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Competence versus Recalcitrance for *in Vitro* Regeneration

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Abstract

Plant regeneration from tissue cultures of many species has been reported so far, but various groups, families and genera are still regarded as recalcitrant, and tools susceptible to distinguish as early in culture as possible the regeneration-competent cells and tissues from those that will never regenerate would be of interest, whatever the regeneration pathway. We have examined various cytological parameters to identify those that might serve as early indicators of cell competence to undergo somatic embryogenesis and/or organogenesis. Water potential of the culture medium declined preceding the onset of embryogenesis while intracellular osmolarity and cell surface increased concomitantly in embryogenic cells. In addition, cellulose accumulated in the walls of non-embryogenic cells while cell walls became thinner with onset of embryogenesis, and diminished further as embryos matured. In parallel experiments, the cytogenetic, proteomic and molecular processes governing the competence for embryogenesis and/or organogenesis from plant cells were studied with *Arabidopsis thaliana*, and more recently also with *Medicago truncatula* and pea, both by flow cytometry, immuno-cytohistology, mono and bidimensional electrophoresis and comparing insertion mutants and RNAis with their respective wild types. The implications of these results when applying biotechnology approaches for grain legume breeding, and in terms of plant regeneration competence in general are discussed.

Keywords: organogenesis, somatic embryos, recalcitrant species, genetics, breeding, legumes

Introduction

Whatever the regeneration pathway, tools permitting an early distinction of regeneration-competent cultures from those that are not would be of interest for in vitro plant regeneration of recalcitrant species, such as the grain legumes (Ochatt *et al.,* 2000a). For instance, in pea, hypernodulating mutants were highly embryogenic while the wild type was not (Ochatt *et al.,* 2000a, b), and grass pea was consistently recalcitrant to embryogenesis (Ochatt *et al.,* 2002). On the other hand, when attempting gene transfer in the model legume species *Medicago truncatula*, the requisite for success was the use of a specific and reduced range of genotypes selected previously as being highly regenerating (Trinh *et al.,* 1998). As a matter of fact, cases of differential responses in terms of regeneration among different genotypes within the same species are commonplace in the literature, and differences between species are even more drastic.

This variability in competence for *in vitro* plant regeneration has handicapped and delayed the exploitation of biotechnology approaches for breeding in many species, and was and still is the subject of research in many laboratories. Unfortunately, it is easier to find descriptive studies than explanatory ones and the distinction between cause and effect or the underlying determinism (be it physiological, genetic, or metabolic) is still a matter of controversy.

The transition from the dedifferentiated status to the embryogenic (Mordhorst *et al.,* 1998; Boutilier *et al.,* 2002) and/or organogenic (Che *et al.,* 2002, 2007; Srinivasan *et al.,* 2007; Atta *et al.,* 2008) status is a complex process which comprises of several phases: dedifferentiation, cell reactivation, cell division and various metabolic and developmental re-programming steps. Studies conducted so far in this domain have addressed the problem in vivo mainly and most frequently for Arabidopsis thaliana, with several genes identified and their roles described.

In this paper, some of the parameters governing the acquisition of regeneration competence by cultured cells and tissues are discussed, whether plants are recovered from organs or somatic embryos, and some indicators of such competence are outlined.

On the fate of somatic embryos and their conversion to plants

The success in producing somatic embryos is often diminished by their lack of germination capacity and conversion to viable plants. One is thus tempted to establish a parallel between the situation *in planta* (moreover, *in silico*) and that in somatic embryos *in vitro.*

In this context, it is now accepted that seed maturation begins with a transition phase during which control switches from maternal to filial (Weber *et al.,* 2005). It has also been discussed recently that the maturation processes

are under the control of the concerted action of a considerable number of signalling pathways, which integrate genetic, metabolic and hormonal signals (Gazzarini and McCourt, 2003; Gutierrez *et al.,* 2007). In this respect, sugars in general and sucrose in particular are major components of the signalling pathway that triggers the onset of the transition phase (Borisjuk *et al.,* 2003; Weber *et al.,* 2005), via a dramatic decrease of free hexose levels in the embryo parallel to a strong and transient increase in sucrose uptake (Gibson 2005). As a result, sucrose signals have been postulated to control storage and differentiation processes thanks to their potential to act on transcriptional and post-transcriptional regulation, through a regulation of metabolic enzymes and a modulation of gene expression and activity (Brocard-Gilford *et al.,* 2003; Gibson 2005; Baud *et al.,* 2005).

