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ORIGINAL PAPER

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Mapping of quantitative trait loci (QTLs) affecting autumn freezing resistance and phenology in *Salix*

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Abstract Quantitative trait locus (QTL) analysis was performed at different time points during cold-acclimation of a tetraploid F_2 *Salix* pedigree. The pedigree (n=92) was derived from a cross between a frost-susceptible diploid female clone 'Jorunn' (*Salix viminalis*) and a frost resistant hexaploid male clone 'SW901290' (*Salix dasyclados*). Freezing resistance, height growth increment and number of new leaves were assessed at days 0, 12, 20, 24, 31 and 42 of a short day–low temperature (SD-LT) hardening regime, while the initiation of shoot tip abscission and shoot tip abscission were measured daily. 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 on stem segments, after exposure to a predetermined freeze-thaw stress. Using a genetic map of the F_2 composed of 432 single-dose AFLP markers, a total of 19 genomic regions controlling freezing resistance (10) and phenological traits (9) before and during cold-acclimation (SD-LT) were identified. The magnitude of the phenotypic variation explained by each freezing resistance locus varied over acclimation time (0–45%), and there was no time point at which all the QTLs could be detected. The single QTL detected for non-acclimated freezing resistance did not reach significance at any time point during cold-acclimation, suggesting an independent genetic relationship between non-acclimated and acclimated resistance to freezing in *Salix*. Five of the loci associated with freezing resistance shared common intervals with loci controlling phenological traits. Of the 14 QTLs controlling autumn freezing resistance and/or phenological traits in the indoors experiment, six (43%) were associated with autumn phenology-related traits, i.e. height increment, dry-to-fresh weight ratio and number of new leaves, measured in the field. A major locus with multi-trait association in both indoor and outdoor experiments was detected.

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Introduction

Low temperature is considered one of the most important environmental factors limiting the growth, development and distribution of plants. In the past few decades considerable research effort has been devoted to the study of physiological and biochemical pathways involved in freezing stress in both herbaceous and woody plants (Weiser 1970; Li 1984; Sakai and Larcher 1987). These efforts, however, have resulted in only modest improvements in freezing tolerance (Thomashow 1998) and frost damage to plants continues to be a major problem with high economic significance. Current advances in molecular biology, however, could provide additional opportunities for a better understanding of the molecular events involved in the development of resistance to freezing in plants. Knowledge of the molecular basis of frost resistance could potentially lead to the development of new strategies to improve plant resistance to freezing and result in increased plant productivity (Thomashow 1998).

Progress in developing superior frost-resistant genotypes could be enhanced if the locations of genes involved in frost resistance were identified. Because adaptive traits like resistance to freezing stress generally exhibit continuous variation, the quantitative trait locus (QTL) mapping approach is appropriate. With this approach, loci controlling frost resistance can be positioned on a genetic map and be further studied in terms of the magnitude of their effects on the phenotype, the parental origins of the

favourable QTL alleles, and the relationships between QTLs controlling different cold-adaptive processes.

Recent genetic studies have illustrated the possibility of mapping QTLs controlling frost resistance in forest tree species. 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 *Pseudotsuga meniziesii* (Douglas fir) (Jermstad et al. 2001), have identified QTLs explaining up to 23% of the phenotypic variation in the mapping population. These results indicate that genes with a considerable effect on resistance to freezing are present in forest tree species. Furthermore, QTL studies from agricultural plants have clearly demonstrated the involvement of major genes in frost resistance (Hayes et al. 1993; Stone et al. 1993; Pan et al. 1994; Galiba et al. 1995; Teutonico et al. 1995).

It is well known that woody plants increase their frost resistance after exposure to short days and low temperatures during the autumn (cold acclimation). Earlier studies have indicated large genetic variability within and between forest tree species in autumn frost resistance (Nilsson and Eriksson 1986; Sutinen et al. 1992). Genetic studies in *Populus* and *Salix* have shown strong genetic control of frost resistance at the autumn stage and negligible genetic effects at non-acclimated and dormant stages (Tsarouhas et al. 2001). Similarly, Aitken and Adams (1996) found notably larger genetic variability in freezing resistance during autumn as compared to winter in Douglas fir. However, no study comparing QTLs between non-acclimated (actively growing) and acclimated stages has been reported for forest trees. Additionally, QTL studies dealing with autumn frost resistance in forest trees, and in plants in general, have been limited to the analysis of resistance to freezing at a fixed phenological stage. A better understanding of the genetic basis of autumn freezing resistance could be achieved by observing the QTL dynamics during cold-acclimation.

