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ASSESSMENT OF MOLECULAR VARIABILITY BETWEEN AND WITHIN VARIETIES BY AFLP IN ROSE

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ASSESSMENT OF MOLECULAR VARIABILITY BETWEEN AND WITHIN VARIETIES BY AFLP IN ROSE

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1. Introduction

Rose is one of the most economically important ornamental species used as landscape and cut-flower plant in the world. In France, rose is one of the main vegetatively propagated ornamental plants, at both cultivation and commercial level. Each year, the Groupe d'Etude et de Contrôle des Variétés et des Semences (GEVES), the French official technique institute responsible for Distinctness, Uniformity and Stability (DUS) testing of plant varieties, registers about 70 new applications for cultivar protection in rose. More than 1300 rose cultivars are maintained in the French official reference collection, used for DUS testing in the context of UPOV (International Union for the Protection of New Varieties of Plants) convention. Traditionally, the DUS testing is based on the observation of morphological characteristics. This becomes insufficient due to (1) the increasing number of patented rose cultivars, (2) the frequent occurrence of mutants from the existing varieties and (3) the narrow genetic base used for variety selection in some cases. On the other hand, the infringement is frequent in rose production. The rose breeders require a faster, powerful and reliable technique for accurate identification of varieties in order to prevent frauds. Moreover, the examination of essentially derived varieties (EDV) by application of the UPOV-convention of 1991 requires also a powerful method to evaluate the genetic conformity between two varieties in litigation.

As a result, molecular markers have been developed to help the identification of rose varieties : RFLP by Hubbard et al. (1992) and Ballar et al. (1995), RAPD by Torres et al. (1993), Cubero et al. (1995) and Reynders-Aloisi and Bollereau (1995), and DNA fingerprint by Vainstein and Ben-Meir (1994). All these reports show the usefulness of molecular markers for variety identification in rose. However, the AFLP technique (Vos et al., 1995) seems more powerful and attractive than RFLP and RAPD to variety identification purposes in vegetatively propagated plants (De Riek et al., 1997 ; Dirlewanger 1997) ; the AFLP tool shows number of advantages : high polymorphism rate per run, low cost of analysis and good reproducibility. So, we have initiated a research program entitled 'Description of genetic variability and variety identification in rose by molecular markers'. The aim of our research was to evaluate the potentials of AFLP and RAPD markers for variety identification in rose, compared with the existing morphological data. In this paper, we present our preliminary results on assessment of the genetic variability of inter- and intra-varieties by AFLP.

2. Materials and methods

2.1 Plant materials

To evaluate the polymorphism of AFLP among modern rose varieties, we chose 13 varieties which show a wide range of morphological variations : type of utilization (landscape or cut-

flower), growth habit, plant height, colour, size and number of flowers, leaf size and etc (Table 1). For reason of confidentiality, the 13 varieties used for this study are coded from 1 to 13.

The intra-variety variability has been examined on two varieties : the first one, coded as TK, from which leaves were sampled on 3 different shoots of 6 plants, giving a total of 18 samples ; the second one, coded as MF, from which leaves have been sampled on 5 clones and each has a different combination of plant origin (France, Spain and The Netherlands) x rootstock (*R. canina inermis, R. indica major* and *R. Manettii*) x rootstock origin (Germany, Maroc, Spain and The Netherland).

To deal with essentially derived varieties, we have tested three cases. The first case concerns an applicant variety **A'** which failed its DUS testing because it was considered by the rose DUS testing experts to be identical to variety **A** which is notoriously known. The second case involves two varieties (**B** and **B'**) morphologically very close ; variety **B'** has two versions, one stable (**B1'**) and the other unstable (**B2'**). The last case concerns variety **C** and its two mutant variants (**C1** and **C2**) ; they have following relationship : **C2** is a mutant of **C1** which is a mutant of variety **C**. For all these studies, the young leaves were harvested from greenhouse growing roses at GEVES Sophia-Antipolis (south of France).

