Regeneration of roots, shoots and embryos: physiological, biochemical and molecular aspects

G.-J. DE KLERK, B. ARNHOLDT-SCHMIDT*, R. LIEBERER**
and K.-H. NEUMANN*

Centre for Plant Tissue Culture Research, PO Box 85, 2160 AB Lisse, The Netherlands
Institute of Plant Nutrition, Justus-Liebig University, Südfenlage 6, 35390 Giessen, Germany*
Institute of Applied Botany, University of Hamburg, Marseiller Str. 7, 20355 Hamburg, Germany**

Abstract

When the proper stimulus are given, somatic plant cells may form adventitious embryos, roots or shoots. The three pathways of regeneration show apparent similarities. They consist of three analogous phases: 1) dedifferentiation (during which the tissue becomes competent to respond to the organogeneic/embryogenic stimulus), 2) induction (during which cells become determined to form either a root, a shoot or an embryo), and 3) realization (outgrowth to an organ or an embryo). The first phase may involve a period of callus growth (indirect regeneration), but often cells present in the explant become competent without cell division or without cell division at a large scale (direct regeneration). In an explant, only very few cells show the organogeneic/embryogenic response. In direct regeneration, the three regenerative pathways start from cells in different tissues. This is most obvious when the different types of regeneration occur in the same explant. The hormonal trigger for the dedifferentiation phase is a general one, probably auxin. During the induction phase, each pathway requires specific hormonal triggers. During the realization phase, hormones should be absent or at low concentration. The successive steps in the regeneration process coincide with events on the molecular and biochemical levels, but so far no coherent picture has emerged. In particular during the early stages of regeneration, research on these levels is hampered by a technical problem, viz., the very low proportion of cells that participate in the process of regeneration. New methods may overcome this problem.

Additional key words: biotechnology, callus, micropropagation, plant hormones, tissue culture.

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; NAA - naphthaleneacetic acid; PAA - phenylacetic acid.
Introduction

In plants, particularly during culture in vitro, somatic cells may be capable of regeneration: when given the proper stimuli they may form adventitious roots, shoots or embryos. Biotechnological breeding and propagation methods depend on this. Genetic engineering, haploid production, etc., include the regeneration of a complete plant from somatic cells. Regeneration of adventitious shoots from, e.g., leaves or buds, forms part of many micropropagation protocols. In the final stage of micropropagation, microshoots are treated with auxin to induce the formation of adventitious roots. Moreover, regeneration of somatic embryos from cell suspensions will, if broadly applicable, revolutionize plant propagation. In practical applications of regeneration, there are two main problems. First, regeneration does occur not at all, only infrequently, or only from cells that are not susceptible for genetic engineering (Colby et al. 1993, Lowe et al. 1993, Geier and Sangwan 1996). Second, plants that are produced via regeneration are often genetically different from the mother plant, a phenomenon referred to as somaclonal variation (Larkin and Scowcroft 1981, De Klerk 1990).

Since in a range of cultivars, the capacities to various pathways of regeneration are correlated (Olesen et al. 1995), the same underlying mechanisms appear to operate to some extent. In the present paper, we deal with the similarities and dissimilarities between the regeneration of roots, shoots and embryos. Dissimilarities concern different types of starting cells and different hormonal triggers, and similarities the analogy of the developmental process in time.

Adventitious root, shoot and embryo formation consist of analogous successive stages

Regeneration is a developmental process consisting of distinct phases. The terms used to describe these phases are not consistent in the literature. In this article, we use the terms "acquisition of competence", "induction" and "realization". Preferably, the phases should be identified on the base of the expression of different sets of genes, but research has not yet progressed that far.

