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**A System to Study Plastid Development in Spruce**

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**Chishih Chu**

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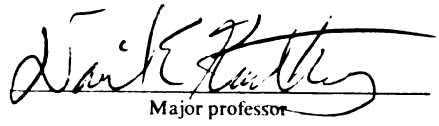
**Doctor of Philosophy** degree in **Plant Breeding and Genetics - Forestry**



Major professor

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# A SYSTEM TO STUDY PLASTID DEVELOPMENT IN SPRUCE

By

Chishih Chu

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**ABSTRACT**

**A SYSTEM TO STUDY PLASTID DEVELOPMENT IN SPRUCE**

**By**

**Chishih Chu**

The objectives of this study were 1) to develop a callus system to study plastid development associated with the change of pigments and gene expression and 2) to map the spruce cpDNA genome by RFLP analysis.

Protocols were developed for the induction and maintenance of white megagametophyte-derived callus and green callus derived from white callus in *Picea glauca*.

Induction of pigmentation changes in callus, from white to green, red, or brown, were observed using different levels of BAP or zeatin. The effects of zeatin significantly differed from those of BAP causing the white callus change to green, but not to red or brown. In contrast, the addition of 2,4-D inhibited the color changes induced by BAP. Glutamate caused browning of white calli, while ALA alone or glutamate and ALA together maintained white color. Heat shock at 37$^{o}$C for an hour altered the responses of white calli to BAP and zeatin by significantly increasing browning while decreasing greening.

Variations were found between green callus and white callus for dry weight and protein concentration. A lack of chlorophyll in white callus and a chlorophyll a/b ratio (1.339) for green callus were observed. Neither white callus nor green callus showed photosynthetic activity. In comparison with needles, green callus and white callus contained an extra DNA band on CsCl density gradients. Differences in polypeptides and differential expressions of five isozymes (IDH, GOT or

AAT, SKDH, G6PDH, and DIAor MNR) were found between green and non-green callus.

The cpDNA genome size is 120.4, 120.2, and 120.2 kb for blue spruce, Engelmann spruce, and white spruce respectively. Spruce cpDNA lacks one of the inverted repeat sequences and has multiple rearrangements when compared to that of radiata pine or Douglas-fir.

**This dissertation is dedicated**

**to my parents,**

**I-Wan Chu and Chun-Ing Thom**

**and**

**to my brothers and sister**

**ACKNOWLEDGEMENTS**

I would like to thank my major professor Dr. Daniel E. Keathley, and my committee members Drs. James Hancock, James Hanover, Amy Iezzoni, and Kenneth Sink for guidance throughout this study.

Thanks to Dr. Michael Stine for the initial instruction in the isolation of cpDNA, and to Dr. Robert Jensen for the gift of tobacco cpDNA subclone library, my close friend Andrew David for the helpful discussion and proofreading of manuscript.

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## Introduction

Tissue culture techniques have been used to propagate superior genotypes, produce disease and herbicide-resistant plants by gene transfer, generate biochemical mutants to study metabolic pathways, and investigate the relationships between gene expression and plant development.

In tissue culture, long-term cultures often result in tissue culture-induced variability (D'Amato, 1977). This somaclonal variation is thought to arise from a combination of different chromosomal abnormalities, such as aneuploidy (Murashige and Nakano, 1967, for tobacco), polyploidy (Bayliss, 1973, 1975, for carrot), or chromosomal translocation (Jill Roth et al., 1989). Changes in DNA methylation have also been associated with the level of auxin in the culture medium, especially 2,4-dichlorophenoxy acetic acid (2,4-D) (LoSchiavo et al., 1989). In addition to nuclear genomic DNA changes in tissue culture, changes in plastid number and their structure have been observed in cells undergoing protoplast culture and plant regeneration (Thomas and Rose, 1983).

Plastids are characterized by their different morphology, pigmentation, and metabolic activities and are typically grouped into functional categories: proplastids, chloroplasts, chromoplasts, amyloplasts, etioplasts, and elaioplasts. In addition, plastid type is closely correlated to the cell type which contains them. Proplastids are the simplest and least differentiated type of plastid and are found in the zygote, root and shoot meristems, and reproductive tissue. Amyloplasts are non-green plastids which accumulate starch and are typically found in storage tissues such as cotyledons, endosperms and tubers. Elaioplasts

accumulate essential oils or fat. Chromoplasts are yellow, orange, or red plastids and are found in petals, fruits, and roots. Chloroplasts, which are the most functionally important and widespread member of the plastid family, contain the site of photosynthesis and occur in all green tissues. Finally, the etioplasts have a highly ordered paracrystalline region, called the prolamellar body, and protochlorophyllide, but no chlorophyll.

In plants, the main pathway of plastid development and interconversion was identified by Whatley (1977, 1978). This interconversion involves changes in proteins, size, internal structure, pigments, and sometimes alternations in DNA methylation-demethylation. The physiology, biochemistry, and genetics of amyloplasts, chromoplasts, etioplasts and chloroplast have been broadly studied (Boyer et al., 1989).

**DNA methylation**

It has been postulated that methylation is involved in the regulation of plastid DNA transcription. This is supported by evidence of different methylation sites in the various plastid types. In comparison to chloroplast DNA, chromoplast DNA and amyloplast DNA are methylated in regions of little or no transcriptional activity (Ngernprasirtsiri et al., 1988a, 1988b; Macherel et al., 1986). In addition, some plastid-specific gene expression has been reported. In the case of ripening tomato fruit, chloroplast-specific mRNA levels continuously decrease (Piechulla et al., 1985, 1986; Piechulla, 1988), while two chromoplast-specific polypeptides are either not detectable or barely detectable in chloroplasts (Hadjeb et al., 1988).

**Pigmentation**

In plants, pigments are usually found in the plastids and vacuoles. The green color of plants is due to chlorophyll, which is located in the chloroplasts. Carotenoids, the yellow to red pigments, are also found in chloroplasts but they are masked by chlorophylls. Flavonoids (anthocyanins and flavones or flavonols) are generally present in vacuoles and contribute colors other than green to plants. These pigments are functionally important for plant survival. Chlorophylls absorb light energy and transfer it to other participants in the photosynthetic process. Carotenoids are involved in trapping light for photosynthesis and probably protect chlorophyll from photo-oxidation. Flavonoid pigments are also important in attracting insects and birds during pollination and seed dispersal. The biosynthesis of these pigments has been well studied. The regulation, limiting factors, and major enzyme involved in the biosynthesis of each pigment are described below.

**A. Chlorophyll biosynthesis**

5-aminolaevulinic acid (ALA) is the universal precursor for the synthesis of chlorophyll and other tetrapyrroles. In higher plants, ALA is synthesized from glutamate, and its formation is the rate-limiting step for pigment accumulation during the greening of dark-grown cells and tissues following exposure to light (Beale and Castelfranco, 1973, 1974). Most of the ALA synthesizing capacity assayed *in organello* is dependent upon the light and dark treatments of the seedling prior to chloroplast isolation (Haung and Castelfranco, 1989). This light regulation was shown to be a low fluence phytochrome response (Haung et al., 1989). After incubation with ALA and then exposure to light, dark-grown cress seedlings (*Lepidium sativum L.*) showed a reduction in the rate of

greening and an inhibition of grana formation (Kittsteiner et al., 1991); thus, indicating that excess ALA inhibits normal plastid development.

**B. Carotenoid synthesis and conversion of chloroplasts to chromoplasts**

Carotenoids are members of the tetraterpenes. Their $C_{40}$ carbon skeletons are built by the successive addition of $C_5$ units to form geranylgeranyl pyrophosphate, a $C_{20}$ intermediate, which then condenses tail-to tail with another molecule of geranylgeranyl pyrophosphate to form phytoene. Phytoene, the $C_{40}$ condensation product, is dehydrated to yield lycopene. Cyclization of both ends of lycopene gives b-carotene.

In the conversion of green fruit to red fruit in *Capsicum annuum*, chlorophyll disappears and the amount of total carotenoids increases, especially the carotenoid precursors phytoene and phytofluene. These precursors were detected at the beginning stage of conversion and their contents increased up to the red stage (Camara, 1978). Changes in other carotenoids such as xanthophyll pigments were also observed. Lutein, the main xanthophyll of green fruits, decreased gradually, while violaxanthin and neoxanthin levels persisted during the ripening period.

**C. Flavonoid biosynthesis**

Flavonoids consist of two aromatic rings joined to form a chromatic structure by a three carbon unit ($C_6$-$C_3$-$C_6$). The phenylpropane residue (ring B and $C_{2,3,4}$) is derived from p-coumaric acid, itself formed via the shikimate pathway. Ring A is basically formed from acetate and is a rather special case of polyketide synthesis. Shikimate dehydrogenase (SKD) is the major enzyme of the shikimate pathway.

**D. Plant growth regulators**

It is known that cytokinins promote chloroplast development (Parthier, 1979) and that in the absence of kinetin plastids do not accumulate chlorophyll (Stetler and Laetsch, 1965). The application of cytokinins restores chlorophyll accumulation as demonstrated in cucumber, where the chlorophyll content of etiolated cotyledons increased 450 percent following cytokinin application (Fletcher and McCullagh, 1971). There is no evidence of an auxin influence on chlorophyll accumulation, chloroplast formation, or replication (Laetsch and Boasson, 1972; Schmerder et al., 1978).

**Physical and genetic mapping of chloroplast DNA**

The chloroplast genome is small relative to plant nuclear genomes and is encoded on a circular molecule. In angiosperms, the chloroplast genome contains two inverted repeats and ranges in size from 120 to 217 kb. Exceptions to this, species lacking one repeat region, are known only in the subfamily Papilonoideae of the legume family (Palmer and Thompson, 1982; Palmer et al. 1987). In gymnosperms, cpDNA maps have been developed for three species Gingko, Douglas-fir, and radiata pine (Palmer and stein, 1986; Strauss et al. 1988). In Douglas-fir and radiata pine, chloroplast genome lacks one inverted repeat region and is approximately 120 kb in size, Gingko differs from these species in having two inverted repeats and genome size 158 kb.

Changes in genome size accure from two processes: changes in the amount of repeated DNA and changes in sequence complexity. Both processes are important in cpDNA evolution (Palmer, 1990). Chloroplast DNA (cpDNA) RFLPs provide a tool for ascertaining amounts and patterns of species introgression (Govindaraju et al., 1988, Szmidt et al., 1988).

The comparison of cpDNA maps between different species is probably the best current procedure for developing phylogenies of plant species.

Generally, chloroplast genes are either single gene or organized and cotranscribed as multigene transcriptional units. Recombination or inversion of cpDNA results in cpDNA variation and typically takes place at the ends of the multigene units. Examples are rps2-atpI-atpH-atpF-atpA and rpoA-rpoB-rpoC1-rpoC2 (Herrmann et al., 1986; Hudson et al., 1987, 1988; Hudson and Mason, 1988).

**Objectives of this study**

The interconversion of plastids involves changes in gene expression for nuclear and cpDNA. Megagametophyte tissue of *Picea glauca* was used as explant to develop a tissue culture system that would allow the study of both these processes and the relationship between plant development and plastid differentiation. Megagametophpyte tissue was chosen because callus derived from megagametophyte tissue lacks pigmentation. A shoot regeneration protocol using megagametophyte-derived callus was developed (Chapter 1).

Factors that induced color change in megagametophyte callus from white to green, red, or brown were studied. These factors included plant growth regulators (cytokinins and auxin), chlorophyll precursors (glutamate and ALA), and heat shock (Chapter 2).

The differences between green and non-green tissue were also studied. These differences included expression of five different isozymes - aspartate aminotransferase (AAT), shikimate dehydrogense (SKDH), isocitate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PDH), and menadione reductase (MNR), as well as physical

characteristics - dry weight, chlorophyll concentration, chlorophyll a/b ratio, photosynthetic activity, protein amount and genomic DNA (Chapters 1 and 3). In addition, the physical map of cpDNA for *Picea engelmannii*, *P. glauca*, and *P.pungens* was determined (Chapter 4).

**Literature cited**


Bayliss, M.W. 1973. Origin of chromosome number variation in cultured plant cells. Nature 246:529-530.

---- 1975. The effects of growth in vitro on the chromosome complement of *Daucus carota* (L.) suspension cultures. Chromosoma (Berl.) 51:401-411.

Beale, S.I. and P.A. Castelfranco, 1973. $_{14}C$ incorporation from exogeneous compounds into 5-aminolevulinic acid by greening cucumber cotyledons. Biochem. biophys. Res. Cummun. 52:143-149.

---- 1974. the biosynthesis of 5-aminolevulinic acid in higher plants. II Formation of $_{14}C$-5-aminolevulinic acid from labeled precursors in greening plant tissues. Plant Physiol. 53:297-303.

Boyer, C.T., J.C. Shannon and R.C. Hardison (eds), 1989. Physiology, biochemistry, and genetics of nongreen plastids. The American Society of Plant Physiologists.

Camara, B. 1978. Les carotenoides du fruit de poivron *Capsicum annunum* pendant sa croissance et sa maturation:evolution des xanthophylles ceoniques. These $3^{O}$ Cucle Univ. Paris VI 80p.

Fletcher, R.A. and D. McCullagh, 1971. Cytokinin-induced chlorophyll formation in cucumber cotyledons. Planta 101:88-90.

Govindaraju, D., D.B. Wagner, G.P. Smith and B.P. Dancik, 1988. Chloroplast DNA variation within individual trees of a *Pinus banksiana* - *Pinus contorta* sympatric region. Can. J. For. Res. 18:1347-1350.

Hadjeb, N., I. Gounaris and C.A. Price, 1988. Chromoplast-specific proteins in *Capsicum annum* Plant Physiol. 88:42-45.

Herrmann, R., P. Westhoff, J. Tittgen and N. Nelson, 1985. Thylakoid membrane proteins and their genes. *In*. Molecular Form and Function of the Plant. G.P.S. Groot and T.C. Hall (eds) Plenum, pp.233-256.

Huang, L. and P.A. Castelfranco, 1989. Regulation of 5-aminolevulinic acid synthesis in developing chloroplasts. I. Effect of light/dark treatments *in vivi* and *in organello*. Plant Physiol. 90:996-1002.

---- 1990. Regulation of 5-aminolevulinic acid (ALA) synthesis in developing chloroplasts. III. Evidence for functional heterogeneity of the ALA pool. Plant Physiol. 92:172-178.

Huang, L., B.A. Bonner and P.A. Castelfranco, 1989. Regulation of 5-aminolevulinic acid (ALA) synthesis in developing chloroplasts. II. regulation of ALA-synthesizing capacity by phytochromone. Plant Physiol. 90:1003-1008.

Hudson, G.S. and J.G. Mason, 1988. The chloroplast genes encoding subu nits of the $H^+$-ATP synthase. Photosynth. Res., 18:205-222.

Hudson, G.S., J.G. Mason, T.A. Holton, B. Koller, G.B. Cox, P.R. Whitfeld and W. Bottomly, 1987. A gene cluster in the spinach and pea chloroplast genomes encoding one $CF_1$ and three $CF_0$ subunits of the $H^+$-ATP synthase complex and the ribosomal protein S2. J. Mol. Biol. 196:283-298.

Hudson, G.S., T.A. Holton, P.R. Whitfeld and W. Bottomley, 1988. Spinach chloroplast rpoBC genes encode three subunits of the chloroplast polymerase. J. Mol. Biol. 200:551-564.

Jill Roth, E., B.L. Frazier, N.R. Apuya and K.G. Lark, 1988. Genetic variation in an inbred plant:variation in tissue cultures of soybean [*Glycine max* (L.) Merrill]. Genetics 121:359-368.

Kittsteiner, U., A. Mostowska and W. Rudiger, 1991. The greening process in cress seedlings. I. Pigment accumulation and ultrastructure after application of 5-aminolevulinate and complexing agents. Physiol. Plant 81:139-147.

Kosinski, G., A.M. Gordon and I.R. Brown, 1988. Isozyme analysis of micropropagated *Betula pendula* Roth. *In*. M. Somatic Cell Genetics of Woody Plants. M.R. Ahuja (ed.) pp 143-145. Hluwer Academic Publishers.

Laetsch, W.M. and R. Boasson, 1972. Effect of growth regulators on organelle development. *In*. Hormonal regulation in Plant growth and development. H. Kaldewey and Y.J. Vardar (eds.) pp.454-465. Verlag Chemie. Weinheim.

LoSchiavo, F., L. Pitto, G. Giuliano, G. Torti, V. NutiRonchi, D. Masrazziti, R. Vergara, S. Orselli, and M. Terzi, 1989. DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypo-methylating drugs. Theor. Appl. Genet. 77:325-331.

Macerel, D., H. Kobayashi, E. Valle and T. Akazawa, 1986. Expression of amyloplast DNA in suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.) FEBS Lett 201:315-320.

Mayer, S.M. and S.I. Beale, 1990. Light regulation of 5-aminolevulinic acid biosynthetic enzymes and tRNA in *Euglena grcillis*. Plant Physiol. 94:1365-1375.

Murashige, T. and R. Nakano, 1967. Chromosome complement as a determinant of the morphogenetic potential of tobacco cells. Am. J. Bot. 54:963-970.

Ngernprasirtsiri, J., H. Kobayashi and T. Akazawa, 1988a. DNA methylation occured around lowly expressed genes of plastid DNA during tomato fruit development. Plant Physiol. 88:16-20.

---- 1988b. DNA methylation as a mechanism of transcriptional regulation in nonphotosynthetic plastids in plant cells. Proc. Natl. Acad. Sci. USA 85:4750-4754.

Palmer, J.D. 1990. Contrasting modes and tempos of genome evolution in land plant organelles. TIG 6:115-120.

Palmer, J.D. and D. Stein, 1986. Conservation of chloroplast genome structure among vascular plants. Curr Genet 10:823-833.

Palmer, J.D. and W.F. Thompson, 1982. Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. Cell 29:537-550.

Parthier, B. 1979. The role of phytohormones (cytokinins) in chloroplast development. Biochem. Physiol. Pflanzen 174:173-214.

Piechulla, B. 1988. Differential expression of nuclear and organelle encoded genes during tomato fruit development. Planta 174:505-512.

