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SPECIAL SYMPOSIUM: *IN VITRO* PLANT RECALCITRANCE

LOSS OF PLANT ORGANOGENIC TOTIPOTENCY IN THE COURSE OF *IN VITRO* NEOPLASTIC PROGRESSION

THOMAS GASPAR[1,2*], CLAIRE KEVERS[1], BADIA BISBIS[1], THIERRY FRANCK[1], MICHÈLE CREVECOEUR[2], HUBERT GREPPIN[2] and JACQUES DOMMES[1]

[1]*Biologie moléculaire et Hormonologie végétales, Institut de Botanique B 22, Univ. de Liège, Sart Tilman, B-4000 Liège, Belgium*
[2]*Physiologie végétale, Univ. de Genève, 3, pl. de l'Université, CH-1211 Genève, 4, Switzerland*

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Summary

The aptitude for organogenesis from normal hormone-dependent cultures very commonly decreases as the tissues are serially subcultured. The reasons for the loss of regenerative ability may vary under different circumstances: genetic variation in the cell population, epigenetic changes, disappearance of an organogenesis-promoting substance, etc. The same reasons may be evoked for the progressive and eventually irreversible loss of organogenic totipotency in the course of neoplastic progressions from hormone-independent tumors and hyperhydric teratomas to cancers. As in animal cells, plant cells at the end of a neoplastic progression have probably undergone several independent genetic accidents with cumulative effects. They indeed are characterized by atypical biochemical cycles from which they are apparently unable to escape. The metabolic changes are probably not the primary defects that cause cancer, rather they may allow the cells to survive. How these changes, namely an oxidative stress, affect organogenesis is not known. The literature focuses on somatic mutations and epigenetic changes that cause aberrant regulation of cell cycle genes and their machinery.

Key words: plant cancer; organogenic totipotency; neoplastic progression; habituation; hyperhydricity; oxidative stress.

Decline in Totipotency in Normal Tissue Cultures

Some plant cells, tissues and organs can be subcultured for many years without visible change, retaining a capacity to regenerate shoots or produce embryos in response to specific stimuli. However, in conventional media, the aptitude for organogenesis from such cultures very commonly decreases as the tissues are serially subcultured (George, 1993; Collin and Edwards, 1998). The reasons for the loss of regenerative ability are still uncertain and may vary in different circumstances. George (1993) advanced three theories to explain the loss of regenerative ability, as detailed in the following sections.

Genetic variation in the cell population. The oldest, and most widely-held, theory is that the culture conditions are mostly chosen to enhance cell proliferation and that cells are preferentially selected for this attribute, which is incompatible with the ability to undergo organogenesis. This also probably means that there is genetic variation in the original cell population and that the selection pressure results in the loss of cells retaining the genetic information for totipotency.

There is also a good indication that deleterious changes are occurring in the cells: there is a progressive increase of cells showing aneuploidy and polyploidy. Although the natural or artificially induced occurrence of cells having aneuploid chromosome numbers does not necessarily prevent morphogenesis in callus, it seems clear that gross variations in the genetic make-up of callus cells will prevent plant regeneration (see reference in George, 1993). The fact that buds or embryos regenerated from aneuploid callus cells show much less obvious chromosomal variation suggests that the process of regeneration occurs from the more normal diploid cells. Moreover, shoots or plantlets regenerated with aneuploid chromosome numbers showed low vigor and died in the course of development. Thus, the proportion of potentially meristematic cells must decline in a subcultured callus, and this is reflected in a reduced totipotency.

The idea is that the introduction of abnormalities into cultured cells is accelerated by encouraging cell division. The various strategies for retaining totipotency all place a limit on the rate of cell division (low auxin levels, reduced temperatures, etc.).

Aneuploidy is just one of the genetic changes that can be induced by cell culture. Other karyotypic alterations may have even more impact on the decline in totipotency. Loss of regenerative ability is not always dependent on the occurrence of karyological diversity; it has been observed in cells remaining strictly diploid.

The existence of a substance promoting organogenesis. Organogenic promoting compounds present in freshly isolated explants

*Author to whom correspondence is to be addressed: Email Th.Gaspar@ulg.ac.be

may slowly diminish during growth *in vitro*. The converse of this hypothesis would be that an inhibitor of regeneration accumulates with time.

An epigenetic change in the cultured cells. Rather than being due to the disappearance by selection of cells possessing the necessary genetic information for organogenesis, loss of regenerative ability may be caused by a changed pattern of gene expression brought about by the cultural environment. This may be an epigenetic phenomenon. One possibility is that genes responsible for organogenesis become hypo- or hypermethylated *in vitro* and their expression is modified. Alternatively, failure to regenerate organs might be induced by a changed pattern of endogenous hormone metabolism, receptivity or transduction pathways.

Plant cells may not only show a gradual loss of regenerative capacity the longer they are cultured, but also an altered organogenic potential. There are many examples in the literature of altered sensitivity to applied growth substances for different organogenic types. There are also examples where regeneration from 'old' cultures yielded abnormal structures. These events could also be explained in terms of a new epigenetic gene expression and/or altered pattern of endogenous hormone metabolism and/or action, although an explanation based on genuine genetic change is also plausible.

Plant Tumors, Cancers and Inbetween Neoplastic Progressions With Progressive Loss of Organogenic Totipotency

Tumors or neoplasia. In neoformed tissues showing abnormal, unlimited growth, these result from increased cell production. The second main characteristic of a tumor is its tendency to 'autonomic' proliferation, which also means the absence of regression with the disappearance or removal of the apparent causes (this makes the distinction with an inflammatory process in medicine). Medicine distinguishes benign or mild tumors from malignant ones: the former are localized and are thus noninvading and limited by a capsule, being formed by a tissue not much different from the normal one; the malignant tumors are not clearly limited, which means that they invade the neighboring tissues. Their cells generally (but not obligatorily) present abnormal cytological and nuclear features. The circulating cancerous metastases are derived from the malignant tumors. There are no known circulating metastases in plants and thus the concept of plant malignant tumors does not exist. The most common plant primary tumors are those appearing locally on infected surfaces. However, there are also so-called Kostoff genetic tumors arising spontaneously in interspecific crosses with apparent incompatibility of the two inherited parental genomes and thus in the absence of external pathogenic agents such as viruses, bacteria or fungi. Such tumorigenesis in interspecific crosses also exists among animals but is rather rare. So-called fully habituated (hormone-independent) tissues in *in vitro* cultures are also generated without introduction of a pathogen.

