PROTOCOLS FOR MICROPROPAGATION OF WOODY TREES AND FRUITS

Protocols for Micropropagation of Woody Trees and Fruits

Edited by

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PREFACE

Micropropagation has become a reliable and routine approach for large-scale rapid plant multiplication, which is based on plant cell, tissue and organ culture on well defined tissue culture media under aseptic conditions. A lot of research efforts are being made to develop and refine micropropagation methods and culture media for large-scale plant multiplication of several number of plant species. However, many woody and fruit plant species still remain recalcitrant to *in vitro* culture and require highly specific culture conditions for plant growth and development.

The recent challenges on plant cell cycle regulation and the presented potential molecular mechanisms of recalcitrance are providing excellent background for understanding on plant cell totipotency and what is more development of micropropagation protocols. Today, the need for appropriate *in vitro* plant regeneration methods is overwhelming both for basic and applied research in order to overcome problems facing micropropagation such as somaclonal variation, recalcitrant rooting in woody species, hyperhydricity, high labour cost, contamination, loss of material during hardening, quality of plant material and polyphenols. For large-scale *in vitro* plant production the important attributes are the quality, cost effectiveness, maintenance of genetic fidelity, and long-term storage. Moreover, the useful applications of micropropagation in various aspects make this technology more relevant for example to production of virus-free planting material, cryopreservation of endangered and elite woody species, applications in tree breeding, afforestation and reforestation. Reforestation is important to prevent the loss of forest resources including timber, biodiversity and water resources, and would require continuous supply of planting material. The majority of world wood products still come from natural and semi-natural forests, but there is a clear trend towards more efficient plantation forestry. Generally, the development of vegetative propagation methods will yield additional profit for plantation forestry by the exploitation of non-additive genetic variation, by providing more homogenous planting material and by compensating potential shortage of improved seed stock.

The fruit trees and shrubs are grown to produce fruits to be consumed both as fresh and as processed forms including juices, beverages, and dried fruits. They are an important source of nutrition, e.g. rich in vitamins, sugars, aromas and flavour compounds, and raw material for food processing industries. Fruit trees have long juvenile periods and large tree size. Moreover, fruit trees are faced with agronomic and horticultural problems in terms of propagation, yield, appearance, quality, diseases and pest control, abiotic stresses and poor shelf-life. The available genetic information in fruit crops is very limited and their genetic improvement has heavily relied on classical breeding and on vegetative propagation of specific cultivars. Furthermore, micropropagation has increasingly been promoted in enhancing the total number of genetically modified fruit plants.

Our previous book entitled *Micropropagation of Woody Trees and Fruits* provided a comprehensive coverage on various aspects on micropropagation of economically important forest and fruit trees. However, it did not exclusively focus

on precise stepwise protocols for plant multiplication. The introductory chapter of this book will cover the present knowledge of plant cell totipotency in the context of the cell cycle and the potential mechanisms of gene silencing in competence and recalcitrance. The follow-up chapters will cover micropropagation protocols of diverse plant species, i.e. the practical examples of plant cell totipotency. The book will provide information on 'organogenesis' approach for plant multiplication, and various applications such as genetic transformation, cryopreservation and others. The chapters are easy to follow including step by step protocols for numerous woody plants. Therefore, the book can be used as a practical handbook in tissue culture laboratories. It will certainly benefit students, researchers, horticulturists, forest geneticists, and biotech companies.

This book has a total of 48 chapters on micropropagation protocols and is divided into three sections: Section A) contains 1–22 chapters on forest and nitrogen fixing trees, Section B) covers 23–40 chapters on fruit trees, and Section C) deals with 41–48 chapters on non-tree plants such as bananas and small fruits. All manuscripts have been peer reviewed and revised accordingly.

We appreciate very much all contributory authors for their contribution in compilation of this book, and for their co-operation in revising their manuscripts and sending them to us well in time. We are thankful to the reviewers for giving their precious time in reviewing manuscripts, and that has helped in improving the quality of the book. Springer publisher has given us the opportunity to edit this book, and we highly appreciate it.

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H. Häggman

Section A

CHAPTER 1

TOTIPOTENCY AND THE CELL CYCLE

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Totipotency. The potential of an isolated undifferentiated plant cell to regenerate into a plant (Cassells & Gahan, 2006).

1. INTRODUCTION

In theory, each diploid plant cell contains the genetic information for the formation of an individual, and so each diploid nucleate cell should be capable of differentiating into a complete individual. Gurdon demonstrated this for animal cells (reviewed in Gurdon, 1974). Working with *Xenopus laevis,* nuclei from intestinal epithelial cells and skin epidermal cells were transferred to enucleated oocytes which were then initiated to develop into mature frogs. A parallel study by Steward showed that individual cells isolated from carrot-derived callus could be cultured to produce individual carrot plants (Steward, 1970). For this to be considered as universal for all plant cells rather than just intermediate callus cells, it needs to be demonstrated that each type of plant cell can give rise directly to whole plants by producing either shoots which can be rooted or roots which develop shoots or somatic embryos. Clearly, the ease with which this can be shown will depend upon the degree of differentiation undergone by each cell type and the degree of gene silencing that pertains together with the readiness with which these aspects can be reversed. Given that xylem elements lose their nuclei on differentiation eliminates them from this possibility, as is likely with sieve elements and their modified structure. Nevertheless, in Solanum aviculare, xylem parenchyma cells in cotyledons can give rise to somatic embryos (Alizdah & Mantell, 1991) whilst the mesophyll cells of both cotyledons and first leaves can give rise to roots though it is not clear if these arise from single cells as is the case of the somatic embryos.

There are a number of cases where the production of plants from single cells can be demonstrated. Thus, the basal cells from the hairs of *Kohleria* will develop into plants (Geier & Sangwan, 1996) whilst adventitious shoots have been reported

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to form from single epidermal cells of a range of species such as *Streptocarpus* (Broertjes, 1969) and *Nicotiana* (De Nettancourt et al., 1971). Equally, somatic embryos can be derived from single cells in either explanted tissues, callus and suspension cell cultures, protoplasts and mechanically isolated cells (reviewed in Gahan, 2007).

At least two factors appear to influence the ability of cells to express this capacity namely, the degree of differentiation and specialization and the impact of one tissue on gene expression in an adjacent tissue. As meristematic cells are left behind by the advancing meristem, they are considered to differentiate in order to form cells with special functions within an organ. Differentiation implies an irreversible state and is suitable to describe changes in most vascular tissue, cork tissue and the development of the woody state. However, in many non-woody plants, roots and shoots this is not necessarily an irreversible process, in which case, the term specialization is, perhaps, more apt. Clearly, in the case of, e.g., cortical parenchyma and collenchyma the ability to enter mitosis is not lost (Esau, 1953; Hurst et al., 1973). Equally, mesophyll cells, epidermal and hypodermal cells can all revert to the mitotic state. Thus, the relative degree of specialization will involve the relative degree of gene silencing in relation to mitosis and the expression of the gene sequences for developing into an individual plant. The second point concerns the impact on the adjacent tissue. This is seen in the studies of Chyla (1974) on *Torenia fourieri* in which the presence of an epidermal layer influenced the subepidernal layers. Culturing the epidermis together with the subepidermal layers resulted in the production of shoots whilst the culturing of the subepidermal layers in the absence of the epidermis resulted in the production of roots.

In many ways, the ability of a single cell to form a shoot or somatic embryo on the way to producing a whole plant will depend upon whether it is competent or recalcitrant. Competence may be defined as the state of a cell in which it is able to respond to epigenetic signals. Determination may then be defined as the state of a – previously competent – cell that has responded to that (those) signal(s) so committing the cell to a particular pathway which will include organogenesis and the production of a somatic embryo. Such epigenetic factors include plant bioregulators, and RNAi. Whether such cells are in a position to respond to epigenetic signals may depend upon the phase of the cell cycle in which they are held. Thus, it is possible that for recalcitrant cells, which may well be specialized, they may be non-cycling and held in G0 in which phase they are unlikely to be able to perceive an epigenetic signal. In contrast, those cells which are cycling and are held in G1, could be susceptible to epigenetic signals.

