Chromosome Microdissection and Microcloning: Technique and Application in the Plant Sciences

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Summary The technique of chromosome microdissection and microcloning, as a bridge between cytogenetics and molecular genetics, has been widely applied and play an important role in many genetic researches, such as chromosome painting probe isolation, genetic linkage map and physical map construction, and expressed sequence tags generation. To promote the further application of this technique in plants, in the present study, firstly, the experimental procedure was summarized. Secondly, the application status of this technique in plant researches was reviewed and the intractable problems of the application in plant researches were elucidated. Finally, the application of this technique in plants was looked into in the future. The results of the present study have obvious significance in promoting the application of chromosome microdissection and microcloning technique in plants.

Keywords Chromosome, Microdissection, Microcloning, Technique, Application.

Chromosome microdissection and microcloning, as a bridge between cytogenetics and molecular genetics, has been widely applied and play an important role in many studies, such as chromosome painting probe isolation, genetic linkage map, and physical map construction, and expressed sequence tags generation. Chromosome microdissection and microcloning, a technique in which the target chromosome/segment is cut or burned by glass microneedles or lasers in an inverted microscope for separation from other chromosomes and the isolated chromosome/segment is then collected and amplified *in vitro* to establish specific DNA libraries (Emmert-buck *et al.* 1996). The specific DNA libraries are mainly established by the direct cloning of the enzyme and the PCR-mediated microcloning technique. This technique was developed and first used to cut the chromosomes of *Drosophila melanogaster* (Scalenghe *et al.* 1981). After the creation of the DNA PCR technique, chromosome microdissection and microcloning developed rapidly. However, it is still mainly applied to studies of human and animal chromosomes (Zadesenets *et al.* 2017, Mahalingam 2018). The application of chromosome microdissection in plant research is limited because (1) the thick cell wall, denser cell cytoplasm, and asynchronous cell division make chromosome preparation difficult in plants and (2) chromosome banding is not perfect in

most plants, so it is very difficult to distinguish the target chromosome (Zadesenets *et al.* 2017). Nevertheless, the technique of chromosome microdissection and microcloning was successfully used in rye chromosomes by Saudery *et al.* (1991), and the cloning of rye DNA was successfully achieved. Since then, the application of this technique in the field of plant research has rapidly developed (Casson *et al.* 2005, Deng *et al.* 2014, Cao *et al.* 2017).

The advantage of chromosome microdissection is that any chromosome or DNA marker can be accurately obtained for any chromosome region according to the research need (Burgemeister 2005). Although the research and application of the chromosome microdissection technique in plants had a late start, with the rapid and strong development of plant genome research, chromosome microdissection will indeed become a powerful tool for the localization and cloning of plant genes (Day *et al.* 2005). It will also play a unique role in elucidating the structure of the plant genome, and studies on plant functional genomics will generate a new research field in plant cytogenetics. In this article, the experimental procedure of this technique was summarized. Then, the application of this technique in the field of plant research was reviewed. Finally, the key scientific problems and research prospects of this technique in plant research were discussed. The purpose of this article is to provide references for further studies and applications of this technique in the field of plant research.

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Table 1. Comparisons of main techniques in the plant chromosome microdissection and microcloning.

