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Genome Mapping of Quantitative Trait Loci in *Salix* with an Emphasis on Freezing Resistance

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Abstract

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Using the genetic map of the backcross family, nineteen quantitative trait loci (QTL) were identified; eleven for growth-related traits and eight for the timing of bud flush. The estimated magnitude of the QTL effect ranged from 12 to 24% of the total phenotypic variance. One QTL for height growth, one for diameter and one for the height:diameter ratio, were found clustered in the same marker interval. One QTL associated with indoor bud flushing coincided with a QTL controlling timing of bud flush in the field. Little evidence was found for QTL stability in height growth over 3 consecutive years.

Ten genomic regions controlling freezing resistance and nine affecting phenological traits were identified during cold acclimation of the F_2 family. The magnitude of the phenotypic variation explained by each freezing resistance locus varied over acclimation time (3 - 45%) and there was no time point at which all the QTL could be detected. The single QTL detected for non-acclimated freezing resistance did not reach significance at any time during cold acclimation suggesting an independent relationship between non-acclimated and acclimated freezing resistance in *Salix*. The determination of QTL position on the map suggested the partial involvement of a common set of genes for autumn freezing resistance and phenology traits. Of the 14 QTL controlling autumn freezing resistance and phenological traits at the indoor experiment, six (43%) were associated with autumn phenology traits *i.e.* total height increment, dry-to-fresh weight ratio and number of new leaves, as measured in the field. A major locus with multi-trait association in both indoor and outdoor experiments was detected. The presence of genes with large effect for growth and adaptation traits suggests that a marker assisted selection breeding scheme could accelerate the breeding process in *Salix*.

Key words: genetic mapping, QTL mapping, genetic markers, freezing resistance, phenology traits, growth traits, *Salix*.

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The mind can conceive only phenomena

but never the essence,

-more strictly, not even the phenomena

but only their interactions.

..... if the mind could not overcome borders, may be my heart could?

from "*The Askitism*" of N. Kazantzakis

Αφιερώνεται

σε όλους τους πολεμιστές του φωτός –με τη λάμψη στο βλέμμα- που πάντα αναζητούν μιαν αιτία,

στους γονείς μου και στην πόλη μου, τη Σπάρτη.

To all warriors of light-with the shiny glance-who always seek for the cause,

to my parents and to my city-Sparta

Abstract

Tsarouhas, V. 2002. Genome mapping of quantitative trait loci in *Salix* with an emphasis on freezing resistance.
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Περίληψη (Abstract in Greek)

Τσαρούχας Βασίλειος, 2002. Χαρτογράφηση γενετικών θέσεων που ελέγχουν ποσοτικούς χαρακτήρες στο γονιδίωμα των ιτιών (*Salix*) με ιδιαίτερη έμφαση στην ανθεκτικότητα θερμοκρασιών κάτω του μηδενός. (Διδακτορική διατριβή. Department of Plant Biology, SLU, Box 7080, Uppsala, Sweden. Agraria 327, ISSN 1401-6249, 91-576-6164-2)

Η σύγχρονη ανάπτυξη της βιοτεχνολογίας μπορεί να συμβάλλει στο καλύτερο γνωστικό επίπεδο του γενετικού ελέγχου και της μοριακής βάσης των ποσοτικών χαρακτήρων στα φυτά. Η παρούσα διδακτορική έρευνα έχει ως κύριο σκοπό τον προσδιορισμό γονιδίων πού ελέγχουν την ανθεκτικότητα στις χαμηλές θερμοκρασίες (<0°C) και την φαινολογία των ιτιών (*Salix*).

Δύο γενετικοί χάρτες σύνδεσης κατασκευάστηκαν για τα γονιδιώματα δυο οικογενειών ιτιών. Ο πρώτος γενετικός χάρτης βασίστηκε στην αναδιασταύρωση τού αρσενικού υβριδογενούς κλώνου Björn (*Salix viminalis* x *Salix schwerinii*) με το θηλυκό κλώνο 78183 (*Salix viminalis*) και περιείχε 325 AFLP και 25 RFLP μοριακούς δείκτες [οι οποίοι RFLP ιχνηθέτες –probes- προήλθαν κυρίως από το γονιδίωμα της λεύκης (*Populus*)]. Ο δεύτερος γενετικός χάρτης περιείχε 433 AFLPs και ήταν βασισμένος στην F_2 οικογένεια των κλώνων Jorunn (*S. viminalis*, ευαίσθητος στους παγετούς) και SW901290 (*S. dasyclados*-like, ανθεκτικός στους παγετούς). Ο μέσος όρος συνολικού μήκους των χαρτών και ο μέσος όρος κάλυψης του γονιδιώματος ήταν αντίστοιχα 2483cM και 72%, ενώ η μέση απόσταση μεταξύ μοριακών δεικτών ήταν περίπου 11cM. Στον πρώτο γενετικό χάρτη, προσδιορίστηκαν δεκαεννιά (19) γενετικές θέσεις που ελέγχουν σε ποσοτικούς χαρακτήρες (QTL), όπως αύξηση και χρόνος εισόδου από το λήθαργο στη βλαστιτική περίοδο. Η επιρροή κάθε γενετικής ή χρωμοσωμικής θέσης (locus) στους χαρακτήρες κυμάνθηκε από 12 ως 24% της συνολικής φαινοτυπικής διαφοροποίησης. Τρείς γενετικές θέσεις βρέθηκαν σε συστάδα, μία με έλεγχο για αύξηση ύψους, μία για διάμετρο και μια για το πηλίκο ύψος/διάμετρος. Καμμία γενετική θέση δεν βρέθηκε για 2 ή 3 συνεχόμενα χρόνια.

Η γενετική ποικιλομορφία επιλεγμένων κλώνων για παραγωγή βιομάζας ιτιών, αλλά και λευκών, όσον αφορά την ανθεκτικότητα σε χαμηλές θερμοκρασίες, βρέθηκε εξαρτημένη από το φαινολογικό στάδιο του φυτού, όπου είχε τη μέγιστη τιμή κατά την αρχική βλαστιτική και προς το τέλος της αυξητικής περιόδου (αρχές-μέσα φθινωπόρου). Στη παρούσα χαρτογράφηση γενετικών θέσεων των ιτιών, μελετήθηκε η ανθεκτικότητα προς το τέλος της αυξητικής περιόδου (αρχές-μέσα φθινωπόρου). Προσδιορίστηκαν δέκα γενετικές θέσεις που ελέγχουν την ανθεκτικότητα σε θερμοκρασίες κάτω των 0°C και εννέα που ρυθμίζουν τα φαινολογικά χαρακτηριστικά της F_2, πριν και κατά τη διάρκεια 6 εβδομάδων εγκλιματισμού (μικρές μέρες/χαμηλές θερμοκρασίες). Η μέτρηση της ανθεκτικότητας βασίστηκε κυρίως σε βλάβες των κυτταρικών μεβρανών όπως καθορίστηκαν με τη μέθοδο της διορροής ηλεκτρολυτών από ιστούς φύλλων. Η επιρροή κάθε γενετικής θέσεως στους χαρακτήρες κυμάνθηκε από 3 ως 45% της συνολικής φαινοτυπικής διαφοροποίησης. Η μοναδική γενετική θέση η οποία προσδιοριστήκε πριν τον εγκλιματισμό δεν βρέθηκε κατά τη διάρκεια αυτού, δείχνοντας έτσι, ότι ο γενετικός έλεγχος της εγκλιματιζόμενης και μη εγκλιματιζόμενης ανθεκτικότητας (στις χαμηλές θερμοκρασίες) είναι, τουλάχιστον εν μέρει, ανεξάρτητος. Η απεικόνιση των γενετικών θέσεων στο χάρτη σύνδεσης φανέρωσε ότι ο έλεγχος της ανθεκτικότητας, σε χαμηλές θερμοκρασίες, και της φαινολογίας κατά τη διάρκεια εγκλιματισμού στο κρύο, γίνεται εν μέρει από κοινά γονίδια. Το 43% των γενετικών θέσεων που προσδιορίστηκαν σε πειράματα εσωτερικού χώρου, βρέθηκε να ελέγχουν τα αντίστοιχα χαρακτηριστικά σε πειραματικές φυτείες ανοιχτού χώρου. Προσδιορίστηκε μια εξαιρετικά μείζονα γενετική θέση (major locus) με πολλαπλό έλεγχο σε ανθεκτικότητα και φαινολογικά χαρακτηριστικά. Συνοπτικά, η εντόπιση μείζονων γονιδίων για αύξηση και προσαρμοστικότητα σε χαμηλές θερμοκρασίες, υπαινίσσεται ότι η επιλογή με μοριακούς δείκτες θα μπορούσε να επιταχύνει την τρέχουσα γενετική βελτίωση των ιτιών.

Λέξεις κλειδιά: γενετικός χάρτης σύνδεσης, γενετική θέση (locus), αύξηση, ποσοτικοί χαρακτήρες, ανθεκτικότητα χαμηλών θερμοκρασιών (< 0 °C), φαινολογικοί χαρακτήρες, μείζονα γονίδια, ιτιά, *Salix*.

Appendix

Papers I-VI

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Tsarouhas V, Kenney WA and Zsuffa L, (2000). Application of two electrical methods for the rapid assessment of freezing resistance in *Salix eriocephala*. *Biomass and Bioenergy,* 19: 165-175.

II. Tsarouhas V, Kenney WA and Zsuffa L, (2001). Variation in freezing resistance during different phenological stages in some *Populus* and *Salix* clones: implications for clonal selection. *Silvae Genetica,* 50: 54-63

III. Tsarouhas V, Gullberg, U and Lagercrantz U, (2002). An AFLP and RFLP linkage map and Quantitative Trait Loci (QTL) analysis of growth traits in *Salix*. *Theoretical and Applied Genetics* (in press)

IV. Tsarouhas V, Gullberg U and Lagercrantz U, (2002). Mapping of Quantitative trait loci (QTL) controlling timing of bud flush in *Salix*. (manuscript)

V. Rönnberg-Wästljung A-C, Tsarouhas V and Lagercrantz U, (2002). A genetic linkage map of *Salix* based on AFLP markers. (manuscript)

VI. Tsarouhas V, Gullberg U and Lagercrantz U, (2002). Mapping of Quantitative Trait Loci (QTL) affecting autumn freezing resistance and phonological traits in *Salix*. (manuscript)

Contents

Abbreviations

List in alphabetical order of the commonly used abbreviations in the text:

AFLP = amplified fragment length polymorphism
CBF = C-repeat binding factor
COR = cold regulated
D = dormant
DM = dry matter
EF = early fall
ERD = early responsive to desiccation
ES = early spring
FTB = flushing of terminal buds
G = growing
IDX_t = index of injury at freezing temperature *t*
KIN = cold induced
LOD = logarithm of the odds
LTI = low temperature-induced
MAS = marker-assisted selection
NAG = new axillary growth
PCR = polymerase chain reaction
QTL = quantitative trait locus/loci
RAPD = random amplified polymorphic DNA
RC_t = relative conductivity at freezing temperature *t*
RD = responsive to desiccation
RFLP = restriction fragment length polymorphism
S = spring
SNP = single nucleotide polymorphism
SRIC = short rotation intensive culture
SSR = simple sequence repeat

Introduction

The present thesis is focused on the identification of genes affecting freezing resistance in *Salix*. Low temperature is considered the most important environmental factor limiting the growth, development and distribution of plants. The lowest temperature on the earth, almost -90 °C, has been measured in Antarctic while minimum temperatures as low as -66 to -68 °C have been recorded in valleys and lowlands in eastern Siberia. Severe frost (minimum annual temperature < -20 °C) is expected to occur on 42 % of the earth's surface while only 1/3 of the total land area is totally safe from frost (Larcher 1995). Freezing temperature stress has an enormous impact on agricultural and forestry deployment practices. Yield losses due to freezing injuries have been estimated to be 14 billion US $ per year world-wide (Steponkus *et al.* 1993). A modest increase in plants freezing resistance of only 1-2 °C is expected to have a significant economical impact on agriculture. For *Salix* growing in short rotation intensive cultures (SRIC) systems, freezing injury is the most significant obstacle for expanding their use in northern countries (von Fircks 1985). Clones used in biomass plantations today can not be grown successfully in northern Sweden or even in central parts of Sweden due to severe frost damage (Larsson 1998). It has been estimated that a single night with frost in central Sweden can cause losses up to 60% of the annual yield (Verwijst *et al.* 1996).

The formation of genotypes with increased freezing resistance would provide more reliable means of minimizing yield losses. Despite of the considerable research effort devoted in the past few decades to studies of physiological and biochemical pathways involved in freezing stress for both herbaceous and woody plants, only modest improvement in freezing tolerance has been achieved (Thomashow 1998). The current advancement of biotechnology, however, could provide additional opportunities for a better understanding of the genetic control and the molecular basis of freezing resistance in plants. This could potentially lead to the development of new strategies to improve plant-freezing resistance and result in increased plant productivity.

One strategy that holds promise for the development of superior frost resistance genotypes is the identification and localization of genes controlling freezing resistance. This work requires the detection of co-segregation of the phenotype with genetic markers on chromosomes. A prerequisite then, is a large number of genetic markers based on differences in DNA sequences among individuals. Until the 1980s it was difficult to look specifically at the DNA sequences of different organisms directly to detect polymorphism. Most work was concentrated on differences at the primary structure of proteins. However, only about 10 % of the DNA is "coding DNA" and hence looking at structural genes limits the variation available for analysis (Kearsey and Pooni 1996). Now-days, new technology has made it easier to identify variation in the DNA both in coding and non-coding regions of the chromosomes and thus made it possible to generate a large number of loci for the construction of high-dense maps.

The present thesis is focused on the mapping of genes involved in freezing resistance in *Salix*. In the following text I will first briefly introduce the basics of mapping genes underlying the so-called complex or quantitative traits, such as freezing resistance, along with physiological and genetic aspects of freezing resistance.

Genetic dissection of quantitative traits

Quantitative traits and molecular technology-new opportunities

For many traits the variation between individuals does not fall into discrete classes in Mendelian proportions but is continuous, showing a gradation from one extreme to the other. Examples from different organisms include traits such as hypertension and body weight in humans, growth rate and leanness in livestock, yield and flower time in plants (Kato *et al.* 1999; Axelsson *et al.* 2001; Andersson 2001). Traits with such continuous variation controlled by many loci and environmental effects are referred to as "quantitative", "polygenic" or "complex". By definition quantitative trait loci (QTL) are genomic regions that differentially affects a quantitative trait. Quantitative traits can be contrasted to qualitative, major gene traits, for which the variation is due to allelic differences at just one or a couple of genes and the effects of allelic differences on the phenotype are sufficiently large relative to differences due to the environment to produce discrete classes between genotypes.

