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CONSTRUCTION OF A BAC-LIBRARY FOR SUNFLOWER

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For my Mother and Father
„Sevgiyle“

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1 INTRODUCTION

1.1 Economic Importance of Sunflower

The cultivated sunflower (*Helianthus annuus* L.) is one of the most important oil crops in the world. The sunflower growing area world-wide increased from 17 Mio. ha in 1990 to 23 Mio. ha in 1999 (FAOSTAT 1999). One tenth of the oil crops growing area belongs to sunflower which is the third important oil crop in the world behind soybean (*Glycine max* L.) and rapeseed (*Brassica sp.*). Sunflower represents the most important oil crop in France, Spain, Italy, Romania, and Bulgaria, which have an ideal climate for sunflower. In Germany, sunflower growing area decreased from 189.000 ha to 32.465 ha in the last six years and winter rapeseed increased due to the more suitable climate for this crop in Germany (FAOSTAT 1999).

The origin of the genus *Helianthus* is in North America (Hugger 1989). The genus belongs to the family *Asteraceae* and consists of 49 species. Schilling and Heiser (1981) divided the genus *Helianthus* in four sections: *Annui*, *Ciliares*, *Agrestes* and *Divaricati*. The haploid number of chromosomes is n = 17. Besides the annual diploid species, a number of tetraploid and polyploid perennial species are known (Seiler 1992). Some of the polyploid species consist of a combination of the three basic genomes A, B and C which have different origins in North America (Anaschenko 1982).

Conventional sunflower types with an oleic acid content of 15 to 25% are especially valuable for human nutrition, because of the high content (70%) of linoleic acid (C 18:2) (Ganßmann and Friedt 1983). The value of sunflower oil for industrial purposes was improved by the development of high oleic sunflower types, with an oleic acid content up to 90% (Friedt and Scheuermann 1991). Because of the temperature stability of the oleic acid it can be used as frying oil, as well as renewable raw material in the chemical industry (Korell and Friedt 1996).

1.2 Relevance of a BAC Library for Sunflower

The importance of sunflower oil for human nutrition and chemical industry, makes sunflower one of the major objects of research. One of the main diseases in most of the sunflower growing areas in the world (Sackston 1981, Viranyi 1992) is downy mildew caused by *Plasmopara halstedii*. The pathogen is world-wide distributed and leads to yield

losses up to 50% (Viranyi 1992). A number of major resistance genes have been either identified in cultivated sunflower or introduced from wild *Helianthus annuus* or other *Helianthus* species (Miller 1992). These dominant resistance genes have been called *Pl* genes. The *Pl2* gene is supposed to be a part of the *Pl6* “gene cluster” and confers resistance to a number of downy mildew races. A high resolution map of the target region of the sunflower genome is essential to arrive at and isolate the gene by using molecular markers. RAPD and AFLP analyses were performed to identify molecular markers, which can be used to isolate the resistance genes against downy mildew by a map-based cloning approach (Brahm and Friedt 1996, Brahm et al. 1998, Röcher et al. 1998).
In sunflower, hybrid breeding is based on a single cytoplasmic male sterility (CMS) source, the PET1-cytoplasm, which was obtained by an interspecific cross between *H. petiolaris* and *H. annuus* (Leclercq 1969). The PET1-cytoplasm is associated with a new open reading frame “*orfH522*” in the 3’-flanking region of the *atpA*-gene and an additional 16 kDa protein (Horn et al. 1996). The restorer line RHA325 has a dominant restorer gene *Rf1* which allows fertility restoration of cytoplasmic male sterile plants (PET1) which is essential for commercial hybrid seed production (Korell et al. 1992).

In the cultivated sunflower three RFLP-maps were constructed (Berry et al. 1995, Gentzbittel et al. 1995, Jan et al. 1998). Construction of a linkage map using the AFLP- and RFLP-techniques and identification of AFLP and RAPD markers tightly linked to the Rf1-gene was performed by Prüfe et al. 1998.

For further genome research e. g. positional cloning and physical mapping large genomic insert DNA clones are now required. Bacterial artificial chromosome (BAC) libraries represent one type of large-insert genomic DNA libraries. Because of the advantages of the bacterial artificial chromosomes over other cloning systems like e.g. yeast artificial chromosomes, BAC have become the most popular tool for cloning large DNA fragments.

1.3 BAC Libraries in Plants

1.3.1 Comparison of Vectors used in the Construction of BAC Libraries

Bacterial vectors developed for cloning large DNA fragments (>100 kb) were derived from plasmids that are capable of replicating long regions of DNA and are maintained at one copy per cell. These features favour the stable replication and propagation of large DNA molecules, including those that contain high levels of repetitive sequences. BAC vectors,

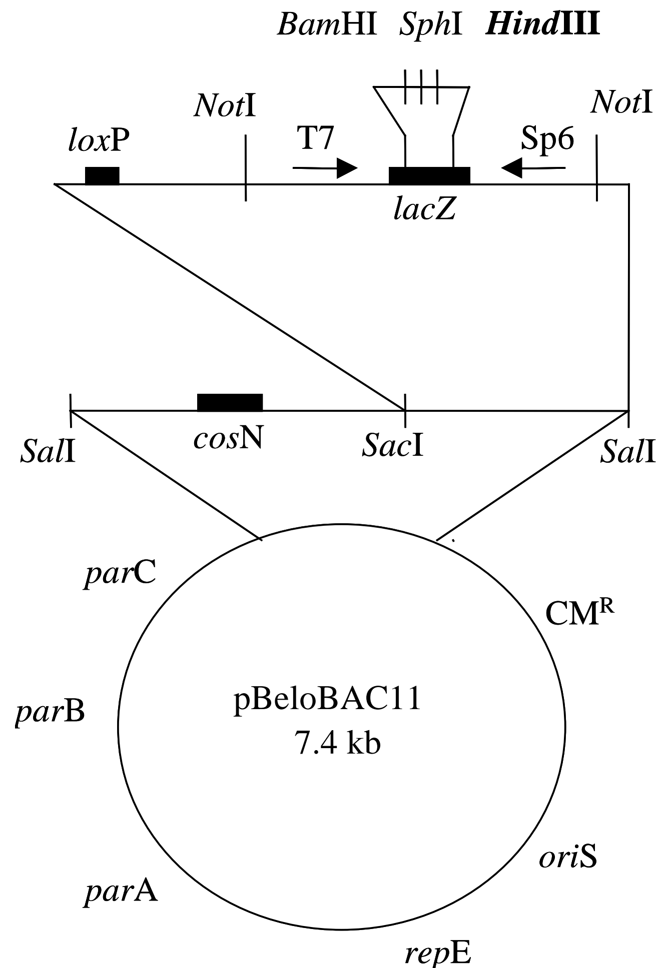

Fig.1.1 Map of the vector pBeloBAC11 used for construction of BAC libraries (Shizuya et al. 1992)

such as pBAC108L, were derived from the F factor of *E.coli* containing minimal sequences needed for the autonomous replication, copy-number control, and partitioning of the plasmid (Birren et al. 1997). The F factor codes for genes that are essential to regulate its own replication and controls its copy number in a cell. The regulatory genes include *oriS, repE, parA,* and *parB.* The *oriS* and *repE* mediate the unidirectional replication of the F factor , and the *parA* and *parB* maintain copy number at a level of one or two per cell.
The most widely used BAC vector, pBeloBAC11, represents the second generation of BAC cloning vectors, which were developed from the pBAC108L (Shizuya et al. 1992). There are three unique cloning sites: *Bam*HI, *Sph*I and *Hind*III, which are flanked by the T7 and SP6 promoters within the *lacZ* gene which allow identification of recombinants by colony colour (see Fig.1.1). These promoters allow preparation of probes from the ends of cloned sequences by *in vitro* transcription of RNA, i.e. generating RNA probes for

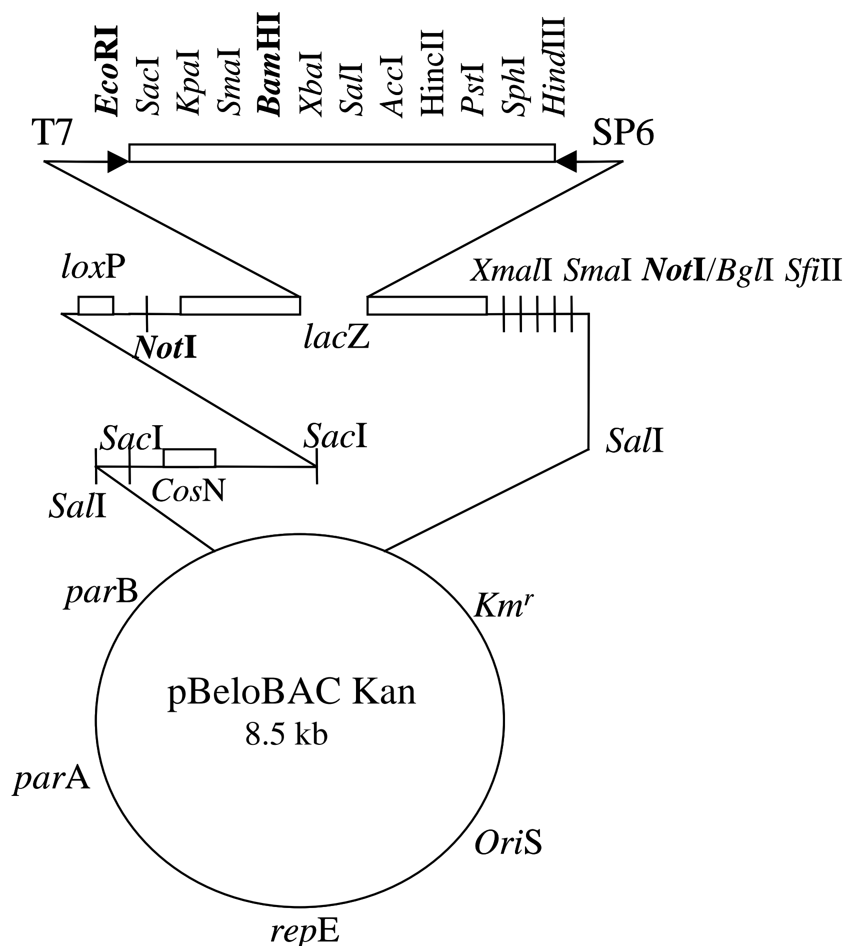

Fig.1.2 Map of the vector pBeloBACKan (Mozo et al. 1998a)

chromosome walking (Sambrook et al. 1989), and DNA sequencing of the insert fragment at the vector- insert junction. The G+C rich restriction sites (*Not*I, *Eag*I, *Xma*I, *Sma*I, *Bgl*I, and *Sfi*I) can be used to excise the inserts of BAC clones. There are two selective markers for cloning purposes: the *lacZ* gene to facilitate recombinant identification with blue and colourless (white) phenotypes and the CMR (chloramphenicol) resistance gene for selection of transformed bacteria.

The blue colour of nonrecombinant pBeloBAC11 clones is not as intense as that obtained with multicopy vectors (Birren et al. 1997). The vector pIndigoBAC, a derivative of pBeloBAC11, produces a more intense blue colour than pBeloBAC11. The cloning site in both pBeloBAC11 and pIndigoBAC is derived from pGEM3Z, which is located within the *lacZ* gene and contains all of the restriction sites of the pGEM3Z multiple cloning site. In pIndigoBAC, the *Eco*RI site present in the chloramphenicol resistance gene of

pBeloBAC11 has been eliminated, which allows to clone *Eco*RI fragments (Birren et al. 1997).

Another vector containing an *Eco*RI cloning site is pBeloBACKan (see Fig.1.2). This modified vector contains a unique *Eco*RI cloning site (Mozo et al. 1998a). A Tn*903*-derived kanamycin resistance gene (Oka et al. 1981) was inserted by blunt-end cloning at the *Eco*RI site present in the chloramphenicol resistance gene in pBeloBAC11, giving pBeloBACKan (Mozo et al. 1998a).

Additional BAC vectors have been derived from pBeloBAC11 like pECSBAC4 (Frijters et al. 1997) which also has a unique *EcoRI* cloning site, and pBACwich (Choi et al. 2000) which has a promoterless hygromycin gene for transformation of plants. With the effectiveness of the *sac*BII gene pBACe3,6 allows positive selection for insert-containing BAC clones (de Jong et al. 1997). It also has a "pUC-link" which allows to increase the copy number of the vector for plasmid purification prior to BAC library construction.

DNA transfer into plants has been accomplished by several methods including *Agrobacterium*-mediated transformation, biolistic bombardment, and microinjection (Hamilton et al. 1996). BAC vectors have been engineered for transformation of large DNA inserts into plant genomes. BIBAC (binary bacterial artificial chromosome) has been designed to replicate in both *E.coli* and *A. tumefaciens* and has all of the features which are required for transferring large inserts of DNA into plant chromosomes (Hamilton et al. 1996). Thus, BIBAC genomic DNA library clones are immediately suitable for transformation of plants. BIBAC test constructs containing 150 kb human DNA were introduced into *A. tumefaciens* strains and used for transformation experiments. The human DNA fragments were shown to be randomly integrated into the tobacco genome (Hamilton et al. 1996). These results open new possibilities for genetic engineering and for plant molecular biology. The recent development of BIBAC vectors capable of transforming plants with large DNA fragments has reinforced the popularity of BACs among plant biologists.

Tab. 1.1 Comparison of different BAC vectors described so far (http://www.genome.clemson.edu)

Name	**Cloning sites**	**Recombinant selection**	**Special Features**	**Reference**
pBAC108l (6.7 kb)	*Hind*III, *Bam*HI	No		Shizuya et al. 1992
pBeloBAC11 (7.4 kb)	*Hind*III, *Bam*HI, *Sph*I	*lac*Z		Kim et al. 1996
pECSBAC4 (9.3 kb)	*Eco*RI, *Hind*III, *Bam*HI	*lac*Z		Frijiters et al. 1997
pBeloBACKan	EcoRI	*lac*Z		Mozo et al. 1998a
BIBAC2 (23.5 kb)	BamHI	*sac*BII	Plant Transformation via Agrobacterium	Hamilton et al. 1996
pBACwich (11 kb)	*Hind*III, *Bam*HI, *Sph*I	*lac*Z	Plant Transformation via Site-Specific Recombination	Choi et al. 2000
pBACe3,6 (11.5 kb)	*Bam*HI, *Sac*I, *Sac*II, *Mlu*I, *Eco*RI, *Ava*III	*sac*BII	High copy number is available	De Jong et al. unpublished

1.3.2 Characteristics of Pulsed Field Gel Electrophoresis (PFGE)

Normal gel electrophoresis of DNA molecules is carried out by placing DNA samples in a agarose or polyacrylamide matrix and migration of the molecules in the gel occurs under a static electric field (Birren and Lai 1993). Under the influence of an electric field, DNA molecules elongate and align with the field and migrate towards the anode by a process termed "reptation". Reptation of DNA through the gel means that the DNA moves like a snake: the head selects the path and the rest of the molecule follows. Normally, molecules larger than 20 kb cannot be separated from each other, because DNA molecules greater than that will have the same cross-sectional area after they align with the electric field. Gels as low as 0.1 % can be used to separate larger molecules with very low voltage gradients, although they are mechanically very difficult to handle and the use of low voltage gradients requires running times of days to weeks. Even under these extreme conditions, separation of DNA molecules larger than a few hundred kilobase pairs is not possible (Birren and Lai 1993).

Klotz and Zimm (1972) demonstrated that, after the removal of an electric field, the elongated DNA molecules relax back to their unperturbed state. The rate of relaxation is dependent on the length of the DNA. Schwartz and Cantor (1984) attempted to exploit this size-dependent relaxation to separate large DNA molecules. Periodically changing the orientation of the electric field would force the DNA molecules in the gel to relax on removal of the first field and elongate to align with the new field. The effectiveness of field switching was shown by separating yeast chromosomes several hundred kilobases in size (Shwartz and Cantor 1984).

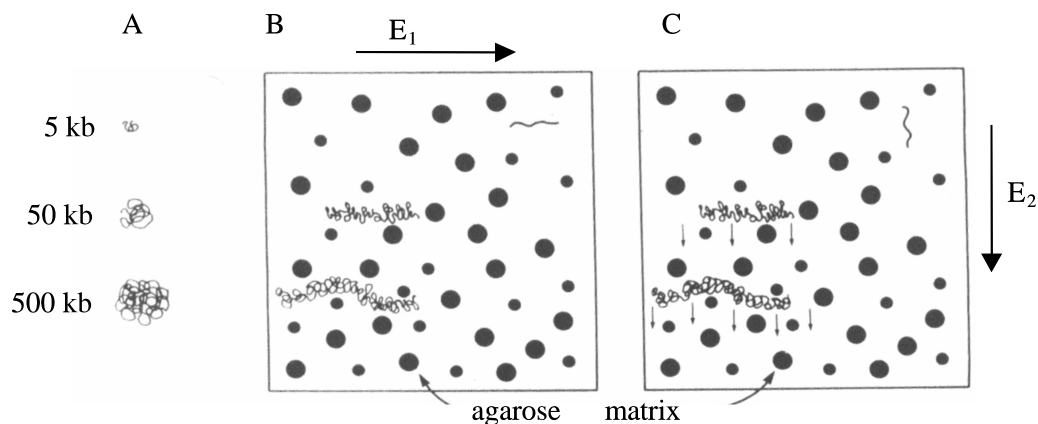

Fig.1.3 Schematic illustration of DNA separation in PFGE (Birren and Lai 1993). When the first electric field (E1) is applied to the gel as in figure A, DNA molecules elongate in the direction of the field and begin to migrate in the gel (B). All molecules are aligned with the field, but the 50 and 500 kb molecules present essentially the same cross sectional area in the gel and thus migrate at the same rate. The first electric field is then removed and a second field (E2), at some angle to the first field, is activated in a new direction (C).

The principle of pulsed field gel electrophoresis (PFGE) separation is shown as schematic illustration in Fig.1.3 and as microscopic observation in Fig. 1.4. The DNA must change conformation, reorients before it can migrate in the direction of the second electric field. The time required for this reorientation has been found to be very sensitive to the length of the molecule. Larger DNA molecules take more time to realign than smaller ones because of the physical barrier of the agarose matrix.

Direct microscopic observation of individual DNA molecules in the gel provided clear pictures of how large DNA molecules move and change direction during PFGE (Schwartz and Cantor 1984). Fig.1.4 shows the movement of a single fluorescently stained phage DNA molecule (164 kb) that is undergoing PFGE on a microscope slide.

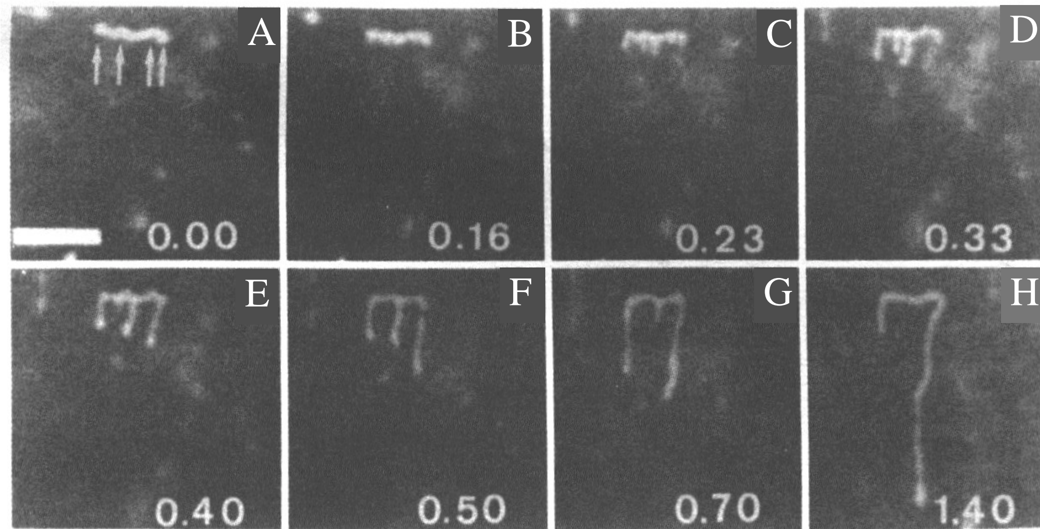

Fig.1.4 Microscopic observation of migration of DNA molecules in PFGE (Birren and Lai 1993). The DNA molecule (164 kb) aligned along the horizontal direction from left to right by the initial electric field (A). The first electric field then is turned off and the second electric field is activated (from top to bottom) (B-G). As can be seen in photographs, the molecule forms kinks (white arrows) as it attempts to align with the second electric field. The different kinks compete to become the new "head" that will lead the migration of the molecule (C-F). One of the kinks eventually wins, and the molecule migrates in the new direction. The longer the DNA molecule, the more kinks are formed and the longer the establishment of a new head and migration in the new direction takes.

For the construction of BAC libraries, PFGE is an essential tool to separate the high molecular weight DNA in a gel. Although, with conventional gel electrophoresis, it is not possible to distinguish molecules larger than 100 kb in a gel, by using PFGE DNA up to 1600 kb can be separated.

1.3.3 Bacterial Strains and Transformation Protocols for BAC Libraries

For construction of a BAC library another necessary tool is electroporation of an appropriate bacteria strain. The method of electroporation was developed for *E.coli* (Dower et al. 1988, Taketo 1988). Tao and Zhang (1998) reported the feasibility of cloning very large fragments of eukaryotic DNA in bacteria using conventional plasmid-based vectors. One conventional plasmid vector (pGEM11), one conventional binary plasmid vector (pSLJ1711) and one conventional binary cosmid vector (pCLD04541) were investigated using the widely applied BAC (pBeloBAC11 and pECBAC1) and BIBAC

(BIBAC2) vectors as controls. The plasmid vector pGEM11 yielded clones ranging in insert sizes from 40 to 100 kb, whereas the two binary vectors pCLD04541 and pSLJ1711 yielded clones ranging in insert sizes from 40 to 310 kb.

Transformation is carried out by electroporation and the transformation efficiency for BACs is about 40 to 1,500 transformants from one µl of ligation product, or 20 to 1,000 transformants/ng DNA (Sheng et al. 1995). There is an inverse relationship between the insert size and the transformation efficiency. It has been demonstrated that a lower field strength (9-13 kV/cm) yields a higher average insert size but a lower number of clones.

Transformation of *E.coli* with DNA by electroporation is an extremely efficient process with DNA that has a size of few kilobase pairs (Birren et al 1997). Transformation efficiencies comparable to those obtained with in vitro packaging and infection can be obtained with DNA as large as 50-80 kb if the appropriate host strain is used. However, many commonly used strains of *E.coli* exhibit a pronounced drop in transformation efficiency with large DNA molecules (Sheng et al. 1995). DH10B (Hanahan et al. 1991) is commonly used as a host because it can be transformed extremely well with large DNA clones and it has the appropriate genotype concerning recombination, restriction, and modification (Sheng et al. 1995). Identical transformation efficiencies for DH10B can be obtained with 7 kb and 80 kb DNA. However, transformation works approximately tenfold less well with 150 kb molecules than with 80 kb molecules, even in DH10B. Therefore, the construction of large-insert bacterial clones by electroporation requires ligation mixtures that are nearly free of small molecules (<30 kb). Even if these molecules represent a tiny fraction of a ligation mixture, they give rise to colonies at a much higher frequency than the larger ligation products. Effective electroporation conditions with large DNA molecules (>80 kb) differ from conditions that produce the highest efficiencies with smaller plasmids (~10 kb) (Birren et al. 1997).

DH10B competent cells (ElectroMAXDH10B cells (BRL,USA)) that are appropriate for BAC cloning can be purchased in a form competent for electroporation. Preparation of the competent cells is a simple process that only entails washing freshly grown cells in 10 % glycerol. The glycerol washes remove the electrolytes in the medium that would cause arcing and thus interfere with electroporation. However, commercially obtained cells eliminate a source of variability in the procedure. If cells are self made, test transformations should be performed to verify the quality of the cells before they are used for valuable ligations.

1.3.4 Comparison of BAC Libraries in Plants

Recently, BAC libraries have become the most widely used cloning system. An overview of some of the plant BAC libraries constructed up to date to is shown in Table 1.1. The number of clones in the BAC libraries varies, because of the different genome sizes of the plant species. The average insert size of BAC libraries ranges from 90 kb to 157 kb. The minimum insert size is 20 kb in *Arabidopsis thaliana* and the maximum is 340 kb in *Sorghum bicolor*. Until today, no sunflower BAC library has been reported.

Tab. 1.2 Overview of some BAC libraries reported in plants. The table was complemented according to CUGI (http://www.genome.clemson.edu).

Plant Species	Average Insert Size	Number of Clones	Reference
Sorghum bicolor	157 kb	13,500	Whoo et al. 1994
Arabidopsis thaliana	100 kb	12,672	Choi et al. 1995
Oryza sativum	125 kb	11,000	Wang et al. 1995
Oryza sativum (japonica)	150 kb	7,296	Zhang et al. 1996
Oryza sativum (indica)	130 kb	14,208	Zhang et al. 1996
Glycine max	147 kb	40,320	Kanazin et al. 1996
Oryza sativum	107 kb	18,432	Yang et al. 1997
Lactuca sativa	111 kb	53,000	Frijters et al. 1997
Glycine max	120 kb	30,000	Danesh et al. 1998
Solanum tuberosum	100 kb	160,000	Kanyuka et al. 1999
Lycopersicum esculentum	125 kb 100 kb	42,272 18,816	Hamilton et al.1999 Folkertsma et al.1999
Lycopersicum pennellii	90 kb	53,760	Hamilton et al.1999
Triticum tauschii	119 kb	144,000	Moullet et al. 1999
Solanum tuberosum	155 kb	23,808	Song et al. 2000

1.3.5 Comparison of Large-Insert Genomic DNA Libraries

In the last several years detailed genetic maps based on restriction fragment length polymorphism (RFLPs) have been constructed for a large number of plants (Tanksley et al. 1995). These maps have a density of markers in excess of one marker per megabase, and thus make physical mapping of defined regions of the genome possible.

Mapping relative to RFLP probes, known as "Chromosome Walking" is theoretically possible. The difficulty with such an approach for most genes is that the closest RFLP hybridisation probe may be several hundred kb or even several Mb way from the target gene (Ordon et al. 2000). Although RFLP maps are now available for many plant species, only a few systems provide the high density of markers required for long range physical mapping using pulsed field gel electrophoresis (PFGE). By PFGE in combination with the digestion of DNA, using rare cutting restriction enzymes, it is possible to bridge these gaps between the markers and to construct long range physical maps (Ganal and Tanksley 1989). With the assembly of genomic contigs using BAC clones, large complex genomes can be sequenced. Thus the application of pulse field gel electrophoresis (PFGE) and large-insert genomic libraries like cosmid (Collins and Hohn 1978), yeast artificial chromosome (YAC) (Burke et al. 1987), bacterial artificial chromosome (BAC) (Shizuya et al. 1992), and PAC (Ioannou et al. 1994) has advanced the development of genome research (Zhang et al. 1996).

Cosmids have been used since the earliest beginnings of plant molecular biology and remain valuable as general vectors for cloning DNA fragments up to approximately 40 kb. Cosmids are basically plasmid vectors and allow DNA to be packaged *in vitro*. Cosmid vectors have also been modified into plant transformation vectors (Zhang et al. 1996).

BACs (Shizuya et al. 1992) and P1-derived artificial chromosomes (PACs) (Ioannou et al. 1994) are relatively new types of cloning vectors available to plant molecular biologists. These BAC vectors permit the cloning of DNA of at least 350 kb in *E.coli*. They are derived from the *E.coli* F factor plasmid, which contains genes for strict copy number control and unidirectional DNA replication (Zhang et al. 1996). Both features promote plasmid maintenance and stability.

The PAC vector shares many features with the BAC system. However, the vector contains the *sacB* gene, which allows a positive selection for recombinant clones. *SacB* encodes the sucrose synthase. When cells are grown in the presence of saccharose, sucrose synthase

will degrade saccharose into levan, which is highly toxic to *E.coli*. The *Bam*HI cloning site is located within the s*acB* gene, and thus disruption of the s*acB* gene by inserting a large DNA fragment allows the growth of transformed cells on media containing saccharose (Ioannou et al. 1994). The size of the PAC vector is approximately 16 kb, whereas the size of the BAC vector ranges between 7 and 8 kb. Because of the big size, PACs have a lower efficiency in shotgun cloning than BACs. Generating large-insert libraries with PACs has largely been superseded by BACs (Zhang et al. 1996).

The another cloning system is YACs (Burke et al. 1987). YAC vectors have been used extensively since 1987, especially for physical mapping, contig construction, and map-based cloning (Giraudat et al. 1992, Martin et al. 1993). The foreign DNA fragments are maintained in yeast as linear chromosomes by the YAC vector. The cloning capacity in YACs is unlimited and primarily depends on the quality and size of the DNA to be cloned. When evaluating the BAC cloning system, a comparison must be made with the YAC system. Table 1.3 shows a comparison between the two most common large DNA fragment cloning systems, YACs and BACs.

Differences between YACs and BACs can be attributed mainly to the host systems. The host cells of YACs are yeast and the host cells of BACs are *E.coli*. Bacteria are easier for isolation of DNA and transformation and divide faster than yeast. Thus, the construction and analysis of BAC libraries and individual BAC clones is easier and faster than YACs. There is also significant difference in transformation efficiencies between yeast and bacteria (10^7 versus 10^{10}) (Zhang et al. 1996).