2. THE CELL CYCLE

The cell cycle is comprised of four major periods termed G1, S, G2 and M where S is the period of DNA synthesis, M is mitosis (Howard & Pelc, 1953) and G1 and G2 refer to gaps in our knowledge (S.R. Pelc priv. comm.). It is now clear that there are many events occurring in G1 and G2 in preparation for S and M, respectively (Alberts et al., 2002). A fifth period, G0, is when the cell leaves the cell cycle for a period of time, e.g. on specialization. For cells to progress round the cycle, there are

a series of checkpoints which enable the cell to monitor its progress before moving to the next step. Such checkpoints include the monitoring of cell size and the environment prior to proceeding from G1 to S, that all DNA has been synthesized before moving from S to G2, cell size and correct environment before leaving G2 to enter mitosis and a further check on the alignment of the chromosomes at the mitotic plate and their attachment to the spindle fibres. Clearly there are additional controls that will be discussed later and in particular how they might affect the states of competence and recalcitrance. Once a cell has passed a specific point at the end of G1, it will enter S and must complete the cycle before being able to enter G1 again. Some cells will be blocked in G2 presumably because the all aspects of the cell and its environment are not adequate for it to pass into M. Lack of carbohydrate substrate is a typical feature causing a both a G1 and a G2 block (Van't Hof & Kovacs, 1972).

According to the studies of milk production by breast cells (Vonderhaar & Topper, 1974) there is a phase within G1 in which hormonal signals could be received by the cells to initiate milk production. This would imply that there is only a very short G1 phase between early and late G1 when the signal might be perceived by plant cells since on leaving M, cells would have an adjustment period prior to electing either to recycle or to enter G0. They could then have a window of time to receive any epigenetic signals prior to reaching the START phase which sees them either differentiate/specialize or enter S (Figure 1).

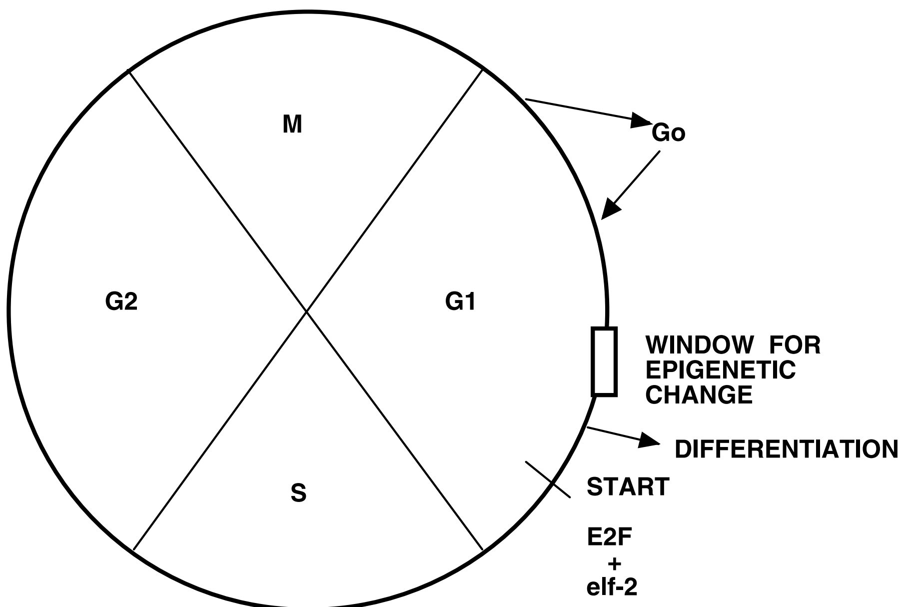

Figure 1. *Diagrammatic representation of the cell cycle with events in G1. M = mitosis; S = DNA synthesis; G1 and G2 – gaps in our knowledge; Go = quiescent phase.*

Two important periods occur prior to entry into S and M providing that the cell is ready to enter these phases. The entries depend upon two complexes being formed and comprising of a cyclin and cyclin-dependent protein kinase (CDK) the product of the gene *cdc2*. There are a number of cyclins of which cyclin B is necessary for entry to M. Of the cyclin Ds, when the gene for cyclin D1 from *Antirrhinum majus* was tested in *N. tabacum*, the cyclin D1 interacted with CDKA and, in contrast to animal cells, appeared to promote both Go/G1/S and S/G2/M progression (Koroleva et al., 2004). In addition, cyclinD2 appears to control the length of G1 whilst cyclin D3:1 appears to be important for the passage from G1 to S in *Arabidopsis thaliana* (Menges et al., 2006). Of the CDKs, CDKF has been found to be plant-specific in addition to CDKD that is homologous with that of vertebrates (Umeda et al., 2005).

Although the cyclinD3:1-CDK complex is necessary to pass from G1 to S, there is also the need for the gene regulatory protein E2F. The E2Fs are conserved transcription factors, of which six have been identified in *A. thaliana* (Sozzani et al., 2006), and which bind to specific gene sequences in the promoters of genes encoding proteins needed for entry to S and to M. The inhibition of E2F can be achieved with retinoblastoma protein (Rb protein) that binds to E2F so preventing it from binding to the promoters and resulting in an inhibition of the progress of the cell cycle. This inhibition can be reversed by the phosphorylation of Rb protein when the latter is released from the E2F. Phosphorylation of the Rb protein and histone H1 appears to be under the control of cyclinD1 associated CDK (Koroleva et al., 2004). The Rb protein-E2F complex can act either by sequestering transcription factors or by recruiting histone deacetylases or repressor proteins. Two forms of E2F have been found in plants, namely E2FA and E2FB. E2FB appears to be more important in Bright Yellow 2 (BY-2) cells from *N. tabacum* for passage from G1 to S (Magyar et al., 2005). The mechanism for the regulation of E2F in plants is not clear. However, in human cells, it has been proposed that the proto-oncogene c-MYC encodes a transcription factor that regulates cell proliferation, growth and apoptosis (O'Donnell et al., 2005). E2F1 is negatively regulated by two miRNAs from a chromosome 13 cluster at which c-Myc acts.

2.1. Quiescent Cell

Cells which are not cycling either can spend a prolonged period in G1 or can leave the cycle and enter a quiescent phase, Go, where they remain until receiving a signal to re-enter G1. A depression of protein synthesis is one feature resulting in the movement from G1 into Go and a non-proliferative state. This passage to Go is assisted by regulation of the gene elF-2. The product of these gene complexes with GTP to mediate the binding of the methyl initiator of t-RNA to the small ribosomal subunit, that binds to the 5' end of the m-RNA and starts scanning (Alberts et al., 2002). Thus, regulation of this gene will impact on translation and hence the overall level of protein synthesis.

2.2. Plant Bioregulators and the Cell Cycle

The correlation between the cell cycle progression and endogenous levels of plant bioregulators was studied in synchronized *N. tabacum* BY-2 cell suspension cultures (Redig et al., 1996). No significant correlation was found for IAA and ABA. However, there were sharp peaks of zeatin and dihydrozeatin at the end of S and during mitosis. Other cytokinins such as N- and O-glucosides of zeatin remained low implying that there was a *de novo* synthesis of zeatin and dihydrozeatin. The role of zeatin in the G2-M transition was further confirmed when the addition to the cultures of lovastatin affected both cytokinin biosynthesis and blocked mitosis. Lovostatin is a competitive inhibitor of HMG-CoA reductase and blocks the mevalonic acid pathway (Metzler, 2001). Of eight different aminopurines and synthetic auxin tested, only zeatin could override the lovastatin inhibition of mitosis (Laureys et al., 1998).

