Plant Cell Reports (1995) 14:335–340

Plant Cell Reports

Somatic embryogenesis of *Prunus subhirtella autumno rosa* and regeneration of transgenic plants after *Agrobacterium*-mediated transformation

Artur da Câmara Machado, Monika Puschmann, Helene Pühringer, Robert Kremen, Hermann Katinger, and Margit Laimer da Câmara Machado

Institute of Applied Microbiology, University of Agriculture and Forestry, A-1190 Vienna, Austria
Dedicated to Franticek Novak

Received 28 July 1994/Revised version received 14 September 1994 – Communicated by H. Lörz

Abstract

Embryogenic lines of *Prunus subhirtella autumno rosa* were established on a modified MS medium supplemented with 1 mg/l NAA, 0.06 mg/l IBA and 0.04 mg/l BA from petioles of axenically grown shoots of adult origin. To induce normal development of plantlets we compared a range of approaches on solid culture media as well as in suspension cultures including treatments with ABA, GA3, zeatin, darkness, and cold. A series of experiments were conducted to follow the temporal pattern of somatic embryo development.

Separation of embryos at different stages of development was carried out by sieving the suspension cultures through nylon nets. While the embryogenic masses were used for further subcultures, well formed embryos were used for germination experiments.

Transformed *Prunus subhirtella* plants were regenerated from somatic embryos by inoculating an embryogenic callus with *Agrobacterium* strain LBA 4404 containing the ß-glucuronidase (GUS) gene on plasmid pBinGUSint. Several putative transformed embryogenic calli were selected for continued proliferation on kanamycin containing media. Finally transgenic plants were regenerated on shoot multiplication medium containing kanamycin. Embryos and plants were shown to express the GUS gene by histochemical assays and northern blot analysis. With a yield of 110 transgenic lines from a single transformation experiment this approach appears ideal for the study of the influence on level of expression caused by different copy number, site of insertion etc. This will be helpful in establishing parameters according to which the best transgenic line for a chosen purpose should be selected.

Abbreviations: BA 6-benzylaminopurine, IBA 3-indolebutyric acid, GA_3 gibberellic acid, NAA 1-naphthylacetic acid, ABA abscisic acid, GUS ß-glucuronidase, NPTII neomycin phosphotransferase II, SDS sodium dodecyl sulphate, SSC standard saline citrate, PEM proembryogenic masses.

Introduction

Bajaj (1984) predicted that somatic embryogenesis, whether by direct induction on the explants (as in coconut) or through callus culture (as in Douglas fir) would be among the most important modes of micropropagation for the future.

Although many herbaceous plants have been shown to regenerate via somatic embryogenesis, reports of success in fruit trees have been few, e.g. *Theobroma cacao* (Adu-Ampomah et al. 1988), *Prunus avium* (Druart 1990), *Vitis* (Mullins et al. 1990), *Citrus* (Vardi et al. 1982), *Coffea* (Neuenschwander and Baumann 1992), *Actinidia* (Oliveira and Pais 1992) and *Juglans* (McGranahan et al. 1988).

However, an efficient protocol for somatic embryogenesis in deciduous fruit trees would be highly desirable for a) developmental studies of somatic embryogenesis at a molecular level; b) rapid multiplication and automated production of rootstocks; c) indirect gene transfer experiments, due to the fact that regeneration takes place from single cells (Polito et al. 1989).

Here we report the development of the principal steps for the scale up of an embryogenic culture of a cherry rootstock, *Prunus subhirtella autumno rosa*, which is of special interest due to its outstanding resistance to cold. Furthermore, by taking advantage of the capacity for repetitive embryogenesis we were able to introduce a marker gene (ß-glucuronidase) into somatic embryos, thus opening new perspectives in fruit tree improvement.

