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## Timing of DNA Extraction from Megagametophytes for PCR during Initial Steps of Seedling Development in *Picea abies* (L.) KARST.

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(Received 10th February 1998)

### Abstract

Within the framework of QTL investigations and subsequent marker aided selection in *Pinaceae*, a need to grow seedlings with saved megagametophytes for DNA extraction has evolved. The present investigation reports a timing experiment in which the optimum stage of seedling development is identified. DNA amount proved to be relative independent of seedling

development, but successful PCR was, probable due to the occurrence of fat and carbohydrates, only possible at relative late stages of seedling development when the role of the megagametophyte as a nutrient source has decreased. Removing the megagametophyte at this stage is not a problem for the germinating seedlings, which then are relying on their own root uptake and photosynthesis.

**Key words:** DNA extraction, megagametophytes, RAPD, *Picea abies*, seedling development.

**FDC:** 165.3/4; 181.525; 174.7 *Picea abies*.

## Introduction

An important field of supportive research for conifer breeding is to map major genes behind breeding objectives. By using DNA-markers for genome mapping it is possible to study linkage between these markers and quantitative trait loci – QTL – in for instance *Picea abies* (L.) KARST.

The PCR based random amplified polymorphic DNA markers – RAPD – appear to be well suited for genetic mapping in *Pinaceae* species, because it is possible to utilize the tiny megagametophytes of individual seeds of a single tree as a mapping population.

*Pinaceae* seeds, when fertilized, consist of a haploid nutritive tissue, the megagametophyte, and the diploid developing embryo. The megagametophytic tissue and the corresponding egg cell – female founder of the embryo – originate from a single mother cell as a product of a meiotic division, and therefore possess identical genotypes (CHAMBERLAIN, 1935).

A main drawback of the RAPD system is the dominant expression (absent/present) of this marker type. Usually there is only two alleles in a certain locus and heterozygotes cannot be distinguished from homozygotes in diploid tissue. This can be circumvented by using haploid tissue. By recording markers in the haploid megagametophytes, we can identify the mother contribution to the genotype of the progeny and we can map the markers of individual mother trees by performing classical linkage studies on recombinations of markers directly expressed in the haploid megagametophytes.

QTL-mapping can then be performed by studying co-segregation of markers in the megagametophytes – the mother contribution – and quantitative traits in the corresponding germinating half-sib or full-sib offspring population, which later develops into juvenile and mature trees (WILCOX et al., 1996).

An important component in such QTL experiments is therefore the survival rate of the seedlings with rescued megagametophytes as well as purity of the extracted DNA from the corresponding megagametophytes.

Although RAPDs are simple to perform, the early experience with amplification of RAPD fragments from megagametophytic DNA did not turn out convincing at least in our laboratory. Often the resultant amplified product was missing or not complete and not reproducible at a sufficient high rate. In order to refine the combined process of RAPD-analyses on saved megagametophytic tissue and secure undisturbed further growth of the corresponding seedling, a developmental stage/PCR experiment was initiated.

The reported study is aiming to uncover the two-sided problem encountered:

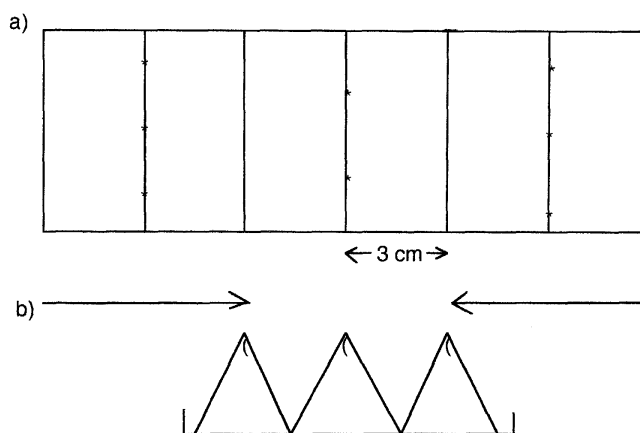
- Development of a DNA extraction method tailored to megagametophytes of germinating seedlings.
- Development of a safe method for growing seedlings when megagametophytes are rescued for DNA extraction.

