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Seasonal shifts in dormancy status, carbohydrate metabolism, and related gene expression in crown buds of leafy spurge

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ABSTRACT

Crown buds of field-grown leafy spurge (*Euphorbia esula* L.) were examined to determine relationships between carbohydrate metabolism and gene expression throughout para-, endo-, and eco-dormancy during the transition from summer, autumn, and winter, respectively. The data indicates that endo-dormancy plays a role in preventing new shoot growth during the transition from autumn to winter. Cold temperature was involved in breaking endo-dormancy, inducing flowering competence, and inhibiting shoot growth. An inverse relationship developed between starch and soluble sugar (mainly sucrose) content in buds during the shift from para- to endo-dormancy, which continued through eco-dormancy. Unlike starch content, soluble sugars were lowest in crown buds during para-dormancy but increased over two- to three-fold during the transition to endo-dormancy. Several genes (*AGPase*, *HK*, *SPS*, *SuSy*, and *UGPase*) coding for proteins involved in sugar metabolism were differentially regulated in conjunction with well-defined phases of dormancy in crown buds. Marker genes for S-phase progression, cell wall biochemistry, or responsive to auxin were also differentially regulated during transition from para-, endo-, and eco-dormancy. The results were used to develop a model showing potential signalling pathways involved in regulating seasonal dormancy status in leafy spurge crown buds.

Key-words: carbohydrate metabolism; dormancy; gene expression; perennial weeds; sugar.

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; CS, cellulose synthase; DAAR, dormancy-associated auxin-repressed; DOY, day of year; Fru, fructose; F6P, fructose 6-phosphate; Glc, glucose; G6P, glucose 6-phosphate; HK, hexokinase; HisH3, Histone H3; Inv, invertase; Suc, sucrose; SuSy, sucrose synthase; SPS, sucrose phosphate synthase; S6P, sucrose 6-phosphate; UDP-Glc, UDP-glucose; UGPase, UDP-glucose pyrophosphorylase; XET, xylogucan endotransglycosylase.

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INTRODUCTION

Leafy spurge is a serious perennial weed causing economic losses to range, recreational, and right-of-way lands in North American plains and prairies (Leitch, Leistritz & Bangsund 1996; Bangsund, Leistritz & Leitch 1999). The perennial nature of leafy spurge is attributed to vegetative propagation from an abundance of underground adventitious buds (more commonly referred to as crown and root buds). Dormancy-imposed inhibition of new shoot growth from crown and root buds is one of the key characteristics leading to the persistence of perennial weeds such as leafy spurge (Coupland, Selleck & Alex 1955). As a result, dormancy optimizes the distribution of shoot emergence over time, and is therefore a key factor that allows weeds to escape control by chemical, cultural, mechanical, and biological control measures (Anderson, Chao & Horvath 2001; CAB 2004). Thus, integrated pest management systems could be improved by gaining a better understanding of environmental and biological factors affecting signalling pathways involved in regulating dormancy status, reproduction, and survival of vegetative propagules.

Leafy spurge crown and root buds are known to demonstrate the three types of dormancy commonly referred to as para-, endo- and eco-dormancy (Lang *et al*. 1987; Horvath *et al*. 2003). Phytohormones, nutrients, and water status have been reported to affect root bud dormancy in leafy spurge (McIntyre 1972; Nissen & Foley 1987; Harvey & Nowierski 1988) and gibberellic acid (GA) has long been known to reverse leafy spurge root bud dormancy (Shafer & Monson 1958). Although auxin-derived signals from meristematic tissue are probably involved in root bud para-dormancy (Horvath 1998), a second, leaf-derived signal dependent on photosynthesis has also been linked to root bud para-dormancy (Metzger 1994; Horvath 1999). The leaf-derived signal is thought to act at the G1/S transition of the cell cycle and may involve sugar perception (Horvath, Chao & Anderson 2002). A preliminary working model for potential signalling pathways reg-

ulating para-, endo-, and eco-dormancy in leafy spurge crown and root buds has been proposed (Horvath *et al*. 2003). In this model, para-dormancy is primarily controlled by polar auxin transport and leaf-derived sugar, via abscisic acid (ABA) inhibition and GA and cytokinin signalling. ABA is the primary signal regulating eco-dormancy, and endo-dormancy is primarily regulated by phytochrome and/or ethylene and might act via a chromatin remodelling epigenetic-like mechanism, or by ABA-mediated growth arrest. Cross-talk between signalling pathways responding to phytohormones, sugars, and environmental- or stress-related stimuli affect plant growth and development (Roitsch 1999) and are also likely to play a role in dormancy status.

Sugars are known to act as signalling molecules that can regulate gene expression and developmental processes in plants (Koch 1996; Jang *et al*. 1997; Sheen, Zhou & Jang 1999; Ho *et al*. 2001). Both sucrose and glucose have been reported to affect signal transduction pathways in plants. Previous studies indicated that feeding either glucose or sucrose inhibited new shoot growth from para-dormant root buds of leafy spurge after decapitation of aerial tissue (Chao, Anderson & Horvath 2001). The studies further showed that GA could reverse sugar-induced inhibition of growth. These data suggest that sugar levels or signalling in root buds of leafy spurge might play a potential role in dormancy status. Environmental factors such as light, drought, and temperature have been linked to sugar levels in plants (Koch 1996). These environmental factors are also known to affect genes encoding proteins that regulate sugar metabolism (Koch 1996; Ciereszko, Johansson & Kleczkwski 2001; Gupta & Sowokinos 2002; Schrader *et al*. 2004), hormone balance (Schrader *et al*. 2004), cell cycle (Schrader *et al*. 2004), and cell wall biosynthesis (Ciereszko *et al*. 2001; Schrader *et al*. 2004).

Seasonal changes in the levels of carbohydrates in roots of perennial weeds have been reported for such species as leafy spurge (Arny 1932; LeTourneau 1957; Lym & Messersmith 1987; Harvey & Nowierski 1988; Cyr & Bewley 1989), dandelion (Wilson, Kachman & Martin 2001), and Canada thistle (Tworkoski 1992). However, little information exists on seasonal shifts in total non-structural carbohydrates, or carbohydrate metabolism in crown or root buds of leafy spurge in relation to dormancy status. The majority of literature pertaining to dormancy regulation in leafy spurge root buds (adventitious buds located on the root tissue) has come from studies done using plants grown under greenhouse conditions (Horvath 1998, 1999; Horvath & Anderson 2000, 2002; Anderson & Horvath 2000, 2001; Chao *et al*. 2001; Horvath *et al*. 2002) or root sections grown under controlled environments (Nissen & Foley 1987; Harvey & Nowierski 1988). Although greenhouse plants have been a valuable resource for the study of signalling events associated with para- and eco-dormancy, so far, induction of endo-dormancy in greenhouse-grown plants remains elusive; even under reduced light or cold-acclimation. Because new shoot growth in spring usually occurs from over-wintering crown buds (adventitious buds located on the underground extension of the stem) (CAB 2004), models based on greenhouse-grown plants need to be correlated to new growth that occurs from buds under field conditions. Thus, to enhance our understanding of how internal biological signals such as sugars and hormones, and external environmental signals such as light and temperature act through specific, overlapping signal transduction pathways to regulate para-, endo-, and eco-dormancy, and to see how our dormancy models hold up under field-grown conditions, we present a comprehensive overview describing seasonal influence on dormancy status, carbohydrate metabolism, and related gene expression in crown buds of leafy spurge grown under field conditions. We also provide a proposed model summarizing key events related to shifts in seasonal dormancy status of leafy spurge crown buds.