Function	Technique	Characteristics	Reference
Chromosome microdissection and collection	Glass micro-needle	In the inverted microscope, the chromosomes were directly cut by glass micro-needles with diameters of less than 0.5 μ m. Low costs. Difficult to operate and master.	Scalenghe <i>et al.</i> 1981
	Laser micro-beam	The chromosome microdissection and collection was conducted by laser micro-beam in a special device. The simplicity of operation, easy to master, and more accuracy in microdissection. Expensive cost and harsh working conditions of the special device.	Casson <i>et al.</i> 2005, Day <i>et al.</i> 2005
	Flow cytometry	After dying with specific dyes, such as Hoechst and chromomycin, the target chromosomes were separated and collected by flow cytometry. A large number of target chromosomes can be separated within a short time. Difficult to separate similar chromosomes and invalid separation of the chromosomal segment.	Cápal <i>et al.</i> 2015, Yang <i>et al.</i> 2017
Chromosome microcloning	Direct cloning after enzyme digestion LA-PCR (Linker-adaptor PCR)	This method can clone the large DNA fragment, but this method is complex, need more careful operations, and requires lots of substrates of 100–200 target chromosomes/segments as the template. Any DNA fragment with the unknown sequence can be amplified. And the constructed library does not need to be separated, and the possibility of pollution reduces obviously and the integrity improves greatly. This method makes the difficulty of chromosome micromanipulation to reduce remarkably and is a quantum leap in the chromosome microdissection and microcloning.	Saudery <i>et al.</i> 1991 Albani <i>et al.</i> 1993, Guo <i>et al.</i> 1998
	DOP-PCR (Degenerate oligonucleotide primed-PCR)	By this method, the chromosome DNA can be directly used as a template to be amplified without the operation steps of the digestion of restriction enzyme, designation of linker and adaptor, and so on. It is very rapid and efficient. The amplified products contain both high repetition and low copy sequences.	Pich <i>et al.</i> 1994, Hobza <i>et al.</i> 2004, Deng <i>et al.</i> 2014
	Southern hybridization	The genomic DNA labeled with certain markers hybridizes with the DNA library of the target chromosome/segment. Alternatively, the insertion fragments in the DNA library of the target chromosome/segment are labeled and used as probes to hybridize with the genome DNA. By this method, the source of the insertion fragments in the library can be preliminarily identified.	Hu <i>et al.</i> 1998, 2004
Screening and identification of chromosomal microcloning	<i>In situ</i> hybridization	The fragments inserted in the DNA library of the target chromosome/segment are used as probes to conduct the <i>in situ</i> hybridization and detect whether the insertion fragments were derived from the target chromosome/segment. By this method, both the specific clones of the target chromosome and the mutual clones of the target chromosome with other chromosomes can be detected.	Guo <i>et al.</i> 1998, Paz <i>et al.</i> 2013
	PCR amplification	When the sequence of both ends of any gene in the target chromosome/segment is known, the specific cloning of the target chromosome/segment can be identified by PCR by using the microdissected chromosome DNA or the insertion fragments in the DNA library as the template.	Zlotina <i>et al.</i> 2016

Table 2. The commercial products for chromosome laser microdissection.

No.	Company	Country	Product	Principle	Comparison
1	Arcturus/Life Technologies	USA	Arcturus XT	A thermoplastic membrane of ethylene-vinyl acetate (EVA) was heated by a low-energy infrared laser pulse, and the targets adhered in the membrane under a microscope.	The product of Arcturus XT is a unique microdissection instrument combining laser capture microdissection (LCM) and ultraviolet (UV) laser cutting. The products of the other three companies all use ultraviolet lasers to accomplish microdissection and target collection.
2	Leica	Germany	LMD6500, LMD7000	The targets were microdissected by an ultraviolet laser micro-beam through a microscope lens and collected by gravity.	
3	Zeiss	Germany	PALM MicroBeam	The targets were microdissected by a focused laser micro-beam and bounced into tubes for collection by the pressure wave of the laser.	
4	MMI (Molecular Machine Industry)	Switzerland	Cell Ector Plus, Cell Cut Plus	The targets were microdissected and collected by the solid-state ultraviolet laser. During the microdissection process, the laser does not contact the targets and only slightly damages the targets.	

Experimental procedures

The chromosome microdissection and microcloning technique can be divided into five steps: 1) preparation of the chromosome specimen, 2) identification of target chromosomes, 3) microdissection and collection of target chromosomes or chromosomal segments, 4) construction of the microcloning library and 5) screening and identification of microclones.

Identification of target chromosomes

The chromosome specimens must be prepared precisely by conventional methods. After the preparation of chromosome specimens, the appropriate methods must be used to identify the target chromosomes. Karyotype analysis and chromosome banding are often used. For example, the meiotic monovalent chromosomes in wheat monosomic lines were identified by karyotype analysis and successfully microdissected (Vega *et al.* 1994). The B chromosomes of rye were identified by chromosome banding and successfully microdissected (Houben *et al.* 2001). However, traditional methods of karyotype analysis and chromosome banding are limited in the identification of chromosomes for microdissection, especially in these species with small and inconspicuous chromosomes.

