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Assessment of age-related polyploidy in *Quercus robur* L. somatic embryos and regenerated plants using DNA flow cytometry

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Abstract

Flow cytometric analysis with 4,6-diamidino-2-phenylindole (DAPI) staining was used to screen for chromosomal changes in *Quercus robur* during *in vitro* culture. The initiated cell lines (1992 until 1999) were maintained via secondary embryogenesis on P24 medium with 0.9 μM 6-benzylaminopurine (BAP) in regular subculture intervals of 6 weeks. Regenerated plants established in the greenhouse and *in vitro* plantlets derived from encapsulated somatic embryos were screened. The embryogenic cell lines were characterized as individual clones by isoenzyme analysis. Flow cytometric relative DNA content analysis of the first screening period revealed that somaclonal variation in form of tetraploidy occurred in two out of 26 tested somatic embryo clones (Alt and Jung). These two clones lost their ability to convert into plantlets. Intraspecific relative DNA content variation including technical variation was below 3 %. In the second screening period, however, 3 out of 37 clones (Alt, E4.31H9 and P3.27H) contained tetraploid cells leading to the assumption that the frequency of tetraploidy seems to be correlated with the duration of *in vitro* culture. No chromosomal differences were detected in regenerated plants. However, tetraploidy occurred in 8 % of the tested clones over a culture period of 7 years.

Additional key words: ploidy level, oak, somaclonal variation, synthetic seeds.

Introduction

The technique of somatic embryogenesis (SE) is well developed for a respectable number of diverse plants including woody plants. This method of plant micropropagation is well established for different oak species such as *Quercus robur, Q. petraea, Q. suber, Q. acutissima, Q. serrata* (Chalupa 1995, Wilhelm *et al.* 1996, Endemann and Wilhelm 1999, Wilhelm 2000). For several years we have investigated the different aspects of somatic embryogenesis, such as initiation (Endemann and Wilhelm 1999) and conversion into plantlets (Wilhelm *et al.* 1999) with the aim of cryopreservation (Tutková 1999), artificial seed production (Prewein, unpublished), and genetic transformation (Wilhelm *et al.* 1996).

In terms of somaclonal variation, the system of somatic embryogenesis in woody plants has long been regarded as a safe method but there is recent evidence that this is not the case. To minimize the risk for genetic variation a rapid screening for possible changes may help to maintain the primary goal of "true-to-type" propagation.

Gallego *et al.* (1997) and Thakur *et al.* (1999) monitored *Q. suber* and *Q. serrata* SE by using RAPD-markers for their genetic stability. Both studies revealed no aberrations in RAPD banding patterns among the tested samples. Fourré *et al.* (1997) and Isabel *et al.* (1996) suggested that RAPD markers fail to detect

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Abbreviations: BAP - 6-benzylaminopurine; CV - coefficient of variation; DAPI - 4,6-diamidino-2-phenylindole; IBA - indole-3-butyric acid; PVP - polyvinylpyrrolidone; SE - somatic embryos, somatic embryogenesis.
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genetic variation. Numerical chromosomal changes which are the most frequently reported changes in broadleaf and conifer species (DeVerno 1995, Nkongolo and Klimazewska, 1995, Bueno *et al.* 1996, Fourré *et al.* 1997, Roth *et al.* 1998) are not seen in RAPD analysis (Fourré *et al.* 1997).

In the present study, initiated somatic embryogenic clones (1992 until 1999) and derived plantlets of *Q. robur* were assessed for age-related chromosomal changes with flow cytometry analysis in two screening periods over 3 years. A set of isoenzymes was used to characterize the initiated clones (included half sibs) in order to distinguish the different genotypes of the cell lines.

