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REVIEW

Can mimicking sexual reproduction solve problems with recalcitrance in in vitro propagation of tree species?

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Abstract: Clonal propagation by in vitro means of adult forest trees is often difficult or not possible with current technology. This problem, generally described as recalcitrance, has been approached from several angles by researchers and reviewers. However, it has not yet been reviewed from the point of view that it may eventually be possible to overcome recalcitrance by mimicking the rejuvenation process that occurs during sexual reproduction. It is suggested that somatic cell nuclear transfer, in a manner similar to the one used for animal cloning, could be helpful. Furthermore, application of controlled stress and autophagy, or inducing apomixes by halting natural or artificially induced meiosis before chromosome segregation, could perhaps assist in overcoming recalcitrance. The discussion below indicates that mimicking the sexual or apomictic process in vitro is worthy of further exploration and should be evaluated as a means of genetically improving tree species, especially those of high economic value. Studies along these lines may also be helpful in improving our knowledge of the epigenetic, cytogenetic, and genomic mechanisms involved.

Key words: autophagy, cytoplasm, micropropagation, rejuvenation, somatic cell nuclear transfer (SCNT).

Résumé : La propagation clonale in vitro d'arbres forestiers adultes est souvent difficile ou impossible avec la technologie courante. Ce problème, généralement qualifié de récalcitrance, a été envisagé sous plusieurs angles par les chercheurs et les réviseurs. Cependant, il n'a pas encore été revisité en considérant qu'il pourrait éventuellement être possible de surmonter la récalcitrance en imitant le processus de réjuvénilisation qui survient durant la reproduction sexuée. On croit que le transfert nucléaire de cellules somatiques comme celui qui est utilisé pour le clonage animal pourrait être utile. De plus, l'application d'un stress contrôlé et l'autophagie, ou l'induction de l'apomixie en stoppant la méiose naturelle ou artificiellement induite avant la ségrégation des chromosomes, pourraient peut-être aider à surmonter la récalcitrance. La discussion indique que l'imitation du processus sexuel ou apomictique in vitro mérite d'être explorée plus en profondeur et devrait être évaluée comme moyen pour améliorer génétiquement les espèces d'arbre, particulièrement celles qui ont une grande valeur économique. Des études dans ce sens pourraient aussi être utiles pour améliorer notre connaissance des mécanismes épigénétiques, cytogénétiques et génomiques impliqués. [Traduit par la Rédaction]

Mots-clés : autophagie, cytoplasme, micropropagation, réjuvénilisation, transfert nucléaire de cellules somatiques.

Introduction

Micropropagation, by either organogenesis or somatic embryogenesis (SE), of adult forest trees is often difficult or still not possible. However, to do so is often a cherished goal because it would provide considerable advantages in tree improvement programs. Tree improvement by breeding is difficult and expensive because of the high degree of self-incompatibility of most tree species, their long life cycle, and the costs associated with breeding and seed orchards (Bonga 2016). Genetic improvement of tree populations, especially of conifers, has been greatly improved by SE initiated from zygotic embryos excised from seed. SE has been effective in tree improvement programs because embryogenic cultures can be cryopreserved, recovered alive after long-term cryopreservation, and then used for mass-producing clonal offspring. While part of each culture is cryopreserved, the other part can provide somatic seedlings for long-term field testing. After completion of the field test, clones that have performed best in the test can be retrieved from cryopreservation and used for mass-producing clonal propagules for reforestation (Lelu-Walter et al. 2013; Park et al. 2016). This technology, if used in combination with tree breeding experiments, allows for selection within a breeding population for clonal lines that will perform better than the average performance of the population obtained by breeding alone as it captures non-additive gains (Lelu-Walter et al. 2013; Bonga 2016; Park et al. 2016). However, even though this procedure is effective in producing genetically improved populations, further improvements are envisaged by cloning adult specimens that have shown their superior worth. Such trees may show not just one but a number of useful traits that, unfortunately, tend to get lost in the reshuffling of genes during meiosis because they are often based on non-additive, specific gene combinations. Therefore, these gene combinations may not show up in any of the improved seed provided by controlled pollinations and thus cannot be captured via the SE process. Furthermore, some of the desired traits may not show up until late in the life cycle of the tree and thus would require excessively long field testing of the clones before the ones with the desired traits could be selected. Therefore, cloning of adult trees is attractive, although, if achieved, it would at least initially still require long-term field testing to make sure that the clones are true-to-type. Once the technology has matured and proven its reliability, it may be possible to dispense with the long-term testing requirement. Unfortunately, efforts to clone adult conifers by in vitro means have so

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far only had limited success (Klimaszewska and Rutledge 2016; Trontin et al. 2016*a*).