Piechulla, B., K.R.C. Imaly, and W. Gruissem, 1985. Plastid gene expression during fruit ripening in tomato. Plant Mol. Biol. 5:373-384.

Piechulla, B., E. Pichershy, A.R. Cashmore and W. Gruissem, 1986. Expression of nuclear and plastid genes for photosynthesis-specific proteins during tomato fruit development and ripening. Plant Mol. Biol. 7:367-376.

Schmerder, B., F. Rabenstein and H. Borriss, 1978. Steuerung des Ergundungsprozesses in den Kothledonen. Biochem. Physiol. Pflanzen. 173:97-113.

Stetler, D. and W.M. Laetsch, 1965. Kinetin-induced chloroplast maturation in cultures of tobacco tissue. Science 149:1387-1389.

Strauss, S.H., J.D. Palmer, G.T. Howe, and A.H. Doerksen, 1988. Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. Proc. Natl. Acad. Sci. USA 86:6030-6034.

Szmidt, A.E., Y.A. El-Kassaby, A. Sigurgeirsson, T. Alden, D. Lindgren and J. E. Hallgren, 1988. Classifying seed lots of *Picea sitchensis* and *P. glauca* in zones of introgression using restriction analysis of chloroplast DNA. Theor. Appl. Genet. 76:841-845.

Thomas, M. R. and R.J. Rose, 1983. Plastid number and plastid structural changes associated with tobacco mesophyll protoplast culture and plant regeneration. Planta 158:329-338.

Whatley, J.M. 1977. Variations in the basic pathway of chloroplast development. New Phytologist 78:407-420.

---- 1978. A suggested cycle of plastid developmental interelations. New phytologist 80:489-502.

## Chapter 1

## White Spruce (*Picea glauca* (Moench) Voss) Megagametophyte Culture


### Abstract

Shoot regeneration of white spruce (*Picea glauca*) mature megagametophyte derived callus was obtained using seeds that had been stored for six years. Megagametophyte calli were induced on MS medium (pH 5.8) supplemented with 2% sucrose, 1.0% agar, 10 uM 6-benzyl-aminopurine (BAP), and 10 uM 2,4-dichlorophenoxyacetic acid (2,4-D) for 3-4 weeks in the dark. Calli were subcultured on the same medium but in either low light (8 $uE/m^2/sec$) or high light (50 $uE/m^2/sec$). Megagametophyte calli remained white when grown under either light intensity.

When transferred to MS medium containing either 1 uM zeatin or the combination of 0.5 uM zeatin and 0.05 uM naphthaleneacetic acid (NAA), white calli produced organized green or green and red regions. Continued subculture and maintenance on the same medium resulted in shoot regeneration from the green regions. A chromosome number of 2n=24 was determined for all tissue types. Differential expression of isocitrate dehydrogenase (IDH), glutamate oxaloacetic acid transaminase (GOT)(also called aspartate aminotransferase; AAT), shikimate dehydrogenase (SKDH), glucose-6-phosphate dehydrogenase (G6PDH), and diaphrase (DIA) (also called menadione reductase; MNR) was observed in different tissues.

## Introduction

Constant progress has been made in the development of micropropagation and tissue culture systems for conifers during the past three decades. La Rue (1950), using *Cycas revoluta* was the first to obtain *in vitro* cell masses from mature gymnosperm megagametophyte tissue. Since that time, tissue culture systems have been developed for many conifer species. The successful *in vitro* propagation of conifers through organogenesis and embryogenesis by the use of cotyledon, hypocotyl, embryo, needle, pollen, and megagametophyte explants, and from cell suspension, and from protoplast cultures has been reviewed by Durzan and Campbell (1974), David (1982), Berlyn et al. (1986), Rohr (1987), Bonga et al. (1988), and Dunstan (1988).

There has been extensive work on the development of tissue culture systems for members of the genus *Picea*. Somatic embryogenesis from immature embryos and cotyledons has been reported in *Picea abies*, (Hakman et al., 1985; Hakman and von Arnold, 1985; Gupta and Durzan, 1986; von Arnold and Hakman, 1986; von Arnold, 1987; Krogstrup, 1986; Lelu et al. 1987), *P. sitchensis* (von Arnold and Woodward 1988), and *P. mariana* (Attree et al. 1990).

In white spruce, plantlet regeneration from different explants has been accomplished. Plantlets were first regenerated from hypocotyl and cotyledon segments (Campbell and Durzan, 1975, 1976). Currently, the successful development of somatic embryogenesis using immature embryos (Hakman and Fowke, 1987; Lu and Thorpe, 1987), mature embryos (Tremblay, 1990) or cultured shoots and cotyledons from stored seeds (Attree et al., 1989) as explants has made mass propagation of particular genotypes possible.

Isozymes have been used as markers to indentify the specific genotypes or hybrids and to study the variation in physiological condition during plant differntiation. Ernst et al. (1987) studied the isozyme pattern of Engelman spruce (*Picea engelmannii)* and blue spruce (*P. pungens*) and Gruber (1990) used phosphoglucoisomerase (PGI-2), which gave different banding patterns, to identify hybrids of Engelmann and blue spruce. In additon, variations in isozyme patterns demonstrate the various physiological and developmental states in intact plants (Scandalios 1974, 1977).

This study reports the development of a tissue culture system for mature megagametophytes of white spruce. Differential expression of isozymes among different explants and change in ploidy were also investigated.

## Materials and Methods

### Tissue culture

White spruce seeds that had been stored at 4$^{o}$C since 1984 were surface-sterilized for 12 minutes in 25% Clorox with 5-6 drops of Tween 20 (Sigma) and washed 3-4 times with sterilized, double distilled water. They were then placed on agar (0.4% or 0.6%) containing 3% sucrose and allowed to germinate in a growth chamber with an 18 hr light/6 hr dark photoperiod at 26$^{o}$C. The megagametophytes used as explants were removed from the seeds 1 to 2 weeks following germination.

Megagametophyte calli were induced on MS medium (pH 5.8) supplemented with 2% sucrose, 10 uM BAP, 10 uM 2,4-D, and either 0.8% or 1.0% agar. Cultures were stored in the dark at 26$^{o}$C. After 3-4 weeks, callus was subcultured on the same medium and stored in either low light (8 uE/m$^{2}$/sec) or high light (50 uE/m$^{2}$/sec) with an 18 hr light/6 hr dark

photoperiod at 26°C. After 6-8 months of subculture on the callus induction medium, white calli were transferred to MS medium (pH 5.8) with 2% sucrose, 0.6% agar, and either 1 uM zeatin or 0.5 uM zeatin and 0.05 uM NAA and then transferred to high light to stimulate shoot regeneration. When green spots appeared in the otherwise white callus, they were subcultured on white callus induction medium to establish green callus.

**Chromosome Analysis**

Chromosome number was examined in shoots, white calli, and green calli derived from white callus using the method of Dyer (1979).

**Isozyme studies**

Isozyme polymorphisms were analyzed in tissues from seeds, seedlings and calli. Tissue samples were homogenized in sample vials containing 200 ul of extraction buffer (100 mM Tris-HCl pH7.5, 5% w/v sucrose, 5% w/v PVP-40, 0.1% v/v 2-mercaptoethanol, 50 mM ascorbic acid, 10 mM diethiothreitol, 10 mM sodium metabisulfate and 0.1% w/v bovine serum albumin). All explants were derived from seeds collected from white spruce accession number 6719-0916. Seedlings and calli were germinated or induced from those seeds. The sample size and types are listed in Figure 3. Filter paper wicks 2 x 20 mm (Whatmann No.3) were dipped in the homogenate and inserted into a vertical slit at the gel origin. Two different samples of each tissue were loaded in each gel.

The composition of electrophoresis buffers and power requirements followed Cheliak and Pitel (1984) and Scandalios (1969). The buffer system for each enzyme: shikimate dehydrogenase (SKDH) EC 1.1.1.25, isocitrate dehydrogenase (IDH) EC 1.1.1.42, glucose-6-phosphate dehydrogenase (G6PDH) EC 1.1.1.49, aspartate aminotransferase (AAT) (also called glutamate aminotransferase; GOT) EC 2.6.1.1, and diaphrase (DIA)

(also called menadione reductase; MNR) and the staining method followed those described by O'Malley et al. (1980) and Conkle et al. (1982). Connaught starch lot 400-1 (Connaught Laboratories, Willowdale, Ontario, Canada) was used to prepare the 20 x 15 x 1.6 cm, 12.5% w/v starch gels.

## Results

### A. Callus induction

Seed germination averaged 82.0%. Initial experiments using combinations of BAP (1 uM, 10 uM and 25 uM) and 2,4-D (1 uM and 10 uM) and 20 explants of each treatment on 0.6% agar solidified medium yielded a low callus induction rate (8.6%). Medium containing 10 uM BAP and 10 uM 2,4-D gave the highest callus induction rate (16.7%). Therefore this combination was selected for the subsequent experiments. Increasing the agar concentration to 0.8% resulted in increased rate of callus induction to 28.2% (22/78) for megagametophytes 1 week after seed germination and 10.5% (4/38) for megagametophytes 2 weeks after seed germination. Megagametophyte callus grown on 0.8% agar stopped growing after two or three subcultures. Increasing the agar concentration to 1% and using megagametophytes 2 weeks after seed germination on either 0.6% or 0.4% agar resulted in callus induction rates of 40.5% (17/42) and 35.7% (15/42), respectively. The megagametophyte calli grew well but slowly after several subcultures. When callus size reached 1 cm in diameter, the calli were maintained in the callus induction medium with the agar concentration changed to 0.6 %.

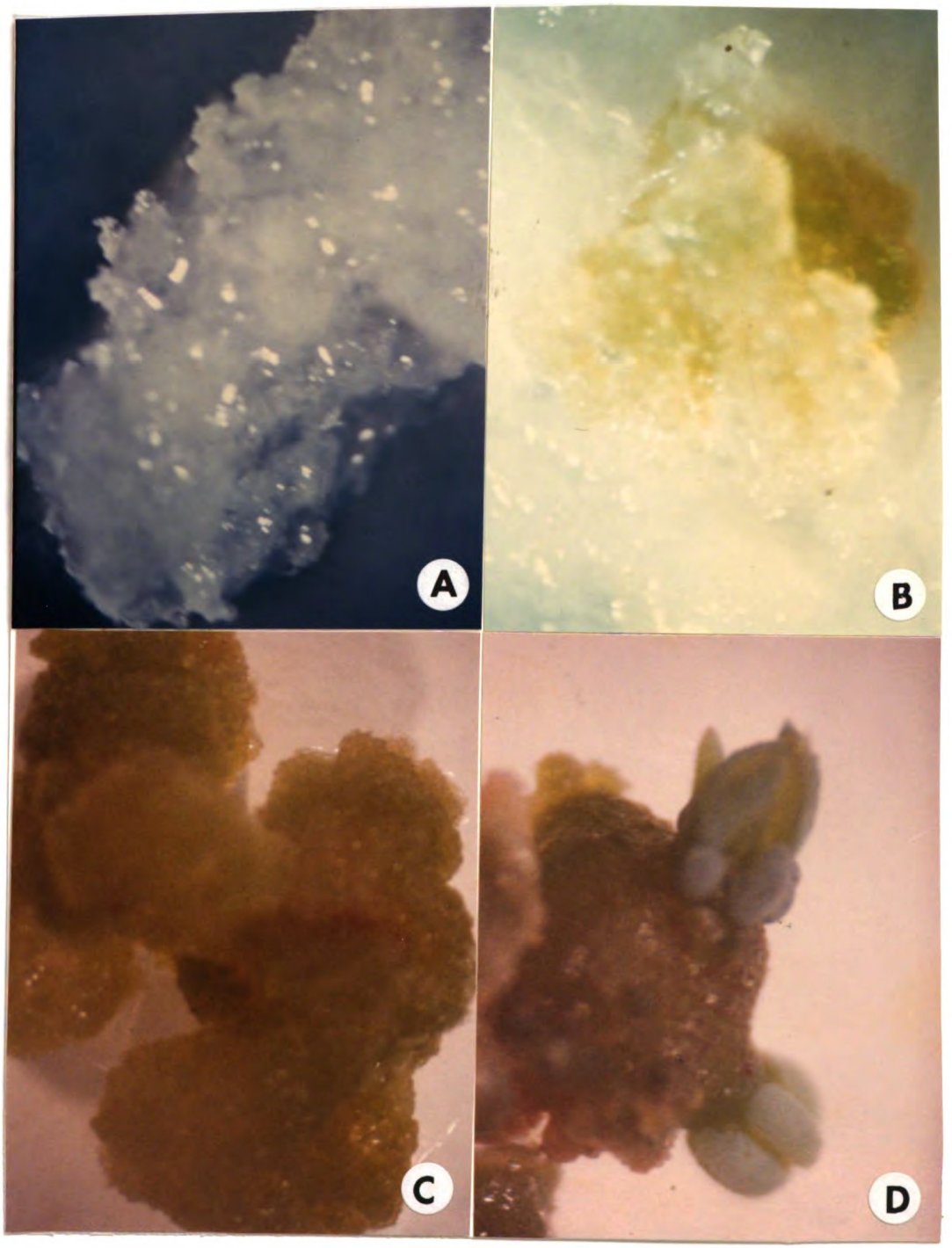


Figure 1. Tissue culture of the megagametophytes of white spruce. A) 1-year-old white callus. B) green regions on white callus after transferring the callus on the shoot regeneration media. C) green callus derived from white callus. D) shoot regenerated from green regions of white callus.

**B. Shoot regeneration**

One to two months following transfer onto MS medium with either 1 uM zeatin or a combination of 0.5 uM zeatin and 0.05 uM NAA, green colored regions developed on the surface of most calli. The surface of these green regions was either smooth or rough. After subculturing for 2-3 months, shoots regenerated from the smooth surface of the green regions (Figure 1). More than forty shoots were regenerated. Actual shoot length was not measured, however, it was noted that shoot elongation ceased when shoots reached approximately 3 mm in length. Root regeneration was attempted using 1/10 strength MS without sucrose and lacking plant growth regulators, but none occurred.

**C. Chromosome Studies**

Despite the fragility of callus tissues and the low frequency of white and green callus cells undergoing cell division, approximately 20-30 callus cells could be found for each tissue type that were suitable for use in counting chromosome numbers. The chromosome number of shoots, white calli, and green calli derived from white callus; was 2n=24 for all cells (Figure 2). However some cells showed chromosome overlapping occurred. Despite chromosome overlap, chromosome number of callus cells were clearly higher than the haploid number of 12, but chromosomal abnormalities such as aneuploidy can not be excluded.

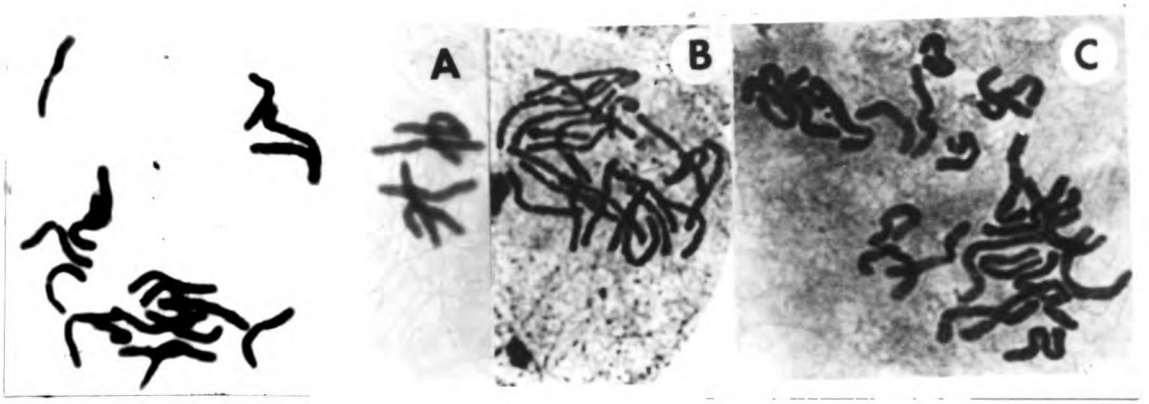


Figure 2. The chromosome complements at mitotic metaphase. Magnification of each picture was 800 folds. (a) shoot cell of seedlings. (b) white callus cell. (e) green callus derived from embryo.

**D. Isozyme studies**

Both the staining intensity of individual bands and the presence or absence of bands for specific isozymes varied among the different tissue types that were used for sample extraction. A schematic representation of the isozyme banding patterns is shown in Figure 3.

The IDH isozyme banding pattern showed that variation among tissues was mainly observed in the faster zone (Figure 3A). The band at Rf=0.20 appeared in all samples and was highly expressed in both green callus types. The band at Rf=0.33 did not appear in white callus and was expressed at a lower level in root, stem, and needle tissues than that observed in green callus. One extra band at Rf=0.23 was present only in samples from green calli derived from embryo.

The AAT isozyme banding pattern showed that two zones of activity were evident on gels (Figure 3B). White calli did not have the band at Rf=0.29. White calli and green calli derived from white calli showed less intensity at band Rf=0.47 than the other tissues. Root, stem, and needle tissues produced low intensity bands at Rf=0.23 and 0.29. Interestingly, an extra band was observed in samples from embryos (Rf=0.53) and both green callus types (Rf=0.25).

Two zones of activity of DIA (MNR) isozyme were observed (Figure 3C). Bands Rf=0.67 and 0.71 did not appear in white callus, embryo, and megagametophyte samples. The band at Rf=0.46 was only observed in seedling samples.

Only one band (Rf=0.17) was observed for SKDH. It was present in all samples except those from white calli (Figure 3D).

Three G6PDH isozyme bands were observed (Figure 3E). In white callus, the band at Rf=0.31 was barely visible, and band at Rf=0.39 was very weakly expressed. Both green calli showed high intensity at band with Rf=0.24. The band shown at Rf=0.39 had the highest staining intensity in megagametophyte samples.

## Discussion

Immature megagametophyte tissue has typically been the explant of choice in studying organogenesis or embryogenesis *in vitro*. The problem with using immature seeds for megagametophyte culture is timing the collection period to coincide with the desired developmental stage. Using mature seeds to develop the megagametophyte culture circumvents that problem and has the advantage of long term storage.