Until recently it has been impossible to locate QTL controlling a quantitative trait. Due to advent of molecular markers and advances in statistical methodologies, it is now possible to map individual QTL to specific chromosomal segments in a genome. This can complement traditional quantitative genetics with a more detailed understanding of the genetic architecture of complex processes such as growth and adaptation. The detection and mapping of QTL allows a more realistic modelling of phenotypic variation and responses to selection (Lande and Thompson 1990). Further, information on genetic markers associated with a QTL can be used to improve selection. Marker-assisted selection (MAS) may be an effective way of introducing desirable genes from one breed to another or of improving selection within a breed (Haley and Andersson 1997). Additionally, the mapping of a QTL opens the way to positional cloning of the gene, as have been achieved for a number of monogenic and a few polygenic traits in plants and animals (Brommonschenkel and Tanksley 1997; Pierre *et al.* 2000; Frary *et al.* 2000; Millan *et al.* 2000; Andersson 2001).

Genetic mapping

The genetic dissection of complex traits requires the development of a genetic map of the species in question. Genomes vary among different organisms in terms of total DNA content, chromosome number, ploidy level, total recombination distance in the genome and number of functional genes. Eucaryotic species are extremely variable with respect to their chromosome numbers. Among the well-studied species, the haploid chromosome number (n) ranges from 4 in a fruit fly to 23 for a

human (Table 1) while for other eukaryotes the chromosome number (n) ranges from 2 (*e.g.* horse threadworm) to several hundreds (e.g ancient adder's tongue fern). Similarly, large variation among organisms can be observed for the amount of the molecular weight (Mbp) and recombination units in genome (Table 1).

Table 1 *Genome size (chromosome number n, base pairs Mbp, and recombination map length cM) for some of the most extensively studied organisms*

Organism	***n***	***Mbp***	***cM***	
Yeast (*Saccharomyces cerevisiae*)	16	14	4.20	
Fruit Fly (*Drosophila melanogaster*)	4	170	280	(Female)
Mouse (*Mus musculus*)	20	3000	1700	
Human (*Homo sapiens*)	23	3000	2800	(Male)
			4800	(Female)
Arabidopsis thaliana	5	100	500	
Rice (*Oryza sativa*)	12	450	1700	
Corn (*Zea mays*)	10	2500	1500	
Loblolly pine (*Pinus taeta*)	12	20000	2000	

Note: the values for map length are average numbers from published data. Due to incomplete genome coverage genome length should be regarded as minimum estimates; Mbp=millions of base pairs (source: Liu 1997).

A large number of genomes have been mapped including the human genome, as well as several animal and plant genomes. The main objectives in the analysis of the human genome (and model plant organisms such as *Arabidopsis*) is an understanding of the genomic organization and its relation to function; complete genome sequence; complete full-length cDNA sequence data; large-scale gene function (Collins *et al.* 1998; Gale and Davos 1998). In contrast, the analysis of most plant genomes is oriented much more towards providing a means for locating genes of economic importance, a theme that is pursued in the present thesis. In this section, I will shortly introduce three requirements for gene mapping: genetic markers, pedigrees and linkage analysis.

Genetic markers

The identification and localization of genes affecting phenotypic traits requires the detection of co-segregation of the phenotype with genetic markers on chromosomes. Genetic markers are characters that show simple Mendelian inheritance and can be used as the landmarks on a genetic map. Phenotypic traits like seed color and seed size (Sax 1923) in plants and blood protein polymorphism in animals (Smithies 1955) were the first genetic markers used in gene mapping. For these type of markers, however, it was necessary for a gene to cause a discrete change in the phenotype of an organism in order to be detected (Tanksley 1993). Even where phenotypic trait-markers were available they could not provided broad

genome coverage. The invention of DNA markers later opened a new era in genome mapping. DNA markers represent DNA sequence polymorphisms that can be readily assayed and that show Mendelian inheritance.

Restriction fragment length polymorphism (RFLP) are DNA markers that are obtained by using restriction endonucleases to precisely cleave a genomic DNA fragment containing a particular DNA sequence. RFLPs yield simple patterns of variation that are readily interpreted as individual loci that contains a specific DNA sequence corresponding to the probe sequence. RFLPs are informative markers as they are usually co-dominant. They have been broadly used to construct linkage maps for many plants important for agriculture and forestry research (Lagercrantz and Lydiate 1996; Bradshaw *et al.* 1994; Devey *et al.* 1994). However, the large amount of high molecular weight genomic DNA required and the multiple step procedure (cloning informative probes, Southern blots, hybridization) make RFLPs laborious for constructing high-density genetic maps. The later development of markers based on the PCR (Polymerase Chain Reaction) assay permitted much faster genotyping of a large number of individuals. One PCR based type of marker is the RAPD (Random Amplified Polymorphic DNA) marker. RAPDs are based on amplification of a small amount of genomic DNA template using a single short primer (Williams *et al.* 1990). RAPDs can provide a significant advance in the construction and saturation of genetic maps. However, their mode of inheritance is typically dominant; the sequence simply amplifies or not, and one copy cannot readily be distinguished from two copies. This means that it is difficult to distinguish a homozygote (+ +) from a heterozygote with one "null" allele (+ –).

Another class of highly polymorphic markers that combines both RFLP and PCR strategies are the AFLPs (amplified fragment length polymorphisms) (Vos *et al.* 1995). With the AFLP technique genomic DNA is first digested to completion with two restriction enzymes: a rare cutter (*e.g.* the six-base recognition enzyme *Eco*RI) and a frequent cutter (*e.g.* four-base recognition enzyme *Mse*I); and then ligated to oligonucleotide "adaptors." The sequence of the restriction site, the adaptor and one extra nucleotide serve as the primer for amplification of the fragments (pre-amplification). Because the amplified fragments are too many to be analysed at that stage a second amplification is made using the primer of the pre-amplification plus a few extra selective nucleotides. In this amplification (selective amplification) only that subset of fragments whose terminal sequences happen to match the selective bases will be amplified. By choosing a suitable number of selective bases, the amplified subset can contain a low number of fragments that can be resolved on a polyacrylamide gel. The frequency of polymorphism of AFLPs may be expected to be similar to that of RFLPs, however, because each AFLP experiment generates many different loci, the probability of finding polymorphic loci is considerably increased. AFLP fragments originate from unique locations in the genome and can be used as landmarks in genetic and physical maps. Their advantage over RAPDs is their high reproducibility (Jones *et al.* 1997) due to stringent reaction conditions during PCR. Further, the larger number of markers generated per AFLP reaction can contribute to rapid construction of high-density maps as well as to the saturation of existing maps. The AFLP technique has recently been used to create genetic maps in several agricultural plants (Becker *et al.* 1995; van Eck *et al.* 1995;

Keim *et al.* 1997; Wang *et al.* 1997; De Riek *et al.* 1999) and forest tree species (*Eucalyptus globulus, Eucalyptus tereticornis*: Marques *et al.* 1998; Norway spruce: Paglia *et al.* 1998: *Larix*: Arcade *et al.* 2000; *Populus*: Cervera *et al.* 2001). However, AFLPs are usually dominant markers. Microsatellites or SSRs (simple sequence repeats), another PCR-based category of markers, offer co-dominant evaluation of a locus. These types of markers are based on PCR-amplification of a genomic region containing a simple repeated sequence (*e.g.* CT*n*) (Morgante and Olivieri 1993). Microsatellites in forest-tree species have been reported for *Pinus radiata* (Smith and Devey 1994), *Eucalyptus* (Brondani *et al.* 2002), *Populus* (Cervera *et al.* 2001). Although SSRs are highly polymorphic and multi-allelic markers, they require considerable effort for development. Another marker system technique, the so-called single nucleotide polymorphism (SNP), has recently been developed. With this technique individual point mutations through the genome can been detected. SNPs are diallelic in populations and they are well adapted to new commercially available fuorescently systems (Kalinina *et al.* 1997), offering possibilities of high automatization in genome mapping (Picoult-Newberg *et al.* 1999). In humans genome there are thought to be over 200 000 SNPs that lie in genes. In plants, the technique has been recently introduced and it is expected to assist the fine mapping efforts.

Pedigrees

Choosing the appropriate pedigree is an important issue in genetic mapping. The population should preferably originate from genetically diverse parents since that can increase the DNA sequence variation in the offspring. The variation at the DNA level is essential to trace recombination events. The more DNA sequence variation, the higher chance to find a large number of polymorphic informative markers. When the aim of the mapping is to search for genes controlling a particular trait, genetic variation of the trait between parents is also important.

The most common types of plant mapping pedigrees include F_2, backcross and recombinant inbred populations. Recombinant inbred populations are produced by inbreeding *e.g.* F_2 progeny, until virtually homozygous lines are produced. Most linkage maps in plants have been obtained from segregating populations derived from crosses between inbred lines. However, inbred lines of homozygous individuals are not available for forest trees and the common type of backcross and inbred F_2 families used for agricultural crops are not feasible (O'Malley 1996). Further, inbred F_2 families or three-generation pedigrees are difficult to obtain in forest tree species due to a significant genetic load and time constrains (Strauss *et al.* 1992). In spite of these limitations, existing three-generation outbred *Pinus taeda* and inbred *Populus* pedigrees have been used as mapping populations (Bradshaw *et al.* 1994; Devey *et al.* 1994). Alternatively, the use of haploid megagametophytes in conifers has allowed the direct analysis of linkage in gametes and the construction of genetic maps (Nelson *et al.* 1993; Plomion *et al.* 1995; Hurme *et al.* 2000). Because most available pedigrees in tree species include two generations, two parents and their offspring, Grattapaglia and Sederoff (1994) have used a two-way "pseudotestcross" mapping strategy to construct single-tree genetic maps for two-generation pedigrees. This strategy is based on the fact that by

crossing two parents of highly heterozygous species (*i.e.* forest tree species) many polymorphic markers will be heterozygous in one parent, null in the other and therefore segregate 1:1 in their progeny as in a testcross.

Linkage analysis

Meiotic recombination is the base of genetic mapping. The longer the distance between two genes on a chromatid, the more likely they are to be separated by crossing over. The probability that two gene loci recombine describes the distance between them on the chromosome. The map that represents the distance between any two points in terms of recombination is called linkage map or genetic map. The distances are measured in centimorgans (cM) (named after American geneticist and Nobel Price winner Thomas Hunt Morgan). The recombination fraction, θ (theta, from the Greek alphabet) can be calculated as the ratio of the number of informative recombinants to the total number of informative meioses. In principle, if θ is less than 0.5, it implies that the loci are linked. The standard way of statistically verifying linkage is the LOD score estimate, first introduced by Morton (1955). LOD stands for "log of odds." The method calculates the probability of obtaining a set of observed results in a family on the basis of independent assortment and the probability of a specific degree of linkage (θ). Then, the ratio (odds) of the two probabilities is calculated, and the logarithm of this number, which is the LOD, is estimated. More specifically:

LOD (θ) = $\log_{10}$ (likelihood at recombination θ / likelihood at recombination fraction 0.5)

Because logarithms are exponents, the LOD score has the useful feature that scores from different matings for which the same markers are used can be added, hence providing a cumulative set of data either supporting or not supporting some particular linkage value. The possibility of double or multiple crossovers between two markers during meiosis cannot be excluded. Therefore, several mapping functions have been developed to deal with double recombinants. Mapping functions represent the mathematical relation between the physical (real) genetic map distance across an interval and the observed percent recombination in the interval. Two extreme mapping functions used in practice are those assuming complete interference (Morgan 1910) and no interference (Haldane 1926) respectively. The Kosambi mapping function (Kosambi 1944), used in the present thesis, provides an intermediate between complete interference and no interference. Due to the large size of the data set required for a comprehensive map, special programs have been developed to handle the linkage analysis. Descriptions and archives of linkage analysis and mapping programs can be found at: ftp://ftp.ebi.ac.uk/pub/software/linkage_and_mapping/(Europe)and http://www.linkage.rockefeller.edu (USA).

QTL mapping

For a long time it has been assumed that quantitative traits are controlled by a large number of genes each with a small effect (Fisher 1930). However, results from QTL mapping studies conducted during the last 15 years have questioned this assumption. QTL analysis in plants and animals has uncovered a surprising number of QTL with large effects (Tanksley 1993; Bradshaw *et al.* 1998; Mackay 2001). The number and the effect of QTL are extremely variable between experiments, depending on the features of the experimental design (Beavis 1994).

Among the most important factors influencing the power of the QTL detection are the type of cross, the trait's heritability and the sample size. For additive QTL an intercross is more powerful than a backcross, while for dominant QTL a backcross can be two-fold more powerful than an intercross. Higher heritability reflects less environmental influence for the trait, which increases the power of QTL detection. Plants that can be vegetatively propagated *i.e. Salix*, hold a valuable advantage for QTL mapping since clonal replications could essentially increase the heritability of a trait (Bradshaw and Foster 1992). Still, an important factor for determining the number and effect of the QTL is the sample size. Small population sizes reduce the power to find QTL and lead to systematic overestimation of additive QTL effects, the so-called "Beavis effect". Simulations suggest that this effect is significantly reduced as experimental sample size reaches 400-500 individuals (Beavis 1994). However, even with large sample sizes all true QTL, especially those with small effect, cannot be detected. It has been shown that it is difficult to locate more than 12 QTL in any given plant population at any time (Hyne and Kearsey 1995).

QTL analysis is mainly based on the association between marker genotypes and phenotypic values. *Single-marker analysis*, *interval mapping* and *composite interval mapping* are among the most common methods used in QTL analysis. *Single-marker analysis* tests the phenotypic values against each locus separately (by using for example, *t*-test, ANOVA and simple linear regression statistics). Typically, the null hypothesis tested is that the mean of the trait is independent of the genotype at a particular locus. Although the *t*-test, ANOVA and simple linear regression analysis are all equivalent to each other when testing for differences in the phenotypic means, they do not provide an estimate of QTL location, or recombination frequency between the marker and the QTL. This is because the QTL effect and the location are confounded and they cannot be estimated separately (Göring *et al.* 2001). With this approach, it is impossible to determine at which side of the significant marker the QTL is located. Furthermore, when a large number of markers are investigated the level of statistical significance that is set can lead to detection of false-positive QTL. If, for example, the level of significance is set to 5%, and there are 100 unique tests, five of the 100 markers would detect QTL incorrectly. Permutation re-sampling (Churchill and Doerge 1994) or a multiple test adjustment such as Bonferroni or Scheffe might sufficiently correct the significant level (Neter *et al.* 1997). The single marker analysis is widely used due to its simplicity (can run with any statistical software) to identify loci that are segregating with a trait. A more extensive approach, the so-called "*interval mapping*", can be used to estimate QTL effects and their map position.

