Integrated physical mapping of the soybean genome: A tool for rapid identification of economically important genes.

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The complex genomes of crops like soybean are rich in highly repeated sequences (55%) that block chromosome walking from linked DNA markers to target genes (Danesh et al., 1998) and confound shotgun approaches to complete genome sequencing (Boysen et al., 1999). In addition, much of the rest of the soybean genome is moderately repeated (25%) or unique but duplicated in two homeologous loci (20%) (Grant et al., 2000). Success of map-based cloning in crop plants depends on the target gene being localized to such a short genetic interval (0.01-0.1 cM) that the markers and target gene are separated from regions rich in repeated sequences (Meksem et al., 2000; 2001). Fine mapping to this degree can be accomplished 10-50 fold more efficiently with a physical map that encompasses the whole genome than by marker saturation and chromosome walking or landing (Zhang and Wing, 1997; Meksem et al., 2001a).

Three types of approach can be used singly or in combination for physical map construction. Physical maps may be constructed by the sequence tagged site (STS) based hybridization to generate sets of contiguous overlapping clones (hereafter contigs; Chumakov et al. 1995; Mozo et al., 1999; Ross et al., 1999). STS based physical maps of metazoans are often refined from radiation hybrid map construction, that preliminarily produce low resolution gene maps (Schuler et al., 1996; Deloukas et al., 1998) but form the basis and provide tools for high density sequence

ready physical map development (McPherson et al., 2001). However, hybridization may provide low resolution and non-robust physical maps when the genome being studied is polyploid or diploidized tetraploid as in the case of soybean. Although preliminary work is promising the ability of overgo probes to be homeolog specific is not yet clearly proven (Han et al., 2000).

Physical maps may be developed from random large insert clones by fingerprinting with restriction enzyme digestion and fragment size estimation by gel electrophoresis (Coulson et al. 1986; Marra 1997; 1999). Fragment size estimation may also be determined by optical mapping (Lai et al., 1999) at least for small genomes. Early researchers used YACs, cosmids and lambda clones for fingerprinting small (Hodgkin et al. 1995) and large eukaryotic genomes (Heding et al. 1992). BAC libraries have facilitated the method by simplifying large insert library development (Marra et al., 1997; 1999; Zhang and Wing 1997; Hong 1997; Gregory et al., 1997; Ding et al., 1999; Klein et al., 2000). Our group (Zhang and Wing 1997; Tao and Zhang 1998; Zobrist et al., 2000; 200a; Tao et al. 2001) and others (Marra et al., 1997; 1999; Klein et al., 2000; Mao et al., 2000; Tingey et al., 2001; McPherson et al., 2001) have demonstrated the feasibility of genome physical map construction from BAC fingerprints for large genomes. Among plants genome encompassing physical maps have been developed for Arabidopsis, rice, sorghum, soybean and corn, in ascending genome size order. Local physical maps around specific loci have been constructed or evaluated by fingerprinting in many large genome crops (Meksem et al., 2000; 2001; Pattochi et al., 2000; Wu et al., 2000) enabling comparison of the method with hybridization and PCR methods for contig building. Using these fingerprints we can distinguish among regions where sequences are repeated in rice (Tao et al., 2001) and soybean (Meksem et al., 1998; 2000; Zobrist et al., 2000; 2000a). Fingerprinting has the advantage of requiring relatively little labor and therefore has low cost load and is useful as an independent test of contigs generated with other methods.

Physical maps can be developed by sequencing the two ends of enough BACs to cover 15 x haploid genomes (Venter et al., 1996; Boysen et al., 1997; Budiman et al., 2000; Marek et al., 2000). A seed BAC is then selected, sequenced in toto, and compared with all BAC end sequences in the database. Putative overlapping

BACs are fingerprinted and used to construct the regional BAC contig physical map. Therefore, the sequence-ready physical map development and genome sequencing are conducted simultaneously by the Sequence-Tag Connector strategy (Boysen et al., 1997; 1997a).

