Title: Selection of valine-resistance in callus culture of Arabidopsis thaliana (L.) Heynh. derived from leaf explants

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SELECTION OF VALINE-RESISTANCE IN CALLUS CULTURE OF *ARABIDOPSIS THALIANA* (L.) HEYNH. DERIVED FROM LEAF EXPLANTS

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ABSTRACT

The selection of valine-resistant mutants was carried out in leaf explant cultures of three *Arabidopsis thaliana* (L.) Heynh. ecotypes: C-24, RLD and Columbia. The valine concentration used for in vitro selection, lethal for seed-growing plants, has not affected callus formation and growth. However, strong inhibition of shoot regeneration ability of calli growing under selection pressure was noticed. In total, 1043 explants were cultured on valine medium and 18 shoots were regenerated with an average frequency of 1.7 shoots per 100 calli. Most R_1 shoots were sterile and seeds were collected from 3 plants. The transmission of valine-resistance to the sexual progeny of these plants was scored and the increased level of valine-resistance was found in progeny of one line – 61C. This line originated from the culture of Columbia leaf explant and displayed tetraploid chromosome number.

KEY WORDS: *Arabidopsis thaliana*, biochemical mutants, in vitro selection, somaclonal variation, valine resistance.

INTRODUCTION

Plant tissue cultures widely used in micropropagation, haploid production, somatic hybridization and transformation are also a valuable source of biodiversity in plant germplasm. Somaclonal variation, as a tool for selection under in vitro conditions provides a unique opportunity to obtain new genotypes. Advantages of tissue culture over classical selection methods are obvious when biochemical mutants are to be recovered. Among them mutants resistant to amino acids, herbicides or antibiotics can be selected by application of positive selection system (Negrutiu 1990).

Although *Arabidopsis thaliana* (L.) Heynh. was recommended many years ago as a model plant in somatic cell genetics (Negrutiu et al. 1975), and many reports on regeneration of *Arabidopsis* plants from tissue culture exist, the number of reports on induction and selection of *A. thaliana* biochemical mutants in in vitro system is limited. The attempts to select biochemical mutants resulted in selection of cell or callus-lines of *A. thaliana* resistant to amino acid-analogues after mutagenic treatment (Negrutiu et al. 1978; Jacobs et al. 1987).

Contrary to many other species, where the phenomenon of somaclonal variation has been described as a new source of variation and applied for in vitro selection of mutants, in vitro generated variation has not been used for mutant selection in *Arabidiopsis* tissue culture. However, the application of *Arabidopsis* tissue culture for in vitro mutant selection seems to be possible as genetically determined somaclonal changes, including chlorophyll mutations, lethal embryos, polyploids (Gaj and Maluszynski 1987) and isozymatic variants (Gaj et al. 1991), were observed in progenies of plants regenerated from somatic callus.

The presented study was designed to estimate efficiency and usefulness of somaclonal variation for development of *Arabidopsis* valine-resistant mutants by selection in callus culture derived from leaf explants. The selection for valine resistance was done following the scheme developed for *Nicotiana* culture, which was proven as an efficient, reproducible and recommended model system in plant cell genetics (Bourgin et al. 1985).

MATERIAL AND METHODS

Plant material

Three ecotypes of *Arabidopsis thaliana* were used in the study: C-24, RLD and Columbia. The explant-donor plants grew from sterilized seeds (70% ethanol for 1 minute followed by 20% solution of commercial sodium hypochloride 'ACE' for 20 minutes and rinsing in sterile water) in glass jars on MS10 agar medium (Murashige and Skoog, 1962) supplemented with 10 g/l sucrose, in the growth-chamber (16 h photoperiod, 3000 lux, 22-25°C).

Abbreviations:
CIM Callus-inducing medium; *2,4-D* 2,4-D-Dichlorophenoxyacetic acid; *2iP* 6 (γ,γ- Dimethylallyl-amino) purine; *IAA* Indole-3-acetic acid; *MS* Murashige and Skoog; *SIM* Shoot-inducing medium

Regeneration of plants from tissue culture

The culture was established from leaf explants of 4-6 week-old plants according to the Feldmann and Marks (1986) method in which regeneration of plants requires a two-step procedure. In the first step the leaf explants were incubated for 5-7 days on a gyratory shaker (120 rpm) in the liquid callus inducing medium (Gamborg and Eveleigh, 1968). The medium called CIM contained micro, macro salts and vitamins; 2.2 mg/l 2,4-D; 0.05 mg/l kinetin and 30 g/l sucrose. Then, the leaf-explants were subcultured on agar shoot inducing medium (SIM) containing Murashige and Skoog's (1962) micro and macro salts; Gamborg and Eveleigh's (1968) vitamins; 5.0 mg/l 2iP; 0.15 mg/l IAA; 30 g/l sucrose and 8 g/l agar. Shoots regenerating during 1-3 months of culture were transferred into tubes with MS10 agar medium, where plants were rooted and seeds from R_1 plants were collected.