Construction of microcloning library

The microdissection and collection of target chromosomes/segments can be conducted by the techniques of glass micro-needles, laser micro-beams, and flow cytometry (Table 1). Currently, there are special laser beam devices (Table 2) for the chromosome microdissection. After microdissection, the obtained chromosomes/segments can be amplified *in vitro* for subsequent studies. The construction of chromosome microcloning libraries has mainly been conducted by two methods: direct cloning after enzyme digestion and PCR-mediated microcloning (Table 1).

Direct cloning after enzyme digestion—Direct clon-

ing after enzyme digestion was widely applied before the construction of PCR. The procedure for this method includes the following steps. First, the target chromosomes/segments were extracted from DNA. Then, the extracted DNA was digested by enzymes after deproteinization and purification. Third, the DNA digested was added to the ligase to link the vector digested by the same restriction enzyme. Finally, the DNA linked to the vector was transformed into the host bacteria to construct the chromosome DNA library. Saudery *et al.* (1991) were the first to apply this method in plant chromosome microcloning and DNA library construction. They cut and separated the B chromosomes of rye by glass micro-needles and successfully microcloned the B chromosomes after extraction, deproteinization, purification, digestion, and so on (Saudery *et al.* 1991).

PCR-mediated microcloning- The number of target chromosomes obtained directly by microdissection is limited. After PCR amplification *in vitro*, additional target chromosomes can be obtained to meet the needs of molecular biology experiments. Since 1989, several PCR-mediated microcloning methods have been developed (Deng *et al.* 2014, Cápál *et al.* 2015). Two of these methods, LA-PCR and DOP-PCR, are used frequently.

LA-PCR (Linker adaptor PCR) involves the following steps. First, the DNA of the target chromosomes/segments was digested by restriction enzymes. Second, according to the base sequence of the sticky end produced after enzyme digestion, one linker and one adaptor were designed, and the mixture of the linker and adaptor can produce the same sticky ends as those of the DNA digested by restriction enzymes. Finally, the PCR amplification was conducted by using the linker as the primer. Albani *et al.* (1993) successfully applied the linker-adaptor system of *Sau3AI* for the PCR amplification of microdissected chromosomes. Guo *et al.* (1998) have also successfully cloned the microdissected B chromosome of rye by LA-PCR with the linker-adaptor system of *Sau3AI*.

DOP-PCR (Degenerate oligonucleotide-primed PCR) refers to the use of a simple degenerate primer for amplification after two PCR reactions (Pich *et al.* 1994). The annealing temperature is low in the first PCR reaction. The chromosomes of bean and wheat were microdissected and successfully amplified by DOP-PCR (Pich *et al.* 1994, Deng *et al.* 2014).

Screening and identification of microclones

The specificity of the constructed DNA library is generally screened and identified by using Southern hybridization, *in situ* hybridization and the PCR amplification of specific DNA segments (Table 1) (Paz *et al.* 2013).

Southern hybridization can preliminarily identify the source of the inserted DNA fragments in the library. Hu *et al.* (1998) successfully proved that the microclones produced from *in vitro* PCR were derived from the maize genome by Southern hybridization of digoxin-labeled maize genomic DNA with single chromosome DNA microdissected from maize. Using the same method, the 4 DNA fragments in the library of the 6B wheat chromosome were proved to be derived from the wheat genome (Hu *et al.* 2004). *In situ* hybridization not only detects the chromosomal source of the inserted DNA fragments in the library and identifies the specific clones of the target chromosome, but it also identifies the mutual clones of the target chromosome with other chromosomes (Paz *et al.* 2013). By *in situ* hybridization, Guo *et al.* (1998) successfully located the PCR products at the terminal of the B chromosome of rye. When the sequence of both ends of any gene in the target chromosome/segment is known, the specific cloning of the chromosome can be identified by PCR. The 4 different DNA fragments of R1, R2, R3 and R4 were successfully proved to be derived from the wheat 6B chromosome by PCR using different SSR markers of the 6B chromosome as primers (Hu *et al.* 2004).

