A genetic linkage map of cucumber (Cucumis sativus L.) combining SRAP and ISSR markers

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Sequence-related amplified polymorphism (SRAP) and Inter-simple sequence repeat (ISSR) markers are both simple and efficient maker systems adapted to many crops and for multiple purposes. In this study a genetic map based on SRAP and ISSR markers was constructed for cucumber (Cucumis sativus L.) based on the segregations of SRAP and ISSR markers in 112 plants of F2 population derived from a cross between two cucumber inbred lines PW0832 and PW0801. In the investigation of polymorphisms with 50 ISSR primers and 132 SRAP primer combinations, 13 (26%) ISSR primers and 26 (20%) SRAP primer pairs were polymorphic generating a total of 109 polymorphic markers of which 48 were ISSR and 61 were SRAP. The average polymorphic bands were four for ISSR and two for SRAP. All the 109 polymorphic markers were scored for segregation of which 86 satisfied the Mandelian segregation ratio of 3:1. These data were used to construct an integrated linkage map for cucumber consisting of 62 loci, distributed in seven linkage groups (LGs) spanning a total of 992.2 cM, with an average distance of 16.0 cM between two adjacent loci. These markers would be very useful tool for marker assisted selection in cucumber breeding as well as for studies in quantitative traits.

Key words: SRAP, ISSR, linkage mapping, cucumber.

INTRODUCTION

Cucumber (Cucumis sativus L.) is one of the most widely cultivated fruit vegetables in many areas in the world and is the fourth most important vegetable crop after tomato, cabbage and onion (Tatiloglo, 1993) It is mostly consumed sliced and raw preferably as salad or pickles. Cucumber is a highly polymorphic species with variations in both vegetative and fruit characteristics (Staub et al., 1997). Despite its large morphological variability cucumber displays a low level of DNA polymorphism (Kennard et al., 1994). This low level of DNA polymorphism has limited the number of polymorphic DNA markers available for cucumber breeding and has been an obstacle in the construction of linkage maps, marker assisted selection and cultivar identification. The genome of cucumber (750 – 1000 cM) is estimated to have seven linkage groups (Staub and Melglic, 1993).

However different linkage groups have been observed in several studies. In their studies to identify linkage groups in cucumber, Knerr and Staub (1992) and Meglic and Staub (1996) assigned 12 and 17 loci respectively to four linkage groups. Their linkages spanned 215 cM and 584 cM, respectively. In another studies using RFLP, RAPD, isozyme, morphological and disease resistance markers Kennard et al. (1994) constructed 50 and 70 points maps with wide and narrow crosses of cucumber respectively. They observed ten linkage groups in each map, spanning 766 and 480 cM for the narrow and the wide crosses respectively. In 2001, Bradeen et al. (2001) expanded and integrated the linkage maps constructed in the previous studies to produce a consensus ten linkage-group map in cucumber. But recent works have all supported the seven group accession. For example Fazio et al. (2003) constructed a genetic map of cucumber with both RIL and F2 populations using 14 SSR, 24 SCAR, 27 AFLP, 62 RAPD and three morphological markers. They constructed seven linkage groups spanning 706 cM with mean
The criteria used for linkage grouping are established by frequencies used to determine the relative distance genetic markers along a chromosome. Recombination arrangements of genes based on the relationship of the markers. Genetic mapping also describes the coding sequences in the genome (Li and Quiros, 2001). They are both PCR-based techniques allowing for the detection of more than one independent band in a single PCR reaction. However, while ISSR is dominant and targets simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome (Nagaoka and Ogihara, 1997), the SRAP markers, which is mainly dominant but with moderate number of co-dominant markers, is aimed at the amplifications of open reading frames (ORFs) which are coding sequences in the genome (Li and Quiros, 2001).

A genetic linkage map represents the relative order of genetic markers along a chromosome. Recombination frequencies are used to determine the relative distance between the markers. Genetic mapping also describes the arrangements of genes based on the relationship of the linkages. Construction of linkage map includes firstly grouping markers and secondly ordering the markers within each group. Linkage grouping is placing markers into linkage groups based on their linkage relationships. The criteria used for linkage grouping are established by testing the independence between two loci in segregating population by means of goodness of fit or log likelihood ratio. Recombination fraction, lod score (base-10 log likelihood ratio) and significant P-value are used as a criteria to infer whether each pair of loci belongs to the same linkage group (Liu, 1998; Larsen, 1979). High resolution genetic maps provide breeders with powerful tools to analyze the inheritance of genes of interest and also for map based cloning (Kumar, 1999). However, previous maps constructed with SRAP markers in cucumber (Pan et al., 2005; Gang et al., 2005) with low number of loci could not offer a high resolution and utility for cucumber breeders.

