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RAPD ANALYSIS OF SOMACLONAL AND NATURAL DNA VARIATION IN *HYPERICUM PERFORATUM* L.

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RAPD analysis of DNA variation in somaclones of *Hypericum perforatum* which passed through one or two cycles of in vitro regeneration revealed great variation among the donor plants from which the somaclones were regenerated. The donor plants represented either seed-derived plants or the seed progeny of first-cycle somaclones. The variation among them may indicate natural genetic variation in *Hypericum perforatum*. In addition, no differences were found in the RAPD profiles of 51 first-cycle somaclones within groups originated from 7 donor plants. This may point to relative stability of the *Hypericum perforatum* genome under given conditions of in vitro regeneration. One polymorphic band was found in 3 of 51 RAPD profiles of second-cycle somaclones, perhaps an effect of repeated in vitro regeneration on DNA changes. Study of DNA variation among 75 *Hypericum perforatum* progenies derived from 8 mother plants indicates the prevalence of the apomictic mode of reproduction. Analysis of 47 seed progenies of diploid somaclones derived from 5 R_3 plants may suggest the sexual mode of reproduction.

Key words: *Hypericum perforatum*, RAPD, intraspecific variation, in vitro regeneration, somaclonal variation, DNA changes.

INTRODUCTION

Hypericum perforatum L. (St. Johns wort, 2n = 4x = 32) contains a wide range of pharmacologically active compounds including hypericin and pseudohypericin, which have significant antiviral and anticancer activity (Diwu and Lown, 1994). To widen genetic variability and to select genotypes with increased content of pharmaceuticals, Čellárová et al. (1992) developed an effective in vitro regeneration system for *Hypericum perforatum*. The system enabled morphological, biochemical, cytogenetic and molecular study of variation among the medicinal plant's somaclones and their seed progenies (Čellárová et al., 1992, 1994; Halušková and Čellárová, 1997; Brutovská et al., 1998). The dependence of some phenotypic traits on somaclone ploidy has been reported; diploid somaclones were characterized by increased hypericin content during several seed generations (Čellárová et al., 1997).

DNA polymorphism amplified by arbitrary primers (RAPD) has proved useful as a genetic marker for different genetic purposes in plants (Williams et al., 1990; Welsh and McClelland, 1990).

This study examines the effect of repeated in vitro regeneration on DNA changes and the level of DNA variation among seed-derived control plants and the seed progenies of diploids derived from tissue culture.

MATERIALS AND METHODS

PLANT MATERIAL, IN VITRO REGENERATION AND CULTIVATION CONDITIONS

First- and second-cycle somaclones of *Hypericum perforatum* were analyzed in order to study the effect of repeated in vitro regeneration on DNA changes. The somaclones were obtained in Experiments 1 and 2 (Tab. 1) by in vitro regeneration of 14

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TABLE 1. In vitro regeneration

Experiment 1			Experiment 2		
No. of seed-derived donor plants	No. of somaclones analyzed	Analysis of donor plant	No. of R_1 seed-derived donor plants	No. of somaclones analyzed	Analysis of donor plant
1	8	yes	1	8	yes
2	8	yes	2	9	yes
3	9	yes	3	7	no
4	4	yes	4	8	no
5	8	no	5	6	yes
6	7	no	6	6	no
7	7	no	7	7	no

donors, of which 7 represented *Hypericum perforatum* cv. Topas plants and 7 represented R_1 plants, the progeny of 5 original R_0 *Hypericum perforatum* somaclones. The somaclones were regenerated from leaves of *Hypericum perforatum* seedlings on basal RM medium (Linsmaier and Skoog, 1965) supplemented with 0.5 mg/ml or 1.0 mg/ml of 6-benzylaminopurine according to a protocol previously described by Čellárová et al. (1992).

Natural DNA variation was analyzed in 75 progenies obtained from seeds of 8 *Hypericum perforatum* cv. Topas mother plants (different from the donor plants in Experiment 1). Also, 47 diploid plants representing R_4 progeny of 2 original R_0 diploid somaclones were derived from seeds of 5 different R_3 plants. The chromosome numbers of the diploids were counted using classical cytogenetic procedures (Brutovská et al., 1998).

Plant seeds were sterilized with 1% solution of $AgNO_3$ for 15 min. Seed-derived plants and shoots after in vitro regeneration were grown on basal RM medium (Linsmaier and Skoog, 1965) without plant growth regulators in a cultivation room at 22˚C and 40% relative humidity under a 16 h photoperiod (1100 lux, 6.8 μmol m^{-2} s^{-1}).