For shoot regeneration, the occurrence of phases was examined by Christianson and Warnick (1983) in leaf explants of Convolvulus. They transferred explants at various times after the start of tissue culture from one medium to another, using three types of media, namely shoot-, root-, and callus-inducing medium (SIM, RIM and CIM, respectively). These media had very different auxin-cytokinin ratios. During the initial period after the start of culture, the hormonal composition could be varied over a wide range (SIM, RIM and CIM gave essentially the same results). After that, a phase occurred during which auxin and cytokinin should be applied in the proper concentrations. In the final, third phase, the hormonal composition could again be varied over a wide range. These results demonstrate the occurrence of three phases:
Phase 1, acquisition of competence: Cells are at first not competent to respond to the organogenic stimulus, but acquire this competence during an initial phase of dedifferentiation.

Phase 2, induction: In the induction phase, cells are responsive to the organogenic stimulus and become determined to form a specific organ, i.e., a shoot. Only during this phase, the hormonal composition of the medium is critical.

Phase 3, realization: When the cells are determined, the new program of differentiation is initiated to produce a shoot.

For shoot formation, this scheme has been confirmed for various species (e.g., Flinn et al. 1988, Attfield and Evans 1991b).

The same scheme can be applied in adventitious root and embryo formation. During a roothing treatment of apple microshoots, De Klerk et al. (1993) applied pulses with either auxin or cytokinin. They found a strongly enhanced responsiveness (inhibition by cytokinin and promotion by auxin) from 24 to 96 h after the start of the roothing treatment, indicating that during this time induction occurs. During this period there is also an enhanced responsiveness to the antiauxin p-chlorophenoxyisobutyric acid (De Klerk 1995). The occurrence of a lag period before stems become sensitive to auxin has been reported by Went (1939) and Mitsushashi et al. (1969). It should be noted that in easy-to-root apple microshoots, phase 2 (induction) is not preceded by cell divisions at a large scale (De Klerk et al. 1995, Jasik and De Klerk 1997).

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Fig. 1. General sequence of developmental phases during regeneration.
After application of 2,4-D to, e.g., hypocotyl explants of carrot, first a non-embryogenic cell suspension is established (De Vries et al. 1988a). This suspension becomes embryogenic only a considerable time after that (in our terminology, the cells first require acquisition of competence and after that are induced to become embryogenic callus). In alfalfa, it was found that a phase of nonembryogenic-callus growth is established and maintained with the auxin PAA (Plusad et al. 1995) or with NAA + BAP (Dudits et al. 1991). By treatment with 2,4-D, cells in these callus cultures are induced to form embryogenic callus (induction phase). In carrot somatic embryogenesis, it has been found early, that auxin has to be removed to allow the development of the embryos from embryogenic cell suspensions (in our terminology the transition from "induction" to "realization"). Embryogenic cultures at the end of the induction phase can be kept proliferating for a prolonged period of time in the presence of 2,4-D. After many subculture cycles, the transition to stage 3 can still be initiated by transfer of the cells to a hormone-free medium. Most investigations on somatic embryogenesis and its molecular basis have been performed using such proliferating embryogenic cultures (e.g., Sung and Okimoto 1981, De Vries et al. 1988a, Komamine et al. 1990).

The results discussed in this section indicate that the succession of steps during regeneration is very similar for root-, shoot- and embryo-formation (Fig. 1). It should be noted that the first stage may involve a period of callus growth (indirect regeneration). Often, though, competent cells already exist in the explant and become competent to respond to the organogenic/embryogenic stimulus after a (short) lag phase without any cell division or without cell division at a large scale (direct regeneration).

Adventitious roots, shoots and embryos develop from different cell types

In addition to zygotic embryogenesis, in planta other cells can also be initiated to perform embryogenesis. These natural occurring detours are confined to tissues of the sexual apparatus. Somatic embryogenesis in vitro, however, may be induced from other plant organs. In particular, in monocotyledonous species, immature embryos are often the best (and often only) source of explants for somatic embryogenesis. In many species, somatic embryos may also develop from seedlings or adult plants. Adventitious shoots may be induced from various organs, although regeneration from roots is usually difficult. Adventitious roots are mostly formed from stems. The formation of lateral roots is similar to adventitious root formation since lateral roots are not derived directly from a root primordium. In perennial plants, juvenile tissue regenerates roots and shoots more easily than adult tissue (Boulay 1987).