Material and Methods

For plant propagation and development of plantlets derived from somatic embryos a medium based on a modified DKV (Driver and Kuniyuki 1984) supplemented with 0.8 mg/l BA and 0.01 mg/l IBA was used. For all other experiments a modified MS was used as basal medium, supplemented with 100 mg/l myo-inositol, different concentrations of sucrose (1 to 7%) and 0.8% agar. All growth regulators, except ABA and GA_3 which were filter sterilized, were added to the medium before autoclaving. Media were adjusted to a pH of 5.7 - 5.8 with 1N NaOH or 1N HCl and autoclaved at 120°C for 20 minutes. Aliquots of 30 ml media were dispensed into Petri dishes (92 x 17 mm, Nunc), 35 ml into 100 ml Erlenmeyer flasks and 70 ml into 250 ml flasks and suspension cultures were exposed to agitation of 110 rpm on a gyratory shaker (Swip, Bühler).

Induction

Donor plants were axenically grown shoots of *Prunus subhirtella autumno rosa*, established from an adult plant and maintained on a

Correspondence to: M. Laimer da Câmara Machado

modified DKV medium. Different explants (leaf discs, petioles and stem internodes) were screened for their ability to produce callus on a range of culture media based on a modified MS supplemented with high concentrations of auxins (NAA, IBA and 2,4-D) (table 1).

Table 1: Growth regulators used for the induction of somatic embryogenesis

HORMONES mg/l / MEDIA	BAP	NAA	IBA	2,4-D
R1E	0.02			2
R2E	0.02			4
R3E	0.4			7
R4E	0.4			10
R5E	0.02	1	0.06	
* R6E	0.04	1	0.06	
R7E	0.04	2	0.06	
R8E	0.02		1	
R9E	0.02		2	
R10E	0.4		1	
R11E	0.4		3	
R12E	0.4	0.06	2	

Subcultures were carried out every 21 days according to the following scheme: from the original cultures, which were maintained throughout the experiment, in each passage part of the callus was placed on MS, and on MS with another hormone concentration and subsequently onto MS, both in solid and liquid phase, e.g. from R6E cultures were placed on R6E, MS and R7E and subsequently to MS, and so on.

Cultures were kept at 23 +/- 2°C either with a 16 h photoperiod provided by cool white fluorescent tubes with an irradiance of 60 umol $m^{-2}s^{-1}$ or in complete darkness, and subcultured monthly.

Development

Direct obtention of plantlets on solid media. Embryogenic callus produced on R6E was used for further trials with the purpose of a direct obtention of plantlets: **1.** MS with ABA in a concentration range from 3.96 to 7.92 mg/l for 4-6 weeks followed by a) MS in darkness for 1 week and then in light and b) MS with 0.5 mg/l GA_3. **2.** R6E in darkness at 4°C or 23°C for 4-8 weeks followed by a) MS with 2-4% sucrose and b) MS with 0,5 mg/l GA_3. **3.** MS with 1-7% (=x) sucrose followed by a) MS with 0,2 mg/l zeatin and b) MS with (x-1%) sucrose. **4.** R6E with 4% sucrose at 23°C for 4-8 weeks in darkness and light followed by MS. **5.** MS with GA_3 in a concentration range from 0.5 to 1 mg/l for 4 weeks. **6.** MS with 0.2 mg/l zeatin.

Development in suspension cultures. Callus from R6E kept in darkness was used as starting material for a series of experiments with suspension cultures in 100 ml flasks in hormone free MS media with 4% sucrose in order to evaluate the time factor during normal embryo development. Every 14 days the supernatant was replaced with fresh culture media. After 4 weeks cultures were passed into 250 ml Erlenmeyer flasks.

To obtain more homogeneous populations of embryo stages, a sieving procedure modified after Giuliano et al. (1983) was applied. Separation of embryos at different stages of development was carried out by sieving the suspension cultures through 3 nylon nets (Thal) with 1000, 500 and 225 µm meshes respectively. The filtrate was subcultured in the same liquid medium (v:v=1:3), while the 3 fractions obtained were placed on liquid and solid MS medium with 2-4% sucrose for further development. The control experiment involved a weekly manual harvesting of embryos at the cotyledonary stage with forceps under a binocular microscope (Wildt). Well formed embryos were used for germination experiments, while the embryogenic masses were used for further subcultures. Cultures were kept at 23 +/-2°C.

