Chapter 12
Meta-Analysis Identifies Potential Molecular Markers for Endodormancy in Crown Buds of Leafy Spurge; a Herbaceous Perennial

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Introduction

Dormancy in Underground Adventitious Buds of Leafy Spurge

Leafy spurge (*Euphorbia esula* L.) is a herbaceous perennial that is not considered invasive in its native range of Europe and Asia. However, after introduction through shipping, commerce, and migration of immigrants in the eighteenth and nineteenth centuries, it has become an invasive weed in North American ecosystems (Chao and Anderson 2004). Reproduction and spread occurs by both seeds and underground adventitious buds (UABs, commonly referred to as crown and root buds; see Fig. 12.1). However, the perennial nature of leafy spurge is attributed to vegetative production from an abundance of UABs that undergo well-defined phases of seasonally induced para-, endo- and ecodormancy (Anderson et al. 2005), which help optimize distribution of new shoots from the soil bud bank over time (Anderson et al. 2010). Because dormancy in UABs involves arrested development of the shoot apical meristems (Horvath et al. 2003; Horvath and Anderson 2009), similar to that reported in buds of perennial tree species (Cooke et al. 2012; Rinne et al. 2010; Rohde and Bhalerao 2007), it is a key factor allowing herbaceous perennial weeds to escape many control measures and periods of severe abiotic stress (Anderson et al. 2001, 2010; Doğramacı et al. 2014).

As defined by Lang et al. (1987), paradormancy and ecodormancy involve growth cessation controlled by physiological and environmental factors, respectively,

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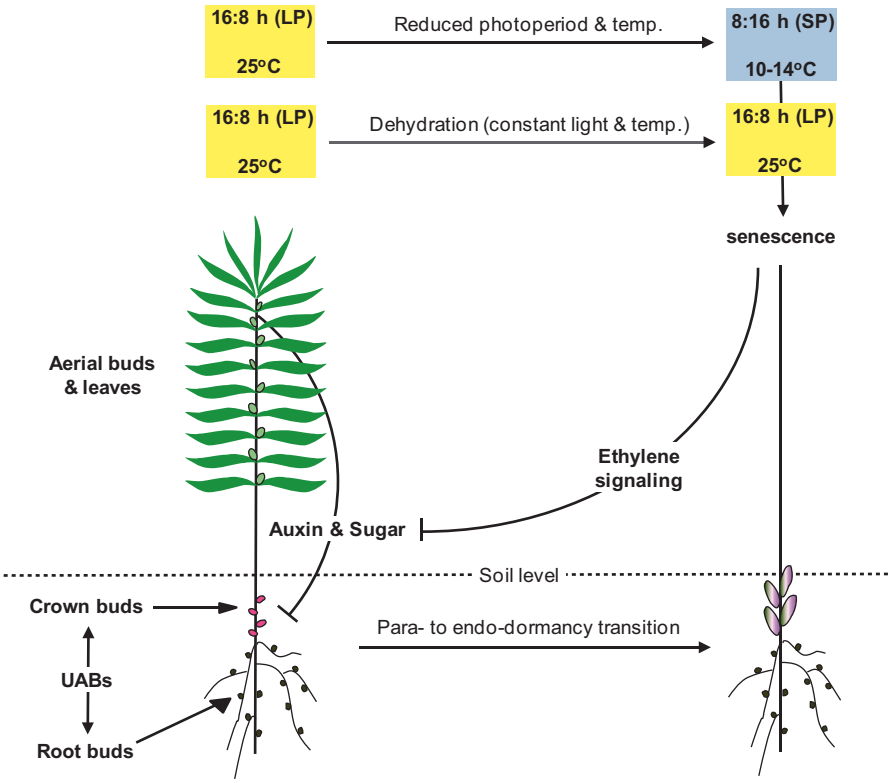

Fig. 12.1 Diagram of leafy spurge anatomy and environmental factors associated with transition from para- to endodormancy. Underground adventitious buds (UABs) are located on the underground stem (crown buds) and on the lateral roots (root buds). Basipetal movement of leaf-derived sugar and auxin under long photoperiod (LP) and growth-conducive temperature maintains paradormancy in UABs. A shift from long to short photoperiod (SP) and summer to autumn temperatures (°C), or extended periods of severe dehydration stress, induces senescence of aerial tissues, reduces sugars and auxin signaling from the aerial tissues, and coincides with a transition of UABs from a state of para- to endodormancy

external to the affected structure, whereas endodormancy involves growth cessation controlled by physiological factors internal to the affected structure. The environmental parameters required for inducing well-defined phases of dormancy in UABs of leafy spurge have previously been determined under field (Anderson et al. 2005) or controlled environmental conditions (Foley et al. 2009). As illustrated in Fig. 12.1, the transition from para- to endodormancy coincides with senescence of the aerial tissues, which is induced by decreasing photoperiod and temperature during the seasonal transition to autumn, or by extended periods of dehydration alone. The transition from endo- to ecodormancy requires an extended period of cold temperatures and usually occurs in late November to early December in the Northern Hemisphere. The discovery that transition from endo- to ecodormancy is also the point at which crown buds become flower competent (Anderson et al. 2005; Foley et al. 2009) provided evidence to support the hypothesis that cross-talk occurs

between mechanisms regulating both dormancy and flowering (Horvath 2009; Horvath et al. 2003). Based on average seasonal bare soil temperatures in Fargo, North Dakota, the transition of leafy spurge UABs from para- to endodormancy generally occurs at ~10–15°C and the transition from endo- to ecodormancy generally occurs at ~0°C under natural field conditions (Anderson et al. 2005).