Murray et al. (1998) proposed that cyclin Ds responded to specific signals and that cyclinD3 was induced by cytokinin. This was further confirmed by the response of cyclinD3 to cytokinin (Riou-Khamlichi et al., 1999). It is clear that passage from G1 to S requires a CDK-cyclin complex and E2F at adequate concentrations which processes appear to be controlled, at least in part, by auxin and cytokinin. Murray et al. (1998) proposed that auxin was able to induce CDK homologues (Figure 2).

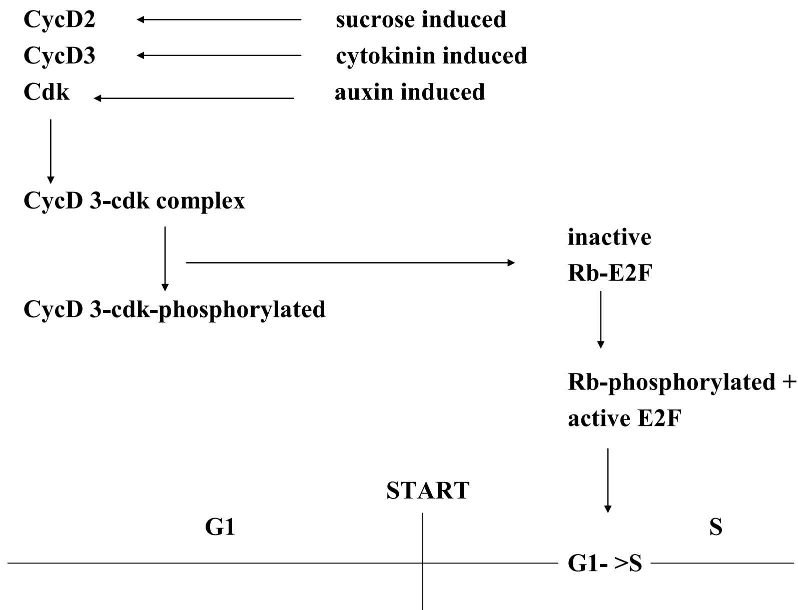

Figure 2. *A speculative model for the control of the G1-S transition. (After Murray et al., 1998.)*

Although jasmonic acid (JA) is better known for its involvement in plant fertility and defense, it has also been linked to a negative regulation of the cell cycle (Swiatek et al., 2004). JA prevents the accumulation of B-type CDKs and the expression of cyclinB1:1 in synchronized *N. tabacum* BY-2 cells, so causing G2 arrest and blocking entry to M. Hence JA could be affecting an early checkpoint in G2.

3. GENE SILENCING IN COMPETENCE AND RECALCITRANCE

It is generally accepted that actively transcribed genes are present in the euchromatin and that genes in the heterochromatin are not (Alberts et al., 2002). Whether the genes are located in either the eu- or the heterochromatin, they will be silenced at specific times. The activation or silencing will be influenced by epigenetic signals and can occur in a number of ways such as (a) complexing into heterochromatin, (b) through methylation, acetylation phosphorylation glycosylation, ADP ribosylation, carbonylation, sumoylation, biotinylation and ubiqutinisation of the histones (Loidli, 2004), methylation and deacetylation of the DNA, (c) RNA interference (RNAi) and (d) the action of retinoblastoma protein.

3.1. Heterochromatin Silencing

The complexing of genes into heterochromatic regions of the chromosomes generally result in gene silencing. In order to protect the euchromatin from being further linked into the heterochromatin, the nucleosome between the heterochromatin and euchromatin becomes modified. Instead of being composed of two pairs each of histones H2A, H2B, H3 and H4, H2A/H2B histones are replaced by H2AZ/H2b molecules. This histone exchange is mediated by the Swr1 complex (Alberts et al., 2002). This prevents the spread of silence information regulator (Sir) proteins into the euchromatin from, e.g., the telomeres; the Sir proteins (Sir2, Sir3, Sir4) binding to the nucleosomes to transcriptionally silence the chromatin. Euchromatin H3 and H4 tails are usually acetylated, but heterochromatin H3 and H4 tails tend to be under-acetylated and are thought to complex with Sir proteins. Sir2 binds initially and helps to form new binding sites for the other Sir protein complexes.

3.2. Methylation and Acetylation

Although methylation, acetylation phosphorylation glycosylation, ADP ribosylation, carbonylation, sumoylation, biotinylation and ubiquitinisation (Zhang, 2003) of the histones can occur in modifying gene activity, little is known about many of these events. The better known include the methylation and deacetylation processes with more known about the former than the latter (reviewed in Loidli, 2004).

Methylation and acetylation of the core histones, H2A, H2B, H3, H4 and the histone variants H2AZ and H3.3 are implicated in gene regulation. Many of the modifications are specific for either euchromatin or heterochromatin, e.g. methylation of histone H3lysine4 for euchromatin and H3lysine9 for heterochromatin. The methylated residues on H3 histone are recognized by special chromo-domain proteins

including HP1, a highly conserved heterochromatin protein. DNA is also methylated at the cytosine residue of triplets CNG and CNN where N can be C, T, A or G. Hence the methylation of both the DNA and the histones can lead to gene silencing with DNA methylation in the heterochromatin having been identified before that of histone methylation and the role siRNA (Lippman & Martienssen, 2004). Methylation of H3 and H4 histones by histone methyl transferases leads to transcriptional activation and repression, depending upon the level of methylation (di- or tri-methylation). To date, although DNA demethylation has been proposed to occur via a family of DNA glycosylases as proteins that can remove DNA methylation and so alleviate silencing (Gong et al., 2002; Chan et al., 2005), no histone demethylases have been identified in plants (Loidli, 2004)

Acetylation is the most extensively characterized type of histone modification. Core histones can be post-synthetically acetylated by histone acetyltransferases and deacetylated by histone deacetylases. However, little is known about acetylation in plants (Loidli, 2004).

The importance of methylation is seen in the studies of tree ageing where the quantification of genomic DNA methylation is being used to identify putative markers of ageing (Fraga et al., 2002a), phase change in trees (Fraga et al., 2002b) and reinvigoration (Fraga et al., 2002c). Indeed, global DNA methylation has been defined as a marker for forestry plant production so permitting an association between culture conditions and a specific epigenetic status.

3.3. siRNA

Short interference RNA (siRNA) is a class of double-stranded RNAs 21-24 nucleotides long. They are formed from dsRNA (double-stranded RNAs) and silence genes in one of three ways. The first is by initiating cleavage of mRNAs with the exact complementary sequences. The second method is by modifying the DNA directly by either complementary RNAi sequences or recruiting inhibitory proteins (Meister & Tuschi, 2004; Novina & Sharp, 2004; Jover-Gil et al., 2005). Finally, they compromise one of the more abundant classes of gene regulatory molecules in multicellular organisms and likely influence the output of many protein-coding genes (Bartel, 2004). They have a number of roles in plants (Baulcomb, 2004) including heterochromatic gene silencing (Lippman & Martienssen, 2004; Jia et al., 2004; Pal-Bhadra et al., 2004; Verdal et al., 2004).

Double-stranded RNAs appear to induce post-transcriptional gene silencing in several plant species apparently by targeting CpG islands within a promoter and inducing RNA-directed DNA methylation (see in Kawasaki & Taira, 2004). In addition, Lippman et al. (2004) have also indicated that siRNAs correspond to sequences of transposable elements in *A. thaliana* in which it is possible that the heterochromatin is composed of transposable elements (McLintock, 1956). Some 90–95% of endogeneous siRNAs correspond to either transposons or repeats that are heavily methylated. Transposons can regulate genes epigenetically though only when inserted within or close to the gene. This could account for the regulation of the chromatin remodelling ATPase DDM1 (Decrease in DNA Methylation 1) and DNA methyltransferase (Lippman et al., 2004), siRNA silencing linked to DNA

methylation and suppression of transcription (Wassenger et al., 1994; Mette et al., 2000; Jones et al., 2001).

3.4. Heterochromatin Formation

Heterochromatin formation has been considered in *A. thaliana* where DNA methylation, H3 methylation, H4 acetylation are implicated (Loidli, 2004). However, such a model does not explain all of gene silencing in the heterochromatin and it is clear that siRNA also has a significant role.

