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Sucrose utilization during somatic embryo development in black spruce: involvement of apoplastic invertase in the tissue and of extracellular invertase in the medium

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Summary

The involvement of apoplastic invertase (Ap Inv) and sucrose synthase (SuSy) in the somatic embryo development of black spruce (*Picea mariana*) was investigated under different maturation conditions. Replacing 6% sucrose with 3% or 1% sucrose in the maturation medium drastically decreased Ap Inv activity and amount in embryogenic tissues. This was accompanied by a decrease in the hexose pool that resulted in a lower starch deposition and protein amount in embryogenic tissues together with a lower embryo production. Conversely, SuSy activity was stable during maturation regardless of the sucrose concentration used in the medium. The presence of an extracellular enzyme responsible for sucrose hydrolysis in the maturation medium was also verified. An immunodetection experiment with anti-acid invertase antibodies revealed the presence of an active 53 kDa polypeptide in the medium, which had a similar molecular mass to that of the Ap Inv polypeptide found in embryogenic tissues. Utilization of sucrose from the medium by the tissues was also studied using labelled ^{14}C-sucrose. Distribution of the radioactivity between tissular sucrose, glucose, and fructose showed that sucrose was diffused into the cell wall of embryogenic tissues and partly hydrolyzed by Ap Inv. These results show that the utilization of sucrose from the medium, the Ap Inv activity in embryogenic tissues, and the release of an active

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invertase into the medium operate together for the utilization of the carbohydrates during somatic embryo development in black spruce.
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Introduction

Sucrose plays a central role in plant growth and development as a primary transport form of carbon and energy in most plant species and also as a regulator of gene expression (see reviews by Chiou and Bush, 1998; Lalonde et al., 1999; Smeekens, 2000). In plant tissues, the first step for sucrose utilization is the cleavage of the glycosidic bond by either sucrose synthase (SuSy; EC 2.4.1.13) or invertase (Inv; EC 3.2.1.26), resulting in the production of hexoses, which are an essential energy source for the accumulation of different storage products and tissue growth (reviewed by Wobus and Weber, 1999). Although invertase and SuSy are both implicated in sucrose hydrolysis, their participation in plant growth and development differs. It is generally assumed that SuSy activity is related to cell wall and starch synthesis (Winter and Huber, 2000), but it may also be associated with sucrose synthesis (Déjardin et al., 1997b; Iraqi and Tremblay, 2001b). Invertases provide hexoses for cellular metabolism (Hubbard et al., 1989; Yelle et al., 1991). They also participate in the control of growth, cell differentiation, as well as seed and embryo development (Weber et al., 1997; Carlson and Chourey, 1999; Tang et al., 1999; Iraqi and Tremblay, 2001b). Although the importance of SuSy and invertases in the regulation of sucrose utilization has been highlighted in angiosperm species, little information is available on the importance of these enzymes to conifer embryo development.

For several coniferous species, sucrose represents the best carbon source to support somatic embryo maturation (reviewed by Jain et al., 1995; Tremblay and Tremblay, 1995; Taber et al., 1998; Iraqi and Tremblay, 2001a). As in other plant tissue systems (George, 1993; Taber et al., 1998) and in spruce embryogenic tissues, sucrose in the maturation medium is hydrolyzed into glucose and fructose, resulting in an increased osmotic pressure of the medium (Tremblay and Tremblay, 1995; Iraqi and Tremblay, 2001a). This increased osmotic pressure was not responsible for somatic embryo maturation since the addition of mannitol (Tremblay and Tremblay, 1995) or the replacement of sucrose by gradually increasing glucose and fructose concentrations in the medium throughout the maturation period (Iraqi and Tremblay, 2001a) resulted in decreased embryo numbers. In the last case, the produced embryos had a lower germination rate with lower protein contents than did embryos produced on a maturation medium containing 6% sucrose. Decreased embryo number and quality also resulted when daily transfers onto fresh medium prevented complete sucrose hydrolysis (Iraqi and Tremblay, 2001a). It was therefore concluded that sucrose hydrolysis in the medium, but not the resulting increase in osmotic pressure or the products of hydrolysis, improved the production of somatic embryos. These embryos contained high levels of storage proteins that led, in turn, to high germination rates. Tremblay and Tremblay (1995) hypothesized that this hydrolytic activity was due to an active extracellular enzyme but no data confirmed this point.