Although the migratory (invasive) cell is not part of the plant's developmental repertoire, secondary tumors arising at some distance from the primary ones, without intervention, have been observed in plants (Braun and Stonier, 1958). Their occurrence may be attributed to the systematic spread of an oncogenic pathogen such as a virus or bacterium, or to the transfer of an oncogenic potential from cell to cell in the absence of the pathogen (Pengelly, 1989). Secondary tumors, therefore, result from separate inductive events and not from the spread of tumor cells with new and more virulent properties.

Another common feature of all plant tumors is the retention of a capacity for regeneration, i.e. the formation of organized structures such as roots or shoots, either spontaneously or after treatment with plant growth regulators. The regeneration of complete plants from a great number of habituated clones, as well as from fully transformed single-cell clones isolated from a young crown gall strain, has been presented as proof that both processes, habituation and *Agrobacterium*-transformation, are reversible (Sacristan and Melchers, 1969, 1977; Hervagault et al., 1991; Syono and Fujita, 1994). The chimeric nature of a tumor, i.e. the presence of normal cells among the hormone-independent cell population, still remains a debatable issue.

The four best-known plant neoplasia include the crown gall disease, Black's wound tumor diseases, the Kostoff genetic tumors and the habituated tissues (for a review, see Gaspar, 1999).

Plant tissue habituation. This phenomenon consists of the acquisition of hereditary capacity for autonomous growth, in the absence of exogenously supplied auxins and/or cytokinins in the tissue culture (Meins, 1982, 1989; Jackson and Lyndon, 1990; Syono and Fujita, 1994). Habituation has been observed, in some cases, as a gradual process while in other cases it occurs abruptly. The phenomenon of habituation bears a striking similarity to tumor transformation in crown gall disease where tumor tissue grows independently of exogenous hormones. This common loss in the requirement for exogenous growth factors has led workers to consider habituated tissues as hormone-autotrophic, i.e. capable of autonomous production of auxins and cytokinins (Meins, 1989). Major production sources of auxins and cytokinins in habituated cells have not been confirmed, however (see reference in Gaspar et al., 1999; Kevers et al., 1999b; Gaspar, 1999). Alternatively, an altered sensitivity to endogenous hormones has been suggested (Mousdale et al., 1985; Szabo et al., 1994), as well as the accumulation of metabolites (dehydrodiconiferyl alcohol glucosides) that could replace cytokinins in the control of cell divisions (Teutonico et al., 1991). Another explanation may come from altered metabolisms of ethylene (Campell and Town, 1991; Köves and Szabo, 1987; Bisbis et al., 1998) and polyamines (Le Dily et al., 1993a,b; Kevers et al., 1997). Alterations in gene expression might result in cell division being independent of hormonal regulation (Campell and Town, 1991). An increased content of diacylglycerol as well as increased levels and turnover of inositol phosphates in habituated tissues, as in animal cancerous cells, might account for the latter hypothesis (Feutry et al., 1995). In most cases, the process of habituation appears to be reversible; habituated cells keep their totipotency, as do genetic tumors (Bayer, 1982). Habituated cells can regenerate roots, buds or somatic embryos. Habituation is thus generally regarded to be an epigenetic phenomenon (Meins, 1982, 1989). It may also be considered as a neoplastic step towards cancer (see below). Habituation mainly concerns the transition of cells into calluses but habituation may also overtake shoots in culture (see below).

Plant cancers. An abundance of literature has presented details of plant tumorous cells with many morphological and biochemical features similar to those of animal cancerous cells, but the concept of plant cancers and of plant cancerous cells remains rather vague.

TABLE 1

CHARACTERISTICS THAT CONSTITUTE A FULLY HABITUATED NONORGANOGENIC SUGARBEET CALLUS CONSISTING OF TRULY CANCEROUS CELLS, IN THE ABSENCE OF INTRODUCED PATHOGENS (ACCORDING TO GASPAR, 1998)

Biological characteristics:
Monoclonal origin
Full hormonal independence *in vitro*
High rate of cell division
Polyploidy and aneuploidy
Reduced cell-to-cell adhesion (friability)
Susceptibility to necrosis
Morphological characteristics:
Deficient cell wall differentiation
Deficient chloroplast and mitochondria differentiation
Big nuclei with irregular shape, with many nucleoli plus micronucleoli
Apoptotic bodies
Biochemical characteristics:
A programmed cell death?
Hyperhydricity
Deficiency of tetrapyrrole-containing compounds
Permanent oxidative stress
Low ethylene production
Accumulation of polyamines
Pentose phosphate pathway and alternative respiration favored
Typical plant cancer trait:
Irreversible loss of organogenic totipotency, i.e. the capacity for such cells to reorganize primary organogenic meristems, at the end of a neoplastic progression