MATERIALS AND METHODS

Plant material

Leafy spurge (Biotype 1984-ND001) plants were propagated and maintained in a greenhouse as previously described (Anderson & Davis 2004). A portion of the leafy spurge greenhouse population was transplanted to a field plot in 1998. Leafy spurge plants were also transferred into 20.3 cm × 40.6 cm smooth-side containers (Nursery Supply, Fairless Hills, PA, USA). The containers with plants were housed inside of PVC pipe with all but the top 5 cm buried into the ground in a second garden plot. The present study was conducted between 2000 and 2004.

To monitor crown bud development (elongation), crown and root material from two plants was randomly dug out of the garden plot on a monthly or bi-weekly basis. Crown buds were photographed and/or stored at –80 °C. To monitor dormancy status under field-grown conditions, leafy spurge plants grown in containers were periodically transferred to the greenhouse at which time the aerial portion of the plants were removed down to the soil line. For each time point, three pots, each containing one plant, were transferred to the greenhouse and the growth of new plant material from crown buds after 30 d was recorded. Since the number of crown buds showing new shoot and stem growth varied per pot, growth of new shoots and stems from each pot was recorded as an average. Plants transferred to the greenhouse were watered daily and maintained at 25 °C under a 16 : 8 h day : night photoperiod.

Greenhouse-grown, whole leafy spurge plants were cold-acclimated at 4 °C for 15 d in a growth chamber. To avoid light-induced (such as a short-day response) gene expression during cold-acclimation studies, lighting was maintained at a 16 : 8 h photoperiod to mimic that of plants grown under greenhouse conditions. Crown buds were collected at 0, 1, 2, 4, 7 and 15 d, immediately frozen in liquid N_2, and stored at –80 °C until extracted. All crown buds, in this study, were collected between 1100 and 1300 h Central Standard time to avoid diurnal variations in gene expression.

Environmental data

Daylight hours for Fargo, ND (rise and set of sun at location: 46°52′ N, 96°47′ W) were obtained from the Astronomical Applications Department, US Naval Observatory, Washington, DC, USA. Average daily bare soil temperatures were obtained from the North Dakota Agricultural Weather Network (http://ndawn.ndsu.nodak.edu/daily). Soil temps, taken at a depth of 10 cm were collected and recorded at a weather station located approximately 1.6 km south of the study site.

Non-structural carbohydrate analysis

Tissue extraction was done following the methods of Gesch *et al*. (2002). Frozen crown buds were ground to a fine powder in liquid N_2. Approximately 250–300 mg of frozen crown bud powder was extracted three times in 4 mL of 80% (v/v) ethanol at 85 °C. Extracts for each sample were combined and all samples brought to 12 mL and then clarified by adding approximately 200 mg of activated charcoal and left to stand overnight at 4 °C. The clarified solution was removed and evaporated at 60 °C overnight, re-suspended in 2 mL of deionized H_2O, filtered (0.45 μm, Whatman, Clifton, NJ, USA), and analysed for Glc, Fru and Suc by high-performance liquid chromatography (HPLC; Agilent Technologies, Foster City, CA, USA) using a Aminex HPX-87 N column (Bio-Rad, Hercules, CA, USA) and a refractive index detector at a flow rate of 0.5 mL min^{-1} in 0.01 M Na_2HPO_4. External standards of Glu, Fru, and Suc were used to standardize the HPLC and were run after every 20 samples as a quality check. The pellet remaining after the hot ethanol extraction was oven dried overnight at 60 °C and used for starch analysis. The dried pellet was incubated with 1 mL of 0.2 N KOH in boiling water for 30 min. After cooling, 0.2 mL of 1 N acetic acid was added and the solution was incubated with 2 mL of acetate buffer (pH 4.6) containing amyloglucosidase (6 units; Roche Diagnostic Corp., Indianapolis, IN, USA) at 55 °C for 1 h. The reaction was terminated in boiling water. After centrifuging at 3500 *g* for 1 min, the resulting supernatant was collected and dried at 60 °C, re-suspended in 2 mL deionized H_2O, filtered (0.45 μm), and assayed for glucose. Starch measurements are reported as glucose equivalents. The carbohydrate extraction and measurement procedures were performed three separate times for each bud sample.

Expression analysis

Total RNA was extracted from leafy spurge crown buds using the method of Chang, Puryear & Cairney (1993) as previously described by Anderson & Davis (2004). Expression analysis by semi-quantitative, reverse transcriptase (RT)-polymerase chain reaction (PCR) was done using DNase-treated, total RNA. Reverse transcription of 2 μg of total RNA was performed using a SuperScript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA (50 ng) was added to 25 μL of a PCR reaction mixture containing 12.5 μL of 2× PCR buffer (Invitrogen), 1 μL of forward and reverse primers (20 pmol, each), and 2.5 U Platinum Taq (Invitrogen). Thermal cycling was performed on a RoboCycler Gradient 96 (Stratagene, La Jolla, CA, USA) with an initial denaturation step of 5 min at 95 °C, followed by 21–35 cycles of 50 s at 94 °C, 1 min at annealing temperature (based on primers used), and 1 min at 72 °C. PCR reactions (25 μL) were separated on a 1% agarose gel and visualized by ethidium bromide staining. Absolute values for gene expression were obtained using a Fluor-S Imager and integrated quantization software (Bio-Rad). Primer sets, annealing temperatures, and cycles used for RT-PCR are listed in Table 1. Primer sequences were generated based on sequence information available from leafy spurge EST-databases (Anderson & Horvath 2001; and unpublished). Sequence data for leafy spurge *SuSy* (accession AW990923), *HisH3* (accession AF239930), and *DAAR* (accession BI961996) are available from the National Center for Biological Information (NCBI) website. Sequence data for leafy spurge *SPS* (CV03012B1G07, CV03016B1C12, CV03020B1D06), *UGPase* (CV03063A1A10, CV03054B1A06, CV03060B1G07), *AGPase* (CV03010B2B06, CV03006B1G02, CV03017A2G08, CV03021A2A11, CV03013A2B05), and *HK* (CV03014A2C05, CV03018A1B11, CV03022A1A05) were obtained from an ongoing leafy spurge EST-database project. Sequence from these clones will be available from NCBI at the end of the project; however, sequences for individual clones are available upon request. Sequence data for *XET* (clone 16–3; strong similarity to XTR-7 from *Arabidopsis* gene *At4g14130*) was obtained from a cDNA library developed from 3 d growth-induced root buds of leafy spurge (Anderson & Horvath 2001).