3.5. Recalcitrance and Heterochromatin

It is clear that in competent cells, elF-2 genes can be upregulated in order to permit a move from Go to G1 and phosphorylation of Rb protein will result in the release of E2F to permit a move from Go to S. Evidently, these events can be triggered by treatment with auxin and cytokinin (Figure 2). The problem arises with recalcitrant cells that fail to respond to plant bioregulator treatments. A possible explanation for this may be found in an extension of the model proposed by Williams & Grafi (2000). As discussed earlier, Rb protein can inhibit E2F so blocking the passage from G1 to S. This process will affect E2F in the euchromatic region of the chromosome, an apparently easily reversible situation. However, it is also possible that the Rb protein, on binding to E2F, brings the euchromatin closer to the heterochromatin. The heterodimer DF-E2F anchors the Rb protein into the promotor region (Figure 3). A direct connection can occur between the Rb protein and a region containing heterochromatin-associated proteins such as CLF (curly leaf) and HP1 (heterochromatic protein 1) proteins from *A. thaliana*. HP1 is found to contain an Rb protein binding motif located at the loop between B-3 short end and the a-helix structure (Figure 4). This loop is a variable region among the different chromodomain proteins which might not affect its 3-D structure. Maize Rb protein has been demonstrated to react with both HP1 and CLF proteins (Williams & Grafi, 2000). Such an interaction can result in the euchromatic E2F target gene being located in close proximity to the heterochromatin. This could result in a packaging into condensed, transcriptionally inactive chromatin (Figure 3).

Such a packaging could lead to recalcitrance which in some cases may be overcome by treatment with plant bioregulators, e.g. an auxin shock induced rooting in York M9 stems (Auderset et al., 1994). Normally, the nucleosome between the heterochromatin and the euchromatin will be modified, histone H2A.Z replacing histone H2A. However, if a closer integration of the portion of euchromatin with the heterochromatin occurs, this would lead to a modification of this nucleosome with H2A replacing H2A.Z again. This would result in the euchromatin becoming more closely integrated into heterochromatin and its genes transcriptionally silenced by Sir proteins binding to the nucleosomes after they have been deacetylated. Thus, E2F genes could be silenced in a way that cannot be readily reversed by plant bioregulators. At present it is not clear how such a reversal could be easily achieved and a variety of new strategies need to be developed.

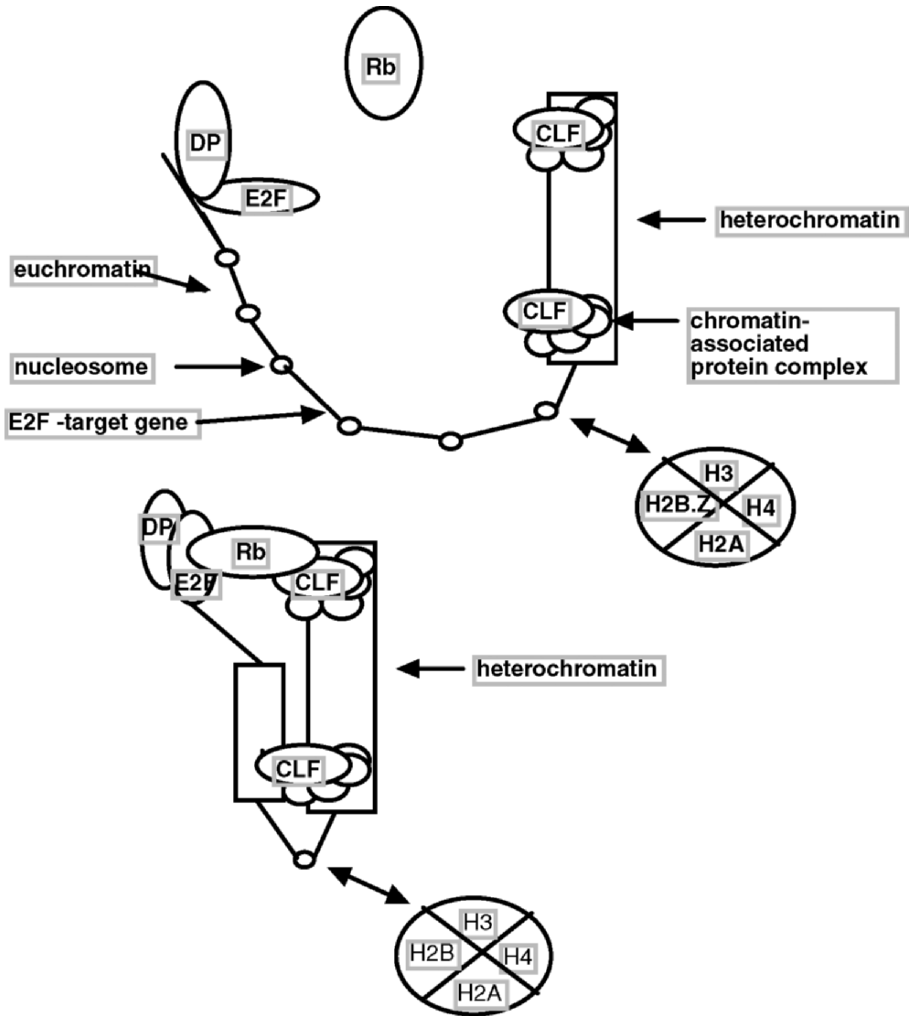

Figure 3. *Diagrammatic representation of possible mechanism by which recalcitrance occurs. Upper figure shows E2F without Rb protein, so activating the target gene in the euchromatin. Lower figure shows an effect of the dephosphorylation of Rb protein which binds to the E2F site and is also linked to CLF protein as a part of the chromatin- associated protein complex on the heterochromatin. This results in the E2F protein being linked to the heterochromatin so drawing the target gene associated nucleosome to be complexed to another chromatin-associated protein complex. CRL = curly leaf protein. (After Williams & Graffi, 2000.)*

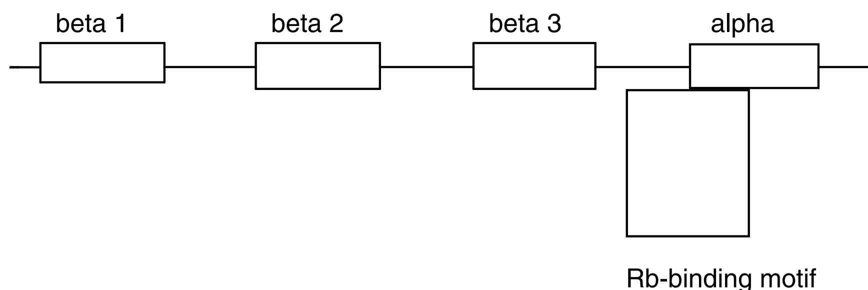

Figure 4. *Model of HP1 chromodomain secondary structure in relation to the Rb-binding motif in Arabidopsis thaliana SET-domain CURLY LEAF protein. This is similar to that from other eukaryote HP1 proteins. (After Williams & Graffi, 2000.)*

4. CONCLUDING REMARKS

In theory, each diploid plant cell is totipotent and contains the genetic information for the formation and differentiating into a complete individual. The degree of differentiation and specialization of the cells as well as the impact of one tissue on gene expression in an adjacent tissue appear to influence the ability of cells to express totipotency. In many ways, the ability of a single cell to form a shoot or somatic embryo on the way to producing a whole plant will depend upon whether it is competent or recalcitrant. Competence may be defined as the state of a cell in which it is able to respond to epigenetic signals such as plant bioregulators and RNAi. Whether such cells are in a position to respond to epigenetic signals may depend upon the phase of the cell cycle in which they are held. Thus, it is possible that for recalcitrant cells, which may well be specialized, they may be non-cycling and held in Go in which phase they are unlikely to be able to perceive an epigenetic signal. In contrast, those cells that are cycling and are held in G1, could be susceptible to epigenetic signals. This chapter has summarized the present knowledge of plant cell totipotency in the context of the cell cycle and the potential mechanisms of gene silencing in competence and recalcitrance. The follow-up chapters will cover micro-propagation protocols of diverse plant species, i.e. the practical examples of plant cell totipotency.