The upper limit of insert-size is 350 kb for BAC cloning (Shizuya et al. 1992). This size is considerably lower than in the YAC system, where human and mouse libraries have been constructed with average insert sizes of greater than 570 kb (Chumakov et al. 1992, Larin et al. 1991). The genome of *E.coli* is about 4 Mb. Therefore, it is unlikely that *E.coli* cannot replicate a 1 Mb BAC. One possible reason why no larger BAC clones have been detected is that there may be a limit to the size of a molecule that can be delivered by electroporation (Zhang et al. 1996).

Tab. 1.3 Differences between Yeast Artificial Chromosomes (YACs) and Bacterial Artificial Chromosomes (BACs) (http://www.genome.clemson.edu).

Features	**YAC**	**BAC**
Configuration	Linear	Circular
Host	Yeast	Bacteria
Copy Number/Cell	1	1-2
Cloning Capacity	Unlimited	Up to 350 kb
Transformation	Spheroplast($\leq 10^7$ Transformants/µg)	Electroporation ($\leq 10^{10}$ Transformants/µg)
Chimerism	Up to 40%	None to low
DNA Isolation	PFGE Gel isolation	Standard Plasmid Minipreparation
Insert Stability	Unstable	Stable

YAC systems include a high degree of chimerism and insert rearrangement which limit the usefulness (Neil et al. 1990, Green et al. 1991, Pierce et al. 1992). The BAC vectors are circular and BAC clones are more stable than linear YAC DNA (Woo and Choi 1997).

Due to BAC clone stability and the easier handling, the BAC cloning system has emerged as the system of choice for the construction of large insert genomic DNA libraries. Additional because of the employing of BAC libraries in a variety of applications, BAC was preferred for the construction of large-insert genomic DNA library in the sunflower.

2 MATERIAL and METHODS

2.1 Plant Material

As plant material the sunflower inbred line RHA325 was used. RHA325 which is an open American inbred line with PET1-plasma, has got two important genes for sunflower that are the *Pl*2-gene conferring resistance to downy mildew and the restorer gene *Rf*1, which is responsible for fertility restoration in the presence of the PET1 cytoplasm. For the construction of BAC library in sunflower, the use of RHA325 line as plant material was decided, because it is one parent for the mapping population in the Institute of Crop Science and Plant Breeding, in Giessen, Germany. With the usage of the restorer line RHA325, it is possible to isolate these two genes from the sunflower BAC library.

2.2 Bacteria and Vector

The *E.coli* strain DH10B was used for the construction of the BAC library (Hanahan et al. 1991) (F-*mrc*A **delta** (*mrr-hsd*RMS-*mcr*BC) **phi**80d*lacZ* **delta**M15 **delta***lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 **delta** (*ara,leu*) 7697 *gal*U *gal*K **lambda**-*rps*L *nup*G), which includes mutations that: (1) block restriction of foreign DNA by endogenous restriction endonucleases (*hsd*RMS); (2) block restriction of DNA containing methylated DNA (5` methyl cytosine or methyl adenine residues, and 5´ hydroxymethyl cytosine)(*mcr*A, *mcr*B, *mcr*C and *mrr*); (3) block recombination (*rec*A1); and (4) take up large DNA (*deo*R) (CUGI 2000). Electroporation competent DH10B cells were prepared in the laboratory or purchased (ElectroMAXDH10B cells (BRL,USA)).

As vectors pBeloBACKan which is 8.5 kb and pBeloBAC11 which is 7.4 kb in size were tested. The vector pBeloBAC11 consists of the elements from the F factor of *E.coli* (*oriS, repE, parA, parB, and parC*) as well as a gene for chloramphenicol resistance (Cm^R), the bacteriophage λ *cosN* site, the bacteriophage P1 *loxP* site, and the multiple cloning site from pGEM3Z, which lies within the *lacZ* gene. Unique restriction sites are shown, including the *Hind*III and *Bam*HI sites used for cloning (Fig 1.1). The orientations of the bacteriophage T7 and bacteriophage SP6 promoters which flank the cloning sites are shown. *Not*I sites which flank the cloning site allow the excision of the insert from vector.

The vector pBeloBACKan was kindly provided from Dr. T. Altmann and Dr. T. Mozo, Max-Planck-Institute for Moleculer Plantphysiology, Golm, Postdam, Germany. The

pBeloBAC11 was modified to contain a unique *Eco*RI cloning site by Mozo et al. (1998a). A Tn*903*-derived kanamycin resistance gene (Oka et al. 1981) was inserted by blunt-end cloning at the *Eco*RI site present in the chloramphenicol resistance gene in pBeloBAC11, giving pBeloBACKan (Mozo 1998a).

Finally the vector pBeloBAC11 was used for the construction of the sunflower BAC library.

2.3 Construction of a BAC Library

BAC libraries are constructed basically by the following steps: First, the high molecular weight DNA (HMW DNA) is prepared. Then the vector is digested with *Hind*III or an another restriction endonuclease and then dephosphorylated to prevent self-ligation. Next, HMW DNA is partially digested with *Hind*III and DNA fragments > 150 kb are size-selected by Pulsed Field Gel Electrophoresis (PFGE). Finally, the vector and digested genomic DNA fragments are ligated and then electroporated into *E.coli*. Recombinant transformants are selected on media containing chloramphenicol, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (IPTG) and X-gal. Recombinant transformants are detected by blue/white selection. In addition, the size of the BAC inserts is assayed by DNA minipreparations followed by the digestion with *Not*I to cut out the DNA insert from the vector followed by PFGE.

2.3.1 Isolation of High Molecular Weight DNA

To construct large insert DNA libraries, methods must be developed to isolate very high molecular weight DNA – megabase-size DNA- from plants.

With care, molecules as large as 500 kb can be prepared in solution using conventional methods. However, the common steps in handling DNA, that is, pipetting and phenol extraction, introduce shear forces that reduce the length of the DNA. The most common methods for plant megabase-size DNA isolation use the preparation of protoplasts or nuclei. To isolate HMW DNA, protoplasts or nuclei must first be embedded in agarose plugs or microbeads. The agarose acts as a solid, yet porous matrix which allows the diffusion of various reagents for DNA purification and subsequent manipulations while preventing the DNA from being sheared (Schwartz and Cantor 1984).

Methods for preparation of HMW-DNA by protoplasts and nuclei were developed for sunflower (Krämer 1998, Özdemir 1998).

2.3.1.1 Isolation from Protoplast

Most protocols for the isolation of megabase-size DNA from plants utilise the protoplast method (Ganal et al. 1989, Honeycutt et al. 1992, Sobral et al. 1990, van Daelen et al. 1989, Wing et al. 1993, Woo et al. 1995). Although the protoplast method yields megabase-size DNA of high quality, the process is costly and labour intensive.

The isolation of protoplast was performed according to Ganal (1996). The quality of leaf material is essential for the protoplast isolation. Sunflower seedlings were grown for two weeks in the greenhouse. Before protoplast isolation plants were stored in the dark room for two days. This is essential to decrease the amount of starch in the protoplast preparation.

First, 10 grams young plant leaves were cut in 1 mm strips parallel to the veins with razor blades in large petri dishes (Fig. 2.1). Then, this leave material was transferred into four large petri dish each containing 25 ml protoplast isolation buffer, to which the enzymes 1% cellulase RS (Cellulase Onozuka RS Tokyo, Japan) and 0.05 % pectolyase Y-23 (Yakult Honcha Co.ltd.) were freshly added (Ganal et. al. 1996). This solution was incubated for 5.5 h, at 28 °C and 50 rpm. During the incubation cell walls will be hydrolysed by the enzymes.

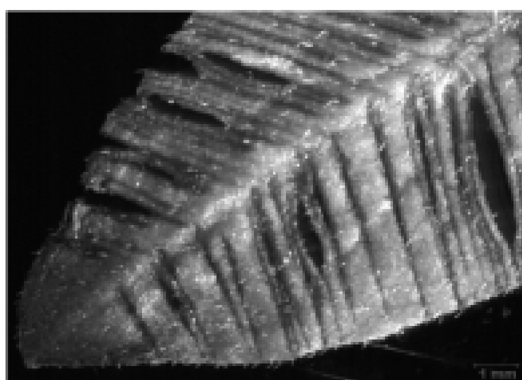

Fig.2.1 Cut young leafs prepared for protoplast isolation. Young leaves were harvested and cut in 1 to 2-mm strips parallel to the veins with razor blades starting near the midvein while leaving the morphology of the leaves intact.

Afterwards that the protoplast solution was filtered through 70 µm sieves. The petri dishes were thoroughly rinsed with protoplast isolation buffer until all protoplast were collected from the bottom of the petri dishes. The protoplasts were transferred into 50 ml falcon tubes and spun down at 200 g (Beckman GS-6) for 10 min. After centrifugation the supernatant was removed carefully. Pellets which did not tightly remain at the bottom of the tubes were collected in two falcon tubes and resuspended in 50 ml of protoplast isolation buffer. The centrifugation step was repeated under the same conditions. The pellets were finally collected in one falcon tube and resuspended in 1 ml protoplast isolation buffer. An aliquot of the resuspended protoplasts was counted to calculate the total number of isolated protoplast. The yield should be approximately 5 to 10^{10} protoplast/ml.

A volume of prewarmed 1% insert agarose (FMC insert agarose), was prepared with protoplast isolation buffer and added in equal volume to the protoplast solution. These solutions were carefully mixed with a cut tip and then transferred to a prechilled plug mould with cut tips. Plug moulds were kept at 4 °C for 10 min to solidify the agarose. The agarose plugs were transferred into lysis buffer, to which proteinase K (Boehringer Mannheim GmbH) was added freshly and incubated at 55 °C with 30 rpm for 24 h. After 24 h, fresh lysis buffer was added and plugs were incubated for another 24 h. This lysis step should be repeated until the blocks obtain a transparent structure. After treatment with proteinase K agarose blocks should no longer be green.

Protoplast Isolation Buffer

0.5 M Manitol	91.10 g	per 1,000 ml
20 mM MES (Morpholinoethane Sulfonic acid)	3.904 g	

Enzymes added

1 % Cellulase	10 g	per 1,000 ml
0.05 % Pectolyase Y-23	0.5 g	

Lysis Buffer

0.5 M EDTA (pH8.0)	90 ml	per 100 ml
10 % Sodium N-Lauroylsarcosinate	10 ml	

Add Proteinase K (1mg/ml) freshly

2.3.1.2 Isolation from Nuclei

An alternative technique is applied for the preparation of high molecular weight DNA from plant nuclei. Some scientists have with varying degrees of success tried to prepare megabase-size DNA from nuclei (Hatano et al. 1992, Kleine et al. 1995). Zhang et al. (1995) have recently developed a nuclei method which works well for several divergent plant taxa. Fresh tissue is homogenized with mortar and pestle. Nuclei are then isolated and embedded as described above for isolation from protoplasts. The quality of the DNA is as good as DNA prepared from protoplasts, but is more concentrated, and was demonstrated to contain lower amounts of chloroplast DNA (Zhang et al. 1995).

The isolation method of megabase-size DNA from plant nuclei was modified from Zhang et al. (1995). Ten grams of the young leaves from two weeks old sunflower seedlings were harvested and immediately transferred into liquid nitrogen. The frozen tissue was grinned into powder in liquid nitrogen with 1 mortar and pestle.

The powdered tissue was quickly transferred into 200 ml of ice-cold chromatin isolation buffer, to which Triton X-100 and ß-mercaptoethanol were added freshly. The solution was swirled with a magnetic stir bar for 20 minutes on ice. After homogenisation of the frozen tissue the mixture was filtered through two layers of cheesecloth into four ice-cold centrifuge tubes. To obtain more nuclei, the cheesecloth was removed and the remaining homogenate was squeezed into the centrifuge tube with gloved hands.

The filtered homogenate was sedimented by centrifugation at 1,200 g for 10 minutes at 4 °C. The supernatant was discarded and the pellet collected in two tubes. The pellets were resuspended in 1-5 ml of ice-cold chromatin isolation buffer with assistance of a small paint brush. After resuspension the volume was adjusted to 25 ml with ice-cold chromatin isolation buffer. The mixture was spun at the same condition used for the first centrifugation. The supernatant was poured off and the pellet was resuspended in 250-500 µl H_2Odd. A volume of 1.5 % Insert Agarose (FMC insert agarose), which was prepared with H_2Odd, was added in equal volume to the nuclei and mixed carefully. Then the mixture was transferred to a prechilled plug mould. The agarose plugs were placed into lysis buffer and incubated at 55 °C with 30 rpm for 24 h. After 24 h, fresh lysis buffer was added and the mixture incubated for another 24 h.

2XChromatin Isolation Buffer		
Tris/HCl pH 7.8	4.84 g	per 1,000 ml
Saccharose	171.2 g	
$MgCl_2x6H_2O$	2.03 g	
KCl	0.745 g	

Chromatin Isolation Buffer (ready for use)		
2x Chromatin Isolation Buffer	250 ml	per 500 ml
Triton X-100	1.25 ml	
ß-Mercaptoethanol	0.5 ml	
H_2O	248.25 ml	

2.3.1.3 PMSF Treatment

The agarose plugs were washed three times in 1xTE containing 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), which inactivates the residual proteinase K, and incubated for 30 minutes at 4 °C. PMSF is very toxic and should be prepared using gloves and a fume hood. After the PMFS treatment the plugs were washed three times in 1xTE at 4°C for 1 h. Finally, the washed plugs can be stored at 4 °C for several months - 1 year without significant degradation.

1xTE		
10 mM Tris pH8.0	1.211 g	per 1,000 ml
1 mM EDTA pH 8.0	0.292 g	

PMSF Stock Solution		
0.1 M PMSF dissolved in isopropanol	1.742 g	per 100 ml

For 0.1 mM PMSF, the stock solution is diluted with 1xTE

2.3.2 Preparation of Vectors

Two different vectors were purified with different methods. The vector pBeloBACKan which was kindly provided from Max-Planck-Institute, Potsdam, was isolated with the QIAGEN plasmid Maxi Kit (QIAGEN Cat#12162, USA). Supercoiled plasmid DNA was further purified by digestion of linear and open circular DNA with DNase Plasmid Safe.

After complete digestion with *Eco*RI vector DNA was dephosphorylated with HK phosphatase (Epicentre).

The pBeloBAC11 was developed by Kim et al. (1996). The pBeloBAC11 was kindly provided by Clemson University. The pBeloBAC11 allows a *lacZ*-based positive colour selection of the BAC clones that have insert DNA in the cloning sites. This vector is the single copy in *E.coli*. For this reason, purification of this vector DNA in large quantity takes some effort. First, bacteria were recovered by centrifugation and lysed by maxipreparation. Then vector DNA was purified by centrifugation on a CsCl density gradient. Next, the purified vector DNA was digested with the restriction enzyme and then dephosphorylated to prevent self ligation, which can increase the efficiency of ligating vector DNA to insert DNA.

2.3.2.1 Preparation of the Vector pBeloBACKan

As the BAC vector pBeloBACKan is single copy plasmid in *E.coli*. A large volume of LB medium should be used. *E.coli* strain DH10B containing the vector pBeloBACKan was streaked on a LB plate containing kanamycin (50 µg/ml) (DUCHEFA), X-gal (40 µg/ml) (Roth), and IPTG (0.2 mM) (Roth) and grown at 37°C overnight. Then, 5 ml of LB medium containing kanamycin (12,5 µg/ml) were inoculated with a single blue colony from the plate and grown for 6-7 hours at 37 °C. Two or 3 litres of LB media were inoculated with 2 ml/litre of culture from the 5 ml first culture. The cells were grown overnight at 37 °C, 200 rpm.

The cells were harvested by centrifugation for 20 minutes, at 4 °C, 5000 rpm (Beckman, JA-10). The plasmid DNA was isolated from the cells by QIAGEN plasmid Maxi Kit according to the manufactories introduction. After centrifugation, the supernatant was discarded and the bacterial pellet was completely resuspended in each bottle with 10 ml of P1 buffer by vortexing. Before using P1 buffer, RNase A (100 µg/ml) had to be added to it. Then, 10 ml of P2 were added. The mixture was mixed gently by inverting the tubes several times and incubated at RT for 5 minutes. Ten ml of prechilled buffer P3 were added and mixed gently by inverting the tubes several times. The mixtures were incubated on ice for 20 minutes. The bottles were centrifuged at 10,000 rpm, 4 °C, for 30 minutes. The supernatant were transferred to new corex tubes. The corex tubes were centrifuged again at 13,000 rpm (Beckman, JA-20), 4 °C, for 15 minutes. By the way, the filters were equilibrated with 10 ml of QBT buffer. After centrifugation, the supernatant was poured

into filters. It was allowed to enter the filters by gravity flow. The filters were washed twice, each time with 30 ml of QC buffer. The plasmid DNA was eluted from the filters with 15 ml of prewarmed (65 °C) QF buffer. The DNA was precipitated with 0.7 volume of ice-cold isopropanol. For precipitation the DNA was kept at –20 °C overnight. The next day, the DNA was centrifuged at 13,000 rpm, 4°C, for 30 minutes. After discarding the supernatant, the pellet was washed carefully with ice-cold 70 % ethanol for 15 minutes and air dried. A pellet corresponding to one l original culture was resuspended in 170 µl of TE buffer.

2.3.2.1.1 DNase Plasmid Safe Treatment

After maxipreparation, the supercoiled plasmid DNA was purified away from the remaining part of *E.coli* chromosomal DNA. The reaction mix was prepared as follows:

Plasmid DNA (pBeloBACKan)	170 µl
10xPlasmid safe buffer	21 µl
ATP (25 mM)	8.4 µl
DNase	10 µl

The reaction mix was incubated at 37 °C overnight. The next day the plasmid safe ATP dependent DNase (Epicentre Technologies) was inactivated by heating at 70 °C for 30 minutes. For precipitation of the DNA, 0.7 volume of isopropanol was added and the mixture was kept at –20 °C for 20 minutes. The mixture was centrifuged at 8,000 rpm, 4 °C, for 30 minutes. After the discarding the supernatant the pellet of plasmid DNA was dried at 37 °C. The pellet was resuspended in 100 µl of H_2Odd at 37 °C and 2 µl of DNA were saved for the control gel of undigested DNA.

2.3.2.1.2 The digestion of the vector pBeloBACKan DNA

The digestion was set up as follows:

pBeloBACKan DNA	100 µl
10X Reaction buffer	15 µl
Spermidine 40 mM	15 µl
*Eco*RI (10 U/µl)	10 µl
H_2Odd	10 µl

This reaction mix was incubated at 37 °C for 5 hours for the digestion by *Eco*RI (Boehringer Manheim GmbH). At the end of the reaction, 2 µl of reaction mix were saved

for digestion test gel. The DNA was precipitated by adding 0.7 volume of isopropanol and by keeping the solution at –20 °C for 20 minutes. The mixture was centrifuged at 8,000 rpm, 4 °C, for 30 minutes. The supernatant was discarded and the pellet of plasmid DNA was dried at 37 °C. The pellet was resuspended in 100 µl of H_2Odd at 37 °C and 2 µl of DNA were saved for a control of self-ligation test.

2.3.2.1.3 Treatment with Phosphatase

After digestion, the dephosphorylation reaction was set up as follows:

digested pBeloBACKan DNA	100 µl
10xTA buffer	15 µl
$CaCl_2$ (50 mM)	15 µl
HK phosphatase (1 U/µl)	10 µl
H_2Odd	10 µl

The reaction mix was incubated at 30 °C for 1.5 h. The HK phosphatase (Epicentre Technologies) was inactivated by heating at 67 °C for 30 minutes. The DNA was precipitated as above. The pellet was resuspended in 70 µl of TE buffer.

2.3.2.2 Maxipreparation of the Vector pBeloBAC11

Another vector pBeloBAC11 was purified by maxipreparation and CsCl density gradient. As the BAC vector pBeloBAC11 is single copy plasmid, it is rather difficult to obtain large amount of vector DNA. Extra care is also necessary to minimise the contamination with *E.coli* chromosomal DNA which represents more than 99 % of the total DNA. However, it is possible to obtain a few micrograms of pBeloBAC11 (7.4 kb) from one litre culture of bacteria, which is usually enough for the construction of BAC library.

E.coli strain DH10B containing the BAC vector (pBeloBAC11) was streaked onto a LB agar plate containing chloramphenicol (12.5 µg/ml) (Boehringer Manheim), X-gal (50 µg/ml) (Roth), and IPTG (25 µg/ml) (Roth) and was incubated at 37 °C overnight. Five ml of Terrific Broth (TB) medium containing 30 µg/ml chloramphenicol were inoculated with a single blue colony from the plate and grown for 8 hours at 37 °C.

Two 3 litre culture flasks containing 750 ml of TB medium with LB medium, Terrific Broth medium chloramphenicol (30 µg/ml), prewarmed to 30 °C, were inoculated with 1

ml/litre of preculture. Cells should be grown to a cell density of approximately $1x10^9$ cells/ml (A600= 1.0-1.2) at 30 °C with shaking (200 rpm) overnight.

The content of each flasks was transferred into 500 ml plastic centrifuge bottles. The cells were harvested by centrifugation at 4 °C for 15 min at 4,000 rpm (in Beckman JA-10, Beckman, USA). After centrifugation nine ml of solution I (without lysozyme) were added to each cell pellet. The pellets were thoroughly resuspended by pipetting the suspension up and down. Then, 1 ml solution I with lysozyme (final concentration of 4 mg/ml) (Boehringer Manheim) was added. The mixture was mixed by swirling and incubated 5 min at room temperature. Twenty ml of freshly prepared solution II were added and mixed well by shaking. The bottles were placed on ice for 10 minutes.

Fifteen ml of solution III were added to the mixture above and thoroughly mixed by shaking. Bottles were kept on ice for 10 minutes. Afterwards a centrifugation step followed at 7,500 rpm (in Beckman JA-10), 4°C, for 25 minutes. Then the supernatant was divided by pipetting into two 50 ml Beckman polypropylene centrifuge tubes. (If the supernatant was not clear, centrifugation can be repeated). For DNA precipitation 0.6 volume of room-temperature isopropanol was added and mixed. Tubes were kept at RT or at –20°C for 15 min. DNA was harvested by centrifugation at RT for 20 min at 6,000 rpm (in Beckman JA-20, Beckman, USA). After discarding the supernatant the pellets were gently washed with 70 % ethanol. Pellets were dried at 37- 42 °C. The final pellets from 2 l TB culture medium were dissolved in 3 ml TE.

LB Medium (pH 7.0)

NaCl	10 g	per 1,000 ml
Bactotrypton	10 g	
Yeast Extract	5 g	
(Bacto Agar (for pouring plates)	16 g)	

Selective LB-Medium

X-Gal 40 µg/ml, Chloramphenicol 50 µg/ml, IPTG 0.2 mM

TB Medium plus Phosphat Buffer

Yeast Extract	24 g	per 1,000 ml
Peptone	12 g	
Glycerol (96 %)	4 ml	

Phosphat Buffer

KH_2PO_4 (0.17 M)	23.1 g	per 1,000 ml
K_2HPO_4 (0.72 M)	125.4 g	

Solution I (Lysozyme Solution)
50 mM Glucose, 10 mM EDTA, 25 mM TrisHCl pH 8.0
Before use 5mg/ml lysozyme is added.

Solution II (NaOH-SDS Solution)
0.2 N NaOH, 1% SDS

Solution III (Potassium acetate)

5M Kac	60 ml	
Acetic acid	11.5 ml	per 100 ml
H_2Odd	28.5 ml	

2.3.2.2.1 Purification of pBeloBAC11 by CsCl Density Gradient

The plasmid DNA was purified from *E.coli* chromosomal DNA by equilibrium centrifugation in CsCl-ethidium bromide gradients.

After maxipreparation, 4.5 g of solid CsCl (caesium chloride, Life Technologies, GibcoBRL) was added to 3 ml vector DNA in a 50 ml falcon tube and mixed at 37 °C until dissolved. Then, 500 µl of 10 mg/ml of ethidium bromide were added. Ethidium bromide is a powerful mutagen and is toxic. Extra special gloves should be worn when working with solutions that contain ethidium bromide. The solution was mixed well by vortexing and centrifuged at 6000 rpm, at RT, for 10 minutes. The supernatant was loaded into a Beckman ultracentrifuge tube by using a pasteur pipette. Above the supernatant the tubes were filled with a 1:1 CsCl/ H_2Odd solution until the first rim. Tubes were balanced with this solution. Then, tubes were filled up with H_2Odd. They were balanced and sealed with a metal hood by heating.

The tubes were placed carefully in a Beckman VTI90 rotor and were covered with special screws for this rotor. The screws were tightened with the equal craft. The mixtures were centrifuged to density equilibrium at 55,000 rpm for 18-20 hours at 20 °C. The next day the supercoiled plasmid and *E.coli* chromosomal DNA bands were viewed with long-wavelength UV light.

Two well-separated bands should be clearly visible (Fig.2.2). A 21-gauge needle was inserted into the top of the tube to allow air to enter. The lower band, which contains the supercoiled vector DNA, was removed by puncturing the side of the tube with a 21-guage syringe needle below the band and carefully the plasmid DNA was drawn into a 1 ml syringe. The contamination with the upper band, which contains chromosomal DNA and open circular vector DNA was avoided. The plasmid DNA was transferred into a 15 ml falcon tube.

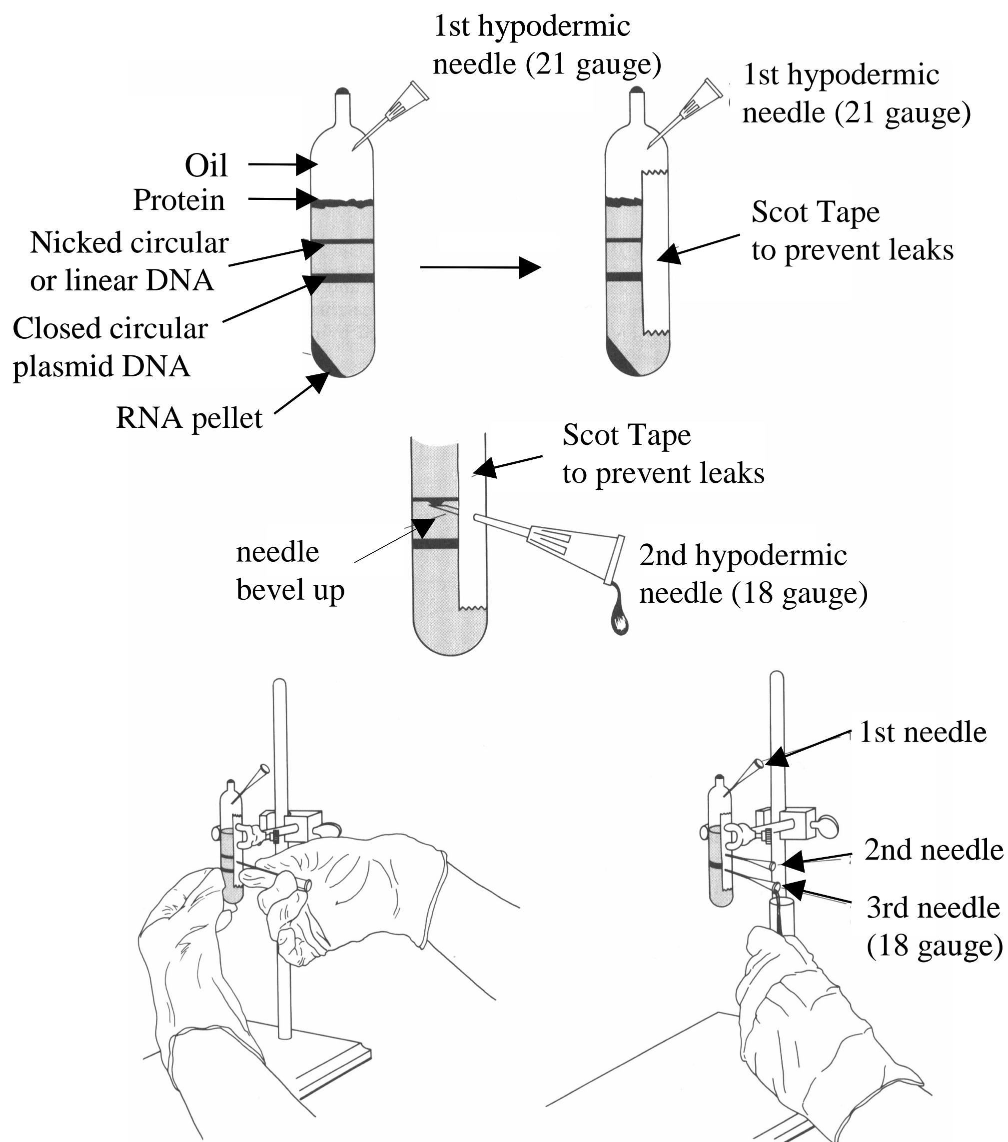

Fig.2.2 The outline of the CsCl gradient steps (Sambrook 1989)

The next step was to remove ethidium bromide from the plasmid DNA by extraction with 2 ml of CsCl/H_2Odd saturated isopropanol. The extraction should be continued with fresh

isopropanol until all visible colour is removed (5-6 times). After all, the pink colour disappears from both of the phases. Then, the extraction should be repeated again twice. The extracted DNA was diluted with 2 volume of H_2Odd and precipitated with 0.6 volume of room temperature isopropanol by centrifugation at 14,000 rpm, 4 °C, for 30 min. The pellet was gently washed with 70 % ethanol, recentrifuged and dried. The DNA pellet was resuspended in 100 µl of TE buffer.

2.3.2.2.2 Digestion of the Vector pBeloBAC11

After purification the vector DNA must be digested with the restriction enzyme to create a cloning site. For this, 40 µl of purified pBeloBAC11 vector DNA were incubated with 3 µl *Hind* III (10 U/µl) (Promega) and 4.8 µl buffer E at 37 °C over night. An appropriate size markers (*Hind*III-digested fragments of bacteriophage λ (MBI, Fermentas Molecular Biology) and 1kb marker were analysed together with 2 µl of the digestion mixture on a 1% agarose (NEEO-Agarose, Roth) in 0.5xTBE buffer.