Table 1. Primers, annealing temperatures, and cycles used for amplifying genes from leafy spurge crown bud total RNA by RT-PCR

Gene	Forward 5′–3′	Reverse 5′–3′	Anneal temp. (°C)	Cycles
AGPase	TTATGTGTTTAGGACCGAGGTTCTTTTA	CATCTCTTCCTATCTTGGCATTCTTGT	55	25
DAAR	GAGGCCCTTAACCATAGATACTGA	GAGACAATGATCAAAACGACACT	50	20
HK	ACAAAAATCATCTTCGGGACAAACAATA	TTCTAATCCCAAAAGCTCACTCACTGC	55	28
HisH3	TTCTCAAGATCAAATGGCTCGTA	CTCAATTAAGCCCTTTCCCCTCTA	50	33
SPS	TTCAGATGCACTTTTAAAACTTGTTG	AACCTGCAGCTTTCATCACATTC	50	30
SuSy	CATTTACTTTGCCTACACCGAGAA	CTCCCCGTTCCTCACCCTGTTCAT	55	25
UGPase	AAAGGACAGAGTGGCAAGGATGGATG	GGTATAAAGATCAGACTGGACAAGAAGC	50	25
XET	NCAGGGAAAAGGAAACAGAG	ATGAAGGGAGAAGATTAGCACT	50	25

Statistical analysis

For gene expression data, both seasons (i.e. 2002–03 and 2003–04) were combined and regression techniques were used to determine whether the dependent variable was significantly affected by day of the year (DOY). Regression analysis was done using the REG Procedure of SAS (SAS Institute, Cary, NC, USA). Data for gene expression were fit to either second- or third-order polynomials. Since the phenological stages of leafy spurge growth and development are relatively consistent by date from year to year, statistical comparison of shoot growth was done by first grouping data for multiple years by the periods of 1 July–23 September, 1 October–20 November, and 1–31 December and performing analysis of variance (ANOVA) using the GLM Procedure of SAS. Crown bud carbohydrate data were analysed for each season using regression techniques (Proc REG, SAS) to determine whether DOY had a significant affect; data were fit to third-order polynomials. To compare carbohydrate levels between dormancy periods within seasons, the data were grouped in the same manner as for shoot growth, with the exception that the third group included measurements from December through to February, and ANOVA was performed (Proc GLM, SAS). Least significant differences (LSD) at the $P < 0.05$ level were used to detect differences between means.

RESULTS

Seasonal influence on growth and development of crown buds

Growth and development of crown buds on field-grown leafy spurge was monitored over a 4-year period (2000–04) in Fargo, ND (Fig. 1). At the beginning of a growing season, new shoot growth usually occurs from over-wintering crown buds. New shoot growth from crown buds subsequently inhibits further shoot development and growth from remaining crown or root buds unless the new shoots are damaged or removed. Shortly after early flowering (usually late April to early June) crown buds that did not initiate new shoot growth degenerated and a new set of crown buds became physically visible in June. Long-day photoperiod is associated with the onset of new crown bud development since maximum photoperiods, in Fargo, ND, occur during June (Fig. 1). During the height of the summer photoperiod, average crown bud length changed very little. However, as photoperiod hours decreased in late summer to autumn (late August to early November), crown buds showed an overall linear growth pattern. Crown buds had an arrested growth pattern from mid-November to January but showed resumed growth from early February through spring. Although photoperiod may play a factor in the signalling events associated with growth and development of field-grown crown buds, it is obvious that winter temperatures experienced in Fargo, ND would affect the growth potential of vegetative propagules.

To overcome the effect of temperature, a portable container system was developed for growing leafy spurge under field conditions that allowed transfer of plants to a controlled greenhouse environment without major disturbance of the root system. Using this portable system, we were able to monitor changes in dormancy status resulting from environmental field-acclimation of plants. Field-grown plants (2001 and 2003 growing season) were periodically transferred to the greenhouse and the above-ground tissue was removed (decapitated) to prevent inhibitory signals from the apical buds and leaves (Horvath 1998, 1999; Horvath *et al*. 2002). Growth of new shoots, from the crown buds, was measured after a 30 d period (Fig. 2a). Plants transferred from the field to the greenhouse between days 182 and 266 (1 July–23 September) all showed similar growth after 30 d, indicating that the crown and root buds

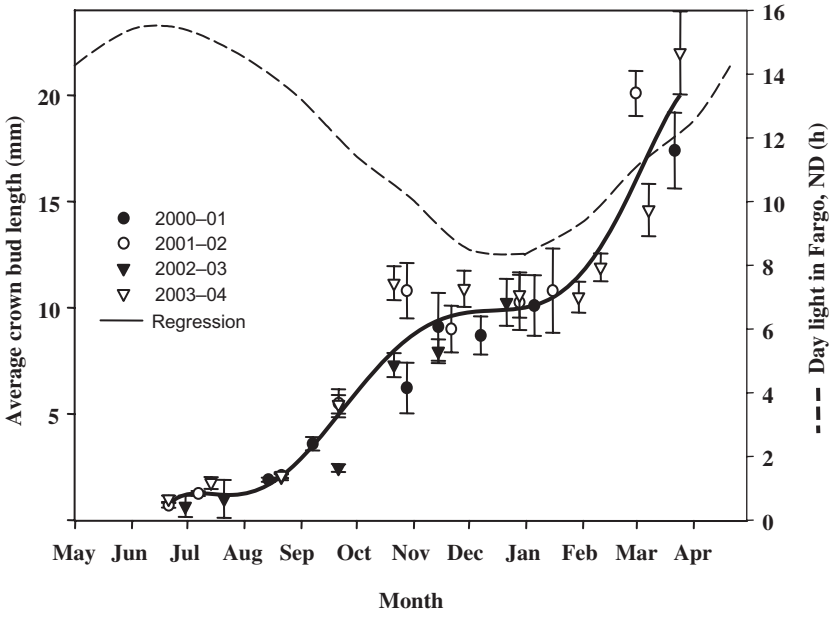

Figure 1. Average seasonal crown bud length from field-grown leafy spurge and sun light hours in Fargo, ND. Individual time points for crown bud lengths represent the average from a minimum of the 10 largest buds from replicate plants. Error bars represent the SE of the average. Buds were collected over four cycles of bud development and are represented by 2000–01 (solid circles), 2001–02 (open circles), 2002–03 (solid triangles), and 2003–04 (open triangles). All data points for average crown bud length were used to develop a trend line (sixth-order regression) which is included only as a visual aid.