This method was first introduced for crosses between inbred lines by Lander and Botstein (1989) and statistically tests for an individual QTL at multiple positions in intervals between markers with known map position and order. The results of the tests are expressed as LOD scores, which compare the likelihood under the null hypothesis (no QTL) with the likelihood of the alternative hypothesis (QTL at the testing position). Because of its ability to separate the effect of a QTL from its position, interval mapping has more power to detect a QTL than the single marker approach especially in cases of large distances between markers. It has been shown that the maximum benefit of interval mapping versus single marker analysis is realized when linked markers are more than 20 cM apart. Conversely, when the map density is high single marker analysis and interval mapping give similar results (Stuber *et al.* 1992). A more powerful statistical approach that accounts other QTL in the analysis as cofactors is the so-called "*composite interval mapping*" approach. It was introduced by Zeng (1993) and it basically extends the idea of interval mapping to include additional markers as cofactors with the aim to reduce variation that is associated with other linked QTL in the genome.

Today, the development of multiple QTL models to identify and localize epistatic QTL has gained much attention (Barton and Keightley 2002). Several workers have proposed methods for mapping of epistatic QTL. Zeng *et al.* (1999) have introduced an interval mapping method to simultaneously map multiple QTL and their interactions in experimental crosses between inbred lines. This method, which is called *multiple interval mapping* (Kao and Zeng 1997; Zeng *et al.* 1999), models two-locus epistasis between all pairs of QTL based on an orthogonal genetic model (Cockerham 1954). It uses maximum likelihood to estimate parameters in a statistical framework based on simultaneous modelling of multiple interacting QTL. However, the method is not a true simultaneous mapping method, since the search algorithm is limited to map QTL in pre-selected genomic regions. A non-parametric method for mapping multiple QTL and their interactions has recently been proposed by Fridlyand (2001). This method attempts to detect QTL regions only and makes no effort to make interferences about the underline genetic model. However, the method need very large population sizes and limits the power to detect QTL with small effects. Sen and Churchill (2001) have developed a general statistical framework that can accommodate multiple interacting QTL based on a Monte Carlo algorithm to implement QTL analysis. Single and pair-wise genome scans are performed to identify regions that exceed randomisation testing thresholds for no QTL versus one QTL and no QTL versus two QTL. New strategies for simultaneous mapping of epistatic QTL using genetic algorithms have also been developed (Carlborg *et al.* 2000; Carlborg 2002).

Marker assisted (MAS) forest tree breeding

Due to long generation time in trees, classical tree breeding is a time consuming process. With recently developed molecular tools, it becomes possible to identify molecular markers that are closely associated with traits of interests. Establishing predicted associations of molecular markers with traits of interest opens a multitude of alternatives in tree improvement. Among others, it provides the basis for accelerated breeding through early selection of new hybrids at the seedling stage, it

allows increased intensity of selection of larger number of progeny and it permits more efficient selection of parents for subsequent breeding programs. Linkage maps generated from DNA markers are now available for several forest tree species (Grattapaglia and Sederoff 1994; Bradshaw *et al.* 1994; Plomion *et al.* 1995; Marques *et al.* 1998; Arcade *et al.* 2000; Cervera *et al.* 2001). QTL affecting traits such as wood density, vegetative propagation traits, stem growth, stem form, spring leaf flush, bud set, frost resistance and pathogen resistance have already been identified in conifers, eucalypts and poplars (Table 2).

Marker-assisted selection (MAS) has the potential to increase genetic gain in breeding programs of forest trees above that realized from conventional population improvement methods (Williams and Neale 1992). Consideration of trait properties in forest breeding suggests that MAS will theoretically be most helpful in direct selection with traits of high economical value and low-heritability traits such as height and diameter growth. However, these traits often show strong GxE interaction and unfavorable genetic correlations with other desirable traits, and are likely to be controlled by a large number of minor genes rather than relative few major ones (Strauss *et al.* 1992). Traits with the highest potential for MAS in nonhybrid tree population might therefore be the highly inherited ones for which phenotypic assays are difficult *i.e.* wood quality, resistance to pathogens and plenological traits. Recent QTL studies on *Populus*, however, indicate that traits like stem volume and height, contrary to previous assumption, are controlled by a few number of genes each with surprisingly large effect (Bradshaw and Stettler 1995). For example, two QTLs explained 45% of the genetic variance in stem volume of two years-old plants. These results might be exceptional due to the mating design (interspecific cross) or the estimates could be biased upwards due to a small population size as has been indicated from simulation studies in maize (Beavis 1994). If, however, genes with a large effect on important traits exist in forest trees, then a MAS breeding scheme could have a valuable effect in accelerating the breeding process in forestry.

MAS in tree breeding is mostly at the research stage and recent results from studies with QTL must be considered as preliminary for predicting the usefulness of MAS in forestry. Forest trees are long life organisms and major changes in the genetic control during their growth can occur. Thus, the expression of a QTL may not be stable across years of growth. Preliminary results from pine suggest that different QTL could be important in different years (Emebiri *et al.* 1998). Similarly, Verhaegen *et al.* (1997) found no QTL to be consistent over 3 years of growth in *Eucalyptus*. However, marker-trait association instabilities over the

Table 2. *An overview of QTL detected in forest trees species*

Species	Traits	Type of Population (size)	QTLs (no)	Range % of phenotypic variance	References
Pines					
(*Pinus radiata*)	Stem height	full-sib (300)	6	1-2	Emebiri et al. 1998
	St. diameter	F_1	7	8-33	
	St. volume	Meg-gam-phytes	4	0-1	
	Wood density	F_1 (80-93)	2	-	Kumar et al. 2000
	Needle/stem	Full-sib	4	8-36	Emebiri et al. 1998
	unit rate	F_1 (93)			
(*Pinus silvestris*)	Bud set	F_1 (92--96)	4	3-13	Hurme et al. 2000
	Frost resistance	Meg-gam-phytes	7	0-11	
	Tree height	F_1 (94)	3	12	Lerceteau et al. 2000
	Trunk diameter		4	9-15	
	Tree volume		4	11-17	
	Frost resistance		2	11-23	
(*Pinus taeda*)	wood spec. gravity	F_1 (177)	5	23[a]	Groover et al. 1994
	height and diameter increm	F_2 (84-171)	21	5-27	Kaya et al. 1999
	wood specific gravity	F_2 (172)	9	5-16	Sewell et al. 2000
	vol %		5	6-12	
	microfibril angle		5	6-12	
	cell wall chemistry		8	5-13	Sewell et al. 2002
Fir					
(*Pseudo-tsuga meniziesii*)	Bud flushing	F_2 (78-190)	33	2-11	Jermstand et al. 2001
	Spring cold-hardiness		15	1-10	
	Fall cold-hardiness		11	2-7	
Poplar					
(*Populus trichocarpa* x *deltoides*)	Height	F_2 (55)	1	26	Bradshaw and Stettler 1995
	Basal area		1	25	
	Height/Diameter		1	33	
	Spring bud flushing		5	28-51	Newcombe and Bradshaw
	septoria resistance		2	61-72	1996
	bud flush	F_2 (346)	6	6-16	Frewen et al. 2000
	bud set		3	6-12	
Eucalypts					
(*E.nitens x E. urophyla*)	microprop. response	full-sib f.	10	7-12	Grattapaglia et al. 1995
	sprouting ability	F_1 (122)	6	7-11	
	rooting ability		4	7-21	
	circumference-breast height	half-sib f.	3	4-7	Grattapaglia et al. 1996
	wood sp. gravity	F_1 (300)	5	3-10	
	dry weight (bark)		5	7-9	
(*E. tereticornis x E. globules*)	pulp yield	F_1(94)	1	1	Marques et al. 1999
	vegetative propagation		21	3-17	
(*E.nites*)	St. height	F_2 (465)	3	10-15	Byrne et al. 1998
	leaf area		2	10-12	
(*E. urophyla x E. grandis*)	Frost tolerance	F_2 (210)	2	7-11	Byrne et al. 1997
	Wood density	F_1 (93-201)	9	6-11	Verhaegen et al. 1997
	Height:diameter ratio		8	5-11	
	Stem growth		8	5-14	

Note: [a] total phenotypic effect of all detected QTL

years, may not be an equally serious constrain for breeding practices in forest tree species with short rotation (*i.e. Populus, Salix*).

In general, MAS may be particularly awarding in interspecific hybrid programs where large disequilibria generated during hybridization and great phenotypic variance segregates in F_2 and in backcross generations. It may also be effective for highly heritable traits that are difficult to assay and for within family selection of weakly heritable traits by using very large population sizes and high selection intensities (Strauss *et al.* 1992). Last but not the least, QTL mapping can be an invaluable tool for understanding basic and unsuspected aspects of the quantitative trait architecture in trees and can undoubtedly enhance the knowledge about the structure, organization and evolution of forest tree genomes.

Low-temperature stress

Low temperature stress can be divided in two types, chilling stress and freezing stress. *Chilling stress* refers to the stress caused by non-freezing temperatures (above 0°C), which largely affects plants from tropical and subtropical origins. This category of plants, the so-called chilling sensitive, includes crop plants like rice, cotton and tomato that can be injured already at temperatures around 10 °C. Because forest tree species are generally not injured at chilling temperatures, although low temperatures may affect growth, chilling stress is not considered in the present thesis. The process of solidification of water and the formation of ice in aqueous solutions is termed freezing. *Freezing stress* is referred to as the stress, which links not directly to the low temperature but to the freezing of water in the plant. The term freezing stress is a misnomer, strictly speaking, since the low temperature is the stress and freezing (when it occurs) is the resulting strain. Therefore, a plant may remain unfrozen though exposed to a low-temperature stress but a plant subjected to a freezing strain is frozen (Levitt 1980).

In nature the condition in which temperatures fall below 0°C is named *frost*. Low temperatures are the result of a negative heat balance of a body, microsite or larger area of the earth's surface. Globally, the energy balance of the earth is the difference between energy input as short-wave irradiation, and energy losses due to the emission of long-wave radiation (Larcher 1995). During daytime, from shortly after sunrise until shortly before sunset, energy flows to the earth's surface and the radiation balance is positive. During the night, the amount of energy lost due to "radiation cooling" is greater, the longer the night and the less the long-wave radiation from the earth's surface is prevented by clouds, fog or other turgidity. Long, clear nights accompanied by low air humidity favour negative energy balance and frost can occur. The geographical latitude of the site concerned, the season, the slope of the terrain, the ratio of day length to night length, topographical variations in net radiation are important factors to radiation balance changes and they affect the occurrence or severity of the frost. Frost can also be caused by energy exchange with the surrounding due to cool-mass movements in a horizontal direction (advection frost). This type of frost is more drastic and is not influenced

by the topography of the site concerned (Sakai and Larcher 1987). Advection frosts have been reported as the most serious kind of frost in Sweden especially during spring and autumn (von Fircks 1994). Since frost always should be regarded in combination with the stage of development of the vegetation, a distinction has to be made according to its time of occurrence. Sakai and Larcher (1987) suggested a distinction between periodic winter frost that occurs when the plants are in a resting stage and episodic frost (not below -5 to -8 °C) that catches the plants during a phase when they are actively growing. Finally, in some environments (*i.e.* equatorial uplands and mountains), there may be frost at any time of the year (Larcher 1995).

The freezing process in plants

The formation of ice in tissues and the appearance of frozen plant cells are well documented in the literature. Extensive studies employing optical microscopy have shown extracellular and intracellular ice formation (Molisch 1897; Sakai 1982). Microcinematographic investigations have reported similar results (Steponkus *et al.* 1982).

Extracellular freezing is defined as the ice formation on the surface of the cell or between the protoplasm and the cell wall (extraplasmatic freezing). Ice is first formed in the extracellular space as the temperature drops below 0°C. This is because the extracellular fluid has a higher freezing point (lower solute concentration) than the intracellular fluid. Formation of extracellular ice is promoted by slow cooling rates (1-2 °C/h), ice nucleators (*i.e.* dust, bacteria) and the lower solute concentration (Palta and Weiss 1993). An important fact upon ice formation is that the vapor pressure of the cell water is higher than that of ice (at the same temperature). Consequently, cell water will diffuse through the plasma membrane to the extracellular ice (Fischer 1911; Palta and Weiss 1993). The movement of water from the cell to the extracelular space results in cell dehydration (Fig. 1). During cold-induced dehydration it has been indicated that membrane lipids form the lamellar-to-hexagonal II phase transition (a type of membrane lesion), which is a major cause of membrane damage upon freezing (Steponkus *et al.* 1993). Due to the loss of water the cell is contracted. After re-warming the cells, if they have not been injured, can soon reabsorb water and regain full turgor, otherwise they would remain collapsed.

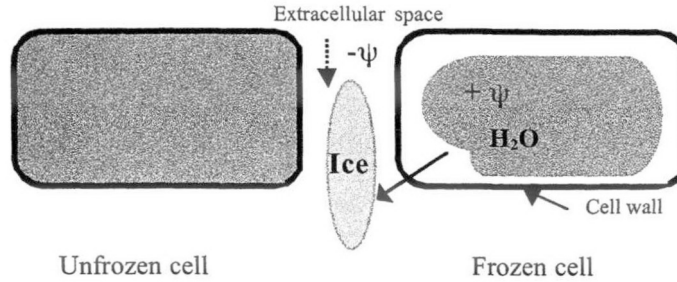

Figure 1. A simple illustration of the cell dehydration during freezing. Because the vapor pressure (ψ) of the cell water is higher than that of ice, when extracellular ice is formed cell water diffuses through the plasma membrane to the extracellular space. The movement of water from the cell to the extracelular space results in cell dehydration.

Although ice is ordinarily formed extracellularly, many observations of intracellular ice formation (*intracellular freezing*) have been made in the laboratory. Intracellular nucleation generally does not occur spontaneously unless the cells are supercooled to at least -10°C (Mazur 1977). At the instant of intracellular freezing, cells are killed as a rule, probably due to the mechanical destruction of biomembranes resulting from the fast growth of ice crystals in the protoplasm (Maximov 1914; Sakai and Larcher 1987). This is in agreement with observations in xylem ray parenchyma of deciduous trees (Quamme *et al.* 1973) and florets in flower buds (Graham and Mullin 1975) after deep supercooling.