To better understand these results, the dynamics of sucrose metabolism enzymes in spruce embryogenic tissues during maturation on a medium containing 6% sucrose was further studied. Throughout the maturation, high levels of cytoplasmic and vacuolar invertase activities corresponded with high tissue levels of glucose and fructose and, more importantly, with high protein and starch accumulation in the embryos (Iraqi and Tremblay, 2001b). At the same time, the cellular level of sucrose showed a four-fold increase while SuSy and SPS activities stayed constant. Iraqi and Tremblay (2001b) suggested that a direct uptake of sucrose from the medium could also be involved in spruce somatic embryo maturation.

The present report aims, firstly, to document the role of a third invertase, the apoplastic invertase (Ap Inv), which is prevalent in heterotrophic tissues such as maize endosperm (Carlson and Chourey, 1999). Ap Inv is also involved in the control of growth and cell differentiation in *Vicia faba* seed (Weber et al., 1997) and carrot embryo development (Tang et al., 1999), but its role in spruce somatic embryo development has never been studied. Secondly, we wanted to confirm the hypothesis of Tremblay and Tremblay (1995) regarding the presence of an extracellular enzyme in the medium during spruce embryo development. Finally, the utilization of sucrose from the maturation medium by embryogenic tissues was followed up by evaluating the incorporation of ^{14}C-labelled sucrose in the medium into sucrose, glucose, and fructose in the tissues. From the data obtained, we suggest that the Ap Inv in the tissues and an extracellular invertase in the medium operated

together for the utilization of the carbohydrates during somatic embryo development in black spruce.

Materials and methods

Plant materials, media and culture conditions

Embryogenic tissue, line M-286, was induced from a mature zygotic embryo of black spruce (*Picea mariana* (Mill.) BSP) and maintained by 14-day subcultures on HLM-1 medium before use (Tremblay, 1990). Basal maturation medium (Tremblay and Tremblay, 1991) was supplemented with 45 μM (±) *cis,trans*-abscisic acid and was solidified with 0.4% (w/v) Gelrite™ gellan gum. Sucrose was filter-sterilized and added to the cooled medium. Culture conditions were as previously described (Tremblay and Tremblay, 1991).

Maturation experiments

To determine the effect of sucrose concentration in the medium on carbohydrate metabolism during the maturation of black spruce somatic embryos, concentrations of 1%, 3%, and 6% (w/v) were compared. Seven days after transfer onto fresh maintenance medium, portions (90 ± 5 mg) of embryogenic tissue were taken and placed in Petri dishes ($100 \times 15\,mm^2$) containing 24 ml of maturation medium. For each treatment, hexose contents, protein levels, and enzyme activities were monitored in the embryogenic tissues on days 0, 7, 14, 21, 28 and 35 of the maturation period. The experiment was conducted as a completely randomized design with three embryogenic tissues per Petri dish (replicate) per treatment. On each sampling date, four Petri dishes were used for dry weight (DW) and sugar analysis, and five Petri dishes were used for protein analysis and enzyme assays. After 10 days of maturation, immunodetection with anti-acid invertase antibodies was performed on protein extracted from embryogenic tissues matured under the different sucrose concentrations. After 35 days of maturation, the number of normal and abnormal embryos was assessed from five Petri dishes. DW of embryogenic tissues was determined after lyophilization. Sugar and protein levels as well as enzyme activities and immunodetection were performed as described below.

To verify if the release of an extracellular invertase was responsible for sucrose hydrolysis in the maturation medium, embryogenic tissue was grown for 6 days in liquid HLM-1 maintenance medium (1.5 g tissue per 40 ml medium) in a 250 ml Erlenmeyer flask on a rotary shaker at 96 rpm. The cells and tissue aggregates were then collected on a 41 μm nylon mesh that was placed onto a 6% sucrose-containing maturation medium. After 10 days, invertase extraction from the medium, immunodetection with anti-acid invertase antibodies, and an enzyme assay were conducted as described below.

An experiment using ^{14}C-labelled sucrose aimed to determine if sucrose present in the maturation medium could be used directly by the tissues. To test this, embryogenic tissues (90 ± 5 mg) were transferred onto a maturation medium containing 6% sucrose. After 10 days, 1 g of embryogenic tissue was removed and placed on 6 ml of the same maturation medium to which $0.2\,\mu Ci\,ml^{-1}$ of uniformly labelled ^{14}C-sucrose (ICN, CA, USA, specific labelling: $672\,mCi\,mmol^{-1}$) had been added. After 5, 10, and 20 min of incubation, unspecific binding was eliminated by extensive washing of the tissues with distilled water until no radioactivity could be detected in recovered water. Then, tissues were frozen in liquid nitrogen before extraction of sucrose, glucose, and fructose for radioactivity measurements. The experiment was conducted with five replicates for each incubation period.