In earlier reviews, this problem, commonly referred to as "recalcitrance", has been looked at from various angles for tree species, i.e., stress application (Bonga et al. 2010; Salo et al. 2016), explant choice (Bonga 2017), application of rejuvenating treatments (Monteuuis et al. 2011), and genetic and metabolic analyses (Trontin et al. 2016*b*). However, one aspect that has attracted only limited attention with regard to clonal propagation is how natural rejuvenation resets the adult sporophyte's developmental program to that of the zygotic embryo during the sexual process. If this process could be mimicked by experimental means, the recalcitrance problem could perhaps be overcome. The objectives of this report are (*i*) to review what the literature has reported regarding the rejuvenation process that occurs during the sexual process and (*ii*) to consider if this knowledge can possibly suggest ways in which rejuvenation of tissues can be achieved experimentally, so that they attain the competence to produce clonal offspring.

Somatic cell nuclear transfer (SCNT)

An early indication of how the phase transition, i.e., the transition from a mature to a juvenile cellular state occurs was observed for animal cells and those of a number of lower organisms. Experiments involving transplantation of a nucleus excised from one cell into another cell from which the nucleus has been removed have indicated that whether a cell behaves in a juvenile or mature fashion is largely determined by the cytoplasm. As far back as the 1960s, it was demonstrated that transplantation of the nucleus of a frog's gut lining cell (i.e., a nucleus that is programmed for expressing a mature type of growth) into a denucleated egg cell (i.e., into the cytoplasm of a cell programmed for juvenile growth) resulted in the reconstituted egg cell initiating early embryo development (Gurdon and Byrne 2003). Further experiments along these lines eventually resulted in cloned mammals, the first one being a sheep named Dolly (Wilmut et al. 1997; Gurdon and Byrne 2003). These experiments demonstrated that the epigenetic program of terminally differentiated cells is not irreversibly fixed, but can be reprogrammed to an embryonic state (Ranganath 2004; Jaenisch and Young 2008).

Genetic information contained in the cytoplasm can have a considerable effect on morphogenetic events. A classic example of that is the observation made during the 1930s by Haemmerling that morphogenetic gradients exist in the unicellular, morphologically complex green algal genus *Acetabularia* (see reviews by Haemmerling (1963), Menzel (1994), and Mandoli (1998)). These algae survive enucleation and subsequently carry on photosynthesis, other metabolic functions, and morphogenesis for up to 40 days (Vanden Driessche et al. 1997). Haemmerling (1963) found that in nucleus-free sections of the algal cell, the apical section showed the highest developmental capacity by regenerating new whorls of branches and a cap involved in sexual reproduction (gametangium), whereas the most basal section had the lowest regenerative capacity. These differences in the behavior of the various cell sections were determined by asymmetrically distributed mRNA and ribosomal RNA in the cytoplasm (Menzel 1994; Mandoli 1998; Serikawa et al. 2001). Because, in the absence of a nucleus, new mRNA is not produced, the cytoplasmic mRNA must be long lived (Menzel 1994). As a further demonstration of the morphogenetic control exerted by the cytoplasm, transplantation of mature *Acetabularia* nuclei into juvenile cytoplasm rejuvenated the nuclei (Berger and Schweiger 1975; Menzel 1994).