It was even claimed recently (Doonan and Hunt, 1996) that plants cannot get cancer, the main reason being the absence of circulating metastases. We are convinced that the opposite is true (Gaspar, 1995, 1998); the problem is a simple question of concept and of adapted definition. The process of habituation (loss of requirement of exogenous growth regulators for sustained growth *in vitro*; see above) and hyperhydric malformations of *in vitro* cultured cells and shoots, respectively, were compared (Gaspar et al., 1995). The conclusion was that both phenomena were taking part in neoplastic progressions (without introduction of pathogens; see below), leading to true cancer cells (in calluses) or to true generalized cancers at the organismal (shoot) level, with (programmed?) death as an ultimate issue. The arguments developed for such plant cancer concepts have been reviewed (Gaspar et al., 1991, 1995) and commented on (Anonymous, 1995). The definition at the cellular level implies an array of characteristics that were found in a unique sugarbeet cell line examined at biological, morphological and biochemical levels (Table 1). All these characteristics are those of animal metastases, including: close to full hormone independence; polyploidy and aneuploidy; complete loss of cell-to-cell adhesion (here essentially due to an over-esterification of pectin; Liners et al., 1994); permanent oxidative stress and accumulation of polyamines. Plant cancer cells evidently cannot become circulating metastases or 'extra cells' (Doonan and Hunt, 1996). However, because normal plant cells have the unique capacity (which animal cells do not) to organize themselves into organogenic or regenerating meristems, the typical plant cancer trait has been precisely defined as the irreversible loss of organogenic totipotency, i.e. the inability of such cells to reorganize primary organogenic meristems at the end of a neoplastic progression. This definition makes a clear distinction with tumors (such as those mediated by pathogens or resulting from genetic transformation), which are chimeric and still organogenic (see above).

Similarly, bud- and meristem-bearing shoots in culture may lose their totipotency by way of a neoplastic progression (involving hyperhydricity and habituation successively), where all the characteristics reported in Table 1 will appear progressively. The rooting capacity will first be lost with the acquisition of hormonal independence, and therefore the lack of sensitivity to the exogenous root-inducing auxin; appearance of stem fasciation will be the first sign of stem meristem dysfunction; increasing brittleness (resulting from loss of cell-to-cell adhesion and communication) will necessarily be followed by necroses of apices (caulogenic meristems) and meristematic leaf borders. When synchronized, these necroses have been considered (Gaspar et al., 1991; Gaspar, 1995) to be a sign of generalized cancer leading to death of the whole organism. Note that the nonmeristematic cells still surviving in contact with the culture medium may continue to form extra cancerous cells and grow into a fully habituated nonorganogenic callus. *In vitro* culture stress conditions may lead to such cancer cases. This means that plants are probably no more resistant to neoplastic transformation than animals, the features simply being different.

Neoplastic progressions. Neoplastic transformation to the fully habituated state is not accomplished in a single step, but occurs gradually. Organogenic tumors are considered to be less advanced than tumors which grow in a chaotic and unorganized fashion. There are several intermediate situations between 'normal' cells of a higher plant and true 'cancerous' cells (see below). These phenomena are known as neoplastic progressions. A neoplastic progression in callus cell cultures is described hereafter and the arguments for habituation as a step are discussed. The same is then done for the process of hyperhydricity.

A so-called normal callus is defined by its apparent 'anarchic proliferation of undifferentiated cells'. This classical definition should be revised taking into account that its growth proceeds through meristematic centers (Floh and Handro, 1985) and thus is not as anarchic as it appears at first sight, and also considering that cells differentiate, into xylem cells for instance (Crèvecoeur et al., 1987). Nevertheless, absence of visible tissue and organ organization has some resemblance to a tumor and, therefore, a normal callus should be considered as a primitive neoplastic growth phase. Hormonal manipulation of this normal callus allows the expression of organogenic potential, notably through bud and root formation. A green fully habituated organogenic callus as described by De Greef and Jacobs (1979) and Kevers et al. (1981b) represents additional neoplastic steps with the acquired independence to auxins and cytokinins and the loss of rooting capacities. Such a callus is only capable of abnormal shoot formation and is very often unable to form roots (Fig. 1). A white, fully habituated callus was isolated from white cell clumps appearing at the surface of the preceding callus (Kevers et al., 1981a). The white habituated callus lacks cell differentiation and is incapable of organizing primary meristems to grow and to form adventitious organs, whatever the treatments.