Levitt (1980) classifies the two types of freezing injury as: (1) primary direct injury due to intracellular freezing; and (2) secondary freeze-dehydration injury due to extracellular freezing. He attributes the former to a direct physical effect of the intracellular ice on the protoplasm, while he explains the latter as the cause of the mechanical stress and solution effects (*i.e.* pH changes, increases in ion concentration), both of which increase with the degree of cell dehydration and contraction. These kinds of freezing injuries are summarized in a scheme below:

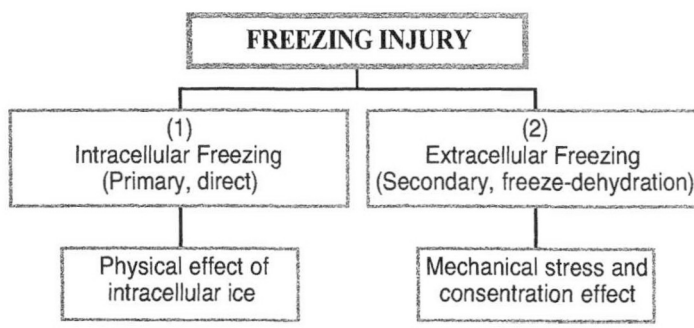

Figure 2. Types of freezing injury (after Levitt 1980).

Visible symptoms of frost injury

The most frequent visible signs of injury due to frost are listed below with a short description essentially as reported in Sakai and Larcher (1987).

Discoloration-Due to destruction of biomembranes, cell contents that were previously confined to their own compartments now come into contact with each other and with oxygen. This leads to coloured reaction products, which adhere to the cell proteins and cell walls. Discoloration in any part of a plant is the most frequent and clearest indication of freezing injury.

Bleaching-Injured tissues of green parts of plants that do not form necrogenic pigments turn pale green, yellow or even white. Furthermore, following a severe winter, chlorophyll formation may remain defective, so that chlorotic leaves are produced.

Tissue shrinking and dieback-decay of frozen tissue leads to the formation of holes in leaves, to constrictions in roots, stems, leaf and flower stalks of herbaceous plants, and to withering and dieback of shoot tips and roots. The necrotic parts of buds dry out.

Rupturing due to the mechanical effects of frost-The pressure exerted on the immediate surroundings as a result of the expansion of ice masses forming in tissue spaces leads, by mechanical means, to tearing of the tissues (frost blisters).

Malformations-Frost damage to leaf and flower primordia in winter buds may result in malformations, flower sterility or the buds may even fail to open altogether.

Heterochronism-Another abnormality that may result from freezing of buds is a delay in the onset of the various stages of development, *e.g.* retarded sprouting and flowering.

Freezing resistance

The freezing resistance includes two main components, *freezing avoidance* and *freezing tolerance*. Freezing avoidance refers to the ability of any tissue of a plant to prevent ice formation. Examples of freezing avoidance include: supercooling (cooling below the cell's freezing point without immediate freezing), absence of free water and lowering of the freezing point by antifreeze substances. On the other hand, freezing tolerance is the ability of living tissues (cells) to resist the internal freezing stress (ice formation) without suffering injury. Freezing tolerance at cell level can be distinguished into two types: (a) avoidance of freeze-induced dehydration; and (b) tolerance of freeze-induced cellular dehydration. In the present thesis, *freezing* resistance refers to results from the laboratory freezing tests, while *frost* resistance refers to results in field conditions. The broader term freezing resistance is used in the present thesis, which refer to both freezing avoidance and tolerance components.

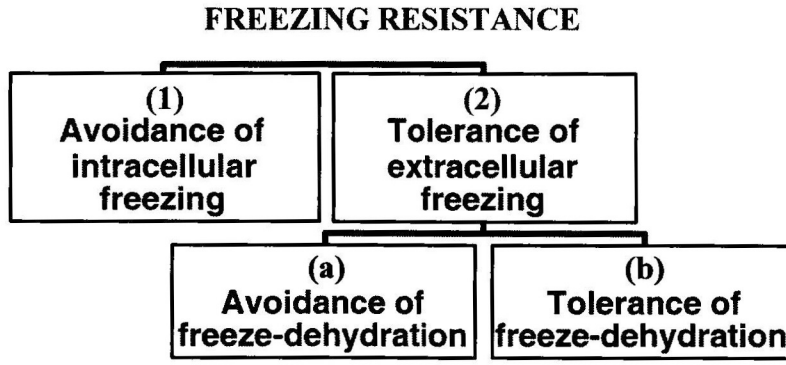

Figure 3. Types of freezing resistance (after Levitt 1980).

Cold Acclimation

Unlike animals, plants are immobile under a changing environment. Therefore plants must develop advanced mechanisms for survival under unfavorable conditions, such as low temperatures. One important process that enhances freezing resistance in plants is the *cold acclimation.* In herbaceous plants, temperatures around 0-5°C trigger the cold acclimation process and enable them to survive freezing. In woody plants cold acclimation is a more complex process and includes more than one stage. It is initiated by short days and cold nights slightly above zero and from there onwards different cold levels produce different degrees of freezing resistance.

The maximum degree of freezing resistance is different among plant species. Generally, over-wintering crops can survive about -30 °C, and annual plants, for example *Arabidopsis thaliana*, can tolerate temperatures down to -10 °C (Levitt 1980; Gilmour *et al.* 1988; Wanner and Junttila 1999). Woody plants can survive temperatures as low as -50 °C after acclimation and some species like *Populus* and *Salix* can withstand the temperature of liquid nitrogen at their dormancy (Sakai 1970).

Molecular changes associated with cold acclimation

When the cold acclimation process (or frost hardiness a term often used for trees) is activated and the plant develops from its growing condition of minimum frost hardiness (summer) to its hardy condition of maximum frost hardiness (winter), major changes take place within living plant cells. In general, cell sap concentration increases with freezing tolerance. The flow of water in and out of cells is facilitated by the increased permeability of the cell membranes and the cytoplasm, which changes from a translucent solution to an opaque gel state (Hazel 1995). These changes reflect the numerous biochemical reactions that occur in the cells during hardening and which have therefore attracted considerable attention. The most

pronounced alterations include changes in membrane composition, expression of cold-induced proteins and accumulation of sugars. All of these changes play an important role during cold acclimation and I will briefly discuss what is known about these responses during the cold acclimation.

Lipid changes

The plasma membrane defines the boundaries of the cell and is considered to be the primary site of freezing stress (Steponkus 1984). While cell membranes are vulnerable to freezing, the stabilization of the plasma membrane is of primary importance because of its central role in cellular behavior during freeze/thaw cycles. Cold acclimation has been shown to decrease the propensity of lamellar-to-hexagonal II phase transitions (a freeze-induced cellular dehydration phase transition) in regions where the plasma membrane is brought into close proximity with various endomembranes (Steponkus *et al.* 1993). Alterations in the lipid composition such as an increase in unsaturated fatty acids and phospholipids have been demonstrated. A higher proportion of phosholipids in the plasma membrane during cold acclimation has been observed in many plant species including rye, oats (Uemura and Steponkus 1994) and *Arabidopsis* (Uemura *et al.* 1995). Using transgenic mutants, in which the lipid composition of the plasma membrane was altered, Sugawara and Steponkus (1990) have demonstrated that an increase in the proportion of di-unsaturated phospholipids contributes to the decrease of incidence for freeze induced formation of hexagonal II phase. The characterization of *fab* and *sfr* mutants (deficient in encoding fatty acid desaturase and putative lipid transfer protein, respectively), also in *Arabidopsis* (McKnown *et al.* 1996), indicates the important role of fatty acid un-saturation in resistance to low (above 0°C) temperature stress, which is the requisite for plants to acclimate.

Cold-induced genes

Many genes have been shown to be induced by low temperature, some of the genes encode proteins with known or predicted activities but most do not. Proteins with known function include the lipid transfer proteins (Hughes and Dunn 1996), fatty acid desaturases (Gibson *et al.* 1994), antifreeze proteins (Antikainen and Griffith 1997) and proteins with a potential role in cold signal transduction (Gilmour *et al.* 1988). Genes induced by low temperature in *Arabidopsis* are the COR (cold regulated) or LTI (low temperature-induced) genes, KIN (cold induced), RD (responsive to desiccation) and ERD (early responsive to desiccation) genes (Thomashow 1998). The COR genes comprise four families, COR6.6, COR15, COR47 and COR78, each of which is composed of two genes that are physically linked in the genome in tandem (Thomashow 1998). In *Arabidopsis*, most of the COR/LTI genes encode highly hydrophilic proteins with repeated amino acid sequence motifs and they have been classified in five pairs: LTI78-LTI65, LTI29-COR47, LTI30/XERO2-XERO1, KIN1-KIN2 and COR15a-COR15B (Welin *et al.* 1995; Rouse *et al.* 1996; Thomashow 1999). It must be noted that at least one gene of each pair could also be induced in response to other stress conditions, like drought and high salinity (Thomashow 1999). Genes like LTI29, COR47, LTI30 and XERO1 are known as dehydrins, a widespread gene family in plants, with a potential role in resistance to freezing and/or dehydration (Close 1997). Dehydrins

have been found in conifers (*Pinus edulis* Engelm) and deciduous trees (*Populus* and *Salix*), indicating that these proteins are also common to woody plants and further research to characterize their regulation and function is needed (Wisniewski *et al.* 1996; Close 1997; Sauter *et al.* 1999).

A well-studied gene family is the COR15 family, which encodes a 15 kDa polypeptide that is targeted to the chloroplast. A basic expression of COR15a in non-acclimated transgenic *Arabidopsis* plants increases freezing tolerance in both chloroplasts and protoplasts by 1 to 2 °C (Artus *et al.* 1996). The COR15a protein is more likely to bind to the inner membrane of the chloroplast envelope and as a result stabilize both the chloroplast and the plasma membranes during freezing (Steponkus *et al.* 1998). As Steponkus *et al.* (1998) stated, the COR15a inhibits freeze-induced formation of hexagonal II phase by altering the intrinsic curvature of the inner membrane of the chloroplast envelope. While the COR15a plays an important role in stabilizing membranes, it poorly contributes in the increase of freezing tolerance at the plant level. However, the recent discovery of a CBF family (transcriptional activators for the COR genes) indicates that the COR15a gene product induces freezing resistance at the whole plant level only when acting in concert with proteins encoded by other known COR genes (Thomashow *et al.* 2001). Furthermore, recent reports from Strand (2000) shows that COR proteins might be important in the initial stress response to low temperature but they may not be as important for the development of full cold hardiness and freezing tolerance. Another family of cold-induced genes is the BLT101/RC12 but its function is not well known. One pair of this family (RCI2A-RCI2B), which encodes small hydrophobic proteins, has been isolated in *Arabidopsis* (Capel *et al.* 1997; Nylander 2000).

Accumulation of solutes

The solutes proline, mannitol, sucrose, glucose and fructose have been found to accumulate during acclimation. The accumulation of monosaccharides and sucrose during acclimation has been shown to occur in the cytosol rather than in the vacuole (Koster and Lynch 1992) and therefore they may contribute to cry-protection. One important role, which solutes might play in freezing resistance, is the osmotic adjustment of the cell. The accumulation of carbohydrates and other solutes alter the osmotic potential of the cell and consequently diminish the difference in water potential between the frozen extracellular space and the solution within the cell. This will result in a reduced water loss from the cell. A two-fold increase of the internal solute concentration could decrease the extent of cellular dehydration by approximately 50% (Steponkus 1984). Sugars and particularly sucrose and its galactosides have been found to have a stabilization effect on membranes during cold induced dehydration (Strauss and Hauser 1986). Fructans, sucrose, glucose and fructose have been shown to accumulate in the extracellular space in winter oats in response to exposure to low temperature (Livingston and Henson 1998). Further, it has been proposed that fructans stored in the crowns of cereals are converted to fructose and sucrose during freezing while they are reversed back to fructans as the crown thaws (Olien and Clark 1993). Sucrose and raffinose are the most accumulated sugars during cold acclimation in *Salicaceae*

(Sauter and van Cleve 1994; Ögren 1999b). Interestingly, the proportion of sugar lost in the spring has been reported to be analogous to dehardening rates after wintering for coniferous seedlings (Ögren *et al.* 1997).

Inheritance of freezing resistance

Freezing resistance is well known as a quantitative genetic trait. The inheritance of frost hardiness has been studied with various agricultural plants for many years. Nilsson-Ehle (1912) was the first who crossed two winter wheat varieties similar in winter hardiness and found transgressive segregation for the character. He concluded that winter hardiness behaved similarly to other quantitative characters controlled by polygenes. Goujon *et al.* (1968), using a monosomic set of winter and spring types of wheat, found that the chromosomes 5A, 2D and 5D carried genes for hardiness, while genes on chromosomes 7A and 1B were responsible for frost sensitivity. Because of the large number of chromosomes that have been shown to influence low-temperature resistance in conventional, non-molecular genetic studies in crops, it has generally been assumed that a large number of genes with small effects and complex interactions determine the phenotypic expression of low-temperature resistance. However, recent QTL studies have demonstrated the involvement of major genes in freezing resistance. For agricultural plants, for instance, Hayes *et al.* (1993) reported the presence of a multilocus cluster (QTL) on barley chromosome 7 with major effect on frost-hardiness component traits. Major genes influencing frost tolerance (*Fr1*) have been mapped on the long arm of chromosome 5A of wheat (Galiba *et al.* 1995). In barley, frost resistance loci accounted for 31 or even 79% of the variation in different years (Pan *et al.* 1994). Studies in diploid *Solanum* spp. and *Brassica spp.*, suggest oligogenic control in non- acclimated freezing resistance and in capacity to acclimate (Stone *et al.* 1993; Teutonico *et al.* 1995). Likewise, genetic studies have shown that major genes controlling frost resistance are also present in forest trees. QTL studies in natural populations of *Eucalyptus* (Byrne *et al.* 1997) and *Pinus sylvestris* (Hurme *et al.* 2000; Lerceteau *et al.* 2000) and in breeding populations of Douglas fir (Jermstad *et al.* 2001), have identified QTL that explain up to 23 % of the phenotypic variation of the mapping population. Therefore, the inheritance of freezing resistance in plants might not be as complex as initially thought. However, how genes so far associated to freezing resistance and to certain biochemical processes controlling freezing resistance will be an important aspect of further studies in plants.