For this study, our aim was to break down the transition process from the non-acclimated to the cold-acclimated state into a series of steps that could be investigated individually. QTL mapping was performed at successive times throughout indoor cold-acclimation of a *Salix* pedigree with the following objectives: to map and estimate the effects of QTLs controlling freezing resistance before and at successive times of cold-acclimation; to compare the genomic regions associated with freezing resistance before and during cold-acclimation; and to relate QTLs for freezing resistance to QTLs controlling important autumn frost resistance components, i.e. growth cessation and shoot tip abscission. The study was based on a tetraploid *Salix* F_2 family derived from one frost-resistant and one frost-susceptible clone. *Salix* species provide a good model system for QTL mapping of autumn frost resistance due to their large variation and the high contrast in their resistance to freezing before and after the development of frost hardiness. Newly expanded shoots of *Salix* can be damaged by exposure to as low as −3°C, while dormant shoots can tolerate freezing temperatures as low as −85°C (von Fircks 1994; Tsarouhas et al. 2001) or even the temperature of liquid nitrogen (Sakai 1970). A strong relationship between growth rhythm and frost hardening has been observed in *Salix* (Ögren 1999b). In general, *Salix* species offer good systems for biological studies due to their small genome sizes (2C=0.76–0.98 pg, Thibault 1998) and to their shorter times to mature to flowering (2 years) compared to other forest tree species like *Populus* (7 years) and *Picea abies* (20 years). We focus our research on the transition process from the non-acclimated to the cold-acclimated stage due to the importance of freezing resistance for biomass production during that stage.

Materials and methods

The mapping pedigree

Two F_1 plants from a cross between the female clone 'Jorunn' (*Salix viminalis*) and the male clone 'SW901290' (*Salix dasyclados*) made in 1995 by Svalöf Weibull AB and collected from Kirov, Russia, were used to produce the F_2 mapping pedigree utilized in the present study. DNA content measurements classified 'SW901290' as a hexaploid in relation to the diploid 'Jorunn' (Rönnberg-Wästljung et al. 2003). The high germination rates (98%) of the F_2 suggested relatively normal F_1 meiosis. The selection of the cross was based on results from freeze tests of the clones prior to F_2 production. The clones 'Jorunn' and 'SW901290' showed the largest contrast in their resistance to freezing ('Jorunn' was highly frost susceptible; 'SW901290' was highly frost resistant) compared to four other parental candidate clones ('Astrid', 'Björn', '78183' and'Bowles H') (Tsarouhas 2002). Following seed germination in a greenhouse, 92 individual plants were selected and planted indoors to provide material for DNA analysis. In the spring of 1999, 96 individuals were vegetatively propagated and placed in the greenhouse for 2 months before being planted in the field. The field trial was planted in a flat valley, 500 m from the river Fyrisån, with an elevation of ~25 m (58°N, 17°E, Pustnäs, Uppsala, Sweden). The trial plantation consisted of ten randomized blocks, each containing a complete set of the 96 genotypes as single-tree plots. During January 2000, plants were cut down to 5–10 cm above the ground.

Phenotypic trait assessment (indoor experiment)

One-year-old shoots were collected from the field in January 2000 and stored in a cold room (−4°C). In the summer of 2000, cuttings 10 cm in length were planted in 2 l pots containing soil (20% clay and 80% peat). After 8 weeks of growth under an 18 h photoperiod (200 μmol/sm^2) and a 25/18°C temperature regime two sets of plants of uniform size (within clones) were selected. In one set, plant material (6 plants per clone) was tested directly for stem and leaf resistance to freezing (time point 0, non-acclimated plants). The other set (6 plants per clone) was treated with a short day (9 h, 200 μmol/sm^2) low temperature (15/4°C) regime (SD-LT) (Junttila and Kaurin 1990) for 8 weeks. During the SD-LT treatment the height growth increment, the number of new leaves, and the freezing resistance of detached leaves were recorded at five time points: (1) 12 days of acclimation; (2) 20 days of acclimation; (3) 24 days of acclimation; (4) 31 days of acclimation; and (5) 42 days of acclimation (Fig. 1). At time point 5, we also assessed the resistance of stems to freezing. For practical simplicity, the six plants of each genotype were kept in a single plot in which plants were rearranged bi-weekly. Although the experiments were performed in controlled-environment rooms with highly homogenous growth conditions, 50 plants (10 clones×5 plants) were evenly