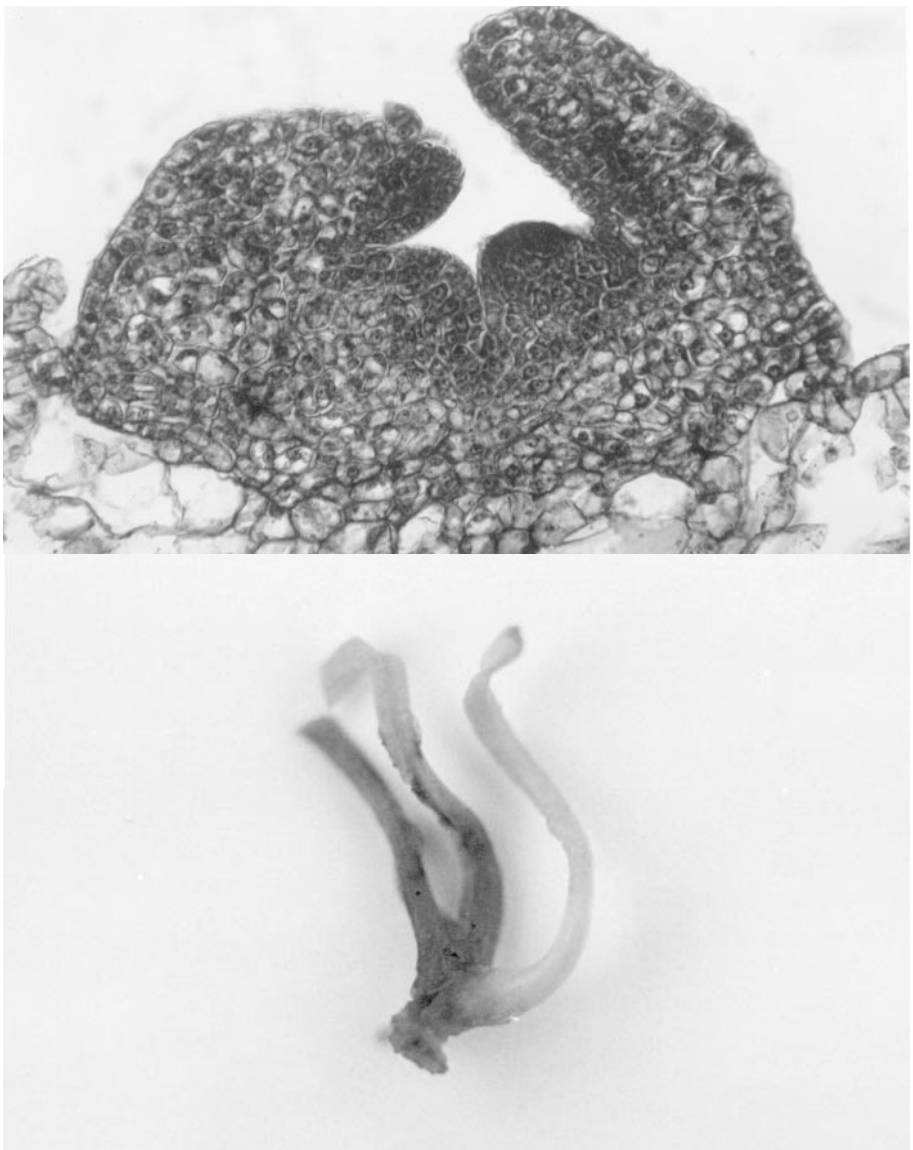

Fig. 1. Dichotomic meristem and derived abnormal shoot (short fasciated stem with abnormal leaves, without roots) from a fully habituated organogenic callus.

Some habituated tissues have already been observed to show a reduced, or lost, organogenic potential (Lutz, 1971; Syono and Fujita, 1994) but the white, nonorganogenic callus with apparent irreversible character described above probably constitutes a rare clone with some more neoplastic progression. It can even be considered as the terminal phase of the neoplastic progression, as confirmed by the series of morphological and biochemical traits similar to those of animal cancerous cells (Table 1). The concept of plant cancer cells thus involves the complete loss of organized and organogenic meristematic structures, i.e. an irreversible loss of totipotency, which makes cancer cells different from those of tumors, where cell differentiation still takes place and where organogenesis is still possible (Braun, 1978).

Vitrification was the term formerly used to characterize the hyperhydric malformations frequently affecting herbaceous and woody plants during their *in vitro* vegetative propagation (under the effect of cytokinins at high concentrations, on soft culture media and in a confined atmosphere with high relative humidity). The hyperhydric shoots appear turgid, watery at their surface and hypolignified. They indeed contain much water in their intercellular spaces and less lignin than normal shoots. The hyperhydric organs are somewhat translucent, in some cases less green than normal, and brittle (Gaspar, 1991; Ziv, 1991). In the most common hyperhydricity cases, organs are not really malformed but cell differentiation is limited which, for woody plants, has led to the consideration of hyperhydricity as a means of rejuvenation (John, 1986; Coumans and Ribet, 1993). In other cases, leaves are malformed, frequently very elongated, wrinkled and/or curled, and brittle. In general, stems of hyperhydric shoots are broad, thick in diameter and with shorter internodes. Necrosis of leaf margins and

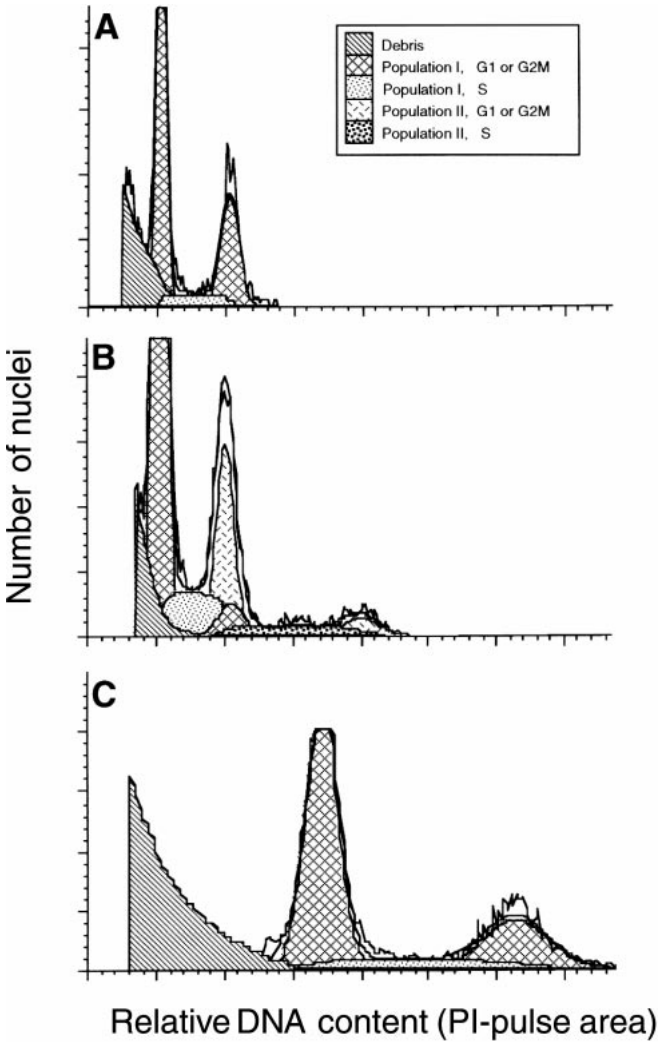

Fig. 2. Representative flow cytometric distributions of propidium iodide-stained nuclei isolated from diploid leaves (A) of a seed-mother sugarbeet plant, habituated organogenic (B) and habituated nonorganogenic (C)-derived cell lines within one or two populations and in each population within G_0G_1, G_2M and S phases (according to Kevers et al., 1999c). The histogram of the normal callus (not shown) is similar to that of the leaves.

of some stem apices may occur (Kataeva et al., 1991). In most cases, hyperhydricity is still reversible at that stage, which means that some apices of hyperhydric shoots placed in nonhyperhydric conditions can function normally and give rise to normal plants.