Materials and methods

Plants: Somatic embryos (SE) of *Q. robur* (44 clones) were induced during the years 1992 - 1999 (Kodym 1993, Rodler 1996, Endemann and Wilhelm 1999). Seventeen clones of SE and encapsulated embryos (Prewein, unpublished) were converted into plantlets. Six oak shoots propagated via organogenesis by axillary budding were used as controls (Table 1). P24 medium (Teasdale 1992) with 0.8 % agar, supplemented with 0.9 µM 6-benzylaminopurine (BAP), was used for proliferation of SE. P24 medium containing 0.9 % agar and 0.9 % BAP was used for maturation of SE. P24 medium containing 0.8 % agar, 1 µM BAP and 0.1 µM indole-3-butyric acid (IBA) was used for germination (Rodler 1996, Wilhelm *et al.* 1999). Cultures were kept at a temperature of 24 ± 1 °C and white light (TLD 36W/33, *Philips*, irradiance 65 µmol $m^{-2}s^{-1}$, 16-h photoperiod).

Isoenzyme analysis: Eighteen SE cell lines of *Q. robur* (50 mg dry mass per each sample) were homogenized in an extraction buffer containing polyvinylpyrrolidone (PVP-10) and 2-mercaptoethanol and then separated by electrophoresis in starch or polyacrylamide gel for the following isoenzyme staining assays: aminopeptidase (AP-B), isocitrate dehydrogenase (IDH-B), phosphoglucose isomerase (PGI-B), acid phosphatase (ACP-C), aspartat-aminotransferase (AAT-B), glutamate dehydrogenase (GDH), formate dehydrogenase (FDH), 6-phosphogluconate dehydrogenase (6PGDH-B) and leucine aminopeptidase (LAP). Enzymes were stained at 35 °C in the dark for 20 - 40 min, as described previously by Hertel and Zaspel (1996) and Hertel (1997).

Flow cytometry analysis: For preparing the nuclear suspension and staining with 4,6-diamidino-2-phenylindole (DAPI) the method of Barany and Greilhuber (1995) was modified by adding 1 % m/v PVP-10 to the isolation buffer (Bharathan *et al.* 1994). In brief, to release nuclei from the cells, cotyledonary embryos of approx. 3 mm size or half of a leaf were chopped with a razor blade together with a young leaf (0.2 to 0.5 cm^2) of the internal standard *Glycine max* cv. Ceresia in isolation buffer (0.6 cm^3 ice-cold 0.1 M citric acid solution containing 0.5 % Triton X-100). The suspension of nuclei was filtered through a 50 µm nylon net in order to remove cell debris. Then the filtrate was stored on ice. Shortly before measurement of the relative DNA-content, the suspension of nuclei was mixed with a 2.5 fold volume of staining solution (5 µg cm^{-3} DAPI and 3 µg cm^{-3} sulforhodamine in 0.4 M sodium hydrogen phosphate). A *Partec PA* (Germany) flow cytometer equipped with a 100 W high pressure mercury lamp, *KG1*, *BG 38*, *UG 1* filters, *TK 420* dicroic mirror and *GG 435* filter for DAPI staining was used. Measurements of the first screening period were performed during continuous propagation over 2 years. The SEs were tested after induction as somatic embryos (26 clones) or conversion into plantlets (12 clones). The relative DNA content of 3-21 SE, derived plantlets or oak shoots was measured per clone and given as index relative to the internal standard (*Glycine max*). 2 - 3 runs per preparation were carried out. During the second screening period (7 month) 37 clones (10 to 80 SE; 10 SE per Petri dish) were analyzed per clone, except for clone P5.27Hb. Each Petri dish contained approximately 2.5 g SE in globular, torpedo and cotyledonary stages. Additionally plantlets obtained from encapsulated SE were screened (5 clones). Each sample was measured twice.

Statistics: For statistical analysis multiple mean tests (Tukey-Kramer and Scheffé) and the *SAS-V.6.12* procedure *GLM* were used.

Results

Isoenzymes as markers have been proved to be very useful for genetic field studies (Hertel and Zaspel 1996). With the enzyme system used, it was possible to characterize the cell lines as genetically different from each other. Somatic embryo clones E4.31.H9/E4.31H10, P5.27.Oa/P5.30H29 and P29H1/P29H17 are halfsibs, differentiation occurred at least at one locus (Table 2).