With complete digestion, a single band of vector DNA should be visible at 7.4 kb. There should be no traces of the uncut supercoiled vector DNA or *E.coli* chromosomal DNA. If the vector is not completely digested , this step needs to be repeated.

10XTBE		
Tris	108 g	per 1,000 ml
Boric acid	55 g	
0.5 M EDTA pH 8.0	40 ml	

2.3.2.2.3 Treatment with Phosphatase

The DNA fragments and the plasmid can circularise during the ligation. Removal of the 5`-phosphate groups with alkaline phosphatase is frequently used to avoid self-ligation and circularisation of the plasmid DNA. During ligation *in vitro*, bactariophage T4 DNA ligase will catalyse the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5`-phosphatase group and the other contains a 3`-hydroxyl group. Recircularisation of plasmid DNA can therefore be minimised by removing the 5´-phosphates from both termini of the linear DNA with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) (Seeburg et al. 1977). However, an insert DNA segment with 5´-terminal phosphates can be ligated efficiently to the

dephosphorylated plasmid DNA to give an open circular molecule. As circular DNA is much more efficient in transformation than linear plasmid DNA, most of the transformants will contain recombinant plasmids (Sambrook et al. 1989)

After digestion of closed circular plasmid DNA, treatment of HK (Heat Killed) phosphatase (Alkaline Phostpatase, Calf Intestinal, Promega) has to be performed to protect recircularization of the linearised plasmid DNA. Therefore, 2.5 µl CaCl (0.1 M), 1 µl buffer and HK phosphatase (1 U/µl) were added to the digestion reaction mix. The mixture is incubated at 30 °C for 1.5 hours.

At the end of the dephosphorylation reaction, HK phosphatase, which must be completely removed if the subsequent ligations should work efficiently, is inactivated by heating the mixture at 65 °C for min.

Then the dephosphorylated DNA was purified by extraction with phenol:chloroform. To 50 µl TE, 45 µl phenol and 45 µl chloroform were added to precipitate the vector DNA and mixed well. The mixture was centrifuged at 13,000 rpm, RT, for 5 minutes. The upper phase was transferred into a new tube. Then, 3 µl NaCl (final concentration 5 mM) and 200 µl 96% ethanol were added to the mixture and placed for 30 minutes, at -20 °C. The solution was centrifuged at 13,000 rpm, 4 °C, for 30 minutes. The pellet was washed with 70% ethanol and again centrifuged. The dried pellet was redissolved in 70 µl TE. The vector was divided into 10 µl aliquots and stored frozen at –20 °C until needed for ligation.

2.3.2.3 Control of Vector

For the control of digestion, digested and undigested vector DNA were loaded on 1% agarose gel (NEEO-Agarose, Roth) in 0.5xTBE buffer. The normal electrophoresis was performed at 100 Volt for 1.5 h. After that, the gel was stained with ethidiumbromide (EtBr) (2 µg/ml H_2Odd) for 15 minutes. The gel was photographed using UV light (254 nm) with a polaroid camera.
However, the extent of dephosphorylation was assayed by performing a self ligation test as follows: The first self ligation reaction mix which included 1 µl of digested vector DNA, 1 µl of 10X T4 ligase buffer, 0.5 µl T4 ligase (1 U/µl) (Boehringer Manheim) and 7.5 µl of H_2Odd was incubated overnight at 15 °C. The second self ligation reaction mix that included 1 µl of digested/dephosphorylated vector DNA, 1 µl of 10X T4 ligase buffer, 0.5 µl of T4 ligase (1 U/µl) and 7.5 µl of H_2Odd was also incubated at 15 °C overnight. The

quality of dephosphorylation was ensured with normal electrophoresis on an agarose gel in 0.5xTBE buffer. If self ligation of the digested vector DNA occurred, it should be seen on the agarose gel as supercoiled form. In the second reaction mixture no self ligation of the digested/dephosphorylated vector DNA should be observed. A linear band should be seen.

2.3.3 Preparation of Insert DNA

The generation of BAC libraries requires high-quality genomic DNA. High-quality DNA can be used to produce BACs containing large inserts. HMW DNA should be partial digested to give clonable fragments of the desired size. After size selection of the DNA to remove small fragments size-selected DNA can be recovered from agarose gels by electroelution (Strong et al. 1997). So insert DNA can be ligated with vector DNA.

2.3.3.1 Partial Digestion with *Hind*III Restriction Enzyme

For BAC cloning, partial digestion should produce genomic DNA fragments ranging from 50 kb to larger than 600 kb (Riethman et al. 1997). Before a large-scale (preparative) digestion can be performed on a new batch of DNA, a pilot experiment must be carried out to establish the digestion conditions that will produce the desired size range of fragments. Therefore the amount of enzyme and time of digestion should be determined empirically.

Control Digestion in 200 µl

DNA	½ Plug(~ 50 µl)
10X buffer E	20 µl
Spermidine (40 mM)	20 µl
Albumin BSA (1 mg/ml)	20 µl
Dithiothreitol (DTT) (10 mM)	20 µl
*Hind*III (10 U/µl)	0.5 U, 1 U, 5 U, 20 U

*Hind*III (Promega) was freshly diluted in distilled water (e.g. 0.5 U, 1 U, 5 U). Reaction mixture was incubated on ice 1 h to allow the diffusion of the restriction enzymes into the agarose plugs. After 1 h, the reaction mixture are transferred to 37 °C for partial digestion and incubated for 15-20 minutes. The reaction was stopped by adding 1/10 volume of 0.5 M EDTA, pH 8.0 and the tubes were placed on ice.

The partially digested DNA plugs were loaded on a 1 % agarose gel (Pulsed Field Certified Agarose, BIO RAD) in 0.5xTBE and the wells were sealed with the same molten agarose as the gel.

Pulsed-field gel electrophoresis was performed under the conditions of 6.0 V/cm, 50-90s pulse, 0.5xTBE buffer, 14 °C, 19 h, in a PFGE Chamber (BIO-RAD, USA). After checking the EtBr-stained gel, the enzyme concentration was chosen giving a majority of DNA fragments ranging from about 300 to 600 kb.

A surprisingly common reason for failure in BAC cloning experiments is that the preparative electrophoresis is performed in an electrophoresis chamber that contains nucleases in the electrophoresis buffer. These nucleases are the result of microbial growth in the chamber. Before any preparative PFGE is attempted, the inside of the chamber should be cleaned with a soft brush and rinse extensively with sterile H_2O. Preparative electrophoresis should also be performed with freshly prepared autoclaved electrophoresis buffer.

2.3.3.2 Partial Digestion with EcoRI Restriction Enzyme

For partial digestion using *Eco*RI as restriction enzyme, there are different possibilities to limit the digestion: (a) competition between *Eco*RI and methylase, (b) limitation by Mg^{2+} concentration, (c) different enzyme concentrations or (d) different incubation times.
Variation of the concentration of the enzyme cofactor Mg^{2+} was performed according to the protocol of Albertsen et al. (1989). One half of a DNA agarose block (approximately 50 µl), 20 µl buffer H without $MgCl_2$, 100 U restriction enzyme *Eco*RI (10 U/µl) (Boehringer Manheim) and H_2Odd to complete the reaction volume to 200 µl, were placed into tubes. Then, the DNA agarose blocks were equilibrated for 2 h on ice. After 2 hours 20 µl of different $MgCl_2$ stock solution (0.1 M, 0.3 M and 0.5 M) were added. The reaction mix was incubated at 37 °C for 1.5 h. The reaction was stopped by adding 20 µl of bluejuice. The DNA blocks were loaded on a 1% pulsed-field agarose gel. PFGE was performed under conditions of 6.0 V/cm, 50-90s pulse, 0.5xTBE buffer, 14°C, for 19 h.

For variation of the concentration of the restriction enzyme *Eco*RI the protocol according to Wing (1997) was used. One half of a DNA agarose block (~ 50 µl), 20 µl of 10X buffer H, 20 µl of BSA (1 mg/ml), 20 µl of spermidine (40 mM), 20 µl of DTT (10 mM) and H_2Odd to complete the reaction volume to 200 µl, were placed into tubes. The reaction mix

was incubated at RT for 1 h. 10 U, 20 U, 40U, 60 U, 80 U and 100 U of *Eco*RI (10 U/µl) were added and the enzyme was allowed to diffuse into the plugs for 1 h on ice. Then, the reaction mixtures were incubated at 37 °C for 1.5 h. The reaction was stopped by adding 20 µl of bluejuice. The DNA blocks were run on an 1% pulsed-field gel under conditions of 6.0 V/cm, 50-90s pulse, 0.5xTBE buffer, 14 °C, for 19 h.

For variation of incubation time with the restriction enzyme *Eco*RI the same protocol as for variation of the concentration of enzyme was used. One half of a DNA agarose block (~ 50 µl), 20 µl of 10x buffer H, 20 µl of BSA (1 mg/ml), 20 µl of spermidine (40 mM), 20 µl of DTT (10 mM) and H_2Odd to complete the reaction volume to 200 µl, were placed into tubes. The reaction mix was incubated at RT for 1 h. However, only 60 U or 80 U of *Eco*RI were added. Then, the enzyme was allowed to diffuse into the plugs for 1 h on ice. The reaction mixtures were incubated at 37 °C for 45 minutes, 1 h, 1.5 h, 3 h, 6 h and overnight. The reaction was stopped with 20 µl of bluejuice. The DNA blocks were run on a 1% pulsed-field gel under conditions of 6.0 V/cm, 50-90s pulse, 0.5xTBE buffer, 14 °C, for 19 h.

Competition experiments between *Eco*RI (Boehringer Manheim) and methylase, DNA was digested with 100 U *Eco*RI and different concentration of methylase (New England, BioLab) according to a modified protocol from Mozo et al. (1998a). First, each ½ DNA agarose block was equilibrated for 2 h at 4°C with 500 µl equilibration buffer which included 50 µl of a modified buffer H (50 mM NaCl, 500 mM Tris pH 8.0, 5 mM $MgCl_2$), 50 µl of BSA (1 mg/ml), 50 µl of spermidine (40 mM), 50 µl of DTT (10 mM) and H_2Odd to complete 500 µl for each DNA block. This equilibration buffer was removed from the DNA blocks. Then, the DNA blocks were incubated for 30 minutes on ice with 150 µl of fresh equilibration buffer with 80 mM SAM (S-Adenosylmethionine, 32 mM, New England, BioLab). 240 U, 400 U, 560 U, 720 U, and 800 U of methylase were added to the reaction mix. The reaction tubes were incubated for 30 minutes on ice. 100 U of *Eco*RI were added to the reaction tubes and incubated for 1.5h at 37 °C. The reaction was stopped by adding 50 mM EDTA. The DNA blocks were loaded on to a 1% pulsed-field agarose gel. PFGE was performed under conditions of 6.0 V/cm, 50-90s pulse, 0.5xTBE buffer, 14°C, for 19h. For optimization of partial digestion all of these methods were tested.

2.3.3.3 Agarase Digestion

The agarose limits the diffusion of the restriction enzyme in to the DNA blocks and influences the restriction of the DNA by enzymes. However, it is possible to digest agarose fibres with agarase.

Fifteen DNA blocks, which were embedded in low melting agarose (BIO RAD low melt preparative grade agarose), were washed twice in 10x agarase buffer plus 1xPA for 10 minutes at RT. The buffer was discarded. The DNA agarose blocks were melted in a water bath at 68°C for 15 minutes. The molten blocks were transferred to another water bath at 40 °C to equilibrate the temperature of the plugs to 40 °C for 5–10 minutes. The volume of the molten blocks was estimated. One block correspended approximately 100 µl. Five units of agarase were added per 1 ml molten plugs. For 15 blocks, 7.5 U agarase (0.8 U/µl, Boehringer Manheim) and 47.5 µl 5x agarase buffer had to be added. The blocks were incubated at 40 °C overnight. The next day the same volume of agarase and agarase buffer was added again. The mixture was incubated again at 40 °C for 6 h. The solution was transferred to dialysis bags (Roth, MWCO:12-14,000) in TE buffer for 1 h at RT and than at 4°C overnight. The solution taken from the dialysis bag with a cut tip and stored at 4°C.

100x PA		
75 mM Spermidine	1.90 g	per 100 ml
30 mM Spermine	0.60 g	
dissolve in H_2Odd and sterile filter		

2.3.3.4 Size Selection of Insert DNA

The size selection of DNA for BAC cloning occurred before ligation. Whether a second size selection is needed depends primarily on the DNA to be cloned. At higher DNA concentrations small fragments which would give rise to small clones can comigrate with larger DNA during electrophoresis (Riethman et al. 1997). Therefore, one or two preparative gel electrophoresis of partially digested DNA are often performed before ligation. The use of a second size selection to eliminate small DNA molecules in the population will further reduce the frequency of clones with small inserts. It can be useful to perform a trial ligation with a portion of the DNA after the first size selection. If analysis of the clones shows the optimum portion of clones with high insert sizes, a second size selection is not necessary.

The amount of enzyme giving the optimum partial digestion is selected from the control digestion and the digestion reaction on a large scale is performed by carrying out a number of reactions (10-20 reactions) under exactly the same conditions as previously determined (same volumes, same tubes, same dilution of enzyme, same enzyme tube, etc.).

The size selection was performed as a single selection and double selection. For single selection, PFGE was performed just under conditions of 6.0 V/cm, 90s pulse, 14 °C, 19 h. The DNA was cut from gel. For second size selection, this gel piece was loaded onto a second gel and PFGE was performed under conditions of 4.5 V/cm, 5s pulse, 14°C, for 15 h. The other alternative is to perform first and second size selection in the same gel. The partially digested DNA was loaded onto a 0.8 % agarose gel in 0.5xTBE. Pulsed-field gel electrophoresis was performed under two different conditions using the same gel. First, PFGE was performed under conditions of 6.0 V/cm, 90s pulse, 14 °C, for 4 h. After 4 hours, conditions were automatically changed to 6.0 V/cm, 6s pulse, for 12 hours. In this way first and second size selection can be performed together in the same gel.
Both sides of the gel containing the size markers were cut and stained with ethidium bromide. DNA fragments ranging from about 300 to 500 kb were cut from gel (according to the DNA size standard, lambda ladder and Saccharomyces cerevisiae, BIO RAD) from the unstained portion of the gel (Fig.2.3).

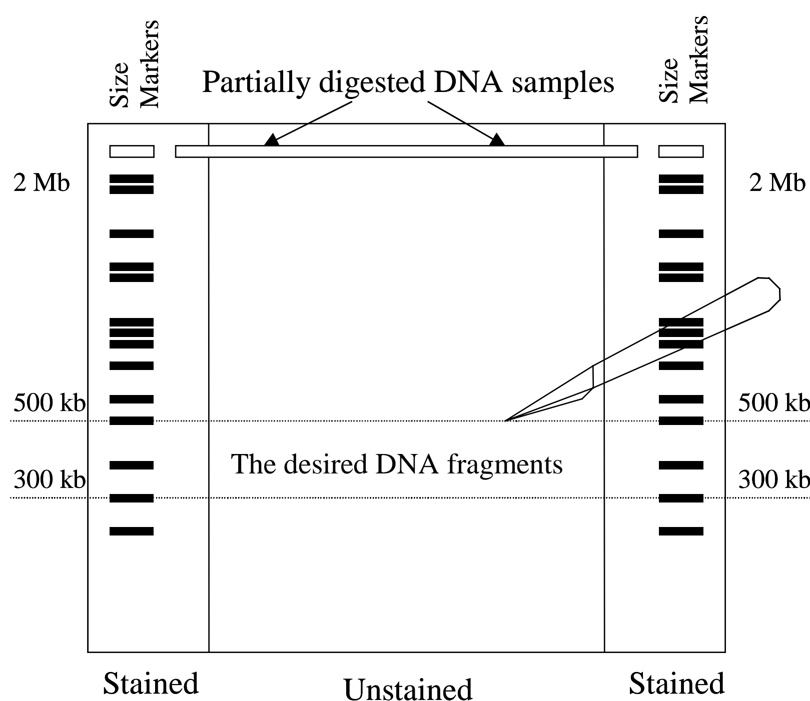

Fig.2.3 Cutting of the DNA fragments from gel

2.3.3.5 Electroelution of Insert DNA

Electroelution was used for the recovery of the DNA fragments from gel slices. The DNA containing agarose slice was placed into a thin dialysis bag. Approximately 500 µl of 1x TAC buffer were filled into the dialysis bag (Roth, MWCO: 12-14,000). Both ends of the dialysis bag were closed with plastic clips ensuring that there were no air bubbles. The dialysis bag was placed transverse to the direction of the current in a large gel chamber and immersed in 1xTAC electrophoresis buffer. Electroelution was performed under conditions of 100 V, 4 °C, for 1 h.

A piece of gel from the gel slice was stained and checked under UV light (254 nm) to ensure that the digested DNA fragments had left the gel and had been eluted into the buffer. If this was not the case, the dialysis bag was placed again into the chamber for new run. It should be placed in the same orientation as before so that the DNA will not run back into the agarose gel. If all DNA had been electroeluted the gel slice was taken out of the dialysis bag. The bag was closed again and put back into the electrophoresis apparatus. The current was reversed for 1 min to release any DNA attached to the dialysis bag into the buffer. Then, about 500 µl buffer including the DNA fragments were taken from the bag with a cut tip. The dialysis bag was washed with 1.5 ml of 1x TE buffer. By this addition, the DNA fragments were diluted in 2 ml. However, for ligation the DNA needed to be concentrated to 150 to 250 µl.

Therefore, the 2 ml of diluted DNA were placed in centrifugation tubes (Amicon) and centrifuged at 4,100 rpm, 4 °C, for 7 min. During centrifugation, the DNA stayed above the filter and was concentrated in 100 to 300 µl buffer. To obtain the DNA, the apparatus was turned upside down and centrifuged again at 1,150 rpm at 4°C for 2 min. The DNA was transferred into 1.5 ml Eppendorf tube with cut tips. The DNA should be immediately used for ligation.

50X TAC Buffer			
Acetic acid		57.1 ml	per 1,000 ml
2 M Tris		242 g	
50 mM EDTA	(0.5 M EDTA, pH 8.0)	100 ml	

2.3.4 Preparation of Competent Cells for Electroporation

There are two ways to obtain stocks of competent *E.coli.* The first option is to purchase frozen competent bacteria from a commercial source. These products are very reliable and

generally yield transformants at frequencies ≥ 10^8 colonies/µg of supercoiled plasmid DNA. However, they are much more expensive than competent cells prepared in the laboratory. Therefore, commercially produced competent cells were only used as a yardstick against which to measure the efficiency of transformation of stocks of competent cells generated in our laboratory or for transformation of a valuable ligations.

The following procedure can yield competent cells of the *E.coli* strains DH10B. Three ml of SOB medium were inoculated with a single colony of bacterial strain DH10B and incubated at 37 °C overnight. One litre of SOB medium was placed in a 3 litre flask and inoculated with 1 ml of the saturated overnight culture. Medium was incubated at 37°C with shaking at 200 rpm. The cell growth was observed by reading the optical density at 600 nm (OD600).

The cells were harvested by centrifugation at 5,000 rpm at 4 °C for 10 minutes in Beckmann 450 ml centrifuge bottles, when the OD600 had reached between 0.6 and 0.8 (i.e., $3x10^8$ to $4x10^8$ cells/ml; usually after 5 hours of growing). Supernatants were decanted. The cells should be placed on ice when they are not in centrifuge. The cell pellets were resuspended in 450 ml of ice-cold 10% glycerol by vortexing. The cells were harvested again by centrifugation at 5,000 rpm at 4 °C for 10 minutes. The supernatants were decanted carefully and pellets were resuspended again in the same volume of 10 % ice-cold glycerol. The cells were harvested again using the same conditions and the supernatants were discarded. Then the pellets were resuspended in a small volume of 10 % ice-cold glycerol and transferred into a 30 ml corex glass tube. The cells were centrifuged at 5,500 rpm, 4 °C, for 10 minutes.

The pellets were resuspended in approximately 200 µl of ice-cold 10 % glycerol for each 450 ml of original culture. The cells were divided into 30 µl aliquots in 1.5 ml microcentrifuge tubes. The tubes were placed in liquid nitrogen for 5 minutes. The cells were transferred immediately to –80 °C and stored until needed for the electroporation.

2.3.5 Ligation

For cloning fragments of foreign DNA carrying identical termini (either blunt-ended or protruding) must be ligated in a linearised plasmid vector bearing compatible ends. During the ligation reaction, the foreign DNA and the plasmid have the capacity to circularise and to form tandem oligomers. It is therefore necessary to carefully adjust the concentrations of

the two types of DNA in the ligation reaction to optimise the number of “correct” ligation products. Optimal ligation conditions should be established by performing several test ligation with different molar ratios of vector DNA to insert DNA.

2.3.5.1 Ligation of Size-Selected DNA to Vector

The optimal molar ratio of vector DNA to size-selected insert DNA was determined by performing test ligations with different amount of insert DNA. The different amounts of DNA (~1-2 ng/µl) (20 µl, 40 µl, 60 µl, 80 µl) were transferred into 1.5 ml microcentrifuge tubes. The volume of ligation was usually defined by the concentration of insert DNA that had been purified from agarose. Each of other components should be added in a minimal volume.

For each tube 1 µl vector DNA (~14-16 ng/µl), 10 µl 10x ligation buffer, 5 µl T4 DNA ligase (1 U/µl) (Epicentre Technologies) and H_2Odd were added. The ligations were performed in a total volume of 100 µl at 15 °C overnight. When the optimal conditions had been defined by transformation and analysis of the resulting colonies, ligations were performed with larger volumes.

The next day 2.5 µl proteinase K (10 mg/ml) was added to the reaction mix. The ligation mixture was incubated at 37 °C for 2 hours. Before dialysis 1 µl of 100xPA was added to 100 µl of the ligation mixture.

2.3.5.2 Dialysis

Salts and other small molecules need to be removed from the ligated vector/insert DNA before electroporation by spot dialysing (drop dialysing).

A 10-cm petri dish was placed where it could stay undisturbed by contact or vibration. Then, 20 ml of 0.5x TE (pH8.0), 1x PA were transferred into the petri dish. A blunt forceps was used to gently place on 0.025 µm filter (Milipore) on top of the TE solution. If more than one sample needs to be dialysed together, the filters can be labelled with pencil or waterproof pen before applying the samples. Either a single sample of 20-400 µl or as many a four 50 µl samples can be dialysed on a single filter. A cut tip was used to slowly pipette the sample onto surface of the filter. The dish was covered. The ligation was dialysed at room temperature for 1 hour. Brief dialysis (< 1 hour) may increase the sample volume if the applied sample contains a high concentration of salt. Prolonged dialysis (> 4

hours) results in loss of sample volume by evaporation. Each dialysed sample of ligated vector/insert DNA was transferred into a separate 1.5 ml microcentrifuge tube. The ligated vector/insert DNA was stored at 4 °C if it was necessary to wait to performed the transformation but transformation should be perform as soon as possible, preferably on the same day as the ligation.

2.3.6 Electroporation of Ligated BAC Vector/Insert DNA

Transformation efficiencies can be optimised by changing various parameters, including the strength of the electrical field, the length of the electrical pulse, and the concentration of DNA. The conditions for the electroporation were used according to Sheng et al. (1995). For each ligation mixture, 1 ml of SOC medium was placed in a separate 15 ml culture tube on ice. The electroporation cuvettes (0.1 cm path length) (Gen Pulser Cuvette BIO RAD) were placed on ice. The DH10B competent cells were allowed to thaw on ice. Then, 30 µl of competent cells were transferred into a separate 1.5 ml microcentrifuge tube for each sample of ligated DNA, and tubes were placed on ice. The following conditions were used for generating BAC clones (Sheng et al. 1995): a capacitance of 25 µF, a resistance of 100 ohms, and a voltage gradient of either 12.5 or 9 kV/cm. The desired conditions were set on the electroporation device. After this 3 µl of the dialysed ligation mixture were added to the tube containing the cells. The cells and ligation mixture were mixed carefully with a cut off tip. The cells were transferred to the bottom of a chilled electroporation cuvette. The outside of the cuvette was wiped to dry with a piece of soft paper. The cuvette was placed in the chamber of the electroporation device and the pulse was applied. Immediately, 1 ml of SOC medium was added to the cuvette.

The cells were transferred into the new microcentrifuge tube using a Pasteur pipette. They were incubated at 37 °C at 200 rpm for 45 minutes. Then 100 µl of the cells were spread on each LB agar plate containing chloramphenicol (12.5 µg/ml), X-gal (50 µg/ml), and IPTG (25 µg/ml). The plates were incubated at 37 °C for at least 18 hours to allow the colour to develop sufficiently to distinguish white colonies from blue.

2.3.7 Blue/White Selection

The cloning site of pBeloBAC11 is within the *lacZ* gene. Recombinant BAC clones in which DNA inserts have disrupted the *lacZ* gene are identified by conversion of colony from blue to white when grown on X-gal and the inducer IPTG (Yanisch-Perron et al.

1985). The blue color of nonrecombinant pBeloBAC11 clones is not very intensive and usually requires at least 18 hours to be clearly discernible. Additional incubation at room temperature or at 4°C will further intensify the color. Whitin two weeks recombinant white clones were either picked manually with sterile toothpicks or robotically (Genetix Qpix) into 384-well microtiter plates containing growth medium.

2.3.8 Storage of BAC Clones

Libraries can be stored as cells or DNA. Large-insert DNA libraries are most commonly stored as individual clones in either 96- or 384-well microtiter plates as glycerol stocks. The BAC library of sunflower was mainly established with 384-well microtiter plates.

For the storage medium part 1 and part 2 were prepared separately. For 10x HMFM 80 ml part 1 and 20 ml part 2 were mixed in a sterile bottle. For each well 5 µl of 10x HMFM and 45 µl of LB medium were added. 384-well microtiter plates were filled manually or by a roboter (Biomek 2000). After picking and incubation for growing, the 384-well plates were stored at –80 °C.

Part 1		
$MgSO_4 7H_2O$	0.09 g	per 100 ml
Trinatriumcitrat $2H_2O$	0.45 g	
$(NH_4)_2SO_4$	0.9 g	
Glycerol	44 g	
Part 2		
KH_2PO_4	1.8 g	per 100 ml
K_2HPO_4	4.7 g	
10x HMFM		
Part 1	80 ml	per 100 ml
Part 2	20 ml	

2.4 Characterisation of BAC Library

Several tests can be done to determine the quality of a BAC library. Two basic tests can be performed to evaluate the genome coverage of a BAC library: (1) estimation of average insert size and (2) detection of average number of clones hybridising with single copy probes.

2.4.1 Minipreparation of BAC DNA

The average insert size of a library is assessed by digestion of the BAC DNA with *Not*I. During library construction or after the library was completed every ligation was tested to determined the average insert size by assaying 20-50 BAC clones per ligation. DNA was isolated from recombinant clones using a standard minipreparation protocol, digested with *Not*I to free the insert from the BAC vector and then separated PFGE.

For BAC minipreparation 3 ml of LB medium containing (12.5 µg/ml) chloramphenicol were inoculated with a single white colony and incubated with shaking (200 rpm) at 37 °C overnight. The overnight culture was transferred to a 1.5 ml microfuge tube. The tubes were centrifuged 13,000 rpm, 4 °C, for 3 minutes. The supernatants were removed. The cell pellets were resuspended by vortexing in 150 µl of solution I. Then, 200 µl of solution II were added and mixed by inversion of the tubes for 8-10 times. The solution should turn translucent. The tubes were incubated for 5 minutes at room temperature. Then, 150 µl of ice cold solution III were added. The tubes were placed on ice and mixed by vortexing.

After incubation for 15 minutes on ice the tubes were centrifuged at 13,000 rpm, 4 °C, for 10 minutes. Then, 400 µl of phenol/chloroform (1:1) were added and mixed strongly by vortexing. Tubes were centrifuged at 13,000 rpm, 4 °C, for 5 minutes. The supernatants (about 375 µl) were removed carefully and transferred to a new microfuge tube. Then, 750 ml of 96% EtOH (prechilled at –20 °C) were added into new tubes and mixed vigorously by vortexing.

The tubes were centrifuged at 13,000 rpm, 4 °C, for 10 minutes. The supernatants were discarded. Again, 500 µl 70 % EtOH were added to the pellet. After centrifugation at 13,000 rpm, 4 °C, for 5 minutes the supernatants were discarded. The pellets were dried until they became transparent for 10 minutes at 55 °C. Then, 16 µl of H_2Odd were added to dried pellets which were solubilised at 37 °C.

The pellets can be stored at room temperature or at 4 C° overnight but the DNA should be analysed within two days. Otherwise, nucleases in the sample may nick the DNA. Physical shearing of the sample (e.g., mixing by vortexing) will also cause large supercoiled BAC DNA to become nicked.

For the digestion, 0.6 µl *Not*I (10 U/µl) (New England, BioLab), 1 µl 10x buffer for *Not*I and 0.4 µl H_2Odd were added to 8 µl of BAC clone DNA. The reaction mix was incubated at 37 °C for 2 hours for digestion.

After digestion reaction mixes were loaded on the 1% PFGE gel electrophoresis. PFGE was performed under condition of 6.0 V/cm, 5-15s pulse, 14 °C, 16 h. The next day, the gel was stained with ethidium bromide. The insert DNA size were determined according to low Range PFGE marker (New England, BioLab).