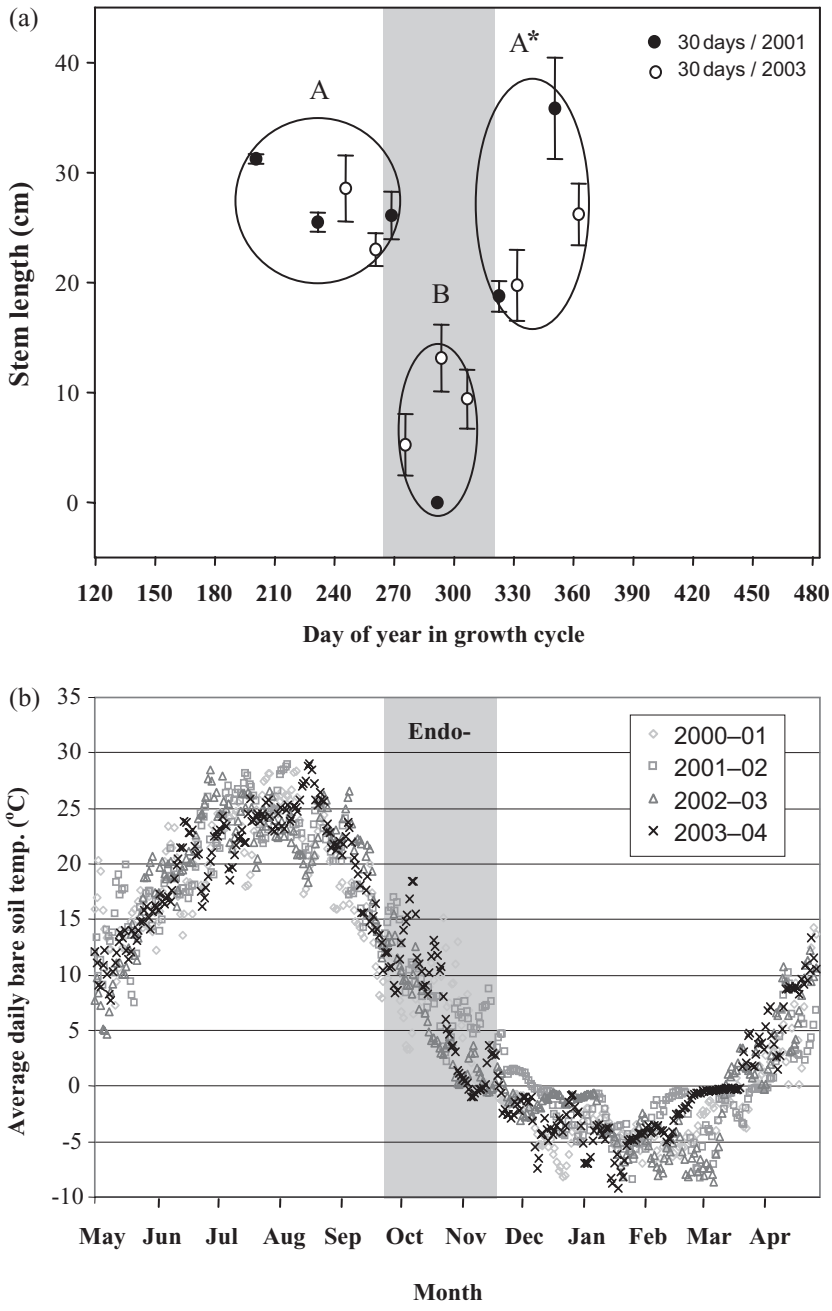

Figure 2. Seasonal shoot growth from crown buds of field-grown leafy spurge 30 d following decapitation of aerial tissue (a) and average daily bare soil temp (b). Values within circles followed by the same letter were not significantly different at the $P < 0.05$ level and were used to estimate the range of para-, endo-, and eco-dormancy. Endo-dormancy is highlighted by grey bar and para- and eco- are represented to the left and right of grey bar, respectively. Data points in circles denoted by an * represent plants which flowered. Average daily bare soil temperatures for Fargo, ND were obtained from the North Dakota Agricultural Weather Network (NDAWN).

had been inhibited from developing into new shoots by para-dormancy. However, plants transferred to the greenhouse between days 274 and 324 (1 October–20 November) showed very little growth from crown or root buds indicating that they had entered a state of endo-dormancy. Plants transferred from the field to the greenhouse between days 335 and 365 (1–31 December) showed growth of new shoots from crown buds. These results indicate that endo-dormancy had been broken, and therefore, growth-inhibition in the field at this time (after day 324 or late November to early December) was due to eco-dormancy.

New shoot growth from plants transferred between days 274–324 (1 October–20 November) were significantly reduced compared with that either prior to or after this time period. These data were used to estimate the range of the endo-dormant period which is highlighted by shading within Fig. 2a. Interestingly, most field-grown replicate plants transferred to the greenhouse in late November or early December (after day 324) were flower competent (Fig. 2a). Average soil temperature at 10 cm during the period of November (in Fargo, ND) range from approximately 4 °C to –3 °C (Fig. 2b). Correlations from this data suggest that either soil temperatures near 0 °C or the accumulated duration of cold temperatures prior to 0 °C are sufficient to break endo-dormancy and induce flowering in field-grown leafy spurge.

Table 2. Seasonal sucrose, hexose, and starch levels in field-grown crown buds of leafy spurge

	2002–2003			2003–2004			
Date	Sucrose (mg g^{-1} FW)	Hexose (mg g^{-1} FW)	Starch (mg g^{-1} FW)	Date	Sucrose (mg g^{-1} FW)	Hexose (mg g^{-1} FW)	Starch (mg g^{-1} FW)
July 16	7.8 ± 2.6	5.7 ± 0.9	22.6 ± 1.4	July 7	16.1 ± 0.4	7.4 ± 0.3	26.6 ± 1.1
Aug 20	12.1 ± 1.5	9.1 ± 1.8	35.0 ± 2.4	Aug 12	20.5 ± 0.6	5.0 ± 0.5	58.7 ± 3.0
Sept 3	14.2 ± 2.0	8.1 ± 1.9	31.3 ± 2.0	Sept 9	23.9 ± 0.5	6.2 ± 0.6	41.8 ± 1.4
Sept 23	18.8 ± 2.3	7.6 ± 2.2	36.7 ± 1.6	Sept 23	17.0 ± 0.4	10.9 ± 0.2	34.0 ± 1.2
Oct 7	*30.1 ± 1.5*	*10.9 ± 1.2*	*39.8 ± 2.3*	*Oct 8*	*27.7 ± 0.5*	*7.9 ± 0.5*	*36.9 ± 2.3*
Oct 15	*45.1 ± 2.4*	*12.1 ± 4.0*	*39.0 ± 4.9*	*Oct 21*	*24.3 ± 0.9*	*5.9 ± 0.7*	*25.1 ± 1.4*
Oct 28	*63.8 ± 2.2*	*9.0 ± 0.6*	*25.0 ± 1.5*	*Nov 3*	*64.5 ± 2.0*	*10.9 ± 0.4*	*0.8 ± 0.2*
Nov 12	*74.9 ± 2.1*	*12.5 ± 0.2*	*5.9 ± 0.3*	*Nov 20*	*49.3 ± 1.5*	*8.2 ± 0.6*	*15.9 ± 0.4*
Dec 16	87.1 ± 1.9	16.7 ± 0.3	1.0 ± 0.1	Dec 1	60.3 ± 1.8	11.0 ± 0.8	2.4 ± 0.3
Jan 27	72.8 ± 1.7	13.4 ± 0.2	0.8 ± 0.3	Jan 15	53.2 ± 6.3	8.3 ± 1.0	0.0 ± 0.0 ‡
Feb 20	74.1 ± 1.0	18.3 ± 0.8	0.3 ± 0.1	Feb 10	56.5 ± 2.2	9.4 ± 0.3	0.2 ± 0.1
Mar 14	73.6 ± 1.4	16.4 ± 0.7	0.7 ± 0.1	Mar 9	39.0 ± 4.2	9.2 ± 1.3	16.4 ± 1.4
Apr 10	30.5 ± 0.6	11.6 ± 0.1	22.4 ± 1.9				
† r^2	0.90 *	0.58 *	0.84 *	r^2	0.74 *	0.29 NS	0.76 *

Growth cycles are indicated as July 2002 to April 2003 and July 2003 to March 2004. Values are the means ± SE, $n = 3$. Italic data denotes endo-dormancy period. † Denotes r^2 values for the regression models and * denotes probability that date had a significant effect on the dependant variable at the $P < 0.05$ level. Data were fitted to either a second- or third-order polynomial and analysed with the REG Procedure of SAS (SAS Institute, Cary, NC, USA). ‡ Starch on 15 January 2004 was below detectable limits with methods used.