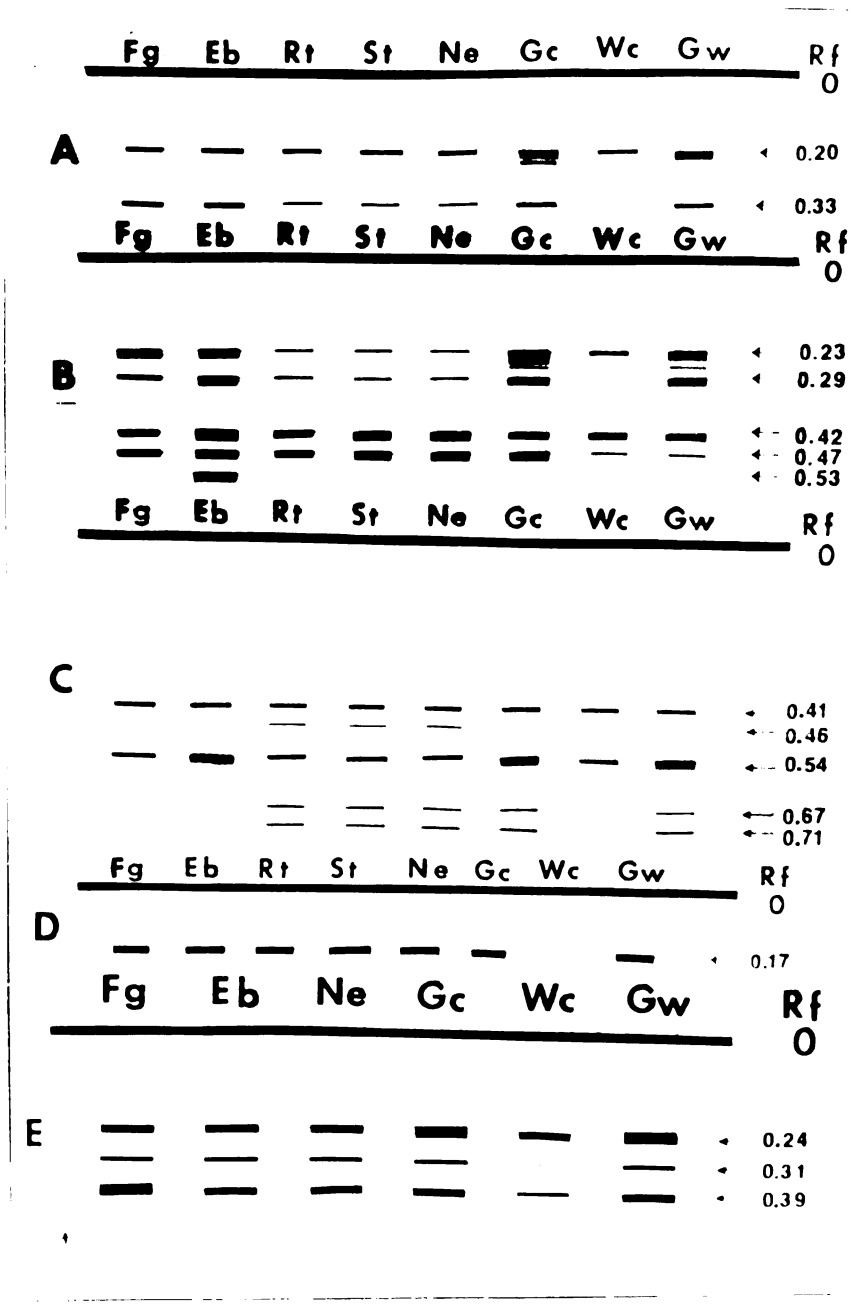


Figure 3. Representative diagrams corresponding to 5 different isozyme loci for different materials. A. IDH isozyme pattern. B. AAT (GOT) isozyme pattern. C. DIA (MNR) isozyme pattern. D. SKDH isozyme pattern. E. G6PDH isozyme patterns. Fg:megagametophytes of 7 seeds. Eb:embryos of 7 seeds. Rt:Roots of 5 seedling. St:shoots of 5 seedlings. Ne:needles of 5 seedlings. Gc:green calli derived from embryo. Wc: white megagametophyte callus. Gw:green calli derived from white callus.

When the agar concentration in the callus induction medium was increased from 0.6 % to 1%, the callus induction rate increased. This suggests that moisture content in the medium may play an important role in developing the success and rate of callus induction, especially during the first few subcultures. The opposite relationship appears to exist during seedling germination where megagametopyte tissues from seedlings germinated on 0.4% agar, overlaid with water on seeds, had a higher callus induction rate than those germinated on 0.6% agar.

The morphogenetic capacity of megagametophyte tissues *in vitro* is often determined by the developmental stage. Immature seeds are often used to initiate megagametophyte cultures. In general megagametophyte tissue of gymnosperms has been far more responsive *in vitro* than the microspore tissue. In this study, shoot regeneration was obtained from callus derived from mature megagametophyte tissue, but shoot growth arrested at about 3 mm in length and the shoots failed to regenerate roots. The attenuated shoot growth may have resulted from many factors, including media that were not suitable for continued shoot development.

The ploidy level of mature megagametophyte tissue is often determined from the callus derived from the megagametophyte tissue. In previous studies, callus from megagametophyte tissue remained haploid after five months in culture (Bonga, 1974, 1981), became dihaploid for almost all of the cells after several months in culture (Berlyn, 1968), or had mixed ploidy levels (70% haploid, 25% dihaploid, and small number of tetraploid) after six months in callus culture (Renfroe and Berlyn, 1986). The diploid chromosome number of white spruce is 2n=24 (Sax and Sax 1933). In the case of mature megagametophyte calli of white spruce, the chromosome number (2n=24) indicated the calli may have originated

from haploid tissues through the nuclei fusion of the multinucleate cells, endomitosis, or chromosome non-disjunction. In this study, it may be that the long subculture period resulted in chromosome doubling, but for whatever reason, all countable samples showed a diploid chromosome number, consequently no haploid white callus cell lines were identified.

Isozyme patterns frenquently vary in intact plants as a function of various physiological and developmental stages (Scandalios, 1974) and similar responses have been demonstrated in cultured cells of tobacco (Lee, 1972a,b). In tobacco, kinetin and zeatin cause the appearance of new isozyme forms, as does gibberellin $GA_3$ in the presence of IAA. During shoot regeneration from callus, changes in the expression of the isozymes peroxidase (Thorpe and Gaspar, 1978) or esterase (Kosinski et al., 1988) were observed. This differential expression of isozymes from shoots regenerated from the same genotype explant indicates that culture conditions can cause epigenetic effects. Moreover, it has been shown that enzymes or isozymes for carbohydrate metabolism associated with plastid type differ between green tissue and etiolated tissues (ap Rees and Entwitle, 1989; Keeling, 1989; Madore, 1990; Frehner et al., 1990). The data from this study showed differential expression of isozymes in different tissues. In white callus, isozyme IDH (band Rf=0.33) and AAT (band Rf=0.29) were absent (Figure 3) in comparison with green calli derived from white calli and other materials. This could be explained by either differential expression of both isozymes or the existence of null alleles in the genotypes used in this study. Given that the green calli were established using white callus explants, it most likely indicates differential gene expression between these two callus types. In addi-

tion, differential expression of isozymes IDH, AAT, DIA (MNR), SKDH, and SKDH was observed in various tissues.

White megagametophyte callus and green callus derived from white callus responded differently when placed on shoot regeneration media. Although shoots arose from small isolated green regions in white calli, no shoot regeneration from green calli derived from white calli has been established. However, white calli and green calli maintain their original color white or green under both high and low light regimes. Furthermore, green calli is inducible from white calli and cytokinins can stimulate chloroplast development (Partier, 1979).

The prospects for mature megagametophyte culture of *Picea glauca* are promising. The success of using mature megagametophyte tissue to produce megagametophyte derived callus and plantlets would be an advantage to tree breeding programs in terms of a long-term supply of haploid derived sources. The lack of root regeneration and failure to obtain continued shoot elongation currently pose significant barrier to the commercial application of this process. Future research should concentrate on the regeneration of plantlets and the mechanisms underlying embryogenesis.

The differential expression of isozymes between white callus and green callus derived from white callus along with the inducible change of white callus to green callus provides an interesting mechanism for studying the genetic regulation of this essential metabolic process in plants.

**Literature Cited**


ap Rees, T. and G. Entwistle, 1989. Entry into the amyloplast of carbon for starch synthesis. *In*. Physiology, biochemistry, and Genetics of Nongreen Plastids. C.T. Boyer, J.C. Shannon and R.C. Hardison (eds.) The American Society of Plant Physiologists. pp49-62.

Attree, S.M., S. Budimir, and L.C. Fowke, 1989. Somatic embryogenesis and plantlet regeneration from cultured shoots and cotyledons of seedlings from stored seeds of black and white spruce (*Picea mariana* and *Picea glauca*). Can. J. Bot. 68:30-34.

Berlyn, G.P., R.C. Beck and M.H. Renfroe, 1986. Tissue culture and the propagation and genetic improvement of conifer:problems and possibilities. Tree Physiol. 1:227-240.

Bonga, J.M. 1974. *In vitro* culture of microsporophylls and megagametophyte tissue in *Pinus*. In vitro 9:270-277.

Bonga, J.M. 1981. Haploid tissue culture and cytology of conifers. coll. Int. Cult. *in vitro* Essences Forestieres. IUFRO, Fontainebleau, France. AFOCEL pp283-293.

Bonga, J.M., P. von Aderkas, and D. James, 1988. Potential application of haploid cultures of tree species. *In*. Genetic Manipulation of Woody Plants. J.W. Hanover and D.E. Keathley (eds.) Plenum Press, New York. pp.57-77.

Borchert, R. 1968. Spontane Dipoidisierung in Gewebekulturendes Megagametophyten von *Pinus lambertiana*. Z. Pflanzenphysiol. 59:389-392.

Campbell, R.A. and D.J. Durzan, 1975. Induction of multiple buds and needles in tissue culture of *Picea glauca*. Can. J. Bot. 53:1652-1657.

---- 1976. Vegetative propagation of *Picea glauca* by tissue culture. Can. J. For. Res. 6:240-243.

Cheliak, W.M. and J.A. Pitel, 1984. Genetic control of allozyme variants in mature tissues of white spruce trees. J. Hered. 75:34-40.

Conkle, M.T., P.D. Hodgskiss, L.B. Nunnally and S.C. Hunter, 1982. Starch gel eletrophoresis of conifer seeds: a laboratory manual. USDA For. Serv. Gen. Tech. Rep. PSW-64.

David, A. 1982. In vitro propagation of gymnosperms. *In*. Tissue Culture in Forestry. J.M. Bonga and D.J. Durzan (eds.) Martinus Nijhoff/Dr. W. Junk Publishers. pp.72-108.

Dunstan, D. 1988. Prospects and progress in conifer biotechnology. Can. J. For. Res. 18:1497-1506.

Durzan, D.J. and Campbell 1974. Prospects for the mass production of improved stock of forest trees by cell and tissue culture. Can. J. For. Res. 4:152-174.

Dyer, A.F. 1979. Investigating Chromosomes. Edward Arnold publisher. 138 p.

Ernst, S.G., D.E. Keathley and J.W. Hanover, 1987. Inheritance of isozymes in seed and bud tissues of blue and Engelmann spruce. Genome 29:239-246.

Frehner, M., J. Pozueta-Romero and T. Akazawa, 1990. Enzyme sets of glycolysis, gluconeogenesis, and oxidative pentose phosphate pathway are not complete in nongreen highly purified amyloplasts of sycamore (*Acer pseudoplatanus* L.) cell suspension cultures. Plant Physiol. 94:538-544.

Gruber, K. 1990. Morphological trait analysis of synthetic *Picea engelmanni* Parry x *P. pungens* Engelm. hybrids. MS thesis. 59p.

Gupta, P.K., D.J. Durzan and B.J. Finkle, 1987. Somatic polyembryogenesis is embryogenic cell masses of *Picea abies* (Norway spruce) and *Pinus taeda* (loblolly pine) after thawing from liquid nitrogen. Can. J. For. Res. 17:1130-1134.

Hakman, I., L.C. Fowke, S. von Arnold and T. Eriksson, 1985. The development of somatic embryos in tissue cultures initiated from immature embryoes of *Picea abies* (Norway spruce). Plant Sci. 38:53-59.

Hakman, I. and S. von Arnold, 1985. Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). J. Plant. Physiol. 121:149-158.

Hakman, I and L.C. Fowke, 1986. Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). Can. J. Bot. 65:656-659.

Keeling, P.L. 1989. Evidence against triose phosphate transport into amyloplasts of developing wheat and maize grain. *In*. Physiology, Biochemistry, and Genetics of Nongreen plastids. C.T. Boyer, J.C. Shannons and R.C. Hardison (eds) The American Society of Plant Physiologists. pp.63-78.

La Rue, C.D. 1950. Regeneration in *Cycas*. Am J Bot 37:674

Lee, T.T. 1972a. Changes in IAA oxidase isozymes in tobacco tissue after treatment with 2,4-dichlorophenoxyacetic acid. Plant Physiol. 49:957-960.

---- 1972b. Interaction of cytokinin, auxin, and gibberellin on perioxidase isozymes in tobacco tissues cultured in vitro. Can. J. Botany 50:2471-2477.

Lelu, M-A., M. Boulay and Y. Arnold, 1987. Obtension de cals embryogenes a Partir de cotyledons de *Picea abies* (L.) Karst. preleves sur de jeunes plantes agees de 3 a 7 jours apres germination. C. R. Acad. Sci. Ser. III Sci Vie. 305:105-109.

Lu, C-Y. and T.A. Thorpe, 1987. Somatic embryogenesis and plantlet regeneration in cultured immature embryos of *Picea glauca*. J. Plant Physiol. 128:297-302.

Madore, M.A. 1990. Carbohydrate metabolism in photosynthetic and nonphoosynthetic tissues of variegated leaves of *Coleus blumei* Benth. Plant Physiol. 93:617-622.

O'Malley, D.M., N.C. Wheeler and R.P. Guries, 1980. A manual for starch gel electrohoresis. Univ. Wis. Dep. For. Staff Pap. no.11.

Renfroe, M.H. and G.P. Berlyn, 1986. Variation in nuclear DNA content in *Pinus taeda* L. tissue culture of diploid origin. J. Plant Physiol. 121:131-139.

Rohr, R. 1987. Haploids (Gymnosperms). *In*. Cell and Tissue Culture in Forestry. J.M. Bonga and D.J. Durzan (eds.) Volume 2 p230-246. Martinus Wijhoff Publisher, Dordrecht, The Netherlands.

Sax, K. and H.J. Sax, 1933. Chromosome number and morphology in the conifers. Jour. Arnold Arbor. 15:255-262.

Scandalios, J.G. 1969. Genetic control of multiple molecular forms of enzymes in plants:a review. Biochem. Genet. 3:37-39.

Scandalios, J.G. 1974. Isozymes in development and differentiation. Annu. Rev. Plant Physiol. 25:255-258.

---- 1977. Regulation of isozyme patterns in plant cell differentiation. *In:* Cell differentiation in Microorganisms, Plants and Animals, International Symposium. Nover, L. and K. Mothes (eds.) pp.467-483. North-Holland, Amsterdam.

Thorpe, T.A. and T. Gaspar, 1978. Changes in isoperoxidases during shoot formation in tobacco callus. In Vitro 14:231-235.

Tremblay, F.M. 1990. Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca* Can. J. Bot. 68:236-242.

von Arnold, S. 1987. Improved efficiency of somatic embryogenesis in mature embryos of *Picea abies*. J. Plant Physiol. 128:233-244.

von Arnold, S. and I. Hakmann, 1987. Regulation of somatic embryo development in *Picea abies*, with emphasis on ABA effects. J. Plant Physiol. 122:261-265.

## Chapter 2

**Factors that effect pigmentation change in white megagametophyte callus of *Picea glauca***


**Abstract**

When grown on MS medium containing 10 uM 6-benzylaminopurine (BAP) and 10 uM 2,4-dichlorophenoxyacetic acid (2,4-D) spruce megagametophyte tissue produced white callus. Transfer of white calli to MS medium containing BAP or zeatin resulted in a change of color to green, red, or brown. The effects of zeatin differed from BAP, causing the color change to green, but not to red or brown. The addition of 2,4-D to the MS medium inhibited the color change induced by the cytokinins, especially the development of green and red regions. White callus also showed different responses to the addition of glutamate and 5-aminolevulinic acid (ALA) to the medium. Glutamate caused browning of white calli, while ALA alone or glutamate and ALA together caused no change in color. Exposure to 37$^{o}$C for an hour also changed the response of white calli to BAP and zeatin by significantly increasing browning while decreasing the development of green regions.

## Introduction

Plastids are characterized by differences in their morphology, pigmentation and metabolic activities. Their differentiation is governed by developmental and environmental factors which alter the gene expression of the plastid and nuclear genomes. Changes in both nuclear and plastid gene expression are involved in plant developmental processes (Piechulla et al., 1986; Piechulla, 1988; Kuntz et al., 1989), and changes in plastid polypeptide composition have been observed during the greening process of etiolated peas (Dietz and Bogorad, 1987).

In plants, the main pathway of plastid development has been identified by Whatley (1977, 1978). The interconversion of plastids includes changes in protein compliments, plastid size, internal structure, and pigments. Cytokinins have been linked to the promotion of chloroplast development (Parthier , 1979), and it has been shown that in the absence of kinetin plastids do not accumulate chlorophyll (Stetler and Laetsch, 1965). This was also convincingly shown in etiolated cucumber cotyledons that the chlorophyll content increased 450 percent after a 14 hour cytokinin pretreatment (Fletcher and McCullagh, 1971). No evidence for an auxin influence on chlorophyll accumulation, chloroplast formation, or replication has been found (Laetsch and Boasson, 1972; Schmerder et al., 1978).