On the other hand, maximum cell volume, responsible in fine of the final size of organs (Ochatt and Moessner 2010), including seed, and their protein storage capacity, is under genetic and environmental determinism (Lemontey *et al.,* 2000). Also, the cell wall properties determine the ability of cells to expand and grow, but also their division competence, another determinant of the total cell number in an organ that, ultimately, influences also its final size. Such phenomena of cell volume limitation have to be linked to specific cytological and biochemical events in order to "date" the duration of the cell division and storage protein accumulation phases in the developing seed. Thus, the role of cell walls has always caught a lot of attention in the cell biology of plant growth (Burk and Ye 2002; Kerstens and Verbelen 2003), while the implication of cell wall composition is presently a matter of intense research (Fagard *et al.,* 2000) and, yet, knowledge on the mechanisms through which the form of plants and organs is established on the cell and tissue levels is still largely inexistent (Frugis *et al.,* 1999).

Unravelling Cytological indicators of embryogenesis competence

Against this background, a number of cytological traits that are susceptible to impact the competence for regeneration through somatic embryogenesis, including the kinetics of cell surface, cell wall thickness and of the medium and intracellular osmolarity at the onset of embryogenesis from cell suspensions and during embryo growth were examined with embryogenic and non-embryogenic cell suspensions of several pea, grass pea and *Medicago truncatula* genotypes (Ochatt 2008; Ochatt *et al.,* 2008; Ochatt and Moessner 2010). Thus, novel cyto-physical parameters served as early indicators of the competence of cells to undergo somatic embryogenesis, and may also be useful to study the kinetics of seed development and storage protein synthesis.

The cell surface of embryogenic cells was significantly larger than that of non embryogenic ones (Ochatt *et al.,* 2008). Interestingly, young cells also were larger than non embryogenic, mature ones. This result held true for all genotypes of all species examined. Then, the cell wall thickness was examined by the end of active cell division and the onset of somatic embryogenesis, to check whether this was the result of cell enlargement or of cell elongation. Differences were evident in the time course of cell wall thickness between young, mature and embryogenic cells, and they were maintained irrespectively of the species and genotype analysed. Thus, cell wall thickness increased slightly through the subculture cycle for young cells, it remained largely constant for mature non-embryogenic cells, and it decreased concomitantly with the end of active cell division and the onset of cell elongation for embryogenic ones. Additionally, an examination of cell wall thickness by day 14 (end of subculture passage) revealed that, for all species and genotypes, this was comparable for young and mature cells, even if young cells tended to have slightly thinner walls, whereas the cell walls of embryogenic cells were consistently and significantly the thinnest of all (Ochatt *et al.,* 2008).

The osmolarity of medium and cells reflects the actual consumption of nutrients from the medium by cells and their internal salt concentration, respectively. When embryogenesis started, the medium osmolarity decreased sharply between the beginning and the end of the subculture passage when embryogenesis was induced, to reach an osmolarity value that was significantly lower than that of the medium alone. Conversely, for cultures of young and mature non embryogenic cells, the osmolarity of the medium did not exhibit any significant evolution throughout the subculture passages, nor when comparing them with each other or with the medium alone. The intracellular osmolarity appeared to be highest for young cells and similar in mature and embryogenic ones. This means that there is more salt concentrated in young non-embryogenic than in embryogenic clones, i.e. that embryogenic cells would have bigger (and/or more) vacuoles than non-embryogenic ones (Ochatt *et al.,* 2008).