When adventitious shoots and roots develop from the same organ, they originate from different tissues (Hartsema 1924, Atthfield and Evans 1991a, Julliard et al. 1992). In cultured carrot petioles, application of IAA results in the formation of adventitious shoots, roots and embryos, but not from the same but from different tissues within the petiole (Neumann and Grieb 1992, Neumann 1995).
1. A few days after the start of culture, an increase of cytoplasm occurs in some originally vacuolised cells near the vascular bundles. After that, these cells divide to produce root primordia and eventually roots.

2. About one week later, an increase in cytoplasm occurs in vacuolised subepidermal cells. These cells develop into somatic embryos.

3. Large parenchyma cells are competent to caulogenesis (5–7 days after the start of tissue culture).

Thus, cells committed to either one of the three pathways of regeneration apparently exist in the original explant. Plant cells are often considered as totipotent, i.e., capable of forming a complete plant (for a recent review see Komamine et al. 1992). The results with carrot petioles show that subepidermal cells are totipotent, but that other cells are only multipotent, i.e., forming firstly only an organ and not a complete plant with both a root and a shoot meristem (compare Neumann 1995, Geier and Sangwan 1996). The limited capability of cells in the original explant to follow only one of the three pathways should be noted. It indicates that to achieve regeneration in recalcitrant species it may be more important to have the right types of cells in an explant than to develop highly refined regeneration conditions (nutrients, hormonal composition of the medium, etc.).

When given the proper stimulus, somatic cells may initiate tumorous growth. Often, cells that are incapable to respond to an organogenic or embryogenic stimulus, become capable during this tumorous growth. Thus, difficult to root genotypes may form roots from cells close to the surface of the callus, after which vascular tissues are formed within the callus to connect the root with the stem (Zhou et al. 1992).

**Triggers**

In their classical paper, Skoog and Miller (1957) report that root, shoot and callus formation in tobacco explants cultured in vitro are brought about by high, low and intermediate auxin/ cytokinin ratios. Since the process of regeneration can be dissected into a series of successive phases, each with its own hormonal requirements, this scheme is apparently too simple.

The first phase, the acquisition of competence, depends upon auxin. It has been shown, that the auxin PAA is not effective in the second phase (induction) of rooting (Went 1939, De Klerk, unpublished and embryogenesis (Fristad et al. 1993), but is, nevertheless, effective during the first phase. In rooting, also the antiauxin "p-chlorophenoxyisobutyric acid, which is inhibitory during the second phase (De Klerk 1995), is promotive during the first phase (Shibaoka 1971, De Klerk, unpublished). Since regeneration often occurs only after wounding, signals related to wounding may also play a role during dedifferentiation (Wilson and Van Staden 1999).

In the second phase, induction, auxin and cytokinin should be supplemented in the appropriate concentrations (for shoot formation: Christianson and Warmick 1983; for root formation: De Klerk et al. 1993). In alfalfa, Dullits et al. (1991) reported that
during the first phase a combination of NAA and kinetin induces tumorous growth. This type of growth continues when these hormones are applied but a (short) treatment with a high dose of 2,4-D starts the embryogenic program. Thus, the embryogenic stimulus seems to be 2,4-D. In carrot, 2,4-D can be replaced by IAA (Schäfer et al. 1988).

In the third phase, realization, the hormonal composition of the medium is less critical. However, the high concentrations of exogenous hormones required for induction, are often inhibitory. Here, other hormones may also play a role, e.g., abscisic acid in somatic embryogenesis.