Solution I (Lysozyme Solution)

50 mM Glucose	0.991 g	per 100 ml
10 mM EDTA	0.292 g	
25 mM TrisHCl pH 8.0	0.303 g	

Solution II (NaOH-SDS Solution)
0.2 N NaOH, 1% SDS

Solution III (Potassium acetate)

5M Kac	60 ml	per 100 ml
Acetic acid	11.5 ml	
H_2Odd	28.5 ml	

2.4.2 The Set of the BAC Library at Filters

The BAC library was set at the filters to screen for the BAC clones. 22 cm x 22 cm Hybond N+ membranes (Amersham, UK) were inoculated using a Q-bot (Genetix, Inc., UK) with 384-well microtiter plates containing BAC clones. The sunflower BAC clones from 72 plates are spotted twice onto one membrane, resulting in 27,648 individual clones on each membrane. The membrane were placed on LB agar plates (Q-Plates, 22x22 cm) containing 12.5 µg/ml chloramphenicol. They were incubated at 37 °C for 12 to 36 hours until colonies of 1 to 2 mm diameter were obtained.

The membranes were removed and placed, the colonies side up, in Solution A (0.5 N NaOH-1.5 M NaCl). The membranes were incubated for 7 minutes. Then, the filters were transferred into Solution B (1.5 M NaCl-0.5 M Tris-HCl). The membranes were incubated for 7 minutes. Then, the membranes were air-dried for more than 1 hour. The membranes were soaked in Solution C (0.4 N NaOH) for 20 minutes. Finally, the membrane were incubated in Solution D (5X SSPE) for 7 minutes and air dried overnight. Now, the membranes were ready to be hybridised against any probe of interest. They can be stored until use for months at RT.

3 RESULTS

3.1 Construction of a BAC library for Sunflower

3.2 Preparation of HMW-DNA from Sunflower

The isolation of large quantities of high-molecular-weight DNA from plants is difficult due to several factors: Plant cells have a very stable cell wall which cannot easily be removed by physical means without damaging the contents of the cell. Also, the largest organelle of a plant cell is the vacuole, which is full of degradative enzymes, including DNases and large amount of secondary metabolites, such as phenolics, which can damage DNA by oxidation (Ganal 1996). DNA needs to be prepared from healthy, growing cells. Therefore, the source of the cells should be a tissue that can be rapidly and easily lysed and also contains minimal amounts of endogenous nucleases.

3.2.1 Isolation of HMW DNA from Protoplasts

The isolation of plant HMW DNA from protoplasts can be subdivided into three steps. First, intact protoplasts need to be liberated from plant tissue or cell cultures via digestion with cell wall degrading enzymes, and to be purified. Protoplasts are then embedded into agarose blocks or beads, and the DNA is purified by extensive digestion with proteinase K. For isolation of HMW DNA from protoplasts, the yield of protoplasts is important to obtain enough high quality HMW DNA. The isolation of good quality protoplasts in a sufficiently high number is the most critical step in the whole procedure. If this is successful, then the protocols should work without any problems. If the protoplast isolation is low in yield and/ or low in quality, the best thing is to start all over again because there is no way to remedy this problem. The quality of the leaf material for protoplast isolation is essential. For sunflower, the plants were grown in the greenhouse in Giessen. For harvesting sunflower leaves the optimal stage proved to be two weeks after germination. It was observed that older plants yielded a lower amount of protoplasts with higher amounts of starch.
Krämer (1998) tested several methods to optimise the yield of protoplasts for isolation of HMW DNA of sunflower (Fig.3.1).

In experiments 1 to 13 HMW DNA was isolated with an enzyme combination of cellulase, driselase and macerozyme R-10 (Burrus et al. 1991). In experiments 14 and 15 the DNA

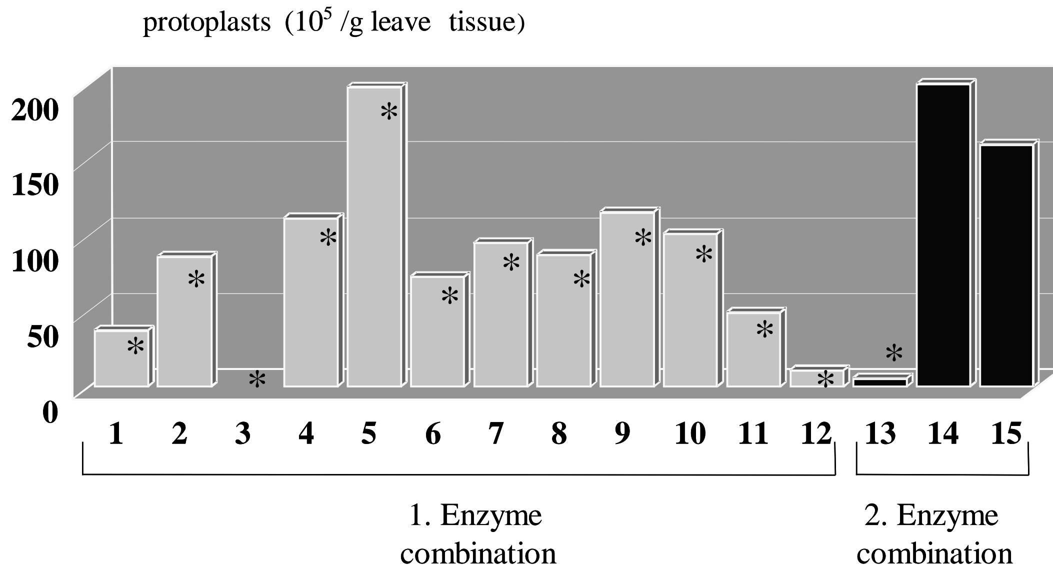

Fig. 3.1 Optimisation of the yield of protoplasts isolated from sunflower seedlings. Stars show methods which include starch in the preparation. For each step the amount of protoplasts, measured as 100,000 protoplasts per g of leaves is given. Different growing conditions were used: 1-2: short day, soil, 3: short day, vermiculite. Modification of methods to release the protoplasts: 4-6: carborund 7: quartz sand, 8: vacuum, 10: pipetting of protoplasts, 11-13: concentration of protoplasts in a ficoll-gradient.

was isolated with a second enzyme combination including cellulase and pectolyase (Ganal et al. 1996). In the experiments 1 to 3 different growing conditions were tested, for example in the experiment 2, plants were grown under short day conditions. In experiment 4 to 8, different methods were tried to release the protoplasts. In experiment 5 and 7 carborund and quartz sand were used to destroy the surface of the leaves. In experiment 5 a high amount of protoplasts was obtained but this preparation also included starch grains. In experiment 8, vacuum was applied during digestion of the leave tissue with macerozyme to release the protoplasts. In experiment 9 to 13, starch grains were removed by physical methods. In all protoplast preparations using the enzyme combination of cellulase, driselase and macerozyme, starch grains were observed (experiments 1 to 13). The experiments 14 and 15 using the enzyme combination cellulase and pectolyase gave better results as higher amounts of protoplasts without starch grains were obtained.

As described two different kinds of enzyme combinations were tested for protoplast isolation. One combination was macerozyme R-10, driselase and cellulase. With this combination the release of starch grains represented a big problem for the protoplast

isolation. During centrifugation steps starch grains sedimented to the bottom of the centrifugation tubes (Fig.3.2 A), and also stayed in the supernatant (Fig.3.2 B). The second enzyme combination was cellulase RS and pectolyase Y23 which hardly released any starch grains and also yielded high amounts of protoplasts (Fig. 3.2 C).

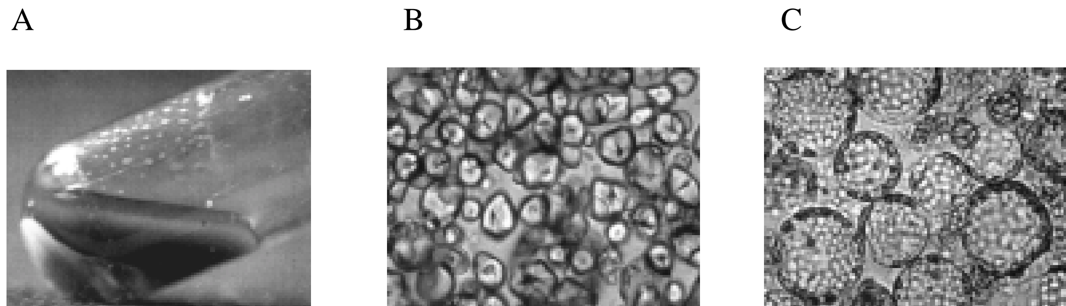

Fig.3.2 Starch grains in HMW DNA preparations from protoplasts. A and B, protoplasts isolated by using an enzyme combination of macerozyme R-10, driselase and cellulase. B, starch grains under the microscope. C, protoplasts without starch grains isolated by an enzyme combination of cellulase RS and pectolyase Y23.

For protoplast isolation starch is the most problematic plant component as starch grains destroy the protoplasts during the isolation steps. Also the digestion of high molecular weight DNA with restriction enzymes is problematic because starch which gives a deeply white colour in the agarose blocks prevents the restriction by endonucleases. By keeping the plants in the dark for 2 days before isolation, or by harvesting very young leaves that are still expanding, the accumulation of starch can be reduced.

The first enzyme combination (macerozyme R-10, driselase and cellulase), was not further used because of the high amount of starch grains observed in this procedure. Thus, the isolation of HMW-DNA from protoplasts to construct a BAC library in sunflower was performed with the second enzyme combination using cellulase RS and pectolyase Y23 which resulted in protoplasts without any starch grains.

During isolation plants cells were incubated with this enzyme combination for 3.5 hours at 28 °C. To increase the yield of protoplasts, incubation time was extended to 5.5 hours according to Ganal et al. (1996). With a longer incubation time, an increase in yield of protoplasts was clearly observed. The yield of protoplasts was increased from 1-2x10^6 protoplasts/gram of leaf tissue to 4-8x10^6 protoplasts/gram of leaf tissue. However, only

0.4 % of the sunflower BAC library was constructed with HMW DNA isolated from protoplasts.

3.2.2 Isolation of HMW DNA from Nuclei

For isolation of HMW DNA from nuclei, the plant material was grown under the same conditions as the plants for isolation of protoplasts.

The procedure for isolation of HMW DNA from nuclei is simple, rapid, and economic. The majority of the DNA prepared is larger than 5.7 Mb in size. In addition, the chloroplast DNA content in the DNA prepared by this technique can be reduced by more than 10 fold compared to HMW DNA prepared from plant protoplasts by currently used techniques (Wing et al. 1994).

After isolation of HMW DNA of sunflower, the quality of MB DNA was checked by using PFGE (Fig.3.3). The size of DNA prepared from nuclei is here about 2.2 Mb. The quality of MB DNA from protoplast isolation is better than from nuclei, because MB DNA from plant nuclei contains large amounts of small DNA fragments which are not desirable for cloning.

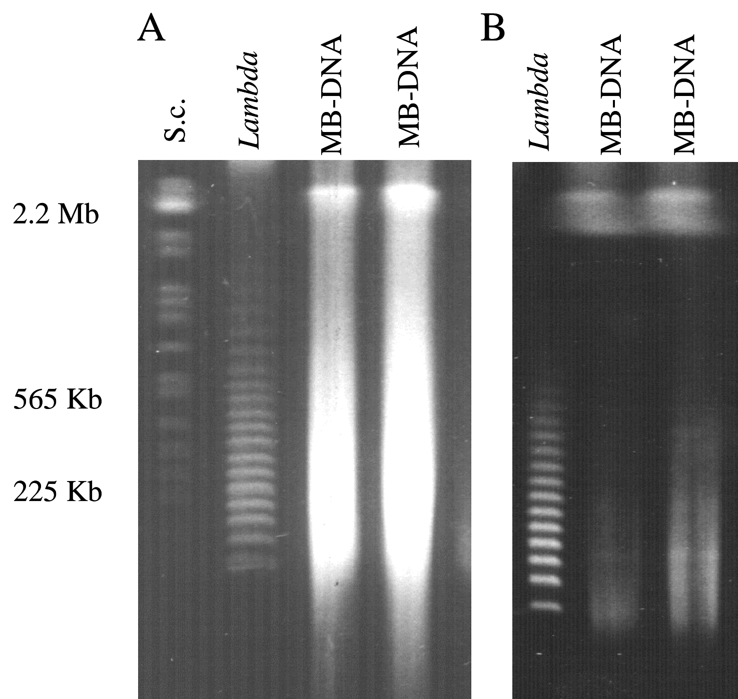

Fig. 3.3 Analysis of HMW DNA isolated by nuclei (A) and protoplast (B) preparations. HMW DNA was separated on a 1 % pulsed field gel (14°C, 50-90 pulse, 19 h, 6 V/cm).

However, isolation of nuclei represents a simple, rapid and economic way to obtain large amounts of HMW DNA for BAC cloning. For elimination of small DNA fragments, pre-electrophoresis systems were used before or after digestion with restriction enzymes.

3.3 Restriction Digestion of HMW DNA

High amounts of starch and agarose can inhibit the digestion of MB DNA by restriction enzymes. For cloning and mapping, MB DNA needs to be digestible. Therefore digestion of isolated HMW-DNA was checked by using different restriction enzymes (Fig.3.4). The sunflower HMW DNA was digested over night with the restriction enzymes *Eco*RI, *Bam*HI, *Eco*RV, *Pst*I, *Xba*I, *Xho*I, *Sac*I, *Hind*III, *Dra*I, *Sal*I, *Not*I and *Bgl*I. In a Southern hybridisation of the PFGE gel with a radiolabelled genomic DNA probe (Clone 24, Mösges 1993) clear signals were observed for all restriction enzymes, apart from *Eco*RI which showed star activity resulting in multiple bands. These results indicate that the sunflower HMW DNA is digestible by different restriction enzymes and suitable for plant genome analysis by PFGE.

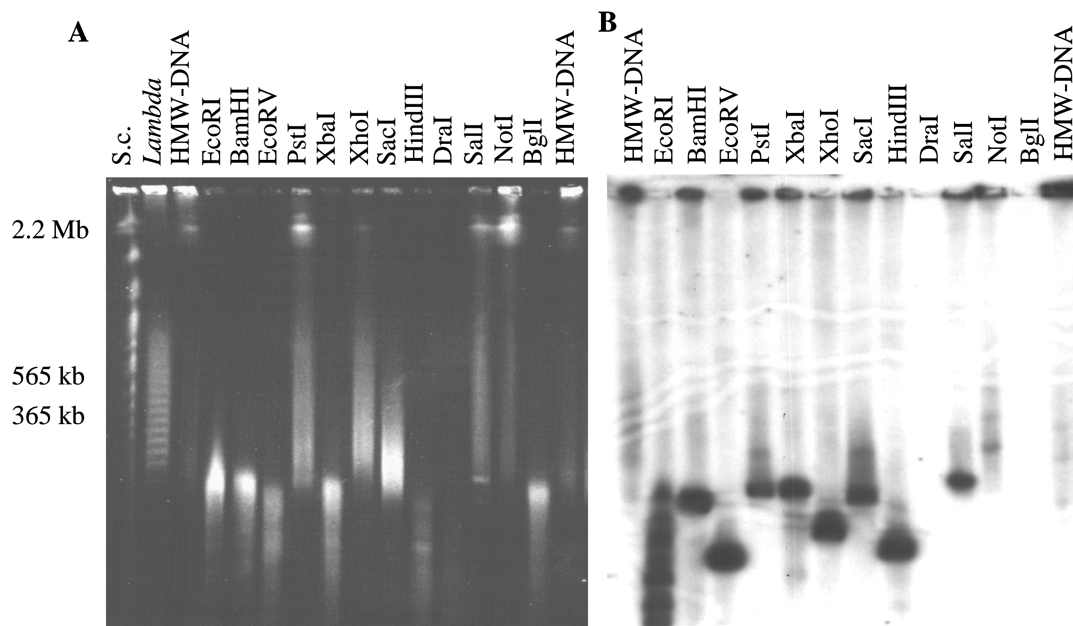

Fig. 3.4 Restriction digestion of sunflower HMW-DNA and Southern hybridisation. The sunflower HMW DNA was digested with different restriction enzymes (A) and hybridised with a radiolabelled genomic sunflower RFLP probe (B). The digested DNA was separated on a 1% pulsed-field gel under conditions of 6.0 V/cm, 50 initial 90 final pulse, 14 C°, 19 h.

3.3.1 Optimisation of the Partial Digestion with *Eco*RI

The vector pBeloBACKan contains a single *Eco*RI restriction site which allows cloning of *Eco*RI fragments. Partial digestion can be achieved by four common techniques using *Eco*RI: (1) varying the concentration of the restriction enzyme (Burke and Olson 1991), (2) varying the time of incubation with the restriction enzyme (Anand et al. 1989), (3) varying the concentration of an enzyme cofactor (e.g. Mg^{2+}) (Albertsen et al. 1989), and (4) varying the ratio of endonuclease to methylase (Larin et al. 1991).

First, the effect of different concentrations of the required cofactor Mg^{2+} was tested. According to Albertsen et al. (1989), more precise and reproducible partial digests are obtained, when a limiting concentration of the cofactor Mg^{2+} is used. This method is especially useful for partial digestions of DNA embedded in agarose. In agarose, diffusion time of the much smaller Mg^{2+} ions is shorter than that of the restriction enzyme (Albertsen et al. 1989). The DNA agarose blocks were first equilibrated with restriction enzyme *Eco*RI and restriction enzyme buffer without $MgCl_2$ for two hours on ice. After 2 hours different concentrations of $MgCl_2$ were added to the reaction buffer. Then, the DNA blocks were incubated at 37°C for 1.5 hours. After separation of the DNA, an optimal partial digestion was not observed with any of the applied Mg^{2+} concentrations (Fig.3.5).

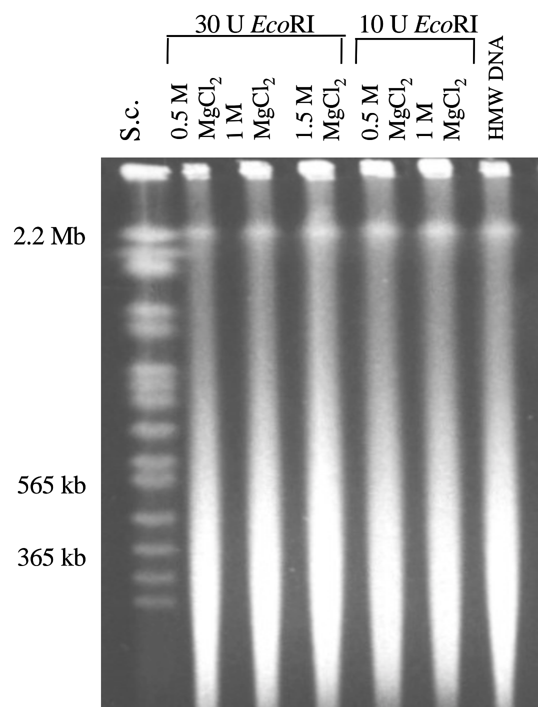

Fig.3.5 Partial digestion of HMW DNA from sunflower with *Eco*RI using different concentrations of the cofactor Mg^{2+}. HMW DNA was digested under the conditions given in the picture. The PFGE was performed at 14 °C, 6 V/cm, 90s pulse, 19 hours.

In addition, different enzyme concentrations of *Eco*RI and different incubation times were tested for partial digestion using the protocol from Wing (1997). For partial digestion incubation time was limited to 1.5 hours for investigating the effect of the different enzyme concentrations. The results of digestion with different enzyme concentrations and different incubation times are shown in Fig.3.6. Digestion of the DNA was observed with this protocol but it was not possible to concentrate DNA fragments between 300 kb and 500 kb.

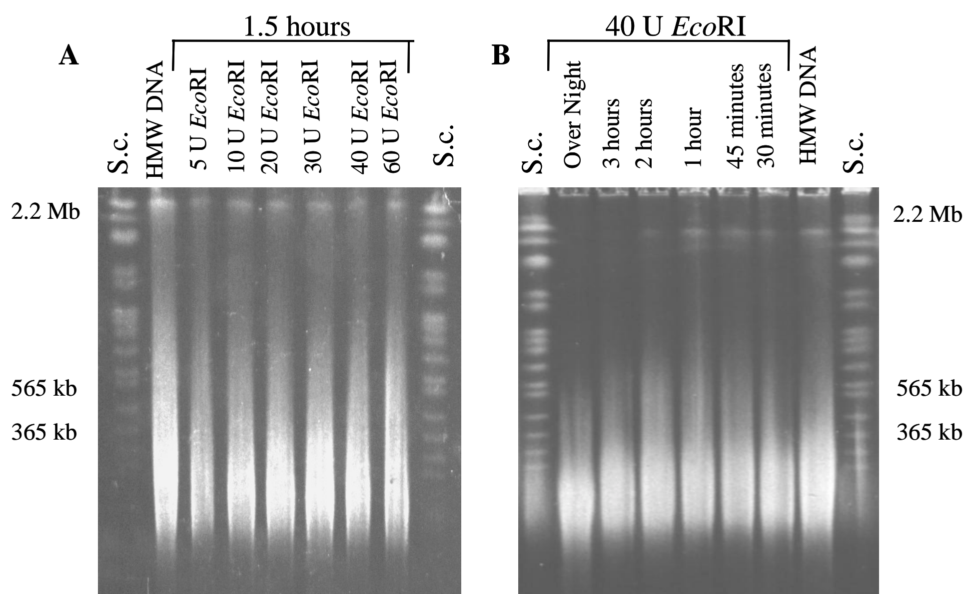

Fig.3.6 Partial digestion of sunflower HMW DNA with different concentrations of *Eco*RI and different incubation times. A, partial digestion with different *Eco*RI enzyme concentrations for 1.5 h, B, partial digestion using 40 U *Eco*RI and different incubation times.

Next, variation of the ratio of restriction enzyme *Eco*RI to methylase was tested. First, the concentration of *Eco*RI was kept at 80 U and different concentrations of methylase (840-800 U) were used to optimise the partial digestion. The effect of the ratios of *Eco*RI to methylase on restriction are shown in Fig.3.7 A. The ratio of 80 U *Eco*RI to 800 U methylase gave the highest amount of DNA fragments in the size range of 300-500 kb. Lowering the concentration of methylase from 800 to 480 clearly increased the digestion of the HMW DNA. No difference in the digestion was observed between the ratio 80 U *Eco*RI to 480 U methylase and 80 U *Eco*RI to 240 U methylase. Second, a ratio of 800 U methylase to 100 U *Eco*RI was used in order to find the ideal ratio of restriction enzyme

*Eco*RI to methylase for partial digestion (Fig. 3.7.B). This ratio gave the best result for partial digestion.

Agarose limits the diffusion of the restriction enzyme in blocks and influences the restriction of the DNA. Therefore, to control the effect of agarose blocks containing HMW DNA were melted and the agarose fibres were digested with agarase.

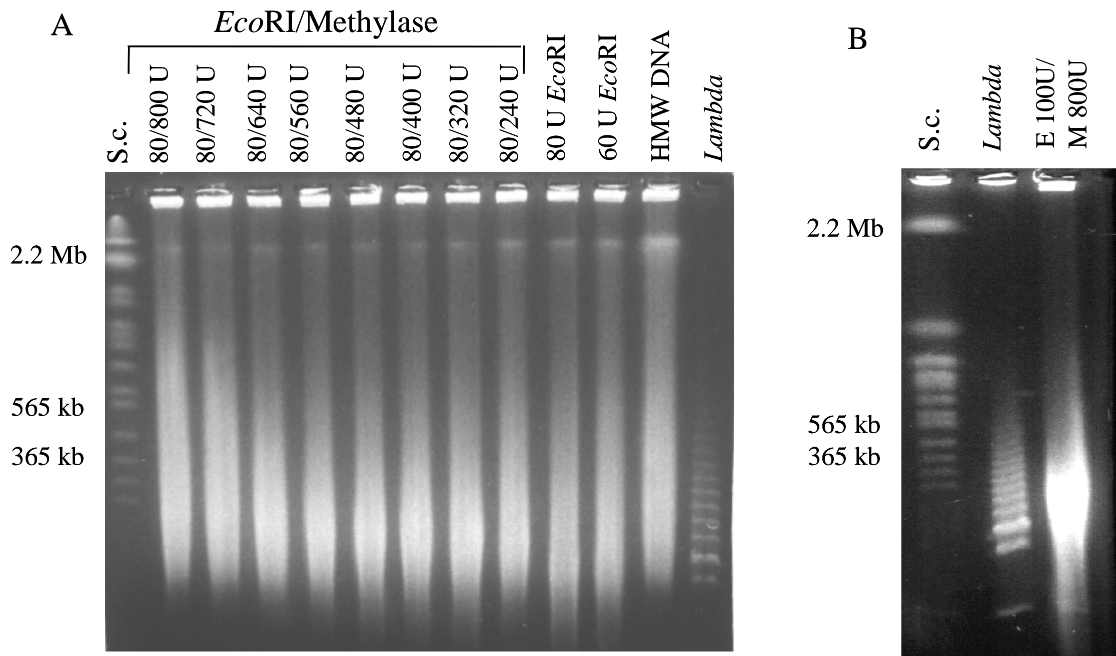

Fig.3.7 Partial digestion of sunflower HMW DNA with varying ratios of restriction enzyme *Eco*RI to methylase. A, the concentrations of methylase and *Eco*RI were changed, B, partial digestion of HMW DNA with a ratio of 100 U *Eco*RI to 800 U methylase.

The HMW DNA could be recovered from agarose gels with some degradation and the DNA was handled as a liquid for digestion. For comparison between partial digestion of HMW DNA released from agarose and HMW DNA embedded in agarose blocks, the same DNA was digested with different *Eco*RI units (Fig 3.8). The HMW DNA released from agarose was more digested than the HMW DNA in plugs with the same units of *Eco*RI. On the other hand, liquid HMW DNA was destroyed and HMW DNA fragments were sheared by melting the agarose. However, HMW DNA molecules in solution are more susceptible to breakage and the liquid DNA can be easily degraded during every steps. Physically shearing of the HMW DNA fragments increases the amounts of small DNA fragments which are not desirable for BAC cloning. Therefore, HMW DNA digested after agarase treatment was not used for cloning.

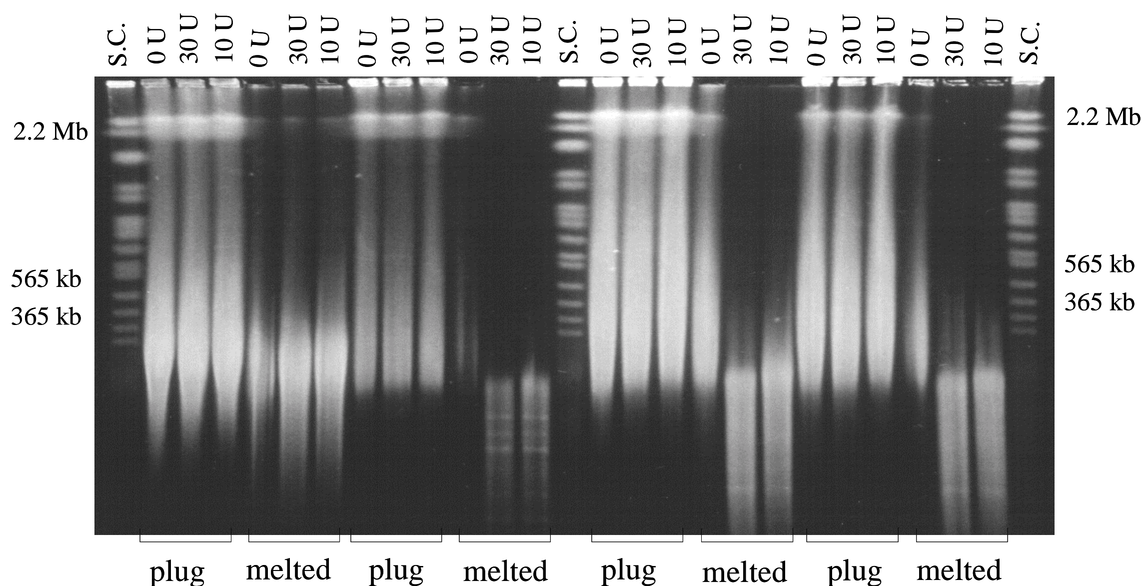

Fig.3.8 Restriction digestion of HMW DNA embedded in agarose plugs or after melting and digestion of the agarose with agarase. The HMW DNA was digested with *Eco*RI and separated on an 1 % pulsed-field gel. The PFGE was performed at 14 °C, 6 V/cm, 90s pulse, 19 hours.

3.3.2 Optimisation of the Partial Digestion with *Hind* III

After changing the vector to pBeloBAC11 which has a *Hind*III cloning site, HMW DNA had to be partially digested with *Hind*III. Digestion progress for *Hind*III was adapted from Wing (1994). Optimising the partial digestion proved to be easier compared to the digestion with the restriction enzyme *Eco*RI. First, the incubation time was varied. In contrast to *Eco*RI, the restriction enzyme *Hind*III needs less time for restriction. Ten or 20 minutes of incubation time gave good results for partial digestion (Fig.3.9). The optimal fragment size was obtained by digestion of the HMW DNA with 5 U *Hind*III for 15 minutes at 37 °C. Most of the digested HMW DNA fragments had a size between 200 kb to 600 kb. The BAC library was constructed by partial digestion using the restriction enzyme *Hind*III.

