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Restriction fragment length polymorphism detected by cDNA and genomic DNA clones in *Stylosanthes*

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Abstract A DNA isolation method suitable for genomic library construction and RFLP analyses of the forage legume *Stylosanthes* was developed. Probes isolated using this method were used to investigate the feasibility of constructing RFLP-based genetic maps in this genus. Two hundred and seventy-one *Pst*I genomic DNA and 134 cDNA clones were analysed against four *Stylosanthes* accessions, including two tetraploids and two diploids, with the use of two restriction enzymes, *Dra*I and *Hin*dIII. The proportion of clones which detected single-copy sequences from the *Pst*I genomic library was higher than that from the cDNA library, but the percentage of clones which detected low-copy sequences was doubled in the latter. There was no significant difference in the level of RFLPs detected by gDNA and cDNA probes, although the level of polymorphism was lower in the diploids. A large proportion of RFLPs seemed to have resulted from mutation/base substitution events, and this was especially the case in diploids.

Key words *Stylosanthes* · DNA isolation · RFLP

Introduction

Restriction fragment length polymorphisms (RFLP) have been widely used to detect DNA polymorphism in plant species. The speed of RFLP mapping depends, to a large degree, on the level of DNA variation available in a given species. Generally, predominantly cross-pollinating species such as maize (Helentjaris et al. 1985) and pearl millet (Liu et al. 1994) harbour abundant RFLPs while predominantly self-pollinating species such as wheat (Devos et al. 1992) and barley (Graner et al. 1991) possess fewer RFLPs. Another factor affecting the detection of RFLPs in a given species is the probe source. Genomic clones detect a much higher level of RFLP in some species such as wheat (Devos et al. 1992), but cDNA clones are superior in others such as lentil (Havey and Muehlbauer 1989). A further factor to be considered in RFLP analysis is the nature of the RFLPs. The predominant source of RFLP, either single-base mutations/substitutions such as in pearl millet (Liu et al. 1994) or deletion/insertion such as in rice (McCouch et al. 1988) will also determine screening strategies in RFLP detection.

The genus *Stylosanthes* consists of approximately 40 species. They may be diploid, tetraploid or hexaploid with a basic chromosome number of x=10 (Cameron 1967). These legume species are grown extensively in tropical regions of the world. Two of them, *S. scabra* and *S. hamata*, form the most significant pasture group in Northern Australia. Previous RFLP work on *Stylosanthes* was severely hindered by the lack of a simple and effective DNA isolation method (Kazan et al. 1993a). In this paper a DNA isolation method for *Stylosanthes* is presented, and experiments which were conducted to determine some factors affecting RFLP analysis in this genus are described.

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Materials and methods

Genetic stocks

Two tetraploid accessions, *S. scabra* cv 'Fitzroy' and *S. hamata* cv 'Verano', and two diploid accessions, *S. viscosa* 33941 and *S. viscosa* 34904, were employed in this study. All four genotypes were obtained from the Australian Tropical Forages Genetic Resources Centre, Division of Tropical Crops and Pastures, CSIRO Cunningham Laboratory. They were selected as potential parents for mapping populations based on a preliminary analysis of 50 randomly amplified polymorphic DNA (RAPD) primers on a panel of 17 genotypes (unpublished data).

DNA probes

All genomic clones used in this study were derived from a *Pst*I genomic library. The library contains 450 clones (designated as SsCS1-

SsCS450) that were isolated from total genomic DNA extracted from leaves of *S. scabra* cv 'Fitzroy'. The methods used for constructing the library were as described by Liu et al. (1994), with the modification that pBluescript II SK^+ was used as the cloning vector.

The 134 cDNA clones used in this study were a subset of the 525 clones (designated as ShCS1001-ShCS1525) isolated from a cDNA library of *S. humilis* (McIntyre et al. 1995) kindly provided by Dr. J. M. Manners (CRC for Tropical Plant Pathology, University of Queensland). The methods used for plaque isolation and insert polymerase chain reaction (PCR)-amplification were those recommended by the manufacturer (Amersham RPN 1713).

RFLP procedures

DNA isolation

Freeze-dried leaf tissue was ground to a fine powder. The powdered tissue (0.5 g) was mixed with 20 ml extraction buffer (100 m*M* TRIS-HCl pH 8.5, 100 m*M* NaCl, 50 m*M* EDTA pH 8.0, and 2% SDS) and incubated at 65°C for 1–2 h, followed by the addition of 7 ml of 5 *M* sodium acetate (pH unadjusted). The mixture was then centrifuged (2,000 *g* for 15 min), and DNA in the liquid phase was precipitated with 0.6 volume isopropanol, rinsed in 70% ethanol and dissolved in 5 ml 1×TE buffer. RNA was removed by digestion with 1 mg/ml RNase A at 37°C for 30 min, and the DNA was precipitated with 2 volumes 95% ethanol following a phenol/chloroform extraction.