At first screening period, two SE clones out of 26 tested (Alt, Jung) were detected in which up to 64 % of the embryogenic cultures were mutated in form of tetraploidy

(Fig. 1). At the beginning of this screening period the clones Alt and Jung were convertible into plantlets. Plantlets regenerated from clone Jung were still diploid. After the first occurrence of tetraploidy, the percentage of tetraploidy increased steadily with length of time in culture. Thus the mutated embryos lost the ability to regenerate into plantlets. Clone Alt exhibited at least 64 % tetraploidy, clone Jung 40 % tetraploidy in SE (Table 1). The extent of significant relative DNA content variation between clones (Tukey-Kramer test) was not

Table 1. Relative nuclear DNA contents of somatic embryo clones, derived plantlets and oak shoots. The values are given as mean of DNA index relative to the internal standard *Glycine max* cv. Ceresia. First screening period.

Clone	Location	Year of induction	Somatic embryos index	SD	*n*	Derived plantlets index	SD	*n*
Schoenbrunn	Schönbrunn	1994	0.7662	0.009	12	-	-	-
Glashaus	Glashaus	1994	0.7638	0.008	18	-	-	-
L9	Leithagebirge	1994	0.7817	0.004	3	-	-	-
Alt 2n (36 %)	Seibersdorf	1992	0.7553	0.008	12	-	-	-
Alt 4n (64 %)	Seibersdorf	1992	1.5300	0.026	21	-	-	-
Jung 2n (60 %)	Seibersdorf	1992	0.7675	0.005	9	0.7656	0.007	7
Jung 4n (40 %)	Seibersdorf	1992	1.5530	0.055	6	-	-	-
E4.31H9	Prater	1995	0.7566	0.130	14	0.7587	0.007	11
E4.31H10	Prater	1995	0.7618	0.005	12	-	-	-
E3.27H	Prater	1995	0.7535	0.011	11	-	-	-
C++2P	Irland	1995	0.7752	0.009	9	0.7706	0.011	10
P3.28H12	Prater	1996	0.7733	0.004	10	0.7785	0.004	12
P5.27Oa	Prater	1996	0.7555	0.003	11	0.7532	0.005	10
P5.28H5	Prater	1996	0.7606	0.005	9	0.765	0.004	11
P5.30H29	Prater	1996	0.7598	0.004	10	-	-	-
I1.28H5	Inzersdorf	1995	0.7647	0.008	13	-	-	-
I2.27Ha	Inzersdorf	1995	0.7677	0.003	10	-	-	-
S28H17	Straßhof	1996	0.7686	0.006	10	0.7673	0.002	10
P5.27Hc	Prater	1996	0.7623	0.004	3	-	-	-
P27O	Prater	1997	-	-	-	0.7683	0.005	10
P28O16	Prater	1997	0.7665	0.005	10	-	-	-
P28O17	Prater	1997	-	-	-	0.763	0.003	10
P28H6	Prater	1997	0.7642	0.005	5	-	-	-
P28H9	Prater	1997	0.7690	0.002	11	0.7693	0.004	10
P28H10	Prater	1997	0.7702	0.007	11	0.7719	0.003	7
P28H12	Prater	1997	0.7706	0.009	10	0.7695	0.003	10
P29H1	Prater	1997	0.7680	0.005	10	-	-	-
P29H17	Prater	1997	0.7677	0.008	12	-	-	-
			Oak shoots					
NL100	Netherlands		0.7638	0.0029	5			
EJS 29	Austria		0.7709	0.0031	7			
EJS 1	Austria		0.7703	0.0055	3			
I95	Prater	1995	0.7433	0.0104	3			
P96/1	Prater	1996	0.7665	0.0007	2			
P96/2	Prater	1996	0.7675	0.0021	2			
P97	Prater	1997	0.7650	0.0036	3			

larger than technical variation as shown in repetitive measurements of the same clone at different times. The highest significant variation was less than 3 % (*SAS - V.6.12* procedure *GLM*). Differences of more than 3 % were only detected when the number of samples was low (*e.g.* clone L9, P527.Hc) or coefficient of variation (CV) was higher than 3 %.

However, tetraploidy was observed in the two oldestclones Alt and Jung which had been continuously subcultured since 1992, *i.e.* 7 % of the SE clones. No ploidy changes were detected in 24 SE clones (93 %) and plantlets regenerated from 12 clones as well as in 7 clones of oak shoot cultures (Table 1, Fig. 2).