Sugar extraction and analysis

Extraction of glucose and fructose from the tissues and HPLC analysis were carried out as previously described (Iraqi and Tremblay, 2001a). Starch was extracted from tissues according to Iraqi and Tremblay (2001a) and the resulting glucose in the extract after starch hydrolysis was quantified with a glucose kit (HK) (Sigma) according to the manufacturer's recommendations. Radiolabelled soluble sugars were extracted and fractionated by HPLC using the same procedure as for soluble sugars. Sugar peaks were separated using sucrose, glucose (BDH Inc., Toronto, Ont., Canada), and fructose (Fisher, Fair Lawn, NJ, USA) as standards. The radioactivity in each fraction was determined in a liquid scintillation counter (Wallac 1217, Turku, Finland).

Enzyme extraction and protein quantification

SuSy was extracted and desalted at 4 °C according to Déjardin et al. (1997a) by resuspending the powdered tissues in $50\,mmol\,l^{-1}$ Hepes buffer, pH 7.5, containing $10\,mmol\,l^{-1}$ $MgCl_2$, 1 mM EDTA,

2 mmol l^{-1} DTT, 10% (v/v) glycerol, 1 mmol l^{-1} PMSF, and 1% (w/v) insoluble PVP (named as standard buffer). The homogenates were centrifuged at 13 000*g* for 15 min and the supernatants were desalted by passage through a 4 ml Sephadex G-25 column pre-equilibrated with 50 mmol l^{-1} Hepes buffer, pH 7.5, containing 10 mmol l^{-1} $MgCl_2$, 1 mM EDTA and 1% (w/v) BSA. Ap Inv (ionically bound) was extracted according to Xu et al. (1996). Briefly, the pellet left after SuSy extraction was washed three times with the standard buffer and the final pellet was resuspended in 1 M NaCl in standard buffer without insoluble PVP. After 4 h of incubation at 4 °C, the homogenates were centrifuged at 13 000*g* for 15 min before use in the enzyme assay.

Invertase from the maturation medium was extracted at 4 °C. The maturation medium under the nylon mesh supporting the embryogenic tissues was collected and homogenized into a fine powder in liquid nitrogen. Invertase was extracted in standard buffer. The homogenates were centrifuged at 13 000*g* for 15 min. The supernatant was precipitated with 80% (w/v) $(NH_4)_2SO_4$ when used for enzyme assay, and with 4% (v/v) trichloroacetic acid when used for immunoblotting. For the latter, the pellet left after centrifugation was directly resuspended in a loading buffer. Soluble and insoluble tissue protein were assessed according to the Bradford method (Bradford, 1976) using BSA as the standard.

Enzyme assay

The SuSy activity was assayed in the direction of sucrose breakdown according to Déjardin et al. (1997a) in a Hepes buffer (pH 6.5) containing 100 mmol l^{-1} sucrose and 2 mmol l^{-1} UDP. Reactions, which were started by the addition of desalted extract, were incubated at 30 °C for 15 min and were stopped by boiling. Control reactions without UDP estimated invertase activity. After centrifugation at 13 000*g* for 3 min, formed hexose (fructose) was measured spectrophotometrically using coupling enzymes according to King et al. (1997). The reaction of coupling enzymes was started with 5 µl ATP (100 mmol l^{-1}), 5 µl NAD (40 mmol l^{-1}), 2 units of Glc-6-P dehydrogenase, 2 units of hexokinase, and 3 units of glucoisomerase. Ap Inv was assayed in a sodium acetate buffer (pH 4.5) containing 100 mmol l^{-1} of sucrose. Reactions, which were started by the addition of extract, were incubated at 30 °C for 15 min. The reaction was stopped by boiling. Control reactions were boiled immediately. The production of hexoses was quantified using coupling enzymes as described above. In all cases it was ensured that activity was linearly related to time and to the amount of extract.