## Materials and Methods

### Handling of plant material

Seeds from clone number V6470 were germinated on sterile filter paper according to the method of KNUDSEN (personal communication), see figure 1.

During the process of early germination megagametophytes were rescued from a sample of at least 5 germinating seedlings



**Figure 1.** – Germination in Petri dishes in folded filterpaper according to the “KNUDSEN method”. Surfaced-treated seeds are placed at regular intervals on sterile filterpaper. a) Seen from above – b) side view. After folding the filterpaper and moistening with water, the germination will be initiated and seedlings develop an erect growth pattern suitable for later transplanting after megagametophyte rescuing.

when they had reached 5 successive stages of development, see figure 2. Stage 1: radicle not seen, stage 2: radicle 1 cm, stage 3: radicle 2 cm, stage 4: radicle 4 cm, and stage 5: full-grown seedling, just before spontaneous release of the megagametophyte. In the different stages mentioned, the seed coat was removed and the megagametophytes were separated from the embryos / seedlings and stored at -80°C in distinct sterile microcentrifuge tubes until later DNA extraction. The seedlings from stage 3 to 5 were transplanted to containers in mini-greenhouses to maintain undisturbed growth and survival.

### DNA extraction, - amplification, and gel electrophoresis

The applied materials and methods are described by SKOV (1998).

## Results

Results of the timing experiment are presented in figure 2.

### DNA-extraction and subsequent PCR of megagametophytic DNA

The following main interpretations of the observations are drawn:

- Total DNA amount in the megagametophytes shown in the second column – “DNA-extraction” – seems to be stable through all investigated stages of seedling development.
- At the early germination stages 1 to 3 detectable amounts of DNA are left in the gel-wells when total DNA is applied on agarose gels for initial electrophoresis. This coincides with poor Polymerase Chain Reactions shown in the third column – “PCR”.

Determination of the DNA concentration using an fluorometric assay gives an estimate of the amount of DNA extracted, but actually tells nothing about the purity of the DNA (HENGEN, 1994).

It is to a certain degree possible to dilute the DNA samples and thereby the contaminants with distilled water and in this way be able to amplify the DNA (data not shown). However results were not reproducible probably due to the poorer genome representation in the reaction mixes prepared for PCR.

Inclusion of a proteinase-K step in the extraction procedure helps overcoming part of the problem with contaminants in the

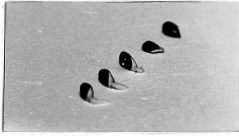
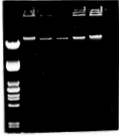
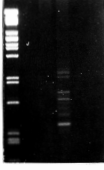
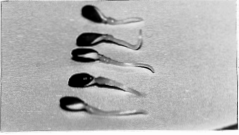

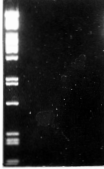
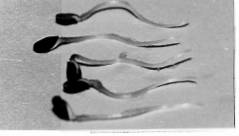
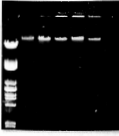
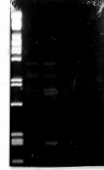
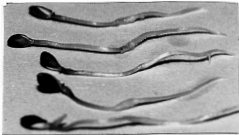
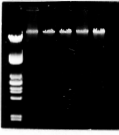
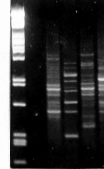
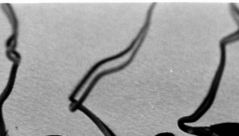

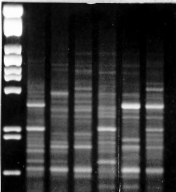
STAGES	MEGAGAMETOPHYTES		SEEDLINGS	
	DNA - EXTRACTION	PCR	SURVIVAL OF TRANSPLANTS (NON STERILE ENVIRONMENT)	REMARKS
1. 				REQUIRE OTHER METHODS
2. 				
3. 			LOW	
4. 			MEDIUM 77 %	PREFERRED STAGE
5. 			HIGH 94 %	

Figure 2. – Optimization of the combined process of: a) DNA – extraction and PCR of megagametophytes. b) Growing seedlings with rescued megagametophytes.