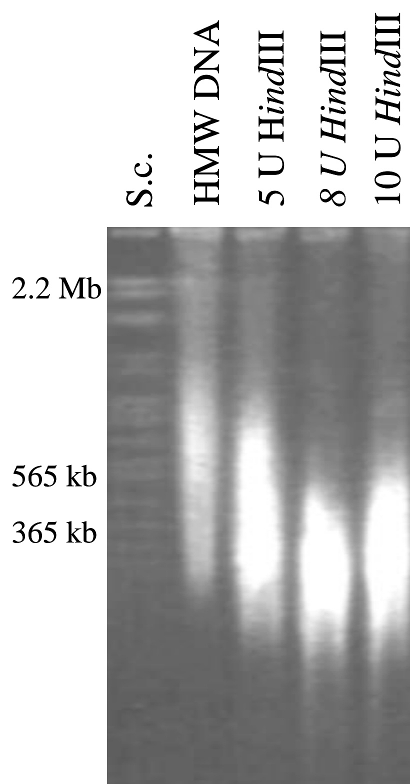

Fig.3.9 Optimisation of the partial digestion with *Hind*III. Sunflower HMW DNA was digested with 5 U, 8 U and 10 U *Hind*III, 15 min, 37 °C. The DNA was separated on a 1% pulsed-field gel.

3.3.3 Pre-electrophoresis

Pre-electrophoresis is performed at conditions that prevent migration of HMW DNA out of the plug whereas other charged molecules and small broken DNA fragments are eliminated from the samples. The different conditions for PFGE were tested to remove small DNA fragments from agarose blocks (Fig. 3.10). When voltage is applied for more than 1 or 2 hours, not only small DNA fragments but also HMW DNA runs in the first gel. Therefore, running time is very important. The best result was obtained under the conditions: 1 h, pulse sec, 5 V/cm, 120°. However, after pre-electrophoresis, the DNA could not be digested. Therefore, pre-electrophoresis system could not be used efficiently.

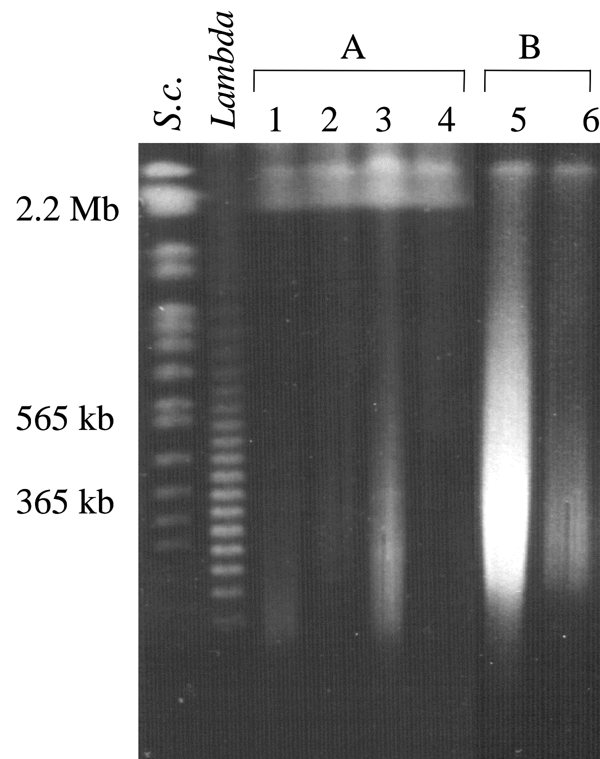

Fig.3.10 Sunflower HMW DNA before and after pre-electrophoresis. A, HMW DNA isolated from protoplasts: 1, 3 before pre-electrophoresis, 2, 4 after pre-electrophoresis. B, HMW DNA from nuclei: 5, before pre-electrophoresis, 6, after pre-electrophoresis. Pre-electrophoresis was performed under conditions of 5 V/cm, 60s pulse, 1 h in 1 % pulsed-field gel.

3.4 Improvement of the Size Selection

The comigration of small fragments with large fragments in PFGE is strongly dependent on the concentration. Samples with lower DNA concentrations have fewer small fragments migrating with the large DNA and may therefore require only a single preparative size fractionation. In addition, the actual length of the DNA molecules in size-fractionated samples often varies significantly from the length predicted on the basis of the molecules migration relative to the molecular-weight markers in a preparative gel. Ethidium bromide also stains on the basis of mass. Thus, when partially digested DNA is viewed after size fractionation, there are actually twice as many 50 kb molecules as 100 kb molecules, if the intensity of the fluorescence in the region at 50 kb is equivalent to that at 100 kb. However, taking these facts into account it is possible to obtain DNA fragments desirable in size.

For size selection, two different gel system were used. In the first gel partially digested DNA was loaded and PFGE was performed under conditions of 6.0 V/cm, 90s pulse, 14 °C, 19 hours. DNA fragments between 200 kb to 500 kb were cut from this gel as shown in

Fig. 3.10 and loaded on a second gel or directly used for ligation (when the second size selection was omitted). The selected DNA fragments were run on a second pulsed field gel under conditions of 4.5 V/cm, 5s pulse, 14 °C, 15 hours. The result of second selection gel is shown in Fig. 3.11.

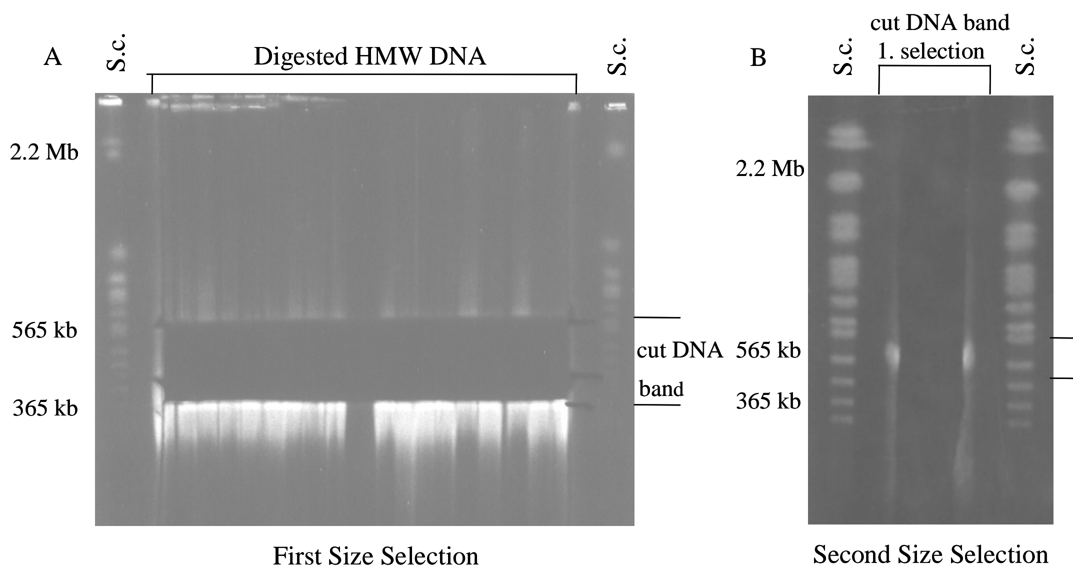

Fig.3.11 First and second size selection of partially digested DNA fragments on two different gels. A, first size selection on a 1 % pulsed-field gel under conditions of 6 V/cm, 90s pulse, 14 °C, 19 hours, B, second size selection, the selected DNA fragments from the first size selection were loaded onto the second 1 % pulsed-field gel. The second size selection was performed under conditions of 4.5 V/cm, 5s pulse, 15 hours.

Another possibility is to perform two size selection in the same gel. After partial digestion DNA was loaded onto a gel and the first PFGE was performed under conditions of 6.0 V/cm, 90s pulse, 14 °C, 4 h and the second PFGE for 12 hours, 6.0 V/cm, 6s pulse (Fig. 3.12). This method is easy and shorter than the other system.

However, DNA fragments obtained by either types of two size selections could not be cloned into pBeloBAC11 with high efficiency.

The major reason for failure in BAC cloning experiments is that the preparative electrophoresis is performed in an electrophoresis chamber that might contain nucleases in the electrophoresis buffer. Therefore, if a preparative PFGE was performed, the inside of the chamber was carefully cleaned with a soft brush and rinsed extensively with sterile H_2O. In addition, freshly prepared and autoclaved electrophoresis buffer was used.

However, using single size selected DNA fragments (200-500 kb) high transformation efficiencies were obtained. For the construction of 98 % of the BAC library for sunflower single size selected DNA was used.

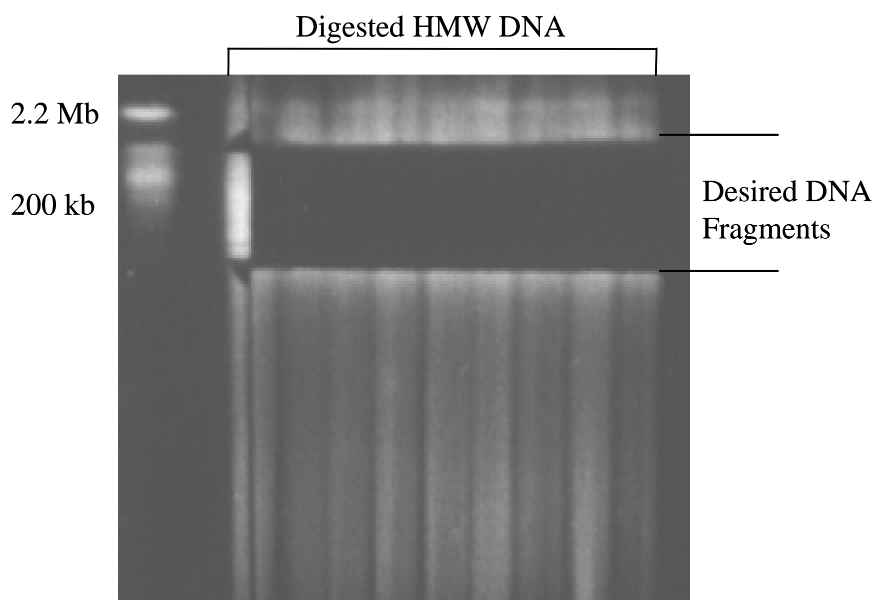

Fig.3.12 First and second size selection in one gel. The first and second size selection on the same gel were performed under conditions of 6 V/cm, 90s pulse, 14 °C, 4 h for the first size selection and then the conditions were changed 6 V/cm, 6s pulse, 12 h for the second size selection. After separating of the DNA, the desired DNA fragments as marked in the picture were cut from the gel for ligation.

3.5 Isolation of Vector DNA

BAC vectors are plasmids. Therefore, the procedure for vector preparation is essentially the same as for conventional plasmid DNA preparation. The most important difference is that a large volume of culture is needed to obtain enough DNA for cloning because BAC vectors like pBeloBACKan and pBeloBAC11 are only present at one to two copies per cell.

3.5.1 Purification of pBeloBACKan by Plasmid Maxi Kit

For the pBeloBACKan, the Qiagen Maxi Plasmid Purification Protocol was used. Plasmid DNA was isolated from the cell pellet by alkaline lysis using the QIAGEN plasmid Maxi Kit (QIAGEN Cat#12162, USA) according to manufacturers specifications for 2 l culture. Vector DNA was eluted from chromosomal DNA. In addition to the purification of the plasmid DNA with the QIAGEN Kit the plasmid safe treatment was performed according

to Mozo (1998a) to obtain the pure plasmid DNA before the digestion of vector with *Eco*RI. After Qiagen maxi plasmid purification, the supercoiled vector DNA was controlled on a mini gel. After linearisation of the vector DNA by digestion with *Eco*RI, the linearised pBeloBACKan DNA was observed with a size of 8.5 kb (Fig.3.13).

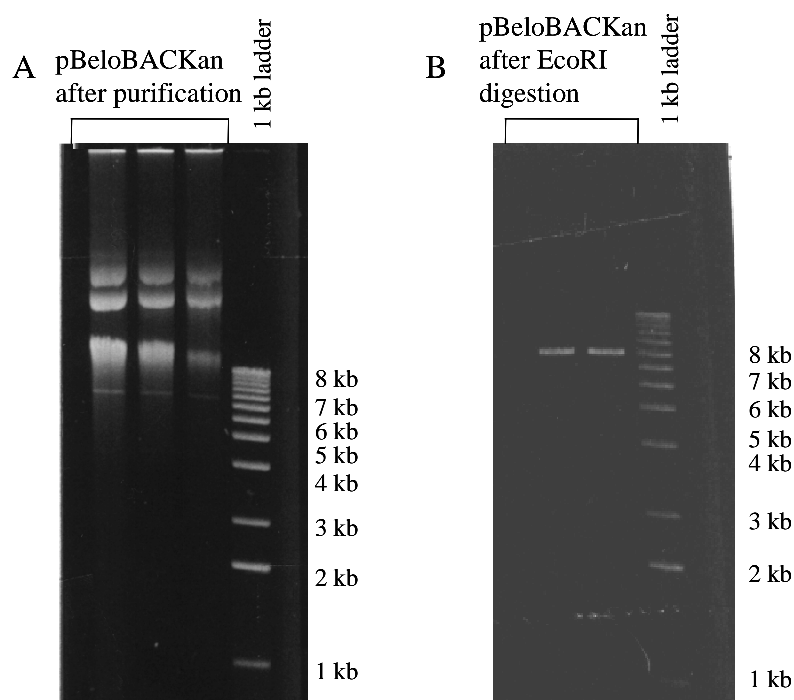

Fig. 3.13 Purification of pBeloBACKan and digestion with *Eco*RI. A, purified vector DNA, B, pBeloBACKan digested with *Eco*RI. The vector DNA was separated on a 1 % agarose gel for 1 h, 100 V.

3.5.2 Purification of pBeloBAC11 by CsCl Gradient

The vector pBeloBAC11 DNA was isolated by alkaline lysis method without any commercial kit. Unlike maxipreparation by Qiagen Kit, Terrific Broth (TB) medium was used for the overnight culture of the bacteria which is richer in nutrients than LB medium. For growing medium, the concentration of chloramphenicol was increased from 12.5 µg/ml to 30 µg/ml. The high concentration of chloramphenicol has the advantage of inhibiting bacterial replication. This reduces the bulk and viscosity of the bacterial lysate and greatly simplifies purification of the plasmid. However, despite the high concentration of chloramphenicol viscosity of the bacterial lysate was not reduced completely. Therefore, after isolation of big DNA pellets from 3 or 4 l medium these could only be dissolved with difficulties in 3 ml of TE buffer.

The plasmid DNA was further purified from the chromosomal DNA by CsCl density gradient. After CsCl density gradient, two bands were observed. The upper band, *E.coli* chromosomal DNA was always more visible than the lower band of supercoiled plasmid DNA.

3.5.3 Control of the Vector Preparation

After purification of the vector, the quality of the plasmid preparation needs to be controlled by digestion with restriction enzymes and ligation. For pBeloBAC11, the vector was cut with *Hind*III. After 2 hours of digestion a single band of vector DNA could be observed in the control gel at 7.4 kb (Fig. 3.14). There were no traces of the uncut supercoiled or nicked open circular vector DNA, nor any smear of *E.coli* chromosomal DNA in the vector preparation.

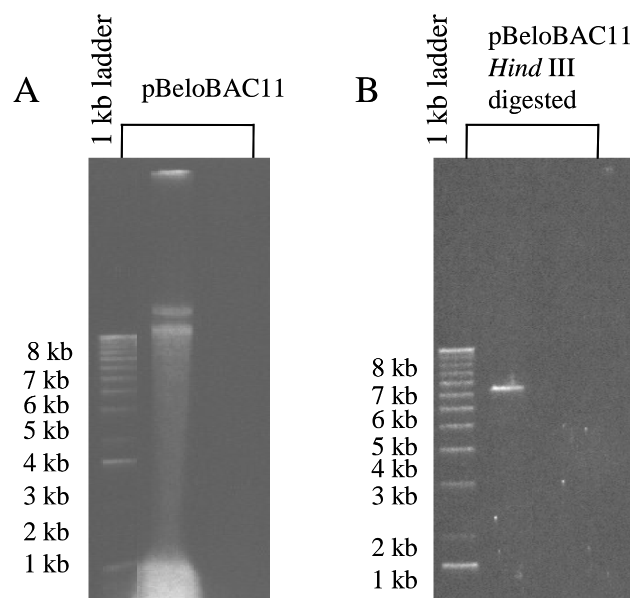

Fig.3.14 pBeloBAC11 DNA after CsCl gradient purification. A, purified pBeloBAC11 vector, B, pBeloBAC11 linearised with *Hind*III. The plasmid DNA was separated on a 0.8 % agarose gel.

After restriction, 2 µl vector DNA were kept to control the quality of the linearised vector DNA. Normally without dephosphorylation, digested vector should self ligate and blue clones should be seen after transformation of *E.coli* cells. This control ligation tests the extent of digestion with *Hind*III and ensures that the ends of the vector DNA have not been degraded. When the vector is self ligated with success, the *lacZ* enzyme is active again.

Using the control ligation reaction, 90 % of the clones obtained after transformation were blue. Degradation is evidenced by the number of white colonies after self-ligation of the digested vector DNA.

After dephosphorylation, the quality of the dephosphorylated vector DNA was also investigated by a test ligation using *Hind*III-digested fragments of bacteriophage λ-DNA which represent a mixture of different sized fragments. After transformation 90 % of white colonies were observed. This result confirmed that the vector was well prepared and suitable for BAC cloning.

3.6 Ligation with the Vector pBeloBAC11

Optimal ligation conditions had to be established by performing several test ligations with different ratios of vector DNA (~14-16 ng/µl) to insert DNA (~1-2 ng/µl) (e.g., 10:1, 1:10, and 1:5). With test ligations, the best result was observed by using ratios between 1:5 and 1:10 vector DNA to size-selected insert DNA. The volume of the ligations is usually dictated by the concentration of size selected insert DNA which had been purified from agarose. Each of the other components should be added in a minimal volume. For obtaining the desired ratio, sometimes 80 µl of insert DNA was used in 100 µl of ligation volume. In most cases, insert DNA concentrations of 20 µl, 40 µl, and 60 µl gave good results.

3.7 Estimation of Transformation Efficiencies

Transformation of *E.coli* with DNA by electroporation is extremely efficient with DNA of a few kilobase pairs in size. When the vector DNA was ligated to *Hind*III-digested fragments of bacteriophage λ-DNA, about 250 colonies of which 90 % were white could be observed after transformation using 2 µl of the ligation, spreading 100 µl of transformed cells.

DH10B was used as host bacteria because it can be transformed extremely well with large and small DNA molecules. Differences in transformation efficiencies were observed between DH10B which were purchased in a form competent for electroporation (Life technologies, Research Genetics) and DH10B which were prepared in our laboratory. When the same ligation was transformed by electroporation into purchased DH10B and in self made DH10B, efficiencies of transformation was extremely higher with purchased

DH10B. With the same ligation, purchased DH10B gave sometimes twice as many colonies (10^6-10^9 transformants/µg of DNA) than self made DH10B (10^4-10^6 transformants/µg of DNA). However, the use of purchased competent cells is very expensive. Therefore, optimization experiments for the construction of the BAC library were performed with self-made competent cells and commercially produced competent cells were only used for valuable ligations.

Different electroporation conditions were tested for effective electroporation. The best results were obtained with the conditions which were optimized by Sheng et al. (1995) for generating BACs larger than 80 kb. These conditions were capacitance of 25 µF, resistance of 100 ohms, and voltage gradient of 9 kV/cm for the 0.2 cm path length of electroporation cuvettes. Also 0.1 cm path length of electroporation cuvettes were used with following conditions: capacitance of 25 µF, resistance of 100 ohms, and voltage gradient of 12.5 kV/cm. With 0.1 cm electroporation cuvettes, transformation efficiency was approximately twofold higher than with 0.2 cm electroporation cuvettes with the same ligation. Therefore, for the construction of the sunflower BAC library 0.1 cm path length of electroporation cuvettes were used.

Small-scale plating of the transformation mixture and analysis of the resulting BAC clones should be performed before the entire transformation mixture is plated. This is especially important when BAC cloning is initiated or when a new DNA sample (either a new preparation of HMW DNA or a new partial digestion of DNA that has been size-selected) is used.
To avoid large-scale platting at densities that are too high (therefore unpickable) or too low (waste of time and resources), various volumes of the transformation mixture (e.g., 25, 100, and 250 µl) were plated and incubated at 37 °C for at least 18 hours. The desired plating density is the one that allows picking of single colonies that are not contaminated by cells from neighbouring clones. The remainder of the transformation mixture was stored at 4 °C for 1-2 days with little (<10 %) loss of viability. For longer storage (up to several months), glycerol was added to the cells to a final concentration of 10% and the cells were stored at –80°C.

The portion of white (recombinant) versus blue (nonrecombinant) colonies was determined. A certain level of background blue colonies is associated with each batch of vector DNA, whereas the number of white colonies varies with the preparation of insert DNA being cloned. The number of white colonies usually ranged from 10 % to 60 % of the

total number of colonies. If the proportion of white colonies was above 40 % of the total number of colonies, the ligation was used for the construction of the sunflower BAC library.

3.8 Characterisation of the BAC Library for Sunflower

The sunflower BAC library was constructed from the restorer line RHA325 using pBeloBAC11 as vector with 12 different ligations and from 6 different HMW DNA preparations. The sunflower BAC library comprises 104,736 clones which are stored in 273 microtiter plates. Each microtiter plates contains 384 individual clones in glycerol stocks. During the construction of the sunflower BAC library 2,148 minipreparations were performed to analyze average of insert size and to control ligations. Therefore about 2 % of the BAC library were controlled by minipreparation. The maximum insert size observed was 270 kb. The average of insert size was found to be 50 kb. The sunflower genome size is 2871-3189 Mb (Arumuganathan and Earle 1991). Therefore the sunflower BAC library covers two sunflower genome equivalents. A genomic library is a random assortment of clones, in which genomic sequences are represented (in the ideal case) by random distribution within the library. In other words, because cloning is largely a random process, some sequences will be cloned several times, and others will be cloned very rarely or not at all. Consequently, it is necessary to construct a library, which must cover two or more genome equivalents, so that it can be assured, within a certain probability, to encounter each sequence at least once.

Part of the BAC library (40,000 clones) was picked with a picking robot Qpix at Max-Planck-Institut für Züchtungsforschung in Cologne. The rest of BAC library was picked with sterile toothpicks by hand in the clean bench. The BAC library was replicated twice with Biomek 2000. Two copies of sunflower BAC library are stored in different –80 °C freezers in the Institute of Crop Science and Plant Breeding, in Giessen. One copy of sunflower BAC library is stored at –80 °C in Max-Planck-Institute in Cologne.

3.8.1 Control of the Stability of the BAC Clones

For the controlling of the stability of the BAC clones, two different BAC clones were used. The insert size of the BAC clones were 72 kb (two bands) and 100 kb. These clones were propagated for 11 and 12 generations. The DNA of 12 generations BAC clones was isolated by minipreparation, *Not*I digested and each generation was loaded on pulsed field

gel (Fig.3.15). During the generation the size of the insert DNA and the vector remained the same. Therefore, the sunflower BAC clones can be considered stabile over at least 11 and 12 generation.

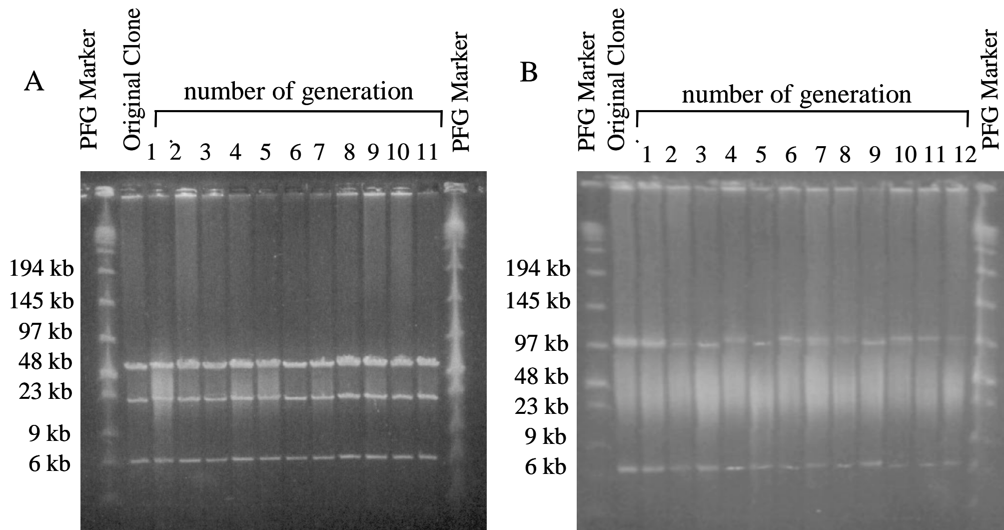

Fig.3.15 Stability of BAC clones over 12 generations. The stability of the BAC clones was controlled by propagation of two different clones with 72 kb (A) and 100 kb (B) insert size. After minipreparation the plasmid DNA was digested with *Not*I and separated on a 1 % pulsed-field gel.

3.8.2 Estimation and Distribution of the BAC Insert Sizes

The use of the BAC system allows pure BAC DNA to be isolated via minipreparation methods. Because BACs are circular, they are relatively resistant to shear-induced breakage during isolation. Hence, handling BACs even as large as 350 kb requires no extraordinary measures. Minipreparations of DNA should not be mixed by vortexing, but the DNA can be pipetted with standard pipette tips without any detectable damage to the DNA.

BAC DNA obtained from an alkaline-lysis minipreparation contains a mixture of nicked (open circular) and supercoiled molecules. The separation of large circular DNA by PFGE is problematic. Very large open circular molecules (>100 kb) cannot be separated in agarose since they become hooked on agarose fibers in the gel matrix and thus remain trapped in the well during electrophoresis. In contrast, large supercoiled molecules (>100 kb) can be resolved by PFGE, but they migrate in a switch-time independent manner. For this reason, these DNA molecules are best separated after they have been linearised. Linearisation is most often accomplished by digesting the DNA with *Not*I.

*Not*I restriction sites occur infrequently in plant DNA but *Not*I cleaves on both sides of the cloning site in the vector pBeloBAC11. Therefore *Not*I excises the insert from the vector and allows the precise size determination of the BAC insert DNA. After digestion with *Not*I and separation of the fragments by PFGE, it is possible to resolve the band of vector DNA and one or more bands of insert DNA, depending on the number of *Not*I sites in the cloned insert (Fig. 3.15).

BAC DNA obtained from minipreparations without organic extraction often contains an inhibitor of the restriction enzyme *Not*I. This problem can be overcome by diluting the DNA during digestion. For example, 5 µl of the resuspended DNA is normally completely digested with 3-5 units of enzyme in a reaction volume of 20 µl, whereas 5-10 units of enzyme may still not allows complete digestion of the same amount of DNA in 10 µl reaction volume.

At least 15-20 white colonies from each ligation mixture used in the small-scale plating were examined to determine the average size of the BAC insert DNA. After determining the size range of clones obtained from a particular ligation, the remainder of the transformation mixture was plated, when the size range of clones was desired.
During this analysis, plates were stored at 4 °C for up to 2 weeks before the colonies were picked. The plates should be sealed to prevent the loss of moisture and should be inspected frequently for fungal contamination.

Fig.3.16 shows a PFGE of *Not*I-digested DNA for determining the insert sizes of a collection of randomly picked BAC clones. Most of these BAC clones show one or two bands derived from the cloned sunflower DNA, thus indicating the presence of zero or only one internal *Not*I site in the cloned sequences, respectively.

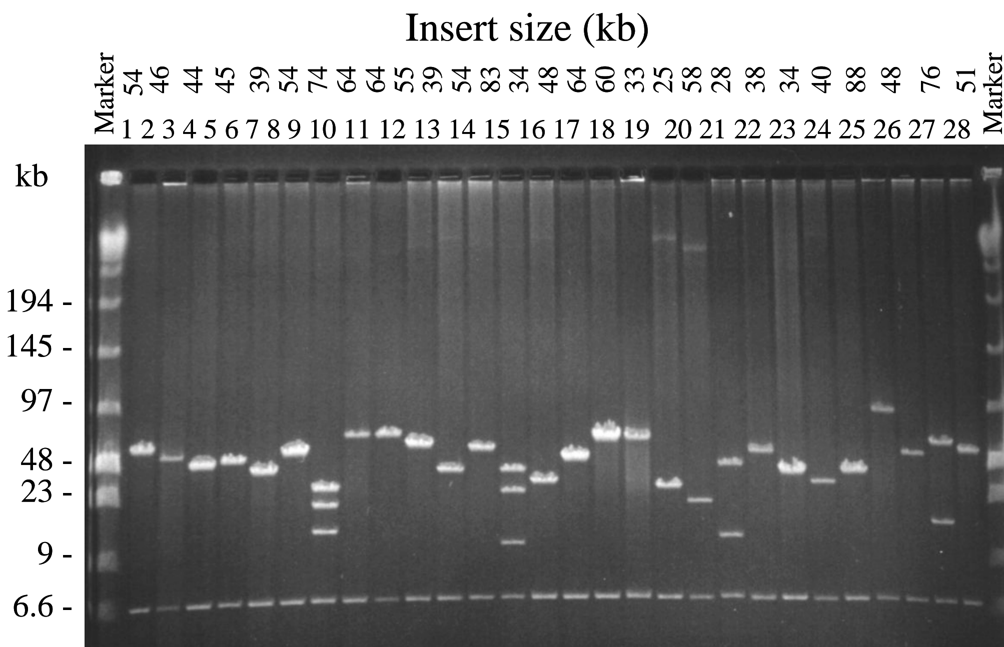

Fig.3.16 Size determination of BAC clones via minipreparation. BAC clones were isolated by an alkaline lysis procedure and digested with *Not* I. Restricted DNA was separated on a 1 % pulsed field gel performed under conditions of 6 V/cm, 5 inital-15 final pulses, 16 hours, 14°C.