The chlorophyll molecule has two major parts, porphyrin and phytol and is a member of the metallotetrapyroles, which have been referred to as "pigments of life" because of their importance in living systems. ALA is the universal precursor for the synthesis of chlorophyll and other tetrapyrroles, and in higher plants ALA is synthesized from glutamate (Beale and Castelfranco, 1973, 1974). ALA formation is the rate-limiting

step for pigment accumulation during the greening of dark-grown cells and tissues when exposed to light (Beale and Castelfranco, 1974). This light regulation was shown to be a low fluence phytochrome response (Huang et al. 1989)

This paper reports inducible changes in the color of white megagametophyte callus of *Picea glauca*. The role of different cytokinins, chlorophyll precursors (glutamate and ALA), and heat shock in this process were investigated using callus cultures grown in a controlled environment.

## Materials and Methods

### Subculture Media

All white megagametophyte callus of *Picea glauca* used for the following experiments was maintained on the MS medium (pH 5.8) supplemented with 10 uM 2,4-D and BAP, 2% sucrose, and 0.6% agar (see chapter 1). Cultures were subcultured at 4 week intervals. All experiments were initiated with callus that had been maintained in culture for at least 8 months.

### Standard Media

White calli were transferred onto MS medium (pH 5.8) containing different sucrose concentrations (0.5%, 1%, 2%, 3%, 4%), and either 0.6% agar or without agar. Changes in coloration were scored after 3 and 7 weeks in culture. A 2% sucrose concentration was chosen as a component of the standard medium (MS medium with 2% sucrose, either with 0.6% agar or lacking agar). All cultures were grown in a controlled environment chamber under an 18 hr light / 6 hr dark photoperiod at 26$^{o}$C. Two

light intensities, high (50 uE/$m^2$/sec) and low (8 uE/$m^2$/sec) were used.

**Scoring Coloration**

Callus coloration was scored as: Red: regions of white callus developing red color; Green: regions of white callus developing green color; Brown: regions of white callus developing brown color; White: no regions developing any other color. An individual callus could be scored for more than one color.

**Plant growth regulators (PGR)**

**Cytokinins:** Eighteen explants of each treatment (six calli per perti-dish) were grown on 20 ml of the standard solid medium with different levels of either zeatin (0.4 uM, 1 uM, and 2.5 uM) or BAP (5 uM, 10 uM, and 15 uM) and either high light or low light intensity.

**Auxin with cytokinin:** A different concentration of 2,4-D (1 uM, 2 uM, 5 uM, and 10 uM) and BAP (4 uM, 5 uM, and 10 uM) and thirty explants of each treatment under high light regimes were used to test the effects of the interaction of BAP and 2,4-D on the color change. After 4 weeks, color change was recorded as green, red or brown color.

**Glutamate and ALA**

Treatments containing liquid standard medium with no glutamate, 20 mM glutamate, 3 mM ALA, and 20 mM glutamate with 3 mM ALA were tested using twenty explants for each treatment (one callus in each 35 x 10 mm petri dish). These calli were grown in 5 ml of liquid medium in the dark. Coloration of calli was scored at 2 and 4 weeks.

The long-term effects of glutamate and ALA were studied by maintaining calli in the same petri dish without subculture for up to nine weeks. Individual white calli were placed in the dark in either 5 ml of

liquid standard medium (sixteen calli per treatment and each in one petri dish) or 20 ml of solid standard medium (twenty-five calli per treatment and five calli per petri dish 100 x 15 mm) with the same treatments. The color was scored at 3, 7, and 9 weeks.

**Heat shock**

White calli were exposed to 37°C for 0 min, 30 min, or 60 min. Fifteen white calli of each treatment (five per petri-dish) were transferred onto standard solid medium with zeatin (0.4 uM, 1 uM, and 2.5 uM) or BAP (5 uM, 10 uM, and 15 uM) under the high light regime. Changes in callus color were scored after 4 weeks.

**Statistical analysis**

The data for color change were analyzed for each experiment using the Multiple General Linear Hypothesis Analysis Program in Systat (version 4.0). The data for each treatment were separated by Duncan's New Multiple-Range Test (Steele and Torrie, 1980) if the MGLH-ANOVA indicated significance.

## Results

White callus underwent color changes in the absence of plant growth regulators in the MS culture medium supplemented with different sucrose concentration. After three weeks in culture no change in color was observed in calli grown on media containing 1% or 2% sucrose. Decreasing the sucrose concentration to 0.5% resulted in 8% browning. Increasing the sucrose concentration to 3% or 4% resulted in an even more pronounced change, with 24% and 27% browning, respectively. Following seven weeks of growth without subculture, brown color was observed at the rate of 24%, 64%, 67%, 61%, and 52% for 0.5%, 1%, 2%, 3%, and 4%

sucrose concentrations, respectively. No other color (green or red) was observed in this experiment.

**A. The effects of plant growth regulators**

**Cytokinins (zeatin and BAP):**

The addition of cytokinins to the standard medium caused a color change from white to green, red and brown (Table 1 and 2). Differences in color change between zeatin and BAP were statistically analyzed. Zeatin and BAP have significantly different effects on the induction of green color, but not on the induction of red and brown color (Table 2). Analysis of the data in Table 1 (ANOVA not shown) indicated there was also a significant interaction between light and cytokinins on color change to red and brown coloration. The BAP treatment averages for red color in high light and low light were 33% and 5%, respectively. The comparable values for the zeatin treatments were 3% and 8%. Calli which did not change color were not scored in this experiment.

Table 1. The effects of cytokinin level and light intensity on color change of white callus.

| Light | Explants | Zeatin(uM) | BAP(uM) | Green | Red | Brown |
|---|---|---|---|---|---|---|
| high | 18 | 0 | 5 | 0.78(14/18) | 0.28(5/18) | 0.56(10/18) |
| low | 18 | 0 | 5 | 0.61(11/18) | 0.17(3/18) | 0.44(8/18) |
| high | 18 | 0 | 10 | 0.61(11/18) | 0.61(11/18) | 0.17(3/18) |
| low | 18 | 0 | 10 | 0.72(13/18) | 0(0/18) | 0.44(8/18) |
| high | 18 | 0 | 15 | 0.83(15/18) | 0.11(2/18) | 0.33(6/18) |
| low | 18 | 0 | 15 | 0.61(11/18) | 0(0/18) | 0.44(8/18) |
| high | 18 | 0.4 | 0 | 0.50(9/18) | 0(0/18) | 0.17(3/18) |
| low | 18 | 0.4 | 0 | 0.50(9/18) | 0.06(1/18) | 0.50(9/18) |
| high | 18 | 1 | 0 | 0.39(7/18) | 0.06(1/18) | 0.28(5/18) |
| low | 18 | 1 | 0 | 0.22(4/18) | 0.06(1/18) | 0.61(11/18) |
| high | 18 | 2.5 | 0 | 0.17(3/18) | 0(0/18) | 0.89(16/18) |
| low | 18 | 2.5 | 0 | 0.28(5/18) | 0.11(2/18) | 0.33(6/18) |

Table 2. Average of green, red, and brown color in white callus grown on solid standard medium containing zeatin or BAP.

| Cytokinin | Green | Red | Brown |
|---|---|---|---|
| Zeatin | 0.34±0.14a | 0.05±0.04a | 0.46±0.26a |
| BAP | 0.69±0.10b | 0.20±0.23a | 0.40±0.13a |

1) Mean ± SE of color induced from white calli.
2) Values followed by different letters are significantly different by Duncan's NMR test (alpha = 0.05).

**The effect of 2,4-D with BAP:**

The addition of both 2,4-D and BAP on the color change of white callus is shown in Table 3. The addition of both growth regulators resulted in a reduction in the amount of color change when compared to calli grown on standard medium containing only cytokinins. Only one treatment (5 uM BAP and 2,4-D) resulted in calli containing any green regions and no red coloration was observed. High levels of 2,4-D were also associated with a reduction of brown color in the calli.

Table 3. The effects of different concentrations of 2,4-D and BAP on the greening and browning of white callus.

| 2,4-D (uM) | BAP (uM) | 2,4-D / BAP | Explant number | White | Green | Brown |
|---|---|---|---|---|---|---|
| 1 | 10 | 0.10 | 30 | 0.83(25/30) | 0(0/30) | 0.17(5/30) |
| 1 | 4 | 0.25 | 30 | 0.97(29/30) | 0(0/30) | 0.03(1/30) |
| 2 | 10 | 0.20 | 30 | 1(30/30) | 0(0/30) | 0(0/30) |
| 5 | 10 | 0.50 | 30 | 1(30/30) | 0(0/30) | 0(0/30) |
| 5 | 5 | 1.00 | 30 | 0.97(29/30) | 0.03(1/30) | 0(0/30) |
| 10 | 10 | 1.00 | 30 | 1(30/30) | 0(0/30) | 0(0/30) |

Table 4. The effects of the Glu and ALA on the color change of the white calli on different media.

| Treatments | Explants | Weeks | Media | White | Brown |
|---|---|---|---|---|---|
| Control | 25 | 3 | Solid | 1(25/25) | 0(0/25) |
| 20 mM Glu | 25 | 3 | Solid | 0.40(10/25) | 0.60(15/25) |
| 3 mM ALA | 25 | 3 | Solid | 0.92(23/25) | 0.08(2/25) |
| 20 mM Glu + 3 mM ALA | 25 | 3 | Solid | 0.96(24/25) | 0.08(2/25) |
| Control | 25 | 7 | Solid | 0.40(10/25) | 0.60(15/25) |
| 20 mM Glu | 25 | 7 | Solid | 0(0/25) | 1.00(25/25) |
| 3 mM ALA | 25 | 7 | Solid | 0.92(23/25) | 0.08(2/25) |
| 20 mM Glu + 3 mM ALA | 25 | 7 | Solid | 0.84(21/25) | 0.16(4/25) |
| Control | 25 | 9 | Solid | 0.36(9/25) | 0.64(16/25) |
| 20 mM Glu | 25 | 9 | Solid | 0(0/25) | 1(25/25) |
| 3 mM ALA | 25 | 9 | Solid | 0.64(16/25) | 0.36(9/25) |
| 20 mM Glu + 3 mM ALA | 25 | 9 | Solid | 0.80(20/25) | 0.20(5/25) |
| Control | 16 | 3 | Liquid | 1.00(16/16) | 0(0/16) |
| 20 mM Glu | 16 | 3 | Liquid | 0.50(8/16) | 0.50(8/16) |
| 3 mM ALA | 16 | 3 | Liquid | 1.00(16/16) | 0(0/16) |
| 20 mM Glu + 3 mM ALA | 16 | 3 | Liquid | 1.00(16/16) | 0(0/16) |
| Control | 16 | 7 | Liquid | 0.25(4/25) | 0.63(10/16)* |
| 20 mM Glu | 16 | 7 | Liquid | 0(0/16) | 1(16/16) |
| 3 mM ALA | 16 | 7 | Liquid | 0.88(14/16) | 0.06(1/16)* |
| 20 mM Glu + 3 mM ALA | 16 | 7 | Liquid | 0.94(15/16) | 0.06(1/16) |
| Control | 16 | 9 | Liquid | 0.25(4/16) | 0.56(9/16)* |
| 20 mM Glu | 16 | 9 | Liquid | 0(0/16) | 1(16/16) |
| 3 mM ALA | 16 | 9 | Liquid | 0.63(10/16) | 0.25(4/16)* |
| 20 mM Glu + 3 mM ALA | 16 | 9 | Liquid | 0.80(12/16) | 0.13(2/16)* |

Control:standard solid or liquid medium.
`*' indicates that remaining calli developed yellow color.

**B. The effects of Glutamate and ALA**

No green regions developed on calli when either glutamate or ALA were added to the standard medium. Red and brown colored regions appeared on the some of the white calli (Tables 4 and 5). However, the glutamate and ALA treatments had different effects on callus color change. Following the addition of glutamate and ALA (Tables 4, 5, and 6); the calli either showed no color change or developed brown regions. No differences in color change were observed among cultures on solid or liquid standard medium. Media augmented with ALA alone or ALA in combination with glutamate resulted in a high percentage of calli that remained white and which rarely became brown (average 14% or 10% respectively). For media augmented with glutamate alone, the duration of the subculture period greatly altered this response. After seven weeks without subculture, 100% of the white calli grown in media containing only glutamate turned brown.

Table 5. Average of white or brown color in white callus grown on media containing glutamate, ALA, or a combination of the two.

| Treatment | Number of Calli | Callus Color | |
|---|---|---|---|
| | | White | Brown |
| Control | 41 | 0.54±0.36a | 0.40±0.32a |
| 20 mM Glutamate | 41 | 0.15±0.23b | 0.85±0.23b |
| 3 mM ALA | 41 | 0.83±0.16c | 0.14±0.14c |
| 20 mM Glutamate + 3 mM ALA | 41 | 0.89±0.09c | 0.10±0.08c |

1) Mean ± SE color induced on white calli.
2) Values followed by different letters are significantly different by Duncan's NMR test (alpha = 0.05).

Browning of calli greatly increased as the number of weeks since the previous subculture increased, but red, brown, or white color of callus was observed in the four week treatment (Table 6). Red color appeared in calli grown on ALA alone or glutamate with ALA within 2 weeks, but disappeared completely after 4 weeks. Also brown and white color increased at same period as red color disappeared.

Table 6. The effects of different concentrations of glutamate and ALA on the color change of white callus in liquid media.

| Medium | Explants | Weeks | White | Green | Red | Brown |
|---|---|---|---|---|---|---|
| Control | 20 | 2 | 1(20/20) | 0(0/20) | 0(0/20) | 0(0/20) |
| 20 mM Glu | 20 | 2 | 1(20/20) | 0(0/20) | 0(0/20) | 0(0/20) |
| 3 mM ALA | 20 | 2 | 0.40(8/20) | 0(0/20) | 0.60(12/20) | 0(0/20) |
| 20 mM Glu + 3 mM ALA | 20 | 2 | 0(0/20) | 0(0/20) | 1(20/20) | 0(0/20) |
| Control | 20 | 4 | 0.10(2/20) | 0(0/20) | 0(0/20) | 0.90(18/20) |
| 20 mM Glu | 20 | 4 | 0(0/20) | 0(0/20) | 0.10(2/20) | 0.90(18/20) |
| 3 mM ALA | 20 | 4 | 0.70(14/20) | 0(0/20) | 0(0/20) | 0.30(6/20) |
| 20 mM Glu + 3 mM ALA | 20 | 4 | 0.75(15/20) | 0(0/20) | 0(0/20) | 0.25(5/20) |

**C. The effect of Heat Shock**

A combined heat shock and cytokinin treatment also resulted in calli of all four color types (Table 7). In the heat shock treatments zeatin and BAP had significantly different effects on the formation of green color and red color, but not brown color. In addition, white calli grown on zeatin remained white more frequently than white calli grown on BAP (36.8% versus 3%) (Table 8). The 30 minute heat shock treatment did not significantly effect callus color (Table 9). However, in calli exposed to 37$^{o}$C for 60 minutes, the percentage of green and red regions decreased while the percentage of brown regions increased significantly.

Table 7. The effect of heat shock, exposure time and cytokinin concentration on color change of white callus.

| Time (min) | Explant # | Zeatin (uM) | BAP (uM) | White | Green | Red | Brown |
|---|---|---|---|---|---|---|---|
| 0 | 15 | 0 | 5 | 0(0/15) | 0.80(12/15) | 0.27(4/15) | 0.07(1/15) |
| 30 | 15 | 0 | 5 | 0.07(1/15) | 0.93(14/15) | 0.27(4/15) | 0.07(1/15) |
| 60 | 15 | 0 | 5 | 0.07(1/15) | 0.33(5/15) | 0(0/15) | 0.67(10/15) |
| 0 | 15 | 0 | 10 | 0(0/15) | 0.93(14/15) | 0.40(6/15) | 0(0/15) |
| 30 | 15 | 0 | 10 | 0(0/15) | 0.93(14/15) | 0.40(6/15) | 0.20(3/15) |
| 60 | 15 | 0 | 10 | 0.07(1/15) | 0.60(9/15) | 0.20(3/15) | 0.60(9/15) |
| 0 | 15 | 0 | 15 | 0(0/15) | 0.93(14/15) | 0.53(8/15) | 0.27(4/15) |
| 30 | 15 | 0 | 15 | 0(0/15) | 1.00(15/15) | 0.80(12/15) | 0(0/15) |
| 60 | 15 | 0 | 15 | 0.07(1/15) | 0.73(11/15) | 0.40(6/15) | 0.73(11/15) |
| 0 | 15 | 0.4 | 0 | 0.33(5/15) | 0.60(9/15) | 0.13(2/15) | 0.13(2/15) |
| 30 | 15 | 0.4 | 0 | 0.20(3/15) | 0.60(9/15) | 0(0/15) | 0.27(4/15) |
| 60 | 15 | 0.4 | 0 | 0.60(9/15) | 0.27(4/15) | 0(0/15) | 0.27(4/15) |
| 0 | 15 | 1 | 0 | 0.27(4/15) | 0.47(7/15) | 0(0/15) | 0.27(4/15) |
| 30 | 15 | 1 | 0 | 0.73(11/15) | 0.13(2/15) | 0.07(1/15) | 0.07(1/15) |
| 60 | 15 | 1 | 0 | 0.47(7/15) | 0.27(4/15) | 0.07(1/15) | 0.40(6/15) |
| 0 | 15 | 2.5 | 0 | 0.33(5/15) | 0.53(8/15) | 0(0/15) | 0.13(2/15) |
| 30 | 15 | 2.5 | 0 | 0.13(2/15) | 0.87(13/15) | 0.20(3/15) | 0.07(1/15) |
| 60 | 15 | 2.5 | 0 | 0.27(4/15) | 0.20(3/15) | 0(0/15) | 0.60(9/15) |

Table 8. Average of white, green, red, and brown color in white callus grown on medium containing zeatin and BAP.

| Cytokinin | White | Green | Red | Brown |
|---|---|---|---|---|
| Zeatin | 0.37±0.20a | 0.44±0.24a | 0.05±0.07a | 0.24±0.17a |
| BAP | 0.03±0.04b | 0.88±0.22b | 0.36±0.22b | 0.29±0.29a |

1) Mean ± SE color induced on white calli.
2) Values followed by different letters are significantly different by Duncan's NMR test (alpha = 0.05).