Germination

Manually harvested embryos were exposed to a series of treatments to induce germination: 1. stratification for 2-3 weeks at 4°C in darkness on solid MS medium with 4% sucrose followed by culture on MS without sucrose, but 1% agar in light or darkness. 2. dessication of embryos treated for 24 h with MS with 0, 1 and 5 mg/l ABA in a sterile Petri dish for 5, 10, 15, 20, 25 and 30 days respectively at room temperature followed by culture on a) MS and b) MS with 0,2 - 1 mg/l zeatin. 3. subculture on a double layer system composed of 20 ml solid MS with 1 mg/l activated charcoal and 4 ml liquid MS with 1 mg/l zeatin (Novak et al. 1989).

Histological studies

Morphological changes during induction and further development of somatic embryos were investigated by light microscopy. For histological studies explants were fixed for 24 h, dehydrated in a graded series of ethanol and toluene and embedded in paraffin wax. 13 µm sections were cut on a rotary microtome and double stained with basic fuchsin and picro indigocarmine acetic acid (Hruby 1933).

Transformation and Selection

Agrobacterium strains and plasmids. In order to check the value of a given explant for transformation experiments it is doubtless advisable to carry out some transformation experiments using marker genes in advance. The most widely used marker gene today is ß-GUS (Jefferson et al. 1987) as it allows a histochemical localization of the transformation events. *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema et al. 1983) containing pBinGUSint (Vancanneyt et al. 1990), carries the marker gene ß-glucuronidase (GUS) besides the NPTII gene for selection. The *Agrobacterium* strain was inoculated from an overnight culture and grown to a density of OD_{600} = 0.6 in LB medium in the presence of 50 $\mu g.ml^{-1}$ kanamycin, supplemented with 20 µM acetosyringone and incubated at 28°C with vigorous aeration in a reciprocally shaking water bath. For co-cultivation, the suspension was diluted 1:50 in MS culture medium.

Cocultivation with Agrobacterium. Embryogenic callus was immersed for 5-10 seconds in the *Agrobacterium* suspension and incubated for 48 h on regeneration medium. Explants were rinsed with sterile half strength MS-medium, blotted dry on sterile filter paper and placed on regeneration medium containing additionally 250 µg/ml carbenicillin to inhibit further bacterial growth.

Determination of the sensitivity of the embryogenic callus towards selecting agents. Embryogenic callus was exposed to 125, 250 and 500 mg/l carbenicillin on one hand and to 6.25, 12.5, 25, 50, 75 and 100 mg/l kanamycin on the other hand to determine the sensitivity towards these antibiotics and selecting agents by the appearance of the cultures and by measuring fresh weight after 4 weeks.

Analysis of the GUS activity. This analysis was performed essentially as described (Jefferson et al. 1987). After 14 days and subsequently at monthly intervals tissue samples were harvested and prepared for the histological enzyme assays. They were exposed to 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-Glu) for 24 h at 37°C and observed for development of indigo dye under the microscope.

Northern blot.

Total RNA was extracted from in vitro grown plantlets by guanidinium thiocyanate extraction followed by CsCl centrifugation (Sambrook et al. 1989). RNA was electrophoresed in a formaldehyde containing 1% agarose gel and transferred to a nylon membrane by capillary blotting (Fourney et al. 1988). The GUS gene excised from pBI201 was labelled with ^{32}P using the Multiprime DNA system (Amersham) and hybridized to the membrane overnight at 65°C using standard methods (Sambrook et al. 1989). The membrane was washed twice each under non stringent (2xSSC + 0.1% SDS; 15 min, 30 min) and stringent (0.2xSSC + 0.1% SDS; 15 min, 30 min) conditions and subsequently exposed to X-ray film for 2d with an intensifying screen. RNA from a non-transgenic *Prunus subhirtella* from the orchard was used as a control.