With respect to the hormonal composition of the nutrient medium, several points should be made: 1) the effect of hormonal supplements to nutrient media can be modified by inorganic nutrients (Preece 1995); 2) the uptake of various hormones by explants from the medium may be very different (for example, in tobacco explants, NAA is taken up six or ten times faster than IAA or BAP - Barendse et al. 1986, Peeters et al. 1991); 3) since hormones are extensively metabolized, the actual concentration of hormones in the tissue has to be considered (for example, petioles of an embryogenic carrot genotype have a four times higher level of IAA during the induction phase than petioles of a non-embryogenic genotype - Li and Neumann 1985); 4) applied hormones may alter the metabolism of endogenous hormones. Michalczyk et al. (1992) demonstrated that exogenous 2,4-D triggers the synthesis of endogenous IAA through a synthetic pathway apparently special for cultured cells.

Regeneration may also occur "spontaneously" without an apparent exogenous trigger. Two examples will be given. Genetic tumors arise spontaneously on plants of certain interspecific hybrids, e.g., in tobacco. When brought in culture, the tumor tissues may show regeneration without addition of plant hormones. In contrast to crown gall tumorous tissue, these cultured genetic tumors have "normal" levels of cytokinin (Nandi et al. 1990) and auxin (Palni and Summons 1987). The second example concerns carrot. In a research program on the production of transgenic carrot plants, in a wild carrot strain hygromycin resistance and in a domestic strain resistance to 5-methyltryptophan were transferred, respectively. Protoplasts of both were fused. From the fusion products, plants were raised via somatic embryogenesis. In some of these plants, somatic embryos regenerated without any special treatment from small tumors occurring on petioles and hypocotyl of seedlings grown on an inorganic agar medium (Chinachit 1991). This could be repeated for several generative generations. On plantlets derived from sexual crosses of the wildtype parents, spontaneous regeneration was never observed. Although other possibilities cannot be ruled out, it seems that the cells had obtained a genetic constitution similar to genetic tumors.

Molecular biology and biochemical characterization, markers

It should be noted that the number of cells involved in the regeneration process is almost always only a very small fraction of the total number of cells of an explant or a cell suspension, especially during the first phases of regeneration. In molecular and
biochemical studies, though, usually all of the cell material is analyzed. Thus, to establish the relevance for regeneration, the molecular and biochemical studies should be accompanied by appropriate analysis on the microscopical level, preferably supplemented by histochemical studies.

**DNA level:** Some authors report DNA amplification during the first hours (1 - 120 h) after inoculation of different tissues from dicotyledonous species (Buiatti 1977, Bassi 1990, Arnholdt-Schmitt 1993). This precedes mitotic activity and may be related to wounding, activation of the cell cycle and/or initiation of dedifferentiation. At a later stage, a decrease of DNA occurs. Such reduction of DNA content per cell (about 50%) has been reported for secondary phloem explants of carrot roots, whereas stable diploid chromosome numbers are being maintained (Mitra et al. 1960, Steward et al. 1964). More recently, it has been found that during the linear growth phase of carrot callus repetitive DNA is strongly reduced (e.g., Schäfer et al. 1978, Dürrszen et al. 1984, Arnholdt-Schmitt 1995, Arnholdt-Schmitt et al. 1995). At the stationary phase of growth of carrot callus after 4 weeks of culture, abundant amplification occurs again, now showing a different genome organization. At the same time, adventitious roots can often be observed. Thus, during the callus phase significant changes in the organization and modification of DNA occur. These are possibly related to developmental events of the regeneration process. In carrot embryogenic cell suspensions, Fujimura and Komamine (1982) demonstrated a reduction of the mean length of replicons shortly before the cell cycle accelerates during the preglobular phase. It remains to be seen whether there is a connection between this observation and the above described quantitative variations in the repetitive DNA fraction in proliferating cells in general (see discussion in Arnholdt-Schmitt 1995). A progressive reduction in DNA was also demonstrated by Giorgetti et al. (1995) during the generation of an embryogenic cell culture from carrot hypocotyle. These authors, however, exclude selective DNA loss and interpret their observations as "somatic meiosis" leading to segregation. Deumling and Clermont (1989) reported a complex pattern of chromosome diminution during cell culture and plant regeneration of Scilla siberica. They concluded that an excessive and specific chromatid loss is a prerequisite for plant regeneration.