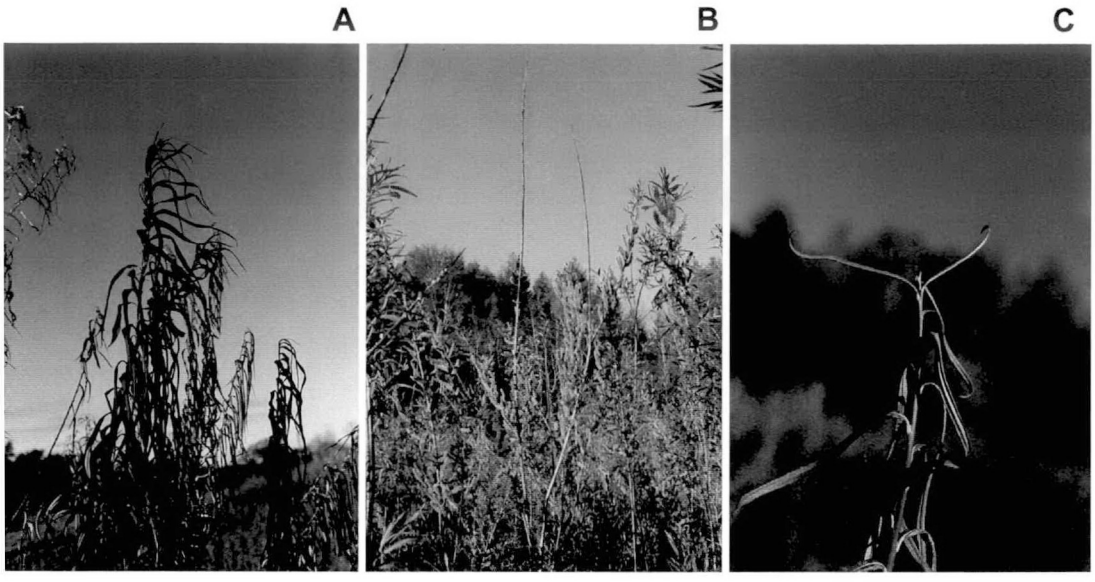

Figure 4. (**A**) Visual signs of frost injury on the apical shoot of *Salix* growing at SRIC systems during the early autumn. Although leaf freezing injury can be visually detected directly after thawing (1-2 days), stem injury is usually apparent early at the following growing season (**B**). Unlike *Populus* (and most forest tree species) in many *Salix* species (*e.g.* the investigated species of the *Vertix* subgenus) autumn conditions cause the shoot apex to abscise, rather to form a typical terminal bud. The shoot tip abscission is shown in picture **C**.

Measurements of freezing resistance

Freezing tests

Valuable information regarding the frost resistance under naturally occurring stress conditions can be obtained by field trials. Due to their simplicity field trials have been used successfully in the past in detecting frost resistance (Fowler *et al.* 1981; Marshall *et al.* 1981; Nilsson and Eriksson 1986). However, the evaluated data sometimes leads to classifications that are neither easy to compare nor simple to interpret due to the fact that frost, sufficiently severe to cause damage, is mostly unexpected and varies on each occasion in severity and duration. Depending on the time at which frost occurs and on the proceeding weather conditions, the plants may be at very different stages of maturity, activity and hardening, all factors, which correspondingly influence their degree of sensitivity to frost.

In order to reduce these limitations, artificial freezing tests can be used. The first use of a refrigerator was reported in 1920 (Glerum 1985). Since that time a considerable improvement in technical support has been achieved. Nowadays commercial freezing chambers with precise programmable microprocessors as temperature controllers and cooling baths with high performance are available.

Freezing at low temperature rates can be easily monitored with multichannel thermocouple type of temperature recorders. Since a large number of plants can be treated at any time with exact and reproducible degrees of freezing stress this method has become popular today in frost hardiness studies in agriculture (Sutka 1981) and forestry (Glerum 1985). In addition, artificial freezing tests have been found to give good agreement with survival in the field in both agricultural and forest tree species (Pellett *et al.* 1981; Guinon *et al.* 1982; Nilsson and Eriksson 1986). Considering QTL studies controlled environments theoretically allow for more firm control of experimental conditions and thus, QTL are more accurately estimated. However, survivals of a "real" life situation are not tested in the lab. Consequently, regardless of available resources, field screens might be used to provide the final measure of the potential effect of QTL on freezing survival.

Methods of assessing freezing resistance

A screening method to measure freezing resistance is important for freezing tests. This method has to meet several criteria, *e.g.* precise differentiation of hardy and unhardy plants, efficiency of detecting clonal or species variation, adaptable to large populations and reasonable expenditure of time and cost. Electrical methods such as electrical impedance and electrolyte leakage are good candidates to meet those criteria. Cell membranes are the most sensitive cell components to freezing stress (Levitt 1980). Changes in function occur in early stage of plant injury and can be used to assess the extent of injury in stressed plant tissues. Most commonly, changes in electrical impedance and electrolyte leakage have been used to detect freezing injury in woody plants (Glerum 1980; Repo 1992; Colombo *et al.* 1995).

Both methods are based on the concept that injured cells are unable to maintain the chemical composition of their contents and release electrolytes through damaged membranes. The electrical impedance of a plant tissue has been found to decrease with cell damage (Glerum 1980; Repo *et al.* 1994). With this method, fine electrodes are inserted directly into the plants and injury can be determined repeatedly after different freeze/thaw treatments. Electrical impedance measurements are simple and non-destructive and offer means for the early detection of plant injury. Electrolyte leakage indicates freezing damage by an increase in a bathing solution's electrical conductivity due to freezing down of plant cell membranes, allowing leakage at ionic contents from the tissue (Levitt 1980). The severity of the injury is proportionate to the amount of electrolytes that diffuse out of the tissue. By comparing the conductivity of uninjured tissue diffusate with that of injured tissue, an estimate of the amount of injury can be made. This method, first developed by Dexter *et al.* (1932) has been proven to be useful in a wide range of situations including test of many tree species like *Picea mariana* (Colombo *et al.* 1995), *Eucalyptus* spp. (Hallam and Tibbits 1988) and *Picea sitchensis* (Nicoll *et al.* 1996).

Phenology of forest trees and its genetic control

Forest tree species are long living organisms and must develop a system to cope with adverse conditions such as low temperature freezing. Trees growing in the Northern hemisphere and particularly in extremely cold zones (*i.e.* Boreal Zone) must tolerate light frosts during summer as well as winter temperatures as low as -40°C. One common strategy that enables trees to survive is the development of frost hardiness (or cold acclimation). The frost hardiness is mainly initiated by environmental factors, of which photoperiod and temperature are the most important. It has been proposed that the process of frost hardening in forest tree species include three stages (Glerum 1985). The first stage occurs in early fall when the decreasing photoperiod becomes noticeable while the day temperatures are still relatively warm, but nights are relatively cool. Photoperiod is considered important at that stage for forest trees. For example, a few long nights might be enough to induce the bud set in Norway spruce (Quamaruddin *et al.* 1995). This stage of hardening is associated with growth cessation, the initiation of terminal buds and, in the case of deciduous hardwoods, the onset of autumn leaf coloration. As this stage proceeds and temperatures fall near or below the freezing point, the plants become increasingly responsive and a large increase in frost hardiness occur (second stage). The third stage of hardening is induced by temperatures of -15 to -50°C and only extremely hardy species are able to attain this third stage (Sakai 1965). This level of hardiness is quickly lost (Weiser 1970). De-hardening (or de-acclimation) is difficult to classify in stages since it is so rapid (Glerum 1985). It requires chilling temperatures between 0°C to +10°C and unlike hardening, which is mainly controlled by photoperiod and temperature, de-hardening is primarily temperature increase dependent.

In *Salix* the lowest level of frost hardiness occurs after bud flush, when newly expanded shoot can be damaged by exposure to -3 °C (von Fircks 1994). Conversely, *Salix* reaches an extremely high frost resistance level in the winter when dormant shoots can tolerate freezing temperature as low as -85 °C (von Fircks 1994) or even the temperature of liquid nitrogen (Sakai 1970). The transition rates between these two extreme levels of resistance are dependent on the genotype. Ögren (1999a) has reported a *Salix* clone originating from northern Sweden, which ceased growth 5 weeks earlier than clones, naturalized in southern Sweden. Likewise, northern ecotypes of *Salix pentandra* have shown 22 hours critical photoperiod for growth cessation while the corresponding southern exhibited critical photoperiod of 15-16h (Junttila 1980). Further, continent-to-coast ecotype differentiation has been reported in frost hardiness initiation for *Salix* (Ögren 1999a).

Traits like growth cessation, time for bud set, abscission of the apical meristem, leaf fall, number of new leaves, water content during the autumn, timing of bud break have been commonly used as indicators for phenology of many forest tree species including *Salix* (Ekberg *et al.* 1985; von Fircks 1994; Ilstedt 1996; Hannerz 1999; Ögren 1999a; b) due to their high correlation with the development of frost hardiness. The genetic variation among phenological traits is large in forest tree

species (Dormling 1973; Bradshaw and Stettler 1995; Hannerz 1999). Phenological traits are not inherited in a simple Mendelian mode, but rather exhibits an additive variation typical of a quantitative trait. Notable examples of the additive mode of inheritance include growth cessation (Junttila 1982) and bud break in *Salix* (Rönnberg-Wästljung 2001), time for bud set and number of new leaves in *Populus* (Howe *et al.* 2000). Heritabilities are moderate to strong for phenological traits, which makes them useful for breeding. In Douglas fir, for example, narrow-sense heritabilities for bud flushing are often in the range of 0.7-0.8, whereas heritabilities for bud set are lower, being around 0.5 (Rehfeldt 1983; Li and Adams 1993; Aitken and Adams 1997). In addition, in an F_2 family of hybrid poplars (*Populus trichocarpa* Torr. and Gray x *Populus deltoides* Barr), broad sense heritabilities for bud flush ranged between 0.80-0.94, depending of the method of estimation (Howe *et al.* 2000). Similarly, Bradshaw and Stettler (1995) reported clonal mean heritability for bud flushing of 0.98 in an F_2 family of hybrid poplars. Although in several genetic studies the polygenic control in freezing resistance is pronounced, QTL studies have clearly demonstrated the involvement of major genes in the control of phenological traits. QTL studies in natural populations of *Pinus sylvestris* (Hurme *et al.* 2000), and in breeding populations of *Pseudo-tsuga meniziesii* (Jermstad *et al.* 2001a), have identified QTL for bud set and bud flushing, respectively, explaining up to 13 % of the phenotypic variation of the mapping population. Further, Frewen *et al.* (2000) have mapped QTL for bud set and bud flush explaining up to 16% of the variation of the traits in an F_2 population of *Populus.*

The study organism

The genus *Salix*, family *Salicaceae*, comprises more than 300 species including trees, shrubs and creeping shrublets (Larsson and Bremer 1991), which are world-wide spread excluding the Australasia and New Guinea. *Salix* can be divided into two subgenera; *Salix* and *Vetrix*, based on the number of stamens and the flowering habit. The subgenus *Salix* includes mainly trees while the subgenus *Vetrix* consists of shrubs (Zsuffa *et al.* 1984). *Salix* is usually found along riversides and in meadows close by the water. In warm temperature countries they are mostly grown in lowlands. With the exception of *S. martiana* all *Salix* species are known as dioecious (Alström-Rapaport 1997). The basic chromosome number is 19 (Blackburn and Harrison 1924) although 22 has been reported for some cases (Wilkinson 1941). Basically many ploidy levels are possible in *Salix*, however, the diploid level (2n = 38) appears as the most predominant in the genus (Zsuffa *et al.* 1984).

Salix has been important for centuries. The first report comes from the work of Theophrastos (312 B.C.), who emphasize the suitability of willows in the construction of army weapons. The first evidence for species selection based on suitability for making baskets from shoots comes from the Romans during the first century (Makkonen 1968). There are no reports showing the extensive use of willows as a crop before the 18th century but the basic principles for cultivating

willows seems to have been the same for the last 2000 years (Pohjonen 1984). At the end of the last century willow cultivation significantly decreased due to the decline of the basket industry. However, the energy crisis in the early seventies caused renewed interest for cultivating willows, this time for biomass production. The systematic breeding of willows started in early 80's by SLU (Sveriges Lantbruksuniversitet) in Sweden. The riparian shrubs, *Salix viminalis*, the closely related *S. dasyclados* and *S. schwerinii* were the primary species used in the breeding program (Gullberg 1993). These species are not native to Sweden but they have been introduced 200 years ago from central Europe. Owing to its fast growth, ease of establishment and wide range of adaptability, *Salix* is the most widely used species for high biomass production in short rotation intensive culture (SRIC) systems (Andersson *et al.* 1983; Zsuffa *et al.* 1993). It has been estimated that a well-irrigated and fertilized willow plantation in southern Sweden can produce up to 36tDMha^{-1} yr^{-1} (Christersson 1987).

Despite their commercial importance and intensive efforts for breeding, little effort has been devoted to the development and use of molecular genetic markers for breeding. Molecular markers *i.e.* isoenzymes, RAPDs and AFLPs have been generated in *Salix* and used for phylogenetic studies (Aravanopoulos 1992; Lin *et al.* 1994; Beismann *et al.* 1997). The first linkage data reported in *Salix* was that of four pairs of isoenzyme loci in *S. viminalis* (Thorsén *et al.* 1997) and a doublet (and possibly a triplet) between isoenzyme loci in *S. exigua* (Aravanopoulos 1998). *Salix* species offer good model systems due to its small genome sizes (2C = 0.8pg; Thibault 1998) and short time to mature for flowering (first or second year) compared to seven in *Populus* and 20 in *Picea abies*. *Salix* spp. can be transformed with *Agrobacterium* (Vahala *et al.* 1989) but no method is yet available to produce transgenic willow plants due to the difficulties in regeneration from single cells. Owing to their well-developed asexual propagation system, *Salix* also offers the possibility of capturing non-genetic variance and increase accuracy of QTL mapping. A large genetic component, at both inter- and intraspecific level, has been reported for most traits related to biomass production *i.e.* growth, dry weight, number of shoots, diameter, specific gravity, bud flushing (Rönnberg-Wästljung and Thorsén 1988; Lin and Zsuffa 1993; Rönnberg-Wästljung and Gullberg 1999). The presence of additive genetic variance in growth traits and timing of bud flush has been well demonstrated in several factorial crossings of *Salix viminalis* (Rönnberg-Wästljung and Gullberg 1999; Rönnberg-Wästljung 2001). However, dominant genetic variance has also been shown for different growth characters (Rönnberg-Wästljung 2001). In contrast to a closely related species, *Populus,* where an extensive effort has been made in QTL mapping, there is no QTL identified yet for quantitative traits in *Salix.* Molecular knowledge on the inheritance of economically important traits will permit the production of improved phenotypes through more effective and less time consuming breeding and parental selection.