Results

Induction

From the different explants compared for their ability to produce callus, petioles proved to be the most responsive tissues and were therefore used as sole explants for all further experiments to obtain embryogenic cell lines. From the 12 different media compared (table 1) after 8 subcultures embryogenic capacity of the callus could be observed (figure 1c) only on medium R6E containing 2 auxins (NAA 1 mg/l, IBA 0,06 mg/l) beside the cytokinin (BA 0,04 mg/l).

The expected positive effects of induction for a short period followed by culture on hormone free media as well as of treatment with higher hormonal concentrations and again removal of the growth regulators were not obtained in any of the variations carried out; callus survived for 1 or 2 subcultures and died subsequently.

The embryogenic callus obtained on R6E originally was quite inhomogeneous. After selective subcultures the callus could be characterized according to its colour: beige, brown and green. Nevertheless in all callus types we could find embryos in all different developmental stages. Callus was green most probably due to a precocious germination of the embryos, tending quite soon towards denaturation to brown callus. The beige, opaque and friable callus was the most proliferating callus type giving rise on its surface to the highest number of embryos and a strong secondary embryo production.

The brown callus contained only few early stages of embryos among a majority of undifferentiated, parenchymatous cells.

Selective subcultures of green, beige and brown callus over several months allowed the isolation of a highly embryogenic callus line, which could be maintained as a highly productive source material through cultivation in darkness. After three years, cultures on R6E still maintained their embryogenic capacity with monthly subcultures both in light and darkness.

Development

Direct obtention of plantlets on solid media. From all the trials described above we conclude that none of the following treatments would cause the normal development of the embryos till the plantlet stage: the zeatin application, the GA_3 treatment, the stratification at 4°C, the ABA treatment. The application of 3.96 mg/l ABA led to the expected increase in the compactness of embryogenic callus and of embryo structures; nevertheless when transferred to MS or MS with GA_3 we observed further abnormal germination and strong formation of anthocyanin. On 7.92 mg/l ABA development was slowed down and ceased after the transfer to MS or MS with GA_3.

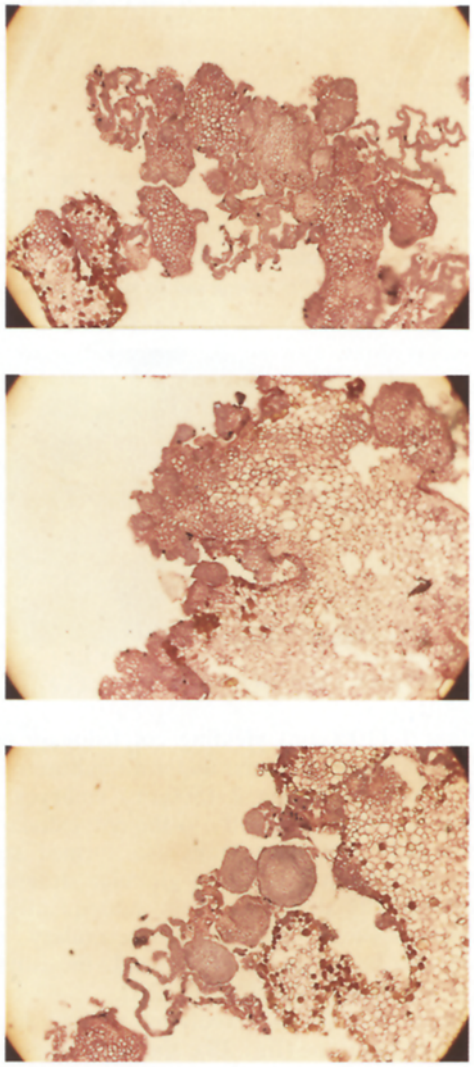
The strongest influence on morphological and developmental changes observed was by the application of different concentrations of sugar. The highest concentration of sugars (6-7%) led to a very slow growth, an opaque coloration and a compact structure of the callus and an inhibition of the formation of single, well formed embryos (fig. 1a).