In our study, we have identified ISSR primers and primer combinations for SRAP that proved highly polymorphic between the two cucumbers inbred parents, PW0832 and PW0801. The paper also describes a genetic linkage map of cucumber using F2 population of the parents.

### MATERIALS AND METHODS

#### Plant material

F2 population of 112 individuals created from F1 seeds from a cross between two inbred lines PW0832 (flood tolerant) and PW0801 (flood sensitive) parental lines was used as the mapping population in this study. The two lines were obtained from the Vegetable Department of School of Horticulture, Yangzhou University, China.

#### DNA extraction

DNA was isolated from young cucumber leaves as described in the protocol of Levi and Thomas (1999). The DNA concentration of samples was measured with visible spectrophotometer (Pharmacia Biotech) at A260. Fifty (50) ISSR and 132 SRAP primers were screened for polymorphism using the two parental lines.

#### SRAP and ISSR analysis

A set of 50 ISSR primers mostly from the University of British Columbia, Canada (UBC set #9) representing di, tri, tetra and penta repeats were used (Table 1). Different concentrations of template DNA and Taq polymerase were tested for optimal amplification products. The optimal amplification mixture of 25 ul contained 100 ng DNA, 1 uM ISSR primer (Sangong Inc.) 0.5 mM dNTPs, 1 mM MgCl2, 1x PCR buffer and 1 U Tag DNA polymerase (Sangong Inc.). PCR amplifications were performed in a Peltier Thermal Cycler PTC-200 (MJ Research) with an initial step at 95°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing temperature of 50°C for 30 s and elongation at 72°C for 2 min. Finally, an additional extension for 10 min at 72°C was used. Amplified DNA products were denatured at 95°C for 5 min and separated by electrophoresis along with marker ladders (Sangon Inc.), for molecular weight determination, in a 6% polyacrylamide sequencing gel containing 7 mol of urea. Each gel was run in 0.5 x TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA, PH 8.0) at 50 W for 4 h and then stained with silver nitrate as described in the protocol by Yun-Tao et al. (2007). Gel images were visualized with a UVP white light transilluminator for band scoring and photographed with UVP Bioimaging System.

For the SRAP marker, two primers were used following the protocol of Ferriol et al. (2003). The two primer type comprised the

### Table 1. The nucleotide sequences of Inter-simple sequence repeat (ISSR) use in the mapping.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC807</td>
<td>(AG)6T</td>
</tr>
<tr>
<td>UBC834</td>
<td>(AG)6YT</td>
</tr>
<tr>
<td>UBC835</td>
<td>(AG)6YC</td>
</tr>
<tr>
<td>UBC840</td>
<td>(GA)6YT</td>
</tr>
<tr>
<td>UBC842</td>
<td>(GA)6YG</td>
</tr>
<tr>
<td>UBC858</td>
<td>(TG)6RT</td>
</tr>
<tr>
<td>UBC862</td>
<td>(AG)6C</td>
</tr>
<tr>
<td>UBC859</td>
<td>(TG)6RC</td>
</tr>
<tr>
<td>UBC887</td>
<td>DVD(TC)6</td>
</tr>
<tr>
<td>UBC882</td>
<td>BVB(AT)7</td>
</tr>
<tr>
<td>A34</td>
<td>GSGC(GT)6</td>
</tr>
<tr>
<td>A35</td>
<td>(AG)6CTT</td>
</tr>
<tr>
<td>N92</td>
<td>(GA)6CC</td>
</tr>
</tbody>
</table>

*Primer motif is bolded.

\[ Y = (C \text{ or } T), S = (G \text{ or } C), R = (A \text{ or } G), D = (A, G \text{ or } T), B = (C, G \text{ or } T) \text{ and } V = (A, C \text{ or } G). \]
forward and reverse primers, the forward primer is 17-20 bp long made up of 14-17 nucleotides rich in C and G and three selective bases at the 3' end. The second primer, the reverse primer has 18 bp made up of 15 nucleotides rich in A and T with three selective bases at the 3' end (Table 2). The forward and the reverse primers amplify the exonic and intronic regions respectively. Each PCR contained a reaction mixture of 25 ul made up of 60 ng of genomic DNA, 200 uM of dNTPs, 1.5 mM of MgCl2, 0.3 uM of each primer, 2.5 ul of PCR buffer, 1 unit of Tag polymerase (Sangong Inc.) and sterile doubled distilled water. Samples were also amplified in a Peltier Thermal Cycler PTC-200 (MJ Research) programmed at 5 min of initial denaturation at 94°C followed by 5 cycles of 1 min denaturing, 1 min annealing at 35°C and 2 min of elongation at 72°C; after these, 30 cycles of 1 min denaturing, 1 min annealing at 48°C ending with an elongation step of 5 min at 72°C. The PCR products were fractionated on 6 % polyacrylamide gel at 50 W for 4 h and stained with AgNO3 (Yun-Tao et al., 2007).