To develop the proposed integrated physical map for soybean, we have developed three large-insert soybean DNA libraries from 'Forrest' (Meksem et al., 2000). We have generated restriction enzyme fragment fingerprints for 90,000 of 112,600 clones to date. Each clone was tested for the content of known genetic markers (Cregan et al., 1999; 1999a; Kassem 2001) and some ESTs and SNPs. We have shown that fingerprints of BACs from other cultivars can be integrated with this data set. The integrated genetic and physical map we are developing will inexpensively and rapidly enable identification of a number of genes underlying QTL of economic importance. Here we report progress toward genetic and physical map integration using microsatellite markers and the web-site at which data can be retrieved, clones submitted for fingerprinting and clones requested.

Materials and Methods

Pools and super-pools of the two BAC libraries: Pools and super-pools of mixed DNA were constructed as Zobrist et al., 2000)

Microsattelite markers screening of the two BAC libraries: Two methods were used as desribed by Zobrist et al., (2000).

Fingerprinting of BACs for physical mapping: Individual BAC DNAs were fingerprinted using the *Hind*III and *Hae*III digestion kit developed for large-insert bacterial clones (the Fpase kit; H.-B.Zhang and Q.Tao, Invention no. TAMUS1228) by methods described previously (Zobrist et al., 2000; Klein et al 2001)

Hybridization with RGAs and overgo probes.

Hybridization probes were prepared from RGAs by amplification of soybean DNA with the gene class specific primers described by Kanazin et al., (1996) and labeled with 32PdATP labeling buffer at 37 c for 30 min (Ross et al., 1999).Cold dATP and dCTP (200 uM) was added and incubation continued for 30 min. Unincorporated nucleotides were removed by Sephadex G50 spin columns, probes

denatured at 94 C for 4 min and added to the hybridization medium. Hybridization was performed at 58 C for 18 h in hybrization solution. The 38,500 clones of the *Eco*RI BAC library were spotted onto nylon filters at 13,824 (36 x 384) clones on seven filters of 24 x 24 cm. After hybridization filters were washed and used to expose film at 70 C overnight.

Overgo primers were selected following a BLAST search to exclude repeat sequences. Oligomers were purchased from Research Genetics and labeled with 32PdATP and 32PdCTP in labeling buffer at 37 c for 30 min (Ross et al., 1999).Cold dATP and dCTP (200 uM) was added and incubation continued for 30 min. Background overgo for E.coli K12 detection were synthesized after Han et al., (2000). Unincorporated nucleotides were removed by Sephadex G50 spin columns, probes denatured at 94 C for 4 min and added to the hybridization medium. Hybridization was performed at 58 C for 18 h in hybrization solution. Filters were washed and used to expose film at 70 C overnight.

Results and Discussion

The soybean bacterial artificial chromosomes (BACs) were generated from cultivar 'Forrest'with *Eco*RI, and the large insert plasmid clones (LICs) with *Hind*II and *Bam*HI. We identified BAC clones that contained microsatellite markers by PCR on pooled DNA samples to generate local integrated genetic and physical maps Figure 1 and 2). Separately we identified sequence tagged sites in groups of 384 by multiplexed hybridization methods. We examined the robustness of these local maps by fingerprinting of DNA from LICs and BACs after digestion and end-labeling with an enzyme kit that contains *Hin*dIII and *Hae*III. The satellite physical map was developed from the *Hin*dIII LIC library. Super-pools were screened with 238 SSR primer pairs that identified 702 positive clones, an average of 2.95 clones per primer from from LICs representing 4.5 genome equivalents. Seventy-three SSR primer pairs were used to identified 163 single positive clones from 38,400 clones and a further 385 primer pairs were used to identify single positive clones from 7,680 clones (about one genome

equivalent). Therefore there are 485 microsattellite anchor available for the soybean physical amp.

Fingerprinting the 163 clones showed that large microsatellite-anchored groups of contiguous overlapping clones (contigs) were formed at the overlap statistic of e^{-18} (about 50% of bands required to overlap). However, many of the clones with large numbers of bands (>50 bands) compared to the mean (35 bands) were not true members of the contigs and should be removed from consideration. Edited anchored contigs would separate into two or three groups at overlap statistic of e^{-23} (about 60% of bands required to overlap). At this stringency fingerprinting was also able to incorporate BAC clones from soybean cultivar Williams in to Forrest derived anchored contigs Figure 3). Integration of these markers provides more than 600 RFLP anchors for the physical map and duplicates many of the microsatellite anchors.