Immunoblotting

Polypeptides, separated by SDS-PAGE (Laemmli, 1970), were electrophoretically transferred onto a nitrocellulose membrane according to Towbin et al. (1979). The transfer was made under constant current with a Mini-Trans-Blot cell (Bio-Rad). The membranes were incubated with anti-acid invertase antibodies (kindly provided by A.H. Kingston-Smith, Kingston-Smith and Pollock, 1996) diluted 1:1000 for tissue immunodetection and 1:500 for medium immunodetection. The membranes were then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase for 1 h at room temperature with gentle shaking. The presence of invertase was revealed by alkaline phosphatase substrate (Bio-Rad) according to the manufacturer's recommendations. The density of the immunodetected band was integrated using NIH 1.61 imaging software (W. Rasband, National Institute of Health, USA) and the signal strength was expressed as a percentage of the highest value.

Results

A decrease in sucrose concentration in the medium affects Ap Inv activity, but not SuSy activity, in embryogenic tissues

Activities of Ap Inv and SuSy were measured in embryogenic tissues during maturation on different sucrose concentrations (Fig. 1). Ap Inv activity revealed significant changes depending on the sucrose concentration used in the medium. In tissues grown on a 6% sucrose-containing medium, the Ap Inv activity increased from an initial value of 170 to 255 nmol mg^{-1} protein mg^{-1} on day 7 and then decreased to 160 nmol mg^{-1} protein mg^{-1} by the end of the maturation period. Decreasing sucrose concentration in the medium to 3% and 1% decreased Ap Inv activity from 170 initially to 75 and 45 nmol mg^{-1} protein mg^{-1}, respectively, after 7 days of maturation. Subsequently, Ap Inv activity sligthly increased from day 7 to day 14 and reached respective minimal values of 60 and 30 nmol mg^{-1} protein mg^{-1} at the end of the maturation period. Regardless of the sucrose concentration in the medium, SuSy activity remained constant at approximately 45 nmol mg^{-1} protein mg^{-1} throughout the maturation period.

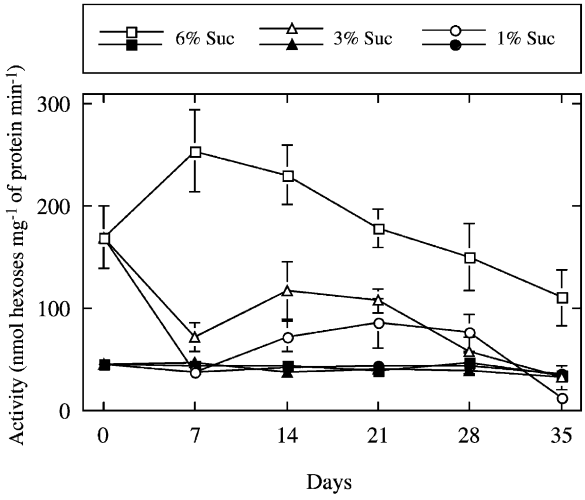


Figure 1. Ap Inv (open symbol) and SuSy (closed symbol) activities in embryogenic tissue of black spruce during maturation on media containing different sucrose concentrations. Bars represent standard deviations ($n = 5$).

A decrease in sucrose concentration in the medium affects Ap Inv amount in embryogenic tissues

Since Ap Inv activity was markedly affected by the sucrose concentration in the medium, Ap Inv amount was investigated in tissues growing on different sucrose concentrations. Immunodetection of Ap Inv with anti-acid invertase antibodies was performed on the insoluble protein fraction extracted from the tissues after 10 days of maturation. For each treatment, the immunoblot showed a 53 kDa band corresponding to Ap Inv in black spruce (Fig. 2A). The strongest Ap Inv signal was recorded in tissues grown on 6% sucrose (Fig. 2B). In tissues matured on 3% and 1% sucrose in the medium, the signal strength of the 53 kDa Ap Inv band was lowered by 30% and 50%, respectively (Fig. 2B).