It has often been stated in the literature that the onset of embryogenesis in isolated cells is accompanied by the establishment of a bipolar type of growth, whereby cells grow in length as opposed to the iso-diametric form characteristic of non-embryogenic cells in active division. However, this had not been quantified in the past and a part of these studies concerned the measurement of the time course of cell surface comparing the different cell suspensions. Our results are only partly in line with published data, as authors in the past have also stated that it is the smaller cells that become embryogenic (Ammirato, 1983), whereas in this work, whatever the original size of cells, once embryogenesis is onset they dramatically increase in surface. In any case, the microscopic observation of cells alone does not permit to reliably ascertain whether these size modifications were due to cell enlargement or to

cell elongation, and the need for measuring other cellular parameters to assess this seemed evident.

Flow cytometry and regeneration competence

Since the first report on flow cytometric studies of plant material thirty five years ago, analysing the nuclear DNA content of field bean (Heller 1973), an ever increasing number of applications of flow cytometry has been developed and applied in plant science and industry, as an ideal means for the analysis of both cells and subcellular particles, with a potentially large number of parameters analysed both rapidly, simultaneously, and quantitatively, thereby furnishing statistically exploitable data, and allowing for an accurate and facilitated detection of subpopulations (Dolezel *et al.,* 2007). It is, indeed, the summation of these facts that has established flow cytometry as a useful tool for the understanding of fundamental mechanisms and processes underlying plant growth, development and function (Ochatt, 2008).

Thus, during extensive research on the induction of plant regeneration from isolated protoplasts of pea, Ochatt *et al.* (2000b) obtained a very large number of independent regeneration events, and with several hundred plants transferred to the greenhouse, which permitted for the first time a detailed study of protoplast-derived tissues and plants aimed at understanding the genetic mechanisms underlying regeneration competence. Given the size of the population to be analysed, this was undertaken by flow cytometry with nuclei isolated from calluses (non-regenerating, organogenic, embryogenic) or leaves of regenerants, for an examination of relative nuclear DNA contents and of peak profiles compared to the profile of the respective mother plants. Most interestingly, out of the large number of materials analysed, it appeared that whenever the calli were competent for regeneration they also presented two DNA peaks only, whereas all tissues that failed to regenerate were consistently abnormal, and showed a non true-to-type DNA content, almost always associated with somatic endoreduplication (a modification of the mitotic cycle with DNA duplication but without mitosis, i.e. the nuclear DNA content is doubled at each cycle to pass from 2C to 4C, then 8C, corresponding to a succession of S phases, but without cell division). Indeed, only rarely would an endoreduplicated callus succeed in undergoing regeneration and, if this occurred, only caulogenesis was observed and resulted consistently in the production of enfeebled shoot buds that ultimately died without rooting. Only non-endoreduplicated protoplast-derived callus tissues were competent for regeneration (Ochatt *et al.,* 2000b). This finding has since helped to increase the percentage of protoplast-derived calluses that regenerate plants in pea and other legumes, as only those calli exhibiting a normal DNA content are now transferred onto the regeneration media, fostering a more efficient use of this approach for breeding of legumes, that could also be applied for other species.

An additional and interesting use of flow cytometry was for the characterization of the genetic base of hyperhydricity, which was found to be systematically associated with flow cytometry profiles comprising of three peaks instead of two for normal tissues (Ochatt *et al.,* 2002). More importantly, when these studies were extended to analysis of somatic embryos with the model legume *Medicago truncatula*, it was found that all such embryos that failed to germinate and convert to plants also possessed abnormal flow cytometry profiles with supernumerary peaks (Ochatt, 2008).