4% sucrose in solid MS media led to an increased production of embryos (fig. 1b), which due to their compact structure and strong aggregation did not separate into single stages when transferred to liquid culture conditions.

On 2% sucrose the obtention of single embryos was possible (fig. 1c) and due to the friable structure of the callus their isolation was facilitated. This was, however, accompanied by instability of the cultures, expressed by the production of different types of callus. This could be compensated by cultivation in darkness.

Darkness induced a more actively growing friable callus with a stronger embryogenic capacity. This callus represents an ideal source material for initiation of suspension cultures, because the isolation of embryos at different stages was facilitated due to its friability.

Even lower contents (1%) of sugar promoted premature germination at an increased rate, by giving rise to morphologically abnormal plantlets accompanied by secondary embryogenesis.



Figures 1 a-c: Histological analyses of beige callus showing the influence of sucrose. Note the compact structure of the callus on 6 % sucrose with the tightly fused embryos (1a) and on 4 % sucrose the high number of embryos on the surface of the embryogenic callus (1b). Their strong aggregation however is limiting for the further development. On 2 % sucrose due to the friable callus structure the obtention of single embryos is possible. As embryo production is satisfactory (1c), this concentration is used for the source material to establish suspension cultures.

Development in suspension cultures. Trials to directly obtain plantlets from embryogenic callus did not show the expected success. It therefore appears to be necessary to stop secondary embryogenesis and to utilize well formed embryos for germination purposes. To control secondary embryogenesis, the first question was: when does it occur? To answer this, we designed an experiment allowing separation of the embryos at the different stages from globular to cotyledonary and observation of the temporal pattern of development in different culture conditions (fig. 2).

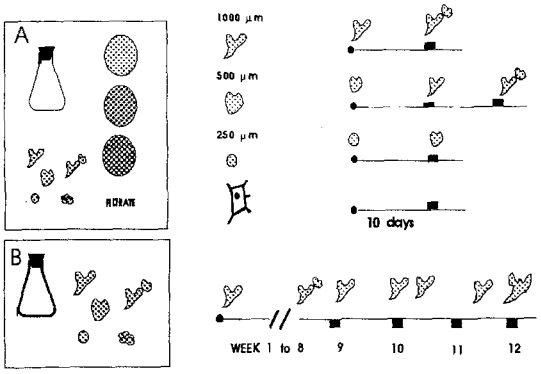


Figure 2: Temporal pattern of somatic embryo development on hormone free media: A) after sieving and B) after manual harvesting.

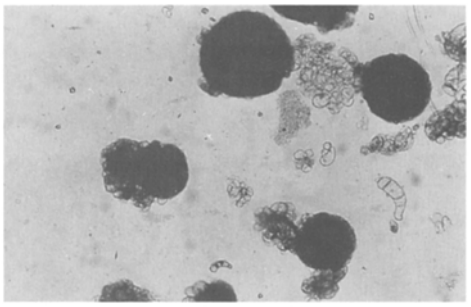


Figure 3: Embryos at a globular stage.

In fraction 1 (1000μm) yielding cotyledonary embryos, secondary embryos appeared under all culture conditions after 7 days. In fraction 2 (500μm) embryos at heart stage first developed correctly to the cotyledonary stage, but about 14 days later they also formed secondary embryos In fraction 3 (225μm) globular structures (fig. 3) developed to the heart and subsequently to the cotyledonary stage, but finally secondary embryos appeared also here; on the other hand, the filtrate soon lost its embryogenic capacity (fig. 2A).