5. REFERENCES

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. (2002) Molecular Biology of the Cell. 4th Edition. Garland Press.

Alizdah, S. & Mantell, S.H. (1991) Early cellular events during direct somatic embryogenesis in cotyldon explants of Solanum aviculare. Forst. Ann. Bot. 647, 257–263

Auderset, G., Gavillet, S., Micheli, J., O’Rourke, J., RIibaux, M. & Moncousin, Ch. (1994) Histological analysis and the evolution of biochemical markers during the in vitro rooting of *Malus domestica* Borkh. ‘Jork 9’. Adv. Hort. Sci. 8, 5–10.

Bartel, D. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Baulcomb, D. (2004) RNA silencing in plants. Nature 431, 356–363.

Broertjes, C. (1969) Mutation breeding of *Streptocarpus*. Euphytica 18, 333–339.

Cassells, A.C. & Gahan, P.B. (2006) Dictionary of Plant Tissue Culture. The Haworth Press, New York.

Chan, S.W.-L., Henderson, I.R. & Jacobsen, S.E. (2005) Gardening the genome: DNA methylation in *Arabidopsis thaliana*. Nat. Rev. Genet. 6, 351–360.

Chyla, H. (1974) Inter-tissue correlations in organ fragments: organogenetic capacity of tissues excised from stem segments of *Torenia fournieri* Lind. cultured separately *in vitro*. Plant Physiol. 54, 341–348.

De Nettancourt, D., Dijkhuis, P., Van Gastel, A.J.G. & Broertjes, C. (1971) The combined use of leaf irradiation and of the adventitious bud technique for inducing and detecting polyploidy, marker mutations and self-compatability in clonal populations of *Nicotiana alata* Link & Otto. Euphytica 20, 508–521.

Esau, K. (1953) Plant Anatomy. John Wiley & Sons, New York.

Fraga, M.F., Canal, M.J. & Rodriquez, R. (2002a) *In vitro* morphogenic potential of differently aged *Pinus radiata* D. Don. Planta 215, 672–678.

Fraga, M.F., Canal, M.J. & Rodriquez, R. (2002b) Phase-change related epigenetic and physiological changes in *Pinus radiata* D. Don. Planta 215, 672–678.

Fraga, M.F., Rodriguez, R. & Canal, M.J. (2002c) Genomic DNA methylation-demethylation during ageing-invigoration of *Pinus radiate.* Tree Physiol. 22, 813–816.

Gahan, P.B. (2007) Adventitious regeneration. In George, E.F., Hall, M.A. & De Klerk, G.-J. (Eds) Plant Propagation by Tissue Culture. 3rd Edition: Volume 1. The Background Springer.

Geier, T. & Sangwan, R.S. (1996) Histology and chimeral segregation reveal cell-specific differences in the competence for shoot regeneration and Agrobacterium-mediated transformation in *Kohleria* internode explants. Plant. Cell Rep. 15, 386–390.

Gong, Z., Morales-Ruiz, T., Ariza, R.R., Roidan-Arjona, T., David, L. & Zue, J.-K. (2002) ROS1, a repressor of transcriptional gene silencing in *Arabidopsis*, encodes a DNA glycosylase/lyase. Cell 111, 803–814.

Gurdon, J.B. (1974) The Control of Gene Expression in Animal Development. Clarendon Press, Oxford.

Howard, A. & Pelc, S.R. (1953) Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome break. Heredity (Suppl) 6, 261–273.

Hurst, P.R., Gahan, P.B. & Snellen, J.W. (1973) Turnover of labelled DNA in differentiated collenchyma. Differentiation 1, 261–266.

Jia, S., Noma, K.-I. & Grewal, S.I.S. (2004) RANi-induced heterochromatin nucleation by the stress-activated ATF/CREB family proteins. Science 304, 1971–1975.

Jones, L., Ratclif, F. & Baulcomb, D.C. (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met 1 for maintenance. Curr. Biol. 11, 747–757.

Jover-Gil, S., Candela, H. & Ponce, M.-P. (2005) Plant microRNAs and development. Int. J. Dev. Biol. 49, 733–744.

Kawasaki, H. & Taira, K. (2004) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature 431, 211–217.

Koroleva, O.A., Tomlinson, M., Parinyapong, P., Sakvarelidze, L., Leader, D., Shaw, P. & Doonan, J.H. (2004) CycD1, a putative G1 cyclin from *Antirrhinum majus*, accelerates the cell cycle in cultured tobacco BY-2 cells by enhancing both G1/S entry and progression through S and G2 phases. Plant Cell 16, 2364–2379.

Laureys, F. Dewite, W., Witters, E., Van Montague, M., Inze, D. & Van Onckelen, H. (1998) Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. FEBS Lett. 426, 29–32.

Lippman, Z. & Martienssen, R. (2004) The role of RNA interference in heterochromatic silencing. Nature 431, 364–370.

Lippman, Z., Gendrel, A.-V., Black, M., Vaughn, M.W., Dedhia, N., McCombie, W.R., Lavine, K., Mittal, V., May, B., Kasschau, K.D., Carrington, J.C., Doerge, R.W., Colot, V. & Martienssen, R. (2004) Role of transposable elements in heterochromatin and epigenetic control. Nature 430, 471–476.

Loidli, P. (2004) A plant dialect of the histone. Trends in Plant Sci. 9, 84–90.

Magyar, Z., De Veylder, L., Atanassova, A., Bako. L., Inze, D. & Bogre, L. (2005) The role of Arabidopsis E2FB transcription factor in regulating auxin-dependent cell division. Plant Cell 17, 2527–2541.

McLintock, B. (1956) Controlling elements and the gene. Cold Spring Harbor Symp. 21, 197–216.

Meister, G. & Tuschi, T. (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343–349.

Menges, M., Samland, A.K., Planchais, S. & Murray, J.A. (2006) The D-type cyclin CYCD3:1 is limiting for the G1-to-S phase transition in *Arabidopsis*. Plant Cell 18, 893–906.

Mette, M.M., Aufstaz, W., van der Winden, J., Matzke, M.A. & Matzke, A.J. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. 19, 5194–5201.

Metzler, D.E. (2001) Biochemistry Vol. 1 2nd Edition Academic Press, New York.

Murray, J.A.H., Feeman, D., Greenwod, J., Huntley, R., Makkerh, J., Riou-Khamlichi, C., Sorell, D.A., Cockcroft, C., Carmichael, J.P., Soni, R. & Shah, Z.H. (1998) Plant D cyclins and retinoblastoma protein homologues. In Francis, D., Dudits, D. & Inze, D. (Eds) Plant Cell Division Portland Press. London.

Novina, C.D. & Sharp, P.A. (2004) The RNAi revolution. Nature 430, 161–163.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V. & Mendell, J.T. (2005) c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839–43.

Pal-Bhadra, M., Leibovitch, B.A., Gandhi, S.G., Rao, M., Bhadra, U., Birchler, J.A. & Elgin, S.R.C. (2004) Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. Science 303, 669–672.

Redig, P., Shaul, O., Inze, D., Van Montague, M. & Van Onckelen, H. (1996) Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. FEBS Lett. 391, 175–180.

Riou-Khamlichi, C., Huntley, R. & Murray, J.A.H. (1999) Cytokinin Activation of Arabidopsis Cell Division Through a D-Type Cyclin Science 283, 1541–1544.

Sozzani, R., Maggio, C., Varotto, S., Canova, S., Bergounioux, C., Albani, D. & Cella, R. (2006) Interplay between *Arabidopsis* activating factors E2FB and E2FA in cell cycle progression and development. Plant Physiol. 140, 1355–1366.