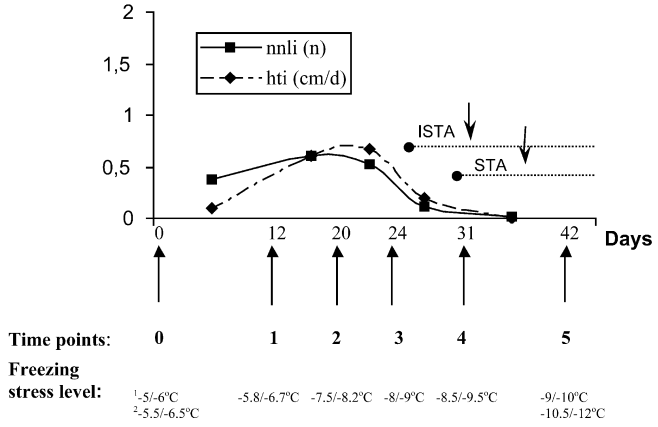

Fig. 1 Sampling points for assessing freezing resistance and phenological traits in the pedigree (F_2) during cold-acclimation. The curves show means of the height growth increment (*hti*, in cm) and the number of new leaves increment (*nnli*) during cold-acclimation. The *arrows* below the graph represent the time points of testing freezing resistance and phenology related traits during cold-acclimation. *Horizontal lines* indicate the time at which initiation of shoot tip abscission (*ISTA*) and shoot tip abscission (*STA*) took place while *filled circles* indicate the start of ISTA or STA and *arrows* the time point that 50% of the family reached ISTA or STA. [1], freezing stress temperature (light/severe stress) for leaf tissues. [2], freezing stress temperature (light/severe stress) for stem tissues

distributed at selected locations in the room to estimate the effect of plant position.

Height growth was measured as the length of the stem (or the tallest stem) from the bottom to the tip of the apex. The height growth cessation between two subsequent time points was estimated by subtracting the heights of the two points. At time point 0 an ink mark was made just below the tip of the stem apex. The number of new leaves (fully expanded) developed above the mark was counted (Howe et al 1995). The initiation of shoot tip abscission and the actual shoot tip abscission were scored daily from the day induced in the first plant. The initiation of shoot tip abscission was scored when discoloration (light green-white) on the upper-most leaf tips (the last developed) of the central shoot apex could be seen. Shoot tip abscission was registered when the upper-most leaf tips (and possibly part of the shoot apex) were dried out and the shoot tip abscission zone was visible.

Freezing resistance was assessed by subjecting leaf tissues to a freeze-thaw stress at each time point, and for the stem tissues at time points 0 and 5 (Fig. 1). In the leaf freezing resistance assessment, three leaves were harvested from the middle third of each plant and placed in tubes containing 5 ml of tap water. The tubes were then transferred into a programmable freezer (Conviron) and incubated for 3 h at −2°C to equalize the temperature within the samples. During the second hour of incubation, ice chips were added to the tubes to initiate extracellular freezing. When the incubation at −2°C was completed the freezer was programmed to cool the plants in the dark, at the rate of −1°C/h. Following a 30 min exposure at two selected temperatures (Fig. 1), a set of tubes was directly transferred in large insulated boxes to a freezer for slow thawing to 2°C. For the stem freezing resistance assessments, two stem segments 7 cm long from the middle third of each plant were harvested and placed in tubes containing 10 ml of tap water. Apart from slightly lower freezing temperatures (Fig. 1), the freezing procedure was similar to that described above for the assessment of leaf freezing resistance. The freezing/thawing rates, as well as the selected freezing temperatures, were determined from a series of pre-screening freezing tests of the F_2 population (essentially as described in Tsarouhas et al. 2001) 2 days prior to each main experiment. Two freezing temperatures were chosen; one to slightly damage the plants and the other to heavily injure them. These temperatures corresponded to LT_{20} and LT_{50} in our experiment (Sakai and Larcher 1987). Because the frost resistance of either 'SW901290' or 'Jorunn' was largely out of the trait distribution in most time points (data not shown), parental frost resistance was not considered in the selection of the test temperatures. One of the selected temperatures resulted in no damage (time point 1), and on other in severe damage of leaf tissues (time point 5). Data from these temperatures were excluded from further analysis.