In some cases, hyperhydric shoot clusters have been described as teratomas with morphological deviations similar to those induced by bacteria (Leshem and Sachs, 1985). Abnormal leaves with deviating phyllotaxis and occasional fasciated stems are produced. These symptoms denote malfunctioning of primary meristems. Different situations may result from the continued subculture of such hyperhydric clusters. It may be the case that the clusters suddenly degenerate through simultaneous necrosis of all meristematic apices. Individual shoots of such clusters do not survive at subculture; they become brown and die. These (humid) apex necroses of hyperhydric shoots are distinct from the other well-known (dry) apex necroses which result from hormone deficiency (Kataeva et al., 1991). In some other cases, the subculture of hyperhydric clusters is still possible but via use of clusters and not by individual shoots. Such explants provide other teratoma-like clusters with more or less distinguishable shoots. They can generally be subcultured and continue to proliferate in the absence of plant growth regulators which means that they became habituated (personal observations on long-term cultures of *Prunus* and *Rhododendron*). Zoglauer et al. (1981) and Pierik (1989) mention other examples of habituated shoot clusters. Shoots from these clusters, insensitive to auxin application, do not root. This may mean that such habituated shoots have lost part of their totipotency, the rooting capacity. In a few cases, habituated shoots form at their bases cauliflower-like or broccoli-like structures, in which true stems and leaves are no longer recognizable. Meristems at that stage have lost their normal way of forming structured stems and leaves, and hyperhydricity is irreversible. Whole-plant structures have disappeared but the habituated 'broccoli-like' mass still increases greatly in volume because of extreme rejuvenation of the cells. Such teratomas, with a progressive loss of organogenic capacity to form stem apices, are thus also the result of a sort of neoplastic progression (Gaspar et al., 1991, 1995). This illustrates plant cancer at the organismal level, where progressive rejuvenation of some cell populations causes the death of the whole organism by rendering meristems incapable of continuing to maintain the normal structures and to play their organogenic and physiological roles. This situation apparently results from a progressive loss of cell-to-cell adhesion which makes the tissues breakable, as in the above case of cancerous friable calli.

On the causes of the loss of organogenic totipotency in cancerous cells

Progressiveness of the phenomenon and difficulty of approach. In cancerous calluses, the inability to organize organogenic, primary meristems was preceded by deficient cell differentiation, dichotomic meristems and leaves or fasciated stems (Fig. 1) in the previous neoplastic steps. Progressive loss of organogenic totipotency in hyperhydric shoots becoming habituated similarly involves deficient cell differentiation (particularly visible at the cell wall level) and malfunctioning of apical and axillary meristems (Leshem, 1983; Bornman and Vogelman, 1984; Leshem and Sachs, 1985) leading to abnormal phyllotaxy and/or fasciated stems. This is followed by decreased axillary proliferation, frequent leaf and apex necroses, reduced rooting ability and, finally, general apex necrosis, resulting in completely translucent and brittle shoots.

The causes of the loss of regeneration ability are difficult to establish precisely because the process is progressive. The causes are apparently different and separate from those at the origin of habituation and during its maintenance. Freshly established habituated cultures are still organogenic, but it might be that organogenesis proceeds from normal cells living in the neighborhood of the habituated ones.

Ploidy level and abnormal mitotic phenomena. Abnormal chromosome numbers may not be the cause of tumor growth (Sacristan and Melchers, 1969; Binns and Meins, 1980), although Kostoff genetic tumors apparently result from a chromosomal imbalance (Braun, 1978) and genomic instability precedes tumorigenesis in animals (Hartwell, 1992). Aneuploidy, polyploidy (illustrated in Fig. 2 for the habituated organogenic and

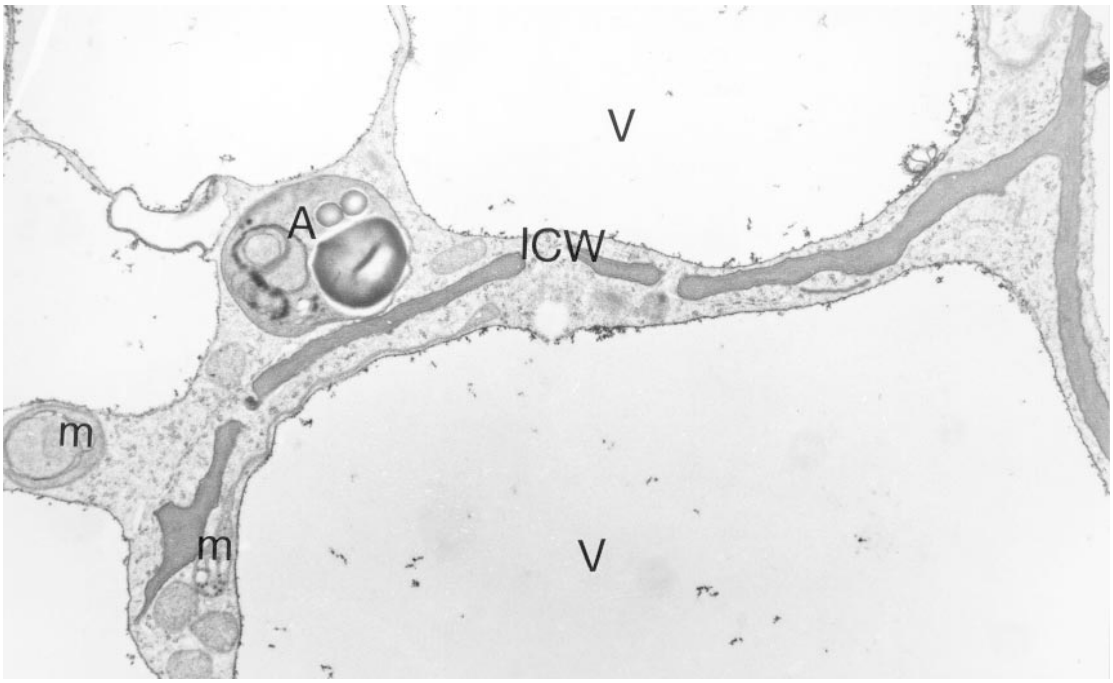

Fig. 3. Electron micrograph (×10 400) showing an incomplete cell wall (ICW) between two cells of the fully habituated nonorganogenic sugarbeet callus (V = vacuole; A = amyloplast; m = mitochondria).