Steward, F.C. (1970) From cultured cells to whole plants: the induction ad control of their growth and differentiation. Proc. R. Soc. B 175, 1–30.

Swiatek, A., Azmi, A., Stals, H., Inze, D. & Van Onckelen, H. (2004) Jasmonic acid prevents the accumulation of cyclin B1;1 and CDK-B in synchronised BY-2 cells. FEBS Lett. 572, 118–122.

Umeda, M., Shimotohno, A. & Yamaguchi, M. (2005) Control of cell division and transcription by cyclin-dependent kinase-activating kinases in plants. Plant Cell Physiol. 46, 1437–1442.

Van't Hof, J. & Kovacs, C.J. (1972) Mitotic cycle regulation in the meristems of cultured roots: the principal control point hypothesis. Adv. Exp. Med. Biol. 18, 15–30.

Verdal, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I.S. & Moazed, D. (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303, 672–676.

Vonderhaar, B.K. & Topper, Y.J. (1974) Role of the cell cycle in hormone dependent differentiation. J. Cell Biol. 63, 707–712.

Wassenger, M., Heimes, S., Riedel, L. & Sanger, H. (1994) RNA directed de novo methylation of genomic sequences in plants. Cell 76, 567–576.

Williams, L. & Grafi, G. (2000) The retinoblastoma protein – a bridge to heterochromatin. Trends in Plant Sci. 9, 239–240.

Zhang, Y. (2003) Transcriptional regulation by histone ubiquitination and deubiquitination. Genes & Development 17, 2733–2740.

CHAPTER 2

MICROPROPAGATION VIA ORGANOGENESIS IN SLASH PINE

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1. INTRODUCTION

Highly efficient and reproducible *in vitro* regeneration systems via somatic embryogenesis or organogenesis are a prerequisite for clonal propagation of elite genotypes of specific plant species and for production of transgenic plants (Becwar et al., 1990; Attree & Fowke, 1993; Tang & Newton, 2003). Although plant regeneration via somatic embryogenesis has been reported in a number of coniferous species, plant regeneration via organogenesis from callus cultures has been obtained in only a few conifers (Hakman & Fowke, 1987; Nørgaard & Krogstrup, 1991; Tang et al., 2004). Routine methods of transformation are still hampered by the lack of readily available, highly efficient, and long-term regenerable cell and tissue culture systems in conifers (Handley et al., 1995; Tang & Newton, 2004).

Currently, a variety of explants have been successfully used for obtaining morphogenesis *in vitro* in conifers (Nagmani & Bonga, 1985; Gladfelter & Phillips, 1987; Tremblay, 1990; Guevin & Kirby, 1997; Salajova et al., 1999; Zhang et al., 1999), of which the most common are immature and mature embryos (Attree & Fowke, 1993; Find et al., 2002; Vookova & Kormutak, 2002). However, developmental progression has been limited to cultures capable of somatic embryogenesis and plant regeneration directly from the explant or via a callus phase using immature embryos (Krogstrup, 1990; Harry & Thorpe, 1991; Jalonen & von Arnold, 1991; Nørgaard, 1997; Klimaszewska et al., 2000). The successful regeneration of somatic embryos and plantlets is achieved using immature embryos (Campbell et al., 1992; Attree & Fowke, 1993; Guevin et al., 1994) as the target tissues in Fraser fir and Nordmann fir. Nevertheless, these explants require that their collection be limited to a special season of the year. In addition, there is a strong genotype dependency involved in tissue culture and efficient regeneration with embryogenesis. Furthermore,

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regeneration efficiency is still low, especially in commercial cultivars, due to various factors affecting the frequency of plant regeneration after transformation and selection (Find et al., 2002; Vookova & Kormutak, 2002). Therefore, a highly efficient regeneration system is needed for the genetic transformation of conifers.

Because of its rapid growth rate, slash pine (*Pinus elliottii* Engelm.) is a valuable southern pine for reforestation projects and timber plantations throughout the south eastern United States. Slash pine is also widely planted in the tropical and subtropical regions over the world. Slash pine is naturally found in wet flatwoods, swampy areas, and shallow pond edges. It can occur in the low sandy soils that are poor in nutrients. Millions of acres of slash pine have been planted and grown in the south eastern United States, where younger trees are harvested for pulpwood. Plant regeneration via somatic embryogenesis from embryogenic callus initiated from immature embryo explants of different slash pine genotypes has been reported (Jain et al., 1989; Newton et al., 1995). However, the development of a significantly improved plant regeneration system through multiple shoot differentiation from callus cultures derived from mature embryos would be valuable to clonal propagation and to genetic transformation in slash pine. In this study, we report the establishment of an efficient plant regeneration system via organogenesis from callus cultures in slash pine. The method presented here will be most useful for future slash pine clonal propagation and genetic transformation programs.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

Mature seeds of genotypes 1177, 1178, 7524, 7556 of slash pine (*Pinus elliottii* Engelm.) are provided by Penny Sieling and Tom Byram (Texas Forest Service Forest Science Laboratory, Texas A&M University, College Station, TX 77843-2585, USA). All seeds are stored in plastic bags at 4℃ before they are used for callus induction. Seeds are washed in tap water for 20 min, then disinfected by immersion in 70% w/w ethanol alcohol for 30 s and in 75% house breach for 15 min, followed by five rinses in sterile distilled water. Mature zygotic embryos are aseptically removed from the megagametophytes and placed horizontally on a solidified callus induction medium in 15 × 100 mm Petri dishes (Fisher Scientific) with 20 ml medium. Make sure the whole embryos are touching the medium. Plates with embryos are incubated in the dark at 23°C.

2.2. Culture Medium

Basal media used in this investigation included BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004) media (Table 1). Plant growth regulators (Table 2) used in callus induction medium include α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and

Table 1. *The basal media used in tissue culture of slash pine. The basal media used for callus induction, adventitious shoot formation, shoot elongation, and rooting included BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004) medium.*

Chemical formula	BMS	DCR	LP	MS	SH	TE
$Ca(NO_3)_2 \cdot 4H_2O$	0	556	0	0	0	556
KNO_3	2,500	340	1,900	1,900	2,500	340
$CaCl_2 \cdot 2H_2O$	200	85	1,760	440	200	85
NH_4NO_3	0	400	1,200	1,650	0	400
$MgSO_4 \cdot 7H_2O$	400	370	370	3,70	400	720
KCl	0	0	0	0	0	1,900
KH_2PO_4	0	170	340	170	0	170
$NH_4\ H_2PO_4$	300	0	0	0	300	0
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	0	8.6	1.0	25.8
$MnSO_4 \cdot H_2O$	16.9	22.3	2.23	16.9	10.0	25.35
H_3BO_3	6.2	6.2	0.63	6.2	5.0	6.2
KI	0.83	0.83	0.75	0.83	1.0	0.83
$Na_2MoO_4 \cdot H_2O$	0.25	0.25	0.025	0.25	0.1	0.25
$CoCl_2 \cdot$ 6H2O	0.025	0.025	0.025	0.025	0.1	0.025
$CuSO_4 \cdot 7H_2O$	0. 025	0.025	0.025	0.025	0.2	0.025
$FeSO_4 \cdot 7H_2O$	27.8	27.8	13.9	27.8	15.0	27.8
NaEDTA	37.3	37.3	0	37.3	20.0	37.3
Myo-inositol	1,000	1,000	1,000	1,000	1,000	1,000
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5	0.5	0.5	0.5
Thiamine HCl	0.1	0.1	0.1	0.1	0.1	0.1
Glycine	0.1	0.1	0.1	0.1	0.1	0.1
Sucrose	30,000	30,000	30,000	30,000	30,000	30,000
Glutamine	0	0	0	0	0	500
Casein hydrolyzate	0	0	0	0	0	500
Gelrite	0	0	0	0	0	3,000
pH	5.7	5.7	5.7	5.7	5.7	5.7

2-isopentenyladenine (2iP). The pH is adjusted to 5.8 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121°C for 20 min. All media are adjusted to pH 5.8 prior to autoclaving for 20 min at 121°C. All tissues are cultured at 23°C. Adventitious shoot induction is conducted in the dark, and adventitious shoot differentiation and proliferation and rooting are conducted at 23°C under a 16-h photoperiod with cool fluorescent light (100 μmol m^{-2} s^{-1}). Each experiment is replicated three times, and each replicate consisted of 50–200 embryos for callus induction, 30–50 pieces of calli (0.5 × 0.5 cm in size) for adventitious shoot formation, and 30–45 elongated shoots for rooting. For shoot proliferation and maintenance, the multiplied shoots of

each clump are cultured in the same shoot formation medium for 6 additional weeks. All cultures are subcultured every 3 weeks.