2.2 DNA isolation and AFLP analyses

Total DNA of all rose samples collected was isolated from frozen leaf tissue according to the CTAB (cetyltrimethylammonium bromide) based procedure described by Rogers and Bendich (1988), with some minor modifications and adaptations.

AFLP analysis was performed using a commercially available kit from Life Technologies (AFLP™ Analysis System I). All the technique procedures used were as in the supplied protocols, and also described in detail by Vos et al. (1995). The enzymes used for DNA digestion were *EcoRI* and *MseI*. Selective amplification was carried out using a combination of one *EcoRI* primer and one *MseI* primer with 3 selective bases each. The *EcoRI* primer was end labelled using [γ-33p]ATP and T4 polynucleotide kinase. PCR reactions were performed in a MJ PTC-100 thermocycler. AFLP fragments were separated by PAGE using a DDH-400-33 gel apparatus (C.B.S. Scientific CO.).

2.3 Data analysis

AFLP Profiles observed in autoradiographs were scored visually. The presence or absence of a band position in a gel lane was coded 1 or 0, respectively. We decided to score only the band positions which were polymorphic at least for 2 out of the 13 varieties studied, and which ranged from 100-500 bp in size. For each AFLP marker, a polymorphic information content (PIC) was calculated with the formula PIC = $1-(P^2+q^2)$ where p and q are allelic frequency, with the assumption that each AFLP fragment correspond to one locus and each locus has two alleles (presence and absence). For purposes of comparison, the simple matching coefficient *SM* (Sneath and Sokal, 1973) as well as *F* index of Nei and Li (1979) were computed to measure the similarity / distance for each pair of the 13 varieties. The *SM* coefficient was computed as : $SMxy = Mxy / n = (n_{11} + n_{00})/n$, where Mxy = number of matches, n_{11} = number of band positions scored 1 for x and y, n_{00} = number of band positions scored 0 for x and y, n = number of band positions scored. *F* index was computed as $Fxy = 2\ Nxy / (Nx + Ny)$, where

*F*xy = similarity index between varieties x and y, Nxy = number of band positions scored 1 for both x and y, Nx = number of band positions scored 1 for x and Ny = number of band positions scored 1 for y.

3. Results

3.1 Polymorphism detected by AFLP on rose varieties

Prescreening of primer combinations was performed using 3 rose varieties. Fifty-six primer combinations, among the 64 possible in the kit, were tested ; almost all gave amplification products. However, only 25 produced scorables fingerprints. Typically, 50 to 100 fragments were amplified per primer combination, with sizes ranging from 50 to 700 bp.

The twelve best primer combinations were then chosen to assess the polymorphism level of AFLP across the 13 rose varieties studied. The results are summarized in table2. According to the criteria defined by us for scoring markers (see material and methods), a total of 322 polymorphic amplification products were scored. The number of polymorphic band positions per primer pair combination varied from 21 to 39, with an average of 26.8. The PIC values of AFLP markers ranged from 0.26 to 0.50, with a mean of 0.40. Among the 12 primer pairs, the mean of PIC values for each primer pair varied from 0.38 to 0.42. More than 50% of the scored AFLP markers had a PIC value superior to 0.40 (Fig. 1). The 13 rose varieties were easily identified by AFLP markers generated by anyone of the 12 primer combinations.

3.2 Genetic similarity and relationships among the varieties

Variety relationships were measured by the computation of similarity indices between varieties (Table 3). Only the AFLP fingerprints generated by 7 out of the 12 primer combinations were used for this computation because of missing data. The simple matching coefficient *SM*, which takes into account both the positive and negative matches, ranged from 0.45 (varieties 5 and 6) to 0.66 (varieties 2 and 13), with an average of 0.56. The *F* index, which takes into account only the positive matches, varied from 0.37 (varietes 1 and 10) to 0.70 (varieties 5 and 9), with a mean of 0.57 which is very similar to that of the simple matching coefficient (Table 2). The obvious low *F* mean value (0.47) for variety 1 could be explained by a very low number of bands scored for this variety, which did not have a prominent influence to the simple matching coefficient of this variety. The two types of indexes measuring the similarity between varieties were well correlated ($\gamma^2 = 0.81$).