Clearly, it would be of interest to determine if adult plants, in particular of tree species, could be cloned by rejuvenation of nuclei obtained from adult specimens implanted into juvenile cytoplasm. Unfortunately, plant egg cells are deeply embedded in the nucellus and thus are difficult to excise in a viable, intact state. Nevertheless, this has been achieved, for example, for maize (Kranz and Scholten 2008), rice (Uchiumi et al. 2007), and wheat (Kumlehn et al. 1997) by the use of cell wall degrading enzymes and microdissection (Kumlehn et al. 1997; Kranz and Kumlehn 1999; Kranz et al. 2008). Male and female gametes thus obtained have been used for in vitro fertilization, which subsequently resulted in embryo development. Kumlehn et al. (1997) implanted excised zygotes of wheat into wheat ovules cultured in vitro. The implanted zygotes developed into normal embryos that eventually formed normal, fertile plants. As stated by Kumlehn et al. (1997), this approach makes zygotes accessible to micromanipulation without affecting embryo development. Holm et al. (2000) demonstrated that isolated barley zygote protoplasts can be microinjected with gene constructs and survive the procedure and grow in vitro into plants. These protoplasts were remarkably stable and could easily be handled and transferred between different environments. This suggests that it may be possible to microinject plant nuclei that are programmed for mature type behavior into denucleated zygote protoplasts in vitro and thus achieve nuclear rejuvenation similar to the one achieved in animal somatic cell nuclear transfer (SCNT) experiments.

If one wishes to attempt SCNT with, for example, conifer species, what would be the best source of juvenile cells? As already stated, access to zygotes or other juvenile cells such as coenocytic proembryos in situ is difficult. A far more accessible source of juvenile cells would be SE cultures at the initiation–proliferation stage. The most suitable cells in these presumably are the large coenocytic ones that closely resemble coenocytic proembryos of zygotic origin. Other cells in these cultures that clearly have the capacity to produce embryos and thus are highly juvenile are the cells that constitute the embryo heads. These, however, are very small and thus perhaps less suitable than the large coenocytic ones. Several cell types in gymnosperm SE cultures that resemble embryonal initials have been described and pictured in detail by Durzan (2012, 2016).

Stress and autophagy

The question that arises is "what is the mechanism involved in the rejuvenation that occurs in the egg cell cytoplasm?" It has long been known that dramatic changes occur in the cytoplasm of meiocytes during the early phases of meiosis by means of autophagy (Heslop-Harrison 1971). Most studies of meiosis and the autophagy occurring during meiosis have been carried out with yeast and animals. However, as these processes are highly conserved among all eukaryotes (Bernstein and Bernstein 2010), observations made with non-plant species are to a large extent applicable to plant species as well and thus deserve attention. Meiosis in yeast can be initiated by stress treatments such as nitrogen deprivation (Nakashima et al. 2006; Bernstein and Bernstein 2010), a process that induces autophagy, a degradative pathway that degrades cytoplasmic proteins, RNA, and organelles and is highly conserved among eukaryotes (Nakashima et al. 2006; Mukaiyama et al. 2009; Brar et al. 2012). This results in a short-lasting reduced expression of ribosomal proteins after the initiation of meiosis (Wen et al. 2016), which corresponds with the early observations by Heslop-Harrison (1971), Dickinson and Heslop-Harrison (1977), and Dickinson (1994) that ribosomes and RNA largely disappear and that mitochondria and plastids de-differentiate into simpler forms during the male and female meiotic prophase in plants. It is not just during early meiosis that selective degradation of specific cytoplasmic components takes place during cell remodeling. For example, degradation of maternal mRNAs and other maternal and paternal components occurs in the zygote as well (Stitzel and Seydoux 2007; Sato and Sato 2013).

With regard to rejuvenation, yeast presents an interesting case. Yeast rejuvenates during its sexual cycle (involving meiosis), as well as during its asexual propagation cycle, i.e., by budding. Yeast

cell lines will go through about 50 mitotic divisions and then will die. At various times during their life-span, the cells will divide asymmetrically and form small, rejuvenated buds. During this division, aging factors that accumulated as the cell line matured are retained in the mother cell, leaving the bud free of them. In the buds, such aging factors as small, extrachromosomal DNA circles, damaged proteins, dysfunctional mitochondria, and defective vacuoles are, therefore, absent (Barral 2010; Denoth Lippuner et al. 2014; Nyström and Liu 2014). This enables the bud to start a new cell line with a cleansed, rejuvenated cytoplasm and to progress through a normal (about 50 divisions long) life-span.