Aims of the study

The present thesis examines the possibility of identifying and localizing genes controlling growth and adaptation traits in *Salix*. Due to the significant importance of frost injury in willows for biomass production, much work behind the thesis was stimulated by the need for genetic dissection of freezing resistance and phenology related traits.

The objectives of the study were to:

1. Construct linkage maps from hybrid *Salix* families using AFLP and RLFP molecular markers

2. Assess variation within such families for characters related to growth, phenology and freezing resistance.

3. Map quantitative trait loci (QTL) affecting the investigated traits.

To assess variation in freezing resistance and to maximize the chance of detecting QTL for freezing resistance traits it was first necessary :

- To search for a rapid, accurate and inexpensive methodology for detecting freezing resistance among clones.
- To study the relation between clonal variation with respect to freezing resistance and the phenological stage of the plant.
- To establish the appropriate mapping pedigree to study QTL affecting freezing resistance and phenological traits.

Materials and Methods

To establish a sufficient methodology for detecting clonal variation within *Salix* families with respect to freezing resistance, data from two experiments (conducted during the author's postgraduate studies at the University of Toronto), was used. The data of this work has been arranged in two papers in the present thesis (Paper I and II).

Methodologies for assessing freezing resistance

Three North-America clones of *Salix eriocephala* were used and two electrical methods, electrical impedance at 1 and 10 kHz alternating current and electrolyte leakage, were evaluated. Differences of electrical impedance to 1, 10 kHz and the ratio (low/high frequency) before and after the freezing treatment were estimated. Electrolyte leakage was expressed as relative conductivity (RC_t) and as an index of injury, IDX_t (see page 10 or Paper I for abbreviations).

The effect of plant phenological stage on clonal variation

The experimental material for this study was: nineteen clones of *Salix* (*S. nigra*[3], *S. viminalis*[7] *and S. eriocephala*[9]) and twenty-one clones of *Populus (P. deltoides*[12], *P. cv angulata x trichocarpa*[4], *P. deltoides x trichocarpa*[2], *P. jackii*[1], *P. cv angulata x balsamifera*[1] *and P. candicans x berolinensis*[1]).

A series of laboratory freezing tests were conducted, using visual assessment and electrolyte leakage to detect freezing injury and survival among clones. Clones were tested at predetermined levels of freezing stress and during seven phenological stages: dormant (*D*), early spring (*ES*), spring (*S*), flushing of terminal buds (*FTB*), new axillary growth (*NAG*), growing (*G*) and early-fall (*EF*) stages (Paper II).

Evaluation of mapping pedigree for studying QTL affecting freezing resistance

Because the aim was to search for QTL controlling freezing resistance, genetic variation of the trait between parents is important. Initially, two full-sib families, produced by Svalöf Weibull AB, along with their parents were tested for variability in autumn freezing resistance. The first family was derived from the cross between the male hybrid clone "Björn" (*S. viminalis x S. schwerinii*) and the female clone "78183" (*S. viminalis*) (fig. 5) while the second from the cross between the male "Jorr" (*S. viminalis x S. viminalis*) and the female clone "78183" (*S. viminalis*). Because of the low variation with respect to freezing resistance found in both families (see Results and Discussion), a third three-generation inbred pedigree was created for mapping QTL affecting freezing resistance. The selection of the parents of the third pedigree was chosen after testing four new candidate parents (clones: SW901290, Astrid, Bowles H. and Jorunn) for freezing resistance against the parents of the previous families (78183, Björn). The four candidate clones were selected from results obtained from the willow field trials during the last ten years in commercial willow breeding in Sweden (S. Larsson, Svalöf Weibul AB; personal communication). Freezing tests were conducted at the non-acclimated as well as at the acclimated stage. Plants growing 8 weeks under 18 h photoperiod and 25/18 °C temperature were treated with LN-LT (long-night (15h)-low temperature (10/4 °C)) regime for 6 weeks. Freezing resistance was estimated on detached leaves frozen in a controlled temperature bath by gradually lowering the temperature (1,5 °C/h) from 2 °C to -4.5 (non-acclimated stage) and from 2 °C to -5.5 or -8.5 °C (acclimated stage). To get an estimate for the potential plant recovery as well as for the re-growth capacity after freezing stress one additional "whole plant" freezing tests (similar to the one described in Paper I) was conducted at the end of the acclimation.

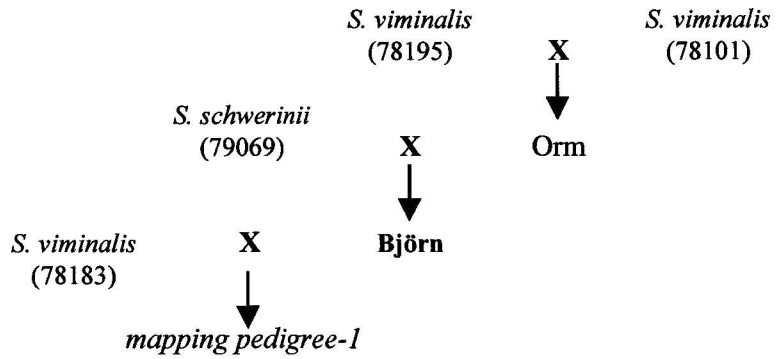

Figure 5. Diagrammatic illustration of the hybrid family (*S. viminalis* x *S. schwerinii*) or mapping pedigree-1.

Application of the genetic markers and QTL mapping

Two genetic marker systems were used, AFLPs and RFLPs. A total of 135 AFLP primer combinations were analysed for the two mapping pedigrees. RFLPs (essentially derived from *Populus* genomic DNA probes) and AFLPs (mainly based on the multicolor fluorescence approach (Perkin Elmer 1995)) were generated for one of the pedigrees while AFLPs (following the manual protocol; Vos *et al.* 1995) were produced for the other pedigree. The genetic maps were constructed according to the pseudo-testcross strategy (LOD ≥ 3) using the MAPMAKER (version 3.0, Lander *et al.* 1987), MAP-MANAGERQTXb (version 10.0, Manly and Olson 1999) and JoinMap (version 2.0, Stam and Van Ooijen 1995) mapping programs. The QTL analysis was based on the composite interval mapping. The details of the methods used of the linkage mapping and QTL analysis are presented in the papers (III, IV, V and VI).

Results and discussion

Methodologies for assessing freezing resistance (I)

Two electrical methods *i.e.* electrical impedance and electrolyte leakage were evaluatedfor the rapid assessment of freezing resistance in *Salix eriocephala.* This study showed that both methods are able to detect freezing injury in *Salix.* Although the electrolyte leakage method detected injury at a higher temperature (-3 °C) comparisons between the methods regarding their sensitivity to detect freezing injury are premature in the present study. Electrolyte leakage was measured on leaf tissues while electrical impedance was used on stem tissue. As the visual assessment showed, leaves were less tolerant tissues than stems at -3 °C (Table 1; Paper 1). Furthermore, impedance was taken in the lower part of the stem which is more frost tolerant compared to the apical part (von Fircks 1994). In the estimation of electrolyte leakage leaf tissue was used for two main reasons: a) it displays a greater increase in conductivity in response to freezing stress than stem tissue and

thus offers a more sensitive test for assessing differences in frost hardiness (Hallam and Tibbits 1988; Webb et al. 1983); and b) it provides a much less destructive method compared to using stem tissue. DZratio (differences of el. impedance of the ratio (low/high) frequencies before and after freezing) was found to be the most sensitive expression of impedance since it detected freezing injury at a higher temperature (-4°C) than DZlow (differences of electrical impedance at 1 kHz before and after freezing) and DZhigh.(differences of electrical impedance at 10 kHz before and after freezing).

Of the two electrical methods tested, only electrolyte leakage was able to detect significant clonal differences in freezing resistance (Table 2; Paper I). The impedance technique has been shown to be a good indicator of differences in freezing resistance between conifer species. However, in this study we were unable to find significant clonal differences in freezing resistance of *S. eriocephala*. These results must be considered as preliminary since only Glerum's electrical impedance protocol was used (Glerum 1980). The fact that only two frequencies (1 and 10 kHz), relatively close, were used may explain the lack of large impedance differences between clones. Modifications of this method may allow measurements that are more effective in detecting clonal variation.

Highly significant correlation between electrolyte leakage and visual leaf injury, with increasing values of RC_t (or IDX_t) associated with increasing likelihood of leaf death were obtained (Table 5; Paper I). Similar correlation between electrolyte leakage from leaf tissue and the visual assessment of leaf injury and survival has also been reported in comparable screening tests for *Eucalyptus* (Hallam and Tibbits 1988). Reversible injury caused by moderate freezing has been demonstrated for onion bulk scales (Palta *et al.* 1977) and *Eucalyptus urnigera* (Paton 1981). However, the highly significant correlation between electrolyte leakage and visual leaf injury obtained in this study suggests that post freezing recovery was not significant for *S. eriocephala* leaf tissues at -3°C and -4°C. Of the three estimated parameters of electrical impedance the DZratio is the most reliable parameter in predicting heavy injury in *S. eriocephala* since it was highly correlated with the visual injury. This may be a result of the DZratio being independent of factors such as tissue size, temperature and moisture content that can influence impedance values (Glerum 1980). Since partial stem injury may have occurred in some plants at -3 and -4 °C, I anticipate that a better correlation at these temperatures might have been seen if electrical impedance had been assessed at several sections of the stem. However, measurements in the upper part of the stem, where stem diameter is 2 mm or less, is not recommended due to the influence of the tissue damages by the electrode penetration in impedance readings (Glerum 1985). Techniques, which would minimise the damage to tissues when inserting electrodes, would make it possible to collect more impedance readings along the length of small and tender tissues *e.g.* apical stem.

In conclusion, the results of this investigation showed that both methods are able to detect freezing injury in *Salix*. This indicates the potential of the electrical methods for the rapid detection of freezing injury in *Salix* plants. However, the electrolyte leakage method detected injury at more levels of freezing stress (-3, -4, -5 °C) than

the impedance (-4, -5 °C), it detected clonal differences in *S. eriocephala* freezing resistance and it was best suited to correlate electrical methods with the visual assessed freezing injury.

The electrolyte leakage method meet the criteria of a rapid, quantitative and non-destructive method for detecting clonal differences in *Salix* with respect to freezing resistance. Modifications of the used electrical impedance method may be essential to allow measurements that are more reliable and effective in detecting clonal variation in freezing resistance of *Salix*.

The effect of plant phenological stage on clonal variation (II)

Significant levels of genetic variation in freezing resistance were present among *Populus* and *Salix* clones important for biomass production. However, there was a significant effect of the plant phenological stage on the clonal variation in freezing resistance. More specifically, of seven phenological stages tested significant clonal variation in freezing resistance was detected only at four stages (S = spring, FTB = flushing of terminal buds, NAG = new axillary growth, and EF = early fall). During dormant and early spring stages, when freezing resistance was greatest, no significant differences in clonal survival were detected. Comparably, at the G stage (growing stage) in which clones exhibited the highest susceptibility to freezing stress, clonal variation was negligible for the estimated index of injury. Significant ($p < 0.05$) clonal variation was only detected at one of the tested freezing temperatures (-3 ° C) in *Salix*, which, accounted for 10% of the total variation. At the EF stage, clonal differences were highly significant ($p < 0.0001$) for the index of injury and accounted for 34% of the variation in *Salix* and 32% in *Populus*. The relatively high proportion of the total variation attributable to clone obtained during the EF stage in both genera may prove to be valid information for clonal selection and improvement with respect to early autumn frost. This phenological stage was chosen as the most appropriate to search for freezing resistance QTL.

The non-significant clonal ranking correlation between the S and EF stages found in *Populus* implies that freezing resistance prior to bud flushing and during the early fall should be considered as separate traits. Furthermore, it suggests that the time of bud break and/or growth cessation are important particulars for optimal freezing resistance. While phenology was not investigated here, it is an important contributor to optimal freezing resistance and should always be considered in the clonal selection for frost resistance. In addition, the clonal rankings elucidate two categories of *Populus* clones with respect to their superiority in freezing resistance among phenological stages: (a) those that maintained their superiority among their phenological stages; and (b) those that exhibited high freezing resistance at specific stages. Therefore two types of selection with respect to freezing resistance for *Populus,* and may be for the close related genus of *Salix*, can be suggested: (a) selection for superior clones which maintain their superiority among phenological stages; and (b) selection for specific set of clones highly resistant to frost at a specific season. Finally, this study confirms earlier evidence (Paper I) that the use of electrolyte leakage in controlled-freezing tests may provide a means of screening large numbers of *Populus* and *Salix* genotypes for resistance to frost.

Evaluation of mapping pedigrees for studying QTL affecting freezing resistance

The artificial autumn conditions resulted in reduction and eventually in cessation of height growth in both families (*S. viminalis x S. schwerinii and S. viminalis x S. viminalis).* Although the hybrid family (*S. viminalis x S. schwerinii*) appears faster growing than the *S. viminalis* family, it ceased height growth earlier (for more details see Lin *et al.* 1998). This indicates that it may be possible to select *Salix* families with superior height growth that also have an acceptable autumn freezing resistance. However, freezing tests showed insignificant within family variation in freezing resistance and no further study in these families were conducted with respect to freezing resistance. Because height growth and phenology were highly significant among clones within the hybrid family (*S. viminalis x S. schwerini*), this family was selected for mapping QTL affecting growth and phenological traits *e.g.* bud break and growth cessation. For simplicity this family is referred to as *mapping pedigree-1.*

Results obtained from the freezing tests on candidate parental clones (see previous section Materials and Methods) indicated significant differences among clones with respect to freezing resistance. The clone SW901290 was highly freezing resistant compared to the other four clones (Astrid, Bowles H. Björn and 78183; Fig. 6). Clones SW901290 and Jorunn exhibited the largest contrast in freezing resistance. Crosses of F_1-individuals from three parent combinations, Astrid x SW901290, Bowles H. x SW901290 and Jorunn x SW901290, produced offspring with germination rates 12, 45 and 98 percent, respectively. The high rates of germability in the F_2s of the SW901290 x Jorunn cross may indicate no major complications in the F_1s meiosis. The clones Jorunn and SW901290 were used as the grandparents of a new established three-generation pedigree (also called *mapping pedigree-2*). The male is a continental genotype of *Salix* (SW901290) collected (Svalöf Weibull AB) from Kirov (Russia) and the female parent is a *S. viminalis* (Jorunn) clone. An F_2 derived from a cross between two F_1 siblings, which were produced by Svalöf Weibull AB, was created (Fig. 7). Notable differences within the family in height rates and morphological characteristics *i.e.* leaf shape and size, were observed. Because phenotypic examination and preliminary phylogenetic data from AFLP fingerprints classified the male clone as *S. dasyclados* (Semerikov, personal communication), the SW901290 clone is considered as *S. dasyclados*- like clone.

