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Adventitious Shoot Regeneration from Apple Leaves – Optimisation of the Protocol and Assessment of Genetic Variation among Regenerants

By

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With 1 Figure

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Summary

VIRŠČEK-MARN M., BOHANEC B. & JAVORNIK B. 1999. Adventitious shoot regeneration from apple leaves – Optimisation of the protocol and assessment of genetic variation among regenerants. – Phyton (Horn, Austria) 39 (1): 61–70, 1 figure. – English with German summary.

An efficient regeneration system and sufficient genetic variation among regenerants are prerequisites for the use of somaclonal variation for apple breeding. Factors influencing in vitro regeneration and development of shoots from leaves of the cultivars Golden Delicious Bovey and Goldspur were studied. 0.2 mg/l TDZ and 0.1 mg/l IBA induced the highest shoot number per leaf and gave the highest number of well developed healthy shoots after dissection and one subculture on multiplication medium. To determine induced genetic variation 39 regenerants of the cultivar Golden Delicious Bovey and 38 regenerants of the cultivar Goldspur were screened with 25 RAPD primers. One regenerant of Golden Delicious Bovey lacked the fragment OPM-02/900 and another regenerant of the same cultivar showed the absence of both OPA-01/850 and OPA11/500 fragments. An additional band, OPX-14/600, was amplified in one of the Goldspur regenerants. On the basis of the estimated amount of genome screened, it can be concluded that the studied regeneration technique induced relatively high genetic variation.

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Zusammenfassung

VIRŠČEK-MARN M., BOHANEC B. & JAVORNIK B. 1999. Sproßregeneration aus Apfelblättern – Optimierung des Versuchsansatzes und Einschätzung der genetischen Variation innerhalb der Neubildungen. – Phyton (Horn, Austria) 39 (1): 61–70, 1 Abbildung. – Englisch mit deutscher Zusammenfassung.

Ein wirksames Regenerationssystem und eine ausreichende genetische Variation innerhalb von Neubildungen sind die Voraussetzungen für die Verwendung von somaclonalen Varietäten zur Apfelzüchtung. Es wurden die in vitro – Regeneration beeinflussenden Faktoren und die Entwicklung von Sprossen aus Blättern der Sorten Golden Delicious Bovey und Goldspur untersucht. 0,2 mg/l TDZ und 0,1 mg/l IBA induzierten die größte Sproßzahl pro Blatt und ergaben nach dem Abschneiden und einer Subkultur auf dem Vermehrungsmedium die höchste Anzahl von gut entwikkelten Sprossen. Um die induzierte genetische Variabilität zu bestimmen, wurden 39 Neubildungen der Sorte Golden Delicious Bovey und 38 Neubildungen der Sorte Goldspur mit 25 RAPD primers gescreent. Einer Neubildung von Golden Delicious Bovey fehlte das Fragment OPM-02/900 und eine andere Neubildung derselben Sorte wies das Fehlen von OPA-01/850 und OPA11/500-Fragmenten auf. Ein zusätzliches Band von OPX-14/600 war in einer der Goldspurneubildungen amplifiziert. Auf der Basis der geschätzten Menge gescreenten Genomes kann geschlossen werden, daß die untersuchte Regenerationstechnik eine relativ hohe genetische Variation induziert.

Introduction

None of the numerous apple cultivars entirely meets the high demands of propagators, growers and the constantly changing apple market. The improvement of only one or a few characteristics of popular varieties is impractical through sexual hybridisation, since apple is a heterozygous crop with high self-incompatibility, a high level of heterozygosity, frequent polyploidy, inbreeding depression, long life cycle and large growth form. Irrradiation of multicellular buds has been used to induce valuable mutants, but only a very limited number of apple cultivars has been obtained with this technique. On the other hand, quite a large number of spontaneous mutants is used in production, but the list of improved characteristics is very narrow. Somaclonal variation may therefore provide an important alternative source of variation for selection of new valuable apple mutants, especially since it has been suggested that novel variants, which cannot be obtained by classical mutation breeding, can be raised through tissue culture (NOVAK 1991).