Table 2. *Procedure for plantlet regeneration in slash pine. The basal media used for callus induction, adventitious shoot formation, shoot elongation, and rooting include BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004).*

Plant growth regulators	Stage of plantlet regeneration			
	Induction	Differentiation	Elongation	Rooting
α-Naphthaleneacetic acid (NAA)	12 μM	0	0	0
Indole-3-acetic acid (IAA)	0	0	2 μM	0.01 μM
Indole-3-butyric acid (IBA)	0	2 μM	0	0.01 μM
2,4-Dichloroxyacetic acid (2,4-D)	15 μM	0	0	0
6-Benzyladenine (BA)	0	3 μM	1 μM	0
Thidiazuron (TDZ)	0	9 μM	0	0
2-Isopentenyladenine (2iP)	6 μM	0 μM	0	0
L-Glutamine	500 mg/l	500 mg/l	400 mg/l	400 mg/l
Myo-Inositol	500 mg/l	500 mg/l	250 mg/l	250 mg/l
Sucrose	30,000 mg/l	30,000 mg/l	20,000 mg/l	10,000 mg/l
Phytagel	4,500 mg/l	4,500 mg/l	5,000 mg/l	5,000 mg/l
PH	5.8	5.8	5.8	5.8
Culture time	6 weeks	6–12 weeks	6 weeks	6 weeks

2.3. Shoot Regeneration and Maintenance

The procedure of plant regeneration involving callus induction, adventitious shoot formation, shoot elongation, and rooting is shown in Table 2. Basal media used for callus induction include DCR, BMS, LP, MS, SH, and TE media. The frequency of callus formation is determined 6 weeks after culture. After calli are transferred onto adventitious shoot regeneration medium consisting of DCR, BMS, LP, MS, SH, and TE media for 6 weeks (Table 1), differentiation is evaluated by the percentage of calli forming adventitious shoots on the medium for a 6-week period.

1. Subculture calli every 3 weeks before the induction of shoot formation.
2. Transfer calli onto shoot formation medium supplemented with IBA, BA, and TDZ for 2–3 subcultures. If more calli are needed, subculture calli 4–6 times.
3. Make sure the whole calli are touching the medium.
4. Culture calli at 23°C under a 16-h photoperiod with cool fluorescent light (100 μmol m^{-2} s^{-1}).
5. Subculture calli with adventitious buds in LifeGuard plant growth vessels (Sigma) every 3 weeks on fresh shoot formation medium.
6. Determine the frequency of calli forming shoots, 6 weeks after calli are transferred onto shoot formation medium.

Among 6 basal media (BMS, DCR, LP, MS, SH, and TE) used in this study, higher frequency (34%–46%) of callus induction is obtained on BMS, SH, and TE, compared to DCR, LP, MSG, and MS. Similar callus induction frequency is obtained in four genotypes of slash pine. The frequency of callus formation increased during 4–6 weeks on fresh callus induction medium supplemented with NAA, 2,4-D, and 2iP. The highest frequency of callus formation is obtained on TE medium. After callus cultures (Figure 1A) are transferred onto shoot formation medium for 6 weeks, frequency of calli forming adventitious shoots is evaluated. Adventitious shoots (Figure 1B, C) are regenerated from callus cultures of four slash pine genotypes on BMS, SH, and TE media, with higher frequency (26%–35%) on SH and TE media and lower frequency (6%–9%) on BMS medium. The frequency of adventitious shoot formation increased during 6–12 weeks on fresh shoot formation medium supplemented with IBA, BA, and TDZ. The highest frequency of callus forming shoots is obtained on TE medium.

2.4. Rooting

Elongated, well-developed individual shoots with more than 8 needles are separated from the mother clumps and transferred onto rooting medium for 6 weeks. After elongated shoots are transferred onto rooting medium, rooting (Figure 1D, E) is evaluated by the percentage of shoots forming roots on the test medium for 6 weeks. Higher rooting frequency (26%–35%) is obtained in four genotypes on SH and TE media, compared to BMS medium (7%–9%).

1. Transfer shoots onto shoot elongation medium supplemented with IBA and BA.
2. Subculture shoots every 3 weeks.
3. Culture shoots at 23°C under a 16-h photoperiod with cool fluorescent light (100 µmol m^{-2} s^{-1}).
4. Subculture shoots every 3 weeks on fresh shoot elongation medium for 6 weeks.
5. Transfer elongated shoots 3–5 cm in height onto rooting medium supplemented with IAA and IBA.
6. Culture the elongated shoots for 6 weeks.
7. Rooting is conducted at 23°C under a 16-h photoperiod with cool fluorescent light (100 µmol m^{-2} s^{-1}).
8. Determine the frequency of shoots forming roots, 6 weeks after shoots are transferred onto rooting medium.
9. Plantlets with roots 2–5 cm in length can then be hardened.

2.5. Hardening

After rooting of adventitious shoots, regenerated plantlets from organogenic calli are treated at 4℃ for 1 week. Regenerated plantlets are then transferred from culture in 125 ml Erlenmeyer flasks into a perlite:peatmoss:vermiculite (1:1:1 v/v/v) soil mixture. For acclimatization, plantlets are covered with glass beakers for 1 week. After acclimatization by decreasing relative humidity to ambient condition over a period of 1 week, plantlets are exposed to greenhouse conditions (Figure 1F).

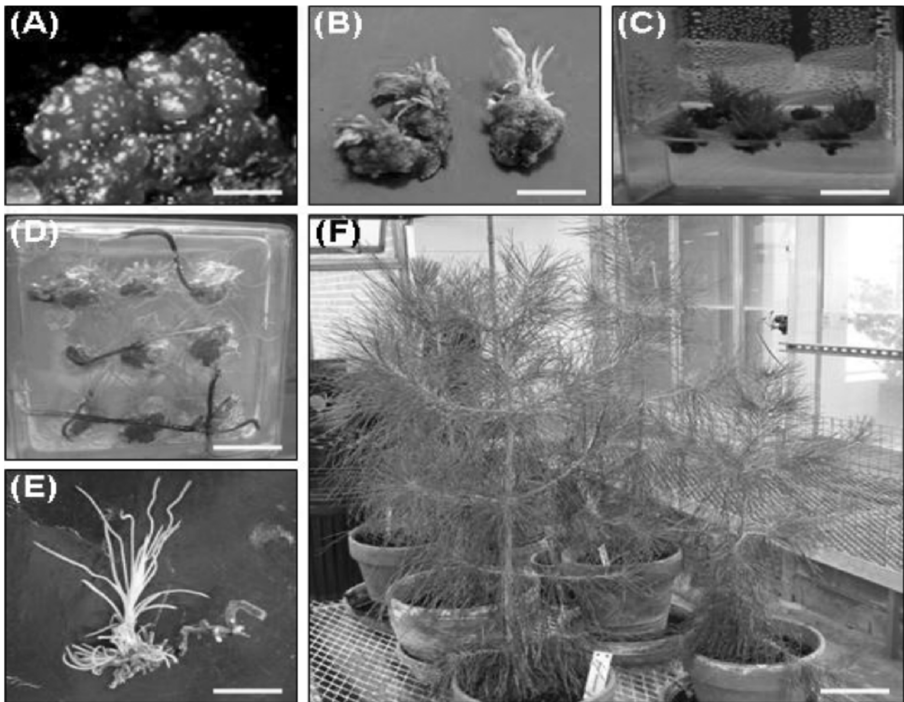

Figure 1. *Plantlet regeneration via organogenesis from callus cultures in slash pine. A) Callus cultures induced from mature embryos cultured for 3 weeks on callus induction medium. B) Clusters of adventitious shoots 6 weeks after callus cultures are transferred into shoot formation medium. C) Clusters of adventitious shoots 9 weeks after callus cultures are transferred onto shoot formation medium. D) Rooting of elongated shoots on rooting medium for 6 weeks. E) Plantlets before transferring into potting soil. F) Regenerated plants established in potting soil in greenhouse for 18 months. (A, bar = 0.5 cm; B, bar = 0.8 cm; C and D, bars = 1.1 cm; E, bar = 2 cm; F, bar = 8 cm.)*