nonorganogenic sugarbeet calli) and chromosome breakage may also not be directly responsible for the diminished organogenic capacities. These events are also found in long-term normal cultures. Somaclonal variants are frequently found among the regenerants. Regenerated plants with aneuploid chromosome numbers frequently show abnormal morphology and sterility (Sacristan and Melchers, 1969). There are, however, indications that regeneration from such cultures preferentially proceeds from the less abnormal cells (George, 1993).

Another difficulty in this study is the coexistence of different cell populations, one deriving from mitotic cells, another from nuclear fragmentations followed by a normal mitotic cycle, etc. Amitotic phenomena have frequently been observed during the culture period (Bennici and Caffaro, 1985). Crèvecoeur et al. (1992) demonstrated incomplete cell partitioning and incomplete cell walls (Fig. 3) in the fully habituated sugarbeet callus (described in Table 1) that irreversibly lost its organogenic potential. *Avena* mesophyll calluses with malformed 'cell-plate-like' structures were described by Hahne et al. (1992). These calluses were not able to regenerate further than an eight-cell stage. In microcalluses derived from protoplasts of sunflower, the loss of a regenerative potential was correlated with incomplete cellular divisions (Keller et al., 1994). Most probably incomplete cell divisions led to an unregulated flux of information between cells that could prevent differentiation and correct cell signaling. In differentiated tissue, completely different adjacent cell types are connected by plasmodesmata, small molecules including hormones and second messengers which guarantee controlled exchange of ions (Robards and Lucas, 1990). It is widely believed that proper cell-to-cell communication is required for growth and developments of plants (Tucker, 1990). Pectins of the friable cancerous sugarbeet callus, characterized in Table 1, are highly methyl- and acetyl-esterified; this probably leads to inability of the cells to associate cooperatively through calcium ions and thus to the loss of cell-to-cell adhesion (Liners et al., 1994).

Phytohormones, tumorigenesis and organogenesis. The comparison of the habituation process with crown gall disease led to the concept that tumorigenesis resulted from the persistent activation of normally repressed biosynthetic systems, namely an increased biosynthesis of auxins and cytokinins (Bayer, 1982). Both hormones, indeed, had been measured at higher levels in some tumors but an examination of the literature does not allow general confirmation of this assumption. The situation is much more complex in the sense that not only quantitative but also qualitative differences have been found between the hormones of normal and tumor tissues. Furthermore, the biosynthetic and catabolic pathways and other associated metabolisms (for instance conjugation) were modified, and alternative molecules playing the same roles (for instance dehydrodiconiferyl alcohol glucosides replacing the cytokinin requirement) might be synthesized (Gaspar et al., 1999; Kevers et al., 1999b). If we accept that organogenesis (at least caulogenesis and rhizogenesis) necessarily depends on an adequate auxin to cytokinin ratio, a strong homeostasis hindering its variation should exist in nonorganogenic cancer cells (Nandi et al., 1990). Curiously this has not been examined. More recently, it has been shown that habituated cells accumulate polyamines as do animal cancer cells (Gaspar et al., 1991) and that this accumulation probably results from altered sugar and nitrogen metabolisms (Le Dily et al., 1993b; Bisbis et al., 1997; Kevers et al., 1997). Polyamines, and more particularly their metabolic pathways, have been found to be closely associated with auxins (and cytokinins) in the induction of organogenic processes (Hausman et al., 1997;

Gaspar et al., 1997; Aribaud et al., 1999). More precisely, the polyamine biosynthetic and catabolic pathways are modified in the habituated, nonorganogenic sugarbeet callus characterized (Table 1) as cancerous (Kevers et al., 1999a). In the same material, ethylene production is very limited, but the sensitivity towards this hormone is increased (Bisbis et al., 1998). The novelty is that the hormonal metabolisms have been found to be interrelated (Gaspar et al., 1999; Kevers et al., 1999b), operating in an integrated manner with other general metabolic pathways as in animal cells (Siedow and Stitt, 1998). Anoxia due to a water layer surrounding the cells in both cancerous callus and hyperhydric shoots might be partly responsible for the metabolic adaptations. How these changes affect organogenesis is not known. The metabolic changes are probably not the fundamental defects that cause cancer; they may confer a common advantage on many different types of cancers that allows the cells to survive (Dang and Semenza, 1999).

Oxidative stress and atypical biochemical cycles. Oxidative stress is one of the causes of cancer initiation in animals (Alberts et al., 1989; Winston, 1990; Halliwell, 1996). Plant tissues in *in vitro* culture are submitted to a series of stresses:

1. wounding due to tissue or organ excision;
2. application of unusual growth regulator concentrations and combinations;
3. excess nitrogen supply;
4. high atmospheric humidity;
5. accumulation of gases in the confined atmosphere of the vessel;
6. osmotic shock due to infiltration of the culture medium into the intercellular spaces.