A detailed screening of the plant material, higher sample numbers (SE per plate) and analysis of the culture conditions (maturation and proliferation medium) as well as on plantlets, propagated via encapsulation was performed during the second screening period. Three

clones (Alt, E4.31H9 and P3.27H) out of 37 showed tetraploidy whereas SE of clone Jung, tetraploid at the first screening period and kept at 4 °C until the second screening, was diploid. Clone Alt, after seven years in culture, indicated the highest percentage of tetraploidy (77 %), compared to the lowest percentage of 5 % of clone P3.27H, (3 years in culture and diploid at first screening period). Clone E4.31H9 was diploid at the first screening period. In the second screening period 45 % of the SE of clone E4.31H9 were tetraploid in proliferation and 25 % in maturation treatments. No tetraploid plantlet was detected in the 23 analyzed regenerated *in vitro* plantlets of clone E4.31H9. Additional measurements of regenerated *in vitro* plantlets from encapsulated SE of cell line C++2P, P3.28H12, P28H9 and P29H17 did not reveal significant differences (α = 0.05) compared to cotyledonary SE on proliferation medium (Table 3). The CV ranged from 1.5 to 2.5 % for immature, translucent SE and > 2.5 % to 4.5 % for mature, opaque SE. Statistical analysis (Scheffé) of 34 SE clones and plantlets obtained from encapsulated SE (5 clones) showed no significant variation at all.

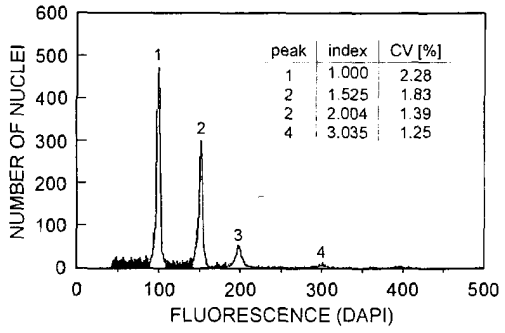

Fig. 1. DNA-histogram of DAPI-stained nuclei of *Q. robur*, tetraploid clone Alt. The values are given as relative DNA index to the internal standard *Glycine max* (index = 1, G1 phase of cell cycle). Relative DNA index of Alt = 1.525, peak 2 = G1, peak 4 = G2.

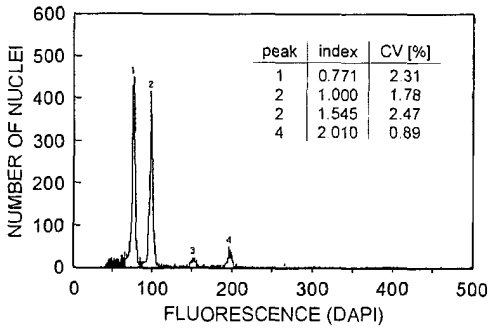

Fig. 2. DNA-histogram of DAPI-stained nuclei of *Q. robur*, diploid clone P3 28.H12. The values are given as relative DNA index to the internal standard *Glycine max* (index = 1, G1 phase of cell cycle). Relative DNA index of clone P3 28.H12 = 0.771 (peak 1= G1), peak 3 = G2.

Table 2. Isoenzyme pattern of different cell lines of somatic embryos of *Q. robur*.

Clone	AP-B	IDH-B	PGI-B	ACP-C	AAT-B	GDH	FDH	6PDGDH-B
Alt	22	66						33
Glashaus	22	46						33
Schönbrunn	66	66	66	11	33	44	12	33
11.28H5	66	44	26	11	33		13	
12.27Ha	66	46	26	11	33	34	13	33
E4.31H9	25	66	66	11	33		33	33
E4.31H10	36	66	66	13	33		33	33
E3.27H	46	44	68	11	33	44	33	33
P5.27Oa	22	66	66	11	33	24	33	33
P5.30H29	24	66	66					23
P3.28H12	46	46	66	11	33	44	13	33
S28H17	26	44	66	11	13			33
P28H9	22	46	66	11	33	44	33	33
P29H1	44	44	16	11		44	33	33
P29H17	46	44	66	13		44	33	33
P28H10	66	46	66	11				
P28O16	66	66	66			44		23
C++2P	36	66	66	11		44	13	33

Table 3. Relative nuclear DNA contents of somatic embryo clones (proliferation, maturation) and derived plantlets obtained from encapsulated somatic embryos. The values are given as means of DNA index relative to the internal standard *Glycine max*. Second screening period.