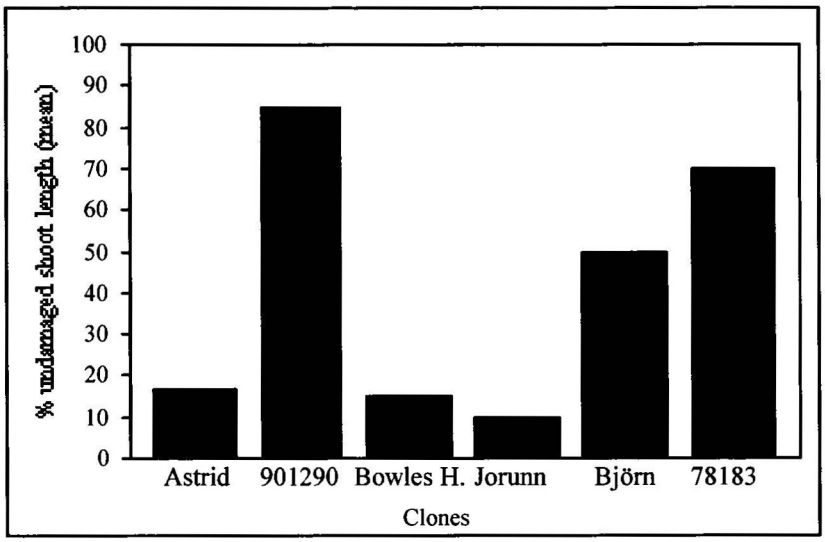

Figure 6 Freezing resistance (% uninjured stem length) of six *Salix* clones subjected to a -8.5°C freezing-thawing stress after four weeks of cold-acclimation (9h photoperiod, 15/5 °C). The freezing stress procedure was applied on intact plants (each clone replicated ten times) as described in Paper I.

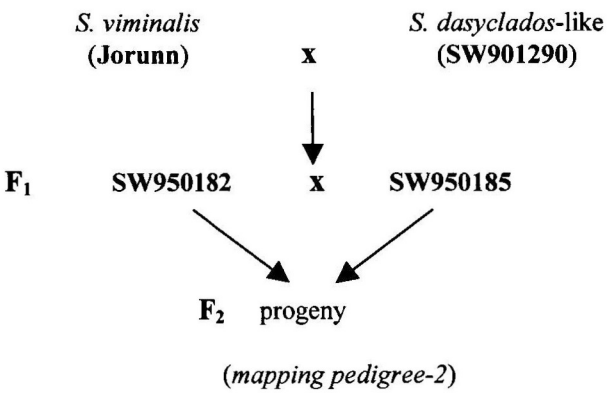

Figure 7. Schematic presentation of the *mapping pedigree-2.*

Genetic Mapping (III, V)

Genetic map 1 (mapping pedigree-1)

A total of 72 AFLP primer combinations were analysed mainly using the multicolor fluorescence approach (Perkin Elmer 1995). Of the 383 AFLP loci scored, 357 loci were heterozygous in one parent and null in the other, while 26 were segregating in

both parents. An illustration of the types of segregation accomplished by the multicolor fluorescence system is shown in Fig. 8 for the primer combinations CGA-AG and CGA- AC (*Eco*RI- *Mse*I). The number of polymorphic (1:1) loci derived from the hybrid parent (*S. viminalis x S. schwerinii*) was considerably higher (18%) than those originating from the *S. viminalis* parent. A total of 70 loci (18 %) showed significantly skewed segregation at the 5% confidence level. There were approximately equal numbers of loci with an excess of bands present as those with a deficit of bands. However, a higher proportion of loci with skewed segregation (5%) was observed in the hybrid parent map. From a total of 50 RFLPs scored (most derived from *Populus* genomic DNA probes), 39 (78%) were segregating in a 1:1 ratio while 11 (22%) were heterozygous in both parents and segregated in a 1:2:1 ratio. Five RFLP loci (10 %) showed significantly skewed segregation at the 5% significance level.

Following linkage analysis, a genetic map composed of 325 AFLP and 38 RFLP, was constructed. The analysis was based on the "two-way pseudo-testcross" mapping strategy and two maps were constructed, one for the hybrid clone Björn *(S. viminalis x S. schwerinii)* and one for the clone 78183 *(S. viminalis)*. The *S. viminalis x S. schwerinii* map (2404 cM) included 217 markers and formed 26 major linkage groups while the *S. viminalis* (1844 cM) consisted of 146 markers placed on 18 major groups (Fig. 1 and 2; Paper III). In addition, eight and 14 additional minor linkage groups composed of less than four markers (doubles and triplets) were obtained in the *S. viminalis x S. schwerinii* and the *S. viminalis* map, respectively. Both maps provided about 70-80 % genome coverage with an average density of markers of 14 cM. To investigate possible homologies between parental maps, 20 AFLPs and 11 RFLPs segregating in a 3:1 or 1:2:1 ratios were included in the linkage analysis. Eight linkage groups homologous between the two maps were detected (Fig. 3; Paper III).

Genetic map 2 (mapping pedigree-2)

A total of 63 AFLP primer combinations were analysed using the manual radioactive AFLP approach (Vos *et al.* 1995). The AFLP scoring was based on the "two-way pseudo-testcross" mapping strategy. Of the 772 AFLP loci scored, 491 loci were heterozygous in one parent and null in the other, while 274 were segregating in both parents. Of the total number of AFLP loci scored, 66 % originated from the grand parent SW901290 only while 15% originated from the grand parent Jorunn (*S. viminalis*). The remaining of the loci were either present or absent in both grandparents. Skewed segregation was observed for 94 markers (12%).

Four hundred and thirty-three AFLP-markers were mapped (at LOD $\geq$ 3.5) in 49 major linkage groups (more than 3 markers). Fifteen of these groups included the following three type of markers: 1) markers segregating from both F_1 parents (3:1 ratio); 2) markers segregating from the male F_1 parent (1:1); and 3) markers segregating from the female F_1 parent (1:1) (Fig. 1; groups I-XV; Paper V). These linkage groups represent a preliminary integrated map between the *S. dasyclados* like and *S. viminalis* genomes. 27 linkage groups were comprised of markers

segregating from both parents (3:1 ratio) and/or markers segregating from either F_1 female or male parent (Fig. 1; groups 1-7, A-P, a-m; Paper V). In addition 24 minor linkage groups (triplets and doublets) were generated. The observed map length, including triplets and doublets, was 3122 cM which, covers 65% of the estimated genome length. The average space between markers was 7.1 cM.

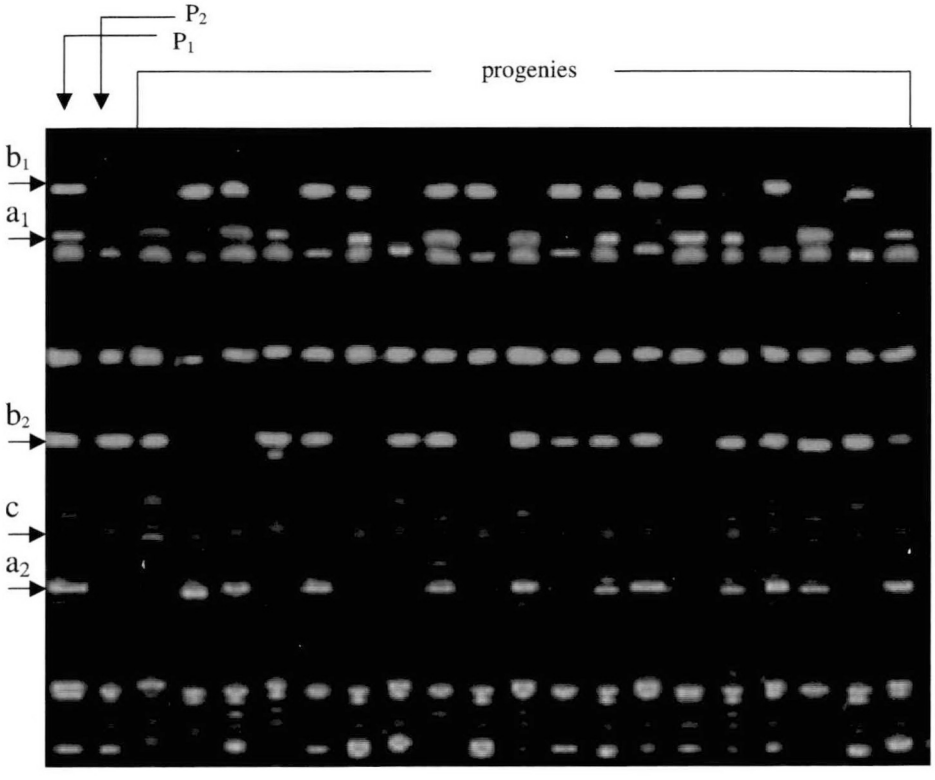

Figure 8. Genetic inheritance and segregation of AFLP markers in the *mapping pedigree-1* as obtained by the multicolor fluorescence genotyping system (ABI-PRISM). The first two lanes P_1 and P_2 indicate the two parents, *S. viminalis* and *S. viminalis* x *S. schwerinii* respectively. The blue and green colors represent the primer combinations CGA-AC and CGA-AG (MseI-EcoRI) respectively. Typical polymorphic AFLP fragments are indicated with arrows. Fragments: a_1, a_2 are heterozygous in one parent and null in the other, segregating in 1:1 ratio primer combination CGA-AC (blue color) ;$_1$his heterozygous in one parent and null in the other, segregating in a 1:1 ratio while b_2 is present in both parents and segregating in a 3:1 ratio primer combinations CGA-AG (green color) ; c is the size standard (red color).

An overview of the results in mapping

The high number of polymorphic loci generated by the AFLP procedure in the present study, facilitate its use for a sufficient and rapid *Salix* genetic mapping. However, the 3:1 markers generated by the AFLP are usually dominant. Dominant markers contain low information content (Ritter *et al.* 1990) and may provide limited information for an unbiased and accurate recombination frequency estimate (Maliepaard 1998; Wu *et al.* 2000). This explains the low percentage of 3:1 placed on the map (*i.e.* only 40% in genetic map 2) which is comparable to other dominant markers systems *i.e.* RAPDs (Verhaegen and Plomion 1996). Several of the RFLP probes revealed multiple loci, suggesting a significant level of genome duplication in *Salix*. Such duplications have earlier been reported for many diploid plant species including *Populus* (Bradshaw *et al.* 1994). In this study no conserved homologous regions were detected between the genomes of the two genera (*Populus* and *Salix*), most likely due to the low number of homologous RFLP markers present on the maps from the two species.

To my knowledge the *Salix* linkage maps presented here are the first reported genetic maps of the *Salix* genome. The expected number of linkage groups (n =19) for a comprehensive linkage map in *Salix* is exceeded and a relative large number of small groups (doublets and triplets) were observed. This indicates the presence of several gaps in the linkage maps. The estimates of the total genome size suggest that an additional 20%-35% of the map length needs to be covered. Gaps in the linkage map may be the result of clustering of AFLP markers. A single combination of restriction enzymes (*Eco*RI and *Mse*I) was used for AFLP analysis that could have resulted in a void of markers in *GC*-rich regions of the genome. Some AFLP loci could also, despite careful checking, contain genotype errors that might obstruct linkage. However, there could also be a biological explanation to the large number of small linkage groups. Early cytological work (Blackburn and Harrison 1924; Wilkinson 1941) reported that some *Salix* species had two basic chromosome numbers, 19 and 22, and Wilkinson (1941) proposed that these differences were due to chromosome fragmentation. The extensive studies of Håkansson (1955), on the other hand, could not confirm this view, but the excessive number of small linkage groups obtained in recent genetic maps of *Populus* (Bradshaw *et al.* 1994; Wu *et al.* 2000) revive the question of a possible biological explanation to the observed high number of small linkage groups observed in *Populus* and the closely related *Salix* genus.

The high level of segregation distortion (18% and 12% for genetic map 1 and genetic 2, respectively) found for AFLP markers was similar to that previously reported in *Eucalyptus* (Marques *et al.* 1998). Skewed segregation ratios in other types of markers *i.e.* RAPDs, RFLPs, have also been observed in molecular analysis of forest tree species (Nelson *et al.* 1993; Bradshaw *et al.* 1994; Barreneche *et al.* 1998). Genetic mechanisms such as expression of genetic load, incompatibility, genetic isolating mechanisms during speciation, and preferential chromosome loss (Bradshaw and Stettler 1994), have been suggested to explain this phenomenon. In the *S. viminalis* map, the skewed loci were randomly mapped across the genome or formed small groups. However, it is interesting to highlight

the localization of 15 of the skewed loci in linkage groups 1, 2, and 10 of the *S. viminalis x S. schwerinii* map (Paper III). The number of skewed markers on these groups showed a significant excess over the Poisson expectations ($p < 0.05$) in a goodness-of-fit-test (data not shown). This indicates the existence of large areas (possibly whole chromosomes) in the hybrid *S. viminalis x S. schwerinii* genome that do not follow Mendelian ratio.

In genetic map 2, the cross-design of the mapping pedigree allowed the origin of the alleles to be identified. Sixty-six % of the scored loci originated from the male grandparent (SW901290) (Table 3; Paper V) while only 15 % of the markers were derived from the female grandparent. High heterozygosity level could be expected in the Russian *S. dasyclados* like clone since it originates from a natural population compared to *S. viminalis* clone "Jorunn" which is an offspring of two generations of breeding. However, we cannot exclude the incident of polyploidy in one of the grandparents. Preliminary results from a flow cytometry study indicate that the *S. dasyclados*-like grandparent might be polyploid showing a two-fold amount of DNA compared to the *S. viminalis* grandparent. Polyploidy for eastern origins of *S. dasyclados* (2n = 76) has been reported earlier (Larsson and Bremer 1991). Differences in ploidy level could also explain the excess in the number of minor groups and the presence of 10 linkage groups completely inherited from the *S. dasyclados* like grand parent (groups XII, XV, 4, 5, 6, B, J, L, O and I; Fig. 1; Paper V). The high seed germination of the F_2s (98%), however, does not support triploid F_1 parents and one tetraploid male grandparent.