3.3 Assessment of intra-variety variability by AFLP

Four primer combinations amongst the 12 best were chosen for this trial. They allowed to generate 194 band positions on the 18 samples of variety '**TK**'. No variation of band position was revealed. This result showed that there was no AFLP variation amongst different plants and different shoots of the same plant for this variety. The same primer pairs were used to examine whether the rootstocks as well as the origin of plant and root stock can create an influence on AFLP fingerprints. The 227 band positions produced by the 4 primer pairs on the 5 clones of variety '**MF**' did not detect any variation among the 5 clones.

3.4 Test of morphologically related genotypes by AFLP

For all the tests, we used the same 4 primer combinations as in 3.3. For the first situation, no difference between **A** and **A'** was observed using 189 scorable band positions. For the second situation, 183 band positions generated have allowed to reveal 5 and 7 differences between **B** and **B1'**, **B** and **B2'**, respectively ; two differences were also observed between **B1'** and **B2'**. As to the third situation, no difference has been detected among **C**, **C1** and **C2** by 190 AFLP band positions.

4. Discussion

Molecular polymorphism among the modern varieties of roses revealed by AFLP in our study was relatively high, with an average of about 27 markers generated per selected primer combination. This number would be still higher if we had scored all the unique band positions (present in only one out of the 13 varieties studied) and those having size between 50 to 100 pb. This result confirms both the high discriminate power of AFLP markers reported in other crop plants (Hongtrakul *et al*, 1997, Qi and Lindhout, 1997, Marsan *et al*., 1998) and the high molecular polymorphism among the modern varieties of roses detected by RFLP (Hubbard *et al*., 1992), RAPD (Cubero *et al*., 1995) and DNA fingerprint (Vainstein and Ben-Meir (1994). The high level of genetic variation in cultivated roses can be explained by the fact that about 10 different species were involved in the breeding of modern varieties of rose. On the other hand, the PIC values of the AFLP markers obtained in our research were relatively high, with a mean of 0.40 (the maximum is 0.50 for a bi-allelic locus), compared with 0.14 reported for sunflower (Hongtrakul *et al*, 1997). The high discriminant power of AFLP markers for varietal identification in rose was also demonstrated by the fact that whichever primer combination selected allowed to identify easily the 13 varieties used.

No molecular intra-variety variation has been detected by the AFLP markers on the 18 samples of variety **TK** examined. This result is in some way what we expect for a vegetatively propagated plant. Likewise, no molecular difference was observed on the 5 clones of variety **MF**, showing no root stock influence on AFLP profiling. The absence of intra-variety variation is anyhow a positive element in favor of the utilization of molecular tools for variety identification and examination of essentially derived varieties. Near 200 band positions have not been able to differentiate the pair **A** and **A'**, and the triplet of **C**, **C1** and **C2**. In fact, there is no difference between **A** and **A'** at the morphological level in the field ; **C1** and **C2** can be differentiated by their flower color only and are climbing mutant of **C**. However, in the other triplet, **B**, **B1'** and **B2'** could be distinguished from each other by AFLP. Morphologically, they are very close except that **B** is a no climbing rose, **B1'** is a stable climbing and **B2'** is an unstable climbing.). About the identification of mutants from their initial variety which is a common problem for most vegetatively propagated plants, the similar results have been reported in pot azalea (De Riek et al., 1997). On the contrary, AFLP markers have allowed to differentiate a mutant from its initial variety in peach (Dirlewanger, 1997). For Ballard et al. (1995), the RFLP and RAPD markers developed have not been able to distinguish Flaming Peace (mutant) from Peace (initial variety) and however have allow to distinguish three other mutants from Peace. Although the molecular markers are in general more discriminate than morphological traits, the probability of the identification of mutants remains very low because the mutations observed concern often one trait often involving only one locus or few loci and the polymorphism of most of molecular markers often origins from the non-coding sequences

(or regions). Using a larger number of AFLP markers with a good coverage of the genome would increase this probability.