2.6. Field Testing

After acclimatization, plantlets are taken out from the LifeGuard plant growth vessels (Sigma) and washed completely in tap water to remove the medium. The washing takes about 30 min. Plantlets are then planted into potting soil. In the first week, plantlets are watered two times a day. After that, they are watered once a day. Survival rate of regenerated plantlets is evaluated 6 weeks after their transfer to soil. More than 90% of the acclimatized plantlets survived in greenhouse.

3. CONCLUSION

The protocol established here is highly reproducible for the production of plantlets via organogenesis in four genotypes of slash pine. Plant growth regulators and the physiological activity of the explants are very important for successfully inducing plant regeneration via organogenesis in pine species. Mature zygotic embryos are good explants for the establishment of highly regenerable multiple shoot cultures of slash pine. The procedure presented here has several advantages over previously published reports of successful embryogenic callus induction from immature embryos. First, seeds of slash pine can be easily provided at any time throughout the year, but immature embryos are only available at the specific season of the year. Second, the process from callus to plant regeneration takes only a few months (8–10 months) which is less than plant regeneration via somatic embryogenesis. Third, plant regeneration from organogenic calli is a simple and highly efficient short-term in vitro regeneration system. There is no difference in the survival rate regenerated plantlets among different genotypes (genotypes 1177, 1178, 7524, 7556) used in this study. Regenerated plantlets produced from six basal media (DCR, BMS, LP, MS, SH, and TE) have very similar survival rates. The plant regeneration protocol established in this investigation may facilitate future research in genetic transformation in slash pine and other conifers.

4. REFERENCES

Attree, S.M. & Fowke, L.C. (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tiss. Org. Cult. 35, 1–35.

Becwar, M.R., Nagmani, R. & Wann, S.R. (1990) Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can. J. For. Res. 20, 810–817.

Boulay, M.P., Gupta, P.K., Krogstrup, P. & Durzan, D.J. (1988) Development of somatic embryos from cell suspension cultures of Norway spruce (*Picea abies* Karst.). Plant Cell Rep. 7, 134–137.

Campbell, M.A., Gaynor, J.J. & Kirby, E.G. (1992) Culture of cotyledons of Douglas-fir on a medium for the induction of adventitious shoots induces rapid changes in polypeptide profiles and messenger-RNA populations. Physiol. Plant. 85, 180–188.

Find, J., Grace, L. & Krogstrup, P. (2002) Effect of anti-auxins on maturation of embryogenic tissue cultures of Nordmann fir (*Abies nordmanniana*). Physiol. Plant. 116, 231–237.

Gladfelter, H.J. & Phillips, G.C. (1987) De novo shoot organogenesis of *Pinus eldarica* Med. *in vitro*. 1. Reproducible regeneration from long-term callus cultures. Plant Cell Rep. 6, 163–166.

Guevin, T.G. & Kirby, E.G. (1997) Induction of embryogenesis in cultured mature zygotic embryos of *Abies fraseri* (Pursh) Poir. Plant Cell Tiss. Org. Cult. 49, 219–222.

Guevin, T.G., Micah, V. & Kirby, E.G. (1994) Somatic embryogenesis in cultured mature zygotic embryos of *Abies-balsamea*. Plant Cell Tiss. Org. Cult. 37, 205–208.

Gupta, P.K. & Durzan, D.J. (1985) Shoot multiplication from mature Doublas fir and sugar pine. Plant Cell Rep. 4, 177–179.

Hakman, I. & Fowke, L.C. (1987) Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). Can. J. Bot. 65, 656–659.

Handley, L.W., Becwar, M.R. & Chesick, E.E. (1995) Research and development of commercial tissue culture system in loblolly pine. Tappi J. 78, 169–175.

Harry, I.S. & Thorpe, T.A. (1991) Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. Bot. Gaz. 152, 446–452.

Jain, S.M., Dong, N. & Newton, R.J. (1989) Somatic embryogenesis in slash pine (*Pinus elliottii*) from immature embryos cultured *in vitro*. Plant Sci. 65, 233–241.

Jalonen, P. & von Arnold, S. (1991) Characterization of embryogenic cell lines of *Picea abies* in relation to their competence for maturation. Plant Cell Rep. 10, 384–387.

Klimaszewska, K., Bernier-Cardou, M., Cyr, D.R. & Sutton, B.C.S. (2000) Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. In Vitro Cell. Dev. Biol.-Plant 36, 279–286.

Krogstrup, T. (1990) Effect of culture densities on cell proliferation and regeneration from embryogenic cell suspension of *Picea sitchensis*. Plant Sci. 72, 115–123.

Murashige, T. & Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15, 473–497.

Nagmani, R. & Bonga, J.M. (1985) Embryogenesis in subcultured callus of *Larix decidua*. Can. J. For. Res. 15, 1088–1091.

Newton, R.J., Marek-Swize, K.A., Magallanes-Cedeno, M.E., Dong, N., Sen, S. & Jain, S.M. (1995) Somatic embryogenesis in slash pine (*Pinus elliottii* Engelm.). In Jain, S.M., Gupta, P.K., Newton, R.J. (Eds). Somatic Embryogenesis in Woody Plants, Volume 3-Gymnosperms. Kluwer, Dordrecht, the Netherlands. pp. 183–195.

Nørgaard, J.V. (1997) Somatic embryo maturation and plant regeneration in *Abies nordmanniana* Lk. Plant Sci. 124, 211–221.

Nørgaard, J.V. & Krogstrup, P. (1991) Cytokinin induced somatic embryogenesis from immature embryos of *Abies nordmanniana* Lk. Plant Cell Rep. 9, 509–513.

Salajova, T., Salaj, J. & Kormutak, A. (1999) Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. Plant Sci. 145, 33–40.

Schenk, R.U. & Hildebrandt, A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199–204.

Tang, W. & Newton, R.J. (2003) Genetic transformation of conifers and its application in forest biotechnology. Plant Cell Rep. 22, 1–15.

Tang, W. & Newton, R.J. (2004) Increase of polyphenol oxidase and decrease of polyamines correlate with tissue browning in Virginia pine (*Pinus virginiana* Mill.). Plant Sci. 167, 621–628.

Tang, W., Harris, L.C., Outhavong, V. & Newton, R.J. (2004) Antioxidants enhance *in vitro* plant regeneration by inhibiting the accumulation of peroxidase in Virginia pine (*Pinus virginiana* Mill.). Plant Cell Rep. 22, 871–877.

Tremblay, F.M. (1990) Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can. J. Bot. 68, 236–240.

von Arnold, S. & Eriksson, T. (1979) Bud induction on isolated needles of Norway spruce (*Picea abies* L. Kast.) grown *in vitro*. Plant Sci. Lett. 15, 363–372.

Vookova, B. & Kormutak, A. (2002) Some features of somatic embryo maturation of Algerian fir. In Vitro Cell. Dev. Biol.-Plant 38, 549–551.

Zhang, C., Timmis, R. & Hu, W.S. (1999) A neural network based pattern recognition system for somatic embryos of Douglas fir. Plant Cell Tiss. Org. Cult. 56, 25–35.