When multiple bands are seen on the pulsed-field gel of *Not*I-digested DNA, the bands may reflect either true internal *Not*I sites or incomplete digestion by the enzyme. To diagnose incomplete digestion from these gels, it is necessary to compare the intensity of the band in correspondence to the vector DNA. Because the yield of BAC DNA from different clones is fairly consistent, the intensity of ethidium bromide fluorescence in the band of vector DNA should be relatively uniform form lane to lane. If no band of vector DNA is visible in the lane but there is a large band giving an intense signal equal to that of large bands in other lanes, it should be assumed that the digestion was incomplete (with the large band corresponding to the vector and insert still joined together). Since ethidium bromide fluorescence is directly proportional to the mass of the DNA in each band, partial digestion should also be suspected if multiple bands are present in a lane, but the differences between their intensities do not correspond to the apparent sizes of the DNA.

BAC clones may yield no visible bands of insert DNA on the gel. These BAC clones can be assumed to contain fragments of insert DNA that are so small that they either run off the gel or are too weakly stained due to the small size. Alternatively, they may be vectors without inserts, which were purified from colonies that resulted from an alteration in the

cloning site (the anticipated frequency of these clones should be known from the control ligations). An alteration in the cloning site is usually a deletion that results from nuclease contamination.

Ineffective digestion with *Not*I is often difficult to recognize and is frequently confused with complete failure to obtain DNA. This is because BAC DNA that is nicked, but not linearised by the enzyme during the incubation at 37 °C will remain in the well during PFGE. In this case, no bands will be visible on the gel, which will also be true if the BAC DNA purification failed. However, if the same minipreparation of DNA is digested with an enzyme less sensitive to inhibition (e.g., *Hind*III), clear bands will be present, thus confirming that BAC DNA was obtained.

In addition, although the low copy number of BAC means that much less DNA can be purified from these vectors than from multicopy vectors, sufficient DNA can be obtained from a few millilitres of bacterial culture for common applications such as restriction analysis, preparation of DNA hybridisation probes, FISH, or PCR.

Distribution of the insert size of randomly selected BAC clones from different ligations used for the construction of BAC library in sunflower is shown in Fig.3.17. A total of 179 clones was investigated after *Not*I digest. The size of the BAC clones varies between 20 kb and 270 kb. The majority of BAC clones carries inserts between 40 kb and 80 kb. Therefore the average insert size was estimated to be 50 kb. But also BAC inserts bigger than 100 kb in size were observed.

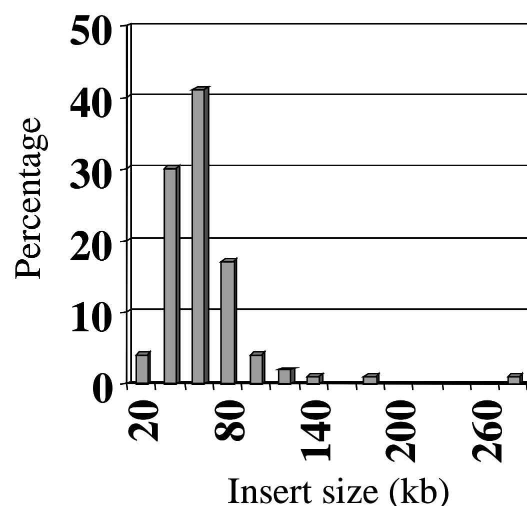

Fig.3.17 Distribution of the insert size in sunflower BAC library estimated according to *Not*I digest.

3.8.3 Preparation of Filters from the BAC library

A set of four filters represents the total sunflower BAC library of 104,736 clones stored in 273x384-well microtiter plates. The clones of plates 1-72, 73-144, 145-216, and 217-273 were spotted onto 22 cm x 22 cm Hybond N+ membranes A, B, C, and D, respectively. All filters were spotted in 8 copies. Each membrane is divided into 6 fields. Each field contains 384 squares (16x24 squares). Within each square there are 24 positions (5x5 square, the middle of the square is left free) where 12 clones are spotted in duplicate. For spotting 0.4 mm dia pins were used by a BioGrid (BioRobotics) in Max-Planck-Institute in Cologne. Each of filters contains 55,296 spots. Because of the duplication, each filter contains only 27,648 individual clones which represents in 72x384-well microtiter plates.

The membranes were placed over night on LB agar plates containing 12.5 µg/ml chloramphenicol at 37°C. After growing the colonies to a size of 1-2 mm in diameter, the BAC library was fixed with different washing steps on the filters. Then the membranes can be stored at room temperature for months. The membranes have been successfully controlled by hybridisation using a cDNA clone for the *sf21* gene in sunflower (Kräuter et al. 1996). Giving four positive signals which is in the range of the expected number of clones by a twofold genome coverage.

4 DISCUSSION

4.1 Large-Insert Genomic DNA Libraries

Large-insert genomic libraries are very important for the isolation and characterisation of genomic regions and genes. The development of tools like pulsed-field gel electrophoresis (PFGE), cosmids (Collins and Hohn 1978), yeast artificial chromosomes (YAC) (Burke et al. 1987), and bacterial artificial chromosomes (BAC) (Shizuya et al. 1992) allow the cloning of large genomic DNA fragments and lead to the physical characterisation of complex genomes and the isolation of many important genes for which only the phenotypes and the map positions were known so far (Zhang et al. 1996).

Cosmids have been the first vectors for cloning large DNA fragments up to approximately 40 kb in plants. The lambda cos sites allowed the DNA to be packaged *in vitro* and to be used for transfection into *E. coli*. Cosmid vectors have also been modified into plant transformation vectors and thus can be efficiently transferred into plants using *Agrobacterium tumefaciens* (Zhang et al. 1996).

With the creation of YACs (Burke et al. 1987), cloning of megabase-sized DNA fragments became possible. YACs have been used for physical mapping, contig construction, map-based cloning, and the isolation of genes (Arondel et al. 1992, Giraudat et al. 1992, Leyser et al. 1993, Martin et al. 1993). The YAC vector system is designed to maintain foreign DNA fragments in yeast as linear chromosomes. The vector system contains all elements of a eukaryotic chromosome, including telomeres, a centromere and a yeast origin of DNA replication, which are required for chromosome maintenance and stability. YAC libraries have been constructed for man (Burke et al. 1987), mouse (Chartier et al. 1992) and plant species, such as *Arabidopsis* (Grill and Somerville 1991, Ward and Jen 1990, Ecker 1990), tomato (Martin et al. 1992), maize (Edwards et al. 1992), barley (Kleine et al. 1993), japonica rice (Umehara et al. 1995), sugarbeet (Eyers et al. 1992), and pepper (Tai and Staskawicz 2000). YACs were used to construct large-scale physical maps (Green and Olson 1990a, Silverman et al. 1991, Wada et al. 1990, Bronson et al. 1991, Coulson et al. 1988, Garza et al. 1989), to walk along large segments of mammalian chromosomes (Silverman et al. 1989), and to examine the structure and function of large genes. Recently, other large DNA cloning vectors have been developed, that are bacteriophage P1 (Sternberg 1990), the bacterial artificial chromosome (BAC) (Shizuya et al. 1992) and the P1-derived artificial chromosome (PAC) (Ioannou et al. 1994).

The BAC vector, developed from an *Escherichia coli* F´-factor plasmid, was shown to stable maintain human genomic DNA fragments of >300 kb (Shizuya et al. 1992). Most BAC vectors possess traditional plasmid selection features such as an antibiotic resistance gene and a polycloning site within a reporter gene. For construction of large-insert genomic libraries, BAC libraries have recently become the most popular large DNA cloning system as BACs have several advantages over YACs and other cloning systems.

When compared BACs versus YACs, the primary similarity between the two systems is that they can, in principle, handle any size of DNA that is cloned into the cloning site. Additionally, both system are maintained as low-copy vectors in the host cell.

The main difference between the two systems represents the host system: yeast versus bacteria the latter divide faster. Isolation of DNA from *E.coli* and transformation of bacteria are also easier than in yeast. These factors contribute to the speed and efficiency for the construction and analysis of individual BAC clones. In contrast, the cloning efficiencies of YAC are low (Smith et al. 1990). Also, it is difficult to isolate cloned DNA compared to bacterial systems, because yeast cells have a rigid cell wall and inserted fragments often comigrate with endogenous yeast chromosomes (Cai et al. 1995).

The transformation efficiency of the host cell is critical for the construction of large-insert DNA library. Under optimal conditions, a 1000-fold difference in transformation efficiencies between yeast and bacteria (10^7 versus 10^{10}) is observed and makes a significant difference during the construction of a complete library. Because of the higher transformation efficiencies for *E.coli*, the amount of size-selected DNA required to construct a complete BAC library is less than needed for a YAC library. Furthermore, BACs may be more efficiently introduced into cells than YACs, because the bacterial artificial chromosome is circular and more stable than the linear YAC DNA (Zhang et al. 1996).

YAC clones often possess insert rearrangements (deletions) or are chimeric, consisting of fragments of DNA from different regions of the target genome (Green and Olson, 1990a), especially in very large insert libraries (>500kb) (Burke 1990, Neil et al. 1990, Green et al. 1991, Anderson 1993, Libert et al. 1993, Venter et al. 1996, Cai et al. 1998). Such clones are unsuitable for sequencing and mapping. The elimination of chimeric clones and clones with rearranged inserts takes long time (Green et al. 1991, Anderson, 1993, Venter et al. 1996). Both chimera and deletions can cause serious problems in chromosome walking

strategies, as walks will be directed into the wrong direction by chimera or are stopped by deletions (Cai et al. 1995).

In the early 1990s, bacterial artificial chromosomes (BAC) have been developed as an alternative to YACs (Shizuya et al. 1992). In particular, BACs are relatively immune to chimerism and insert rearrangements (Woo et al. 1994, Cai et al. 1995, Kim et al. 1996, Boysen et al. 1997, Venter et al. 1996, Venter et al. 1998). The human and plant DNA have been shown to be quite stable in BAC vectors after 100 generations (Shizuya et al. 1992, Woo et al. 1994). Especially, the F factor genes (par A and par B) that prevent more than one BAC from being simultaneously present a bacterium, are responsible for the stability of BAC inserts (Willetts and Skurray, 1987, Shizuya et al. 1992, Cai et al. 1998). The manipulation of cloned DNA in BACs is easier than in YACs because supercoiled, circular plasmid DNA is resistant to shearing. However, a disadvantage of F factor-based system for chromosome walking is that the average size of cloned DNA inserts is smaller than in YACs (Shizuya et al. 1992).

Consequently, BACs are preferred to YACs for construction of large-insert DNA libraries and for physical mapping and sequencing (Cai et al. 1998, Kelley et al. 1999).

4.2 Isolation of High Molecular Weight DNA

Recent developments of PFGE technology (Schwartz and Cantor, 1984, Carle et al. 1986, Chu et al. 1986) and cloning systems for large DNA fragments, such as YACs (Burke et al. 1987) and BACs (Shizuya et al. 1992), has led to valuable information about complex genomes of higher organisms and to the isolation of genes known only by map position and phenotype (Woo et al. 1995). In most higher organisms there is a gap of approximately two orders of magnitude between distances of markers on genetic linkage maps and physical distances determined by cloning, chromosome walking or DNA sequencing. This gap has been closed by the technique of pulsed field gel electrophoresis and large-insert DNA cloning system. PFGE makes it possible to separate and analyse fragments of DNA as large as several million base pairs (Ganal and Tanksley, 1989). A prerequisite for such investigations is DNA of high molecular weight (megabase-size) and high quality. It is more demanding to isolate such DNA from plants than from mammalian cells. Because plant cells have a rigid cell wall, the most common methods for plant megabase-size DNA

isolation include isolation of protoplasts or nuclei, embedding of protoplasts or nuclei in an agarose matrix, cell lysis and protein degradation (Woo et al. 1995)

The techniques for the isolation of megabase-size DNA have been developed for some plant species, like tomato, barley, rice, soybean and sorghum (Ganal and Tanksley 1989, Daelen et al.1989, Sorensan 1989, Sobral et al. 1990, Siedler and Graner 1991, Honeycutt et al. 1992, Hatano et al. 1992, Guidet and Langridge 1992, Wing et al. 1993, Zhang et al. 1995, Woo et al. 1995).

4.2.1 Preparation of HMW DNA from Protoplasts

For protoplast isolation the quality of the leaf material is essential. There are basically two ways to grow plants for protoplast isolation that are *in vitro*-grown plants and greenhouse-grown plants. Each of these has its own advantages and disadvantages.

In vitro-grown plants are good for protoplast isolation because they are grown under uniform conditions and high humidity. The major disadvantage is the large amount of work required to maintain such plants in sufficient numbers under sterile conditions. Ganal et al. (1996) observed two critical points with *in vitro*-grown plants. One is that such plants usually contain large amounts of starch. The other point is that leaves from such plants are very tender (and small) and require short times (1-3 h) of incubation with macerating enzymes. Thus, the digestion should be monitored very carefully to avoid overdigestion.

The main advantage of greenhouse-grown plants is that large amounts of tissue are readily available at any given time without major efforts. This is essential for large-scale isolation of protoplasts. The disadvantage is that the material is not entirely uniform in its digestibility due to varying growth conditions. Such plants require longer times of incubation with macerating enzymes.

For the high molecular weight DNA isolation from sunflower, plants were grown in the greenhouse, under high humidity to keep the leaves more tender and uniform. The optimal stage for harvesting leaves was observed to be two weeks after germination. It was shown that leaves of the older sunflower plants were more difficult to digest and contained more starch. Expanding leaves were preferred to mature leaves because the vacuole is smaller, the leaves softer, and the amount of starch few older.

Furthermore, the plant cell walls make it difficult to prepare HMW DNA from plant tissues. Therefore, the cell walls must first be removed before the cells are embedded in

agarose. Therefore, the most widely used methods in plant systems for preparation of HMW DNA involve the isolation of protoplasts by cell wall hydrolysis and subsequent embedding of the protoplasts in agarose (van Daelen et al. 1989, Ganal and Tanksley 1989, Cheung and Gale 1990, Honeycutt et al. 1992, Wing et al. 1993, 1994). Since the cell wall structure varies for different plant species, a method for protoplast isolation of one species can often not be directly used for the protoplast isolation of another species (Zhang et al. 1995). Therefore, the isolation of HMW DNA from protoplasts had to be first optimised for sunflower (Krämer 1998).

According by in own experiments an enzyme combination of cellulase and pectolyase was used for the isolation of protoplasts from sunflower because the starch grains represent a big problem for protoplast isolation. If the cells contain large starch grains, these can destroy the protoplasts during centrifugation. Furthermore, large amounts of starch in agarose blocks prevent restriction enzymes from digesting the high-molecular-weight DNA. Large amounts of starch are accumulated if the plants are grown under light high-intensities or if the tissues are too old. Consequently, starch accumulation can be reduced by growing the plants under lower light intensity, by keeping the plants in the dark for 1-2 days before protoplast isolation, or by harvesting leaves from very young plants whose leaves are still expanding (Ganal et al. 1996).

The concentration of the DNA is also important for the suitability of HMW DNA for BAC cloning. If the DNA concentration is too low, it will not be recovered efficiently during the preparative steps and will not produce enough clones. If he DNA concentration is too high, some small DNA fragments will be inseparable from the larger molecules during preparative electrophoresis and thus lead to the production of clones containing small inserts (Birren et al.1997).

The isolation of reasonable amounts of high-molecular-weight DNA from protoplasts depends on the protoplast quality. A preparation with large amounts of protoplasts usually has some debris from lysed protoplasts. The yield of protoplast must be higher than $1x10^6$ protoplasts/ g of leaf tissue. A yield of approximately $5\text{-}10x10^6$ protoplasts/g of leaf tissue is typical for tomato and other plants (Ganal et al. 1996). For sunflower, the yield was approximately $2\text{-}8x10^6$ protoplasts/g of leaf tissue.

The size of high-molecular-weight DNA isolated from plant protoplasts is usually in the range of a few million base pairs (Ganal and Tanksley 1989, Woo et al. 1995). The

sunflower HMW DNA prepared by protoplast isolation method was larger than 2 Mb in size and readily digestible with restriction enzymes.

4.2.2 Preparation of HMW DNA from Plant Nuclei

The sunflower HMW DNA was prepared from protoplasts and cell nuclei. However, 99.6 % of the sunflower BAC library was constructed with HMW DNA isolated from nuclei.

Protoplast isolation on a large scale is extremely time-consuming, costly and tedious. Furthermore, HMW DNA prepared from plant protoplasts can contain a significant amount of chloroplast and mitochondria DNA (Martin et al. 1992, Wing et al. 1993, Woo et al. 1994), which could potentially mislead chromosome walking studies using libraries constructed from such HMW DNA due to homology between some organellar and nuclear DNA sequences (Timmis and Scott 1983). It was shown that 6-8 % of the tomato YAC clones (Martin et al. 1992, Wing et al. 1993) and 14 % of sorghum BAC clones (Woo et al. 1994) constructed from protoplasts originated from chloroplast DNA. In the presence of cloned chloroplast DNA, more clones are needed to be generated and screened in order to have an equal chance for isolation a particular nuclear DNA clone (Zhang et al. 1995). Therefore, only 0.4 % of the sunflower BAC library were constructed from the HMW DNA isolated from protoplasts.

For HMW DNA preparation of wheat and rye, Guidet and Langridge (1992) directly embedded the crushed tissues of wheat and related species in agarose plugs as an alternative to protoplast isolation. However, the HMW DNA prepared by this method was mixed with unlysed intact cells and tissue debris that might affect the access of restriction enzymes to the DNA. Hatano et al. (1992) isolated nuclei from rice germ tissues and embedded the nuclei in agarose plugs. The quality of HMW DNA prepared by this method was greatly improved, however, it is very difficult to obtain a sufficient quantity of germ tissue of many species for HMW DNA preparation.

Zhang et al. (1995) isolated HMW DNA from plant nuclei by physically breaking plant cell walls. Plant tissues was homogenised either by blending with a kitchen blender or by grinding in liquid nitrogen with mortar and pestle. The isolation of nuclei in this procedure is simple, and the HMW DNA embedded in microbeads is as easy manipulated as DNA in aqueous solution without significant shearing and the majority of the DNA prepared is over 5.7 Mb in size. This method was used in several divergent plant taxa, including

grasses (wheat, sorghum, maize), legumes (soybean, bean), vegetables (cauliflower, squash, eggplant, watermelon, pepper), and trees (peach, walnut, willow) (Zhang et al. 1995).

For sunflower the HMW DNA from plant nuclei was also isolated according to Zhang et al. (1995) with some modifications. The majority of the sunflower DNA isolated by this method was larger than 2 Mb in size. The sunflower HMW DNA prepared from nuclei was suitable for plant genome analysis by pulsed-field gel electrophoresis and for the construction of a BAC library.

4.3 Partial Digestion of HMW DNA

For BAC cloning, the HMW DNA was prepared and DNA of the desired size range isolated. With the desired DNA fragment size increasing fewer manipulations of the DNA can be tolerated. DNA fragmentation utilises two general approaches: 1) physical shearing or, 2) partial digestion with a restriction enzyme that cuts relatively frequently within the genome. Since physical shearing is not dependent upon the frequency and distribution of particular restriction enzyme sites, this method should yield the most random distribution of DNA fragments (Ward and Jen 1990). However, the ends of the sheared DNA fragments must be repaired and cloned directly or restriction enzyme sites must be added by ligation of synthetic linkers. These steps may damage the HMW DNA and lead to lower yields of clonable DNA. These required subsequent steps to clone DNA fragmented by shearing convince most researchers to fragment DNA by partial restriction enzyme digestion. The advantage of partial digestion is that no further enzymatic modifications of the ends of the restriction fragments are necessary (Wing and Choi 1997). Four common techniques can be used to achieve reproducible partial digestion of HMW DNA: 1) varying the concentration of the restriction enzyme (Burke and Olson 1991), 2) varying the time of incubation with the restriction enzyme (Anand, et al. 1989), 3) varying the concentration of an enzyme cofactor (e.g. Mg^{++}) (Albertsen et al. 1989), and 4) varying the ratio of endonuclease to methylase (Larin et al. 1991).

The sunflower HMW DNA was digested applying these four techniques with *Eco*RI which has a unique restriction site in pBeloBACKan. However, digestion with *Eco*RI was not successful. For Ganal et al. (1996), problems in cutting of HMW DNA can occur if not enough lysis buffer, which is used for cell lysis and protein degradation after isolation of HMW DNA, was added or if the DNA in the lysis buffer cannot be accessed by the

enzymes due to high concentrations of protoplasts in the blocks or the presence of large amounts of starch. Problems also arise if any solution used for handling the HMW DNA was contaminated by nucleases. A few molecules of DNase are sufficient to degrade high-molecular-weight DNA to sizes that are no longer useful for pulsed-field gel electrophoresis. This means that solutions (restriction enzymes, buffers, spermidine, etc.) that are successfully used for conventional DNA digests might not work for high-molecular-weight DNA (Ganal et al. 1996).

If the partial digestion was successful with *Eco*RI, a second problem occurred: cloning and transformation efficiencies with this DNA were low. The restriction enzyme *Eco*RI is an endonuclease that can show a high percentage of star activity. This might be the reason for the low cloning and transformation efficiency. Therefore, the vector pBeloBACKan was not used to construct the sunflower BAC library.

Instead, the vector pBeloBAC11, which has a unique *Hind*III restriction enzyme site was used. The sunflower HMW DNA was successfully partial by digested with *Hind*III. To optimise partial digestion with *Hind*III, different incubation times and different enzyme units were used. Finally, The sunflower BAC library was constructed with partially digested DNA using *Hind*III which showed a high cloning and transformation efficiency

Furthermore, the application of large-insert genomic DNA libraries require much more than simple analysis of restriction endonuclease digested DNA fragments on agarose gels. For these applications, the DNA fragments are cut, ligated, transformed. The handling of these sensitive and often very small biomolecules requires specific proactive laboratory procedures to prevent the loss or contamination of valuable samples. In addition, the DNA fragments may require desalting, buffer exchange, or elution from agarose during any of a number of steps.

For ligation, the DNA fragments in agarose gels are not adequate. Therefore, numerous methods were developed to purify DNA fragments away from the agarose gel matrix (Birren et al. 1997). Using Low Melting Point (LMP) agarose is a method for the purification of DNA from agarose gels. The separation properties of this agarose not as good as normal agarose, and the gel is very fragile. However, the major problem is that the large DNA molecule can not be protected from physical shearing during melting procedure.

Electroelution is a technique used for the recovery of nucleic acid material, particularly DNA, from agarose or polyacrylamide gels. The typical “in-house” method consists of sealing the cut gel piece containing the DNA in dialysis tube containing a suitable buffer. Then the dialysis tube is placed in buffer in an electrophoresis tank and voltage is applied to electroelute the DNA out of the gel. During the construction of the sunflower BAC library this method was successfully used to recover the sunflower DNA for the ligation

4.4 The Comparison of the pBeloBAC11 and pBeloBACKan

Unlike plasmids used for cloning DNA fragments of a few kilobases, such as pUC vectors (Yanisch-Perron et al. 1985), bacterial vectors intended for cloning large DNA fragments (>100 kb) are derived from molecules that ordinarily replicate long regions of DNA and are maintained at a very low copy number, approximately one copy per cell (Birren et al. 1997). BAC vectors are derived from the F factor of *E.coli*. The F factor naturally occurs as a 100-kb molecule, although F´ molecules that have incorporated portions of the *E.coli* genome can be ten times this size (Low 1972). BAC vectors contain the minimal sequences needed for the autonomous replication, copy-number control, and partitioning of the plasmid. The proteins required for these functions are encoded by genes *oriS, repE, parA, parB*, and *parC* and are derived from the F factor.

First, the vector pBeloBACKan which is a modified vector from pBeloBAC11 was used to construct a sunflower BAC library. For the development of pBeloBACKan, pBeloBAC11 (Kim et al. 1996) was modified to contain a unique *Eco*RI cloning site. Then, a Tn903-derived kanamycin resistance gene (Oka et al. 1981) was inserted by blunt-end cloning at the *Eco*RI site present in the chloramphenicol resistance gene in pBeloBAC11, resulting in pBeloBACKan (Mozo et al. 1998).

The pBeloBACKan was used to construct the IGF BAC library of *Arabidopsis thaliana* (Mozo et al. 1988). An *Arabidopsis thaliana* P1 library (Liu et al. 1995) and two BAC libraries (Choi et al. 1995, Wang et al. 1996) were established with other vectors, which differ greatly in clone sizes and genomic representation respectively. However, Mozo et al. (1998) used a different plant tissue as DNA source and another restriction enzyme for the generation of DNA fragments as compared to the P1 (Liu et al. 1995) and the two other BAC libraries (Choi et al. 1995, Wang et al. 1996). The IGF BAC library consists of

10,752 recombinant clones carrying inserts (generated by partial *Eco*RI digestion) of an average size of about 100 kb.

For sunflower, this success could not be obtained in constructing a BAC library with the vector pBeloBACKan which was kindly provided by Dr. Thomas Altmann and Dr. Theresa Mozo, MPI for Molecular Plantphysiology, Golm, and partial *Eco* RI digestion. Therefore, the common BAC vector pBeloBAC11 was used to construct the sunflower BAC library.

Different BAC libraries have already been constructed with BAC vector pBeloBAC11: for *Arabidopsis* (Choi et al. 1995, Wang et al. 1996), for bovine (Cai et al. 1995), for rice (Wang et al. 1995, Zhang et al. 1996), for soybean (Salimath and Bhattacharyya, 1999, Tomkins et al. 1999b), for *Medicago* (Nam et al. 1999), for sugarcane (Tomkins et al. 1999b), for tomato (Folkertsma et al. 1999), for potato (Song et al. 2000) for man (Kim et al. 1996), and for the pig (Suziki et al. 2000). The pBeloBAC11 is a 7.4 kb construct derived from a mini-F plasmid (O´Connor et al. 1989) and contains a gene for chloramphenicol resistance as a selectable marker. The cloning site of pBeloBAC11 is within the *lacZ* gene. Recombinant BACs in which DNA inserts have disrupted the *lacZ* gene can be identified by the conversion of colony colour from blue to white when grown on X-gal and the inducer IPTG (Yanisch-Perron et al. 1985).

The isolation of sufficient amounts of vector DNA for library construction required the growth of more than one liter of bacterial cell culture, because the BAC vector exists as a single copy in each *E.coli* cell. In addition, more effort was needed to purify the BAC vector from the *E.coli* chromosomal DNA, because of the large excess of *E.coli* DNA relative to the BAC vector DNA. For the sunflower BAC library pBeloBAC11 was purified successfully on a CsCl density gradient. The theoretical maximum yield of pBeloBAC11 from a litre of cells grown in LB medium is approximately 10 µg, with actual recovery being less. An entire library can be constructed from a few hundred nanograms of BAC vector DNA.

For success of construction of BAC library, it is essential that the pure plasmid DNA is obtained. Separation of plasmid and chromosomal DNAs by equilibrium centrifugation in CsCl (Caesium Chloride)-ethidium bromide density gradients depends on differences between the amounts of ethidium bromide that can be bound to linear and closed circular DNA molecules (Cantor and Schimmel 1980). Ethidium bromide binds to DNA by

intercalating between the bases, causing the double helix to unwind. This leads to an increase in the length of linear DNA molecules and to the introduction of compensatory superhelical turns in closed plasmid DNAs. Eventually, the density of these superhelical turns becomes so great that the intercalation of additional molecules of ethidium bromide is prevented. Linear molecules, which are not constrained in this way, continue to bind more dye until saturation is reached (~ 1 ethidium bromide molecule for every 2 base pairs). This differential binding of dye, results in different the buoyant densities of linear and closed circular DNA molecules in CsCl gradient containing saturating amounts of ethidium bromide (Sambrook et al., 1989). Purification of pBeloBAC11 by CsCl density gradient allowed to obtain pure plasmid preparation which could be used to construct the sunflower BAC library.

After CsCl gradient density, the vector was ligated with DNA fragments which had been generated by partial *Hind*III digestion. With a high efficiency, the ligated vector/insert DNA could be used to transform competent DH10B cells to construct the sunflower BAC library.

4.5 Characterisation of the BAC Library

Cloning of large genomic DNA into bacterial artificial chromosomes (BACs) provides a new approach for the analysis of genomes of higher organisms. For map based cloning of genes, like the *Pl2*-gene for downy mildew or the restorer gene *Rf1* in sunflower (3,000 Mb genome size) large DNA insert libraries are required for chromosome landing and chromosome walking strategies. In sunflower (*Helianthus annuus* L.), a BAC library was constructed from the restorer line RHA325 using pBeloBAC11 as vector.

The sunflower BAC library contains 104,736 clones, which were constructed from *Hind*III partially digested fragments that were once size-selected on a pulsed-field gel. To test the utility of the BAC library for map-based cloning and physical mapping, the BAC library was analyzed for the distribution of the insert size. To determine the distribution and the average insert size of the BAC library, DNA was isolated by minipreparation from 179 randomly picked clones. The sunflower BAC library has an average insert size of about 50 kb and represents approximately 2 genome equivalents. The insert size ranges from 20 to 270 kb and more than 65 % of the clones have inserts of 45 kb or larger than that.