Clone	Location	Year of induction	Somatic embryos index	SD	*n*	Derived plantlets index	SD	*n*
Alt 2n (23 %)	Seibersdorf	1992	0.7586	0.0018	7	-	-	-
Alt 4n (77 %)	Seibersdorf	1992	1.5463	0.0088	23	-	-	-
Jung	Seibersdorf	1992	0.7752	0.0042	20	-	-	-
Glashaus	Glashaus	1994	0.7723	0.0096	20	-	-	-
L2	Leithagebirge	1994	0.7676	0.0071	30	-	-	-
L5	Leithagebirge	1994	0.7571	0.0074	30	-	-	-
L9	Leithagebirge	1994	0.7752	0.0050	20	-	-	-
C++2P	Irland	1995	0.7721	0.0058	40	0.7736	0.0063	22
E3.27H	Prater	1995	0.7672	0.0122	10	-	-	-
E4.31H9 2n (55 %)	Prater	1995	0.7609	0.0042	22	0.7596	0.0070	23
E4.31H9 4n (45 %)	Prater	1995	1.5468	0.0094	18	-	-	-
E4.31H9 2n (75 %) M	Prater	1995	0.7711	0.0030	30	-	-	-
E4.31H9 4n (25 %) M	Prater	1995	1.5538	0.0100	10	-	-	-
E4.31H10	Prater	1995	0.7662	0.0040	20	-	-	-
I1.28H5	Inzersdorf	1996	0.7807	0.0050	10	-	-	-
I2.27Ha	Inzersdorf	1996	0.7682	0.0071	20	-	-	-
I2.27Hb	Inzersdorf	1996	0.7657	0.0037	20	-	-	-
I2.27Hd	Inzersdorf	1996	0.7716	0.0042	10	-	-	-
P3.27H 2n (95 %)	Prater	1996	0.7808	0.0080	19	-	-	-
P3.27H 4n (5 %)	Prater	1996	1.5880	-	1	-	-	-
P3.28H11	Prater	1996	0.7748	0.0069	20	-	-	-
P3.28H12	Prater	1996	0.7758	0.0069	20	0.7700	-	1
P5.27Hb	Prater	1996	0.7650	0.0037	2	-	-	-
P5.27Hc	Prater	1996	0.7805	0.0114	20	-	-	-
P5.27Hd	Prater	1996	0.7689	0.0111	20	-	-	-
P5.27Oa	Prater	1996	0.7634	0.0062	20	-	-	-
P5.27Ob	Prater	1996	0.7623	0.0073	20	-	-	-
S28H17	Straßhof	1996	0.7754	0.0050	10	-	-	-
P28H1	Prater	1997	0.7697	0.0036	20	-	-	-
P28H3	Prater	1997	0.7803	0.0062	20	-	-	-
P28H5	Prater	1997	0.7616	0.0056	20	-	-	-
P28H8	Prater	1997	0.7697	0.0040	20	-	-	-
P28H9	Prater	1997	0.7684	0.0034	40	0.7655	0.0037	20
P28H10	Prater	1997	0.7685	0.0054	20	-	-	-
P28H14	Prater	1997	0.7725	0.0064	20	-	-	-
P28O16	Prater	1997	0.7751	0.0044	20	-	-	-
P29H1	Prater	1997	0.7770	0.0100	30	-	-	-
P29H10	Prater	1997	0.7601	0.0051	20	-	-	-
P29H16	Prater	1997	0.7635	0.0046	20	-	-	-
P29H17	Prater	1997	0.7712	0.0073	20	0.7657	0.0051	5
P26H1	Prater	1999	0.7777	0.0098	10	-	-	-
P26H2	Prater	1999	0.7672	0.0096	30	-	-	-