The leaf freezing resistance assessment was based on observed alterations in membrane transport function using the electrolyte leakage method as described in Tsarouhas et al. (2000). The percentage of induced leakage expressed as an index of injury at temperature T (IDX_T) was calculated as:

$$IDX_T = 100(RC_T - RC_o)/(1 - RC_o),$$

where RC_T is the fractional release (c_T/c_{Tk}) of electrolytes from freeze-treated samples (C_T=specific conductance of leachate from samples frozen at temperature T; c_{Tk}=specific conductance of leachate from samples frozen at temperature T and then heat-killed), and RC_o is the fractional release (c_o/c_{ok}) of electrolytes from unfrozen samples (c_o=specific conductance of leachate from unfrozen samples; c_{ok}=specific conductance of leachate from unfrozen but heat killed samples).

Stem frost resistance was visually evaluated. The amount of flushing, rooting nodes and discoloration of normally green tissues in each segment was recorded. Injury to each stem tissue was recorded as the percentage (to the nearest 10%) of damaged tissue. An average score of both temperature treatments with the scale 1–6 was given, where 1, 2, 3, 4, 5, 6, corresponded to 0, 20, 40, 60, 80, 100% on average of flushing, rooting and green colour of stem segments.

Field experiment

The total height growth increment and the number of new leaves on the tallest shoot of each plant were assessed at three time points during the autumn of 2000 (23 August, 15 September and 28 September). The recording procedure was as for the indoor experiment. Dry-to-fresh weight ratio of the second-tallest shoot of each plant was assessed on 26 September 2000. A shoot sample 0.5 m long at the weight equilibrium point of the shoot was taken. Fresh (the same day of harvest) and dry weights (after 3 days at 105°C) for each shoot were assessed (Rönnberg-Wästljung and Gullberg 1999).

Map information

On the basis of 92 F_2 individuals ($4n$=19), a linkage map with 432 AFLP marker loci was constructed using single dose AFLP markers segregating in 1:1, 3:1 and 1:2:1 ratios. Two maps, one for each F_1 parent, were constructed according to the 'two-way pseudo-testcross' mapping strategy. The female map (1,640 cM) included 248 markers and formed 35 major linkage groups (>3 markers) while the male map (1,820 cM) consisted of 269 markers placed on 37 major groups (>3 markers). In addition, 22 and 21 minor groups (doublets and triplets) were obtained in the female and male maps respectively. Using the AFLP markers that segregated in both parents (3:1 and 1:2:1) eight homologous groups from both parents were obtained. Both maps provided about 47% coverage (on average). Details of this map are described in Rönnberg-Wästljung et al. 2003.

QTL mapping

QTL analysis was performed using the above-mentioned genetic map. Clonal means and linkage data on 92 progenies were initially analysed for significant loci-trait associations (P<0.05) using a

simple regression model implemented in the MAP-MANAGER-QTXb14.0 program (Manly et al. 2001). Linkage groups with significant loci-trait associations were further analysed by composite interval mapping with QTL Cartographer Version 1.14 (Basten et al. 2000). The background loci (2–4 loci) were selected using SRMAPQTL of QTL Cartographer. The empirical statistical thresholds for an overall significance level of 0.05 were determined by performing 1,000 permutations of the data set for each trait. The marker loci flanking QTLs detected from the indoor experiment were tested for significance with phenotypic data measured in the field. Clonal mean heritabilities were calculated as:

$$H^2 = \sigma_c^2/\left(\sigma_c^2 + \sigma_e^2/b_c\right),$$

where σ_c^2, σ_e^2 are the clone and error variance components, respectively, and b_c is the coefficient for σ_c^2 from the clone expected mean square calculated by the standard least squares model of an analysis of variance (JMP 3.0, 1994). The components of the variance explained by the detected QTLs were estimated from an analysis of variance (ANOVA) on the most significant ($P<0.01$) and most pronounced (present at more than one acclimation point or freezing temperature) set of loci controlling resistance to freezing. In the ANOVA model, the locus closest to each QTL was included as a random factor. The total phenotypic variance explained by all the QTLs for each trait was determined from the R^2adj (coefficient of determination) of the ANOVA model. Phenotypic values of IDX_T (index of injury at freezing temperature T) were deviated from normal distributions and logarithmic transformations were used to improve normality. No effects of room position were detected and no further data normalizations were made.

Results

Phenotypic traits

Both the mean values for freezing resistance and the phenological traits of the F_2 family were strongly affected by the short day and the low temperature (SD-LT) regime. Before acclimation the proportion of electrolytes which leached from leaf cells (estimated by IDX_T) increased 14% after freezing at −5.0°C. However, following 31 days of SD-LT (time point 4) a 3.5°C lower freezing temperature (−8.5°C) resulted in an almost equivalent proportion (11%) of leached leaf cell electrolytes (Table 2). Between 20–24 days of cold-acclimation (at time points 2 and 3) the pedigree showed a rapid decrease in height growth (Fig. 1). The initiation of shoot tip abscission and actual shoot tip abscission occurred later than the cessation of growth. After 31 and 37 days of SD-LT, initiation in shoot tip abscission and shoot tip abscission respectively had occurred in 50% of the plants (Fig. 1). At time point 5 all plants had terminated their height growth while about 98% had initiated shoot tip abscission and 87% had already set the abscission zone (data not shown). Height growth continued much longer in 'Jorunn' (Fig. 2) than in 'SW901290'.The parent 'SW901290' also hardened more rapidly than the other parental clone 'Jorunn' and developed a higher degree of freezing resistance at the end of the acclimation regime (Fig. 3). Offspring (F_2) values for almost all traits were between the corresponding values of the two parents (data not shown). Clonal mean heritabilities (H^2) of freezing resistance were consistently high (80–98%) before and throughout acclimation (Table 2). Phenological traits in general exhibited lower H^2 values (56–92%). In the field experiment, the height growth increment between 23 August and 15 September was considerably higher (0.5 cm/day) compared to the corresponding increment between 15 September and 28 September (0.08 cm/day).

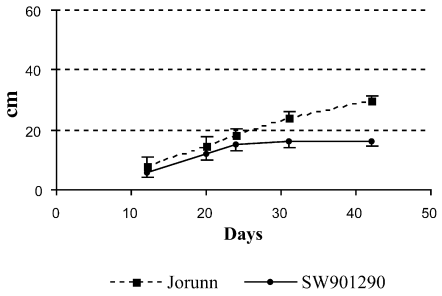

Fig. 2 Height growth increment (*hti*) during cold-acclimation between the two parental clones

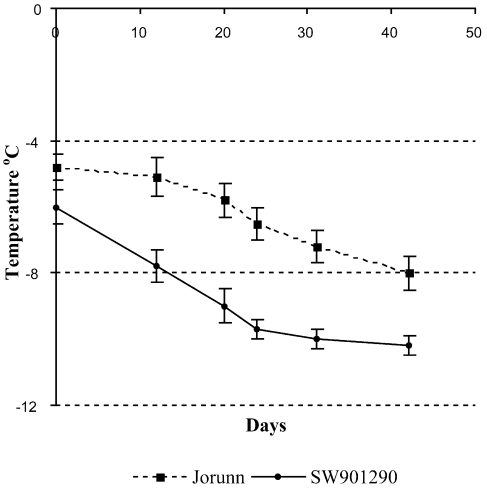

Fig. 3 The freezing resistance of the two parental clones during cold-acclimation expressed as LT_5