Many studies have shown that the state of (de-)differentiation is related to DNA methylation (e.g., Riggs and Crisleps 1990, Palmgren et al. 1991). Cells of different tissues of the carrot plant are also characterized by specific DNA methylation patterns (Arnholdt-Schmitt 1992, Arnholdt-Schmitt et al. 1995). During the linear growth phase of carrot callus, the extent of genome methylation increases and after that again decreases during the stationary phase. These changes may be related to concurrent developmental events in the callus. Changes in DNA methylation patterns have also been reported by other authors (e.g., LoSchiavo et al. 1989, Vergara et al. 1990). Auxin increases DNA methylation reversibly (LoSchiavo et al. 1989) whereas kinetin leads to block changes in DNA methylation (Arnholdt-Schmitt et al. 1995). An increase in genome methylation was suggested to be necessary to disorganize a foregoing cell program (LoSchiavo et al. 1989). Fractionation of cell types of an embryogenic carrot cell line indicated a characteristic low genome methylation level.
of a fraction enriched in precursor cells of somatic embryos (Palmgren et al. 1991). Although the causal relationships concerning DNA methylation are still obscure, the effect of methylated cytosine residues in the DNA are probably mainly due to interferences with DNA-protein binding by that influencing the regulation of genome activities (transcription, replication, rearrangements). DNA methylation is therefore supposed to be a candidate for a genome marker system for (de-)differentiation.

RNA and protein level: Typical changes of gene expression during the early stages of differentiation have been reported (for review see Sterck and de Vries 1993). Using mRNAs isolated from polysomal fractions, Zimmermann et al. (1993) isolated 30 carrot cDNA clones by subtractive hybridization that are enhanced in globular embryos in comparison to seedlings including 24 new clones until then undetected by other methods.

In cultured carrot peintoles, newly synthesized and Coomassie-blue stained proteins have been analyzed with two dimensional electrophoresis (Grieb et al., unpublished); ca. 280 stained and/or 14C-leucine-labeled proteins are present. According to their presence during the different periods of culture, various groups have been distinguished. Some proteins are synthesized during the whole experimental period and therefore probably represent household proteins. The synthesis of other proteins, though, is specific for a particular stage. Thus, a hierarchically and sequentially organized program of initiation and termination of the synthesis of proteins goes along with the initiation of organogenesis and somatic embryogenesis in cultured peintoles. The occurrence of one group corresponds with excessive increase of cytoplasm and the first cell divisions in those subepidermal cells from which the somatic embryos regenerate. It should be noted that (as shown by histoautoradiograms of the 14C-leucine labeled peintoles) abundant protein synthesis is restricted to those areas in the peintole in which regeneration occurs (Mashayekhi and Neumann, unpublished). In carrot peintoles, proteins excreted into the medium have not yet been analyzed but there is extensive evidence for the correlation of excreted proteins, in particular glycoproteins, with differentiation of somatic embryos (Gavish et al. 1991, Kreuger and van Holst 1992, Hendriks and De Vries 1995, Mordhorst et al. 1995, Von Arnold et al. 1995, Donmon et al. 1995).

Contents and activities of enzymes: Differences in (iso)enzyme activities in explants of recalcitrant and easy-to-regenerate explants at the start of culture have only infrequently been reported. In apple microshoots, De Klerk et al. (1994) found a correlation between the activity of a specific basic peroxidase component of a microshoot at the time of transfer to rooting medium and the number of roots that are formed.

Various enzymes have been reported as suitable markers for the phases in regeneration, in particular peroxidases for rooting (Gaspar et al. 1992, Calderon-Balliccia 1994) and somatic embryogenesis (Wochok and Devoress 1994), and esterases for embryogenic and non-embryogenic callus (Coppens and Dewitte 1990). Recently, the activity and activation pattern of polyphenoloxidases in Euphorbia pulcherrima were used for the characterization of the embryogenic status of cell
suspension cultures (Grotkass et al. 1995). A significant increase in polyphenoloxidase activity of carrot callus cultured in the phase of root formation, combined with the appearance of new multiple forms indicates that these enzymes may be used as markers for differentiation processes (Habaguchi 1977).

