DEVELOPMENT OF SEQUENCED TAGGED MICROSATELLITE SITE (STMS) MARKERS IN AZALEA

UTILISATION DES MARQUEURS MICROSATELLITES A SEQUENCES ETIQUETEES CHEZ LES AZALEES

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Abstract

A genomic library was constructed from DNA of two azalea genotypes: a Belgian pot azalea *R. simsii* hybrid Mevr. Van Belle and a Chinese *R. simsii* from Daoxian. An enrichment of microsatellite containing sequences was performed as in Van de Wiel *et al.* (1999). Fragments were sequenced and primers were designed that allow the amplification of the microsatellite repeat. About 220 microsatellite containing clones were selected from the enrichment procedure. Mainly dinucleotide repeats and some trinucleotide repeats were found. The selected primers were tested in a small set of reference varieties to check their value (specificity and polymorphic rate) and to set up the PCR-conditions. Five primer pairs have been tested, two of them gave a specific and polymorphic pattern. They were further screened by radioactive PCR on a selection of 5 plants from the azalea breeders gene pool which included the two genotypes used library construction. These 2 STMS markers uniquely identified the 5 plants.

1. Introduction

Azaleas belong to the genus *Rhododendron* of the family of *Ericaceae*. The azaleas were first classified by Linneaus in Species plantarum (1753) as *Azalea indica.* Azaleas can be divided in two groups, 1°) deciduous azaleas which belong to the subgenus *Pentanthera* and 2°) evergreen azaleas which belong to the subgenus *Tsutsusi* (Chamberlain and Rae, 1990). In Belgium *Rhododendron simsii* hybrids also known as Belgian pot azaleas are an important export product. Identification based on morphological and physiological traits is not always clear and is labour intensive. In *Rhododendron* spp. till now some DNA-marker analysis is done. Feasibility of *Rhododendron* DNA for RAPD–analysis has been evaluated (Iqbal *et al*., 1994). Phylogenetic relationships of *Rhododendroideae* are measured using *rbc*L and *mat*K sequencing data (Kron, 1997). Introgression of *R. kaempferi* into *R. kiusianum* is studied by (Kobayashi *et al*., 1998) using PCR on cp-DNA.

DNA markers, especially AFLP (De Riek *et al*., 1999) and STMS, can give complementary data to the morphological and physiological data.

2. Materials and methods

2.1. DNA extraction

The DNA of 2 plants (*R. simsii* hybrid Mevr. Van Belle and *R.simsii* Daoxian) was used for constructing the genomic library. For testing the developed microsatellites the DNA of those 2 plants plus the DNA of 4 other plants (R. *simsii* “Knut Erwen”,

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"Ambrosiana", the Hirado hybrid "Heiwa-no-hikari", and R. *simsii* Daoxian) was used. For DNA extraction, young leaf material was harvested in the greenhouse and immediately frozen in liquid nitrogen. The leaves were either directly ground in liquid nitrogen or stored at -80 °C until lyophilisation. After 48 h lyophilisation the material was ground in a mechanical mill (Culatti). DNA-extraction was done using a two-step protocol, modification of the protocol described by Greenwood *et al*., (1989). The second extraction buffer was changed to improve yield for small amounts of leaf material (De Riek *et al*., 1999).

2.2. Isolation and of the microsatellites

A genomic library, prepared from a *Alu* I digest of R. *simsii* hybrid Mevr. Van Belle was enriched for GA/GT, TGT/GTG/GAG/GCT/TGTT/GATA and TCT/CGT/AGT/TGA/GTAT containing DNA-fragments, according to Van de Wiel *et al*. (1999). The enriched fragments were cloned into a pBluescript SK+ vector and transformed into *E*. coli DH5α. Glycerol stocks were made for longterm storage. Plasmid DNA extraction was performed with the GFX™ Micro Plasmid Prep Kit (Amhersham Pharmacia Biotech).

Sequencing analysis was done using universal T3 and T7-pimers with the Big Dyes Terminator Kit (PE Biosystems) on a ABI Prism™ 377 DNA XL upgrade Sequencer.

2.3. PCR reactions and detection of the amplified fragments

Forward and reverse primers were developed using Primer 0.5 (Whitehead Institute for Biomedical Research). Amplifications were performed in a total volume of 20 µl which contained 10 ng genomic DNA, 0.1µM of each primer, 100 µM deoxyribonucleotides, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.125% (w/v) BSA and 1.25 U AmpliTaq (PE Biosystems). The unlabelled and radioactive PCR reactions were performed in tubes using a Perkin Elmer 9600 thermocycler with cycling conditions as follows: 1 cycle of 94°C for 5 min and 35 cycles of 55°C for 1 min, 72°C for 2 min, and 94°C for 1 min. After the final cycle, one cycle of 55°C for 1 min and 72°C for 7 min was added. PCR products were electrophoresed using 2,5 % agarose gels and ethidium bromide staining for a preliminary evaluation. To obtain higher resolution polyacrylamide gel electrophoresis (PAGE) on vertical gels (6% polyacryamide, 7.5 M urea, Tris-borate-EDTA buffer) with a Sequi-Gen GT Sequencing cell (BioRad) was performed. Here the DNA bands were labelled, by PCR using a γ-ATP^{33} kinated forward primer. The sizes of the PCR-products were determined by loading a Sequa Mark ladder (Research Genetics) near the samples on the gel.

3. Results

Two hundred and twenty microsatellite containing clones derived from the genomic bank of the R. *simsii* hybrid Mevr. Van Belle were sequenced. Of these 125 clones contained microsatellites (Table 1). The other 95 clones contained no repeat or resulted in bad sequencing reactions.