The control cultures, involving manual harvest of cotyledonary embryos (fig. 2B), did not cease producing embryos, but after 4 subcultures showed a significantly decreased rate of secondary embryogenesis.

These observations suggested that the impulse for secondary embryogenesis was generated by the cultures themselves and depended strongly on a certain dilution factor. We could harvest weekly up to 200 normally developed embryos from 70 ml suspensions (fig. 4).

These observations suggested that secondary embryogenesis must have been already initiated at a very early stage of primary embryo development and can be sustained only in the presence of embryogenic masses.

Indeed in histological analyses we could find globular embryos already showing development of secondary embryos (fig. 5), even if they were apparently morphologically normal.

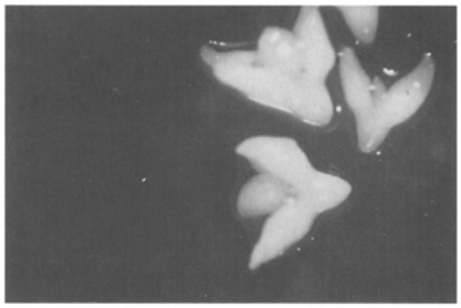


Figure 4: Embryos at a cotyledonary stage.

Germination

Manually selected embryos exposed to 3 weeks at 4° C in darkness, though maintaining their morphological shape did not show any kind of further development, when transferred to normal culture conditions.

The application of zeatin at the cotyledonary stage alone or in combination with a dessication step is able to promote germination. Of all the treatments applied, a long term dessication (30 days at room temperature) yielded the highest number of viable plantlets (data not shown).

Transformation and selection

In the experiments to determine the sensitivity of embryogenic callus to the selecting agents, no influence of the different carbenicillin treatments could be detected by optical evaluation. Only the fresh weight determination showed a reduction in growth at 500 mg/l carbenicillin (Puschmann 1993). Therefore 250 mg/l were chosen for further selection.

Kanamycin at 25 mg/l caused a strong reduction of growth, which was more pronounced from 50 mg/l on, which seems to be typical for *Prunus* (Laimer et al. 1992). However embryogenic structures were inhibited only from 75 mg/l onwards (Puschmann 1993). Therefore, 75 mg/l was chosen, as under these conditions only transgenic tissue retained its embryogenic potential. Selection pressure was visible on R6E containing 75 mg/l kanamycin after the first three weeks. Approximately one third of the putative transformed callus turned brown and squashy and were mainly negative in the GUS assay, where only single cells were stained blue (data not shown). However those calli that continued with a beige appearance and strong growth also showed some GUS positive cells on the surface (fig. 6). The different intensity of single cells may be due to different levels of expression of the introduced gene.

Taking advantage of the capacity for repetitive embryogenesis of the cultures, however, this could be used to obtain after some subsequent subcultures under selection conditions (6 weeks) some explants showing increased areas of transformed tissue (fig. 7).

After 21 weeks of subcultures we obtained cultures existing only of transformed cells, thereby giving rise to fully transformed embryos (fig. 8) and subsequently to fully transformed plantlets of *Prunus*, as could be detected by GUS assay (fig. 9) and Northern blot analyses (fig. 10).

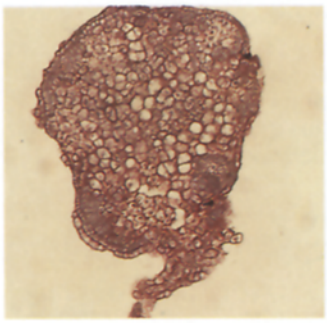


Figure 5: Somatic embryo at the late globular stage with distinct regions of secondary embryogenesis.

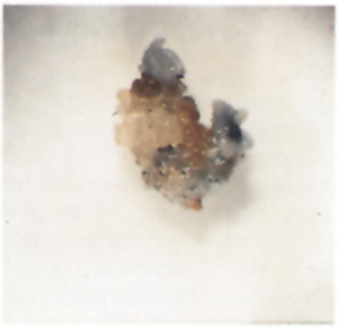


Figure 6: Embryogenic tissue of *Prunus subhirtella* three weeks after transformation showing few spots of cells expressing the marker gene GUS.