Table 9. Average of white, green, red, and brown color in white callus at different heat shock exposure time.

| Time | White | Green | Red | Brown |
|---|---|---|---|---|
| 0 min | 0.15±0.17a | 0.72±0.02a | 0.22±0.22a | 0.14±0.12a |
| 30 min | 0.19±0.28a | 0.74±0.33a | 0.29±0.28a | 0.11±0.10a |
| 60 min | 0.26±0.23a | 0.40±0.22b | 0.11±0.16a | 0.54±0.18b |

1) Mean percent ±SE color induced on white calli.
2) Values followed by different letters are significantly different by Duncan's NMR test (alpha = 0.05).

**Discussion**

In order to give an overview of the factors and their effects on color change, the results of the preceding experiments have been summarized in Table 10.

Table 10. Summary of factors and their effects on color change in white callus.

| Factors | Color | | |
|---|---|---|---|
| | Green | Brown | Red |
| BAP and Zeatin (Table 1) | Yes, but different effects between BAP and zeatin | Yes, but same effect between BAP and zeatin | Yes, same as for brown color |
| 2,4-D (Table 3) | No, inhibition of BAP effects on greening | Yes, but only in high BAP:2,4-D ratio | No |
| Glutamate | No | Yes (about 100%) | No |
| ALA or Glu+ALA (Tables 4 and 6) | No | Yes (15-36%) | Yes, it showed within two weeks, but changed to either brown or white later |
| Time at 37°C (Table 7) | reduced greening at 60 min | increased browning at 60 min | no difference among exposed periods |

It has been reported that increasing the sucrose concentration in the nutrient medium alters the production of phenolics (Knobloch and Berlin, 1980; Suzuki et al., 1981; Khouri et al., 1986). In this study, a high sucrose concentration and a long culture period resulted in an increase in brown colored regions in the calli. Sucrose concentrations at 3% and 4%, but not 1% or 2%, increased browning of white callus after a 3-week period. The percentage of white calli turning brown also increased for all sucrose concentrations when cultures were allowed to grow for seven weeks without subculture. Therefore, a 2% sucrose con-

centration and a 4-week subculture were selected to minimize the effects of browning due to these factors.

Parthier (1979) suggested that cytokinins alter gene expression for plastogenesis. In one study, thirty-nine polypeptides decreased and twenty-three polypeptides increased in concentration during the greening process (Dietz and Bogorad, 1987). In this work, the greening of white megagametophyte-derived callus was inducible by the addition of either BAP or zeatin. However, the response differed significantly for the two plant growth regulators. BAP was more efficient at inducing the formation of green regions (Table 1). This appears to indicate the involvement of cytokinins in the induction of chlorophyll biosynthesis and probably chloroplast development in white callus of white spruce.

Previous work has shown that both the quality and intensity of light, such as blue light and UV-B light (280-320 nm), influence the production of phenolics in cultured tissue (Suzuki et al. 1981; Duell-Pfaff and Wellmann, 1982; Knobloch et al., 1982). In gymnosperms, chloroplast development can occur in either the light or dark. Our results demonstrate that white callus grown at either high or low light intensities had a similar response to the BAP and zeatin treatments when scored for the induction of green regions. This is consistent with evidence from other studies indicating that chloroplast development may be trigged either by light through photoreceptors, such as phytochrome, or by plant growth regulators, such as cytokinins (Lerbs et al., 1981; Mohr, 1984).

The type and concentration of auxins and cytokinins, as well as their relative ratios in the culture medium, play a role in the biosynthesis and accumulation of secondary metabolites. An increase in the

concentration of phenolics has been reported in the presence of low levels of auxins, especially 2,4-D (Kadkade, 1982). In carrot cells, pigment accumulation was inhibited by low levels of 2,4-D, but was stimulated by 0.01-1.0 uM of kinetin, BAP, or zeatin (Ozeki and Komaine, 1981). In our work the presence of auxin (2,4-D ) seems to counteract the effects of the cytokinin for color induction (Table 3). When the control medium (MS medium with 10 uM BAP and 10 uM 2,4-D) was used to culture white calli and green calli, neither white calli nor green calli changed color. This indicates that cytokinins tend to initiate and auxins inhibit the greening process. Once the greening process begins, the addition of auxins does not reverse it.

The browning of white callus in media containing either glutamate or ALA or both glutamate and ALA differed (Tables 4, 5 and 6). This indicates that there may be a different pathway for glutamate and ALA metabolism in white callus. The interaction of the metabolites of ALA and glutamate increased the red colored regions in the white calli but these then changed to brown or back to white after a short period (Table 6). This reddening process appeared to be independent from the other color changes since it was not observed in any brown or green colored regions of the calli. The turnover rate for red color to white or brown was fast and generally required only one to two weeks. Whether the brown calli found in the different treatments are derived from red calli or directly induced from white calli is still unknown.

In glutamate and/or ALA treatments, no green coloration has observed in these treatments (Tables 4 and 6). Apparently, white calli did not synthesize any chlorophyll, even after being treated with chlorophyll precursors glutamate and ALA. This indicates that the greening

process induced by the cytokinins is blocked after the step(s) of ALA biosynthesis in the chlorophyll biosynthesis pathway in white callus. Alternatively it is possible that the genes for chlorophyll synthesis are regulated by cytokinins through major regulatory protein(s), although these experiments do not allow this question to be addressed . Furthermore, green and brown color existed alone or together when white calli were grown in the presence of cytokinins and glutamate and ALA (Tables 1, 4, 6, and 7). It is possible that different pathways exist in white callus for the color change to either green or brown, yet chlorophyll biosynthesis either promoting or inhibiting brown coloration is still unknown.

In plants, heat hardening and heat shock protein (HSP) synthesis are concomitant phenomena, and a good correlation has been found between HSP synthesis and thermotolerance (Lin et al., 1984; Sachs and Ho,, 1986). Shifts from the optimal growth temperature result in a stress for plants and, even if not lethal, affect their development (Christianson, 1982). Plastid HSP synthesis fluctuates through the day and is controlled in a circadian fashion (Otto et al., 1988). However, the effect of plastid HSPs is still not understood. In this case, heat shock changed the response of the white callus to BAP and zeatin. A significant increase in browning, and decrease in greening, took place when white calli were exposed to 37$^{o}$C for 60 min. It is possible that changes in the response of white callus to cytokinins resulted from the breakdown of the cytokinins, or the expression of HSPs.

The objective of this paper was to understand the factors that effect the color change of white megagametophyte callus of *P. glauca*. This research showed that cytokinins can induce greening, and that

auxins inhibit the greening process. Furthermore, we have shown that color change in white callus is inducible, but how that change occurs is still a question. Because green callus can be induced from white callus, this provides a system for studying chloroplast development. More research is needed to evaluate the differences between white and green calli at the DNA, RNA, and protein levels before this will lend any real understanding the regulation of chloroplast development and pigmentation in plants.

**Literature Cited**


Beale, S.I. and P.A. Castelfranco, 1973. $^{14}C$ incorporation from exogeneous compounds into 5-aminolevulinic acid by greening cucumber cotyledons. Biochem Biophys Res Commun 52:143-149.

---- 1974. The biosynthesis of 5-aminolevulinic acid in higher plants. II Formation of $^{14}$-5-aminolevulinic acid from labeled precursors in greening plant tissues. Plant Physiol 53:297-303.

Christianson, M.N. 1982. World environmental limitations to food and fiber culture. In: Breeding plants for less favorable environmetns. Christianson, M.N. and C.F. Lewis(eds.) New York: John Wiley, pp1-11.

Dietrz, K. and B.L. Bogorad, 1987. Plastid Development in *Pisum sativum* Leaves during Greening. Plant Physiol. 85:808-815.

Duell-Pfaff, N. and E. Wellmann, 1982. Involvement of phytochrome and a blue light photoreceptor in UV-B induced flavonoid synthesis in parsley cell suspension cultures. Planta 156:213-217.

Fletcher, R.A. and D. McCullagh, 1971. Cytokinin-induced chlorophyll formation in cucumber cotyledons. Planta 101:88-90.

Huang, L., B.A. Bonner, and P.A. Castfranco, 1989. Regulation of 5-aminolevulinic acid (ALA) synthesis in developing chloroplasts. II. Regulation of ALA-synthesizing capacity by phytochrome. Plant Physiol. 90:1003-1008.

Kadkade, P. 1982. Growth and podophyllotoxin production in callus tissues of *Podophyllum peltatum*. Plant Sci. Lett. 25:107-115.

Khouri, H., R.K. Ibrahim and M. Rideau, 1986. Effects of nutritional and hormonal factors on growth and production of anthraquinone glucosided in cell suspension cultures of *Cinchona succirubra* Plant Cell Rep. 5:423-426.

Knobloch, K. and J. Berling, 1980. Influence of medium composition on formation of secondary comounds in cell suspension cultures of *Catharanthus roseus* Z. Naturforsch., C: Biosci. 35:551-556.

Knobloch, K., G. Bast and J. Berling, 1982. Medium-induced and light-induced formation of serpentine and anthocyanins in cell suspension cultures of *Catharanthus roseus* Z. Phytochemistry 21:591-594.

Kuntz, M., J.-L. Evrard, A. d'Harlingue, J.-H. Weil and B. Camara, 1989. Expression of plastid and nuclear genes during chromoplast differentiation in bell pepper *capsium annuum*) and sunflower (*Helianthus annuus*). Mol. Gen. Genet. 216:156-163.

Laetsch, W.M. and R. Boasson, 1972. Effect of growth regulators on organelle development. *In*: Hormonal Regulation in Plant Growth and Development. eds. H. Kaldewey and Y.J. Vardar pp.453-465. Verlag Chemie, Weinheim.

Lerbs, S., N.L. Klyachko, E.G. Romanko, O.N. Kulaeva, R. Wollgiehr and B. Parthier, 1984. Gene expression in cytokinin- and light-mediated plastogenesis of *Cucurbita* cotyledons: ribulose-1,5-bisphosphate carboxylase/oxygenase. Planta 162:289-298.

Lin, C.Y., J.K. Roberts, and J.L. Key, 1984. Acquisition of the thermotolerance in soybean seedlings: synthesis and accumulation of heat shock proteins and their cellular localization. Plant Physiol. 74:152-160.

Mohr, B. 1984. Phytochrome and chloroplast development. *In*. Chloroplast Biogenesis. N.R. Barker and J. Barker (eds.) pp.305-347. Elsevier, Armsterdan.

Ozeki, Y. and A. Komamaine, 1981. Induction of anthocyanin synthesis in relation to embryogenesis in a carrot suspension culture. Physiol. Plant. 53:570-577.

Parthier, B. 1979. The role of phytohormones (cytokinins) in chloroplast development. Biochem. Physiol. Pflanzen 174:173-214.

Piechulla, B. 1988. Differential expression of nuclear- and organell encoded genes during tomato fruit development. Planta 74:505-512.

Piechulla, B., E. Pichershy, A.R. Cashmore and W. Gruissem, 1986. Expression of nuclear and plastid genes for photosynthesis-specific proteins during tomato fruit development and ripening. Plant Mol. Biol. 7:367-376.

Sachs, M.M. and T.D. Ho, 1986. Alteration of gene expression during environmental stress in plants. Annu Rev Plant Physiol 37:363-376.

Schmerder, B., F.Rabenstein, and H. Borriss, 1978. Steuerung des Ergundungsprozesses in den Kothledonen nachgereifter *Agrostemma*-Embryonen durch Phytohormone. Biochem. Physiol. Pflanzen. 173:97-113.

Stetler, D. and W.M. Laetsch, 1965. Kinetin-induced chloroplast maturation in cultures of tobacco tissue. Science 149:1387-1389.

Suzuki, H., T. Matsumoto, T. Kisaki and M. Noguchi, 1981. Influence of cultural conditions on polyphenol formation and growth of Amacha cells (*Hydrangen macrophylla*). Agric. Biol. Chem. 45:1067-1077.

Whatley, J.M. 1977. Variation in the basic pathway of chloroplast development. New Phytologist 78:407-502.

---- 1978. A suggested cycle of plastid developmental interrelations. New Phytologist 80:489-502.

**Variations in physical characteristics, proteins, and DNA between green and non-green callus cultures of *Picea glauca***

**Abstract**

Variations in callus dry weight and protein concentration were found between white calli and green calli derived from white calli. The chlorophyll a/b ratio for green calli was 1.339. White calli lacked chlorophyll. Both green calli and white calli showed absorbency profiles from 200 to 300 nm and a strong peak at 320 nm, but green calli had additional absorbancy peaks at 410 nm, 430 nm, 470 nm, and 670 nm. No measurable photosynthesis activity occurred in either white or green calli. In comparison with needles, green calli and white calli contained an extra DNA band on CsCl density gradients. There was also differential expression of polypetides among white calli, green calli derived from white calli, and needles. Comparison of HpaII and MspI restriction patterns detected differences in genomic DNA methylation among tissue types.

## Introduction

Chloroplasts and mitochondria are theorized to have originated endosymbiotically with subsequent transfer of genes from these organelles to the nucleus resulting in the dependence of both organelles on the nuclear genes for the synthesis of essential polypeptides. Chloroplast DNA encodes approximately 60-100 polypeptides and the remaining 80-90% of plastid proteins are encoded by the nuclear genome (Sugiura, 1989; Taylor, 1989). Organelle development and genetic regulation are also largely under nuclear control (Attardi and Schatz, 1988; Taylor, 1989).

Plants have five major types of plastids: proplastids, chloroplasts, chromoplasts, amyloplasts, and etioplasts. All plastids can develop directly from proplastids. The reverse process, that is, the simplification of plastid structure back from a specialized form to the proplastid form, is conditional upon cell division and the stage of plant development or differentiation (Whatley, 1977, 1978).

The regulation of plant development and plastid interconversion is accomplished at several levels. Changes in both nuclear and plastid gene expression are involved in this developmental process (Piechulla et al., 1986; Piechulla,1988; Kuntz et al., 1989). Transcriptional and post-transcriptional regulation may play a major role in the interconversion of plastids. Light also has a profound influence on morphogenesis and biochemical differentiation of plastids and regulates the expression at both transcriptional and post-transcriptional levels through light-photoreceptor interactions (Thompson, 1988). Furthermore, some plastid and nuclear genes, such as *psbA and rbcS*

are temporally and spatially expressed during plant development (Fiebig et al., 1990).

The functions of plastids include the biosynthesis of amino acids and fatty acids, nitrate assimilation and photosynthesis. Photosynthetic reactions, which provide metabolic energy, are not present in non-green plastids. Enzymes for carbohydrate metabolism have differential expression between green and non-green tissues (ap Rees and Entwistle, 1989; Keeling, 1989; Madore, 1990; Frehner et al., 1990). In ripening tomato fruit, the chloroplast-specific mRNA levels continuously decrease (Piechulla et al., 1985; Piechulla et al., 1986; Piechulla, 1988), while two chromoplast-specific polypeptides (Mr 35 and 58 kd) are either not detectable or barely detectable in chloroplasts (Hadjeb et al., 1988). Obviously, different plastid types have various mechanisms that regulate their metabolism.

DNA methylation has been postulated as a mechanism that controls the transcription of genes thus regulating gene expression. In studies of plastid DNA, chromoplast DNA and amyloplast DNA, but not chloroplast DNA, are methylated in regions containing genes that are not expressed or are expressed at low frequency (Ngernprasirtsiri et al., 1988a, 1988b; Macherel et al., 1986).

This study is a attempt to better understand plastid development through the characterization of differences among green and non-green tissues of white spruce. Differences in physical characteristics, proteins, and genomic DNA were studied and discussed.

## Materials and Methods

Culture conditions for white calli and green calli were described previously in Chapter 1.

**Relative moisture content**

The relative moisture content of white calli and green calli was measured by weighing calli before and after placement in an oven at 80°C for 24 hours. Six samples of each tissue type were measured.

**Chlorophyll measurement and total protein content**

Photosynthesic activity of white calli and green calli (ca. 5g each) was measured using a Port Photosynthesis System (LI-COR, INC). Approximately 10 g of callus tissue was homogenized at speed 7-8 for 20 seconds with a Teckmar Tissuemizer in 150 ml of grinding medium (Stine et al. 1988). The homogenate was centrifuged at 12,000 rpm for 15 min in a Sorvall GS-3 rotor. Distilled water (40 ml) was added to suspend the pellet and blended for 4 seconds with the Teckmar Tissuemizer. Acetone (160 ml) was then added and blended for 10-20 seconds at speed 7-8, after which the homogenate was centrifuged at 10,000 rpm for 5 min in a Sorvall GSA rotor. The supernatant was used to measure chlorophyll concentration and chlorophyll a/b ratio. The pellet was used to measure the total protein content.

**A. Chlorophyll measurement** Chlorophyll content and the chlorophyll a/b ratio were determined by measuring the $A_{663}$ and $A_{645}$ of the supernatant respectively. Three samples of each supernatant material were measured. Wavelengths from 200 nm to 800 nm were used to measure the absorbency spectra using a Perkin-Elmer photospectrometer and a P-E Lamda 4B program.

**B. Protein determination** Approximately 1-2 mg of each air-dried pellet was used to estimate the protein concentration by Lowry assay kit (Sigma) and $A_{750}$. Three samples of each tissue were measured.

**Protoplast isolation**

Protoplasts were isolated from white and green calli. Calli (5-10 g) were slowly ground through a 100 um nylon or iron mesh in 12 ml of protoplast isolation medium (5% mannitol, 2% sucrose, 5% sorbitol, 18 mM $CaCl_2$, 15 mM $CaSO_4$, 0.4% cellulase (Karlan Chemical), 0.2% macerase (Calibiochem), and 0.3% hemicellulase (Sigma)). After incubation for 5-6 hours at 70 rpm in the dark, protoplasts were concentrated at 1,000 rpm for 5 minutes in a Sorvall HB-4 rotor. The pellet was resuspended in a 8% mannitol solution.