Evaluation of valine concentration for in vitro selection

Seeds of studied genotypes were germinated in aseptic conditions on MS10 medium in the presence of different valine concentrations (0; 2.5; 5.0; 7.5 and 10 mM). Valine was filter-sterilized and added to medium sterilized by autoclaving and cooled down to 60°C. Fifty seeds were sown per combination (genotype x valine concentration) and the ability of plants to germinate and develop was estimated.

In vitro selection and confirmation of valine-resistance

Following culture on CIM medium, leaf-explants were cultured on SIM medium supplemented with valine, on which they grew for 2-4 subcultures, i.e. until green shoots appeared. The subculture onto fresh SIM medium was performed every 4 weeks.

10 mM of L-valine (Serva) was used for in vitro selection of valine-resistant forms. R_1 shoots regenerated on this medium were transferred onto MS + 10 mM valine medium for rooting and seed collection. The resistance to valine was tested in the next progeny (R_2) by planting the seeds of R_1 plants on MS + 10 mM valine medium.

RESULTS AND DISCUSSION

Evaluation of valine concentration for in vitro selection

Preliminary experiments designed to choose the valine concentration suitable for in vitro selection showed that all amino acid concentration tested i.e. from 2.5-10.0 mM decreased seed germination ability and caused changes in morphology and fertility of developing plants.

Although at the lowest concentration of valine tested (2.5 mM), a slight reduction in germination ability of seeds and slower growth of plants was already observed, the development of plants from some seeds could be observed even on media containing valine as high as 7.5 mM. The plants developing in the presence of 5.0-7.5 mM valine showed abnormal morphology. The so-called "cabbage-shape" was most frequently observed (numerous, larger rosette leaves which gradually became white and did not form any stems). The 10 mM concentration of valine was used for in vitro selection. On this concentration some seeds were able to germinate (e.g. 76% for Columbia), but all developing seedlings soon bleached and died.

Efficiency of leaf explant culture on valine-free media

When the selection agent (valine) was not employed the callus forming ability and efficiency of plant regeneration was very high for all genotypes tested (Table 1). Over 90% of leaf explants cultured on valine free media induced callus growth and 69.8 to 76.8% of the calli developed shoots, thus proving the high efficiency of the applied regeneration system.

TABLE 1. Callus formation and plant regeneration frequency in culture of *Arabidopsis thaliana* leaf explants.

Genotype	Number of explants	Frequency of explants forming		Average number of shoots per callus
		calli %	shoots %	
C-24	474	92.2 ± 4.0	69.8 ± 12.2	4.4 ± 1.7
Columbia	289	95.6 ± 5.1	70.6 ± 10.7	4.0 ± 1.9
RLD	328	96.0 ± 4.3	76.8 ± 12.7	3.9 ± 1.1
Total	1091	94.6 ± 1.7	72.4 ± 3.1	4.1 ± 0.2

In vitro culture response under valine-selection

In spite of a the very strong toxic effect of 10 mM valine on germination and growth of *A. thaliana* plants, no distinct influence of this concentration was noticed on callus-formation ability of cultured leaf-explants (Table 2). The percentage of explants forming callus in the presence of 10 mM valine varied from 88.3 to 92.4%, depending on genotype.

TABLE 2. Callus formation efficiency in culture of *Arabidopsis thaliana* leaf-explants under selection on valine medium.

Genotype	No. of explants	Percentage of explants forming calli
C-24	488	88.3 ± 7.9
RLD	436	91.2 ± 6.0
Columbia	238	92.4 ± 8.3
Total	1162	90.6 ± 1.7

Some slight differences were noticed in morphology of calli cultured in the presence of valine. In contrast to green control callus tissue, calli grown on valine-media mostly remained yellow-white and very rarely developed green spots.