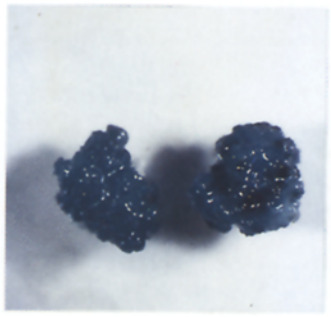


Figure 7: Fully transformed cluster of somatic embryos of *Prunus subhirtella* after several months on selective media.

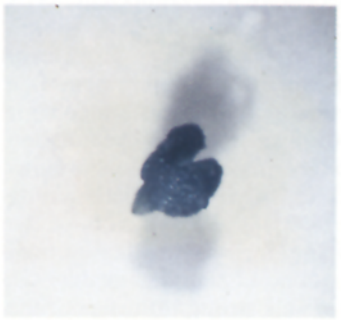


Figure 8: Fully transformed cotyledonary embryo of *Prunus subhirtella*.

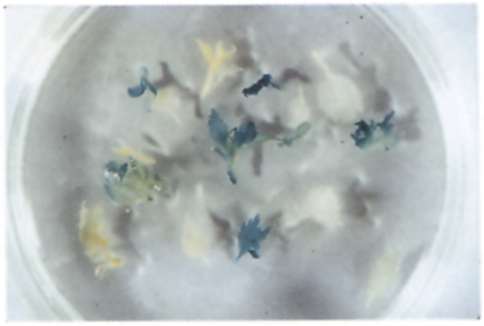


Figure 9: Homogeneously transformed plantlets of *Prunus subhirtella* derived from somatic embryos as compared to the non-transformed control (white).

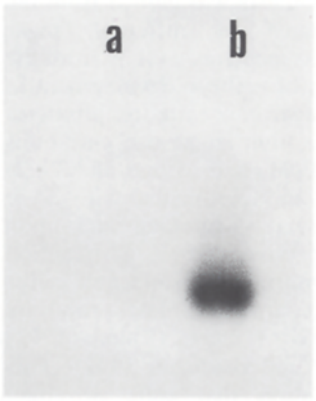


Figure 10: Northern Blot of transgenic Prunus (lane A) as compared to control (lane B).

Discussion

Although somatic embryogenesis has been induced in woody plants, on the whole juvenile plant material has been used as source (Ammirato 1989), e.g. punctured leaves of 1-2 y old poplars (Park & Son 1988), primary leaves of seedlings of horse chestnut (Dameri 1986) and apple (Liu et al. 1983), anthers of oak (Jörgenson 1988) and immature and mature embryos of conifers (Von Arnold 1990) and peaches (Hammerschlag et al. 1985).
Besides the reports of somatic embryogenesis from young leaves of a Sapindus tree (Desai et al. 1986) and from roots of *Prunus incisa x serrula* (Druart 1981, 1990) our regeneration report is one of the few to include adult explant material from a fruit tree rootstock, thereby offering prospects of automation of propagation.
From the 12 different media compared, only R6E induced embryogenesis. Even media with small variations on the hormonal concentrations, e.g. R5E and R7E, could not trigger the embryogenic response during the same induction period. The use of both auxins, NAA and IBA, seemed necessary to induce the embryogenic capacity in the callus. Similar observations were reported by Dameri et al. 1986 in horse chestnut, where the use of NAA and 2,4-D was required.