Methods for restriction enzyme digestion of DNA, gel electrophoresis, Southern transfer and hybridization were as described by Sharp et al. (1988), with the modifications that Hybond N^+ nylon membranes (Amersham) were used. Megaprime DNA labelling systems (Amersham) were used for probe labelling with the modification that labelled DNA was denatured by adding 0.1 volume 4 *N* NaOH. After hybridization the membranes were washed sequentially with 2×SSC/1% SDS, 1×SSC/1% SDS, and 0.2×SSC/1% SDS for 10 min each at 65°C. The sizes of fragments detected by each clone were estimated by comparing them to lambda-*Hin*dIII standards. For re-use, filters were stripped by two washes in a boiling solution of 0.1×SSC/0.5% SDS for 10 min each.

Statistical analysis

The relationship between probe length and level of RFLPs was investigated using a generalized linear model (Nelder and Wedderburn 1972). Probe length was used as an explanatory variable, with a binomial error distribution and logit link function. The significance of the regression test was based on the change in deviance associated with the explanatory variable.

Results and discussion

DNA isolation

Isolation of DNA from *Stylosanthes* is very difficult, and it is suspected that this is due to the high polysaccharide content (Kazan et al. 1993a). It is not clear whether compounds other than polysaccharides are involved. Once plant cells are disrupted these contaminants form a sticky gelation in which nucleic acids become embedded. To separate DNA from these contaminants, several existing DNA isolation methods, including those of Bernatzky and Tanksley (1986), Doyle and Doyle (1987), Sharp et al. (1988), Fang et al. (1992), Maliyakal (1992) and many of their derivatives, 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 (polyvinylpyrrolidone) or NaCl as recommended in many DNA extraction methods. Even after CsCl-EtBr centrifugation the DNA was difficult to digest (Kazan et al. 1993a). However, it was found that when freeze-dried samples were used most of these contaminants could be precipitated in a low isopropanol (0.1–0.2 volume) or a high salt condition as described in the Materials and methods section. Following this protocol DNA with moderate yield (40–90 µg DNA per gram fresh weight of leaf material) and a concentration range between 250 and 610 µg/ml was obtained. The DNA was successfully used for genomic library construction and Southern hybridization analysis (see below). It was noted, however, that this protocol failed to work when fresh tissues were used.

The *Pst*I genomic and the cDNA library

It has been demonstrated that genomic libraries generated using methylation-sensitive restriction enzymes enrich single-copy sequences. One of the most widely used restriction enzymes for genomic library construction in plants is *Pst*I. This enzyme also proved to be successful in *Stylosanthes*. On the basis of calculations from hybridization patterns using two restriction enzymes, *Dra*I and *Hin*dIII, 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 portion 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 (66%).

Level of polymorphism detected by cDNA and genomic DNA probes

It would be expected that genomic probes should be more powerful to detect RFLPs because of a low level of conservation at these sites. However, previous results are not consistent on this issue, and whether gDNA or cDNA clones are superior appears to depend on the species. The first 100 gDNAs and all 76 cDNAs which generated simple RFLP patterns were analysed. RFLP data were collected on a band-by-band basis for all the probe-enzyme combinations. As shown in Table 1, the level of RFLPs revealed by gDNAs was similar to that detected by cDNAs.

It should be noted that the level of RFLPs between the two tetraploid genotypes could be underestimated. As shown in Fig. 1, a typical single-copy probe hybridized to one band only from a diploid genotype and hybridized to two bands from a tetraploid genotype. RFLPs could be unambiguously scored only when both bands of one tetraploid differed from those of the other (Fig. 1A). The situ-

Table 1 Levels of polymorphism and average fragment sizes detected by cDNAs and gDNAs

Probe source	Restriction enzyme	Polymorphism[a]		Fragment length[a] (kb)	
		Tetraploid	Diploid	Mean	SE
cDNA	*Dra*I	46% (a)	20% (a)	3.1 (a)	1.3
	*Hin*dIII	46% (a)	14% (a)	5.6 (b)	2.4
gDNA	*Dra*I	48% (a)	18% (a)	2.9 (a)	1.2
	*Hin*dIII	50% (a)	13% (a)	5.6 (b)	2.7

[a] Values followed by the same letter (a or b) were not significantly different at 5% level

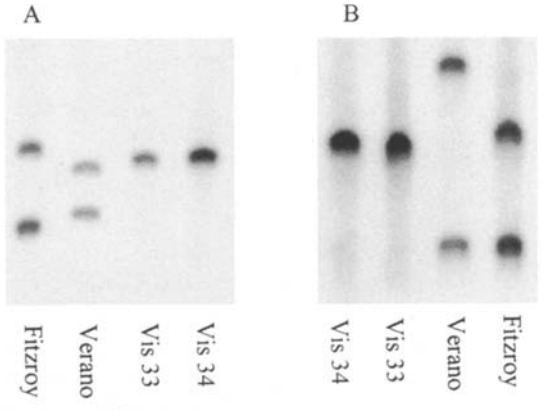

Fig. 1A, B Autoradiograms derived from probing with SsCS141 (**A**) and ScCS220 (**B**) to *Dra*I-digested DNAs from two tetraploid genotypes (Fitzroy and Verano) and two diploid genotypes (Vis 33 and Vis 34) (see text for explanation)