Contrary to the slight effect of valine on callus induction and growth, the strong suppression of regeneration ability of the callus was noticed on SIM-valine medium (Table 3). From the total number of 1043 calli derived from leaf explants grown under selection, only 8 (0.8%) calli regenerated

TABLE 3. Efficiency of shoot regeneration in *A. thaliana* callus culture under selection on valine medium.

Genotype	No. of cultured calli	Shoot regenerating calli		No. of regenerated shoots	
		No.	%	total	per 100 cali
C-24	425	2	0.5	4	0.9
RLD	398	5	1.3	13	3.3
Columbia	220	1	0.5	1	0.4
Total	1043	8	0.8	18	1.7

18 shoots at the frequency 1.7 per 100 calli. This frequency is rather high in comparison to 0.15% of wheat calli producing plants resistant to hydroxyproline (Dorffling et al., 1993). Selection of valine resistance in callus culture of 70 maize tissue clumps (Hibberd, 1988) resulted in 10 presumptive resistant callus lines from which only two fertile plants were regenerated. The efficiency of selection in tissue culture is lower than in cell culture, where up to 30% of variant cell lines resistant to valine was reported (Maliga, 1984).

Although in total 18 shoots were regenerated on selection medium, the vast number of them failed in growth after transfer into rooting medium and only 3 developed seeds. The seed-set of these plants was very low, except for plant 61C derived from Columbia genotype from which 94 seeds were collected.

Confirmation of valine-resistance in the progeny of regenerants – R_2

Out of 3 lines tested in R_2, only the progeny of 61C plant, displayed increased tolerance to valine, in comparison to control plants, but the expression of tolerance was lower than of R_1 regenerant. Seedlings of the other two tested progenies, mostly bleached shortly after germination on MS10-valine medium or, less frequently, developed short, sterile stems.

Only two fertile R_2 plants were obtained on valine-medium out of 94 seeds collected from 61C R_1 plant. The remaining plants displayed abnormal, "cabbage-shape" morphology and were sterile. The seed-set of the two fertile plants was very low as only 15 seeds were collected, from which, in the next progeny (R_3), 8 plants developed. All these plants, in presence of 10 mM of valine, showed morphological abnormalities, such as bleaching and "cabbage shape." Only one of them, despite some morphological abnormalities, developed a few flowers. The cytogenetic analysis of this plant revealed its tetraploid chromosome number (2n=20). The cross between this plant and the wild form (Columbia), which was a pollen donor, was carried out, but no seeds developed.

The analysis of regenerants' progeny revealed some problems related to the selection of biochemical mutants in in vitro systems. First of all, difficulties in the confirmation of selected trait in sexual progeny of regenerants were encountered. To explain this phenomenon, the epigenetic determination of valine-resistance in some regenerants must be considered. Epigenetic variation, an unstable, transient alteration in gene expression that can be reversible and not expressed in progeny of regenerated plants, is a common phenomenon observed in regenerated in vitro plants (Bhaskaran et al. 1987; Smith et al. 1993).

It must also be considered that some valine-resistant clones could be mutants but with a low level of resistance (Nielsen et al. 1985). This explanation seems important in our diploid selection system in which only dominant mutations could be recovered. In the event of genetic determination of valine-resistance, dominant mutation displays lower level of expression in comparison to the recessive one (Bourgin et al. 1985). Additionally, if the mutation was related with deficient valine uptake, inability of resistant plants to mature their seeds due to a defect in amino acid transport (Marion-Poll et al. 1988) explained the low fertility of selected regenerants (only 3 out of 18 developed seeds) and low transmission of the valine resistance (valine tolerant plants were only observed in the progeny of one of these plants). Heterozygous regenerants would develop sensitive to valine seeds. Maturation of seeds carrying mutated allels could be limited on media with high valine concentration due to low expression of dominant mutation and defect in amino acid transport caused by this mutation.

Recovery of valine-resistant lines from diploid culture was found to be much less efficient than from haploid culture as was indicated by Vunsh et al. (1982) in *Nicotiana sylvestris* protoplast culture. In culture of 0.5 million haploid protoplasts plant lines from 20 resistant callus were obtained while from almost 2 million diploid protoplasts cultured, only 1 valine-resistant callus was selected. Resistant plants regenerated from this callus were found to be tetraploid and a decreased level of resistance to valine was observed in their progenies. Similarly, in our selection system, performed in culture of 1043 diploid calli, transmission of valine resistance displayed only one of the selected plants. The plant was tetraploid and its progeny presented a decreased tolerance to valine.