DNA ISOLATION

Hypericum perforatum leaf DNA was isolated from plants with 5–6 pairs of foliage leaves using the method of Haberer et al. (1996), modified by Halušková (1997). The amount of DNA in 1 μl of TE solution was evaluated electrophoretically by comparing band intensities with lambda DNA (100 ng/μl) as the standard.

RAPD AMPLIFICATION

Forty RAPD primers (30 Roth and 10 Life Technologies) were tested for amplification with *Hypericum perforatum* DNA as the template. Two of them (Roth) generated reproducible RAPD profiles and were employed for our purposes: 160–10 (5' – GCA GAC TGA G – 3') and 360–10 (5' – GAG CAG GCT G – 3'). Amplification reactions were performed in 25 μl reaction mixtures containing 1 × PCR reaction buffer (Advanced Biotechnologies), 2 mM $MgCl_2$ (Advanced Biotechnologies), 0.2 mM dNTP mixture (Advanced Biotechnologies), 0.5 μM RAPD primer, 0.5 U Taq-DNA-polymerase (Advanced Biotechnologies) and 1 μl (20–50 ng) *Hypericum perforatum* template DNA. The reaction mixtures were supplemented to 25 μl with sterile deionized water and overdropped with 30 μl sterile mineral oil (Sigma). Amplification reactions using primer 160–10 were performed with a Techne PHC-3 thermocycler, and those using primer 360–10 with a Techne Progene thermocycler. The conditions for amplifications were as follows: denaturing step at 94˚C for 5 min, followed by 40 cycles of denaturing step at 94˚C for 1 min, primer annealing step at 36˚C for 1 min, and elongating step at 72˚C for 2 min. The reactions were accomplished by a final elongating step at 72˚C for 5 min. After amplification the reaction mixtures were analyzed electrophoretically on 1% agarose gels and in 1 × TAE buffer at 5 Vcm^{-1}. *HindIII*-digested lambda DNA (0.5 mg/ml) was used as the size marker. The gels were stained in ethidium bromide buffer (2 μg/ml), evaluated under UV (254 nm) and photographed through a red filter.

RESULTS

No DNA changes were revealed by comparison of the RAPD profiles generated with primer 160–10 within the groups of first-cycle somaclones originated from each of the 7 donor *Hypericum perforatum* plants. The RAPD profiles of somaclones derived from 3

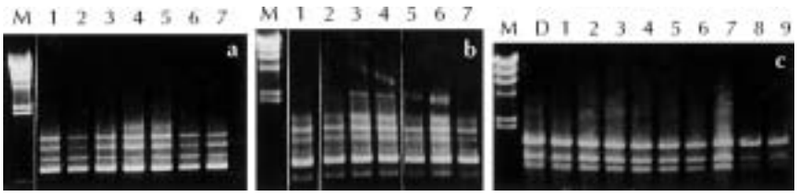

Fig. 1. RAPD amplification of *Hypericum perforatum* DNA with primer 160–10: (**a–b**) 7 first-cycle somaclones regenerated from donor plants 6 and 7 (RAPD profiles of donor plants not present), (**c**) 9 first-cycle somaclones regenerated from donor plant 3 (D). M – size marker (*HindIII*-digested lambda DNA).

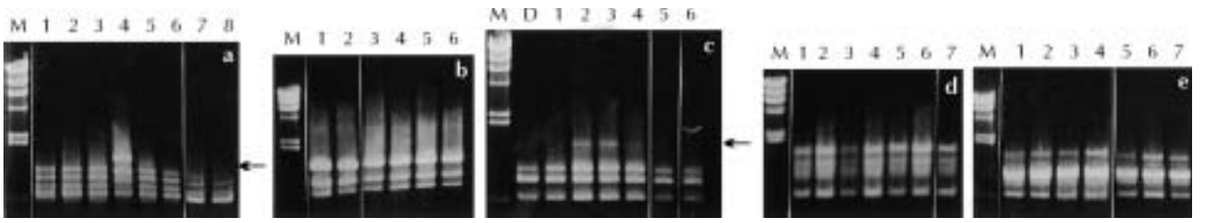

Fig. 2. RAPD amplification of *Hypericum perforatum* DNA with primer 160–10: (**a,b,d,e**) 8, 6 and 7 second-cycle somaclones regenerated from R_1 plants 4, 6, 3 and 7 (RAPD profiles of donor plants not present), (**c**) 6 second-cycle somaclones regenerated from R_1 plant 5 (D). Arrows indicate polymorphic bands present in three second-cycle somaclones. M – size marker (*HindIII*-digested lambda DNA).