Another interesting aspect was that the use of 2,4-D, a well known inducer of somatic embryogenesis since the first description of the process in carrot by Steward (1958) and Reinert (1958) did not induce the expected results. With a few other reports for woody species, e.g. *Actinidia* (Oliveira and Pais 1992), *Prunus incisa x serrula* (Druart 1981) and *Coffea canephora* (Hatanaka 1991), where 2,4-D was not effective, this is quite an exception, as 2,4-D is still the most generally used inducer of somatic embryogenesis, even though some negative effects due to high concentrations have been described (Park and Son 1988). As the concentration shift treatment, which is also a common protocol to induce somatic embryogenesis, did not work with all the media tested, we assume that besides very specific hormonal balances the induction of somatic embryogenesis in fruit trees also requires a longer period. Histological analyses of the embryogenic callus revealed that embryos in all developmental stages. This is in accordance with the observations of Lo Schiavo et al. (1991) who very carefully characterized their embryogenic carrot system. Therefore an embryogenic cell culture, in the presence of growth regulators consists of two types of cells, which are to a large extent cycling:

globular embryos in the presence of auxin dedifferentiate into callus, and new proembryogenic masses (PEM) are formed from vegetative cells all the time. Auxin is necessary to start embryogenesis by generating PEM, on the other hand, it inhibits normal embryo development by causing secondary embryogenesis. This is the dilemma of differential responsiveness to auxin by different cells present in the population, which we circumvented by the sieving procedure in combination with hormone free medium. Also Komamine et al. (1991) mention that the determination for embryogenesis occurs already at some very early stages of embryogenic clusters, and that auxin is necessary for "competent" cells to express their totipotency, while it will inhibit embryogenesis in later phases. This is cleary demonstrated in our control experiments on hormone free medium, where embryogenesis could be maintained for several years. Finally now the system allows examination of the question of the relative importance of hormone concentration versus cell sensitivity in determining morphogenesis (Trewawas 1993). The completion of the single stages is a prerequisite for any further normal development, which in hormone containing media was not possible due to the continuous strong influence of the induction medium, leading to secondary embryogenesis. Further evidence for this hypothesis was gained from the comparison of the influence of different sugar concentrations in hormone free media as well as from the observation of isolated stages of embryo development in suspension cultures. A low sugar concentration promotes premature germination, while high sugar concentrations lead to maturation. Emons (1991) and Min et al. (1992) also reported that on hormone free media with 6 % sucrose germination was severely retarded and upon transfer to 2 % or 3 % respectively 90 % germination occured. It has also been observed that attached callus tissue provides a factor that stimulates leaf primordia formation in somatic embryos of maize (Emons 1991) and coffee (Neuenschwander und Baumann 1992). This correlates with our observation that cells or small aggregates passed through the filtration step lost their embryogenic potential. The combination of two main factors, the passage from induction medium at 23°C in the dark to a hormone free medium with 4% sucrose in combination with a manual harvest of embryos at a cotyledonary stage, finally led to a normal development of embryos.

As for germination of normal plantlets, it seems clear that the quality of embryos is the crucial factor, which in our opinion includes a certain size as well as a correct morphological shape. To allow this regular development and growth of the embryo control of the process of secondary embryogenesis is necessary. Repetitive somatic embrogenesis in *Arachis* (Durham and Parrott 1992) could be interrupted by a 10 day dessication treatment, which lead to an increase in normal germination. We observed in our suspension cultures that repetitive somatic embryogenesis decreases only after 3 months in hormone free media and leads to the production of morphologically normal embryos, which we use for germination experiments.

Recent improvements in tissue culture technologies have made it possible to obtain dessication tolerant somatic embryos (Gray 1987, Senaratna et al. 1990), which, like most orthodox seeds, can undergo dehydration without loss of viability. These successes provide both good material for studies of plant embryogenesis and a promising future for artificial seed production (Xu and Bewley 1992). Due to the single cell origin of somatic embryos (Polito et al. 1989) this system has a great value for the production of transgenic fruit trees.

Acknowledgements

We would like to thank J. Hammond (USDA, Beltsville) for critical reading of the manuscript. This work has been supported by the BMLFW project L606/90 and the BMWF project 30.460.

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