From those 125 clones, 70 (52 dinucleotide and 18 trinucleotide repeats) were useful for primer development.

So far, 5 primers have been developed using dincleotide repeat containing sequences as template. The developed primer pairs were tested on a selection of 5 plants, including the cultivars used to construct the genomic library.

The PCR products were first detected on agarose gels. From the five primer pairs used, two resulted in a good pattern, without smears or non-specific bands.

Those two primer pairs (AZA-002 flanking an $(AC)_8(TC)_8(AC)_{17}$-repeat and AZA-003 flanking a $(TG)_{15}$-repeat) were further used for radioactive detection on

polyacrylamide gel electrophoresis (PAGE). The higher resolution resulted in a better separation of the different alleles. With the 2 primer pairs it was possible to distinguish the 5 plants from each other (Figure 1).

4. Discussion

From the microsatellite containing clones mainly dinucleotide and some trinucleotide repeats were found, using the enrichment procedure like described above. One of the problems encountered for dinucleotide repeats is stuttering of the *Taq* DNA-polymerase, which results in shadow bands. With the 2 primer pairs AZA-002 and AZA-003 some stuttering was observed. This can be reduced by the choice of the repeat used for primer development, optimising the PCR-program or using a hot start enzyme. In the near future hot start enzymes will be tested. Also detection by fluorescent PCR and automatic analysis with genotyping software will be evaluated to obtain higher troughput. In a next step the whole genepool of Belgian pot azaleas will be screened using microsatellites.

DNA-markers become more and more a helpful tool for several crops. In the case of azalea, DNA-markers can be useful for identification purposes. Because in Belgium the pot azaleas are mainly an export product, an identification system on the juvenile, non-flowering, plant stage is of interest. Also, for the protection of commercial azalea cultivars and for granting Plant Breeders Rights DNA-markers can be unambiguous tool. To introduce DNA-markers for these purposes, a quick and inexpensive detection system is required. Once developed, a set of microsatellite markers can satisfy those applications.

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References

Chamberlain D.F., and Rae S.J., 1990. A revision of Rhododendron IV Subgenus Tsutsusi. Edinburgh J. Bot., 47:89-200.

De Riek, J., Dendauw J., Mertens M., De Loose M., Heursel J., and Van Bockstaele E. 1999. Validation of criteria for the selection of AFLP markers to assess the genetic variation of a breeders' collection of evergreen azaleas. Theor. appl. Genet., 99:1155-1165.

Kobayashi N., Kawashima T., and Arisumi K., 1998. Introgression in Japanese evergreen azaleas (*Rhododendron kiusianum* and *R. kaempferi*). Proc. Third. Symp. On New Floricultural Crops. Acta Hortic., 454, ISHS 1998.

Kron K.A. ,1997. Phylogenetic relationships of Rhododendroideae (Ericaceae). Am. J. of Bot., 84,7: 973-980.

Greenwood M.S., Hopper C.A., and Hutchison K.W., 1989. Maturation in Larch. I. Effect of age on shoot growth, foliar characteristics and DNA methylation. Plant physiol., 90:406-412.

Iqbal M.J., Gray L.E., Paden D.W., and Rayburn A.L., 1994. Feasibility of Rhododendron DNA profiling by RAPD analysis. Plant Varieties and Seeds, 7, 59-63.

Van de Wiel C., Arens P., and Vosman B., 1999. Microsatellites retrieval in lettuce (*Lactuca sativa* L.). Genome, 42: 139-149.

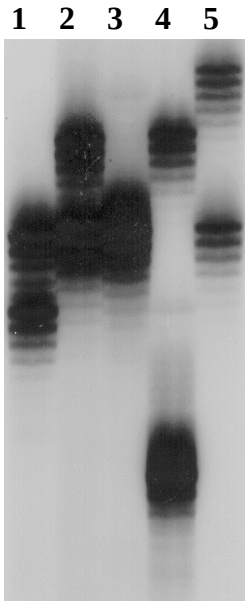
Tables

1. Types of di- and trinucleotide repeats isolated from azalea.

REPEAT TYPE	NUMBER OF CLONES	%
Dinucleotide		
(AC)	30	57.7
(TG)	10	19.2
(CT)	2	3.8
(AC) (TC)	4	7.7
(AG) (TG)	4	7.7
Rest	2	3.8
Sub Total	52	100
Trinucleotide		
(CCA)	42	57.5
(TGC)	12	16.4
(CAG)	6	8.2
(CAA)	1	1.3
(GAC)	1	1.3
(TAG) (TCG)	5	7
(CTG) (TTG)	3	4.2
(TAC) (GAC)	2	2.8
(CAG) (CAA)	1	1.3
SubTotal	73	100
Grand Total	**125**	

Figures

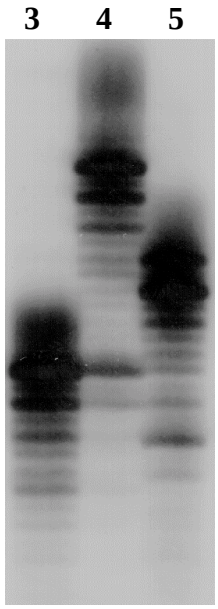
1. Fraction of STMS-pattern of five plants with two microsatellite marker using PAGE.



← ± 260 bp

1 = *R. simsii* Knut Erwen
2 = *R. simsii* Ambrosiana
3 = Hirado Heiwa-No-Hikari
4 = *R. simsii* Mevr. Van Belle
5 = *R. simsii* Daoxian

Primerpair AZA-002
$(AC)_8(TC)_8(AC)_{17}$



← ± 170 bp

Primerpair AZA-003
$(TG)_{15}$

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