E

BMT/7/9
ORIGINAL: English
DATE: November 6, 2001

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA-PROFILING IN PARTICULAR

Seventh Session
Hanover, Germany, November 21 to 23, 2001

DEVELOPMENT OF SSR ANALYSIS STRATEGY FOR VARIETAL IDENTIFICATION IN SUNFLOWER

prepared by experts from France

DEVELOPMENT OF SSR ANALYSIS STRATEGY FOR VARIETAL IDENTIFICATION IN SUNFLOWER

David ZHANG, GEVES, France
BioGEVES, Domaine du Magneraud, BP.52, F-17700 SURGERES
Email: david.zhang@geves.fr; Tel. +33-(0)546-683-036, Fax: +33-(0)546-683-100

Abstract: The presentation will centre on (1) the establishment of a SSR analysis system in sunflower using a sequencer and a fluorescent labelling system, (2) the assessment of SSR polymorphism across a set of 10 inbred lines, (3) the establishment of a set of 100 SSR markers which are being used for the evaluation of inter- and intra-line variability among sunflower inbreds in GEVES, in the context of a research program involving the GEVES and the French inter-professional association AMSOL, and (4) some results of our program in progress.

CONTEXT OF RESEARCH

The DUS testing in sunflower presents the following characteristics in France:

- a big reference collection (about 600 parental inbred lines),
- a lack of discriminating morphological traits permitting to establish reliable groupings of lines,
- a important interaction between genotype x place x year,
- 2 experimental places (La Miniere and Le Magneraud) where all the reference collection and the new applicants are implanted,
- an important number of new applications (about 100 new inbred lines each year),
- 2 years testing.

Because of this situation, it becomes more and more difficult to assess DUS in sunflower with precision and to limit the cost of DUS testing. That's why we initiated the present research program, which is supported financially by the French Ministry of Agriculture, for three years from November 1999. The inter-professional association AMSOL which groups sunflower breeders in France, is our partner in this research project.

OBJECTIVE OF THE STUDY

Our project aims to:

- establish a SSR analysis system in sunflower,
- propose a set of SSRs relevant for variety identification purpose,
- assess the inter-line and intra-line variability by SSR markers on a subset of inbred lines selected from the French reference collection used for sunflower DUS testing,
- optimise the implantation of reference collection, by grouping the sunflower lines based on molecular data,
- improve the efficacy and the accuracy of the DUS testing by integrating molecular data.

MATERIALS

The molecular variability between lines and within line will be assessed respectively by 100 and 50 SSR markers.

For the study of inter-line variability, 178 lines have been chosen, in which there are 100 female lines and 78 male lines. Among these inbreds, most of them are original material; however, particular materials are also included: (1) the male-sterile and fertile forms of a same inbred line, (2) the modified version for mildiou resistance and the susceptible version, (3) different supplies of reference seed lots of a same line.

For the study of intra-line variability, 4 lines have been chosen and 30 individuals per line will be analysed.

A total of 1,111 SSR primer pairs developed in a collaborative research program "CARTISOL" are available for our study. Only a subset of these SSR primer pairs was chosen. At first, we tested the quality of amplification of the chosen primer pairs on 4 lines; then the polymorphism of screened SSR was assessed on 10 lines.

METHODOLOGY

Genomic DNA used for SSR analysis was isolated using the Plant DNeasy Mini kits (Qiagen), from frozen or lyophilised leaf tissues.

PCR reaction was performed in 10µl containing 1X buffer, 0.125mM dNTP, 3mM $MgCl_2$, 0.25µM forward and reverse primers each, 0.5U AmpliTaq Gold and 10ng template DNA.

Amplification was carried out with a "Touchdown" program, which contains (1) 10 mn at 95°C, (2) 10 cycles of 30 seconds at 94°C, at 64°C and at 72°C each, with decreasing 1°C of the annealing temperature every second cycle from 64°C to a touchdown at 55°C, (3) 30 cycles of 30 seconds at 94°C, at 55°C and at 72°C each, and (4) 10mn at 72°C.

Gel electrophoresis is run using an automated DNA analysis system LI-COR 4200S, on a 5% denaturing acrylamide gel.