ation is less clear when only one of the two copies from one tetraploid differed from those of the other (Fig. 1B). A½ score was given to this type of polymorphism (one of the two copies was polymorphic) based on the assumption that the two "monomorphic" bands are allelic, as are the two polymorphic ones. However, the possibility exists that the two bands which have the same molecular weights are not allelic. Each of the two "monomorphic" sequences may be allelic to one of the polymorphic sequences, i.e. not one but both copies were polymorphic. Similar situations may exist even when neither of the two bands from one genotype appeared to be polymorphic to those of the other genotype. Even segregation analysis could not establish the allelic relationships between these bands in such situations.

The average fragment sizes detected by cDNAs and gDNAs may be different in some species, and the larger fragments detected by cDNA clones could have directly contributed to the higher level of polymorphism detected by them (Havey and Muehlbauer 1989; Miller and Tanksley 1990). However, cDNAs failed to hybridize to larger restriction fragments in *Stylosanthes* (Table 1), and no association between probe length and polymorphism was found (r^2=0.015; SE=1.938; P=0.32). The lack of association between probe length and its ability to detect RFLP has also been observed in tomato where it was believed to be due to an insufficient range in the sizes of probes used (Miller and Tanksley 1990). The probe range used in this study (0.4–2.5 kb) was even smaller.

Nature of RFLP

A survey of the RFLPs observed between the two diploid *Stylosanthes* accessions indicated that mutation/base substitution events seem to be a more important source of RFLPs. Forty percent of RFLPs were detected by both restriction enzymes, 30% by *Dra*I only and another 30% by *Hin*dIII only. Apparently there was no difference in the percentage of RFLPs detected by the two restriction enzymes, although the average fragment size detected by *Hin*dIII (5.6 kb) was nearly twice as large as that by *Dra*I (3.0 kb).

A similar survey was conducted for RFLPs observed between the two tetraploid genotypes. Here 59% of RFLPs were detected by both restriction enzymes, 21% by *Dra*I only and another 20% by *Hin*dIII only. Such results suggested that more RFLPs resulted from insertion/deletion events in tetraploids than in diploids. One of the possible explanations for this would be that tetraploids are likely to be more tolerant to large fragment insertion/deletion events. However, as discussed above, mis-classifications may exist in tetraploids, and it was not clear what effect this would have on the analysis of the nature of RFLPs.

Comparison of the level of polymorphism detected by RFLP and RAPD

The same two tetraploid genotypes had been analysed previously for DNA variation using RAPD (Kazan et al. 1993b). The level of polymorphism detected by RAPD (34%) was significantly lower (P<0.01) than the level of RFLPs detected by either gDNAs (49%) or cDNAs (46%). Similar results have been obtained in other species, such as chrysanthemum (Wolff et al. 1994). One of the possible explanations for the different levels of polymorphisms detected by RFLP and RAPD is that the two marker systems may target different regions of the genome. The former, especially with the use of cDNAs and *Pst*I gDNAs, hybridize primarily to single- and low-copy sequences, but a large portion of the RAPD bands may derive from repeti-

tive sequences (Williams et al. 1990; Devos and Gale 1992). Another possible explanation for this difference may reside in the difference in the average DNA fragment sizes detected by the two marker systems. The average fragment size detected by RFLP is several times larger than that by RAPD. Thus, the former should have more chance to detect those polymorphisms due to insertion/deletion events.

Conclusions

A simple DNA isolation method for *Stylosanthes* is described in this paper. When this protocol is used, a large number of samples can be processed with moderate DNA yields. The DNA was shown to be suitable for genomic library construction and RFLP analysis. However, freeze-dried tissues are crucial to this method. Also, the DNA quality usually obtained was not high and this may cause inconvenience in endonuclease digestion and electrophoretic separation. Thus, optimization of methods which could overcome these problems would be desirable.

A large proportion of RFLPs, detected by either genomic or cDNA probes, were revealed by only one of the two restriction enzymes. This indicated that mutation/base substitution provides an important source of RFLPs in *Stylosanthes*. Thus, the use of more restriction enzymes in RFLP analysis may be very effective in this genus.

Both cDNAs and *Pst*I gDNAs were efficient in detecting RFLPs in *Stylosanthes*. On a band-by-band basis, the levels of RFLP revealed by *Pst*I gDNA and cDNA clones were similar. However, the proportion of probes detecting low-copy sequences in the cDNA library was double those in the *Pst*I genomic library. Consequently, cDNAs should be more efficient in mapping experiments because, on average, a larger number of loci can be mapped per labelling. These low-copy sequences would also be preferable in some other applications such as fingerprinting and chromosome evolution studies. However, unambiguous single-copy sequences would be much easier to use in some other applications such as the tagging of genes of agricultural importance.

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