The question now arises, can the simplification of the cytoplasm that occurs early during meiosis and during other rejuvenating events be induced in somatic cells by experimentally induced autophagy? Stress will do so but presumably in a manner that is far less specific than that of the autophagy that occurs during meiosis. Different forms of stress-induced autophagy occur throughout the life-span of organisms and have been researched, especially with yeast, but are similar among all eukaryotes (Díaz-Troya et al. 2008). In budding yeast, more than 40 autophagy-related genes have been identified, and most of these are conserved from yeast to humans. A transcription factor (Ndt80) has been identified in yeast that triggers rejuvenation (Denoth Lippuner et al. 2014), whereas other factors involved have been described by Nyström and Liu (2014).

Stress often plays an important role in the initiation of SE and androgenesis (reviewed by Maraschin et al. 2005). It is of interest to note that 2,4-D, which is commonly used to initiate SE, causes oxidative stress (Fehér et al. 2003), which may be the reason for the effectiveness of this compound in SE initiation. Stress can be applied in many forms. In yeast, nutrient deprivation will initiate meiosis and autophagy (Nakashima et al. 2006) and thus rejuvenation. Stressful situations, e.g., storage at below-freezing temperatures of dormant buds of *Larix decidua* Mill. for several months, have resulted in the formation of embryo-like structures (Bonga 1996), and microsporophylls of *Pinus resinosa* Ait., stored at 2–4 °C for 6 months, showed a high percentage of multicellular pollen (Bonga 1974), which indicates that they potentially may be capable of androgenesis.

SE or androgenesis often proceeds from cells that contain primitive organelles. Sangwan and Sangwan-Norreel (1987) noted that among 22 androgenic and four non-androgenic species, the androgenic ones had immature pollen with primitive proplastids, whereas the non-androgenic ones displayed well-developed amyloplasts and starch grains. During the initiation of androgenesis, organelle-free regions have been observed in the cytoplasm of many species, accompanied by a large decrease in the number and size of starch granules and lipid bodies and a decline in the number of ribosomes (Maraschin et al. 2005). Similarly, this occurred during the development of haploid embryos of *Larix decidua* that had originated from haploid megagametophytes in vitro (Rohr et al. 1989).

As reviewed by Seguí-Simarro and Nuez (2008), most of the genes differentially expressed during androgenesis belong to the following categories: (*i*) those involved in stress adaptation; (*ii*) those that suppress the gametophytic program, which involves cytoplasmic cleaning; and (*iii*) those that start the embryogenic program. They further comment that abscisic acid (ABA) may be involved in the upregulation of genes active in androgenesis and that a cell cycle arrest period, inhibition of starch biosynthesis, and a decreased protein level are needed for the reorganization that leads to embryogenic commitment. In addition, during androgenesis, the cytoplasm becomes more alkaline, and genes involved in lipid, carbohydrate, and protein synthesis are upregulated. It is of interest to note that in yeast aging, the pH of the vacuoles gradually changes from alkaline to acidic, indicating that pH plays a role in the juvenile to adult transition (Denoth Lippuner et al. 2014).

The story so far may have left the impression that cytoplasmic de-differentiation is essential in overcoming recalcitrance. This, however, is not always the case. Organogenesis or embryogenesis is often initiated without the dramatic cytoplasmic de-differentiation that occurs during meiosis. To give one example, SE of *Picea abies* (L.) Karst. originates from small subpopulations of small cytoplasm-rich cells (Filonova et al. 2000). There clearly are several different pathways that lead to SE, and the autophagy described above is only one of them.

Potential ways of solving recalcitrance problems

Are there practical ways to mimic the de-differentiation that occurs during meiosis in somatic cells of individuals that are recalcitrant to cloning? As discussed above, the technology to excise plant egg cells and zygotes and to keep them alive in vitro exists, which suggests that somatic cell nuclear transfer, i.e., the transfer of a nucleus programmed for mature growth behavior into a juvenile cytoplasm, may be possible. However, this may be difficult; nuclear transfer as practiced with animal cells has only a low success rate (Gurdon and Byrne 2003). To my knowledge, such transfer has not yet been attempted with plant cells and, in all likelihood, will be difficult to achieve.