ISSR and SRAP amplifications were repeated at least twice and only bands reproduced were scored for analysis. Nomenclature for the primer that amplified more than one polymorphic band, subscripts 1, 2, 3, etc (starting from the lowest to the highest molecular weight band) were assigned after the primer name.

<table>
<thead>
<tr>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me1 TGA GTC CAA ACC GG ATA</td>
<td>Em1 GAC TGC GTA CTA CGA ATT AAT</td>
</tr>
<tr>
<td>Me2 TGA GTC CAA ACC GG AGC</td>
<td>Em2 GAC TGC GTA CTA CGA ATT TGC</td>
</tr>
<tr>
<td>Me3 TGA GTC CAA ACC GG AAT</td>
<td>Em3 GAC TGC GTA CTA CGA ATT GAC</td>
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<tr>
<td>Me4 TGA GTC CAA ACC GG ACC</td>
<td>Em4 GAC TGC GTA CTA CGA ATT TGA</td>
</tr>
<tr>
<td>Me5 TGA GTC CAA ACC GG AAG</td>
<td>Em5 GAC TGC GTA CTA CGA ATT AAC</td>
</tr>
<tr>
<td>Me6 TGA GTC CAA ACC GG ACA</td>
<td>Em6 GAC TGC GTA CTA CGA ATT GCA</td>
</tr>
<tr>
<td>Me7 TGA GTC CAA ACC GG AGC</td>
<td>Em7 GAC TGC GTA CTA CGA ATT CAA</td>
</tr>
<tr>
<td>Me8 TGA GTC CAA ACC GG ACT</td>
<td>Em8 GAC TGC GTA CTA CGA ATT CAC</td>
</tr>
<tr>
<td>Me9 TGA GTC CAA ACC GG AGG</td>
<td>Em9 GAC TGC GTA CTA CGA ATT CAG</td>
</tr>
<tr>
<td>DC1 TAA ACA ATG GCT ACT CAA G</td>
<td>Em10 GAC TGC GTA CTA CGA ATT CAT</td>
</tr>
<tr>
<td>OD3 CCA AAA CCT AAA ACC AGG A</td>
<td>Em11 GAC TGC GTA CTA CGA ATT CTA</td>
</tr>
<tr>
<td>SA4 TTC TTC TTC CTG GAC ACA AA</td>
<td></td>
</tr>
</tbody>
</table>

The forward and reverse sequence-related amplified polymorphism (SRAP) primer information in the study. (Kosambi, 1944). The “Error-detection” command was employed to identify errors in marker scoring after which putative errors were retested. The map was drawn according to the program developed by Liu and Meng (2003).

RESULTS AND DISCUSSION

ISSR and SRAP markers analyses

A total number of 50 ISSR primers and 132 SRAP primer combinations were used for analysis. Five ISSR primers and 15 SRAP primer combinations failed to amplify products of sufficient quality for analysis. However, for the remaining 45 ISSR primers and 117 SRAP primer combinations, 17 (37.8%) and 32 (27.7%), respectively, showed polymorphisms of which 13 of the ISSR primers and 26 of the SRP primer combinations were reproducible enough for marker analysis (Tables 3 and 4). Each of these 13 ISSR and 26 SRAP polymorphic primers produced at least one scorable polymorphic DNA band which was visible enough for detection and scoring. In total there were 109 scorable polymorphic bands made up of 48 ISSR bands and 61 SRAP bands which were used as markers in this study. Figure 1 gives a representative gel photograph of the analyses of UBC834 (ISSR) and OD3EM5 (SRAP). These primers detected six and five polymorphism bands respectively which indicate their level of polymorphism in this study. Both ISSR and SRAP were considered as dominant markers in this study and therefore scored for their presence or absence.

There were 206 bands generated across all 13 ISSR primers with primer UBC807 yielding the highest number of products (27 bands) and primer A35 the least (10 band). However, among the SRAP markers ME1EM5 had the highest number of bands (20) and the least was recorded in SA4EM8 with seven bands.

For number of polymorphisms bands, primer UBC840 was the only primer that generated eight polymorphic bands while ISSR primers UBC834, UBC887 and SRAP...
### Table 3. Primers used for ISSR analysis and their polymorphism levels.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer type</th>
<th>No. of amplified bands</th>
<th>No. of Polymorphic bands</th>
<th>% polymorphism</th>
<th>Mapped markers</th>
<th>Origin of amplicon PW0832</th>
<th>PW0801</th>
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<tbody>
<tr>
<td>UBC807</td>
<td>ISSR</td>
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<td>ISSR</td>
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<td>0</td>
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<td>ISSR</td>
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<td>1</td>
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<td>0</td>
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<td>ISSR</td>
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<td>0</td>
</tr>
<tr>
<td>N92</td>
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<td>2</td>
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<tr>
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<td></td>
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<td>48</td>
<td>-</td>
<td>30</td>
<td>21</td>
<td>9</td>
</tr>
</tbody>
</table>

ISSR = Inter-simple sequence repeat.