It has been clearly shown that tissues facing such stresses react by adapting their defense enzymes and soluble reductants to counteract the activated forms of oxygen generated in response to the external stresses (Hagège et al., 1992; Franck et al., 1995, 1998). Time-course changes in these defense systems show that tissues or organs that will grow normally recover a normal metabolism after the stress reactions; those that do not recover completely will become abnormal. This appears to be the case in hyperhydric shoots that adapt their superoxide dismutase activity but are unable to adapt the H_2O_2 detoxifying ascorbate peroxidase system (Franck et al., 1995). There are indications of cell 'destabilization' through such stresses, for instance through the abundance of free fatty acids and of thiobarbituric acid-reactive substances (malondialdelyde) in the cancerous callus, where a decrease of the unsaturated level of fatty acids might be indicative of high lipid peroxidation (Arbillot et al., 1991). Thiobarbituric acid-reactive substances are considered to be mutagenic agents in animal cells (Vaca et al., 1988; Dianzani, 1989). A major question is whether such compounds contribute to the transformation of some of the hormone-dependent normal cells into habituated cells and/or some of the habituated organogenic cells into nonorganogenic ones, or whether they simply contribute to maintain the habituated and/or nonorganogenic state.

There are indications that the plant cancerous cells (as characterized in Table 1) are under permanent stress (Le Dily et al., 1993a) and that light might further stress these achlorophyllous cells (Kevers et al., 1995). The accumulation of polyamines in cancerous callus cells as well as in hyperhydric shoots (Kevers et al., 1997) might also be indicative of stresses (Smith, 1985). Do polyamines control the level of peroxides through their membrane-stabilizing properties (Altman et al., 1977) or through their free radical scavenging effect (Drolet et al., 1986) or, on the contrary, do they generate hydrogen peroxide through oxidation (Angelini and Federico, 1989)? Do polyamines maintain the cancerous state, or do they combine with nitrites to form carcinogenic nitrosamines (Hildrum et al., 1976)? Apparently, the same questions have been posed for animal cancerous cells (Porciani et al., 1993). How polyamines, by themselves or through their metabolism, affect organogenesis is another open question (Aribaud et al., 1999). Our view (Gaspar et al., 1998, 1999; Kevers et al., 1999b) is that the cancerous cells, during their neoplastic progression, have been constrained to adopt atypical metabolisms and that they are unable to escape from these interrelated cycles. This would explain the irreversibility of their state, and possibly their programmed cell death by apoptosis. A constitutive release of H_2O_2 has been recently detected in a wide range of human tumor cells. The same apparently holds true in plant cancerous cells (Fig. 4). This H_2O_2, depending upon its concentration, either stimulates DNA replication and cell proliferation or increases the occurrence of apoptosis (Burdon, 1996).

Epigenetic changes followed by genetic alterations. Cell cycle genes and machinery. Spontaneous tumor formation in certain *Nicotiana* hybrids should be caused by aberrant regulation of specific genes rather than by gene mutation (Bayer, 1982) and, similarly, habituation (at least while maintaining its reversibility) has been claimed to be an epigenetic phenomenon (Meins 1982, 1989). In plants as in animals, cancer, at the end of a neoplastic progression, appears to involve several independent accidents with cumulative effects (Alberts et al., 1989). The idea that hormonal control of DNA methylation participates in the regulation of transcription and cell differentiation has proved to be true and very stimulating (see references in Lambé et al., 1997). Alteration in DNA methylation patterns and gene expression has been related to abnormal phenotypes (Finnegan et al., 1998). Also, the concept has been raised that alterations in proto-oncogenes might be the transforming principles during the neoplastic progressions (Persinger and Town, 1991; Hagège, 1993). The finding of specific proteins in habituated and plant cancerous tissues might be an indirect confirmation (Tacchini et al., 1995; Mérillon et al., 1995; Droual et al., 1998). How these alterations affect organogenesis is still unclear, but the past few years have produced experimental evidence supporting the notion that the cell cycle machinery is commonly targeted in oncogenesis. In the absence of migration of cells, only cell division and expansion determine plant morphogenesis. Because cell division mainly occurs in meristematic regions, the identity of a cell that leaves the meristematic region is determined mostly by the position of the cell relative to the position of its neighboring cells. In animals, cell identity is mostly determined by cell lineage. These basic differences indicate that the plant cells have developed mechanisms of growth regulation and developmental switches clearly distinct from those of yeast and animals. Therefore, one can expect that growth-promoting (cyclins, cyclin-dependent kinases or CDK, CDC25 phosphatases, etc.) and growth-inhibitory genes (tumor-suppressor genes) in plants do not exert their functions in the same way as in animals. On the other hand, the high degree of evolutionary conservation of at least parts of these cell cycle regulatory genes argues for basic, common functions that may be unravelled in plants. The recent identification of cyclin D and retinoblastoma (pRb) homologs from plants suggests