An efficient regeneration system from somatic tissue or cells via organogenesis and/or embryogenesis is a prerequisite for the use of somaclonal variation for selection of improved cultivars. In apple, the most studied in vitro regeneration technique is adventitious shoot formation from leaves. Regeneration of several scion and rootstock cultivars of apple has been reported and proved to be efficient for some genotypes (THEILER-HEDTRICH & THEILER-HEDTRICH 1990, HANKE & al. 1991, YEPES & ALDWINCKLE 1994). However, data about the induced genetic variation among regenerants is

scarce. Recently, RAPD technique has been successfully used to identify genetic variation in some other crops (AKASHI & al. 1994, BOHANEC & al. 1995, OROPEZA & al. 1995).

The aims of the present work were to optimize induction procedure for adventitious shoot regeneration and primarily to analyse the degree of genetic variation among regenerants using RAPD markers.

Abbreviations: BAP = 6-benzyladenine, GA = gibberellinic acid, IBA = 3-indole butyric acid, RAPD = Random Amplified Polymorphic DNA, TDZ = thidiazuron

Material and Methods

1. Adventitious organogenesis

In vitro cultures of cultivars Goldspur and Golden Delicious Bovey were established from nodal segments of actively growing shoots 19 to 24 months prior to excision of leaves for regeneration experiments.

The basal medium consisted of full strength Murashige Skoog macro- and microelements, 100 mg/l myo-inositol, 2.0 mg/l thiamine-HCl, 1.0 mg/l nicotinic acid, 1.0 mg/l pyridoxine-HCl and 30 g/l sucrose. Cultures were grown at $24 \pm 1°$ C and 16 hours of photoperiod. Petri dishes were wrapped in aluminum foil for the first 10 days.

1.1 Study of the effects of BAP vs. TDZ on shoot regeneration of cultivars Goldspur and Golden Delicious Bovey (Exp. 1)

The first four fully unfolded leaves from the apical end of *in vitro* grown shoots were taken, cut three times across the midrib and placed with the adaxial side onto the basal medium (pH 5.6) supplemented with 0.1 mg/l IBA, solidified with 6 g/l of agar. Cytokinin content varied according to treatment. Media T1, T2 and T3 contained 0.2, 0.02 and 0.002 mg/l TDZ and media B1, B2 and B3 contained 10, 5 and 1 mg/l BAP, respectively. All 4 leaves from the same shoot were placed in the same Petri dish (50 mm in diameter), containing 8 ml of medium.

After 38 days, the number of shoots per leaf was counted. The developing shoots were cut off and individual shoots were transferred to test tubes on proliferation medium. The proliferation medium was a basal medium (pH 5.8) supplemented with 1.0 mg/l BAP, 0.5 mg/l GA, 0.1 mg/l IBA and 6 g/l of agar. After 19 days on the proliferation medium, the numbers of healthy, hyperhydric and necrotic shoots, as well as shoots overgrown by callus, were recorded.

1.2 The addition of coconut milk to the medium for shoot regeneration of cultivars Goldspur and Golden Delicious Bovey (Exp. 2)

In experiment 2, the effect of coconut milk on the regeneration was studied. The first two fully unfolded leaves were sliced transversely across the leaf lamina into 2–3 mm wide segments. The upper segments were discharged and the remaining segments (3 segments per leaf) were placed in 50 mm Petri dishes containing 10 ml of the medium with the adaxial surface in contact with the medium. 0.2 mg/l TDZ, 0.1 mg/l of IBA, 15% (v/v) coconut milk and 8 g/l of agar (pH 5.8) were added to the basal medium. The addition of coconut milk was omitted in the control.

1.3 Comparison of agar vs. agar + Gerlite as solidifiing agents for regeneration and proliferation media (Exp. 3)

In the third experiment, the influence of gelling agents on the regeneration of healthy shoots was studied. Leaf segments were prepared and inoculated as in experiment 2, except that larger Petri dishes (90 mm) with 13 ml of medium were used. The basal medium (pH 5.8) was supplemented with 0.2 TDZ, 0.1 IBA, 8 g/l agar (A) or 4 g/l agar and 1 g/l Gelrite (A+G). Formed shoots (undetached from leaf segments) were transferred to the proliferation medium containing basal medium (pH 5.6) supplemented with 1.0 mg/l BAP, 0.5 mg/l gibberellinic acid (GA), 0.1 mg/l IBA, solidified with the same gelling agent as used for regeneration. 100 ml Baby food jars (Sigma) with 25 ml of the medium were used for proliferation. Data were recorded 51 days after inoculation.