Discussion

Flow cytometric analysis proved to be a quick and reproducible tool to assess polyploidy in *Q. robur*. DAPI staining was used because of its uniform staining of the oak genome (Zoldos *et al.* 1998) and because of its high resolution without the requirement of RNAse treatment. To count one chromosome of oak (2n = 24) a coefficient of variation (CV) of 2 % is needed. PVP added to the isolation buffer reduced the CV to 1 - 3 %, especially in leaves that are rich in phenolic compounds. Under these conditions, flow cytometry may be used for unambiguous detection of aneuploidy as shown previously in *Abies* (Roth *et al.* 1998). In our case the occurrence of aneuploidy (except B-chromosomes) may be excluded because of the low CV (1 - 3 %) and the low technical

variation (< 3 %) of the flow cytometer.

As our investigations showed, polyploidy occurred in SE clones with an increasing percentage of tetraploidy over the screening time. Clones Alt and Jung, respectively, showed no tetraploidy at the beginning of the first screening period. This indicated that a prolonged stress induced by *in vitro* culture caused these tetraploid cells. DeVerno *et al.* (1999) suggested that somatic embryogenic tissues might be composed of a mixture of altered and unaltered cells. Clone Jung showed tetraploidy at first and diploidy at second screening period. There, the diploid SEs in the mixture might have became more dominant over the tetraploid ones during subcultering.

To our knowledge, tetraploidy in somatic embryo clones of *Q. robur* has not been described previously. Our findings are in accordance with the preliminary report on SEs of *Q. canariensis* (Bueno *et al.* 1996), where tetraploidy was reported after 14 months of continuous subculture.

In the case of chromosomal instabilities, polyploidy is the most commonly observed genetic change (Geier 1991). The disposition for this effect may pre-exist in cell culture on the origin of somatic embryos. Prolonged time in culture (Sharp *et al.* 1980, Berlyn *et al.* 1986, Tremblay *et al.* 1999) or any unfavorable condition or substance that affects or blocks plant metabolism, growth, or development (Puigderrajols *et al.* 1996, Lichtenthaler 1998, Zegzouti and Favre 1999) may also result in polyploidisation. Furthermore, somatic embryogenesis of oak could be of unicellular or multicellular origin depending on the applied growth regulator (Puigderrajols *et al.* 1996, Zegzouti and Favre 1999) which could lead also to somaclonal variation.

Polyploidisation as a result of endomitosis is believed to occur in the absence of cell division, resulting in numerous copies within each cell and is shown in diverse plants (Joubès and Chevalier 2000). Polyploidisation as an effect of restitution of mitosis may occur as a consequence of damage of the mitotic spindle as a failure in the stress response of the cell (Parsell and Lindquist 1994). Smirnova *et al.* (1998) emphasized the importance of relative independence of chromosomal and microtubular cycles, and the influence of these cycles on the progress of mitosis. Extreme desynchronization led to aberrant mitosis while cells with moderately desynchronized cycles were functional.

Our results show that frequency of tetraploidy seems to be correlated with the duration of *in vitro* culture. After induction of somatic embryogenesis the cycles of subculturing and repetitive or secondary somatic embryogenesis can last over many years. We hypothesize that in these highly mitotic tissues the prolonged stress of *in vitro* culture may have caused these tetraploid cells by restitution of mitosis and mitotic spindle failures.

Conclusion: In oak, conventional propagation via organogenesis deals very often with a low rooting ability. Seed storage is limited to two years due to high susceptibility to infections related to the high water content of the acorns. Therefore, somatic embryogenesis has become important for *in vitro* propagation. For maintaining the primary goal of "true-to-type" propagation in oak SE it is essential to monitor the genetic stability with flow cytometry analysis during the process of SE in order to produce high quality SEs, which are convertable into plantlets. Our results show that tetraploidy occurred in 8 % of the tested clones over a culture period of 7 years. Prolonged *in vitro* culture over years should be minimized by applying alternative storage systems such as artificial seeds or cryopreservation.

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