### Table 4. Primers used for SRAP analysis and their polymorphism levels.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer type</th>
<th>No. of Amplified bands</th>
<th>No. of Polymorphic bands</th>
<th>% polymorphism</th>
<th>Mapped markers</th>
<th>Origin of amplicon PW0832</th>
<th>PW0801</th>
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<td>ME1EM5</td>
<td>SRAP</td>
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<td>SRAP</td>
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<td>0</td>
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<td>ME2EM5</td>
<td>SRAP</td>
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<td>22.2</td>
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<td>1</td>
<td>0</td>
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<td>ME2EM7</td>
<td>SRAP</td>
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<td>OD3EM2</td>
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SRAP = Sequence-related amplified polymorphism.
primer SA4EM10 generated six polymorphisms each. However, ISSR primers UBC835, UBC858 and SRAP primers ME1EM9, ME3EM3, ME4EM3, ME4EM5, ME5EM4, OD3EM11 and DCIEM4 yielded one polymorphic bands each. Overall, there were 545 bands and 109 (20%) polymorphisms generated between the two parents, of these 48 (44%) were ISSRs and 61 (46%) were SRAPs. Although, this is the first studies in cucumber mapping both ISSR and SRAP markers together, the results presented in our studies agrees with previous studies that demonstrate the reproducibility of bands profile generated by SRAP (Li and Quiros, 2001) and ISSR (Fang and Rose, 1997) markers when PCR products are fractionated on polyacrylamide gel.

Generally, dinuclotide ISSR primers produced the highest number of bands and were frequent in the cucum-
cucumber genome than the higher repeats. Also, the 3'-end anchor primers of ISSR yielded the highest number of bands in our study, than the 5'-end anchored primers. This is in contrast with observation by Bornet and Branchard (2001) that primers anchored at 5'-end generates higher number of fragments because of their broader specificity. The plausible explanation of our results might be due the degree of density of these repeats in the cucumber genome. This is supported by the fact that the higher the density of repeats in a genome, the more specific primers and stringent PCR conditions needed to optimized resolution on a gel (Fang et al., 1997).

For the SRAP marker, the number of polymorphic and scorable bands was not as high as expected. For example the range of polymorphic bands per primer was 1 - 5 with an average of 2 bands, which is relatively smaller compared with the ISSR marker with an average of 4 bands (Figure 1, Tables 3 and 4). Although the advantages of both ISSR and SRAP markers stems from their simplicity and reproducibility, high number of polymorphisms and the fact there is no need for prior knowledge of DNA sequence, their major limitations could be lack of the number of useful primers that could generate useful polymorphism as observed in our studies and supported by Cekic et al. (2001) and Nagoaka and Ogihara (1997).
Map construction and marker segregation

Seventy-six (86) dominant markers of the 109 that fitted the expected 3:1 Mendelian ratio (P<0.05) were used in the linkage analysis of which 62 markers made up 30 ISSR and 32 SRAP markers were assign to seven linkage groups (Figure 2). The 23 polymorphic markers were excluded from the linkage analysis because of segregation distortions. The linkage map had 62 loci spanning a total length of 992.2 cM with an average genetic distance of 16.0 cM between adjacent markers. However, with this large average distance, greater saturation would be needed for practical application especially for marker assisted selection (MAS). This is because the presence of a tight linkage (<10cM) between a trait and genetic marker may be beneficial for MAS in order to increase the benefit to be derived from selection (Staub et al., 1996).

We could not compare our linkage map with the previously published SRAP linkage map of cucumber by Pan et al., 2005 and Gang et al. (2005) because few markers were common. Gang et al. (2005) constructed molecular linkage map with seven linkage groups spanning 1164.2 cM in length with an average genetic distance of 12.6 cM.

Conclusion

In conclusion, this study has identified primers that generated substantial polymorphism in our population. The study therefore demonstrated the use of ISSR and SRAP markers as potential tools for linkage mapping in cucumber. The degrees of polymorphism exhibited by both markers clearly demonstrate their usefulness in genetic analysis of cucumber. The ISSR-PCR products can be cloned and sequenced to convert the ISSR markers in this study into sequence-characterized amplified regions (SCARS) this would increase the detection of co-dominance to improve the utility of the results of ISSR analysis in this study. Also, amplified fragments from SRAP markers could be recovered from the acrylamide gel, re-amplified and sequenced. These sequences could then be compared with other sequences in the databases for annotation. We further speculate that because of their simplicity and accessibility these markers may rapidly become an invaluable tool for cucumber genome analysis.

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