With respect to markers, two aspects should be discussed: 1) a marker should have a one-to-one correspondence with a developmental process. Thus, for critical evaluation of the usefulness of a marker, situations should be created in which the developmental process does not occur, or occurs at a different rate or to a different extent. In all these situations the marker should show the appropriate changes. De Klerk et al. (1990) found a similar change of total peroxidase activity in shoots treated with auxin (and developed roots) or both auxin and cytokinin (and did not root, but only developed callus), indicating that total peroxidase activity is not a proper marker for the various phases in root regeneration. 2) As has been noted before, the cells that participate in the regeneration process are only a minor fraction of the total number of cells of the explants. The changes in peroxidase activity during rooting have been found in homogenates of whole (micro)cuttings. The specific peroxidase-course during rooting is thought to reflect the state of the whole cutting (Gaspar, personal communication). Often, though, the biochemical processes required as markers are likely to occur only in the cells participating in the regeneration process. Thus, it should be shown by histochemical techniques that a marker is characteristic for these cells. Recently, this was shown for a proline-rich protein that is thought to be a marker for the (absence of) rooting in Hedera (Sanchez et al. 1995).

**End products of the reactions:** Differences in enzyme activity may also be detected by the occurrence of the end products of the enzymatic reaction. Short-lived starch grains occur in the very early stages of regeneration of shoots (Mangat et al. 1990) and embryos (Williams and Maheshwarian 1986). In slices cut from apple microshoots and treated with auxin, short-lived starch grains appear during the dedifferentiation phase in a ring consisting of cells of the vascular bundles and primary rays. These cells enter division and from those in the primary rays, root primordia may develop (Jásik and De Klerk 1997). When auxin is not supplied, starch grains are formed at a much later time. Differential uptake of carbon sources from the medium of suspension cultures has been used as a biochemical marker for embryogenic cultures (Callehaut et al. 1987).

Various other biochemical and physiological features have been correlated with regeneration. Endogenous IAA levels show characteristic fluctuations during the rooting process (Gaspar et al. 1992). The concentrations of polyamines, especially of putrescine and spermidine are higher in embryogenic than in non-embryogenic cells and media of suspension cultures (Altman et al. 1992). Inhibition of polyamine synthesis reduces the number of embryos, whereas addition of polyamines to inhibition-supplemented cultures restores embryo formation at the original level. Accumulation of ethylene is less in embryogenic suspensions than in non-embryogenic cultures (Wann et al. 1989, Hatanaka et al. 1995), as well as the amount of glutathione. The redox status of cells, characterized by the ability to reduce
Fe$^{3+}$ is far higher in non-embryogenic cells. Phenolics are a very heterogeneous group of substances, interacting with intra- and intercellular processes, e.g., with auxin metabolism. The phenolic content is used in woody plants to differentiate between juvenile and adult phases and thus serves as a marker for the ability to root formation (Jay-Allemand et al. 1988).

Conclusion

A major drawback for the study of regeneration is the absence of very sensitive analytical techniques. At present, such techniques are being developed. The differential display technique may help to isolate genes that are involved in the early phases of regeneration. In advanced hormone-detection techniques, the tissue sample may be as small as 1 mg (Ribiwicky et al. 1995). Another technique that is frequently used to study the mechanisms underlying plant development, is the isolation of mutants. Because of its short generation cycle, this research focuses on *Arabidopsis thaliana*. Screening of an *Arabidopsis* mutant population for mutants in adventitious shoot or embryo formation, however, is laborious requiring various steps for each mutant plant. Moreover, *Arabidopsis* is rather recalcitrant in shoot and embryo regeneration. For adventitious root formation, such mutant isolation is now being undertaken. In the coming years, examinations using these new methods will result in many new insights in the processes of adventitious shoot, root and embryo formation.

References


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