cre-\textit{lox} recombination system and for functional analysis by targeted recombination in vivo.

In this report, the operation of the in vitro and in vivo components of the Cre-\textit{lox} system in the tomato genome is demonstrated. We have used the natural tendency of short-range transpositions of maize \textit{Ds} elements to efficiently place \textit{lox} sites on different closely linked loci on the genetically well mapped tomato chromosome 6 [40]. We demonstrate the in vitro release of unique segments of this chromosome and show that these fragments can be specifically rearranged \textit{in planta} in a predictable manner.

Materials and methods

\textbf{DNA constructs}

Yeast integrative plasmid pJS210 was constructed by amplification of a 1.1 kb internal \textit{ABP1} fragment from \textit{Saccharomyces cerevisiae} strain AB 1380, with primer sequences \textit{5'}-CCCGAGATCCCTCCCTCCATTGCTGACGGG - 3' and \textit{5'}-GCAGATCCCGCTTGTGTTGTTGACATCTCGC - 3' and insertion of the \textit{BamHI}-digested amplification product into the \textit{BamHI} site of pYIPlac204 [13] that was equipped with \textit{lox} oligonucleotide cloned into the \textit{HindIII} site. Plasmid pJS211 was constructed by inserting a 2.2 kb \textit{HindIII} genomic \textit{CRY1} fragment (kindly provided by Dr J. Friesen [14]) into the \textit{HindIII} site of pYIPlac211 [13], previously equipped with \textit{lox} oligonucleotide in the \textit{EcoRI} site.

The plasmid pCEL535 for expression of Cre recombinase in \textit{Escherichia coli} was obtained from Dr P.J.J. Hooykaas (State University of Leiden, Netherlands) and consisted of the Cre gene containing its own translational start and stop codon, PCR-amplified from \textit{P1} lysogenic \textit{E. coli} and cloned between the \textit{EcoRI} and \textit{SphI} sites of pUC19.

The binary vector plasmid pD3 was constructed from the \textit{Ds}-containing plasmid pTT281 [31], in which the \textit{HindIII/AseI} fragment containing the GUS gene was replaced by a \textit{HindIII/EcoRI} fragment containing a \textit{CaMV 35S-BAR-nos3'} fusion of the \textit{BAR} gene described in [6] to create the plasmid pCTP103. Into the unique \textit{HindIII} site of this plasmid, a synthetic oligonucleotide was inserted that contained the \textit{lox} sequence and a \textit{NorI} recognition site, to create the plasmid pMH1737. pMH1737 was linearized at its \textit{BgII} site and inserted into the leader of a \textit{CaMV 35S-hygrromycin phosphotransferase (HPT)-nos3'} gene fusion present on plasmid pMH2057. pMH2057 was constructed by subcloning a \textit{tms2} gene from pTT283 [31] into pUC19 as a 3.7 kb \textit{PstI/SphI} fragment. The \textit{CaMV 35S-HPT-nos3'} fusion, also derived from pTT283, was included in pMH2057 as a 2.2 kb \textit{SphI} fragment. Insertion of pMH1737 into pMH2057 yielded the plasmid pMH3030. pMH3030 was linearized at its \textit{KpnI} site and inserted into the \textit{KpnI} site of the binary vector pCGN1548 [23] that contained a \textit{lox} oligonucleotide in its \textit{BamHI} site. The total T-DNA construct was designated D3, the binary vector pD3.

Binary vector plasmid pMH2626 was constructed by first subcloning a 3.4 kb \textit{SacI/HindIII} fragment of pED23, containing the \textit{CaMV 35S-Cre-nos3'} fusion (kindly provided by Dr D.W. Ow, PGEC, Albany, CA [5]), into pIC19R to obtain pMH303. The fragment was transferred as a 3.4 kb \textit{HindIII/Xhol} fragment into \textit{HindIII/Sall} sites of pBin19, and the resulting plasmid was designated pMH2626.

\textbf{Transgenic yeasts}

To construct transgenic \textit{S. cerevisiae} strains yJS210 and yJS211, plasmids pJS210 and pJS211 were linearized by cleaving the unique \textit{BglII} or \textit{BgII} sites respectively within the cloned yeast genes, and used to transform strain AB 1380 by the LiCl method [2]. yJS212 was constructed by retransformation of yJS211 with plasmid pJS210. Correct single-copy integrations were selected by Southern analysis.