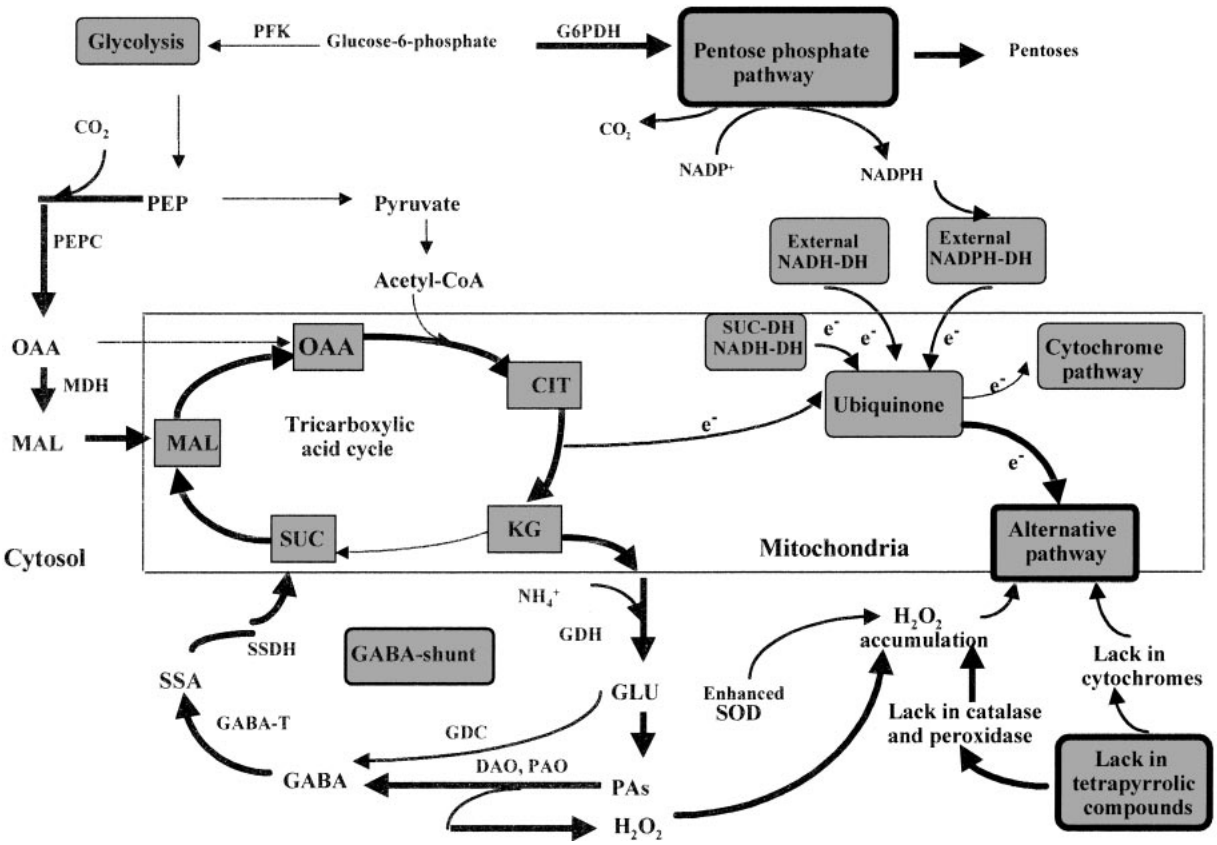

Fig. 4. Illustration of the atypical biochemical cycles (nonphotosynthetic CO_2 fixation, favored pentose-phosphate pathway, polyamine accumulation and degradation, GABA-shunt, alternative respiratory pathway, deficient synthesis of tetrapyrrole-containing compounds) of the fully habituated cancerous sugarbeet cells, showing the constitutive H_2O_2 accumulation (from polyamine degradation and enhanced superoxide dismutase activity together with reduced catalase and peroxidase activities). According to Gaspar et al. (1998) and Kevers et al. (1999b). PFK = phosphate fructokinase; G6PDH = glucose-6-phosphate dehydrogenase; PEPc = phosphoenolpyruvate carboxylase; OAA = oxaloacetate; MAL = malate; SUC = succinate; KG = α-ketoglutarate; CIT = citrate; GLU = glutamate; PAs = polyamines; GABA-T = γ-aminobutyrate transaminase; NADPH = nicotinamide adenine dinucleotide phosphate (reduced form); MDH = malate dehydrogenase.

some conservation with mammalian components; however, the level of sequence homology between the mammalian and plant proteins is relatively low and restricted to certain protein domains (see references in Strnad et al., 1999).

The net result of the cell cycle scenario may not only be growth and differentiation, but also tumor development and apoptosis. This view reflects the evidence accumulated over the last 5 yr in the mammalian field, namely that the vast majority of, and quite likely all, tumors have suffered one or more defects that derail the cell cycle machinery. Such defects can either target components of the cell cycle apparatus itself, including the checkpoint mechanisms that ensure fidelity and orderly progression through the cell cycle phases, thereby protecting genome integrity, or target elements of the upstream signaling cascades, whose effects eventually converge to trigger cell cycle events (see references in Strnad et al., 1999).

Although the concept of cancer as a disease of the cell cycle implies that every tumor is defective in one or more aspects of cell cycle control, it clearly does not mean that oncogenesis targets only the cell cycle clock. Development of a tumor also appears to require aberrations in the cell death machinery and cell–cell and/or cell–matrix interactions that cooperate with cell cycle defects. The above concept simply regards cell cycle deregulation as an essential step in the process of multistep tumorigenesis.

In terms of the molecular pathogenesis of cancer, cell cycle defects can either represent the initial, predisposing event, or contribute to tumor progression. Examples of cancer-predisposing alterations include germ-line mutations of CDK inhibitors, while many of the known cell cycle defects result from somatic mutations or even epigenetic changes that may occur during the early or later stages of tumorigenesis (Strnad et al., 1999). The same may be true for plants. However, in contrast to vertebrates, only limited data on cell cycle genes and their involvement in tumorigenesis have been obtained from plant research.

Conclusions

Plant tissues and organs in *in vitro* culture may thus undergo neoplastic progressions, leading to true cancer cells, with

habituation and hyperhydricity as intermediate steps. Along these steps, there is a progressive loss of organogenic totipotency, terminating with a complete inability of cancer cells to reorganize organogenic primary meristems. The most common reasons evoked for the loss of regenerative ability are the same as those given for the decreased organogenic potential in long-term normal cultures: genetic variation in the cell population, epigenetic changes, disappearance of organogenesis-promoting substances, etc. However plant cancer cells, as with animal cells at the end of a neoplastic progression, have probably undergone several genetic accidents with cumulative effects. They are indeed characterized by atypical biochemical cycles from which they are apparently unable to escape. The metabolic changes are probably not the primary defects that cause cancer, rather they may allow the cells to survive. How these changes, namely an oxidative stress, affect organogenesis is not known. Current investigations focus on somatic mutations and epigenetic changes that cause aberrant regulation of the cell cycle machinery.

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