QTLs for freezing resistance identified before and throughout cold-acclimation

A total of ten genomic regions controlling freezing resistance were identified before and during cold-acclimation (Table 1). QTLs were detected at all time points and freezing temperatures in the experiment, except at time point 1. Five QTLs were detected at single time points while the others were present at two or more time points. For QTLs present at several time points, the magnitude of the phenotypic effect varied during acclimation. Three of the ten freezing resistance QTLs affected stem freezing resistance at time point 5, and two of these were also associated with leaf freezing resistance. Two freezing stress temperatures were used to assess freezing resistance at each time point, and in

Table 1 QTLs for freezing resistance and phenological traits. Freezing resistance QTLs at time point 5 are shown for both leaves (5a) and stems (5b). In the field experiment the numbers 1, 2 and 3 indicate the date (23/8, 15/9 and 28/9) of trait assessment. *ISTA*: days to initiate shoot tip abscission; *STA*: days to shoot tip abscission; *hti*: height increment (numbers indicate the two successive time points of the increment); *nnli*: number of new leaves increment; *dwt*: dry-to-fresh weight ratio

Locus closest to the QTL	Indoor experiment																		Field experiment			
	Freezing resistance											Phenological traits										
	Time points and tested temperatures (°C)																					
	0		1	2		3		4		5a	5b											
	−5	−6	−6.7	−7.5	−8.2	−8	−9	−8.5	−9.5	−10	−11.25	ISTA	STA	hti 2-1	hti3-2	hti 4-3	nnli 3-2	nnli 4-3	dwt	hti 2-1	hti 3-2	nnli 3-2
618						*		*			*											
262										*										*		
F155	***	**																				
F1519							*															*
314											*	**		*		**						
578				***				***		*					*							
585													*									
3016												**		*				*		**		*
148				***	**	***	***	**	***	*	*					*		*	*			*
C2919															*		*					
F72										*									**			
F255																		*				
296				***	*	*		*		**					*				*			
2912				**				**					*									

*$P<0.05$, **$P<0.01$, ***$P<0.001$

Table 2 Variance components (VC %) for the most significant ($P<0.01$ in more than one time point or freezing temperature) freezing resistance loci and their corresponding VC % values for the phenological traits. Stem freezing resistance (5b) was detected by visual scoring within a scale of 1–6; T_1 and T_2 indicate the two selected temperatures of the freezing test (see Fig. 1 for the corresponding temperature values). $R^2adj\%$ represents the total phenotypic variance explained by all QTLs for each trait as determined from the R^2adj of the ANOVA model, VC(*cl*)% represents the proportion of the genetic variation attributed to each genotype in the pedigree as estimated by an one-way ANOVA model using the "clone" as random effect

Locus closest to the QTL	Indoor experiment																			Field experiment		
	Frost resistance during different acclimation time points											Phenological traits										
	0		1	2		3		4		5a	5b											
	T_1	T_2	T_1	T_1	T_2	T_1	T_2	T_1	T_2	T_2	T_1, T_2	ISTA	STA	hti 2-1	hti 3-2	hti 4-3	nnli 3-2	nnli 4-3	dwt	hti 2-1	nnli 3-2
F155	25***	19**	9	0	0	0	0	0	0	0	0	2	0	3	0	1	0	0	0	0	2
2912	1	0	3	14**	6	3	0	8**	1	6	2	14	3*	0	1	0	0	0	5	4	1
578	0	2	0	5***	0	3	8	8***	0	5*	0	14	1	0	1*	0	0	0	0	18	0
148	1	0	2	19***	19**	36***	43***	34**	45***	3*	8*	5	0	1	0	4*	1	24*	11*	1	24*
296	0	3	6	10***	10*	12*	2	5*	3	20**	3	10	0	1	19*	0	1	0	3*	5	6
$R^2(adj)\%$	26	22	18	47	34	50	52	59	46	30	12	38	4	5	20	4	2	23	17	22	29
VC(*cl*)%	67	68	64	64	80	79	80	80	80	74	95	45	70	34	48	33	33	40	24	25	33
H^2 (%)	96	95	80	89	95	94	96	95	95	93	98	80	92	70	75	63	70	79	56	73	72
Mean	14	31	12	11	26	12	28	11	29	25	2.9	30[a]	38[a]	4.8[a]	2.5[a]	1.6[a]	2.1[a]	0.7[a]	0.5	12[a]	0.06[a]
Std error	0.4	0.5	2.0	0.9	1.3	0.8	1.3	0.7	1.2	1.0	0.2	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.002	0.5	0.001