2. RAPD analyses

2.1 Plant material

DNA was extracted from in vitro grown single shoots of 38 regenerants of cultivar Goldspur and 39 regenerants of cultivar Golden Delicious Bovey, all raised in experiment 2. For control, DNA samples of donor cultivars were taken both from micropropagated (axillary) single shoots as well as from young leaves from orchard.

2.2 DNA extraction and preparation, RAPD analyses

The CPBB protocol, described in detail by Virščeк-Marn & al. 1996, was used for DNA extraction and preparation and RAPD analyses, except that the amplification reaction volume was reduced from 50 µl to 25 µl. For the DNA amplification of cultivar Goldspur and its regenerants, the annealing temperature in all 40 cycles was also changed from 30 s to 45 s. The following single decamer primers (Operon Technologies Inc., Alameda, California) were used: OPA-01, OPA-05, OPA-11, OPB-10, OPB-17, OPD-20, OPE-06, OPM-02, OPM-18, OPX-01, OPX-02, OPX-04, OPX-06, OPX-08, OPX-09, OPX-11, OPX-12, OPX-13, OPX-14, OPX-15, OPX-16, OPX-17, OPX-19. Additionally single decamer primers OPB-04 and OPB-20 were used for RAPD analyses of cultivar Goldspur and its regenerants and OPB-11, OPX-18 for cultivar Golden Delicious Bovey and its regenerants.

Amplifications were repeated if any differences were observed among RAPD patterns and regenerants and/or their mother cultivars.

3. Statistical analyses

In experiments 1 and 3 the number of shoots per leaf was analysed by analysis of variance. Statistically significant differences among cultivars and among media were tested with Duncan's multiple range test at $p = 0.05$.

Results

1. Influence of cytokinin source and concentration (Exp. 1)

The results of experiment 1 are presented in Table 1. Statistical analysis of the number of shoots per leaf after 38 days showed significant

Table 1

Influence of cytokinin source and concentration:
T1 = 0.2 mg/l TDZ + 0.1 mg/l IBA
T2 = 0.02 mg/l TDZ + 0.1 mg/l IBA
T3 = 0.002 mg/l TDZ + 0.1 mg/l IBA
B1 = 10 mg/l BAP + 0.1 mg/l IBA
B2 = 5 mg/l BAP + 0.1 mg/l IBA
B3 = 1 mg/l BAP + 0.1 mg/l IBA
on regeneration of apple cultivars Goldspur and Golden Delicious Bovey (Exp. 1)

		GOLDSPUR						GOLDEN D. BOVEY					
Days after inoculation		T1	T2	T3	B1	B2	B3	T1	T2	T3	B1	B2	B3
38	No. of vessels	12	11	12	8	7	12	12	12	12	8	12	12
38	No. of shoots per leaf	2.50	0.20	0.0	0.13	0.57	0.02	1.04	0.27	0.0	0.03	0.13	0.17
38	% of regenerating leaves	79.2	13.6	0.0	6.3	39.3	2.1	60.4	25.0	0.0	3.1	8.3	10.4
57	% of hyperhydric shoots	76.7	55.6	0.0	100.0	68.8	100.0	86.0	69.2	0.0	100.0	50.0	50.0
57	No. of healthy shoots per leaf*	0.25	0.05	0.0	0.0	0.18	0.0	0.10	0.08	0.0	0.0	0.06	0.08

* Total no. of shoots minus shoots, overgrown by callus, hyperhydric and necrotic shoots

differences between cultivars. The induction of shoots was better on the leaves of cultivar Goldspur. On average for both cultivars, 0.2 mg/l TDZ + 0.1 mg/l IBA was significantly superior to all other tested hormone combinations. Differences among other media were not statistically significant, but apart from the medium containing 0.2 mg/l TDZ + 0.1 mg/l IBA, the highest number of regenerants of the cultivar Goldspur was observed in treatment B2 (5 mg/l BAP + 0.1 mg/l IBA) and the highest number of regenerants of the cultivar Golden Delicious Bovey was found on T2 medium (0.02 mg/l TDZ + 0.1 mg/l IBA). No shoots were initiated on the leaves placed on the medium with 0.002 mg/l TDZ + 0.1 mg/l IBA. After 19 days on the proliferation medium, 50% or more of the transferred shoots showed symptoms of hyperhydricity (thick, translucent and brittle stems and leaves).