A decrease in sucrose concentration in the medium affects hexose and starch accumulation in embryogenic tissues

Accumulation of glucose and fructose in black spruce embryogenic tissues was assessed during the 35-day maturation period on three sucrose concentrations (6%, 3% and 1%; Fig. 3). Both the profile and the level of hexose accumulation were affected by decreasing sucrose concentrations in the medium. This was especially marked in tissues maturing on a medium containing 1% sucrose where no peaks of glucose and fructose were observed after 7 days of maturation, in contrast to tissues

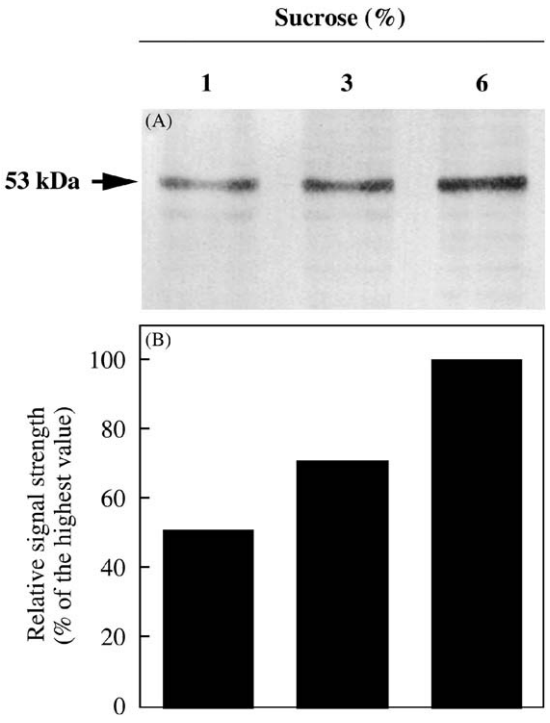


Figure 2. Western-blot analysis showing the amount of Ap Inv in embryogenic tissues of black spruce matured in presence of different sucrose concentrations. (A) Immunoblotting with anti-acid invertase antibodies performed on insoluble protein extract (3 μg) from embryogenic tissue matured for 10 d on 1%, 3%, and 6% sucrose-containing medium. (B) Bar diagrams show densitometric values expressed as a percentage of the highest signal value.

maturing on 3% and 6% sucrose-containing media (Fig. 3A and 3B). When considering starch accumulation in the tissues, no difference was observed between 3% and 6% sucrose-containing media, except at the end of the maturation period when the starch level dropped significantly on 3% sucrose (Fig. 4). When tissues were matured on a medium containing 1% sucrose, starch could not accumulate and remained at about the initial level all through the maturation period.

A decrease in sucrose concentration in the medium affects soluble and insoluble protein accumulation in embryogenic tissues

During maturation, accumulation of soluble and insoluble proteins in embryogenic tissues varied according to the sucrose concentration in the medium (Fig. 5). High sucrose concentration resulted in high soluble and insoluble protein levels whereas low sucrose concentration resulted in low soluble and insoluble protein levels.

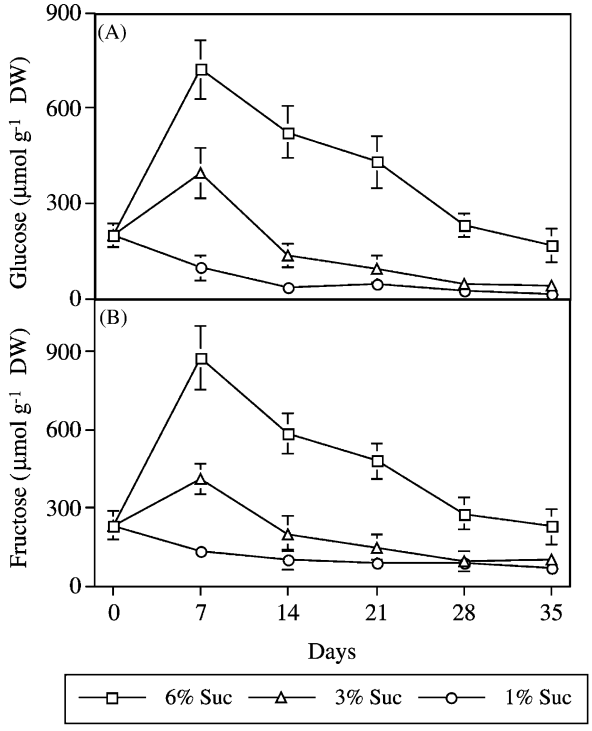


Figure 3. Glucose (A) and fructose (B) levels in embryogenic tissue of black spruce during maturation on media containing different sucrose concentrations. Bars represent standard deviations ($n = 4$).