Applications in plant studies

Construction of a specific DNA library for a single chromosome/segment

After chromosome microdissection, the length of the DNA fragments obtained by PCR amplification is generally ~150–2000 bp, which is suitable for constructing a specific DNA library (Liu *et al.* 2004, Hwang and Lim 2011, Shibata *et al.* 2017). Until recently, the DNA library for a single whole chromosome has been constructed in oat (Emmert-buck *et al.* 1996), corn (Hu *et al.* 1998), and lily (Hwang and Lim 2011). A DNA library for chromosomal segments has been constructed by the same method, such as the short arm of 1H chromosome of barley (Schondelmaier *et al.* 1993) and the long arm of the 6B chromosome of common wheat (Hu *et al.* 2004).

Construction of a specific probe pool for a single chromosome/segment

The product obtained from the PCR of the microdissected chromosome/segment is the probe pool. The probe pool combined with fluorescence *in situ* hybridization (FISH) is a powerful technique for studying the chromosome structure and its variations, such as chromosome translocation, inversion (Taguchi *et al.* 2016, Yanagi *et al.* 2017). Deng *et al.* (1999) successfully set up a specific probe pool for the fourth pair of chromosomes of rice, and the specificity was identified by FISH. This research laid the foundation for screening the new specific molecular markers from the DNA library of a single chromosome in rice.

Development of new molecular markers tightly linked to the target genes

Molecular marker-assisted breeding must have a sufficient number of molecular markers that are tightly linked to the target genes. However, effective molecular markers that can be utilized for breeding are currently lacking, obviously limiting the further development and application of molecular marker-assisted breeding (Doležel *et al.* 2014). Thus, it is important and significant to develop new molecular markers for breeding. The length of the microdissected DNA fragment is typically 1–3 Mb when using the chromosome microdissection technique. Thus, screening molecular markers from the DNA library for the target chromosome/segment constructed by chromosome microdissection and microcloning will become an extremely effective method for developing new molecular markers tightly linked with the target genes (Cao *et al.* 2017). Moreover, from the specific DNA library, the efficiency of developing new molecular markers can be greatly improved (Seifertova *et al.* 2013). Based on the microdissection of the short arm of barley 1H chromosome, the genetic linkage map of molecular markers for the chromosomal segment was constructed after microcloning, and one RFLP marker tightly linked to the gene (*Ma*) for resistance to powdery mildew was screened (Schondelmaier *et al.* 1993). The long arm of the barley 3H chromosome was microdissected and collected, and one moderately repetitive sequence and one highly repetitive sequence were obtained after microcloning (Bush *et al.* 1995). The results provided a foundation for the development of microsatellite markers in barley.

Construction of a dense linkage map matching the physical map

The efficiency of developing new molecular markers can be greatly improved by microdissection and microcloning, which is extremely significant for the construction of a dense and homogeneous genetic linkage map. To date, molecular linkage maps have been constructed for many plant species, such as rice, soybean, corn, and

tobacco, but except for a few species of rice, the genetic linkage map of most plants is highly unsaturated, and the distribution of genetic markers in the linkage group is not homogeneous (Doležel *et al.* 2014). The development of new molecular markers from the DNA library of the target chromosome segment by chromosome microdissection and microcloning can increase the density of genetic linkage maps and resolve the problem of the nonhomogeneous distribution of molecular markers.

Also, there is an important problem, that is, the genetic linkage map does not match its physical map, whether in the traditional genetic linkage map or the molecular marker linkage map. Occasionally, the distance of genes showing in the genetic linkage map greatly differs from the physical distance in the chromosome. Routinely, the relationship of the genetic linkage map with its chromosomal physical map was conducted by chromosome terminal deletion (Doležel *et al.* 2014). However, the breeding of the chromosome terminal deletant takes several years, and the number of chromosome terminal deletants that can breed is limited. Thus, new techniques to resolve the relations of the genetic linkage map with its chromosomal physical map are urgently needed. By chromosome microdissection and microcloning, specific molecular markers of the target chromosome segment can be obtained, which can help quickly realize the relationship between the genetic linkage map and its physical map. In addition, the single-copy molecular markers of STS, obtained from the specific DNA library of the target chromosome/segment constructed by chromosome microdissection and microcloning, can be used to construct both of the genetic linkage map and the physical map. And the corresponding relationship between the genetic map and physical map can be achieved by this method.