2. Influence of coconut milk (Exp. 2)

No shoots developed on the regeneration medium with coconut milk, whereas the control regeneration medium without coconut milk produced 1.43 shoots per leaf of cultivar Goldspur and 1.52 regenerants per leaf of cultivar Golden Delicious Bovey.

3. Influence of gelling agents (Exp. 3)

The results of experiment 3 are presented in Table 2. After 51 days, the differences in the number of regenerated shoots per leaf between the two media and between the two cultivars proved to be statistically significant. In contrast to experiment 1, Golden Delicious Bovey gave better results. The combination of agar and Gelrite proved to be superior to agar alone. Except for Goldspur on medium solidified with the combination of agar and Gelrite, the percentage of hyperhydricity was very similar in all treatments.

4. DNA analyses

139 scorable bands were amplified with 25 primers in cultivar Golden Delicious Bovey and its regenerants with unchanged RAPD patterns. Fragments OPA01/850 and OPA11/500 were absent in the regenerant Bovey-12. Fragment OPM02/900 was amplified in 38 regenerants of Golden Delicious Bovey and the donor cultivar, but was missing in the regenerant Bovey-37 (Fig. 1).

In cultivar Goldspur and 37 regenerants with identical RAPD patterns, 133 fragments were recorded. One regenerant showed the presence of an additional band OPX14/600.

The average length of the amplified fragments was around 1500 bp.

Discussion

1. Adventitious shoot regeneration

BAP and TDZ are the most frequently used and studied cytokinins for adventitious regeneration of apple shoots. In many apple cultivars, TDZ was superior to BAP. Also in our experiment 1, TDZ in a concentration of 0.2 mg/l proved to be superior for adventitious regeneration of cultivars Golden Delicious Bovey and Goldspur to all tree tested BAP concentration.

Table 2

Influence of agar (A) and agar + Gelrite combination (A+G) on regeneration of apple cultivars Goldspur and Golden Delicious Bovey (Exp. 3)

Days after inoculation		GOLDSPUR A	GOLDSPUR A+G	G. D. BOVEY A	G. D. BOVEY A+G
51	No. of vessels	17	19	16	19
51	No. of shoots per leaf	0.09	2.21	1.38	5.58
51	% of regenerating leaves	5.9	84.2	62.5	97.4
51	% of hyperhydric shoots	0.0	36.9	45.5	41.5
51	No. of healthy shoots per leaf	0.09	1.39	0.75	3.26