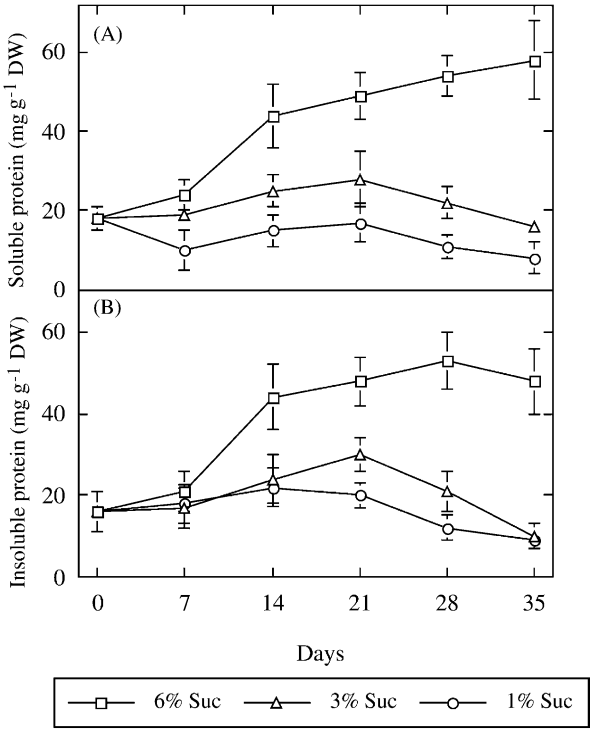


Figure 5. Soluble (A) and insoluble (B) protein levels in embryogenic tissues of black spruce during maturation on media containing different sucrose concentrations. Bars represent standard deviations ($n = 5$).

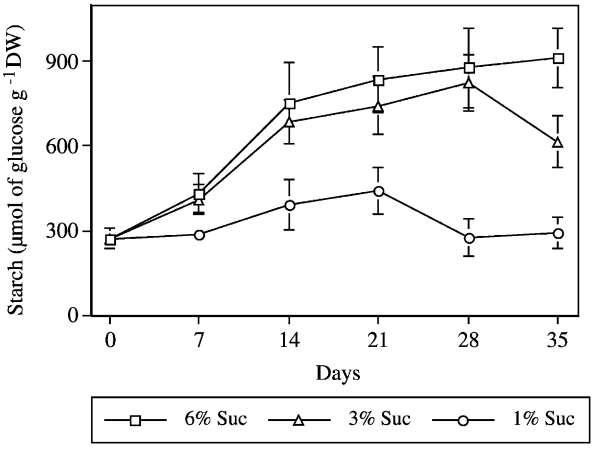


Figure 4. Starch accumulation in embryogenic tissues of black spruce during maturation on media containing different sucrose concentrations. Bars represent standard deviations ($n = 4$).

A decrease in sucrose concentration in the medium affects embryo development

The highest number of normal embryos (42) was observed on embryogenic tissues matured on a 6% sucrose-containing medium (Table 1). When 3% and 1% sucrose were used in the maturation medium, the number of normal embryos decreased significantly to 18 and 4, respectively. Moreover, the ratio of normal:abnormal embryos significantly decreased with decreasing sucrose concentration in the maturation medium.

An active invertase is released into the maturation medium

Immunodetection of Ap Inv with anti-acid invertase antibodies was performed on proteins extracted from the 6% sucrose-containing medium after 10 days of maturation. The immunoblot showed a 53 kDa polypeptide band (Fig. 6, lane 1) having a molecular mass similar to that of the Ap Inv polypeptide found in embryogenic tissues (Fig. 2). This polypeptide was not detected in the control medium incubated without tissue under the same conditions (Fig. 6, lane 2). The invertase released into the maturation medium showed an activity of $750 \pm 260\,\text{pmol}\,\text{g}^{-1}$ of medium min^{-1}. No activity was found in the control medium.

Table 1. Effect of sucrose concentration in the maturation medium on black spruce somatic embryo production

Sucrose concentration (%)	Number of somatic embryos		
	N	*A*	*N:A*
6	42[a]	15[a]	2.8[a]
3	18[b]	13[a]	1.4[b]
1	4[c]	7[b]	0.6[c]

Embryo production was recorded after 5 weeks of maturation in terms of numbers of normal (*N*) and abnormal (*A*) embryos, and ratio (*N:A*). Data in columns displaying the same letters are not significantly different at $P \leqslant 0.05$ according to Bonferroni's test. Each datum is the mean of five replicates.