**Sucrose gradient separation of needle and protoplast solutions**

The protoplast solution was passed through an 18 gauge needle 3-4 times. Needle tissue (10 g) was ground for 20-30 seconds at speed 7-8 in grinding buffer (Stine et al. 1988) with a Techmar Tissuemizer. The homogenate was filtered through 1 layer of 100 um mesh nylon and 2 layers of Mirocloth (Calibiochem.) and centrifuged at 12,000 rpm for 15 minutes in a Sorvall GS-3 rotor. Grinding buffer (10-15 ml) was added to resuspend the pellet. Protoplast and needle solutions were loaded on a discontinuous sucrose gradient, (0.7M, 1.1M, 1.4M, and 1.6M) with a sucrose gradient buffer of 50 mM Tris, 6% sorbitol, and 25mM EDTA at pH 7.5. Organelles and cell particles were separated at 12,000 rpm for 1 hour in a Sorvall HB-4 rotor. Each interface was collected and an equal amount of $T_{50}E_{20}$ (50 mM Tris pH8.0 and 20 mM EDTA pH 8.0) was added. The solutions were centrifuged at 11,000 rpm for 10 minutes in a Sorvall HB-4 rotor and the pellets were used for SDS-PAGE electrophoresis.

**SDS-PAGE Electrophoresis**

Distilled water (0.4 ml) was added to dissolve each pellet and then equal amounts of 2x sample load buffer (0.12 M Tris, 6%(w/v) SDS,

10% 2-mercaptoethanol, 20%(v/v) glycerol, and 2 mg/ml bromophenol) was added. The solution was boiled for 2 min before loading on the gel. Sample volumes ranged from 1-10 ul. A 15% acrylamide separating gel and 6% stacking gel (Laemmli 1970)were used to separate proteins. The gel size was 15cm x 15cm x 0.15 cm. Constant current (3 mA) was applied until the blue dye front reached the bottom of the gel (approximately 5 hours). Bio-Rad silver stain plus was used to visualize the proteins under conditions described by the manufacturer (Bio-Rad laboratory).

**Total DNA isolation**

Total DNA isolation from needles followed the protocol described by Neale et al. (1986). The method used to isolate the total DNA of green calli and white calli was modified from Ausubal et al. (1987). Callus tissue (12 g) was ground to fine powder in liquid $N_2$ with a prechilled mortar and pestle. The powder was transferred into 250 ml plastic bottles and 67 ml of extraction buffer (50 mM Tris pH 8.0, 10 mM EDTA pH8.0, 1M NaCl, 3 mM cysteine, 1 mM ascorbic acid, and 50 ug/ml proteinase K (Boehringer Mannheim)) was added to dissolve the powder. The homogenate was then filtered through a 100 um nylon mesh. Sarkosyl (20 ml of 5% sarkosyl) was added and the solution was incubated for 3 hr at 55$^{o}$C, and then centrifuged for 10 min at 4,000 x g, 4$^{o}$C in a Sorvall GSA rotor. Isopropanol (0.6 volume) was added to the supernatant and gently mixed. Total DNA was precipitated for 15 min at 8,000 x g, 4$^{o}$C in a Sorvall GSA rotor. DNA purification and precipitation followed methods described by Stine et al. (1988).

**Southern analysis**

DNA was digested with restriction enzymes under the conditions specified by the manufacturer (Boehinger Mannheim). A 0.6 % (w/v)

agarose gel (Sigma) in TBE buffer and 1-1.2 V/cm constant power were used to separate DNA fragments. Zeta-probe blotting membrane (Bio-Rad) was used for DNA alkaline blotting (Bio-Rad Laboratory). Petunia cpDNA S8 and maize mtDNA coxII clones were used as probes. Probes were labeled using random primer labeling according to the manufacturer's (Boehringer Mannheim Biochemicals) directions. Prehybridization, hybridization and post hybridization washeswere performed according to Bio-Rad instructions for Zeta-Probe membrane. Autoradiography was carried out at -70°C with DuPont Cronex Lightning plus intensifyingscreens and Kodak X-Omat XARS x-ray film.

**Results**

**A. Physical characteristics**

The relative moisture content, chlorophyll content and protein concentration of green calli and white calli are summarized in Table 1. Green calli differed significantly from white calli not only in chlorophyll content but also in dry weight and protein concentration. Green calli on average weighed 1.4 times more than white calli. The ratio of total protein amount between green and white callus was 4:1 in fresh weight and 2:1 in dry weight. In white calli extraction, absorbency occurred in the $A_{663}$ and $A_{645}$ regions, but no definite peak was observed and no chlorophyll was detected (Table 1 and Figure 1). The chlorophyll a/b ratio of green calli was 1.339. Neither callus types showed the evidence of photosynthetic activity. The absorbency peaks of green callus were at 680nm, 470nm, 430nm, and 410nm. Both callus types shared an the absorbency peak at 320nm and from 200 nm to 320 nm (Figure 1).

Table 1. Relative moisture content, chlorophyll content, and protein concentration of white and green calli

| | Green calli | White calli |
|---|---|---|
| Relative moisture | 0.9384 | 0.9651 |
| Dry weight (g/g of fresh weight) | 0.0616 | 0.0349 |
| Photosynthetic rate | 0 | 0 |
| Chlorophyll conc. (ug/g of fresh weight) | 47.4650 | 0.0315a |
| Chlorophyll a/b | 1.3390 | 2.3360a |
| Total protein (mg/g of fresh weight) | 6.0850 | 1.4736 |

a:the value of white callus was calculated as formulas for the measurement of chlorophyll and chlorophyll a/b ratio.

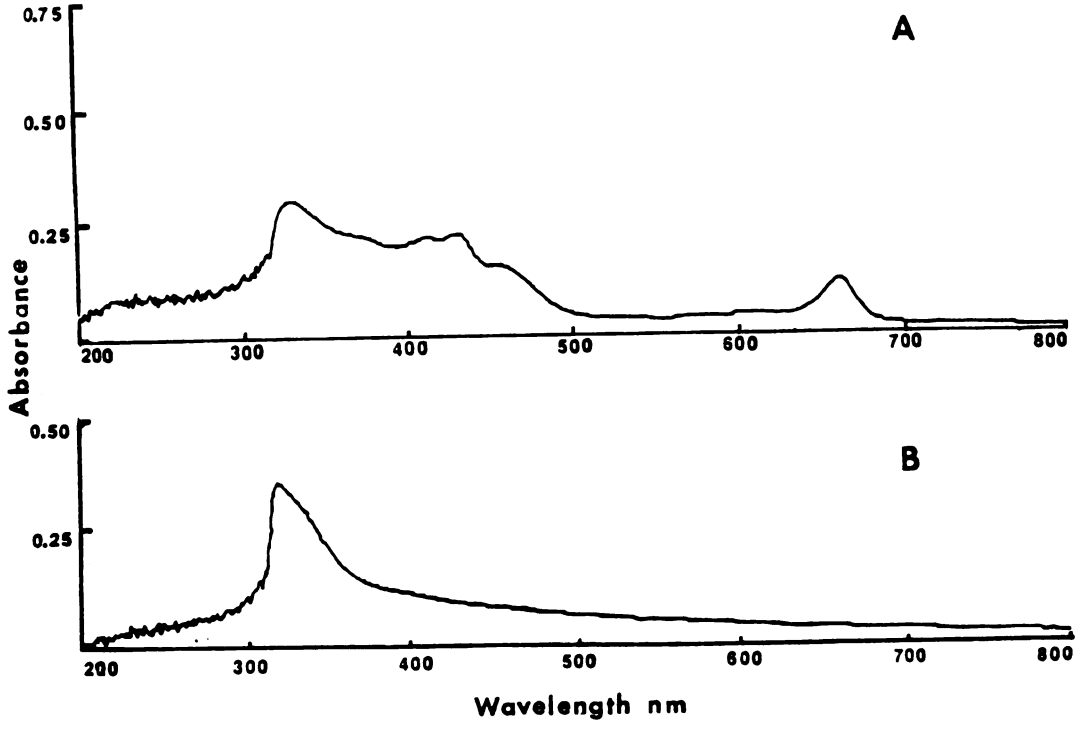


Figure 1. The absorbency spectrum of acetone-water extraction. A. The spectrum of green callus. B. The spectrum of white callus.

**B. Protein analysis**

Differences in polypeptides were found among needles, green callus, and white callus (Figure 2). There was little polypeptides variation among samples from the different interfaces of the sucrose gradients. In samples from white callus, no polypeptides (Figure 2. a and b regions) were observed in samples from the extraction solution-0.7 M and 0.7-1.1 M interfaces, but a number of bands were found in samples from the 1.1-1.4 M and 1.4-1.6 M interfaces. In green callus, the polypeptides migrating to the a and b regions of gel were present in samples from all interfaces of the sucrose gradients. In the sucrose gradient the extraction solution-0.7 M interface, white calli did not contain polypeptides of 45, 38, and 32 kd that were observed in samples from needles. Samples from green calli and needles were more similar. The only difference between needles and green callus was that needle samples produced a band at 38 kd and green callus samples showed a similar band at 39 kd.

**C. DNA Studies**

After ultracentrifugation of total DNA, two strong DNA bands were detected in samples from needles (a third band was either faint or undetectable) and three DNA bands were visible in samples from green calli and white calli. When white spruce cpDNA and the bottom DNA band (band 3) of white calli were digested with restriction enzymes PstI or SacI, the restriction band patterns differed, especially in the 16-24 kb range (Figure 3A). DNA from the third band did not hybridize to the maize mitochondrial CoxII DNA used as a probe (Figure 3B).

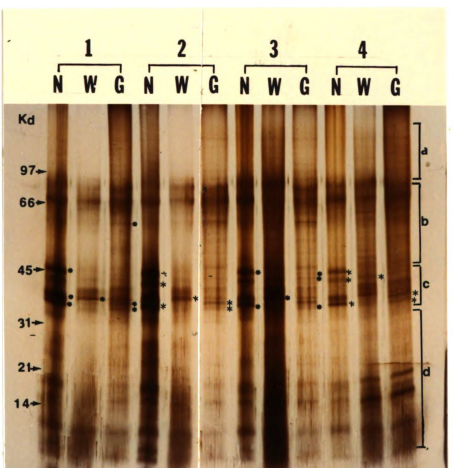


Figure 2. Silver staining SDS-PAGE gel of polypeptides from extracts, which were separated by discontinuous sucrose, of needles, green callus and white callus. 1:interface between loading solution and 0.7 M. 2: interface between 0.7 M and 1.1 M. 3: interface between 1.1 M and 1.4 M. 4; interface between 1.4 M and 1.6 M. N: needles. W:white callus. G:green callus.

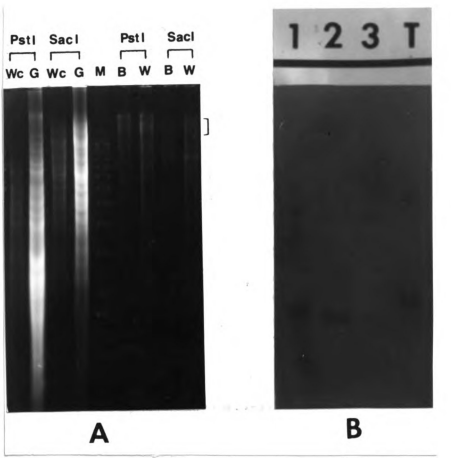


Figure 3. The megagametophyte callus DNA banding pattern. A. PstI or SacI-cut DNA was fractionated by electrophoresis in 0.6% agarose gel with TBE buffer.B. Band 1, 2, and 3 was digested with PstI and hybridized with maize mitochondrial CoxII probe. T:Total DNA. W: White spruce. B: blue spruce. G:Green callus. Wc:White callus.

**D. DNA methylation**

Four different methylation sensitive restriction enzymes: HaeIII, HpaII, MspI, and NciI, were used to study the methylation pattern of genomic DNA. These restriction enzymes and the methylation patterns they fail to cleave are listed in Table 2.

Table 2. Methylation sensitive restriction enzymes and the methylation sequences which will not be cleaved.

| | Restriction enzymes | | | |
|---|---|---|---|---|
| | HaeIII | HpaII | MspI | NciI |
| Sequences not cleaved | GG$^{m5}$CC | C$^{m5}$CGG<br>$^{m5}$CCGG | $^{m5}$CCGG | C$^{m5}$CGGG<br>C$^{m5}$CCGG |

The nuclear genomic DNA pattern for all tissue and callus types was constant for each restriction enzyme, but differed among the restriction enzymes (Figure 4). In contrast to the restriction patterns of HpaII, MspI, and NciI, nuclear genomic DNA was obviously digested by restriction enzyme HaeIII, even though HaeIII is also a methylation sensitive enzyme. Digestion of total DNA by restriction enzymes HpaII and MspI, (which are isoschizomers and differ only in the methylation position of sequences they will not cleave) resulted in different dispersion patterns among samples from needles, green callus and suspension cells. The nuclear genomic DNA patterns of green callus and suspension cells were much more widely dispersed in MspI-cut DNA when compared to HpaII-cut DNA. The nuclear genomic DNA patterns of needles were the same for both restriction enzymes (Figure 4, a region).

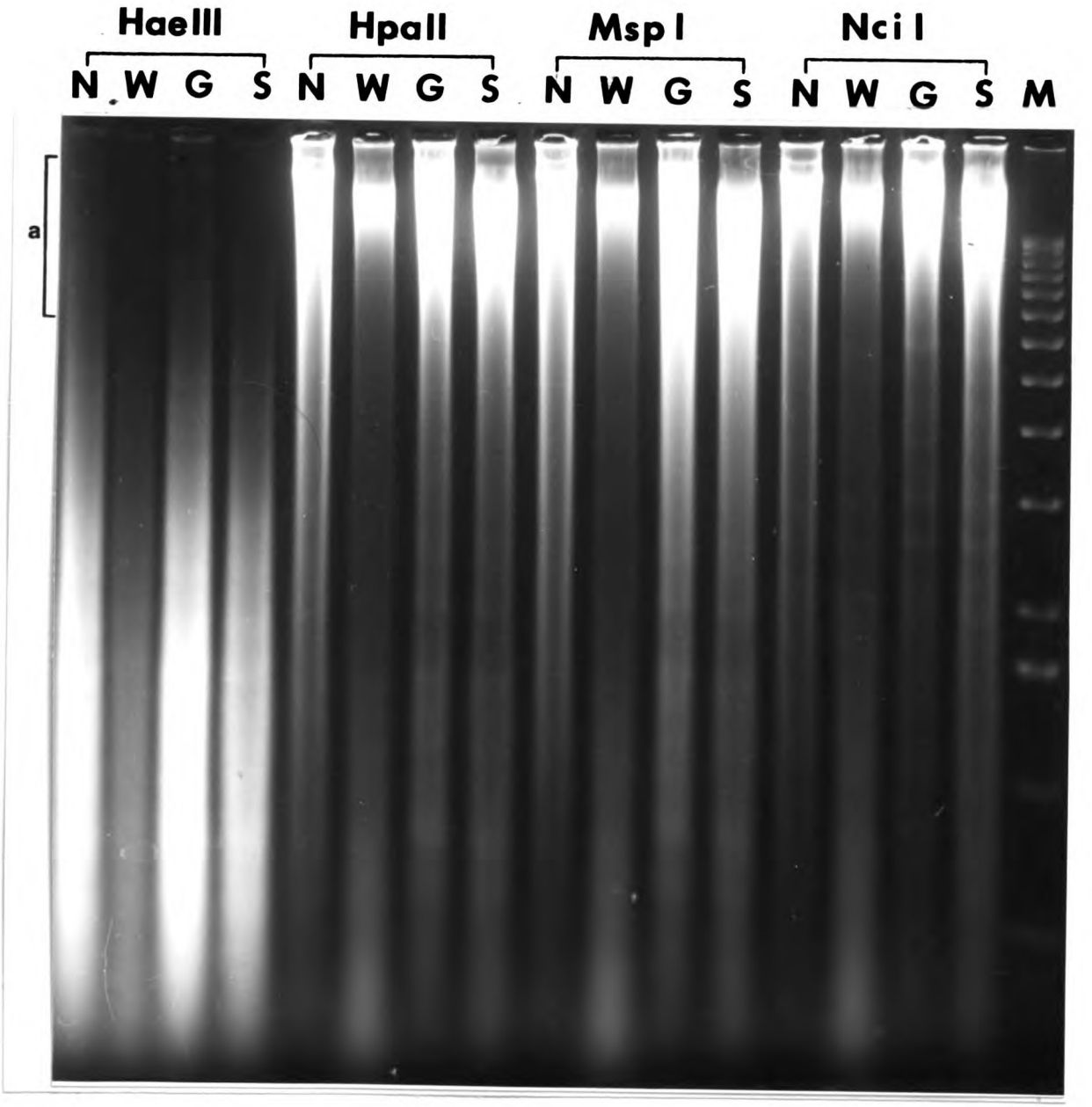


Figure 4. Genomic DNA methylation pattern. Total DNA was digested by methylation-sensitive restriction enzymes: HaeIII, HspII, MspI, and NciI. Ethidium bromide was used to stain DNA. N:Needles. S:suspension cells. G:green calli derived from white callus. W. White calli. M:1 kb ladder. Medium for suspension cell culture was same as callus induction medium without agar. DNA concentration in each tissue type is same for all restriction enzymes.

**Discussion**

Plastid development require the regulation and expression of both genomic and plastid genes. These studies showed that white calli and green calli differ not only in chlorophyll biosynthesis but also in protein concentration (Table 1). Total protein concentration of green calli was four times more than that in white calli and there was a 50% increase in dry weight. The difference in total protein amount may contribute to the difference in dry weight. These differences are also apparent in the lower enzyme concentration and shorter time to isolate protopasts from white calli. It is probable that this indicates a difference in cell wall components for white versus green callus cells.

The most striking difference between green callus and white callus was the absence of detectable chlorophyll in white callus (Figure 2). The presence of the absorbency peak around 320 nm may indicate that some water or acetone soluble polypeptides exist in both green calli and white calli. Similarly some secondary metabolites may exist in the acetone-water extraction solution and cause the absorbency peak from 200 nm to 300 nm (Figure 1). An analysis of this acetone-water extraction solution may provide a clue to understanding the variations of secondary metabolites between green calli and white calli.