A more promising approach may be to try to improve stress applications. A variety of stresses have been applied to achieve embryogenesis or androgenesis. However, the resulting embryo induction rates are often low, presumably because the percentage of cells in which the stress treatment has succeeded in achieving the degree of cytoplasmic cleaning required to reprogram the cell is low. Where stress application has failed to achieve the desired effect, the failure presumably was due to the cytoplasmic de-differentiation being either insufficient to achieve initiation or too drastic, thus killing the cell. If so, the stress treatment may require extensive fine tuning to achieve higher induction rates.

A more targeted approach may be needed, involving, for example, ectopic expression of some of the genes involved in meiotic, apomictic, and, in particular, androgenic autophagy. Genes involved in starch formation and autophagy play a role in the initiation of androgenesis (Maraschin et al. 2005). In immature pollen, i.e., the stage when they are competent to initiate androgenesis, starch formation genes are repressed, but they are active after the first pollen mitosis. Repression of these genes blocks pollen maturation, i.e., gametophyte development, and this may initiate androgenesis. This suggests that in situations where recalcitrance is a problem, manipulating these genes may be of assistance. Similarly, the role of genes involved in proteolytic processes (reviewed by Maraschin et al. 2005) is of interest in that respect. In SE initiation, de-differentiation is accompanied by an increase in gene expression of proteases associated with the ubiquitin – 26S proteasome proteolytic pathway and associated genes are involved in androgenesis. This suggests that manipulation of the expression of these proteolytic genes could perhaps eventually be useful in overcoming recalcitrance. As stated by Maraschin et al. (2005), when the stress-induced de-differentiation phase is completed, key regulatory genes involved in embryo development come into play. Ectopic expression of these genes has led to spontaneous embryo formation, albeit only in experiments with young plants, not with adult ones. In conclusion, ectopic expression of genes involved in autophagy and embryogenesis may be one way to overcome recalcitrance in some cases.

There are other approaches to consider. From the above, it is clear that meiosis and stress play important roles in rejuvenation. This leads to the question "can meiosis be induced artificially in explants obtained from recalcitrant adult trees and, if halted before chromosome segregation, can this result in clonal propagation?" Meiosis occurs spontaneously in some plant tissues in vitro by a process called somatic meiosis, leading to SE. However, because of meiotic segregation, this results in variations in the off-

spring (Yihua et al. 2001; Giorgetti et al. 2011) and, therefore, the offspring is not truly clonal and not true-to-type. However, this type of SE is not universal, and in most cases, SE appears to arise by pathways other than by somatic meiosis. Nevertheless, it is worthwhile to investigate if meiosis can be induced artificially in tissues of species that do not normally show somatic meiosis. Meiosis has been initiated by several means. For example in yeast, it has been started by starvation (Nakashima et al. 2006), whereas hypoxia triggered it in maize (Kelliher and Walbot 2012).

Another approach would be the following scenario. In cultures of immature ovules, could meiosis be halted at the end of the meiotic prophase and the still-diploid egg cell be turned into a cell behaving as a zygote? This unreduced cell would then form an embryo by a process akin to the diplospory type of apomixes. The potential application of this approach has been discussed (Naumova 2008; De La Fuente et al. 2013; Yan et al. 2017) and has been reviewed with regard to recalcitrance in the clonal propagation of tree species, in particular conifers, by Bonga et al. (2010). The occurrence of apomixes and meiosis, as well as formation of unreduced egg cells in gymnosperm embryogenic cultures and the implication of these in in vitro clonal propagation, has also been described and illustrated in detail by Durzan (2016).

In conclusion, it would be worthwhile to try to mimic the sexual or apomictic process experimentally in attempts to overcome persistent recalcitrance in tree species, in particular of adult specimens. However, all suggested approaches are still in the experimental realm, and presumably, none of them will easily be amenable to large-scale, industrial application in the near future. However, the potential benefits of success warrant further exploration.

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