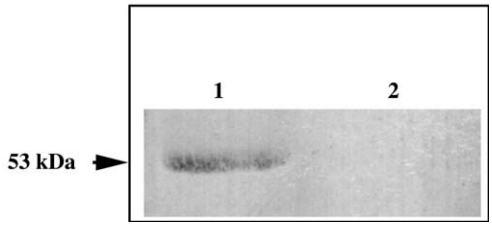


Figure 6. Presence of an active invertase in the maturation medium. Immunoblotting with anti-acid invertase antibodies was performed on protein extract from the 6% sucrose-containing medium after 10 days of maturation. (1) medium incubated in the presence of black spruce embryogenic tissue and (2) control medium without tissue.

Direct uptake of sucrose from the maturation medium by embryogenic tissues

To determine if embryogenic tissues are able to take up intact sucrose directly from the medium, the distribution of radioactivity between sucrose, glucose, and fructose was followed in embryogenic tissues after 5, 10, and 20 min of incubation in the presence of ^{14}C-labelled sucrose. A preliminary experiment had shown that, within this time frame, sucrose hydrolysis is not detectable in the maturation medium incubated in the presence of embryogenic tissues. A direct uptake of sucrose from the medium was confirmed by the presence of radioactive sucrose in embryogenic tissue even after 5 min of incubation (Fig. 7). The results also showed that glucose and fructose were radio-labelled in equal proportions after 5, 10, and 20 min of incubation.

Discussion

In the present study, we show that Ap Inv in the tissues, together with the release of an active

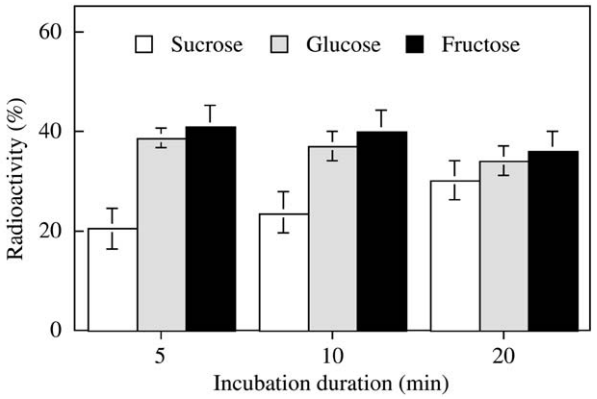


Figure 7. Distribution of radioactivity in sucrose, glucose and fructose in embryogenic tissue after 5, 10, and 20 min of incubation on a maturation medium containing 6% of sucrose and 0.2 μCi ml^{-1} of uniformly labelled ^{14}C-sucrose (specific labelling: 672 mCi mmol^{-1}). Total incorporation (100%) was calculated as the sum of the radioactivity in the sucrose, glucose and fructose fraction. Bars represent the standard deviation ($n = 5$).

invertase into the medium, contribute to the sucrose utilization during somatic embryo development in black spruce.

The role of Ap Inv and SuSy during the maturation of black spruce somatic embryos was studied by modifying the sucrose concentrations in the maturation medium. Ap Inv activity decreased to near minimal values after 7 days of maturation when 6% sucrose in the maturation medium was replaced by either 3% or 1% sucrose (Fig. 1). This decrease in sucrose concentration also affected the amount of Ap Inv in the tissues (Fig. 2). These results show a direct relation between the concentration of sucrose in the maturation medium and the amount and activity of Ap Inv in the tissue, confirming for black spruce that sucrose not only acts as a carbon source for embryogenic tissues during the maturation process but also functions as a factor modulating the genes coding for the enzymes involved in the carbohydrate metabolism (reviewed by Koch, 1996). As reported for other plant systems (Heineke et al., 1992; Herbers and Sonnewald, 1998; Weber et al., 1998; Tang et al., 1999; Hajirezaei et al., 2000; Kim et al., 2000), the decrease of Ap Inv activity and amount that was observed when 6% sucrose in the medium was replaced by 3% or 1% sucrose, was paralleled by a decrease of the hexose pool in the embryogenic tissues (Fig. 3). Such a decrease appears to alter storage deposition and embryo development in black spruce as shown by the decrease of soluble and insoluble proteins and starch (Figs. 4 and 5), as well as a decrease in embryo growth (Table 1). Conversely, the activity

of SuSy, another enzyme able to hydrolyze sucrose, remained low and constant during the maturation period, independent of the sucrose concentration in the medium (Fig. 1). These results suggest that, in addition to soluble invertases (Iraqi and Tremblay, 2001b), Ap Inv may also contribute to the control of tissue hexose pool.