QTL mapping (III, V, VI)

Mapping QTL for growth related traits (III)

The genetic map 1 was used to identify quantitative trait loci (QTL) affecting growth related traits. The study demonstrates that it is possible to detect loci controlling genetic variation for growth traits in *Salix* using molecular-linkage maps and furthermore, to characterize these loci with respect to their map position and phenotypic effect. Eleven QTL were identified; seven QTL for height growth, one QTL for stem diameter, one QTL for height: diameter ratio, one QTL for number of vegetative buds during flowering time and one QTL for number of shoots. The estimated magnitude of the QTL effect ranged from 14 to 22% of the total phenotypic variance. It is often assumed that growth related traits are complex with large number of genes involved, each having a small effect on the phenotypic variance (polygenic control). In this study we were able to detect moderate to large-effect QTL for growth related traits. Interestingly, 32 % of the phenotypic variance for height growth in 1999 (first year after harvest) was explained by two QTL. Thus, our results agree with other QTL studies in trees showing that quantitative traits such as height, diameter and number of shoots may in part be controlled by a few genes of large effect. Bradshaw and Stettler (1995) have reported that 30% of the phenotypic variance for 2-year stem volume growth in *Populus* was explained by two QTL in an F_2 family. Further, major-effect QTL controlling vegetative propagation traits have been reported in *Eucalyptus* in a study using the pseudo-testcross strategy (Grattapaglia *et al.* 1995). In these QTL studies major-effect QTL were detected in interspecific crosses. However, the present QTL dissection study

was partially based on an intraspecific cross (QTL detected on the *S. viminalis* map). This suggests that genomic regions with relatively large effect on quantitative inherited traits *i.e.* growth traits, can also be detected within species.

One QTL associated with height growth and one affecting the height:diameter ratio, were overlapping in the same marker interval with the QTL affecting stem diameter. This suggests that height and diameter growth in *Salix* share common genetic components. Similar QTL clustering has earlier been reported in *Populus* for stem basal area and sylleptic branches (Bradshaw and Stettler, 1995) and in *Eucalyptus* for height: diameter ratio and vigor (Verhaegen *et al.* 1997).

QTL stability over years was estimated for traits measured in multiple years. Generally, QTL were only significant in a single year although two QTL for height growth were close to reach the significance level in two consecutive years. In general, changes in QTL over the years are expected in forest trees (Bradshaw and Grattapaglia 1994) since stand development takes several years. Quantitative genetic studies for growth traits in *Salix* indicate low genetic correlation over years (Rönnberg-Wästljung and Gullberg 1999) which is in agreement with our QTL dissection results. The changes of the magnitude of the genetic control over years may not be surprising for the *Salix* SRIC system considering the drastic changes in the shoot-root growth relationship over years. In our analysis, plants had equal shoot and root age at the years 1997 and 1998 (shoot: root=1:1 and 2:2 respectively) but not at the following years when the new shoots emerged from the 2-year-old rootstocks. On the basis of our annual height growth analysis, however, two of the significant QTL (ht00, L. Group 12, map: *S. viminalis x S. schwerinii* and ht98, L. Group 2, map: *S. viminalis*) were very close to reach significance (LOD = 2.7, 2.9) in two successive years. This indicates some partial QTL stability over the years for height growth QTL which due to environmental variation among years, relatively small population size and high threshold levels (Beavis, 1994), might not have been evident in our experiment. Partial stability of QTL expression has been detected for 2-year growth in *Eucalyptus* (Verhaegen *et al.* 1997) and *Pinus radiata* (Emebiri *et al.* 1998).

Mapping QTL for timing of bud flush (IV)

Salix species important for biomass production are among the earliest leaf flushing forest tree species. When *Salix* plants lose their hardiness during spring, newly flushed buds and shoots are particularly sensitive and unable to tolerate temperatures lower than -3 °C (von Fircks 1994). This indicates high risk of biomass losses due to frost damage and suggests the need of characterizing *Salix* genotypes for late bud flushing.

In the present thesis, spring bud flushing was recorded outdoors in two consecutive years 1998 and 1999, and indoors in the spring of 1998 for mapping pedigree 1. The bud flushing data showed a significant clonal variation within the mapping pedigree. The clonal variance component for the timing of bud flush range between 10-28 %, depending of the section that bud flush was measured. Clonal mean heritabilities ranged between 0.48 and 0.72. In general, bud flush was under

relatively moderate genetic control compared to studies in the close related genus *Populus*. In an F_2 family of hybrid poplars (*Populus trichocarpa* Torr. and Gray x *Populus deltoides* Barr), for example, broad sense heritabilities for bud flush ranged between 0.80-0.94, depending of the method of estimation (Howe *et al.* 2000). Similarly, Bradshaw and Stettler (1995) reported heritability for bud flush 0.98 in an F_2 family of hybrid poplars. However, low to moderate broad-sense heretabilities in timing of bud flush have also been reported for Belgian poplar clones tested in Sweden (Ilstedt 1996). The rapid warming in the spring, particularly in 1998 (Fig 2; Paper IV), may have influenced the precision of the clonal variation assessment. I anticipate also higher levels of inheritance if I had studied an F_2 of more diverse parents.

Interestingly, the correlation between time of bud flushing and height growth was very low suggesting that there is a potential for clonal selection to improve early bud flushing in *Salix* without reducing annual yield. These results are in agreement with reports from *Picea sitchensis* seedlings (Cannell *et al.* 1985).

Using a *Salix* linkage map composed of 325 AFLP and 38 RFLP markers (III), five QTL and three unmapped markers controlling timing of bud flush were detected. Four QTL were detected from the field experiment while one QTL and three unmapped markers were identified in the indoor experiment. The QTL associated with indoor bud flushing showed coincidence with one of the QTL detected from the field data. The QTL explained 10-21% of the phenotypic variance which, is in concordance with previous QTL effects on others adaptive traits (Paper IV) again suggesting the presence of genes with large effect on the phenotype.

Mapping QTL for autumn freezing resistance and phenology traits (VI)

Phenotypic measurements

The mean values for freezing resistance and related traits of the studied F_2 family (*mapping pedigree-2*) were strongly affected by the short photoperiod and the low temperature (SD-LT) regime. Before acclimation the proportion of electrolytes, which leached from leaf cells, (estimated by IDX_I) increased 14% after freezing to -5.0 °C. However, following 31 days of SD-LT a 3.5 °C lower freezing temperature (-8.5 °C) resulted in an almost equivalent proportion (11%) of leached leaf cell electrolytes. Between 20-24 days of cold acclimation the pedigree showed a rapid total and top height growth decrease (Fig. 1: PaperVI). The shoot tip abscission initiated later than the apical growth cessation. After 31 and 37 days of SD-LT, initiation of shoot tip abscission and shoot tip abscission had occurred on 50% of the plants, respectively. At the end of the experiment all plants had terminated height growth while about 98% had initiated shoot tip abscission and 88% had set a terminal bud. The parent "SW901290" of our pedigree hardened more rapidly than the other parental clone "Jorunn" and developed a higher degree of freezing resistance (1.5 °C) at the end of acclimation (Fig. 3; Paper VI). Height growth also continued much longer in "Joruun" (Fig. 2; Paper VI). Clonal mean heritabilities (H^2) of freezing resistance were consistently high (80-98%) before and throughout acclimation.

QTL mapping

The mapping pedigree 2 was used to identify quantitative trait loci (QTL) for freezing resistance and phenological traits at different time points during cold acclimation. Freezing resistance, total height growth increment, number of new leaves, and top height increment were assessed at days 0, 12, 20, 24, 31 and 42 of a short days - low temperatures (SD-LT) hardening regime while bud set initiation and shoot tip abscission were measured daily. Total height increment, dry-to-fresh weight ratio and number of new leaves were also measured in a replicated field trial. Freezing resistance was determined from electrolyte leakage of leaf tissues and from visual injuries stems segments, after exposure to a predetermined freeze-thaw stress.

This study clearly demonstrated the potential of mapping loci controlling cold adaptation traits *i.e.* autumn freezing resistance and phenological traits, in *Salix*. Using a genetic map of the F_2, composed of 485 AFLP markers, ten genomic regions affecting freezing resistance before and during cold acclimation were identified, supporting the polygenic nature of freezing resistance. In this study there was no time point at which all the QTL could be detected indicating that assessing freezing resistance at a single time-point during cold acclimation would underestimate the number of QTL controlling freezing resistance. The estimated effect of different QTL also seemed vary over the acclimation period. A QTL on linkage group 8 (148) was detected at 4 time points and reached its maximum effect at points 3 and 4 (Table 2; Paper VI). This variation in the QTL effect is probably not due to differences in stress temperature used over time points - the means of the proportional cell leakage (IDX_t) showed limited variation between time points (within freezing stress level; Table 2; Paper VI). The observed effects of individual QTL (3-45%) suggest that major genes are involved in the development of freezing resistance during cold acclimation. For instance, the major QTL for freezing resistance (148) explained a large amount of the variation in the family (up to 45%) as expressed by IDX_t (Table 2; Paper IV). Freezing resistance QTL with even larger effects (up to 79 %) have been reported in agricultural plants (Hayes *et al.* 1993; Pan *et al.* 1994; Galiba *et al.* 1995). However, the QTL effects obtained in the present study are much higher than those observed in studies of freezing resistance of *Eucalyptus*, *Pinus silvestris* and Douglas fir (Byrne *et al.* 1997; Hurme, *et al.* 2000; Lerceteau *et al.* 2000; Jermstad *et al.* 2001b). This could partly be attributed to low environmental variability in the controlled experiment in our study, as estimates of clonal heritability were high for most traits (Table 2; Paper VI). The alleles corresponding to the freezing resistance QTL originated from the Russian parent of the pedigree (SW901290). This suggests that the introduction of the Russian and Siberian material to the present *S. viminalis* germplasm might hold great prospects of improving frost resistance, as has already been observed from field trials (Larsson 1998).

The single QTL detected for non-acclimated freezing resistance (before acclimation) did not reach significance at any time point during cold acclimation suggesting an independent relationship between non-acclimated and acclimated freezing resistance in *Salix*. The largely independent relationship between non-

acclimated and acclimated freezing resistance is also supported by the low phenotypic correlation obtained between these traits in the present study ($r = 0.10$). Previous quantitative genetic analysis in some *Populus* and *Salix* clones, indicated a strong genetic control of freezing resistance at the autumn stage but negligible effects at the non-acclimated stage (Paper V). However, differences in frost resistance between actively growing *Salix* clones in late summer have also been reported (Ögren 1999a). Similar results have been recorded in agricultural species such as *Brassica* and diploid *Solanum* species where non-acclimated freezing resistance and capacity to acclimate were genetically independent (Stone *et al.* 1993; Teutonico *et al.* 1995).

Traits like timing of growth secession, timing of shoot tip abscission, and dry-to-fresh weight ratio have been widely used to estimate autumn frost hardiness in forest tree species due to their close relationship with freezing resistance. The number of new leaves has also been used as an alternative indicator of bud set in *Populus* due to its close relationship with the bud set (Howe *et al.* 2000). Because the present plant material (and may be other willow species) forms shoot tip abscission instead of a typical terminal bud during autumn, shoot tip abscission was used as an alternative to the bud set. Five of the loci associated with freezing resistance shared common intervals with loci controlling traits related to autumn phenology. Of the 14 QTL controlling autumn freezing resistance and phenological traits at the indoors experiment, six (43%) were associated with autumn phenology trait, *i.e.* total height increment, dry-to-fresh weight ratio and number of new leaves, measured in the field. The co-localization of genomic regions controlling autumn specific phenological traits and freezing resistance observed in the present study provide more direct evidence that these traits are partly controlled by a common set of genes in *Salix*.

It must be noted that the number of QTL identified in the present thesis represents a minimum number of QTL for the investigated traits. More QTL most likely remain undetected due to incomplete genome coverage of the linkage map and due to the relatively small mapping population size. However, this limitation in power may partly have been compensated by the use of clonal replication of individuals in the mapping population.

Conclusions and future perspectives

The present thesis illustrates the potential of mapping QTL for growth and adaptation traits in *Salix*. The QTL mapping provided some insights into the genetic basis of the investigated traits. It was shown that major genes, at least in part, affect growth, freezing resistance and phenology related traits in *Salix*. From the determination of QTL position on the map it was suggested that highly correlated traits are partly controlled by a common set of genes in *Salix*. The presence of common genes between freezing resistance and phenological traits indicates that physiological markers *i.e.* dry weight content, growth secession rates, number of new leaves, could replace the more laborious freeze tests in selection practices for freezing resistant genotypes. Because a large proportion of the QTL controlling freezing resistance and phenological trais detected under control-conditions associated with phenological traits under autumn field conditions, lab-screening for markers linked to adaptation traits may be meaningful before field evaluation. The use of the electrolyte leakage method detected variation within families and identified genomic regions affecting freezing resistance. The QTL with the largest effect in freezing resistance (148), along with its involvement in several physiological processes during cold acclimation, indicates that this locus may play a major role in the development of autumn freezing resistance in *Salix*. The non-acclimated and acclimated freezing resistance are at least in part controlled by different genes suggesting that these two trait components should be treated separately in breeding practices. The QTL instability across three-years of growth for height growth was pronounced suggesting special consideration for future MAS practices in *Salix*.

The indication that major genes are involved in growth and adaptations traits may suggests that a MAS breeding scheme could have a valuable effect in accelerating the breeding process in *Salix*. First, it would be important to test for the presence of the detected QTL in other *Salix* families. This has major implications for the transferability of the QTL among breeding programs that operate with different populations. Further, it would be valuable to further examine for possible QTL changes across different environments. In principal, alleles and allelic combinations affecting variation can change with the biophysical conditions of the environment. QTL analysis across environments may capture some of the "environment specific" QTL affecting growth. In view of the above, initial successes from MAS are more likely to come from working with QTL that have some consistency across both environments and populations. However, this does not mean that the most significant long-term advances will necessarily come from these simple applications of identified QTL.

Genes induced by low temperature in plants are an essential part of freezing resistance (Thomashow *et al.* 2001), even if no direct function in freezing resistance has been proved for many of these genes. Candidate genes for freezing resistance are available in plants including woody species like *Populus* and *Salix* (Wisniewski *et al.* 1996; Sauter *et al.* 1999). The mapped QTL controlling freezing resistance and phenology related traits here might correspond to such genes. Thus,

the potential co-location of candidate genes with known function with the detected QTLs holds promise for a better understanding of the physiology associated with freezing resistance in *Salix*. Conversely, cold-induced genes with unknown function mapped near or within QTLs associated with freezing resistance (or phenological traits) could provide some information on their role.

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