Comparing the sunflower BAC library with other plant BAC libraries, the average DNA insert size of the sunflower BAC library is relatively small. For example, BAC libraries for *Arabidopsis* (Choi et al. 1995, Mozo et al. 1998), *medicago* (Nam et al. 1999), tomato (Folkertsma et al. 1999), and common bean (Vanhouten and MacKenzie 1999) have an average insert size of 100 kb, and the wheat BAC library (Lijavetzky et al. 1999) 115 kb. For the soybean BAC libraries, average insert sizes reach 105 kb (Salimath et al. 1999), 120 kb (Danesh et al. 1998), and 136 kb (Tomkins et al. 1999b). For a rice BAC library (Wang et al. 1995) 125 kb, sugarcane (Tomkins et al. 1999a) 130 kb and for the potato BAC library of Song et al. (2000) 155 kb were reported. The sorghum BAC library reported by Woo et al. (1994) has the largest average insert size, with 157 kb.
However, BAC cloning for DNA fragments of 80 kb and smaller is an extremely efficient process. For some applications, clones of this size are sufficient and an entire library can be generated rapidly. In general, difficulties in cloning are encountered when attempting to increase the size of the cloned fragments. According to Birren et al. (1997), the primary obstacles that limit the efficiency of cloning large DNA molecules as BACs appear to be (1) the increased frequency at which larger DNA molecules are damaged during their preparation and purification, (2) the preferential cloning of smaller DNA molecules that contaminate preparations of size-selected DNA, (3) the relative difficulty of ligating large DNA molecules to the vector DNA, and (4) the selective effects of electroporation, which introduce a bias against transformation of cells with large DNA.

By size selection small molecules (<80kb) must be eliminated because they will be cloned with a higher efficiency than the desired larger molecules, thus resulting in a small average insert size for the library. The main part of sunflower BAC library was constructed from single size selected DNA fragments. These single size selected DNA fragments can contain small DNA fragments, which can be cloned easier than the desired larger fragments. The use of single size selected DNA fragments is probably the reason for the small average insert size of the sunflower BAC library. Although the sunflower BAC library has a small average insert size, it is nevertheless suitable to analyze the sunflower genome.

With a second size selection, the small DNA fragments can be removed from desired larger fragments. It can be useful to perform a trial ligation with a portion of the DNA after the first size selection. If analysis of the clones reveals a high proportion of clones with small inserts, a second size selection with the remaining DNA will be necessary. For the

sunflower BAC library, a second size selection was also performed. About 2 % of the BAC library were constructed from double size selected DNA fragments. However, the second size selection reduced the transformation efficiency to a level where no library could be constructed anymore.

For the removal of the small DNA fragments pre-electrophoresis represents an alternative method. By using a short pulse field gel electrophoresis (1 h, 60s pulse, 5 V/cm, 120°) a separation of these undesired low molecular DNA fragments from the large fragments is possible (Edwards et al. 1992). The DNA fragments treated by pre-electrophoresis were also used for the construction of the sunflower BAC library.

Osoegawa et al. (1998) also explained that pre-electrophoresis of immobilized high-molecular-weight DNA removes inhibitors of the cloning process. Sizing DNA fragments twice within a single gel effectively eliminates small restriction fragments, thus increasing the average insert size of the clones and ensuring a relatively uniform insert size.

As HMW DNA is sensitive to physical shearing, the recovery of the size selected DNA from agarose is also important for the average insert size. Size-selected DNA can be recovered from agarose gels either by electroelution (Strong et al. 1997) or by using the enzyme agarase. Agarase completely digests the agarose fibers, but the agarose must be melted before the fibers can be digested efficiently. However, the steps in which the agarose is melted and the DNA handled as a liquid sample instead as a solid sample in agarose can be extremely damaging to the large DNA molecules. Electroelution system is more rapid and easier than using the enzyme agarase. This system requires fewer steps than the agarase system, therefore it is less likely for the DNA to be damaged. Whenever HMW DNA is handled as a liquid, pipetting should be minimised and performed slowly with wide-bore tips (Riethman et al. 1997). For the sunflower BAC library the size selected DNA was recovered from gel slides by electroelution to protect the DNA from physical shearing. HMW DNA molecules in solution are particularly susceptible to breakage by shearing because of the large length and the narrow width of the chains. Salts or polyamines included in samples containing DNA offers protection from breakage during handling because these compounds induce condensation of the DNA molecules (Riethman et al. 1997). Polyamines proved to be useful in increasing the average size of clones during YAC construction (Connelly et al. 1991). During electroelution of the sunflower HMW DNA, polyamines were added to protect the DNA in the electroelution buffer. However, this did not increase the transformation efficiency.

4.6 Applications of BAC Libraries

The BAC, system has recently become the most preferred large-insert DNA library system. Until now constructed BAC libraries have been employed in a variety of applications (Ordon et al. 2000).

For example, BAC-end sequencing is a useful technique used in association with chromosome walking to construct contigs. Direct sequencing from BAC DNA templates is used to obtain the sequences at the ends of the cloned DNA, by using primers that anneal to the BAC vector sequence and are oriented towards the insert (Marra et al. 1996, Boysen et al. 1997). The sequences generated by BAC-end sequencing represent regions of insert DNA adjacent to insert sites (“BAC ends”). Once BAC end sequences for a particular clone have been obtained, probes based on these end sequences can be used to screen the BAC library and find clones that overlap with the starting clone (i.e., chromosome walking). When applied on a large scale, probing libraries with BAC end sequences can lead to relatively rapid construction of physical maps. In chromosome walking, the end sequences of a “starter” clone, typically associated with an EST or RFLP marker, are used to probe colony blots/grids. DNA fingerprints of positive clones are compared to the fingerprint of the starter clone, and those exhibiting a minimal amount of overlap with the starter are grouped into a contig with this clone. A contig is a set of clones containing partially overlapping pieces of insert DNA that collectively represent a continuous stretch of genomic DNA.

BAC clones can also be used for chromosome landing (Tanksley et al. 1995). Since chromosome walking can be complicated by the occurrence of chimeric or rearranged HMW clones, and moreover in plants with large and complex genomes by the amount of DNA, the frequency of repetitive DNA and the non-finding of useful end clones, chromosome landing (Tanksley et al. 1995) is the most promising strategy to establish the physical coverage of the region of interest (Schwarz et al. 1999). Thus, one or more molecular markers are required within a physical distance from a target gene that is less than the average insert size of the YAC or BAC library being used for screening with the relevant markers. The detection of flanking molecular markers on a single genomic clone allows landing on the target gene without walking (Büschges et al. 1997, Schwarz et al. 1999).

The suitability of BACs as DNA sequencing/PCR templates has led to the development of BAC-end sequencing (Venter et al. 1996, Boysen et al. 1997, Rosenblum et al. 1997), supported advances in STS-based mapping (Venter et al. 1996, Venter et al. 1998), and provided a means to quickly search well-defined genomic regions for phenotypically significant genes (Bouck et al. 1998).

The “fingerprinting” by restriction digestion is a basic tool for analysing BACs, and for constructing physical maps. Fingerprinting protocols result in a list of sized fragments that are produced after digestion of a clone with one or more restriction enzymes. A comparison of fingerprints among different clones is used to (1) determine the relative sizes of the clones, (2) determine the extent of overlap among the clones, (3) determine the order of clones and the position of their ends on a physical map, and (4) detect any rearrangements of the genomic sequences contained within the clones. Nearly all the restriction fragments present in a clone can be observed by digesting the DNA with enzymes that cut the BAC DNA less frequently (such as *Hind*III or *Eco*RI), separating the products on agarose gels, and then staining the fragments with ethidium bromide. The majority of bands produced by digesting BACs with enzymes that cleave 6-base recognition sites will be smaller than 15 kb, i.e., conventional agarose gel electrophoresis, rather than PFGE, will resolve the fragments (Mara et al. 1997). In BAC-based physical mapping, DNA fingerprints of BAC clones can be compared. Those clones which have considerable overlaps in their fingerprint pattern can be grouped together into contigs (Marek and Shoemaker, 1997, Marra et al. 1997).

The use of BACs as large insert DNA cloning vector combined with the development of methods for high-throughput DNA fingerprinting (Marra et al. 1997), construction of contigs (Gillet et al. 1996, Soderlund et al. 1997, Ding et al. 1999), BAC-end sequencing, and STS-based mapping have helped investigators to bridge gaps between DNA markers in physically-large genomes. Consequently, many interesting and important genes have already been isolated (Wang et al. 1996, Nakamura et al. 1997, Yang et al. 1997, Cai et al. 1998, Danesh et al. 1998, Yang et al. 1998, Folkertsma et al., Moullet et al. 1999, Nam et al. 1999, Patocchi et al. 1999, Salimath and Bhattacharyya 1999, Sanchez et al. 1999). High-throughput physical mapping already has resulted in the construction of BAC contigs encompassing entire chromosomes and/or complete chromosome sets, e.g. in *Arabidopsis thaliana* (Mozo et al. 1999).

In addition, many of the DNA probes used to make genetic maps can be localised on specific BACs, providing a means of superimposing genetic maps directly onto BAC-based physical maps (Yang et al. 1997, Mozo et al. 1999). This feature also facilitates map-based cloning of genes responsible for specific phenotypes (Danesh et al. 1998, Nam et al. 1999, Patocchi et al. 1999, Sanchez et al. 1999). Map-based cloning represents a combination of physical mapping and genetic mapping in order to isolate a gene involved in a particular phenotype. Basically, genetic mapping is used to determine where on the molecular map the gene is located. Once two markers which closely flank the gene have been determined these can be used to identify BAC clones. When the maximum physical distance between the flanking markers is larger than the average insert size, new markers, closer to the gene, need to be found, or the physical distance between the available markers must be covered using a large insert genomic library (BAC, PAC, YAC). Physical mapping allows to build up a contig containing the DNA between the two markers. This contig, which presumably contains the gene of insert, can be further evaluated.

Furthermore, BACs have been successfully employed as probes for fluorescence *in situ* hybridization (FISH) (Cai et al. 1995, Hanson et al. 1995, Jiang et al. 1995, Lapitan et al. 1997, Gomez et al. 1997, Zwick et al. 1998, Godard et al. 1999). FISH-based localisation of cloned DNA sequences on chromosomes allows molecular and physical maps to be directly superimposed onto the framework of chromosomes, and subsequently provides useful information on the relationship between chromosome structure, DNA sequence and recombination (Peterson et al. 1999).

The assembly of genomic contigs using BAC clones will be the primary strategy for sequencing large complex genomes (Peterson et al. 2000). One of the most difficult obstacles in contig assembly is the closing of the gaps separating linearly related contigs along a chromosome. Several phenomena may account for these gaps, such as the presence of repetitive DNA sequences, insufficient coverage of the BAC libraries in certain chromosomal regions, or not enough DNA markers in certain genomic regions because of low recombination rates. These gaps have been present in almost every assembled contig reported (Zachgo et al. 1996, Umehara et al. 1997). It is almost impossible to size such gaps using standard fingerprinting or other molecular techniques. The FISH techniques using different targets, provide valuable tools for estimating the physical sizes of such gaps (Jackson et al. 1998).

Also the success of FISH mapping using large genomic DNA clones provides an alternative approach to map small DNA probes. The strategy is to isolate a large insert DNA clone by screening a library using a small DNA probe, then to map the large clone on chromosomes using FISH. Several laboratories have recently demonstrated that consistent *in situ* hybridisation signals can be generated on plant chromosome using large insert DNA clones as probes (Hanson et al. 1995, Fuchs et al. 1996, Lapitan et al. 1997, Ohmido et al. 1998, Song et al. 2000).

There are two approaches to characterise the structure and organisation of large genomic DNA fragments cloned in BACs. The first is the classical restriction mapping method. With restriction enzyme digestion, gel electrophoresis and gel-blot hybridisation, different restriction sites and subclones can be mapped within the BAC inserts. The second approach is to sequence the entire BAC using the “shotgun” sequencing method. Although sequencing provides the most definitive results, it is expensive and it is still difficult to assemble sequence data if the insert contains highly repetitive sequences. Therefore, new cost-effective and simplistic methods to physically analyse BAC inserts are desired. Jackson et al. (1999) developed a fast and economic technique to analyse the inserts of individual BAC molecules using FISH. By digital mapping of individual BAC molecules using FISH, DNA fragments can be digitally mapped on individual circular BAC molecules to determine their physical locations relative to the cloning vector. The latter authors demonstrated the utility of this technique for analysing the distribution and physical relationship of different repetitive elements, and for localising small single-copy subclones within BAC inserts (Jackson et al. 1999).

The advantage of BAC libraries over other types of large-insert DNA libraries, can be seen in the variety of possible applications of BACs which nowadays makes this system the most popular tool for cloning large DNA fragments.

In sunflower, a BAC library was constructed which now allows to apply this tool for physical mapping and gene isolation. Special emphasis will be on the application for map-based cloning of two genes which are present in the inbred line RHA325 which had been used as plant material for building the BAC library. These genes are the restorer gene *Rf1* and the *Pl2* gene conferring resistance to downy mildew (*Plasmopara halstedii*). The constructed BAC library represents an essential tool for further genome analysis and molecular breeding in sunflower.

5 SUMMARY

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops of the world. Because of the importance of sunflower oil, both, for human nutrition and industrial purposes, this oil crop is one of the major objects of research. At the present time, breeding research mainly focuses on biotechnology and genomics. In genome research, large insert DNA clones represent a valuable tool for the molecular characterisation of genomes. Pulsed field gel electrophoresis, yeast and bacterial artificial chromosome cloning techniques have allowed to manipulate DNA in the megabase size scale. These techniques efficiently permit cloning of large DNA fragments, which is essential for construction of genomic libraries by yeast artificial chromosomes (YAC) or bacterial artificial chromosomes (BAC), physical mapping of whole genomes, and analysis of large genomic or chromosomal regions.

Because BAC libraries are not associated with various disadvantages of YAC libraries, they have recently become the most useful tool for cloning large DNA fragments. Compared with YAC cloning, the advantages of BAC libraries are for example, high cloning efficiency, stable insertion of DNA, low amount of chimeric clones, efficient electroporation of BAC DNA into *E.coli* host, simple manipulation and isolation of the insert DNA.

An essential prerequisite for BAC cloning is the preparation of high molecular weight DNA (HMW DNA). Most of the widely used methods in plant systems for preparation of HMW DNA involve the isolation of protoplasts using cell wall hydrolysis and embedding of the protoplasts in agarose. An alternative method is the preparation of HMW DNA from plant nuclei. For sunflower which has a genome size of 3000 Mb, both methods for the isolation of high molecular weight DNA were successfully developed using leaf material from two weeks old seedlings.

Two BAC cloning vectors, pBeloBACKan and pBeloBAC11, which have an unique cloning site for *Eco*RI and *Hind*III, respectively, were tested for the construction of a BAC library for sunflower. Partial digestion of HMW DNA was obtained by varying the incubation time, the amount of enzyme, the concentration of Mg^{2+} as cofactor and the ratio of *Eco*RI to methylase. However, using *Eco*RI and pBeloBACKan as vector BAC cloning could not be successfully performed. Therefore, pBeloBAC11 was used in a second

approach. Partial digestion with *Hind*III only needed to be optimised with regard to the amount of restriction endonuclease and incubation time. Size fractionation of digested DNA fragments by PFGE allowed to isolate fragments between 200 and 500 kb for BAC cloning.

Using pBeloBAC11 and HMW DNA from nuclei preparations, a BAC library of sunflower was constructed for the restorer line RHA325, which is an open American inbred line based on the PET1-cytoplasm. This sunflower line also carries the *Pl2*-gene for resistance against downy mildew.

The current BAC library comprises 104,736 clones. A number of 179 BAC clones were picked at random and analysed by minipreparation for estimation of the average insert size, which turned out to be 50 kb. The insert size of clones varied between 20 kb and 270 kb with the majority of clones between 40 and 60 kb. According to the average insert size, the sunflower BAC library covers a 1.7x genome equivalent. The stability of BAC inserts was demonstrated by two clones with a size of 72 and 100 kb for 11 and 12 generations. The whole BAC library was spotted in duplicate on four filters, each carrying 55,296 clones. Hybridisation of these filters against a cDNA clone (*sf21*) revealed four positive clones which is in the expected range of genome coverage.

The BAC library is now available for map-based cloning approaches of the resistance gene *Pl*2 and the restorer gene *Rf*1 which require a large insert DNA library for chromosome walking and chromosome landing. In addition, the constructed BAC library represents an essential tool for any further genome analyses e.g. fluorescence in situ hybridisation (FISH) in sunflower.

6 ZUSAMMENFASSUNG

Die Sonnenblume (*Helianthus annuus* L.) ist weltweit eine der bedeutendsten Ölpflanzen. Die Erforschung des Sonnenblumengenoms hat aufgrund der großen Bedeutung des Sonnenblumenöls für die menschliche Ernährung und für die chemische Industrie eine hohe Priorität. In diesem Zusammenhang stellen Genbanken mit großen genomischen DNA-Inserts ein neues methodisches Hilfsmittel für die Genomforschung bei Pflanzen dar. Pulsfeld-Gelelektrophorese (PFGE), YAC´s und BAC´s ermöglichen die Bearbeitung von Megabasen-DNA. Diese Techniken erlauben die effiziente Klonierung großer DNA-Fragmente, die für die Konstruktion genomischer Bibliotheken, die physische Kartierung ganzer Genome, und die Analyse von großen Genombereichen oder chromosomalen Regionen erforderlich sind.

Zur Gruppe der Genbanken mit großen genomischen DNA-Inserts gehören Bacterial artificial chromosome (BAC)-Bibliotheken und yeast artificial chromosome (YAC) Bibliotheken. Im Vergleich zu den YAC-Bibliotheken besitzen die BAC-Bibliotheken weniger Nachteile und sind daher heute die am weitesten verbreitete Methode zum Zwecke des Klonierens großer DNA-Fragmente. Vorteile der BAC-Bibliotheken sind z.B. die hohe Klonierungseffizienz, die stabile Insertion der DNA, ein geringes Ausmaß an chimären Klonen, einfaches und effektives Transformieren der Bakterienzellen (*E. coli*) mit der BAC-DNA und die einfache Isolierung der DNA aus den Bakterienzellen.

Essentielle Voraussetzung für die BAC-Klonierung stellt die Präparation hochmolekularer DNA dar. Überwiegend wird zur Präparation hochmolekularer (high molecular weight, HMW) DNA die Isolierung von Protoplasten, unter Zuhilfenahme der Hydrolyse von Zellwänden und anschließender Einbettung der Protoplasten in Agarose, verwendet. Eine Alternative stellt die Präparation der HMW-DNA aus Zellkernen dar. Für die Sonnenblume, die eine Genomgröße von 3000 Mb hat, wurden beide Methoden zur Isolierung hoch molekularer DNA erfolgreich optimiert. Als Ausgangsmaterial dienten dabei Blätter von zwei Wochen alten Sonnenblumenkeimlingen.

Zwei BAC-Klonierungsvektoren, pBeloBACKan und pBeloBAC11, die jeweils eine einzige Klonierungsstelle für *Eco*RI bzw. *Hind*III besitzen, wurden im Hinblick auf die Erstellung einer BAC-Biblioethek für die Sonnenblume untersucht. Ein partieller Verdau der HMW-DNA konnte durch Veränderung der Inkubationszeit, der Menge an Enzym, der Konzentration von Mg^{2+} als Cofaktor und des Verhältnisses von *Eco*RI zur Methylase

erreicht werden. Allerdings konnte mit *Eco*RI und pBeloBACKan keine erfolgreiche BAC-Klonierung durchgeführt werden. Deshalb wurde in einem zweiten Ansatz pBeloBAC11 eingesetzt. Der partielle Verdau mit *Hind*III mußte hier nur im Hinblick auf die Menge an der Restriktionsendonuclease und Inkubationszeit optimiert werden. Die Größenselektion der verdauten DNA-Fragmente über PFGE erlaubte die Isolierung von Fragmenten mit einer Größe von 200 bis 500 kb für die BAC-Klonierung. Unter Verwendung von pBeloBAC11 und HMW-DNA aus Kernpräparationen konnte eine BAC-Bibliothek der Sonnenblume für die Restorerlinie RHA325, die eine offene amerikanische Inzuchtlinie basierend auf dem PET1-Plasma darstellt, erstellt werden. Dieser Sonnenblumenlinie trägt außerdem das Resistenzgen *Pl2* gegen den Falschen Mehltau (*Plasmopara halstedii*).

Die aktuelle BAC-Bibliothek besteht aus 104.736 Klonen. Zur Größenbestimmung der Inserts wurden 179 BAC-Klone, die zufällig ausgewählt wurden, mit Hilfe der Minipräparation analysiert. Die Größe der Inserts liegt zwischen 20 und 270 kb, und die durchschnittliche Größe der Inserts beträgt 50 kb. Demnach repräsentiert die BAC-Bibliothek 1,7 Genom-Äquivalente. Die Stabilität der BAC-Inserts wurde anhand von zwei Klonen mit einer Größe von 72 und 100 kb über 11 bzw. 12 Generationen demonstriert. Die gesamte BAC-Bibliothek wurde doppelt auf vier Filter gespottet. Jeder dieser Filter trägt 55.296 Klone. Die Hybridisierung dieser Filter gegen einen cDNA-Klon (*sf21*) erlaubte die Identifizierung von vier positiven Klonen. Dies liegt in der erwarteten Größenordnung der Genomabdeckung.

In der näheren Zukunft sollen mit Hilfe der map-based-cloning Strategie die Gene *Pl2* und *Rf1* aus der Linie RHA325 isoliert werden. Die in der vorliegenden Arbeit erstellte BAC-Bibliothek ist eine essentielle Grundlage für diese weiterführenden Experimente (chromosome landing, chromosome walking) zur Isolierung dieser Gene und aber auch für andere künftige Analysen des Sonnenblumengenoms z.B. Fluoreszenz *in situ* Hybridisierungen (FISH).

7 REFERENCES

ALBERTSEN, H.M., PASLIER, D.L., ABDERRAHIM, H., 1989. Improved control of partial DNA restriction enzyme digest in agarose using limiting concentration of Mg. Nucleic Acids Res. 17, 608.

ANAND, R., VIASANTE, A., TYLER-SMITH, C., 1989. Construction of yeast artificial chromosome libraries with large insert using fractionation by pulsed-field gel electrophoresis. Nucleic Acids Res. 17, 3425-3433.

ANASCHENKO, A. V., 1982. Centres of genome origin and centres of the greatest diversity of Helianthus species and fotus. Vestnik sel´skochozjajstvennoj nauki, Moskva 6, 47-53.

ANDERSON, C., 1993. Genome shortcut leads to problems. Science 259, 1684-1687.

ARONDEL, V., LEMIEUX, B., HWANG, I., GIBSON, S., GOODMAN, H. M., and SOMERVILLE, C. R., 1992. Map-based cloning of gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. Science 258, 1353-1355.

ARUMUGANATHAN, K. and EARLE, E. D., 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. 9, 208-218.

BERRY, S. T., LEON, A. J., HANFREY, C. C., CHALLIS, P., BURKHOLZ, A., BARNES, S. R., RUFENER, G. K., LEE, M., CALIGARI, P. D. S., 1995. Molecular marker analysis of *Helianthus annuus* L. 2. Construction of a RFLP linkage map for cultivated sunflower. Theor Appl. Genet. 91, 195-199.

BIRREN, B., LAI, E., 1993. Pulsed Field Gel Electrophoresis, A Practical Guide. Academic Press, INC.

BIRREN, B., GREEN, E.D., KLAPHOLZ, S., MYERS, R.M., RIETHMAN, H., ROSKAMS, J., 1997. Bacterial Artificial Chromosomes. *In Genome Analysis: A laboratory manual. Vol. 3 Cloning Systems* (ed. B. Birren et al.), pp. 242-295. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

BOUCK, J., MILLER, W., W., GORRELL, J.H., MUZNY, D., GIBBS, R. A., 1998. Analysis of the quality and utility of random shotgun sequencing at low redundancies. Genome Res. 8, 1074-1084.

BOYSEN, C., SIMON, M. I., HOOD, L., 1997. Fluorescence-based sequencing directly from bacterial and P1-derived artificial chromosomes. BioTechniques 23, 978-982.

BRAHM, L., FRIEDT, W., 1996. Identifizierung von RAPD-Fragmenten mit Kopplung an die Resistenz gegen den Falshen Mehltau (Rasse 2) der Sonnenblume. Vortr. Pflanzenzüchtg. 32, 106-108.

BRAHM, L., RÖCHER, T., HORN, R., PRÜFE, M., KÖHLER, H., FRIEDT, W., 1998. Mapping downy mildew resistance in sunflower. ISA Symposium III Sunflower Downy Mildew, 13th-14th January, Fargo, ND, USA.

BRONSON, S. K., PEI, J., TAILLON-MILLER, P., CHORNER, M. J., GERAGHTY, D. E., CHAPLIN, D. D., 1991. Isolation and characterisation of yeast artificial chromosome clones linking the HLA-B and HLA-G loci. Proc. Natl. Acad. Sci. USA 88, 1676-1680.

BURKE, D. T., CARLE, G. F., OLSON, M. V., 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236, 806-811.

BURKE, D. T., 1990. YAC cloning : options and problems. GATA 7, 94-99.

BURKE, D. T., OLSON, M. V., 1991. Preparation of clone libraries in yeast artificial chromosome vectors. Methods in Enzymology 194, 251-270.

BURRUS, M., CHANABE, C., ALIBERT, G., BIDNEY, D., 1991. Regeneration of fertile plants from protoplasts of sunflower (*Helianthus annuus L.*). Plant Cell Report 10, 161-166.

BÜSCHGES, R. HOLLRICHER, K., PANSTRUGA, R., SIMONS, G., WELTER, M., FRIJTERS, A., VAN DAELEN, R., VAN DER LEE, T., DIERGARDE, P., GROENENDIJK, J., TÖPSCH, S. VOS, P., SALAMINI, F., SCHULZE-LEFERT, P., 1997. The barley *mlo* gene: a novel control element of plant pathogen resistance. Cell 88, 695-705.

CAI, L., TAYLOR, J. F., WING, R. A., GALLAGHER, D. S., WOO, S.–S., DAVIS, S. K., 1995. Construction and Characterisation of a Bovine Bacterial Artificial Chromosome Library. Genomics 29, 413-425.

CAI, W. -W., RENEKER, J., CHOW, C. –W., VAISHNAV, M., and BRADLEY, A., 1998. An anchored framework BAC map of mouse chromosome 11 assembled using multiplex oligonucleotide hybridisation. Genomics 54, 387-397.

CANTOR, C. R., SCHIMMEL, P. R., 1980. Biophysical chemistry. Part III: The behaviour of biological macromolecules. Freeman Publications, San Francisco. p.p. 1251

CARLE, G. F., FRANK, M., OLSON, M. V., 1986. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. Science 232, 65-68.

CHARTIER, F. L., KEER, J. T., SUTCLIFFE, M. J., HENRIQUES, D. A., MILEHAM, P., BROWN, S. D., 1992. Construction of a mouse yeast artificial chromosome library in a recombinant-deficient strain of yeast. Nat. Genet. 1, 132-136.

CHOI, S., CREELMAN, R. A., MULLET, J. E., and WING, R. A., 1995. Construction and characterisation of a bacterial artificial chromosome library of *Arabidopsis thaliana*. Plant Mol. Biol. Rep. 13, 124-128.

CHOI, S., BEGUM, D., KOSHINSKY, H., OW, D. W., 2000. A new approach for the identification and cloning of genes: the pBACwich system using cre/lox site-specific recombination. Nucleic Acids Res. 28(7), E19.

CHU, G., VOLLRATH, D., DAVIS, R. W., 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science 234, 1582-1585.

COLLINS, J., HOHN, B., 1978. Cosmids: a type of plasmid gene-cloning vector that is packageable *in vitro* in bacteriophage lambda heads. Proc. Natl. Acad. Sci. USA 75, 4242.

CONNELLY, C., Mc CORMIK, M.K., SHERO J., HIETHER, P., 1991. Polyamines eliminate an extreme size bias against transformation of large yeast artificial chromosome DNA. Genomics 10, 10-16.

COULSON, A., WATERSTON, R., KIFF, J., SULSTON, J., and KOHARA, Y., 1988. Genomic linking with yeast artificial chromosomes. Nature 335, 184-186.

CUGI, 2000. The Clemson University Genomics Institute Homepage, http://www.genome.clemson.edu

DAELEN, R. A. J. v., JONKERS, J. J., and ZABEL, P., 1989. Preparation of megabase-sized tomato DNA and separation of large restriction fragments by field inversion gel electrophoresis (FIGE). Plant Mol. Biol. 12, 341-342.

DANESH, D., PENUELA, S., MUDGE, J., DENNY, R. L., NORDSTROM, H., MARTINEZ, J. P., YOUNG, N. D., 1998. A bacterial artificial chromosome library for soybean and identification of clones near a major cyst nematode resistance gene. Theor. Appl. Genet. 96, 196-202.

DING, Y., JOHNSON, M. D., COLAYCO, R., CHEN, Y. L., MELNYK, J., SCHMITT, H., SHIZUYA, H., 1999. Contig assembly of bacterial artificial chromosome clones through multiplexed fluorescence-labeled fingerprinting. Genomics 56, 237-246.

DOWER, W.J., MILLER, J.F., RAGSDALE, C.W., 1988. High efficiency transformation of *E.coli* by high voltage electroporation. Nucleic Acids Res. 16, 6127.

ECKER, J. R., 1990. PFGE and YAC analysis of the *Arabidopsis* genome. Methods, 1, 186-194.

EDWARDS, K. J., THOMPSON, H., EDWARDS, D., DE SAIZIEU, A., SPARKS, C., THOMPSON, J. A., GREENLAND, A. J., EYERS, M., and SCHUCH, W., 1992. Construction and characterisation of a yeast artificial chromosome library containing three haploid maize genome equivalents. Plant Mol. Biol. 19, 299-308.

EYERS, M., EDWARDS, K., SCHUCH, W., 1992. Construction and characterisation of a yeast artificial chromosome library containing two haploid *Beta vulgaris* L. genome equivalents. Genome 121, 195-201.

FAOSTAT, 1999. Faostat Statistics Database. http://apps.fao.org.