Polyploids are frequently observed among *A. thaliana* plants regenerated in vitro (Gaj and Maluszynski 1987; Altmann et al. 1994) and reflect natural endopolyploidization existing in somatic tissues of this species (Galbraith et al. 1991). Increased number of chromosomes in line 61C cannot be excluded as a reason for higher resistance of this form to toxic concentration of valine.

Another phenomenon which has to be considered in relation to the lack or decrease in valine-resistance in sexual progenies of selected plants, it is the regeneration of sensitive plants from leaky or highly unstable callus clones (Grandbastien et al. 1989). However, in their experiment with *Nicotiana* culture, the selection was performed only at the level of callus growth. In the case of our selection system, the need for continuous valine pressure appeared. Valine was employed during the whole regeneration period and the regeneration from wild-type, through "escaping" cells is less probable. The continuos presence of valine probably negatively interfere with regeneration capacity of the callus cells and might be responsible for lower efficiency of recovery of valine-resistant regenerants in comparison to *Nicotiana*.

The valine resistance selection system established for haploid protoplast culture of *Nicotiana* (Bourgin, 1978) and found far less effective in somatic callus culture of *A. thaliana*, indicated the difficulty in transposing the experience gained with one plant regeneration system for use with another one. Widholm (1988) pointed out that frequently, due to unknown reasons, culture systems of some species and even genotypes are more difficult to manipulate than other and called this phenomenon "the artistic nature" of in vitro system. The culture conditions (medium composition, genotype), since they influence the physiological reaction on which the selection mechanism is based, may be very important according to results of Escandon and Hahne (1991). Moreover, the successful in vitro selection can be achieved only for some genotype as found in rice callus culture Adkins et al. (1995).

The obtained results have not supported Bourgin's et al. (1985) suggestion that valine resistance can be an easily selected trait in in vitro culture of plants. It seems that in the case of *Arabidopsis thaliana* a more effective method to induce valine resistant mutants was the screening of M_2 population of EMS-mutagenised seeds (Wu et al. 1994) or directed mutagenesis of *Arabidopsis* gene encoding acetolactate synthase (Hervieu and Vaucheret 1996).

The undertaken study showed that in leaf culture of *Arabidopsis*, plants with resistance to toxic concentration of valine can be selected. The efficiency of this process and stability of selected traits seem to be influenced by many factors, as media composition, type and ploidy of culture, regeneration system but also time and type of selection pressure (continuous or not). Moreover, our results constitute an additional ar-

gument for the necessity of testing in vitro selected traits in the next progeny as frequent lost of variant character can be observed due to many in vitro factors.

According to Duncan et al. (1995) in the optimal strategy for the exploitation of somaclonal variation, in vitro selection should be omitted. They found in vitro selection ineffective to increase the number of stress tolerant variants of sorghum. To study this interesting hypothesis screening of valine resistant plants in progeny of *Arabidopsis* regenerants derived without any selection pressure is under investigation.

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SELEKCJA OPORNOŚCI NA WALINĘ W KULTURZE KALUSA *ARABIDOPSIS THALIANA* (L.) HEYNH. OTRZYMANEGO Z EKSPLANTATÓW LIŚCIOWYCH

STRESZCZENIE

Selekcję mutantów opornych na walinę przeprowadzono w kulturze eksplantatów liściowych trzech ekotypów *Arabidopsis thaliana* (L.) Heynh: C-24, RLD i Columbia. Stężenie waliny zastosowane w selekcji, letalne dla roślin wyrastających z nasion, nie wpływało na inicjację i wzrost kalusa. Zaobserwowano natomiast silne zahamowanie regeneracji pędów z kalusa rosnącego na pożywce z waliną. Ogółem, w kulturze 1043 eksplantatów liściowych poddanych selekcji in vitro, otrzymano 18 pędów. Średnia częstotliwość regeneracji pędów w warunkach selekcji wynosiła 1,7 pędów na 100 kalusów. Większość roślin R1 była sterylna, nasiona zebrano z trzech roślin. Analizowano oporność na walinę w potomstwie generatywnym wyselekcjonowanych regenerantów. Podwyższony poziom oporności na walinę stwierdzono w jednej linii – 61C. Linia ta pochodziła z kultury eksplantatów liściowych genotypu Columbia i posiadała tetraloidalną liczbę chromosomów.

SŁOWA KLUCZOWE: *Arabidopsis thaliana*, mutanty biochemiczne, oporność na walinę, selekcja in vitro, zmienność somaklonalna.