Seasonal effect on crown bud carbohydrate content

To test the hypothesis that sugar may be involved in the inhibition of root buds, we monitored the level of starch and free soluble sugars (sucrose and hexose) in crown buds of field-grown plants over two seasonal cycles. Starch levels were greater during the period of para-dormancy (July to 23 September) and early endo-dormancy (Table 2). Overall, a significant decrease in starch was observed during eco-dormancy in 2002–03 compared to 2003–04 when a significant decrease in starch was observed during all three phases of dormancy (Table 3). At the transition from para- to endo-dormancy, an inverse shift in starch and free soluble sugars began to occur. As starch levels decreased, total soluble sugars increased until reaching maximum levels in November to December and remained elevated through the eco-dormant period (December to March). Although hexose content increased during the endo- and eco-dormant phase in 2002–03 ($r^2 = 0.58$; Table 2), which was significant during all three phases of dormancy (Table 3), it was not repeated in 2003–04 (Tables 2 & 3). Instead, the overall trend for increased free soluble sugars was mainly attributed to sucrose ($r^2 = 0.90$ and 0.74 for 2002–03 and 2003–04, respectively). In both 2002–03 and 2003–04, a significant increase was observed for overall sucrose levels during all three phases of dormancy (Table 3). As crown buds began to show increased growth after over-wintering, the eco-dormant period, a corresponding shift back to increased levels of starch and decreased levels of soluble sugars (mainly sucrose) occurred (Table 2).

Seasonal transcript profiles linked to carbohydrate metabolism

Since seasonal development of crown buds involves shifts in starch to sucrose within crown buds, one would expect that genes encoding proteins for metabolism of soluble sugars would be differentially regulated. Figure 3 shows the

Table 3. Sucrose, hexose and starch contents of leafy spurge crown buds as a function of bud dormancy status for two growing seasons

	2002–2003			2003–2004		
Dormancy status	Sucrose (mg g^{-1} FW)	Hexose (mg g^{-1} FW)	Starch (mg g^{-1} FW)	Sucrose (mg g^{-1} FW)	Hexose (mg g^{-1} FW)	Starch (mg g^{-1} FW)
Para [a]	13.2 c	7.9 c	31.8 a	19.7 c	7.4 b	41.6 a
Endo	53.3 b	11.3 b	26.5 a	41.4 b	8.2 ab	19.7 b
Eco	78.0 a	16.1 a	0.7 b	56.7 a	9.6 a	1.9 c

[a] Designations of para-, endo-, and eco-dormancy are the same as those used in Fig. 2a. Carbohydrate data were grouped into para- (July to September), endo- (October to November), and eco-dormancy (December to February) for comparison. Values are LS means; values within columns followed by the same letter are not significantly different at the $P < 0.05$ level.

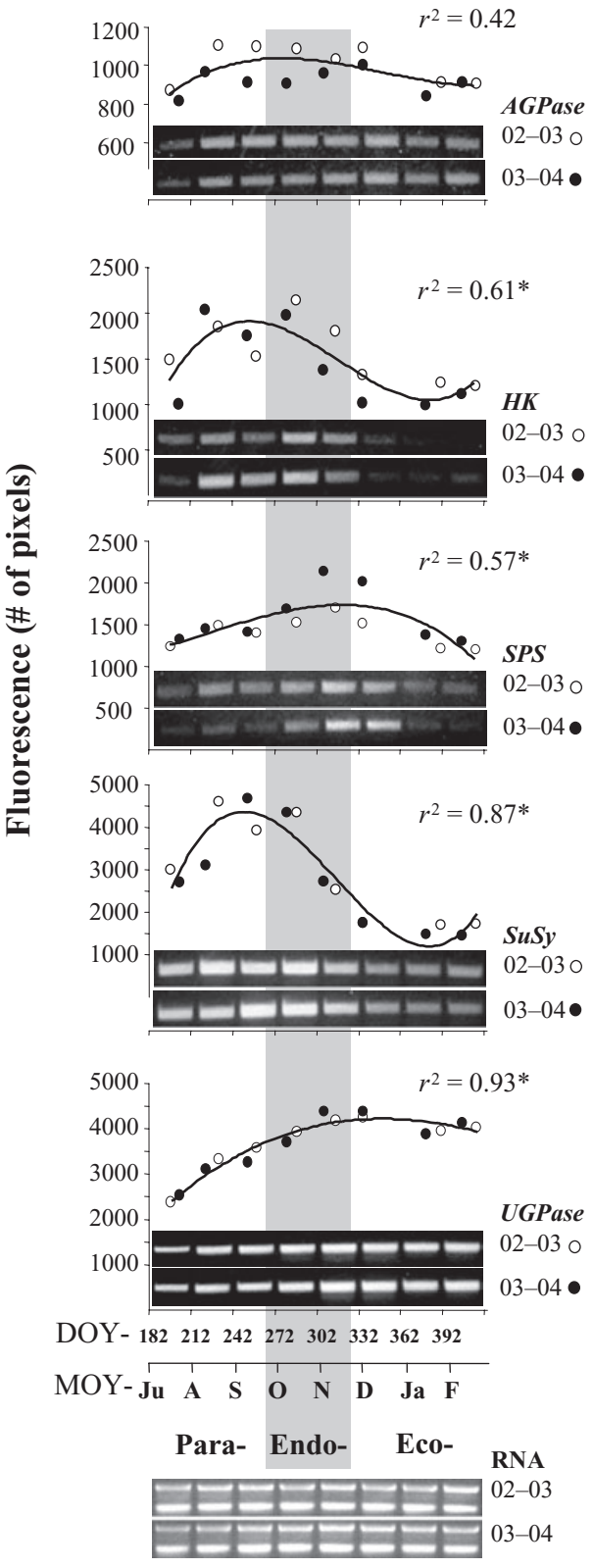

Figure 3. Seasonal transcript expression of carbohydrate metabolism related genes in crown buds of field-grown leafy spurge plants. *AGPase* (ADP-glucose pyrophosphorylase), *HK* (hexokinase), *SPS* (sucrose phosphate synthase), *SuSy* (sucrose synthase), *UGPase* (UDP-glucose pyrophosphorylase). *Denotes that DOY had a significant effect at the $P < 0.05$ level.

transcript expression profiles for *AGPase* (gene encoding protein involved in a key step towards starch production), *HK* (gene encoding protein important for catalysing the phosphorylation of hexoses such as Glc), *SPS* (gene encoding protein that catalyses the conversion of UDG-Glc and F6P to S6P), *SuSy* (gene encoding protein important for catalysing the conversion of Suc to UDP-Glc and Fru), and *UGPase* (gene encoding protein catalysing both the forward and reverse conversion of UDP-Glc to G1P). The forward and reverse pathways of sucrose to starch production are summarized in Fig. 4 and are provided as a source of reference.