FRIEDT, W. and SCHEUERMANN, G., 1991. Aktuelle Ziele und Methoden der Sonenblumenzüchtung. Raps 9(2), 96-103.

FRIJTERS, A. C. J., ZHANG, Z., VAN DAMME, M., WANG, G-W., RONALD, P. C., MICHELMORE, R.W., 1997. Construction of a bacterial artificial chromosome library containing large *Eco*RI and *Hind*III genomic fragments of lettuce. Theor. Appl. Genet. 94, 390-399.

FOLKERTSMA, R. T., SPASSOVA, M. I., PRINS, M., STEVENS, M. R., HILLE, J., GOLDBACH, R. W., 1999. Construction of a bacterial chromosome (BAC) library of *Lycopersicon esculentum cv*. Stevens and its application to physically map the *Sw*-5 locus. Molecular Breeding 5, 197-207.

FUCHS, J., KLOOS, D.-U., GANAL, M. W., SCHUBERT, I., 1996. In situ localisation of yeast artificial chromosome sequences on tomato and potato metaphase chromosomes. Chromosome Res. 4, 277-281.

GANAL, M. W., TANKSLEY, S.D., 1989. Analysis of tomato DNA by pulsed-field gel electrophoresis. Plant Mol. Biol. Rep. 7, 17-27.

GANAL, M. W., 1996. Isolation and analysis of high-molecular-weight DNA from plants, In: Nonmammalian Genomic Analysis: A Practical Guide. Academic Press Inc., San Diego, 61-73.

GARZA, D., AJIOKA, J. W., BURKE, D. T., HARTL, D. L., 1989. Mapping the Drosophila genome with yeast artificial chromosomes. Science 246, 641-646.

GENTZBITTEL, L., VEAR, F., ZHANG, Y. X., BERVILLE´, A., 1995. Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 90, 1079-1086.

GILLET, W., HANKS, L., WONG, G. K. -S., LIM, R., OLSON, M. V., 1996. Assembly of high-resolution restriction maps based on multiple complete digests of a redundant set of overlapping clones. Genomics 33, 389-408.

GIRAUDAT, J., HAUGE, B. M., VALON, C., SAMILE, J., PARCY, F., GOODMAN, H. M., 1992. Isolation of the *Arabidopsis* AB13 gene by positional cloning. Plant Cell, 4, 1251-1261.

GODARD, S., OUSTRY-VAIMAN, A., CRIBIU, E. P., GUERIN, G., 1999. FISH mapping assignment of two horse BAC clones containing HMS41 and HTG3 microsatellites. Anim. Genet. 30, 233.

GOMEZ, M. I., ISLAM-FARIDI, M. N., WOO, S. -S., SCHERTZ, K. F., CZESCHIN, Jr. D., ZWICK, M. S., WING, R. A., STELLY, D. M., PRICE, H. J., 1997. FISH of a maize *sh2*-selected sorghum BAC to chromosome of *Sorghum bicolor*. Genome 40, 475-478.

GREEN, E. D., OLSON, M. V., 1990a. Chromosomal region of the cystic fibrosis gene in yeast artificial chromosomes: A model for human genome mapping. Science 250, 94-98.

GREEN, E.D., RIETHMAN, H.C., DUTCHIK, J.E., Olson, M.V., 1991. Detection and characterisation of chimeric yeast artificial-chromosome clones. Genomics 11, 658-669.

GRILL, E., SOMERVILLE, C. R., 1991. Construction and characterisation of a yeast artificial chromosome library of *Arabidopsis* which is suitable for chromosome walking. Mol. Gen. Genet. 226, 484-490.

GUIDET, F., LANGRIDGE, P., 1992. Megabase DNA preparation from plant tissue. Meth. Enzymol. 216, 3-12.

GUZMAN, P. and ECKER, J. R., 1988. Development of large DNA methods for plants: molecular cloning of large segments of *Arabidopsis* and carrot DNA into yeast. Nucleic Acids Res. 16, 11091-11105.

HAMILTON, C. M., FRAY, A., LEWIS, C., TANKSLEY, S. D., 1996. Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc. Natl. Acad. Sci. USA 93, 9975-9979.

HANSON, R. E., ZWICK, M. S., CHOI, S., ISLAM-FARADI, M. N., Mc KNIGHT, T. D., WING, R. A., PRICE, H. J., STELLY, D. M., 1995. Fluorescent *in situ* hybridisation of a bacterial chromosome. Genome 38, 646-651.

HATANO, S., YAMAGUCHI, J., HIRAI, A., 1992. The preparation of high-molecular weight DNA from rice and its analysis by pulsed-field gel electrophoresis. Plant Sci. 83, 55-64.

HONEYCUTT, R. J., SOBRAL, B. W. S., McCLELLAND, M., ATHERLY, A.G., 1992. Analysis of large DNA from soybean (*Glycine max* L. Merr.) by pulsed-field gel electrophoresis. Plant J. 2, 133-135.

HORN, R., HUSTEDT, J. E. G., HORSTMEYER, A., HAHNEN, J., ZETSCHE, K., FRIEDT, W., 1996. The CMS-associated 16 kDa protein encoded by *orfH522* in the PET1 cytoplasm is also present in other male sterile cytoplasms of sunflower. Plant Mol. Biol. 30, 523-538.

HUGGER, H., 1989. Sonnenblumen: Züchtung, Anbau, Verarbeitung. Verlag Eugen Ulmer, Stuttgart.

IOANNOU, P. A., AMEMIYA, C. T., GARNES, J., KROISEL, P. M., SHIZUYA, H., CHEN, C., BATZER, M. A., DE JONG, P. J., 1994. A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nature Genet. 6, 84-89.

JACKSON, S. A., DONG, F., JIANG, J., 1999. Digital mapping of bacterial artificial chromosomes by fluorescence in situ hybridisation. The Plant Journal 17 (5), 581-587.

JAN, C. C., VICK, B. A., MILLER, J. F., KAHLER, A. L., BUTLER, E. T. III., 1998. Construction of an RFLP linkage map for cultivated sunflower. Theor. Appl. Genet. 96,15-22.

JIANG, J., GILL, B. S., WANG, G. -L., RONALD, P. C., WARD, D. C., 1995. Metaphase and interphase fluorescence *in situ* hybridisation mapping of the rice genome with bacterial artificial chromosome. Proc. Natl. Acad. Sci. USA 92, 4487-4491.

KELLEY, J. M., FIELD, C. E., CRAVEN, M. B., BOCSKAI, D., KIM, U.–J., ROUNSLEY, S. D., ADAMS, M. D., 1999. High throughput direct end sequencing of BAC clones. Nucleic Acids Res. 27, 1539-1546.

KIM, U. J., BIRREN, B. W., SLEPAK, T., MANCINO, V., BOYSEN, C., KANG, H. L., SIMON, M. I., SHIZUYA, H., 1996. Construction and characterisation of a human bacterial artificial chromosome library. Genomics 34, 213-218.

KLEINE, M., MICHALEK, W., GRANER, A., HERRMANN, R. G., JUNG, C., 1993. Construction of a barley (*Hordeum vulgare* L.) YAC library and isolation of a *Hor*1-specific clone. Mol. Gen. Genet. 240, 265-272.

KLEINE, M., CAI, D., HERMANN, R: G., JUNG, C., 1995. Physical mapping and cloning of a translocation in sugar beet (*Beta vulgaris L.*) carrying a gene for nematode (*Heterodera schachtii*) resistance from *B. procumbens*. Theor. Appl. Genet. 90, 399-406.

KLOTZ, L.C., ZIMM, B.H., 1972. Size of DNA determined by viscoelastic measurements: results on bacteriophages, Bacillus subtilis and Escherichia coli. J Mol. Biol.,72(3), 779-800.

KORELL, M., FRIEDT, W., 1996. Anbauerfahrungen mit “high oleic” Sonenblumen auf Stillegungsflächen. Raps 14(2), 82-85.

KRÄMER, M., KÖHLER, H., FRIEDT, W., 1998. Entwicklung einer Megabasen-Technik für die Sonnenblume (*Helianthus annuus*). Vortr. Pflanzenzüchtung 42.

LAPITAN, N. L. V., BROWN, S. E., KENNARD, W., STEPHENS, J. L., KNUDSON, D. L., 1997. FISH physical mapping with barley BAC clones. Plant J. 11, 149-156.

LARIN, Z., MONACO, A., LEHRACH, H., 1991. Yeast artificial chromosome libraries containing large insert from mouse and human DNA. Proc. Natl. Acad. Sci. USA. 88, 4123-4127.

LECLERCQ, P., 1969. Une sterilite male cytoplasmique chez le tournesol. Ann. Amelior Plantes 19, 99-106.

LEYSER, H. M. O., LINCOLN, C. A., TIMPTE, C., LAMMER, D., TURNER, J., ESTELLE, M., 1993. *Arabidopsis* auxin-resistance gene *AXR*1 encodes a protein related to ubiquitin-activating enzyme E1. Nature, 364, 161-164.

LIBERT, F., LEFORT, A., OKIMOTO, R., WOMACK, J., GEORGES, M., 1993. Construction of a bovine genomic library of large yeast artificial chromosome clones. Genomics 18, 270-276.

LIJAVETZKY, D., MUZZI, G., WICKER, T., KELLER, B., WING, R. A., DUBCOVSKY, J., 1999. Construction and characterisation of a bacterial artificial chromosome (BAC) library for the A genome of wheat. Genome 42 (6), 1176-1182.

LIN, Y.-R., DRAYE, X., QIAN, X., REN, S., ZHU, L. –H., TOMKINS, J., WING, R. A., LI, Z., PATERSON, A. H., 2000. Locus-specific contig assembly in highly duplicated genomes using the BAC-RF method. Nucl. Acids Res. 28 (7), E23.

LIU, Y. G., MITSUKAWA, N., LISTER, C., DEAN, C., WHITTIER, R. F., 1996. Isolation and mapping of a new set of 129 RFLP markers in *Arabidopsis thaliana* using recombinant inbred lines. Plant J. 10, 733-736.

LOW, K. B., 1972. *Escherichia coli* K-12 F´ factors, old and new. Bacterial Rev. 33, 587-607.

MAREK, L. F., SHOEMAKER, R. C., 1997. BAC contig development by fingerprint analysis in soybean. Genome 40, 420-427.

MARRA, M., WEINSTOCK, L. A., MARDIS, E. R., 1996. End sequence determination from large-insert clones using energy transfer fluorescent primers. Genome Res. 6, 1118-1122.

MARRA, M. A., KUCABA, T. A., DIETRICH, N. J., GREEN, E. D., BROWNSTEIN, B., WILSON, R. K., McDONALD, K. M., HILLIER, L. W., McPHERSON, J. D., WATERSON, R. H., 1997. High throughput fingerprint analysis of large–insert clones. Genome Res. 7, 1072-1084.

MARTIN, G. B., GANAL, M. W., TANKSLEY, S. D., 1992. Construction of a yeast artificial chromosome library of tomato and identification of cloned segments linked to two disease resistance loci. Mol. Gen. Genet. 233, 25-32.

MARTIN, G. B., BROMMONSCHENKEL, S. H., CHUNWONGSE, J., FRARY, A., GANAL, M.W., SPIVEY, R., WU, T., EARLE, E. D., and TANKSLEY, S. D., 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262, 1432-1436.

MILLER, J. F., 1992. Update on inheritance of sunflower characteristics. In: Proc. 13th Int. Sunflower Conf., Pisa, Italy, 905-945.

MOULLET, O., ZHANG, H. -B., LAGUDAH, E. S., 1999. Construction and characterisation of a large DNA insert library from the D genome of wheat. Theor. Appl. Genet. 99, 305-313.

MOZO, T., FISCHER, S., SHIZUYA, H., ALTMANN, T., 1998a. Construction and characterisation of the IGF *Arabidopsis* BAC library. Mol. Gen. Genet. 258, 562-570.

MOZO, T., DEWAR, K., DUNN, P., ECKER, J. P., FISCHER, S., KLOSKA, S., LEHRACH, H., MARRA, M., MARTIENSSEN, R., MEIER-EWERT, S., ALTMANN, T., 1999. A complete BAC-based physical map of the *Arabidopsis thaliana* genome. Nature Genet. 22, 217-275.

MÖSGES, G., 1994. Molekulargenetische und isoenzymatische Untersuchungen an Sonnenblumen (*Helianthus annuus* L.) unter dem Aspekt der Markerselektion und

Charakterisierung von Linien and Hybriden. Dissertation, Justus-Liebig-Universität Geißen, 118 pages.

NAKAMURA, S., ASAKAWA, S., OHMIDO, N., FUKUI, K., SHIMIZU, N., KAWASAKI, S., 1997. Construction of an 800-kb contig in the near-centromeric region of the rice blast resistance gene *Pi-ta*^{2} using a highly representative rice BAC library. Mol. Gen. Genet. 254, 611-620.

NAM, Y.-W., PENMETSA, R. V., ENDRE, G., URIBE, P., KIM, D., COOK, D. R., 1999. Construction of a bacterial artificial chromosome library of *Medicago truncatula* and identification of clones containing ethylene-response genes. Theor. Appl. Genet. 98, 638-646.

NEIL, D.L., VILLASANTE, A., FISHER, R.B., VETRIE, D., COX, B., TYLER-SMITH, C., 1990. Structural instability oh human tandemly repeated DNA sequences cloned in yeast artificial chromosome vectors. Nucl. Acids Res. 18, 1421-1428.

O´CONNOR, M., PFEIFER, M., BENDER, W., 1989. Construction of large DNA segments in *Escherichia coli*. Science 244, 1307-1312.

OKA, A.,SUGISAKI, H., TAKANAMI, M., 1981. Nucleotide sequence of the kanamycin resistance transposon Tn*903*. J. Mol. Biol. 147, 217-226.

ORDON, F., KÖHLER, H., PELLIO, B., FRIEDT, W., 2000. Recombination: From Genetic Towards Physical Distances - High Resolution Mapping of Plant Resistance Genes. Prog. Bot. 61, 37-53.

OSOEGAWA, K., WOON, P. Y., ZHAO, B., FRENGEN, E., TATENO, M., CATANESE, J. J., DE JONG, P. J., 1998. An improved approach for construction of bacterial artificial chromosome libraries. Genomics 52 (1), 1-8.

ÖZDEMIR, N., KÖHLER, H., KRÄMER, M., FRIEDT, W., 1998. Preparation of high molecular weight DNA from sunflower. Proc. 4th Europ. Conf. on Sunflower Biotechnology, 20-23 October, Montpellier, France.

PATOCCHI, A., VINATZER, B. A., GIANFRANCESCHI, L., TARTARINI, S., ZHANG, H. -B., SANSAVINI, S., GESSLER, C., 1999. Construction of a 550 kb BAC contig spanning the genomic region containing the apple scab resistance gene *Vf.* Mol. Gen. Genet. 262, 884-891.

PETERSON, D. G., LAPITAN, N. L. V., STACK, S. M., 1999. Localisation of single- and low-copy sequences on tomato synaptonemal complex spreads using fluorescence *in situ* hybridisation (FISH). Genetics 152, 427-439.

PETERSON, D.G., TOMKINS, J.P., FRISCH, D.A., WING, R.A, PATERSON, A.H., 2000. Construction of plant bacterial artificial chromosome (BAC) libraries: An illustrated guide. Journal of Agricultural Genomics 5, (www.negr.org/research/jag).

PIERCE, J.C., SAUER, B., STERNBERG, N., 1992. A positive selection vector for cloning high molecular weight DNA by the bacteriophage P1 system: Improved cloning efficiency. Proc. Natl. Acad. Sci. USA 89, 2056-2060.

PRÜFE, M., BRAHM, L., FRIEDT, W., HORN, R., 1998. Erstellung einer kombinierten Genomkarte aus AFLP- und RFLP-Markern bei der Sonnenblume. Vortr. Pflanzenzüchtg. 42, 128-130.

RIETHMAN, H., BIRREN, B., GNIRKE, A., 1997. Preparation , manipulation, and mapping of HMW DNA. In Genome Analysis: A laboratory manual. Vol. 1 Analysing DNA (ed. B. Birren et al.), pp. 83-248. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

RÖCHER, T., BRAHM, L., FRIEDT, W., 1998. The genetics of different sources of sunflower downy mildew resistance . Proc. 4th Europ. Conf. On Sunflower Biotechnology, 20-23 October, Montpellier, France.

SACKSTON, W. E., 1981. Downy mildew of sunflower. In: The downy mildews. Spencer (ed.). Academic Press, London, New York, San Francisco, 545-575.

SALIMATH, S. S., BHATTACHARYYA, M. K., 1999. Generation of a soybean BAC library, and identification of DNA sequences tightly linked to the *Rps1*-k disease resistance gene. Theor. Appl. Genet, 98, 712-720.

SAMBROOK, J., FRITSCH, E. F., MANIATIS, T., 1989. Molecular Cloning, A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

SANCHEZ, A. C., ILAG, L. L., YANG, D., BRAR, D. S., AUSUBEL, F., KHUSH, G. S., YANO, M., SASAKI, T., LI, Z., HUANG, N., 1999. Genetic and physical mapping of *xa13*, a recessive bacterial blight resistance gene in rice. Theor. Appl. Genet. 98, 1022-1028.

SCHILLING, E. E., HEISER, C. B., 1981. Infrageneric classification of *Helianthus* (Compositae). Taxon 30, 393-403.

SCHWARTZ, D. C., CANTOR, C. R., 1984. Separation of yeast chromosome-sized DNAs by pulsed-field gradient gel electrophoresis. Cell 37, 67-75.

SCHWARZ, G., MICHALEK, W., MOHLER, V., WENZEL, G., JAHOOR, A., 1999. Chromosome landing at the *Mla* locus in barley (*Hordeum vulgare* L.) by means of high-resolution mapping with AFLP markers. Theor. Appl. Genet. 98, 521-530.

SEEBURG, J. R., SHINE, J., MARTIAL, J. A., BAXTER, J.D., GOODMAN, H.M., 1977. Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. Nature 270, 486.

SEILER, G. J., 1992. Utilisation of wild sunflower species for improvement of cultivated sunflower. Field Crops Research 30, 195-230.

SHENG, Y., MANCINO, V., BIRREN, B., 1995. Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucl Acids Res 23, 1990-1996.

SHENG, Y., MANCINO, V., BIRREN, B., 1995. Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucl. Acids Res. 23: 1990-1996.

SHIZUYA, H., BIRREN, B., KIM, U.-J., MANCINO, V., SLEPAK, T., TACHIIRI, Y., SIMON, M., 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. USA 89, 8794-8797.

SIEDLER, H., GRANER, A., 1991. Construction of physical maps of the *Hor*1 locus of two barley cultivars by pulsed-field gel electrophoresis. Mol. Gen. Genet. 226, 177-181.

SILVERMAN, G. A., YE, R. D., POLLOCK, K. M., SADLER, J.E., KORSMEYER, S. J., 1989. Use of yeast artificial chromosome clones for mapping and walking within human chromosome segment 18q21.3. Proc. Natl. Acad. Sci. USA 86, 7485-7489.

SILVERMAN, G. A., JOCKEL, J. I., DOMER, P., H., MOHR, R. M., TAILLON-MILLER, P., KORSMEYER, S. J., 1991. Yeast artificial chromosome cloning of a two-megabase-sized contig within chromosomal band 18q21 establishes physical linkage between BCL2 and plasminogen activator inhibitor type-2. Genomics 9, 219-228.

SMITH, D., SMYTH, A. P., MOIR, D. T., 1990. Amplification of large artificial chromosomes. Proc. Natl. Acad. Sci. USA 87, 8242-8246.

SOBRAL, B. W. S., HONEYCUTT, R. J., ATHERLY, A. G., McCLELLAND M., 1990. Analysis of rice (*Oryza sativa* L.) genome using pulsed-field electrophoresis and rare cutting restriction endonucleases. Plant Mol. Biol. Rep. 8, 253-275.

SODERLUND, C., LONGDEN, I., MOTT, R., 1997. FPC: A system for building contigs from restriction fingerprinted clones. CABIOS 13, 523-535.

SONG, J., DONG, F., JIANG, J., 2000. Construction of a bacterial artificial chromosome (BAC) library for potato molecular cytogenetics research. Genome 43 (1), 199-204.

SORENSEN, M. B., 1989. Mapping of the *Hor*2 locus in barley by pulsed-field gel electrophoresis. Carlsberg Res. Commun. 54, 109-120.

STERNBERG, N., 1990. Bacteriophage P1 cloning system for the isolation, amplification, and recovery of DNA fragments as large as 100 kilobase pairs. Proc. Natl. Acad. Sci. USA 87, 103-107.

STRONG, S.J., OTTA, Y., LITMAN, G.W., AMEMIYA, C.T., 1997. Marked improvement of PAC and BAC cloning is achieved using electroelution of pulsed-field gel separated partial digests of genomic DNA. Nucl. Acids Res. 25, 3959-3961.

SUZUKI, K., ASAKAWA, S., IIDA, M., SHIMANUKI, S., FUJISHIMA, N., HIRAIWA, H., MURAKAMI, Y., SHIMIZU, N., YASUE, H., 2000. Construction and evaluation of a porcine bacterial artificial chromosome library. Anim. Genet. 31(1), 8-12.

TAI, T., STASKAWICZ, B. J., 2000. Construction of a yeast artificial chromosome library of pepper (*Capsicum annuum* L.) and identification of clones from the *Bs2* resistance locus. Theor. Appl. Genet. 100, 112-117.

TAKETO, A., 1988. Sensitivity of *Escherichia coli* to viral nucleic acid. 17. DNA transfection of *Escherichia coli* by electroporation. Biochim. Biophys. Acta 949, 318.

TANKSLEY, S. D., GANAL, M. W., MARTIN, G. B., 1995. Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet. 11, 63-68.

TIMMIS, J. N., SCOTT, N. S., 1983. Sequence homology between spinach nuclear and chloroplast genomes. Nature, 305, 65-67.

TOMKINS, J. P., YU, Y., MILLER-SMITH, H., FRISCH, D.A., WOO, S. S., WING, R. A., 1999a. A bacterial artificial chromosome library for sugarcane. Theor. Appl. Genet. 99, 419-424.

TOMKINS, J. P., MAHALINGAM, R., SMITH, H., GOICOECHEA, J. L., KNAP, H. T., WING, R. A., 1999b. A bacterial artificial chromosome library for soybean PI 437654 and identification of clones associated with cyst nematode resistance. Plant Mol. Biol. 41, 25-32.

UMEHARA, Y., INAGAKI, A., TANOUE, H., YASUKOCHI, T., NAGAMURA, Y., SAJI, S., OTSUKI, Y., FUJIMURA, T., KURATA, N., MINOBE, Y., 1995. Construction and characterisation of a rice YAC library for physical mapping. Plant Breeding 1, 79-89.

VANHOUTEN, W., MACKENZIE, S., 1999. Construction and characterisation of a common bean bacterial artificial chromosome library. Plant Mol. Biol. 40 (6), 977-983.

VENTER, J. C., SMITH, H. O., HOOD, L., 1996. A new strategy for genome sequencing. Nature 381, 364-366.

VENTER, J. C., ADAMS, M. D., SUTTON, G. G., KERLAVAGE, A. R., SMITH, H. O., HUNKAPILLER, M., 1998. Shotgun sequencing of the human genome. Science 280, 1540-1542.

VIRANYI, F., 1992. Downy mildew of sunflower. In: Plant disease of international importance. Vol. II, Diseases of vegetables and oil seed crops. Chaube, Kumar, Mukhopadhyay, Singn (eds.). Prentice Hall Inc., New Jersey, USA, 328-344.

WADA, M., LITTLE, R. D., ABIDI, F., PORTA, G., LABELLA, T., COOPER, T., DELLA VALLE, G., D´URSO, M., SCHLESSINGER, D., 1990. Human Xq24-Xq28: Approaches to mapping with yeast artificial chromosomes. Am. J. Hum. Genet. 46, 95-106.

WANG, M., LAI., E., 1995. Pulsed field separation of large supercoiled and open-circular DNAs and its application to bacterial artificial chromosome cloning. Electrophoresis 16, 1-7.

WANG, G. -L., HOLSTEN, T. E., SONG, W.-Y., WANG, H. -P., RONALD, P. C., 1995. Construction of a rice bacterial artificial chromosome library and identification of clones linked to the Xa-21 disease resistance locus. Plant J. 7 (3), 525-533.

WANG, G. -L., WARREN, R., INNES, R., OSBORNE, B., BAKER, B., RONALD, P., 1996b. Construction of an *Arabidopsis* BAC library and isolation of clones hybridising with disease-resistance, gene-like sequences. Plant Mol. Biol Rep. 14, 107-114.

WARD, E.F., JEN, G. C., 1990. Isolation of a single copy-sequence clones from a yeast artificial chromosome library of randomly-sheared *Arabidopsis thaliana* DNA. Plant Mol. Bio. 24, 561-568.

WILLETTS, N., SKURRAY, R., 1987. Structure and function of the F factor and mechanism of conjugation. In Neidhardt, F. C. (ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. American Society for Microbiology, Washington, D.C., Vol. 2, pp. 1110-1133.

WING, R. A., RASTOGI, V. K., ZHANG, H. B., PATERSON, A. H., TANKSLEY, S. D., 1993. An improved method of plant megabase DNA isolation in agarose microbeads suitable for physical mapping and YAC cloning. Plant J. 4, 893-898.

WING, R. A., ZHANG, H.-B., TANKSLEY, S.D., 1994. Map-based cloning in crop plants: Tomato as a model system I. Genetic and Physical mapping of *jointless*. Mol. Gen. Genet. 242, 681-688.

WING, R. A., CHOI, S., 1997. Cloning and analysis of large DNA molecules. Cold Spring Harbor Laboratory manual.

WOO, S.–S., JIANG, J., GILL, B. S., PATERSON, A. H., WING, R. A., 1994. Construction and characterisation of a bacterial artificial chromosomes library of *Sorghum bicolor*. Nucl. Acid. Res. 22, 4922-4931.

WOO, S.–S., RASTOGI, V. K., ZHANG, H.B., PATERSON, A.H., SCHERTZ, K. F., WING, R., 1995. Isolation of megabase-size DNA from *sorghum* sand applications for physical mapping and bacterial and yeast artificial chromosome library construction. Plant Mol. Biol. Rep. 13, 82-94.

WU K.-S., TANKSLEY S.D. 1993. PFGE analysis of the rice genome: Estimation of fragment sizes, organisation of repetitive sequences and relationships between genetic and physical distances. Plant Mol. Biol. 23, 243-254.

YANG, D., PARCO, A., NANDI, S., SUBUDHI, P., ZHU, Y., WANG, G., HUANG, N., 1997. Construction of a bacterial artificial chromosome (BAC) library and identification of overlapping BAC clones with chromosome 4-specific RFLP markers in rice. Theor. Appl. Genet. 95, 1147-1154.

YANG, D., SANCHEZ, A., KHUSH, G. S., ZHU, Y., HUANG, N., 1998. Construction of a BAC contig containing the *xa*5 locus in rice. Theor. Appl. Genet. 97, 1120-1124.

YANISCH-PERRON, C., VIEIRA, J., MESSING, J., 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

ZHANG, H., ZHAO, X., DING, X., PATERSON, A.H., WING, R. A. 1995. Preparation of megabase-size DNA from plant nuclei. Plant J. 7,175-184.

ZHANG, H.-B., CHOI, S., WOO, S.-S., LI, Z., WING, R. A., 1996. Construction and characterisation of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. Molecular Breeding 2, 11-24.

ZHANG, H.-B., WOO, S.-S., WING, R. A., 1996. BAC, YAC and Cosmid Library Construction. Plant Gene Isolation: Principles and Practice (eds. Foster G. D. and Tweil D.), John Wiley and Sons Press, 75-99.

ZWICK, M. S., ISLAM-FARIDI, M.N., CZESCHIN, D. G., WING, R. A., HART, G.E., STELLY, D. M., PRICE, H. J., 1998. Physical Mapping of the *liguleless* Linkage Group in *Sorghum bicolor*: Using rice RFLP-selected sorghum BACs. Genetics 148, 1983-1992.

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Fig.3.17 Distribution of the insert size in sunflower BAC library estimated according to *Not*I digest

9 LIST OF ABBREVIATIONS AND MEASURMENT UNITS

AFLP	Amplified Fragment Length Polymorphism
ATP	adenosin-5´-triphosphat
BAC	Bacterial Artificial Chromosome Library
BIBAC	Binary Artificial Chromosome Library
BSA	bovines serumalbumin
cDNA	copy Deoxyribonucleicacid
CMS	Cytoplasmic Male Sterile
CMR	chloramphenicol
CsCl	caesium chloride
DNA	Doexyribonucleicacid
DTT	dithiothreitol
EDTA	ethylendiamintetraacetat
EST	Expressed Sequence Tags
FISH	Fluorescence *in situ* Hybridisation
g	gram
h	hour
HK	heat killed
HMW DNA	high molecular weight DNA
IPTG	isopropyl-á-D-thio-galactopyranoside
kb	kilobase
l	litre
LB	Luria-Bertani medium
M	molar
Mb	megabase
MB-DNA	megabase DNA
min	minute
mg	milligram
ml	millilitre
mM	millimolar
nm	nanometer
OD600	optical density at 600 nm

PAC	P1-Derived Artificial Chromosome
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PMSF	phenylmethyl sulfonly fluoride
RADP	Random Amplified Polymorhic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleicacid
rpm	revolution per minute
SDS	sodiumdodecylsulfate
STS	Sequence Tagged Site
TB	Terrific Broth
TBE	Tris/Borate/EDTA buffer
TE	Tris/EDTA buffer
U	unit
V/cm	volt per centimeter
YAC	Yeast Artificial Chromosome
µF	micro Farad
µg	microgram
µl	microlitre
µm	microme

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