*$P<0.05$, **$P<0.01$, ***$P<0.001$

[a] Means of ISTA and STA expressed in days. Means of height increment (*hti*) are presented in cm and of the new leaf increments (*nnli*) in numbers (*n*)

general more QTLs were detected using the higher temperature (Table 1). The most predominant freezing resistance QTL (148) was present at four time points and both freezing temperatures, and it explained up to 45% of the phenotypic variance (Table 2). This major QTL was not detected before acclimation (time point 0). In contrast, one out of ten QTLs associated with freezing resistance was detected before acclimation only (Table 1) and explained around 20% of the phenotypic variance in non-acclimated resistance to freezing. No QTLs were detected for stem freezing resistance at the time point 0.

QTLs for autumn phenology traits

A total of nine QTLs were identified for autumn phenology traits in the indoor experiment (Table 1). Interestingly, five of the nine QTLs were located in genomic regions with QTLs affecting freezing resistance. The predominant freezing resistance QTL (148) explained over 20% of the variation for the number of new leaves (*nnli4-3*), but showed marginal effect on height growth increments (Table 2). The QTL associated with freezing resistance before acclimation (F155) showed no association with QTLs for autumn phenological traits. Conversely, one genomic region (identified by locus 3016), where QTLs for several growth rhythm traits were identified, showed no effect on freezing resistance (Table 1).

QTLs detected in the field experiment

Of the 14 QTLs regions detected for autumn freezing resistance and/or phenological traits in the indoor experiment, 6 (or 43%) were associated with phenological traits measured in the field (Table 1). Each of the three QTLs regions detected for dry-to-fresh weight ratio was also associated with freezing resistance in the indoor experiment. One of these was the major freezing resistance QTL (148) that also showed an association with the number of new leaves, not only indoors but also in the field.

Discussion

QTLs controlling freezing resistance during cold-acclimation

This study has clearly demonstrated the potential of mapping loci controlling cold adaptation traits, i.e. autumn freezing resistance and phenological traits, in *Salix*. Despite the fact that the QTL analysis was limited by using single-dose DNA markers, several QTLs were detected. Ten genomic regions affecting freezing resistance before and during cold-acclimation were identified, supporting the polygenic nature of frost resistance. Our results also indicate that assessment of freezing resistance at a single time point during cold-acclimation would underestimate the number of QTLs, since there was no time point at which all the QTLs could be detected. The estimated effect of different QTLs also seemed to vary over the acclimation time. QTL 148 was detected at four time points and reached its maximum effect at time points 3 and 4 (Table 2). 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).

The observed effects of individual QTLs suggest that major genes are involved in the development of resistance to freezing during cold-acclimation. For instance, the QTL for freezing resistance on locus 148 explained a large amount of the variation in the family (up to 45%) as expressed by IDX_T (Table 2). Frost resistance QTLs 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). The QTL effects obtained in the present study are higher than those observed in studies of frost resistance in *Eucalyptus*, *Pinus sylvestris* and *Pseudotsuga meniziesii* (Douglas fir) (Byrne et al. 1997; Hurme et al. 2000; Lerceteau et al. 2000; Jermstad et al. 2001). This could partly be attributed to the use of an interspecific cross (with parents contrasting in frost resistance) and to the low environmental variability in the controlled experiment in our study, as estimates of clonal heritability were high for most traits (Table 2). It should be noted that the assessment of resistance to freezing was mainly based on leaf tissues. Leaves are annual in *Salix* and are likely to be more sensitive than stems (von Fircks 1994; Tsarouhas et al. 2000). Therefore, QTLs involved in the development of leaf frost resistance in autumn may not necessarily predict the overall performance or survival of the mapping family. From the point of view of biomass production, however, where the yield is more important than survival, genes associated with leaf frost resistance on a growing meristem are certainly essential.