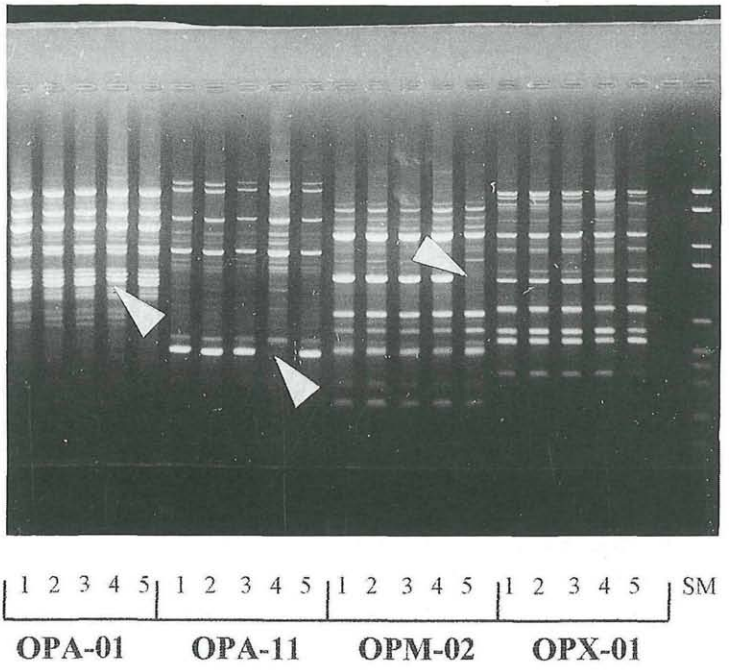

Fig. 1. RAPD patterns amplified with primers OPA-01, OPA-11, OPM-02 and OPX-01 in cultivars Golden Delicious Bovey from in vivo and in vitro and 3 of its regenerants: 1: Golden Delicious Bovey from in vivo, 2: Golden Delicious Bovey from in vitro, 3: Regenerant Bovey-3 without detected variation in RAPD patterns, 4: Regenerant Bovey-12 lacking fragments OPA01/850 in OPA11/500 (both marked by an arrow), 5: Regenerant Bovey-37 lacking the fragment OPM02/900 (marked with an arrow), SM: size marker VI (Boehringer Mannheim).

This results are in accordance with findings of THEILER-HEDTRICH & THEILER-HEDTRICH 1990 and HANKE & al. 1991. A high percentage of hyperhydric shoots was recorded on all tested treatments of experiment 1. The production of hyperhydric shoots presented a major problem also in our previous work (VIRŠČEK-MARN 1994) and has been reported by other authors (PAWLICKI & WELANDER 1994, CABONI & al. 1996), therefore in experiments 2 and 3 the influence of coconut milk and gelling agent on the amount of hyperhydrycity was tested. Coconut milk, which was successfully used to overcome the problems of hyperhydricity by BURIKAM & al. 1988, completely prevented regeneration of shoots in our experiment 2. These results are in contrast with the findings of AL-KHAYRI & al. 1991, who found positive effects of coconut milk on callus development and regeneration on leaf disk of spinach. The combination of Gelrite and agar, tested in our experiment 3, did not significantly reduce the amount of hyperhydricity, as

suggested by PASQUALETTO & al. 1986, but suprisingly had a positive effect on regeneration. Upon our opinion the number of healthy shoots produced on Gelrite and agar solidified medium (up to 6.5 healthy shoots per vessel) allows efficient selection of somaclones.

2. Assessment of genetic variation among regenerants

For exploitation of somaclonal variation for breeding purposes, a high amount of genetic variation is desirable. In virtually all other uses of plant cell and tissue culture, somaclonal variation is highly undesirable. Knowledge about the amount of variation to be expected from the application of different in vitro techniques is therefore very important.

In our work, the RAPD technique was chosen for assessment of genetic variation of regenerants because it is a time and cost effective technique, which does not involve radioactivity. It also requires only a small amount of DNA for analysis, which is especially important for studies of in vitro plants, where large quantities of the plant material are rarely available.

By using 25 primers, 136 bands (average of both cultivars) with a mean length of approx. 1500 bp were obtained, which makes altogether 0.2 million bp. The size of apple genome, estimated by flow cytometry, is 4.5 pg (MARIE & BROWN 1993), which corresponds to approx 2.10^9 bp. Less than 0.01% of the apple genome was therefore screened. 4 mutations were detected in 77 regenerants screened, that is 0.05 mutation events per regenerant. WEEDEN & al. 1994 consider RAPD markers to be randomly distributed over the genome, so a similar frequency of mutation events is highly probable in the unscreened portion of the genome. Approximately 500 mutations can thus be expected per regenerant. Assuming a random distribution of mutation events, that makes 15–25 mutations in structural genes. Based on our results, we conclude that the amount of somaclonal variation is high, and that changes in agronomically important characteristics are highly probable. This results are in accordance to reports of practical application of somaclonal variation in apple (ROSATI & al. 1990, DONAVAN & al. 1994).

To our knowledge this is the first report of molecular analyses for detection of somaclones in apple. Based on our and published results we conclude that adventitious organogenesis is a promising technique for selection of improved individuals, especially in combination with in vitro selection and molecular markers linked to agronomically important characteristics of apple.

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