Conversion of Suc to UDP-Glc by SuSy, or Glc to G6P by HK, would be consistent with pathways favouring starch production (Fig. 4). However, UDP-Glc is also an important intermediate in the pathway to cellulose production (Kawagoe & Delmer 1997) that would be required for production of secondary cell walls during the growth and development of crown buds. During the para-dormant phase of crown bud development, data for transcript levels of both *SuSy* and *HK* showed up-regulation (Fig. 3). Transcript levels for *SuSy* and *HK* remained high during the transition of buds into endo-dormancy but gradually decreased prior to transitioning into eco-dormancy, at which time transcript levels remained low.

Based on the level of Suc in field-grown endo- and eco-dormant crown buds (Table 2), UGPase may play an important role in converting G1P to UDP-Glc. Since crown buds continue to show growth and development through October and November (Fig. 1), a post-senescence period when photosynthate is no longer transported to the crown buds, the conversion of G1P (presumably from starch utilization) to UDP-Glc would be required. *UGPase* expression was lowest in crown buds during the early para-dormancy phase but showed a gradual increase in transcript levels that remained high during the transition to both endo-and eco-dormancy (Fig. 3). *UGPase* is known to be cold-induced (Ciereszko *et al*. 2001). Data presented in Fig. 5 demonstrated that leafy spurge *UGPase* is also cold-induced in crown buds. The increased expression during endo- and eco-dormant periods would be consistent with cold-induction, but the increase during August and September would not.

Expression of *SPS* in crown buds, although less dramatic than observed for *UGPase*, also peaked during the endo-dormant and early eco-dormant phase (Fig. 3). This pattern of expression would be in line with the elevated levels of soluble sugars observed in crown buds during the endo- and eco-dormant phases. Transcript levels for *AGPase* show a somewhat constitutive pattern of expression during all phases of dormancy (Fig. 3). This data may indicate that environment and sugar stimuli had little effect on *AGPase* expression.

Seasonal transcript profiles for dormancy-related genes

HisH3, a marker for S-phase progression, has been used extensively to monitor cell cycle progression in leafy spurge

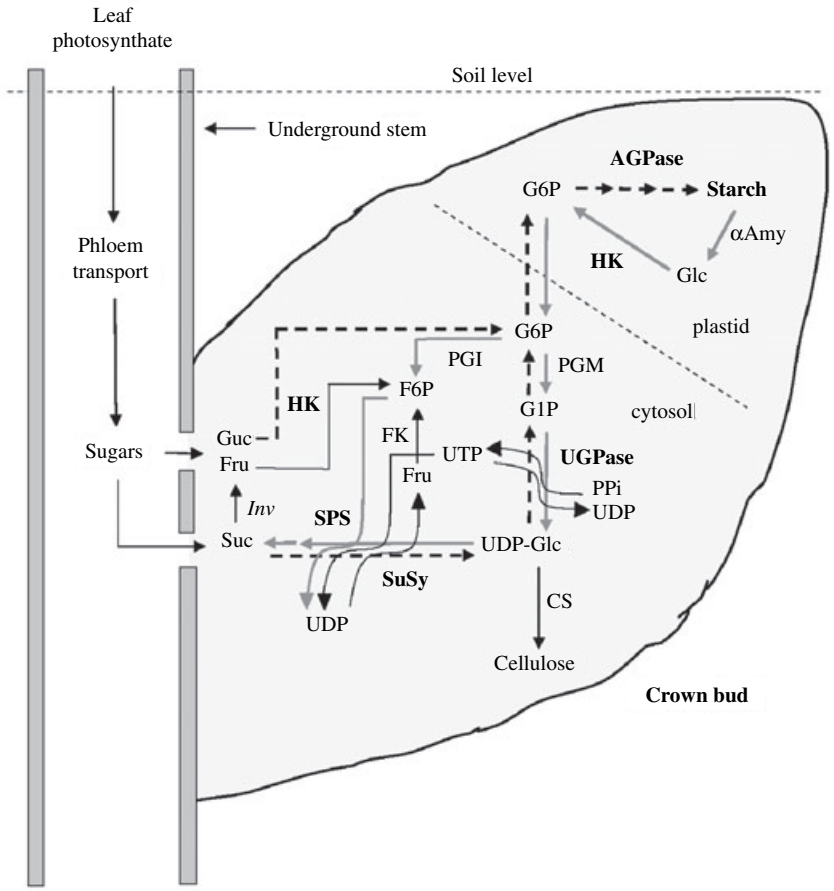

Figure 4. Summary diagram of potential carbohydrate pathway in crown buds of leafy spurge. Dashed lines with arrows represent preferred pathway for starch biosynthesis. Solid grey lines with arrows represent preferred pathway for sucrose metabolism. Diagram does not contain all intermediates, cofactors, or enzymes and only provides an overview to follow key steps in the process.

root buds (Anderson & Horvath 2001; Horvath & Anderson 2002; Horvath *et al*. 2002) and is affected by sugars, environmental stimuli, phytohormones, and cross-talk between signal transduction pathways (Anderson *et al*. 2001; Horvath *et al*. 2003). In this study, *HisH3* transcript levels in crown buds during para-dormancy and most of endo-dormancy were up-regulated, but were down-regulated during the later stages of endo-dormancy and through eco-dormancy (Fig. 6). The results for *HisH3* expression indicate that inhibition of growth during endo-dormancy is not a consequence of reduced transcript availability. However, the down-regulation of *HisH3* during November and December is probably temperature induced. Cold-acclimation of greenhouse-grown plants did result in a marked down-regulation of *HisH3* transcript between 4 and 7 d (Fig. 5).

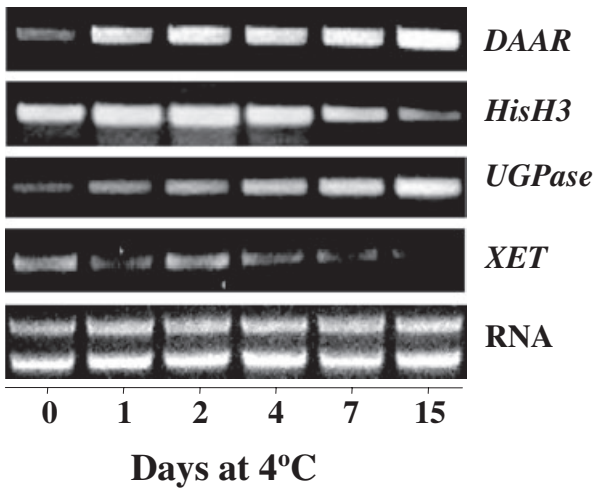

Figure 5. Transcript expression obtained from cold-acclimated crown buds of 4-month-old greenhouse-grown leafy spurge. *DAAR* (dormancy-associated auxin-repressed), *HisH3* (histone H3), *UGPase* (UDP-glucose pyrophosphorylase), *XET* (xyloglucan endotransglycosylase).

In this study, a dormancy-associated, auxin-repressed (*DAAR*) gene was used as an indicator of auxin levels in crown buds of field-grown leafy spurge. As suspected, transcript levels of *DAAR* were down-regulated during early para-dormancy (Fig. 6). As the above ground tissues senesced during the autumn, the transcript levels of *DAAR* increased and were greatest during the endo-dormant phase. These results indicate that auxin levels in crown buds are probably reduced as crown buds transition from para- to endo-dormancy. Low temperature may have an effect on auxin content in crown buds. Transcript levels of *DAAR* in cold-acclimated crown buds of greenhouse-grown leafy spurge increased after 1 d and remained elevated through 15 d (Fig. 5).