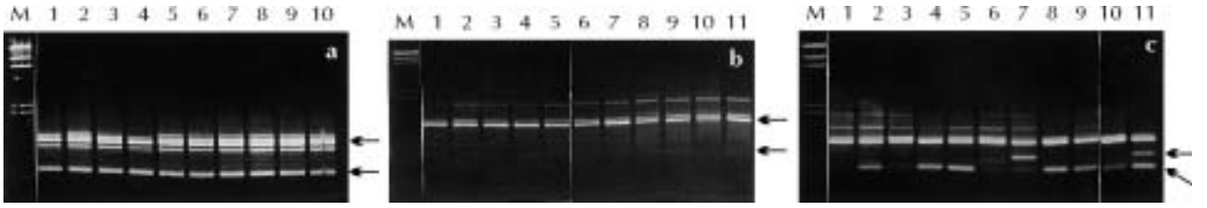

Fig. 3. RAPD amplification of *Hypericum perforatum* DNA with primer 360–10: (**a,b**) 10 and 11 progenies derived from 2 different mother plants, (**c**) 11 diploid progenies derived from one R_3 mother plant. Arrows indicate two bands polymorphic between progenies derived from different mother plants (a and b) and two further polymorphic bands present in the RAPD profiles of diploid progenies derived from the same mother plant. M – size marker (*HindIII*-digested lambda DNA).

different donor plants, demonstrating 3 types of RAPD profile found among first-cycle somaclones, are shown in Figure 1a-c. Only clear, unambiguous and reproducible amplification bands were used for evaluation of the results. The polymorphism of the faint bands present in the RAPD profiles of somaclones 3, 4, 5 and 6 (Fig. 1b) was not considered.

One intensive and one weak polymorphic band were identified in the RAPD profiles of 3 second-cycle somaclones (somaclone 4, Fig. 2a; somaclones 2 and 3, Fig. 2c). Three types of RAPD profile were revealed among the second-cycle somaclones derived from different R1 donor plants (Fig. 2 a–e).

RAPD analysis with primer 360–10 in *Hypericum perforatum* control seed-derived plants revealed identical RAPD profiles within progenies derived from each of 8 mother plants (Fig. 3a,b). The RAPD profiles of all progenies derived from one mother plant lacked two bands (Fig. 3b) that were present in the RAPD profiles of all the other progenies. Two bands that were polymorphic among the RAPD profiles of progenies derived from the same mother plant were identified in *Hypericum perforatum* diploids (Fig. 3c).

DISCUSSION

The results of RAPD analysis indicate that the given conditions of in vitro regeneration appeared not to induce changes in the analyzed DNA sequences of *Hypericum perforatum* plants that passed through one cycle of in vitro regeneration. The polymorphisms observed in 3 of 51 second-cycle somaclones suggest an effect of repeated in vitro regeneration on DNA changes. The use of more RAPD primers to study induced DNA variation might provide more complete information.

The differences in the RAPD profiles of somaclones regenerated from different donor plants may be due to DNA variation among the donor plants themselves, indicating natural genetic variation in *Hypericum perforatum*. We suppose that it may be due to the assumed alloploid origin (Robson, 1981) and facultative apomictic mode of reproduction of the medicinal plant as well. Sexual events occur in 3–7% of *Hypericum perforatum* plants, and more than 90% of the progeny arise from unreduced eggs by apomixis (Noack, 1939; Brutovská et al., 1998). Homogeneity of the amplification profiles in *Hypericum perforatum*, evidencing the predominance of an apomictic mode of reproduction, was identified using RAPD by Arnholdt-Schmitt (2000). RAPD markers were also used to verify sexual and apomictic offspring of intraspecific crosses in *Hypericum perforatum* (Steck et al., 2001).

Our analysis of natural *H. perforatum* DNA variation revealed homogeneity of RAPD profiles within progenies derived from each of 8 mother *H. perforatum* plants. The RAPD profiles of 11 daughter plants, differing from those of the rest of the progenies, may refer to the sexual origin of the plant from which they derived. The results support findings on the prevalence of the apomictic mode of reproduction in *H. perforatum* (Arnhold-Schmitt, 2000; Steck et al., 2001). The heterogeneity of the RAPD profiles in diploid progenies derived from the same mother plant may suggest a sexual mode of reproduction.

Although only 2 of 40 RAPD primers tested in this study suited our purposes, they appeared to be sensitive enough to identify induced DNA changes and natural DNA variation in *H. perforatum*. No more primers generated reproducible results, pointing to the limitations of RAPD amplification for study of DNA variation in this species. ISSR, micro- or minisatellite primers will be employed in future work on this problem.

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