An additional use of flow cytometry is to measure the cell content of various subcellular substances such a cell wall fractions (Ochatt, 2008) and apoptotic markers bound to cell compartments (Cvikrová *et al.,* 2003; Loureiro *et al.,* 2006). As far as the analysis of cell wall fractions is concerned, and linked to studies above linking cell wall thickness to embryogenic competence (Ochatt *et al.,* 2008). Ochatt (2008) carried out studies with *Medicago truncatula* cells and tissues. Previously, Gendreau *et al.* (1997) had demonstrated that the phenomenon of endoreduplication precedes cell elongation in the hypocotyls of *Arabidopsis thaliana*. Thus, when an 8C peak appears very early on, it would imply the beginning of the embryogenesis phase resulting in a modification of cell shape, with cells elongating by the establishment of a bipolar type of growth as opposed to the iso-diametric form characteristic of non-embryogenic cells in active division (Ammirato, 1983). However, despite recent studies aimed at distinguishing between cell enlargement and cell elongation with *Arabidopsis* (Sugimoto *et al.,* 2001) roots, no explanation to this process within embryos other than that given by Ochatt *et al.* (2008) is available in the literature. The determination of cell wall thickness is rather straightforward, as after staining cells with Calcofluor White, this dye intercalates and binds to the β 1-4 bonds in the cellulose molecule emitting a bright blue fluorescence after excitation under UV. This can then be quantified by measuring the cell wall thickness of stained cells with the aid of a computarized image acquisition program (Ochatt and Moessner 2010) or by flow cytometry assessments.

Storage protein accumulation and embryogenesis competence

Gallardo *et al.* (2006) have recently developed a strategy that permits the development of embryos *in vitro* in a way comparable to that observed *in planta*, and allows also the study of the division phase of embryo growth and that of the subsequent phase of storage protein accumulation (seed filling) in *Medicago truncatula*, most recently extended also to pea. It is now possible to modify the composition of the culture medium practically at will (in terms of hormones or of specific nutrients), and then flow cy-

tometry is a choice approach to assess how this affects the potential for cell division (growth) or endoreduplication (storage accumulation) of the embryo cells and at different developmental stages, thereby permitting to develop an *in vitro* modelling system of seed growth and filling. Interestingly, only somatic embryos having entered the storage accumulation phase can develop, and once the endoreduplication process is attenuated, they will germinate and convert to plants, as demonstrated by Benlicha *et al*. (2007), who associated the strategy developed by Gallardo *et al*. (2006) to immunological analyses, and showed the time course accumulation of various storage protein fractions in germinating embryos and developing seeds (but also of ABA in the non germinating embryos).

Stress induces anrogenesis and regeneration in general

The summation of results obtained by a number of authors, clearly suggests that the pyramiding of various stress factors may hold the key to eliminating the recalcitrance for plant regeneration through embryogenesis from a number of tissues.

If one takes only the case of androgenesis for the sake of concision, this is particularly evident as no less than four different stress factors, and sometimes even five, were needed before the first successful recovery of double haploids was attained in pea (Ochatt *et al.,* 2009) and chickpea (Grewal *et al.,* 2009). These were a cold stress pretreatment of flower buds prior to the excision and culture of microspores or anthers, centrifugation, electro-stimulation, osmotic stress and sonication.

A temperature stress was previously observed as beneficial for a large number of species (Maluszinski *et al.,* 2003). As for centrifugation, already in the seventies this was used with tobacco (Tanaka, 1973) and *Datura innoxia* (Sangwan-Norreel, 1977) anthers to improve embryo production; as much more recently with chickpea by Grewal *et al*. (2009).

Concerning electroporation, when a cell is exposed to an electric field pores are formed through an enhancement of its transmembrane potential (Cole, 1968, Neumann and Rosenheck, 1973) which depends on the cell radius, the electric field strength delivered and the angle between the normal vector of the membrane and the direction of the electric field applied (Chang, 1992). For more than two decades, the application of an electroporation treatment has been known to improve division and initial proliferation competence of protoplasts (Rech *et al*., 1987) and tissues (Rathore and Goldsworthy, 1985), coupled with an enhanced regeneration ability of the resulting callus tissues in species as distant apart as *Solanum dulcamara* (Chand *et al*., 1988), Colt cherry (Ochatt *et al*., 1988) and barley (Mordhorst and Lörz, 1992), and with an increased DNA synthesis in treated protoplasts of members of the genera *Prunus* and *Solanum* (Rech *et al*., 1988). Few groups have electroporated intact, walled cells, including suspension-cultured cells of maize (Sabri *et al*., 1996) and, in some cases, microspores of various species. Among the latter, Laurain *et al*. (1993) reported a faster development of embryos from electroporated microspores of *Gingko biloba*, while similar results were also obtained in tobacco (Mishra *et al*., 1987), maize (Fennel and Hauptman, 1992), *Brassica napus* (Jardinaud *et al*., 1993), asparagus (Delaitre *et al*., 2001), pea (Ochatt *et al*., 2009) and chickpea (Grewal *et al*., 2009). Of interest in the report by Ochatt *et al*. (2009) with pea microspores, was that the electrical parameters fostering the proliferation of undifferentiated tissues from the cultured microspores differed from those inducing somatic embrygenesis.