It is interesting that no photosynthesis was observed in green callus. The reason for this may be 1) green callus does not photosynthesize and fix carbohydrate because the sucrose in the culture medium provided a carbon source (feed-back effect); 2) a low chlorophyll a/b ratio (1.3990) and different absorbency spectra from chlorophyll a and b (Figure 1) indicates that the photosynthetic apparatus may not be well developed; 3) light intensity (20 uE/$m^2$/sec) may not be high enough to

stimulate photosynthesis in callus tissue; or 4) polypeptides necessary for photosynthesis may not be completely expressed in green callus (Figure 2). In one dimensional SDS PAGE gels, polypeptide differences occurred among needles, green calli, and white calli (Figure 2). These variations in polypeptides clearly indicate that the expression of genes needed to maintain the cell functions differs according to plant tissue type, developmental stage, and environmental conditions.

Qualitative variations in nuclear DNA result from the changes in the amount of repetitive DNA together with changes in cell developmental patterns (Buitti, 1977; Bassi et al., 1984). A heavier satellite DNA band in CsCl density gradients has been observed in dedifferentiating tissues, but not in differentiating tissues, of *Vicia faba*. This suggests the amplification of G+C rich nuclear DNA sequences during the cell dedifferentiation process (Natali et al., 1986). In the present study, three DNA bands appeared in samples from both callus types after CsCl density centrifugation. This indicates the possible amplification of nuclear DNA sequences occurs in callus, because band 3 DNA neither hybridized to mitochondrial DNA nor showed the same banding pattern as chloroplast DNA (Figure 2B and 2C).

Despite the variation between green callus and white callus, plant growth regulators play an important role in the regulation of the greening process from white to green calli. Green calli can be induced from white calli by the addition of cytokinins (either zeatin or BAP) or inhibited by the addition of auxin (2,4-D) (Chapter 2). In addition, auxins are known to increase genomic DNA methylation. Cytokinins have no known effect on DNA methylation (LoSchiavo et al. 1989). Therefore, the greening process observed in the present study results from

the activation or inhibition of specific genes that regulate the expression of nuclear DNA through the methylation or demethylation of nuclear or plastid DNA in response to the plant growth regulators .

In green plant, up to 30% of total cytosine residues of genomic DNA are comprised of 5-methylcytosines ($m^5C$). The $m^5Cs$ occur predominantly in CpG dinucleotides, but also in CpNpG trinucleotides (Shapiro, 1968, Gruenbaum et al., 1981), and the $m^5C$ content is both species- and tissue-specific (Sharpiro, 1968; Vanyushin, 1984). To detect differences in methylation we used the isoschizomer restriction enzymes MspI and HpaII, which recognize a difference in methylation position within restriction sequences. No methylation differences in nuclear genomic DNA were observed within tissue types for each restriction enzyme. However, variation of nuclear genomic DNA methylation was observed between tissue types. More of the spruce nuclear genomic DNA sequences from green callus and cell suspension cultures than those from needles are methylated at the 3' cytosine of sequences ($C^{m5}CGG$) cut by MspI, as was evidenced in the broader dispersion of DNA from those tissues when digested with MspI as opposed to HpaII, the most complete digestion was by HaeIII, suggesting that nuclear genomic DNA may be less methylated or not methylated at the 5' end cytosine ($GG^{m5}CC$) (Figure 4). It has been reported that DNA methylation is considered to play an important role in the epigenetic control of gene transcription (Holliday, 1987). It is possible that the differences in genomic DNA methylation patterns between needles and green callus may be a result of the addition of 2,4-D to the culture medium, given that 2,4-D is known to increase DNA methylation levels (LoSchiavo et al. 1989). It is unknown whether this in-

creased methylation level is responsible for the lack of photosynthetic activity in green calli.

In this report, differences in dry weight, protein quality, protein quantity were observed between green and white calli. Variations in polypeptides, total genomic DNA and nuclear genomic DNA methylation pattern was observed between needles and green and white calli. These are a clear indication of differential gene expression among those tissues. Although these differences were documented, additional research will be needed to establish the mechanism governing the greening of white calli.

**Literature cited**


Ausubal, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, 1987. Current protocols in molecular biology. greene Publishing Associates and wiley-Interscience.

ap Rees, T. and G. Entwistle, 1989. Entry into the amyloplast of carbon for starch synthesis. *In:* eds. C.T. Boyer, J.C. Shannon, and R.C. Hardison. Physiology, Biochemistry, and Gentics of Nongreen Plastids. The American Society of Plant Physiologists. pp.49-62.

Attardi, G and G. Schatz, 1988. Biogenesis of mitochondria. Annu. Rev. Cell Biol. 4:289-333.

Bassi, P., P. G. Cioini, R. Cremonini, P. Seghizzi, 1984. Underreprentation of nuclear DNA sequences in differentiating root cells of *Vicia faba*. Protoplasma 123:70-77.

Buiatti, M. 1977. DNA amplification and tissue cultures. In: Reinert, J., Y.P.S. Bajaj. (eds) Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. pp.358-464. Berlin-Heidelberg-New York:Springer.

Fiebig, C., H. Neuhaus, J. Teichert, W. Rocher, J. Degenhardt, and G. Link, 1990. Temporal and spatial pattern of plastid gene expression during crucifer seedling development and embryogenesis. Planta 181:191-198.

Frehner, M., J. Pozueta-Romero, and T. Akazawa, 1990. Enzyme sets of glycolysis, glucoeogenesis, and oxidative pentose phosphate pathway are not complete in nongreen highly purified amyloplasts of sycamore (*Acer pseudoplatanus* L.) cell suspension cultures. Plant Physiol. 94:538-544.

Givan, C.V. 1980. Aminotransferase in higher plants. *In:* B.J. Miflin, (ed.) The Biochemistry of Plants: A Comprehensive Treatise. Academic Press. New York, pp329-357.

Gruenbaum, Y., T. Naveh-Many, H. Cedar, and A. Razin, 1981. Nature 292:860-862.

Hadjeb, N., I. Gounaris, and C.A. Price, 1988. Chromoplast-specific proteins in *capsicum annum* Plant Physiol. 88:42-45.

Holliday, R. 1987. Science 238:163-170.

Keeling, P.L. 1989. Evidence against triose phosphate transport into amyloplasts of developing weat and maize grain. *In:* eds. C.T. Boyer, J.C. Shannon, and R.C. Hardison Physiology, biochemistry, and genetics of nongreen plastids. The American Society of Plant Physiologists. pp63-78.

Kuntz, M., J-L. Evrard, A. d'Harlingue, J-H. Weil, and B. Camara, 1989. Expression of plastid and nuclear gene during chromoplast differentiation in bell pepper (*Capsicum annuum*) and sunflower (*Helianthus annuus*). Mol. Gen. Genet. 216:156-163.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

LoSchiavo, F., L. Pitto, G. Giuliano, G. Torti, V. Nuti, Ronchi, D. Marazziti, R. Vergara, S. Orselli and M. Terzi, 1989. DNA methylation of embrogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypo methylating drugs. Theor. Appl. Genet. 77:325-331.

Macherel, D., H. Kobayashi, E. Valle and T. Akazawa, 1986. Expression of amyloplast DNA in suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.). FEBS Lett 201:315-320.

Madore, M.A. 1990. Carbohydrate metabolism in photosynthetic and non-photosynthetic tissues of variegated leaves of *Coleus blumei* Benth. Plant Physiol. 93:617-622.

Natali. L., A. Cavallini, R. Cremonini, P. Bassi and P.G. Cioini, 1986. Amplification of nuclear DNA sequences during induced plant cell dedifferentiation. Cell Differentiation 18:151-161.

Neale, D.B., N.C. Wheeler, and R.W. Allard, 1986. Paternal inheritance of chloroplast DNA in Douglas-fir. Can. J. For. Res. 16:1152-1154.

Ngernprasirtsiri, J., H. Kobayashi, and T. Akazawa, 1988a. DNA methylation occured around lowly expressed genes of plastid DNA during tomato fruit development. Plant Physiol. 88:16-20.

--- 1988b. DNA methylation as a mechanism of transcriptional regulation in nonphotosynthetic plastids in plant cells. Proc. Natl. Acad. Sci. USA 85:4750-4754.

Piechulla, B. 1988. Differential expression of nuclear- and organelle encoded genes during tomato fruit development. Planta 174:505-512.

Piechulla, B., E. Pichershy, A.R. Cashmore, and W. Gruissem, 1986. Expression of nuclear and plastid genes for photosynthesis-specific proteins during tomato fruit development and ripening. Plant Mol. Biol. 7:367-376.

Shapiro, H.S. 1968. *In*. Sober, H.A. (ed.) Handbook of Biochemistry. pp. H39-48. CRC Press, Cleveland.

Stine, M., B.B. Sears and D.E. Keathley, 1989. Inheritance of plastids in interspecific hybrids of blue spruce and white spruce. Theor. Appl. Genet. 78:768-774.

Sugiura, M. 1989. The chloroplast chromosomes in land plants. Annual Rev. Cell Biol. 5:51-70.

Taylor, W.C. 1989. Regulatory interactions between nuclear and plastid genomes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:211-233.

Thompson, W.F. 1988. Photoregulation: diverse gene responses in greening seedlings. Plant, Cell and Enviroment 11:319-328.

Vanyushin, B.F. 1984. *In*. Trauntner, T.A. (ed.) Methylation of DNA. pp. 99-114. Springer-Verlag, New York.

Whatley, J.M. 1977. Variation in the basic pathway of chloroplast development. New Phytologist 78:407-502.

---- 1978. A suggested cycle of plastid developmental interrelations. New Phytologist 80:489-502.

## Chapter 4

## Physical Mapping of *Picea* chloroplast DNA

**Abstract**

The cpDNA genome of *Picea engelmannii*, *P. glauca*, and *P. pungens* are 120.2, 120.2, and 120.4 kb respectively. No difference in cpDNA size cpDNA was observed between white spruce and Engelmann spruce. Small size difference in the blue spruce chloroplast genome versus that found in white and Engelmann spruce may be the result of a deletion, insertion or duplication. A comparison of spruce cpDNA with known maps of radiata pine and Douglas-fir indicated the loss of an inverted repeat region in spruce cpDNA. Sequence homology was observed between chloroplast and mitochondrial DNA fragments in spruce.

## Introduction

Paternal inheritance of cpDNA has been demonstrated in the genera *Pinus* (Neale and Sederoff 1989, Wagner et al. 1987, 1989), Picea (Stine and Keathley, 1990; Stine et al., 1989; Sutton et al.,1991; Szmidt et al., 1988), *Pseusotsuga* (Neale and Sederoff, 1988; Neale et al., 1986), and *Larix* (Szmidt et al., 1987). Occasionally, biparental inheritance of cpDNA occurs, as in *Pinus monticola* (White, 1990).

In general, the organization of cpDNA in gymnosperms differs from that in angiosperms in that the former only has one copy of the inverted repeat region (Lindolm et al., 1988; Strauss et al., 1988) and has a genome size of approximately 120 kb. The only known exception to this is *Ginkgo biloba*, which has two inverted repeats and a genome size of 157 kb (Palmer and Stein, 1986).

RFLPs of cpDNA provide a tool for ascertaining the amount and pattern of introgression between species. Govindaraju et al. (1988) found variation in cpDNA within trees of a *Pinus banksiana* - *P. contorta* sympatric region and suggested that there is increased variability in the organelle genome in regions of sympatry. Szmidt et al. (1988) used this technique to classify seedlots of *Picea sitchensis* and *P. glauca* in zones of introgression.

The phylogenetic diversity of the gymnosperms and the ecological and economic importance of the *Pinaceae* justify a closer analysis of the variation in the spruce chloroplast DNA genome. Currently, the best procedure for developing phylogenies is to map and compare the cpDNA of related species. The physical and genetic map of chloroplast DNA has been determined for *Ginkgo biloba* (Palmer and Stein, 1986) and both Douglas-fir and radiata pine (Strauss 1988).

This chloroplast DNA mapping study increases our understanding of chloroplast DNA variation among members of the *genus Picea*. Physical maps of *Picea pungens*, *P. glauca*, and *P. engelmanii* were constructed by analyzing RFLP's of spruce cpDNA probed with *Petunia* and tobacco cpDNA clones.

## III. Materials and Methods

### DNA preparation

Needles from blue spruce, Engelmann spruce, and white spruce were collected and washed thoroughly with distilled water. The procedure and media for chloroplast DNA isolation was the same as described by Stine et al. (1989), except sucrose gradients, concentrations were changed to 80%, 66%, 52%, 38%, and 10%. Chloroplasts were collected from the 52%-38% interface. Digestion of cpDNA samples with restriction endonucleases was carried out according to the directions supplied by the manufacturer of each enzyme. Chloroplast DNA was subjected to either single restriction endonuclease digestion with PstI, SacI, or SmaI, or double restriction endonuclease digestion with PstI and SacI or SacI and SmaI.

### Electrophoresis

DNA fragments were separated by standard agarose gel electrophoresis techniques as described by Maniatis et al. (1982). The DNA samples were loaded to give approximately equal concentrations of cpDNA. Agarose gels were 0.6% (w/v) and ethidium bromide was incorporated into both the gel and the TBE buffer (0.089 M Tris, 0.089 M boric acid, 2.0 mM EDTA, pH 8.0). High molecular weight markers ranging from 8 to 50 kb (Sigma) and a 1 kb ladder ranging from 0.1 to 12 kb (Boehringer Mannheim

Biochemicals) provided molecular markers for each gel. Electrophoresis conditions were 1 V/cm for 40 hr for the single digested DNA and 1.5 V/cm for 16 hr for the double digested DNA. Following electrophoresis, the DNA in the agarose gels was transferred to Zeta-Probe blotting membranes (Bio-Rad) using the alkaline blotting procedures as described by the manufacturer.

**Southern analysis**

Probes were labeled using random primer labeling according to the manufacturer's (Boehringer Mannheim Biochemicals) directions. A bank of cloned cpDNA of petunia and tobacco was obtained from Dr. J. Palmer and Dr. R. Jansen. Prehybridization and hybridization steps were performed according to Bio-Rad instructions for Zeta-Probe membranes. Post hybridization washes were as follows: the hybridization filter was washed once for a 10-20 seconds at room temperature with 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2 and 0.5% SDS. This was followed by two washes with 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 1% SDS and 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 0.2 SDS at 65$^{o}$C for 30 min each. Autoradiography was carried out at -70$^{o}$C with DuPont Cronex Lightning plus intensifying screens and Kodak X-Omat XAR5 X-ray film. The exposure time ranged from 2 days to 1 week depending on the radiation intensity on the Zeta-Probe membranes. 0.1% SSC and 0.05% SDS were used to strip membranes of labeled DNA.

**Construction of cpDNA physical map**

Following hybridization of single and double digested cpDNA with total tobacco subclones, homologous sequences between tobacco cpDNA clones and spruce cpDNA were compared and analyzed. By arranging the overlapping spruce cpDNA restriction fragments with the labeled tobacco cpDNA clones, a spruce cpDNA physical map was constructed.

**Transverse alternative field electrophoresis (TAFE)**

The procedure and media used to prepare cell homogenate from needles followed the methods of Stine et al. (1989). The homogenate was centrifuged at 2,000 rpm for 3 minutes in a Sorvall GS-3 rotor. The supernatant was then centrifuged at 4,250 rpm for 10 minutes in a Sorvall GS-3 rotor, and that supernatant was centrifuged at 10,000 rpm for 10 minutes in a Sorvall GS-3 rotor. The pellets from the 2,000 rpm, 4,250 rpm, and 10,000 rpm centrifugations were used to make agarose plugs as described by the manufacturer (Beckman). 0.8% LE agarose and running conditions of stage I: 30 minutes, 250 volts, and a field alternation period of 90 seconds; Stage II 10 hours, 250 volts, and a field alternative period of 45 seconds were applied to separate the DNA. The gel was soaked in 0.2 N HCl and slowly shaken at 70 rpm for 12 minutes. Southern blotting was performed as previously described.

**Results**

Petunia cpDNA clones and maize mtDNA clone pZmE1 (Fox and Leaver, 1981), which contains the CoxII gene, were used to probe the DNA separated by the TAFE method. Two DNA bands of approximately 60 kb and 120 kb hybridized to the probes (Figure 1B and 1C). The hybridizing bands were identical in size regardless of whether the chloroplast or mitochondrial probes were used. When DNA separated at the same centrifugation forces, but subjected to CsCl density gradients, was probed with the cpDNA probe, a smeared region from 50 kb to 200 kb was observed (Figure 1D).

The cpDNA restriction fragment sizes of spruce were determined by electrophoresis followed by comparison to DNA molecular size markers

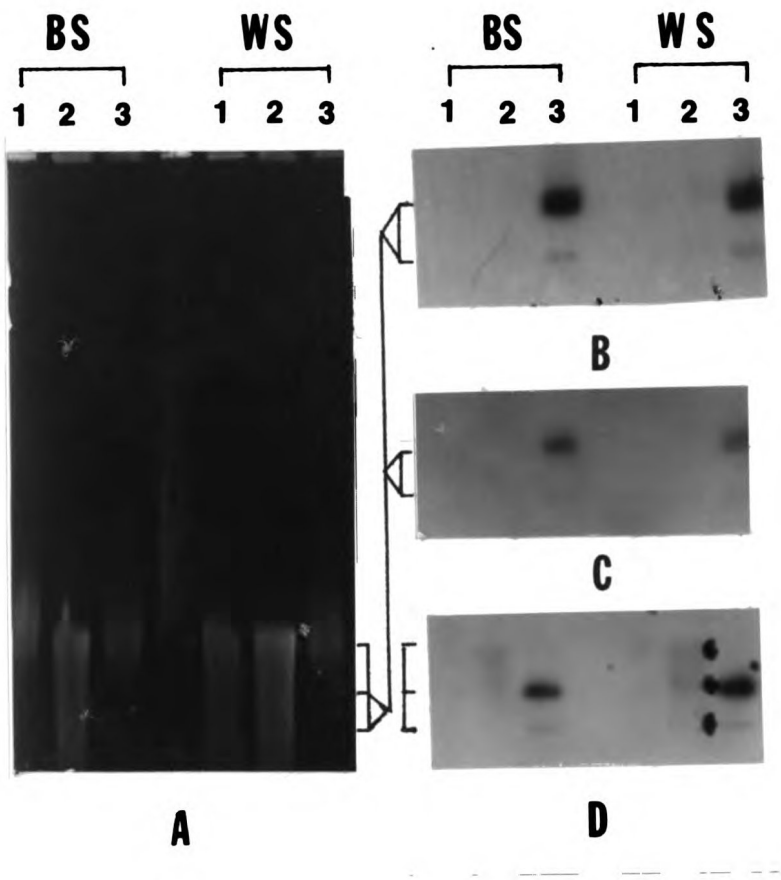


Figure 1. Southern blot analysis of DNA which was isolated at different centrifuge forces and separated by TAFE electrophoresis. 1A. Blue spruce and white spruce DNA was separated by TAFE. 1B. DNA of 1A was hybridized to mtDNA clone CoxII. 1C. After stripping radioactive labeled DNA of 1B, DNA was rehybridized to petunia cpDNA clone p8. 1D. DNA after CsCl density gradient was hybridized to cpDNA clone P3. BS:blue spruce. WS:white spruce. 1:2,000 rpm. 2:4,250 rpm. 3:10,000 rpm.