Electrophoresis of extracted DNA shows a stable amount of DNA in megagametophytes independent of development stage. At early stages 1 to 3, a detectable amount of DNA are left in the gel-wells, which coincides with a poor PCR amplification.

early stages of seed germination. GRATTAPAGLIA et al. (1992) made the same conclusions in their investigation on megagametophytic DNA from different Central American pine species.

#### *Survival of the transplanted seedlings*

Survival improved from an unacceptable low level at stage 3 to a near perfect level at stage 5.

The main conclusion then is that germination stage 5 is the preferred stage for megagametophyte collection. At this stage

we combine the ideal stage for DNA-extraction with the most undisturbed seedling development after transplanting, which is a key point for our future attempts on using RAPD markers for mapping QTLs.

#### **Discussion**

The observed stability of DNA amount during germination coupled with the apparently increasing purity of the extracted DNA during successive stages of the process, supply us with some insight into the biochemical metabolism during seed

germination.

The pathway of transformation of fat into carbohydrates in germinating seed is described by various textbook authors, e.g. by THOMAS (1972), BEEVERS (1980), and HUANG (1987).

During the early stages of germination, the amount of fat and possibly carbohydrates may still be so high that it explains our observation of contamination of the extracted DNA, so detectable amount of DNA is left in the wells and the polymerase chain reaction is not running successfully.

In late stages of germination, nutrients in the form of fat and carbohydrates are removed from the megagametophytes, whereas DNA, which does not have this function, is left relative undisturbed for extraction in a much cleaner form.

The rapid metabolism during seed germination may be the cause for the general finding that megagametophytic DNA was the most sensitive tissue type tested in Norway spruce to utilize in the RAPD assay (SKOV, 1998).

#### Acknowledgements

This investigation forms part of the project 'Identification of DNA

markers suitable for genome mapping in *Picea abies* (L.) KARST.' initiated by the Hørsholm Arboretum and financed by the Danish Research Council. The laboratory part was carried out in the period 1991 to 1994 at The Risø National Laboratory with instruments financed by The Royal Veterinary and Agricultural University. I am grateful to VIBEKE SIMONSEN and HUBERT WELLENDORF for reviewing the manuscript.

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## A Partial Linkage Map of *Picea abies* Clone V6470 Based on Recombination of RAPD-markers in Haploid Megagametophytes

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(Received 7th October 1996)

#### Abstract

By applying a RAPD assay to the haploid megagametophytes of Norway spruce, a low-density linkage map of a cloned member of the current breeding population has been constructed. The map involves allocation of 61 marker loci to 13 linkage groups as well as 22 markers to 11 single-pair groups; 62 markers remained un-linked. In total, the 82 markers cover 1385 KOSAMBI cM map units. This corresponds to an average distance between marker loci of 23.8 cM (range 2.8 to 57.6). The size of linkage groups varied between 38 cM and 213 cM. In three cases the map order of the markers was judged inconsistent. A data set is provided with multipoint analyses of each linkage group with LOD scores of 'best' and 'best alternative' map in relation to each other. Another dataset is provided with pair-wise map distances within linkage groups based on two-point analyses with the appropriate LOD scores. These data sets should facilitate construction of a 'consensus map' for this species. Perspectives for further developments for mapping of forest trees are outlined.

*Key words:* *Picea abies*, linkage maps, RAPD-markers, megagametophytes.

*FDC:* 165.3; 165.441; 161.6; 174.7 *Picea abies*.

#### Introduction

Recent development of DNA-markers based on Polymerase Chain Reactions (PCR) has allowed researchers to embark on mapping of the until recently mostly unknown genomes of conifers.

The aim of the present investigation is to test, if RAPD markers (WILLIAMS et al., 1990) are suitable for intermediate to high density genome mapping in our specific organism Norway spruce *Picea abies* L. (KARST.), in which a number of tree improvement programs are operational.

Prerequisites for embarking into such investigations are reliable methods and laboratory protocols to the degree where confidence exists, including the following points, i) DNA extraction, ii) tissue expression, iii) MENDELian or other types of inheritance, and iv) repeatability of observations within and between laboratories. Contradicting evidence has been found concerning the reproducibility of RAPD-markers between laboratories (PENNER et al., 1993). We have, in Norway spruce, investigated this array of points based on our own laboratory conditions (SKOV, 1998a and b) complemented with one other laboratory environment (SKOV, 1998c) and found the behaviour