In plants, sugars not only function as substrates for growth but also affect sugar-sensing systems that initiate changes in gene expression. Both abundance and depletion of sugars can enhance or repress the expression of a wild variety of genes, including those involved in photosynthesis, stress responses, nitrogen metabolism, secondary metabolism, storage reserve synthesis, and carbohydrate metabolism (Koch, 1996; Rolland et al., 2002). In particular, induction of gene expression has been related to the level of phosphorylation, by hexokinase, of the free hexoses resulting from sucrose hydrolysis (Heineke et al., 1992; Roitsch et al., 1995; Umemura et al., 1998; Kingston-Smith et al., 1999). The cleavage of sucrose by invertase produces two hexoses that might contribute to the hexokinase signal whereas SuSy produces only one hexose molecule (Kingston-Smith et al., 1999; Sturm and Tang, 1999). Thus, sucrose cleavage by Ap Inv may also stimulate the maturation of black spruce somatic embryos by providing the required hexose pool that may contribute, after phosphorylation by hexokinases, to storage deposition and embryo development. In addition to our previous findings which showed that soluble invertases were involved in the maturation of spruce somatic embryos (Iraqi and Tremblay, 2001b), the present study shows that Ap Inv also contributes to the regulation of somatic embryo development in black spruce.

Since the 6% sucrose-containing medium was optimal for black spruce embryo production (Table 1), this maturation condition was selected for a more intensive study of sucrose utilization by the tissues. Sucrose hydrolysis in the medium is indispensable for high production of somatic embryos in spruce (Tremblay and Tremblay, 1995; Iraqi and Tremblay, 2001a). It has been hypothesized that this sucrose hydrolysis probably results from the presence of an active extracellular enzyme in the medium (Tremblay and Tremblay, 1995). Among the enzymes implicated in sucrose hydrolysis, only invertase is considered as extracellular (George, 1993). To determine if sucrose hydrolysis in the maturation medium is due to the presence of an extracellular invertase, an immunodetection experiment was carried out using anti-acid invertase antibodies. The immunoblot revealed a signal for a 53 kDa polypeptide (Fig. 6A), having a similar molecular mass to that of the Ap Inv polypeptide detected in embryogenic tissues of black spruce (Fig. 2). In addition, enzyme assays showed that this extracellular invertase was active (Fig. 6B). These results confirm that invertase was released into the medium and was responsible for sucrose hydrolysis in the 6% sucrose-containing medium. However, this does not exclude the uptake of sucrose by the tissue, as previously suggested by Iraqi and Tremblay (2001b). The incorporation of ^{14}C-labelled sucrose in embryogenic tissues was then examined in follow-up procedures. After 5, 10, and 20 min of incubation, the presence of labelled sucrose was recorded in the embryogenic tissues (Fig. 7). In addition, sucrose was hydrolyzed in the tissues as evidenced by the appearance of labelled glucose and fructose (Fig. 7). Interestingly, the same proportions of labelled glucose and fructose were found in the tissues (Fig. 7). This symmetrical distribution of labelled glucose and fructose can only arise when sucrose is hydrolyzed in the cell wall by Ap Inv since a symplastic hydrolysis would have resulted in an asymmetrical distribution of labelled glucose and fructose due to hexose isomerase activities (Damon et al., 1988; Yelle et al., 1988; N'tchobo et al., 1999). Therefore, the high Ap Inv activity measured in embryogenic tissues (Fig. 1) together with the symmetrical distribution of the labelled hexoses demonstrate that sucrose diffuses through the cell wall where it is partly hydrolyzed. Measurements of the uptake rates for glucose, fructose, and sucrose are now needed to assess the relative contributions of the sugar uptake via their respective transporters.

In summary, the results of this study show that under high sucrose concentration, the stimulation of accumulation and activity of Ap Inv and the release of an active invertase into the medium contribute to sucrose utilization by black spruce embryogenic tissues. Under these conditions, sucrose diffuses into the cell wall of the embryogenic cells where it is partly hydrolyzed by Ap Inv. Sucrose can also be hydrolyzed in the medium due to the release of an extracellular invertase. Thus, the utilization of sucrose from the medium, the high Ap Inv activity in embryogenic tissues, and the release of an active invertase into the medium might operate together in the carbohydrate utilization necessary for black spruce somatic embryo development.

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