Reproducibility of the AFLP markers has been reported to be good (Jones et al., 1997). This was confirmed indirectly by our results that no AFLP profiling variation was observed on 18 samples of the variety **TK** as well as on 5 samples of variety **MF**. However, one should avoid to score the faint bands which could be sometimes not reproducible (results not showed). Moreover, different DNA isolation techniques could influence AFLP profiling (results not showed). Problems of this kind might be avoided by using a standardized technique for DNA isolation and by analyzing double or even triple samples for each variety. This precaution would be necessary in the juridical context, for example, in a litigation for infringement or EDV.

All of our results show that AFLP profiling technology can be a very useful and powerful tool for both variety identification and genetic conformity assessment in rose. Compared with other molecular marker systems assayed in rose, AFLP strategy shows many advantages : it is easier than RFLP to realize ; it is cost efficient due to its high polymorphism rate (markers) per run and it is more reliable than RAPD. In the future, we are going to assess a larger set of rose varieties by AFLP markers. The data generated by AFLP will be compared with those produced by RAPD as well as the morphological data available.

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Table 1. Main characteristics of the 13 rose varieties used.

Varieties	Origin	Commercial Use	Plant Growth type	Growth habit	Plant height	Flower colour	Petal number	Fragrance
1	GB	Garden rose	Shrub rose	bushy	tall	apricot	126-135	very strong
2	DE	mixed	Bed rose	bushy	medium	medium yellow	-	absent or very weak
3	FR	Garden rose	Shrub rose	Broad bushy	medium	medium pink	-	Very weak
4	FR	mixed	Dwarf rose	narrow bushy	very short	yellow and orange (streaked)	94-102	weak
5	FR	mixed	Bed rose	narrow bushy	medium	red and white (streaked)	65-75	weak
6	FR	Garden rose	Bed rose	from narrow bushy to bushy	tall	mauve	28-35	very strong
7	FR	Garden rose	Shrub rose	bushy	tall	from white to clear pink	42-56	absent or very weak
8	FR	Garden rose	Bed rose	narrow bushy	medium	medium red	28-39	weak
9	FR	Garden rose	Bed rose	bushy	medium	dark red	31-36	very strong
10	FR	Cut flower	Bed rose	narrow bushy	medium	brown red	20-24	medium
11	FR	Garden rose	Dwarf rose		very short	orange red	46-59	absent
12	FR	Garden rose	Bed rose	narrow bushy	tall	yellow blend	32-36	weak to medium
13	FR	Garden rose	Shrub rose	bushy	short	red and white (streaked)	55-62	weak

