DEVELOPMENT AND APPLICATION OF RFLP AND STS IN *STYLOSANTHES*

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Summary. RFLP markers have wide applications in genetics and plant breeding but have not been used with *Stylosanthes* because of the difficulty associated with DNA isolation from this genus. A simple and effective DNA isolation method for *Stylosanthes* has been developed, and the DNA isolated using this method was successfully used for constructing a *Pst*I genomic library. Four hundred and fifty clones have been isolated from this library. Together with another 525 clones from a cDNA library, they have been used in RFLP analysis and some 300 single copy RFLP clones have been identified. Some of the single-copy gDNA clones were sequenced and STS primer pairs developed. These single-copy RFLP clones and STS primers are being used for linkage map construction, intraspecific variation and interspecific genetic relationship studies.

INTRODUCTION

Genetic markers can be employed to determine genetic relationships between species and genetic variation within species (9). Such knowledge is essential for efficient collection and utilization of genetic stocks. Genetic markers, especially when assembled into genetic maps, can be used to determine the accurate locations of genes encoding agronomically important traits and provide 'tags' for selection in plant breeding programs (1). Chromosome segments inherited by individuals can be tracked by following different marker alleles. Thus it is possible to combine chromosome segments with high breeding values into one desired background (1). Comparison of the genetic maps among related species allows chromosomal rearrangements to be defined (3). This can enhance our understanding of chromosome evolution, and thus help to design appropriate strategies to transfer genes between these species. In addition, accurate mapping of flanking chromosome regions around a gene may also facilitate its isolation and cloning for subsequent detailed molecular and genetic transformation studies (1). In this paper we outline the latest progress of the genetic marker work on *Stylosanthes* in our laboratory, and discuss the potential applications of these marker systems in genetic studies and breeding of *Stylosanthes* species.

RFLP ANALYSIS

DNA extraction

Restriction fragment length polymorphisms (RFLPs) have been widely used to detect DNA polymorphism in plant species. However RFLP work on *Stylosanthes* has been severely hindered by the lack of a simple and effective DNA isolation method, and it is suspected that this is due to the high polysaccharide content (4). It is not clear whether compounds other than polysaccharides are involved. Once plant cells are disrupted, a sticky gelatin forms in which nucleic acids become embedded. To separate the DNA, several existing DNA isolation methods were attempted. However, good quality DNA could not be isolated by any of these methods. The contaminants could not be removed by CTAB (cetyltrimethylammonium bromide), PVP (polyvinylpyrrolidine) or NaCl as recommended in many DNA extraction methods. Even after CsCl-EtBr centrifugation the DNA was difficult to digest (4). However, when samples were freeze-dried most of these contaminants could be precipitated in a high salt condition (1/3 volume of 5M potassium acetate). Following this protocol DNA with moderate yield (40-90 ug DNA per gram fresh leaf material) and a concentration range between 250 and 610 ug/ml was obtained. The DNA was successfully used for genomic library construction and Southern hybridization analysis.

Probe development and RFLP analysis

It has been demonstrated that genomic libraries generated using methylation-sensitive restriction enzymes enrich single-copy sequences. One such enzyme, *Pst*I, was used to construct a *Stylosanthes* genomic library using the genotype *S. scabra* cv Fitzroy. Four hundred and fifty clones (designated as SsCS1-SsCS450) were isolated from this library. In addition, another 525 clones (designated as ShCS1001-ShCS1525) were isolated from a cDNA library of *S. humilis* (5).

The first 100 gDNAs and 76 cDNAs which generated simple RFLP patterns were analyzed. RFLP data were collected on a band-by-band basis for all the probe-enzyme combinations. This analysis showed that the levels of RFLPs revealed by gDNAs in *Stylosanthes* (48% between *S. scabra* and *S. hamata* and 15% between two *S. viscosa* accessions) were similar to those detected by cDNAs (46% and 17% respectively).

On the basis of calculation from hybridization patterns using two restriction enzymes, *Dra*I and *Hind*III, 66% of the 271 gDNA clones detected single-copy sequences, 19% detected low- (2-4) copy sequences and 15% detected high- (5 or more) copy sequences. As expected, only a small proportion of cDNA clones (4%) detected high-copy sequences. However, compared with the *Pst*I gDNA clones, twice as many cDNA clones (40% versus 19%) detected low-copy sequences. The proportion of cDNA clones which detected single-copy sequences (56%) was even lower

($p < 0.05$) than that of the *Pst*I gDNA clones.

Unlike RAPD (randomly amplified polymorphic DNA) and other multi-locus markers, most single-copy RFLP probes hybridize to a single fragment from a diploid genotype, and hybridize to two fragments from a tetraploid genotype (Fig.1A). This property makes RFLP markers highly transferable between crosses, i.e., linkages established between RFLP markers and genes in one cross can be used in other crosses. Further, unlike many PCR-based marker systems, many RFLP

probes isolated from one species can be used in a wide range of related species. This property makes RFLP a valuable tool for interspecific gene transfer and comparative mapping experiments.