Localization and cloning of genes

Specific probes obtained by microcloning is an effective method to clone new genes (Roux *et al.* 2014, Cao *et al.* 2017). The 1D chromosome of wheat was microdissected and separated in 2009, and the gene fragments for the subunit (1Dx5) of hmw-glutenin (HMW-GS) were cloned after the microcloning (Tian *et al.* 1999). Moreover, the microdissection and microcloning of a specific chromosome carrying target resistant genes can be used for gene cloning to achieve target resistance. Wan *et al.* (2000) used glass needles to microdissect and separate pairs of chromosomes carrying the BYDV (barley yellow dwarf virus) resistance gene in the wheat-*Thinopyrum* alien additional line TAI-27. Then, based on the successful microdissection of a chromosome carrying anti-BYDV genes in the wheat-*Thinopyrum* alien additional line TAI-27, the cloning of the resistance genes was effectively conducted (Jiang *et al.* 2004).

Applications in plant evolutionary genetics

The specific DNA library for the target chromosome/segment, constructed by chromosome microdissection and microcloning, can be used as probes to conduct the micro-FISH (Zlotina *et al.* 2016, Han *et al.* 2017, Kosyakova *et al.* 2017). By this method, the differentiation and homology of the target chromosome/segment between species can be clarified and their evolutionary relationship can be established, thus providing a new way to study plant evolutionary genetics and comparative genomics. Zhang *et al.* (2006) constructed a probe pool of the centric region of the rye B chromosome by chromosome microdissection and microcloning, and by FISH, the highly homologous DNA of the centric region between rye A and B chromosomes was identified. Houben *et al.* (2001) studied the origin and evolution of the rye B chromosome based on the DNA library construction of B chromosome/segment by chromosome microdissection and microcloning. The results showed that the B chromosome was derived from the A chromosome but lacked the repetitive sequence family contained in the heterochromatin region of the A chromosome (Houben *et al.* 2001).

Intractable application to plant research

Difficult preparation of high-class chromosome specimens

The preparation of chromosome specimens is the most fundamental and key technology affecting chromosome microdissection. However, the solid cell wall and nonsynchronous cell division often result in the unsatisfactory dispersion of chromosomes with an unclear background in the preparation of chromosome specimen, seriously affecting the effect of chromosome microdissection. Another major concern for the successful application of chromosome microdissection and microcloning was the extensive depurination of the chromosomal DNA caused by acid treatment during sample fixation in the chromosome preparation. Currently, depurination can be reduced or avoided by improving the chromosome preparation procedure.

Obvious difficulties in the identification of target chromosomes

The ploidy of the plant genome is multiform, the variation of chromosome structure is remarkable, and the gene sequence is also not highly conservative (Doležel *et al.* 2014). Thus, in plants with small similar chromosomes, it is impossible to accurately identify the target chromosomes and their fine structures. Although the technique of chromosome banding is helpful for the identification of target chromosomes, this technique can destroy the DNA structure because of plant chromosomes with a high ratio of DNA and a low ratio of protein (Doležel *et al.* 2014), detrimentally constructing the complete DNA library for the target chromosome.

Thus, the application of chromosome banding for chromosomal identification in chromosome microdissection and microcloning is limited.

The huge workload of screening the chromosome DNA library

The DNA content of plant chromosomes with a large number of highly repetitive sequences is high (Seifertova *et al.* 2013). For example, the genome content (*C* value) of wheat is 1.6×10^{11} bp, and the average of each chromosome is 0.8×10^{10} bp, with approximately 80% repetitive sequences (Hu *et al.* 2004). If the length of each insert fragment is 1 kb, then the specific DNA library for each chromosome will contain more than 8×10^7 clones. In addition, wheat is an allohexaploid with the chromosome complement of AABBDD ($2n=6x=42$). Thus, the specific DNA library for all 42 chromosomes of the wheat genome will contain 3.36×10^9 clones, and screening the chromosome DNA library will be an immense task.