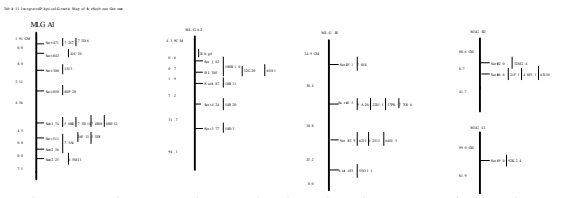
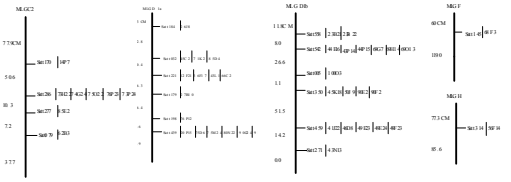


Fig. 1: The anchored physical map of Soybean



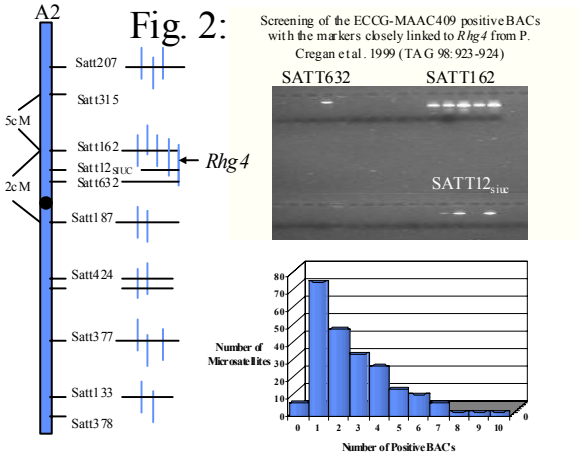
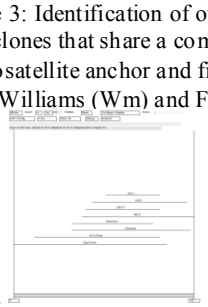
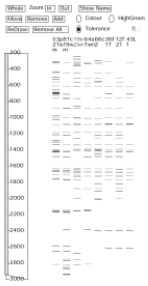


Fig. 2:

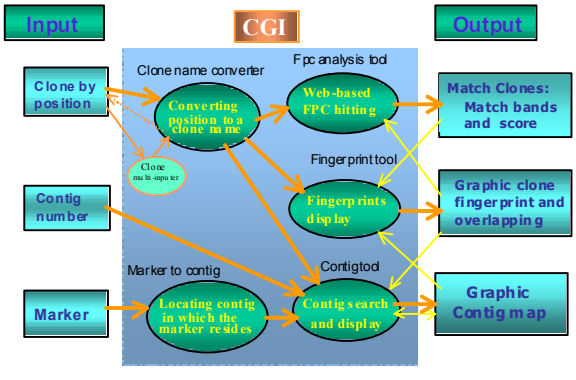
Figure 3: Identification of overlapping clones that share a common microsatellite anchor and fingerprint from Williams (Wm) and Forrest (F).





Group	MLGD1a	MLG G	MLG G	MLG J
Marker	Satt221	Satt217	Satt288	Satt183
Forrest Fpase positive	4	1	2	1
Williams Fpase positive	5	3	2	2
Anchored but Fpase negative	2	2	1	2

Figure 4: A Bioinformatic Tool Showing Input-Output Paths for Contig Access



From 50,000 fingerprinted BACs and LICs we generated 4,500 contigs that were predominantly short, containing 2-4 clones and encompassed about 500 Mbp (Figure 4) . Hybridization to the BAC library with eight classes of resistance gene analogs identified 1,860 hybridizing clones, that formed 99 contigs from 2-46 BACs each that jointly encompass 22 Mbp (wu et al., 2001). Overgo probes were effective in identifying 50% of the RGAs as unique genes on specific contigs (not shown). End sequencing of BACs and LICs identified new gene fragments in soybean that could be positioned on the genetic map. Therefore, the BACs and LICs that are anchored and located on the physical map are ready to nucleate marker saturation, genome sequencing and gene discovery methods for gene rich regions.

Updates to the physical map, the clones and the libraries are available through requests to SIUC at http://www.coalab.siu.edu/genome/ and TAMU at Physical Mapping sections of http://hbz.tamu.edu/. This work was supported by the NSF project 99872635 and the ISPOB project 98-24-198-3. We thank Dr R. Shoemaker for making available the BAC clones from the cultivar Williams.

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