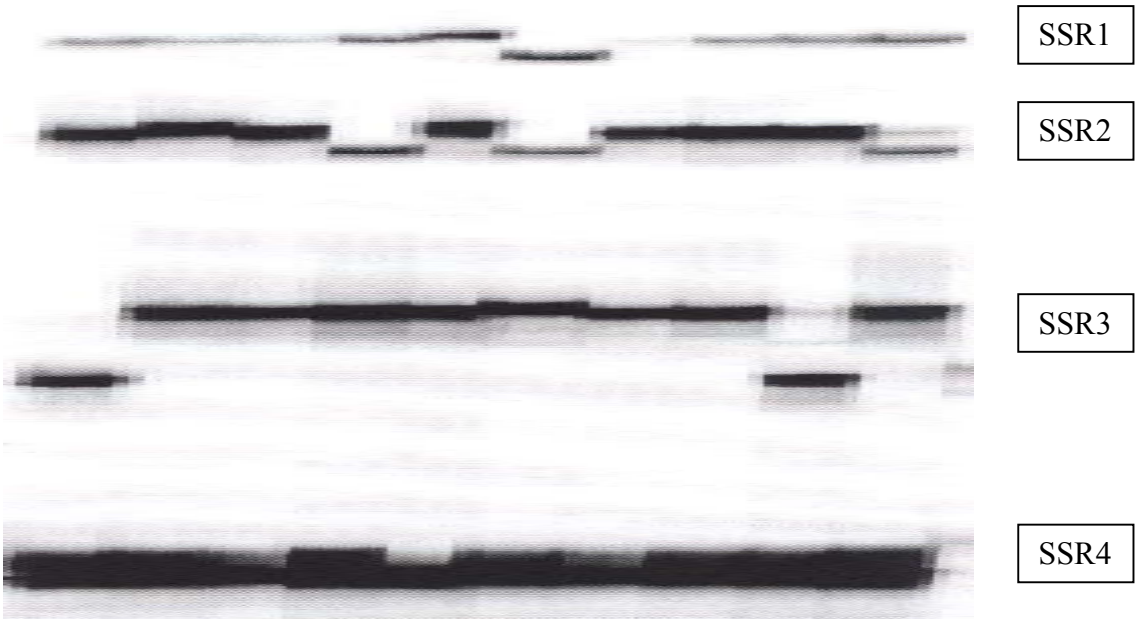
Primers are labeled by fluorescence dyes 700 or 800. A tailed primer strategy is applied in order to reduce the cost of primers labeling. For this, the sequence for a M13 forward primer is added to the 5'-end of one primer in each marker set. During the PCR, an Infrared (IR)-labeled M13F primer is added as a third primer to the SSR reaction to incorporate the label. In this approach, only one labeled primer is required for a family of markers containing M13 tails. To be able to utilise all the two dyes available, a second tail is also developed.

ESTABLISHMENT OF SSR ANALYSIS CONDITIONS

The Plant DNeasy mini kits (Qiagen) works well in sunflower, which allows to extract sufficient DNA in quality as well as in quantity for SSR analysis in multiplexing, from about 30 mg of powdery from lyophilised leaves. It should be indicated that the multiplexing PCR needs higher quality of DNA than simplex PCR.

The described PCR reaction as well as amplification conditions allow a successful amplification for the majority of the SSR tested (323 in total). Multiplexing PCR was established essentially based on the size of alleles of SSRs. Two to five SSR markers can be amplified together (Fig. 1). The tailed primer strategy works also very well; it needs to label only two tails d for all the SSR tested.

Figure 1. Gel image showing the results of a multiplexing PCR in sunflower



Electrophoresis of all amplified SSRs is run on a 5% gel using the LI-COR automated sequencer. The products of two sets of PCR from two different dyes labeling can be mixed by equal volume and loaded together on a same gel. Each gel can be reloaded until 3 times. A multi-channel syringe is used for sample loading.

Gel images and SSR data analyses are performed by using respectively the Gene ImagIR and RFLPscan softwares.

ESTABLISHMENT OF A SET OF SSR MARKERS FOR VARIETAL IDENTIFICATION IN SUNFLOWER

Among the 1,111 SSR primer pairs available, we tested a subset of 323 which were considered as the best ones in term of amplification quality as well as of polymorphism level, according to the information available. We established a set of 100 SSR markers, based on the following criteria: single locus, codominant, clear profiling and easy for data scoring. The information concerning the polymorphism level of these 100 SSRs obtained across a set of 10 public lines (5 female lines: HA89, H52, HA372, HA383 and HA821; 5 male lines: RHA274, RHA377, RHA801, PAC2 and RHA266) is summarised in the table 1. The number of alleles per SSR ranged from 2 to 6, with an average of 3.1. The PIC (Polymorphism Information Content) value of this set of SSRs varied from 0.18-0.78, with a mean of 0.53. This set of SSRs is using for the assessment of variability between lines as well as within lines, scheduled in our research project.

Table 1: Characteristics of the 100 SSR markers maintained

Alleles per SSR	**Number of SSRs**	**Total of alleles**
2	31	62
3	39	117
4	24	96
5	4	20
6	2	12
Total	**100**	**307**

INTERLINE VARIABILITY REVEALED BY SSR MARKERS: PARTIAL RESULTS

Until now, some partial results have been obtained on a subset of 90 lines analysed by 14 SSRs. The number of alleles par SSR varied from 2 to 6, with an average of 3.8 which is higher then that observed on the 10 public lines. The value of PIC () ranged from 0.28 to 0.81, with a mean of 0.57 (Table 2).

Table 2. Polymorphism of 14 SSRs revealed on 90 sunflower lines

SSR	**Number of alleles**	**PIC**
ORS7	4	0.58
ORS10	2	0.28
ORS303	2	0.37
ORS307	4	0.57
ORS309	2	0.5
ORS310	6	0.72
ORS342	4	0.35
ORS607	4	0.74
SSL3	4	0.55
SSL9	6	0.81
SSL13	4	0.53
SSL231	3	0.59
SSL241	4	0.66
Mean	**3.8**	**0.57**

WORK IN PROGRESS

As scheduled in our 3 years' project, the work is going on in GEVES. This concerns the analyses of:

- for inter-line variability assessment, 178 lines x 100 SSR,
- for intra-line variability assessment, 4 lines x 30 individuals / line x 50 SSR.

A ring trial is also in progress, which consists in testing the 100 established by GEVES, by other laboratories, using the same 10 public lines and the DNA supplied by GEVES. The participating laboratories are free to use their own protocols and conditions. This test aims to assess the robustness of the SSRs. Four laboratories from seed companies, members of AMSOL, are involving in this ring trail.

All the work of the project will be ended in October, 2002.

Acknowledgements:

This program is supported by the French Ministry of Agriculture for 3 years, from 1999 to 2002. The French inter-professional association AMSOL is our partner in this research project.

[End of document]