In the current study the highly resistant parent 'SW901290' is a northern (Kirov, Russia) hexaploid *Salix dasyclados* genotype. All the QTL alleles resulting in increased freezing resistance originated from this parent. This indicates the ability to introduce favourable QTLs from a wild *Salix* source, and further suggests that the introduction of Russian and Siberian *Salix* material to the present *S. viminalis* germplasm has great potential for the improvement of frost resistance, as has already been observed in field trials (Larsson 1998).

A QTL for non-acclimated freezing resistance

A single QTL for freezing resistance (F155) was detected before acclimation (time point 0). This QTL did not reach significance during cold-acclimation (Table 1), indicating an independent genetic control of non-acclimated and acclimated frost resistance in *Salix*. The observed inde-

pendent 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, data not shown). Previous quantitative genetic analysis in *Populus* and *Salix* indicate strong genetic control of resistance to freezing at the autumn stage, but negligible effects at the non-acclimated stage (Tsarouhas et al. 2001). Similar results have been found in agricultural species such as *Brassica* and diploid *Solanum* species where non-acclimated frost resistance and the capacity to acclimate were genetically independent (Stone et al. 1993; Teutonico et al. 1995). However, notable differences in frost resistance between actively growing *Salix* clones in late summer have also been recorded (Ögren 1999a).

QTLs for autumn phenology traits

There is a strong link between growth rhythm and frost hardening in both angiosperms and gymnosperms (Deans and Harvey 1996; Hurme et al. 1997). Traits like timing of growth cessation, 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 frost resistance. A correlation between frost resistance, growth cessation and dry-to-fresh weight ratios of stems has been observed in *Salix* (Ögren 1999b). The number of new leaves has also been used as an alternative indicator of bud set in *Populus* due to its close relationship with bud set (Howe et al. 2000). Because *Salix* (shrub willows) exhibits shoot tip abscission instead of a typical terminal bud during autumn conditions, shoot tip abscission was used as an alternative to bud set.

QTL mapping of autumn frost hardiness components i.e. growth cessation, dry-to-fresh weight ratios and shoot tip abscission could provide a better genetic understanding of the development of autumn resistance to freezing in *Salix* and allow better manipulation of trait components towards the development of superior frost resistant genotypes. The co-localization of genomic regions controlling autumn-specific phenological traits and freezing resistance was pronounced in the present study. Five of the ten loci associated with resistance to freezing shared common intervals with loci controlling phenological traits. This provides more direct evidence that these traits are partly controlled by a common set of genes and confirms that physiological markers, i.e. growth cessation, shoot tip abscission and number of new leaves are important predictors of the development of freezing resistance in *Salix*. The large proportion of common QTLs (43%) detected between indoor and field autumn conditions increase the level of reliability of the QTLs detected and suggests that laboratory screening for molecular markers associated with cold adaptation traits could make a reliable contribution to field evaluations.

This study represents a first attempt to map QTLs in a polyploid *Salix* family. The difficulty of determining all possible dosages of each marker (and/or QTL) and the complexity of assessing chromosomal pairing relations during meiosis complicate QTL mapping of polyploids. To date there is no statistical model available that considers all of these factors, although novel approaches for QTL detection in polyploids have been initiated (Doerge and Craig 2000; Ma et al. 2002). Despite this limitation the results of this study undoubtedly encourage further QTL mapping in polyploid *Salix*. Several QTLs which explained a large amount of the variation in the family (up to 45%) with respect to freezing resistance and phenological traits were detected. Due to the use of dominant single-dose DNA alleles only, some of these QTLs could represent the same locus in homologous genomic regions, which would result in an overestimate of the number of QTLs detected. On the other hand, other factors probably bias the number of QTLs downwards. The size of the mapping population (92) was relatively small, which limits the power to detect QTLs with small effects (Beavis 1994) and the genome coverage of the linkage map is not complete. In the present study, the limitation in power was partly compensated by the use of clonal replication of individuals in the mapping population. Addition of multiallelic SSR markers and incorporation of more realistic polyploid models for linkage analysis and QTL mapping will improve our understanding of the genetic architecture of frost resistance in *Salix*. More information on the stability of the detected QTLs over environments and populations is certainly also important.

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