Expression of a potential marker gene involved in cell

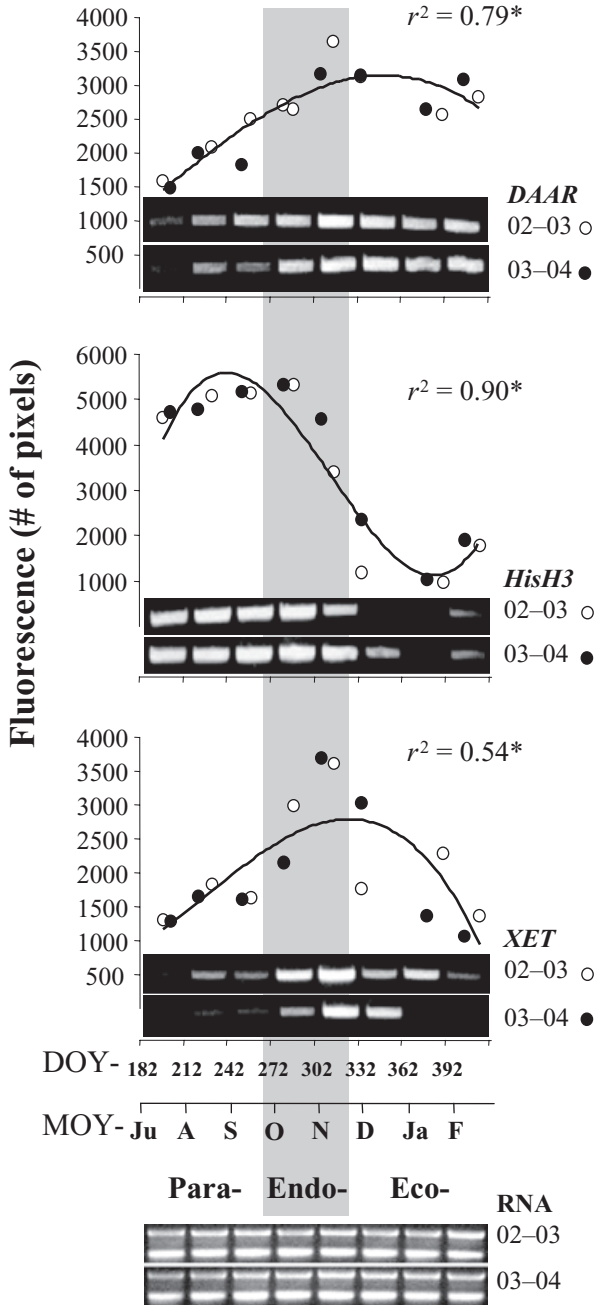

Figure 6. Seasonal transcript expression of dormancy-related genes in crown buds of field-grown leafy spurge plants. *DAAR* (dormancy-associated auxin-repressed), *HisH3* (histone H3), *XET* (xyloglucan endotransglycosylase). *Denotes that DOY had a significant effect at the $P < 0.05$ level.

wall biochemistry has been reported to be up-regulated during endo-dormancy (Anderson *et al*. 2004; Schrader *et al*. 2004). We have identified a *xyloglucan endotransglycosylase* (*XET*) that is specifically up-regulated during the endo-dormant phase in crown buds of field-grown leafy spurge (Fig. 6). Cold-acclimation studies indicate that the up-regulated expression of *XET* is probably not induced by low temperature (Fig. 5).

DISCUSSION

The transition of crown and root buds of leafy spurge from para-dormancy into endo-dormancy appears to be closely linked to senescence of the above-ground foliar tissues. Often, temperature, nutrient and water availability during this transition period are conducive to growth. Thus, the endo-dormant period appears to play an important role in preventing both crown and root buds from differentiating into new shoots prior to transitioning into eco-dormancy. Yet, at the same time, it is the cold temperatures required for eco-dormancy that seem to break endo-dormancy. These phenomena suggest a complex network of signal transduction pathways that are linked by environmentally induced responses. The results of this study indicate the potential for tissue-specific sugar transduction pathways similar to those suggested in potato (Geigenberger, Stitt & Fernie 2004) and support the hypothesis that cross-talk between sugar-dependent signal transduction pathways and other signalling pathways is probably involved in regulating dormancy (Horvath *et al*. 2003).

Previous studies using greenhouse-grown leafy spurge indicated that two separate signals requiring polar auxin transport and photosynthate production are involved in para-dormancy regulation (Horvath 1998, 1999; Horvath *et al*. 2002). Reduced auxin transport has also been implicated to play a role in the endo-dormant phase of cambial cells of trees (Schrader *et al*. 2004). Increased expression of DAAR (Fig. 6), a marker for auxin content, is consistent with reduced auxin transport/levels in crown buds and probably plays a signalling role in the transition from para- to endo-dormancy. The requirement for photosynthate production in regulating para-dormancy in root buds was interpreted to involve sugar perception (Horvath *et al*. 2002). Chao *et al*. (2001) have shown that as little as 30 mM sugar (glucose or sucrose) will inhibit the growth of new leafy spurge shoots from root buds. Consistent with the findings of Horvath (1999), they also demonstrated that this inhibition could be overcome by addition of GA. Cross-talk between GA, ABA, and sugar suggests increased sucrose would be antagonistic to GA perception and should increase ABA perception (Horvath *et al*. 2003; Sheen *et al*. 1999). Such a shift in hormone ratios should be inhibitory to cell cycle progress at G1 and lead to arrest of cell division. However, our results suggest that a marker (*HisH3*) for S-phase progression is more sensitive to reduced temperature than shifts in whole bud sugar levels.

Since greenhouse-grown leafy spurge crown and root buds can not be induced to transition from para- to endo-dormancy, understanding the cross-talk involved in regulating this transition has only been speculative (Horvath *et al*. 2002, 2003). Data obtained from this study was used to propose a new model (Fig. 7) involving cross-talk among light- (photosynthate transport), phytohormone- (auxin/GA/ABA), sugar-, and stress- (cold) signalling pathways and their correlative affect on cell cycle, sugar-dependent and independent signalling, altered gene expression, and possibly chromatin remodelling during well-defined phases