The elicitating effects of osmotic pressure on androgenesis have been known for a long time, first in the *Brassicaceae* (Lichter, 1982; Dunwell and Thurling, 1985; Lionneton *et al*., 2001) and other species (Delaitre *et al*., 2001), and more recently also in legumes (Grewal *et al*., 2009; Ochatt *et al*., 2009). The positive effect of a changing medium osmolarity on the onset of embryogenesis, was previously observed also with cell suspensions of pea and other grain legumes (Ochatt *et al*., 2008), and it is interesting to underline that, working with canola microspores, Dunwell and Thurling (1985) reported that a medium with 17% (w/v) sucrose generated the same osmotic potential as did an anther homogenate, and induced androgenesis.

The genetic determinism of regeneration competence

More than 50 years since Skoog and Miller (1957) described the hormonal control of organ regeneration in plants, there is still little insight on the primary determinants of the capacity of undifferentiated cells to regenerate shoots, and little is understood about the mechanisms through which the auxin/cytokinin balance exerts its effects. It is generally accepted that organogenesis is composed of three sequential phases: acquisition of ability to recognize hormonal or other signals by cells that commit them to a particular developmental program, such signals induce a process where competent cells alter their developmental fate, and finally differentiation to form determinate organs, during which the inductive signals are no longer required (Hicks, 1994; Sugiyama 1999). Although experimental procedures for *in vitro* organogenesis vary among species, the first phase is generally initiated by culturing on an auxin-rich callus-inducing medium (CIM), then explants are cultured on a shoot-inducing medium (SIM) or root-inducing medium (RIM) that contains a specific auxin/cytokinin ratio.

The genetic determinism for the differences in regeneration capacity is still poorly understood though, and a number of genes have been identified that positively influence the competence of plant cells for somatic and/or adventitious shoots formation (Banno *et al.,* 2001; Boutilier *et al.,* 2002; Srinivasan *et al.,* 2007; Che *et al.,* 2007).

The production of shoots from roots must involve considerable genetic programming. The magnitude of gene expression changes during shoot regeneration from roots can be estimated from microarray analyses, such as in studies that compared gene expression patterns between roots and shoots (leaves) in mature *Arabidopsis* (Che *et al.,* 2002; Cary *et al.,* 2002). Recently, using gene-profiling methods, genes that are up-regulated during this process of regeneration were identified (Che *et al.,* 2002). Most of these genes have been identified in *Arabidopsis* and many of them encode transcription factors or proteins involved in signal transduction.

Several studies have demonstrated a positive correlation between cytokinin levels and outgrowth of axillary buds on the basis of phenotypes in mutants and transgenic plants in which cytokinin levels were altered (Chaudhury *et al.,* 1993; Helliwell *et al.,* 2001; Catterou *et al.,* 2002). In addition, molecular and genetic studies have led to the identification and characterization of genes controlling the establishment of polarity, tissue differentiation and the elaboration of patterns during embryo development (Long *et al.,* 1996). The analysis of gene expression has become increasingly important for understanding how genetic profiles affect cell phenotype and function. Overexpression of such genes efficiently substitutes for plant hormones such as cytokinins or auxins, or enhances the regeneration responsiveness of plant tissues to these hormones. By comparing the two cytokinin overproducing mutant phenotypes of *Arabidopsis thaliana*, *Hoc* (Catterou *et al.,* 2002) and *Amp1* (Chaudhury *et al.,* 1993) compared with their respective wild types, these effects can be illustrated and their respective regeneration competence modulated, as this competence was accompanied by a precocious up-regulation of the *WUSCHEL* gene. (Assoumou-Ndong *et al.,* 2010).

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