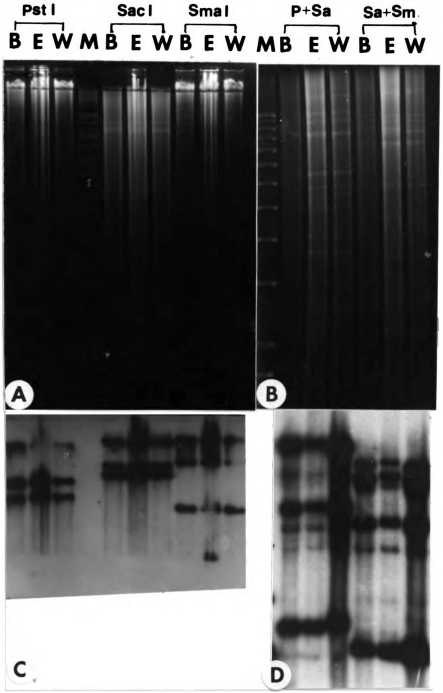


Figure 2. Southern analysis of single and double digested spruce cpDNA. A. Restriction patterns of single digested spruce cpDNA. B. Restriction patterns of double digested spruce cpDNA. C. Hybridization of cloned, $^{32}$-P labeled *Petunia* cpDNA P10 to gel indicated in Fig. 1A. D. Hybridization of cloned, $^{32}$-P labeled *Petunia* cpDNA P1 to the gel indicated in Fig. 1B. B:blue spruce. E:Engelmann spruce. W: white spruce. M:molecular size marker.

(Figure 1 and Table 1). However, repetitive sequences within the spruce cpDNA and homologous sequences between spruce cpDNA and mtDNA (Figure 1) caused problems in constructing the physical map. Forty-two tobacco cpDNA subclones ranging from 1.0 to 6 kb were used to finally create the cpDNA physical map by comaparing the hybridization of single and double digested cpDNA and homolougous sequences of cpDNA fragments.

The cpDNA size of *P. pungens, P. engelmannii, and P. glauca* is about 120.4, 120.2, and 120.2 kb respectively (Table 1). The variation of cpDNA among the different spruce species could be visualized in SacI- and SmaI-cut fragments, but not in PstI-cut fragments. No cpDNA variation in size was detected between white spruce and Engelmann spruce in this study. Values for those two species are reported jointly in Table 1. Variation in total cpDNA between blue spruce and white and Engelmann

Table 1. Spruce cpDNA restriction fragment size.

| *P. pungens* | | | *P. engelmanni and P. glauca* | | |
|---|---|---|---|---|---|
| PstI | SacI | SmaI | PstI | SacI | SmaI |
| 23.7 | 21.4 | 32.3 | 23.7 | 21.4 | 32.3 |
| 21.5 | 17.2 | 21.6e* | 21.5 | 17.2 | 21.4e* |
| 18.7 | 10.6 | 13.7 | 18.7 | 10.6 | 13.7 |
| 16.4a* | 10.6 | 11.6 | 16.2a* | 10.6 | 11.6 |
| 11.4 | 9.6 | 11.3 | 11.4 | 9.6 | 11.3 |
| 8.1 | 9.6 | 9.9 | 8.1 | 9.6 | 9.9 |
| 6.5 | 9.4d* | 5.8 | 6.5 | 9.2d* | 5.8 |
| 5.8 | 9.2 | 3.1 | 5.8 | 9.2 | 3.1 |
| 4.8 | 9.2 | 3.1b | 4.8 | 9.2 | 2.3, 0.8b |
| 2.0 | 4.4 | 2.5c | 2.0 | 4.4 | 2.1, 0.4c |
| 1.5 | 4.2 | 1.9 | 1.5 | 4.2 | 1.9 |
| | 2.7 | 1.2 | | 2.7 | 1.2 |
| | 1.1 | 1.2 | | 1.1 | 1.2 |
| | 0.6 | 0.8 | | 0.6 | 0.8 |
| | 0.6 | 0.4 | | 0.6 | 0.4 |
| 120.4(kb) | 120.4(kb) | 120.4(kb) | 120.2(kb) | 120.2(kb) | 120.2(kb) |

Note: same letter indicates the RFLP polymorphism of cpDNA among speices. '*' indicates same cpDNA fragments which showed variation among species.

spruce was 0.2 kb. Following hybridization of single and double digested DNA with cpDNA probes, a physical and genetic map of spruce cpDNA was constructed (Figure 3).

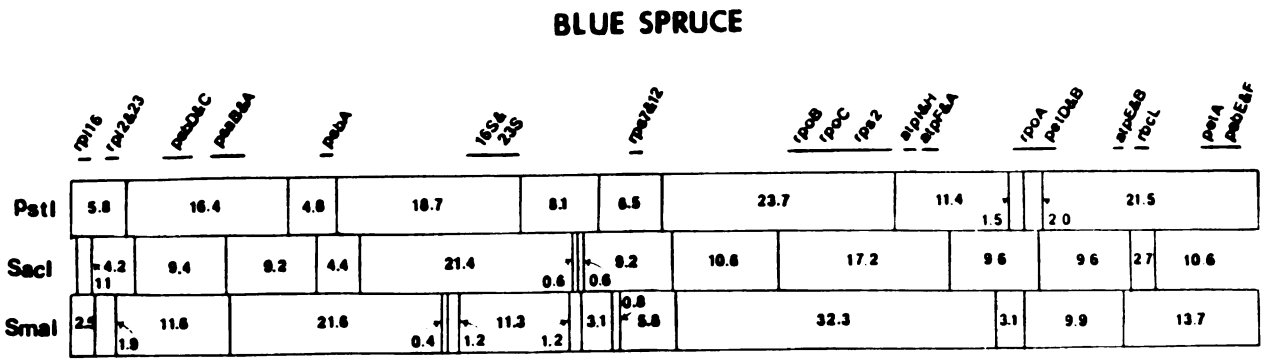


Figure 3. Physical and genetic map of blue spruce cpDNA.

## Discussion

Identical banding patterns were found for DNA separated by different centrifugation forces and TAFE when the DNA was probed with petunia cpDNA or maize mitochondrial CoxII clones (Figure 1). Both hybridizations showed strong signal for DNA in the pellet resulting from centrifugation forces between 4,250 rpm and 10,000 rpm. It has been found that maize mtDNA has regions that are homologous to the cpDNA inverted repeat (Stern and Palmer, 1984). In this study, spruce cpDNA and maize CoxII DNA appeared to share some sequences.

The TAFE method is designed to separate large DNA fragments and whole chromosomes. The 120 kb band from southern analysis of the TAFE gel may be cpDNA because the estimation of spruce cpDNA size is 120.4 kb

for blue spruce and 120.2 kb for white spruce separately (Table 1). However the size of the lower band is estimated at 60 kb band. This band could be supercoiled cpDNA or mtDNA, or plasmids which share some sequence homology with cpDNA and mtDNA. Circular dimers and concatenated dimers of cpDNA have been found in the higher plants (Tewari and Meeker 1979). However they do not appear to be present in the DNA analysis in this study since no bands were located in the 240 kb region of the TAFE gel.

The variation of cpDNA among these spruce species is very low, (Table 1 and Figure 3) and may be only the result of few deletions or duplications or insertions. This may suggest that their divergence is recent event. Morphological similarity between blue spruce and Engelmann spruce, coupled with a smaller geographic range and a reduced variability in the reproductive structure of blue spruce has been taken as an indication that blue spruce is a relatively recent derivation of Engelmann spruce (Daubenmire, 1972). However, paternal inheritance of cpDNA and maternal inheritance of mt DNA in spruce (Stine et al. 1991), the variation of cpDNA and mtDNA between blue spruce and Engelmann spruce (Table 2), and unidirectional interspecific hybridization of blue spruce and Engelmann spruce (only when Engelmann spruce is used as female parent) (Ernst et al. 1990, Fechner and Clark 1969), indicate

Table 2. Similarity of cpDNA and mtDNA among spruces.

| DNA | Blue spruce | Engelmann spruce | White spruce |
|---|---|---|---|
| cpDNA | different | same | same |
| mtDNA | same | different | same* |

*data from probing with maize CoxII clone (personal communication with Andrew David).

that blue spruce and Engelmann spruce may not be as closely related to each other as suggested. Gene sequencing of conserved regions such as 16S and 23S rDNA will help resolve this issue.

It is well documented that the inverted repeat typically found in angiosperms is absent in conifers (Lidholm et al., 1988; Strauss et al., 1988). Our data also showed the absence of the large inverted repeat in spruce cpDNA, resulting in the small size (120 kb) of the chloroplast genome when compared to most other land plants. It has been proposed that the loss of a large repeat sequence increases the frequency of cpDNA rearrangements (Palmer and Thompson 1982). A cpDNA rearrangement of nearly 50 kb in size between Douglas-fir and radiata pine has been documented. This variation may have resulted from the recombination of the repetitive sequences within the cpDNA (Tsai and Strauss 1989). A comparison of cpDNA maps for radiata pine and blue spruce indicates

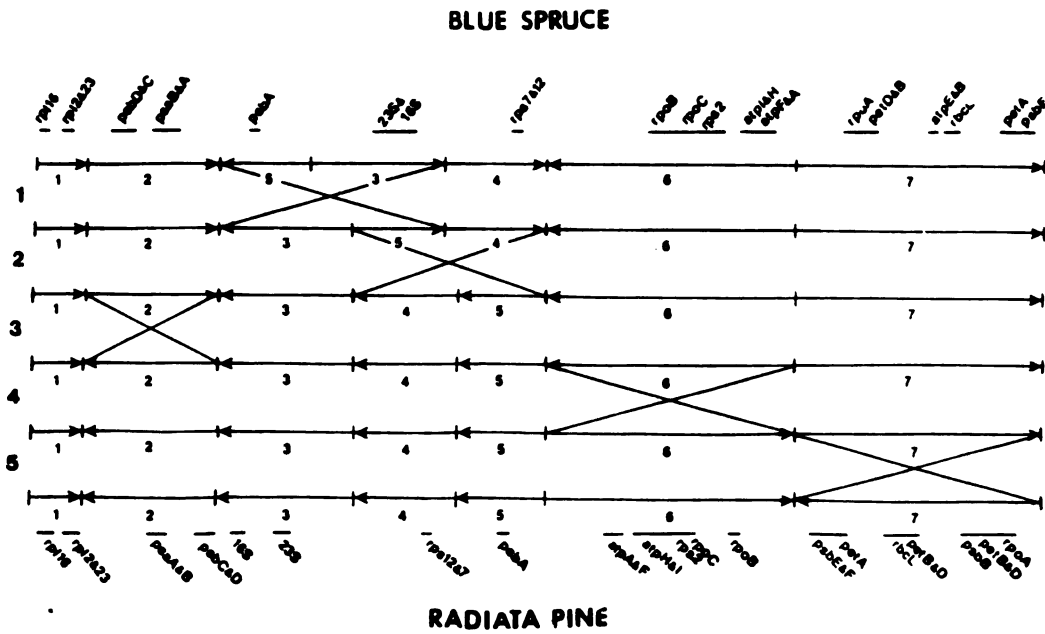


Figure 4. Arrangement of homologous sequences in blue spruce and radiata pine chloroplast genome. cpDNA map of radiata pine is from Strauss et al. 1988. Number above or below maps indicate fragment blocks. Block points are approximate. Number on the left side indicate the steps of inversions between radiata pine and blue spruce.

that several rearrangements (at least 5 inversions) of the cpDNA have occurred (Figure 4).

In the Pinoid group: *Picea*, *Pinus*, and *Pseudotsuga*, a study of seed protein immunology indicated that *Picea* is distant from the other two genera due to a greater time of divergence (Price and Olson-Stojkovich, 1987). Douglas-fir may have arisen from a pine-like ancestor 50 million years ago (Herman, 1985) and the first evidence of spruce in the fossil record dates back 65-135 millions years ago (Florin, 1963). The estimated divergence of the cpDNA sequence between Douglas-fir and radiata pine is 3.8 percent (Strauss et al., 1988). The cpDNA map of spruce is dramatically different from the cpDNA map of Douglas-fir and radiata pine. The cpDNA map analysis among Douglas-fir, radiata pine and spruces supports the seed protein study that H*Picea* has diverged from other two genera and evolved for a considerable time.

**Literature cited**


Daubermire, R. 1972. On the relation between *Picea pungens* and *Picea engelmannii* in the Rocky Moutains. Can. J. Bot. 50:733-742.

Ernst, S.G., J.W. Hanover and D.E. Keathley, 1990. Assessment of natural interspecific hybridization of blue and Engelmann spruce in southern Colorado. Can. J. Bot. 68:1489-1496.

Fechner, G.H. and R.W. Clark, 1969. Prelimiary observations on hybridization of Rocky Mountain spruces. *In*. Proceedings of the 11th meeting of the Commitee on forest Tree Breeding in Canada, MacDonald colledge, Que., August 8-10,1968.Canadian Forestry service. Department of the Environment. Ottawa. pp.237-247.

Florin, R. 1963. The distribution of conifers and taxad genera in time and space. Acta Horti Bergiani, 20(4):122-312.

Fox, T.D. and C.J. Leaver, 1981. The *Zea may* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TAG codons. Cell 26:315-323.

Govindaraju, D., D.B. Wagner, G.P. Smith and B.P. Dancik, 1988. Chloroplast DNA variation within individual trees of a *Pinus banksiana* - *Pinus contorta* sympatric region. Can. J. for. Res. 18:1347-1350.

Hermann, R.K. 1985. The Genus *Pseudotsuga*: Ancestral History and Past distribution (Forest Research Lab., II Oregon State Univ., Corvallis, OR), Spec. Publ. 2b.

Lindolm, J., A.E. Szmidt, J-E. Hallgren and P. Gustafsson, 1988. The chloroplast genomes of conifers lack one of the rRNA-encoding inverted repeats. Mol. Gen. Genet. 211:6-10.

Neale, D.B., N.C. Wheeler, and B.W. Allard, 1986. Paternal inheritance of chloroplast DNA in Douglas-fir. Can. J. For. Res. 16:1152-1154.

Neale, D.B. and R.R. Sederoff, 1988. Inheritance and evolution of conifer organelle genomes. *In*: J.W. Hanover and D.E. Keathley (eds) Genetic manipulation of woody plants. Plenum Press, New York, pp251-264.

---- 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in lobblly pine. Theor Appl Genet 77:212-216.

Palmer, J. and D.B. Stein, 1986. Conservation of chloroplast genome structure among vascular plants. Curr. Genet. 10:823-833.

Palmer, J. and W.F. Thompson, 1982. Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. Cell 29:537-550.

Price, R.A. and J. Olson-Stojkovich, 1987. relationships among the Genera of Pinaceae: an immunological comparison. Syst. Bot. 12:91-97.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophesis. J. Mol. Biol. 98:3683-3687.

Stern, D.B. and J.D. Palmer, 1984. Extensive and widespread holmologies between mitochondrial DNA and chloroplast DNA in plants. Proc. Natl. Acad. Sci. USA 81:1946-1950.

Stine, M., B.B. Sears, and D.E. Keathley, 1989. Inheritance of plastids in interspecific hybrids of blue spruce and white spruce. Theor Appl genet 78:768-774.

Stine, M. and D.E. Keathley, 1990. Paternal inheritance of plastids in Engelmann Spruce X blue spruce hybrids. J. Hered. 81:443-6.

Stine, M., A. David and D.E. Keathley, 1991. Inheritance of plastids and mitochondria in spruce. The Plant genetics News letter. p2-4.

Strauss, S.H., J. Palmer, G.T. Howe. & A.H. Doerksen, 1988. Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. PNAS 85:3898-3902.

Sutton, B.C.S., D.J. Flanagan, J.R. Gawley, C.H. Newton, D.T. Leater, Y.A. Kassaby, 1991. Inheritance of chloroplast and mitochonrial DNA in *icea* and composition of hybrids from introgression zones. Theor. Appl. Genet. 82:242-8.

Szmidt, A.E., T. Alden, and J.E. Hallgren, 1987. Paternal inheritance of chloroplast DNA in *Larix*. Plant Mol. Biol. 9:59-64.

Szmidt, A.E., Y.A. El-Kassaby, A. Sigurgeirsson, T. Alden, D. Lindgren, and J.-E. Hallgren, 1988. Classifying seed lots of *Picea sitchensis* and *P. glauca* in zones of introgression using restriction analysis of chloroplast DNA. Theor Appl Genet 76:841-845.

Tewari, K.K. and R. Meeker, 1979. Chloroplast DNA: Structure, Information Content, and Replication. Molecular Biology of Plants. pp.93-137.

Tsai, C. and S.H. Strauss, 1988. Dispersed repetitive seuences in the chloroplast genome of Douglas-fir. Curr. Genet. 16:211-218.

Wagner, D.B., G.R. Furnier, M.A. Saghai-Maroof, S.M. Williams, B. P. Dancik, and R.W. Allard, 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. USA 84:2097-2100.

Wagner, D.B., D.R. Govindaraju, C.W. Yeatman, and J.A. Pitel, 1989. Paternal chloroplast DNA inheritance in a diallel cross of jack pine (*Pinus Banksiana* Lamb.) J. Hered. 80:483-485.

White, E.E. 1990. Chloroplast DNA in *Pinus monticola*. Theor Appl Genet 79:251-255.