Unavoidable contamination of cytoplasmic DNA

PCR-mediated cloning of microdissected chromosome DNA made chromosome microdissection and microcloning more practicable. However, it is difficult to completely avoid DNA contamination from the cytoplasm, foreign species, and non-target chromosomes. The amplification of non-target DNA will greatly affect the quality of chromosome-specific DNA libraries. Generally, in plant chromosome microdissection and microcloning, DNA contamination can come from the following three sources: (1) foreign species, (2) non-target chromosomes, and (3) cytoplasmic DNA (Zhou and Hu 2007). DNA contamination by foreign species can be controlled by strict disinfection and sterilization of instruments and chemical reagents. DNA contamination by non-target chromosomes can also be avoided by careful operation during microdissection. However, the contamination of cytoplasmic DNA is a serious concern. Due to the particularity of plant cells, it is unavoidable that the slides of plant chromosome specimens will contain remnants from the cytoplasm and cell wall. Furthermore, the contamination of cytoplasmic DNA is almost unavoidable in chromosome microdissection and microcloning (Zhou and Hu 2007). Hu *et al.* (1998) proposed a method of adding a drop of 50% ethanol to the target chromosome to microdissect, thus reducing the contamination by cytoplasmic DNA. Also, using a single chromosome/segment as a template to conduct the PCR amplification is an effective means to reduce the contamination of cytoplasmic DNA (Yang *et al.* 2017).

Plant chromosome microdissection and microcloning technique: the future

The chromosome microdissection and microcloning technique has been widely used in the field of plant mo-

lecular genetics (Zhou and Hu 2007). The location and cloning of genes and the construction of genetic linkage and physical maps are based on the operations of chromosomes. Thus, the chromosome microdissection and microcloning technique developed and combined with other advanced techniques in molecular biology, such as PCR, molecular marker development, and chromosomal painting, will have further and broader applications in plant research fields, such as gene location and cloning, specific probe development, constructing genetic linkage maps, and chromosomal evolution.

Although whole-genome sequencing has rapidly developed in plants since the start of the genome project, a serious problem in plant genome sequencing remains (Doležel *et al.* 2014, Zhang *et al.* 2006). The huge and polyploid genomes with high heterozygosity contain highly repetitive sequences and repeated fragments of whole/parts of genes, and accurate assembly after sequencing became an impossible task in plants. However, the sequence of a single chromosome can easily solve this intractable problem (Deng *et al.* 2018). Therefore, the chromosome microdissection technique can produce a single chromosome and will play an important role in the whole genome sequencing of plants in the future.

Although the flow cytometry to separate single chromosome are fast and accurate, the difficult and invalid to separate similar chromosomes limit the further application in genetics. Along with the improvement of FISH technique, especially, the development of specific DNA probes labeled fluorescent dyes and foundation of phase metaphase chromosome hybridization technique in liquids, the flow cytometry combined with FISH has been successfully applied in the separation of similar chromosomes. Kosyakova *et al.* (2017) successfully separated the similar chromosomes of humans by the flow cytometry combined with FISH technique. So it can be expected that the flow cytometry with FISH technique will be widely applied in the separation and collection of similar chromosomes in plants.

Although there are still several problems in the application of chromosome microdissection and microcloning in plants, with the continuous improvement of chromosome preparation, the development of enzyme digestion and PCR technique, the integration of glass micro-needles and laser microbeams, and the production and application of more sophisticated machines, the automatization of chromosome microdissection will be greatly improved, the microdissection will be more accurate, and the efficiency of microdissection and microcloning will increase markedly. Thus, we predict that chromosome microdissection and microcloning will play an important role in the whole genome sequencing, evolutionary genetics, molecular marker-assisted breeding, development of chromosome-specific probes, and gene location and cloning of plants.

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