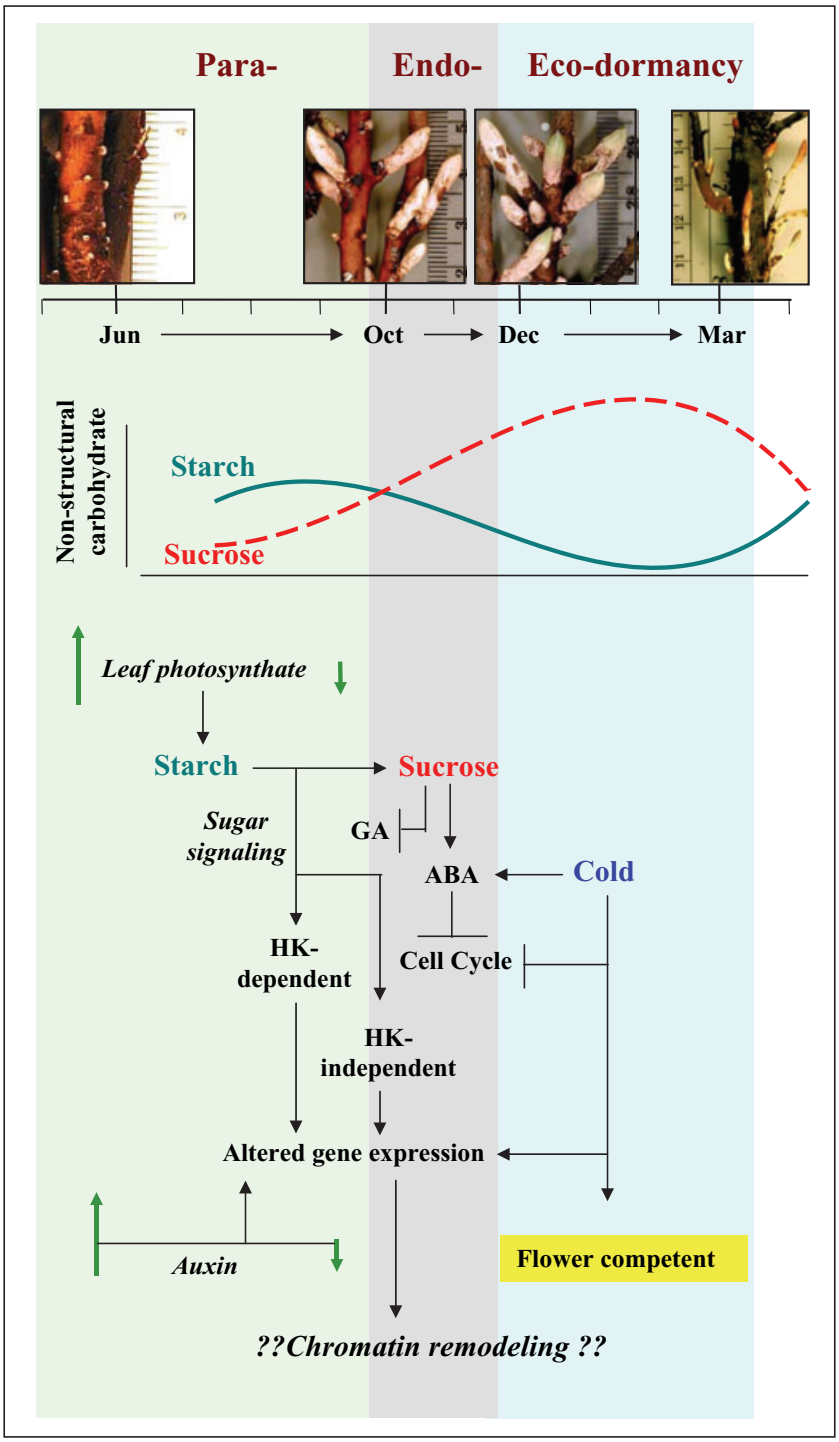

Figure 7. Proposed model for dormancy-associated factors in crown buds of leafy spurge. Picture at top represent crown buds of leafy spurge. Starch and sucrose concentrations are only provided as a reference to trends in seasonal concentrations in field-grown leafy spurge crown buds. Green arrows represent increased (pointing up) or decreased (pointing down) leafy photosynthate transport or auxin perception. Crossed lines represent blocks. ABA, abscisic acid; GA, gibberellic acid; HK, hexokinase.

of dormancy in leafy spurge crown buds. Although whole organ carbohydrate levels and gene expression do not address spatial patterns of development (i.e. meristematic regions) or a specific leaf-derived sugar signal, the seasonal trends observed for sugar levels and gene expression within buds do suggest that some proteins involved in sugar metabolism pathways, such as HK, could be acting as sugar sensors involved in sugar-dependent regulation of gene expression (Jang *et al*. 1997; Smeekens & Rook 1997; Sheen *et al*. 1999). Others, such as SuSy, could also be activated by protein kinases or phosphatases linked to sugar or other signalling pathways (Roitsch 1999; Sheen *et al*. 1999; Ciereszko *et al*. 2001; Geigenberger *et al*. 2004). Since *HK* expression was up-regulated during para-dormancy and the transition into endo-dormancy in crown buds, it is tempting to hypothesize that a hexose-dependent HK signalling pathway might play a role in regulating expression of transcripts involved in dormancy status. Since sugar levels are

low and starch levels are greatest in para-dormant buds, and similar to the seasonal trends previously reported in roots of leafy spurge (Arny 1932; Cyr & Bewley 1989; Harvey & Nowierski 1988; LeTourneau 1957; Lym & Messersmith 1987), one might also speculate that conversion of photosynthate-derived sugar to starch could enhance hexose-dependent HK signalling (see Fig. 4). Such a signalling pathway could account for the leaf-derived signal involved in para-dormancy (Horvath 1999; Horvath *et al*. 2002, 2003). However, HK independent signalling pathways have also been reported (see Sheen *et al*. 1999) and could also be playing a role.

In addition to sugars playing a potential signalling role in dormancy status, pathways involved in sugar metabolism also provide intermediates for other important processes required for growth and development. The endo-dormant period in crown buds was marked by specific cell expansion (Fig. 1) and up-regulation of *xyloglucan endotransglycosylase* (*XET*) (Fig. 6). Increased expression of XET during endo-dormancy in cambial cells of poplar was also reported by Schrader *et al*. (2004). During cell expansion, XETs are involved in the loosening and rapid reinforcement of cell wall structures (Eckardt 2004). These data may indicate that shifts in cell wall biochemistry also play an important role during endo-dormancy and may have some influence on the cell-to-cell communication phenomenon proposed by Rinne, Kaikuranta & van der Schoot (2001).

Finally, the proposed model (Fig. 7) also indicates that both flowering competency and growth competency in the crown buds of field-grown plants are induced by near freezing temperatures (Fig. 2b). Induction of flower competency by cold is known to involve chromatin remodelling of specific genes involved in flower development such as *FLOWERING LOCUS C* (Bastow *et al*. 2004; Sung & Amasino 2004; He & Amasino 2005). Thus, it will be interesting to see what role chromatin remodelling may have on dormancy status in leafy spurge crown and root buds or if there is any conservation of mechanisms between flower and growth competency.

CONCLUSIONS

Transition from para- to endo-dormancy in crown buds appears to coincide with post-senescence and was correlated with shifts in starch, sucrose, and auxin content. Expression profiles of genes encoding proteins involved in carbohydrate metabolism showed differential regulation patterns consistent with pathways favouring starch production during para-dormancy and conversion back to soluble sugars during endo- and eco-dormancy. In agreement with previous reports (Chao *et al*. 2001, Horvath *et al*. 2002), the elevated levels of sucrose in crown buds during endo- and eco-dormant periods appear to be consistent with inhibition of new shoot growth. Cold temperatures break endo-dormancy and appear to induce flower competency in crown buds. These data may suggest the involvement of chromatin remodelling mechanisms playing important roles during endo- to eco-dormancy. Further studies will be required to determine what effects, if any, sugar and hormone perception and cross-talk might play in signalling pathways affecting chromatin remodelling. Although transcript levels can be an indicator of physiological status, further biochemical assay studies will be important to back up this data.

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