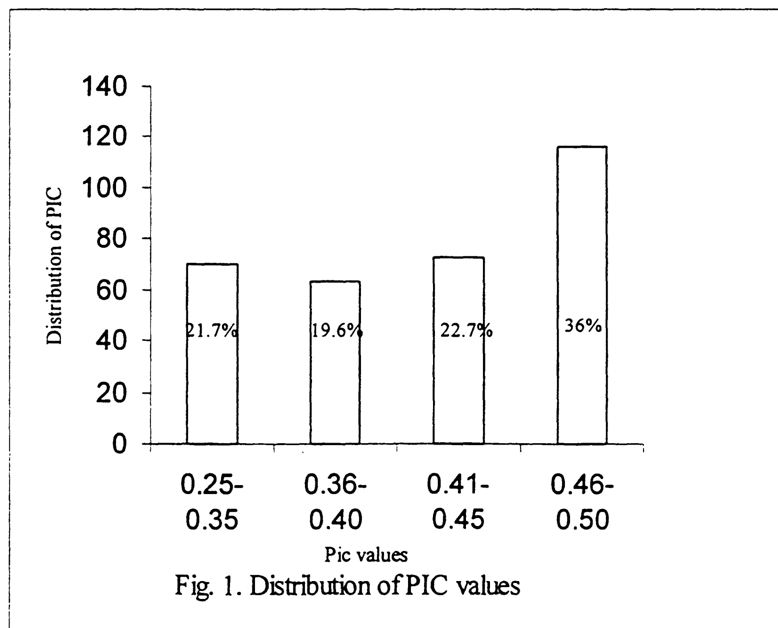

Fig. 1. Distribution of PIC values

Table 2. AFLP polymorphism revealed by 12 primer combinations on 13 rose varieties.

Primer combination	Polymorphic bands scored	Number of varieties assayed	Varieties identified	Mean of PIC
E-ACC / M-CAT	25	13	13	0,43
E-ACC / M-CTA	21	13	13	0,35
E-ACC / M-CTC	21	13	13	0,40
E-ACA / M-CTA	29	13	13	0,41
E-ACA / M-CTG	30	12	12	0,37
E-ACA / M-CAC	31	12	12	0,42
E-ACA / M-CAg	23	12	12	0,42
E-ACA/M-CAT	24	12	12	0,38
E-AAC/M-CAA	39	13	13	0,41
E-AAC/M-CAC	27	12	12	0,41
E-AAC/M-CAg	29	12	12	0,37
E-ACT / M-CAT	23	13	13	0,41
Total	**322**	**13**	**13**	
Mean	**26.8**			**0,40**

Table 3. Similarity indexes computed using 188 AFLP markers generated by 7 primer combinations for the 13 rose varieties analyzed: Simple matching index (Sneath and Sokal, 1973) (below diagonal) and F index of Nei and Li (1979) (above diagonal).

Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	Mean
1		0,51	0,38	0,45	0,49	0,40	0,52	0,52	0,50	0,37	0,51	0,47	0,47	*0,47*
2	0,56		0,61	0,59	0,66	0,56	0,55	0,61	0,63	0,56	0,63	0,57	0,68	*0,60*
3	0,51	0,62		0,56	0,56	0,47	0,50	0,52	0,54	0,53	0,50	0,51	0,63	*0,53*
4	0,55	0,59	0,61		0,51	0,51	0,55	0,55	0,55	0,52	0,51	0,55	0,59	*0,54*
5	0,55	0,63	0,58	0,52		0,47	0,48	0,57	0,70	0,54	0,56	0,62	0,62	*0,57*
6	0,48	0,54	0,50	0,52	0,45		0,62	0,60	0,52	0,57	0,57	0,62	0,58	*0,58*
7	0,60	0,54	0,54	0,57	0,47	0,63		0,61	0,54	0,59	0,58	0,58	0,54	*0,57*
8	0,59	0,59	0,55	0,56	0,55	0,60	0,61		0,59	0,62	0,54	0,58	0,59	*0,58*
9	0,52	0,58	0,53	0,52	0,66	0,47	0,51	0,54		0,62	0,54	0,62	0,62	*0,60*
10	0,46	0,54	0,56	0,54	0,52	0,57	0,60	0,62	0,57		0,58	0,56	0,62	*0,59*
11	0,57	0,61	0,53	0,52	0,54	0,56	0,58	0,53	0,49	0,57		0,55	0,65	*0,60*
12	0,53	0,54	0,53	0,55	0,60	0,61	0,58	0,56	0,57	0,54	0,53		0,59	*0,59*
13	0,54	0,66	0,65	0,60	0,61	0,58	0,55	0,59	0,57	0,62	0,59	0,57		**0,57**
Mean	*0,54*	*0,59*	*0,56*	*0,54*	*0,55*	*0,57*	*0,57*	*0,57*	*0,55*	*0,58*	*0,56*	*0,57*	**0,56**	

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