About 650 RFLP probes from the gDNA and the cDNA libraries have been individually screened against *S. scabra* cv. Fitzroy and *S. hamata* cv. Verano, the two parents from which the mapping population was generated. Some 300 of these probes detected polymorphism between the two parents, and these probes have been used for constructing a genetic map. The map will be used to identify tags for genes conditioning anthracnose resistance in *Stylosanthes*. By selecting for the tags, it will be possible to combine several resistance genes into a single cultivar, and thus improve the capacity of the cultivar to prevent new fungal races from damaging the pasture.

STS ANALYSIS

Recent work on different plant and animal species indicates that PCR-based genetic marker systems with specific primers appear to be highly genome specific. For example, the SSR (simple sequence repeats or microsatellite) primers designed using gene sequences of storage proteins in

hexaploid wheat only generated PCR products from one of the three genomes (2), and the STS

(sequence-tagged-sites) primers generated in chrysanthemum often amplified a single fragment

from each of the polyploid genotypes (11). Similarly a large percentage of SSR primers designed for several mammal species failed to amplify products from related species (7). Such genome specific markers would lose much of their power in interspecific introgression studies and comparative mapping experiments because they are unlikely to be transferable between species. However such PCR-based genome specific markers can be very powerful in intraspecific gene tagging, and in genome identification studies.

To assess the usefulness of STS in *Stylosanthes*, 20 sets of STS primers were generated, 15 from randomly selected single-copy *Pst*I genomic clones, and the other five from two known gene sequences, *shst2* and *shst3* (8). These primer sets were analysed against 26 genotypes representing 13 different *Stylosanthes* species. Thirteen of these primer pairs amplified successfully, and they showed a low level of genome specificity. Of the 338 entries (26 genotypes by 13 primer sets), PCR amplification was not successful (little or no products) in only ten cases. This may simply indicate that the different *Stylosanthes* species are less divergent, even when compared to the differences between the constituent genomes of allopolyploid species such as wheat (2) and chrysanthemum (11). The conservation between *Stylosanthes* species appears to be close enough to allow most STS primers designed for use in one species to be used for another. Compared to Southern blot analysis, STS, like other PCR-based marker systems, is much simpler and more convenient once primers become available. Further, as shown in Fig. 1B, STS detected a high level of interspecific variation (39.5% pair-wise of all primer-genotype combinations), and most STS primers behaved like single-copy RFLP probes, i.e., they generated one band from diploid genotypes and two bands from tetraploid genotypes. Thus STS can be very valuable in many interspecific applications including species identification.

These STS primers were used to study *S*. sp. aff *scabra*, an undescribed taxon showing affinities with the widely cultivated *S. scabra*. This analysis provides strong evidence that *S*. sp. aff *scabra* might be one of the diploid progenitor of the allotetraploid *S. scabra.* Cytological examinations confirmed that *S*. sp. aff *scabra* is indeed a diploid species. If this finding is correct, it should be possible to artificially synthesize *S. scabra* using pre-selected *S*. sp. aff *scabra* and *S. viscosa* (the other progenitor) accessions. These artificial *S. scabra* genotypes could be used directly, or, more likely, be used in breeding programs. By doing so the genetic variation existing in the two diploid progenitor species would become available in improving the allotetraploid *S. scabra*.

Use of STS in intraspecific work, however, can be problematic. This is because only a low level of intraspecific polymorphism has been detected by this marker system (3.0%). Unlike SSR which targets highly variable sequences of a genome, STS is non-selective with respect to the level of polymorphism. Further, compared with RFLP, STS generates much shorter fragments. Thus STS would have less chance of detecting polymorphism caused by insertion/deletion events. *Stylosanthes* is not alone in this regard. Similar results were obtained in barley (10) and even in some highly polymorphic species such as pearl millet (6). Methods to remedy such a problem include the use of a high-resolution electrophoresis system, restriction enzyme digestion of PCR products (9), or design of allele-specific primers (12).

CONCLUSION

A simple DNA isolation method was developed in our laboratory. Processing of a large number of samples produced DNA of moderate yield and sufficient quality for genomic library construction and RFLP analysis. A genomic library was constructed and 450 clones isolated. In addition, 525 cDNA clones were isolated from a cDNA library. These clones were used for RFLP analysis and some 300 single copy clones identified. In addition, a set of STS primers was developed. These markers are being used to tag anthracnose resistance genes, and to investigate interspecific relationships and intraspecific variation. This work will greatly enhance our knowledge of the genetics and evolution of the *Stylosanthes* genus and provides new opportunities for improvement of disease resistance and plant adaptation.

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