A report by Mason et al. (2014) provides evidence that the preference for partitioning of leaf-derived sugar to growing shoot tips plays a pivotal role in regulating axillary bud outgrowth in pea (*Pisum sativum* L.). Specifically, their results indicate that sugar, not polar auxin transport from the apical meristem, is the early signaling mechanism regulating axillary bud outgrowth through repression of *BRANCHED1* (*BRC1*) by sucrose, at least in an annual species such as pea. These results are somewhat consistent with a leaf-derived signal also being involved in regulating paradormancy in UABs of leafy spurge (Horvath 1999; Horvath and Anderson 2000) as illustrated in Fig. 12.1. However, starch appears to be the main total non-structural carbohydrate (TNC) observed in leaves, stems, and UABs of leafy spurge during the paradormant period of June–September (Gesch et al. 2007). During autumn-induced senescence, photosynthetic capacities of aerial tissues dissipate and UABs transition from para- to endodormancy (Fig. 12.1) and the amount of TNC decrease in aerial shoots (leaves and stems) and increase in UABs (Gesch et al. 2007). However, in UABs, the increase in TNC is also paralleled by a shift from starch to sucrose (Anderson et al. 2005). Collectively, these results provide support for a leaf-derived sugar signal regulating dormancy in both annual and herbaceous perennial species, but sucrose appears to induce, not repress, bud dormancy in UABs of leafy spurge. This observation is further supported by a study demonstrating that exogenous application of sucrose to leafy spurge roots inhibited initiation of new shoot growth from paradormant UABs, whereas application of gibberellic acid (GA) was able to override this inhibitory effect (Chao et al. 2006).

However, auxin still appears to have a role in the regulation of paradormancy through apical dominance, because UABs of leafy spurge will not initiate new vegetative shoots unless all aerial tissues, including leaves, stems, and all meristems, are removed, whereas removing just leaves from aerial tissue of leafy spurge induced expression of GA-responsive (*GA-STIMULATED ARABIDOPSIS*), glucose-responsive (*BINDING PROTEIN*), and cell cycle (*HISTONE H3* and *CYCLIN D3-2*) genes in UABs (Horvath and Anderson 2002; Horvath et al. 2002, 2005). These studies led to a proposed model (Horvath et al. 2002, 2003) for two organ-specific signals to regulate paradormancy in UABs that include (1) a photosynthetic-dependent, leaf-derived signal (sugar) impacting GA perception to block the G1/S phase of the cell cycle and (2) meristem-derived signaling (auxin) inhibiting the G2/M phase of the cell cycle.

Leafy Spurge as a Model for Studying Well-Defined Phases of Dormancy

Global transcriptome profiling provided a comprehensive approach to investigate components of molecular mechanisms during well-defined phases of environmentally induced dormancy in UABs. Indeed, development of Euphorbiaceae-specific

microarrays (>23,000 elements) from EST databases (Anderson et al. 2007; Lokko et al. 2007) led to the first reports describing molecular processes for well-defined phases of dormancy in invasive weeds under field (Horvath et al. 2006, 2008) or controlled environments (Doğramacı et al. 2010). To eliminate environmental variability under field conditions, standardized growth chamber conditions for follow-up transcriptome studies included exposing 3-month-old greenhouse-propagated plants to a ramp down (RD) in photoperiod (16-h → 8-h light) and temperature (27 → 10˚C) over 12 weeks. This treatment induced a para- to endodormant transition in UABs, whereas an additional 8–12 weeks of vernalizing cold treatment (5–7˚C) induced a transition from endo- to ecodormancy (Foley et al. 2009). However, leafy spurge plants exposed to a RD in temperature alone (RDt; 27 → 10˚C) under a constant photoperiod of 16 h (Doğramacı et al. 2013) or a RD in photoperiod alone (RDp; 16-h → 8-h light) at a constant temperature of 26ºC (Foley et al. 2009) did not induce endodormancy in UABs. Additionally, exposing leafy spurge plants to 14 days of continuous dehydration induced a transition from para- to endodormancy in UABs (Doğramacı et al. 2014), whereas short-term (3 days) dehydration induced growth competence in UABs, which were previously forced into endodormancy by the RDtp treatment (Doğramacı et al. 2011).

In other perennial systems, growth cessation, bud set, and bud dormancy in *Populus spp*. (Welling et al. 1997), birch (*Betula papyrifera*; Downs and Bevington 1981), and grape (*Vitis riparia*; Fennel and Hoover 1991) are influenced by photoperiod, whereas in apple (*Malus spp.*), pear (*Pyrus spp.*; Heide and Prestrude 2005), and grape (*V. vinifera*; Fennel and Hoover 1991), they are influenced by temperature alone, but in peach (*Prunus persica*), apricot (*P. mume*; Yamane 2014), sour cherry (*P. cerasus*), and sweet cherry (*P. avium*; Heide 2008), they are influenced by both photoperiod and temperature. More comprehensive reviews describing the influence of environmental factors on growth cessation, bud set, and induction and release of bud dormancy in perennial systems including forest trees, fruit trees, shrubs, vines, and forbs are available (Anderson et al. 2010; Cooke et al. 2012; Horvath 2009; Horvath et al. 2003; Rios et al. 2014; Rohde and Bhalerao 2007; Tanino et al. 2010). However, because the transition to endodormancy is critical for inhibiting new vegetative shoot growth from UABs during autumn, when conditions can still be conducive for growth, our focus is to identify molecular processes involved in induction and maintenance of endodormancy. Results from such studies could provide new targets and insights for enhancing integrated weed management programs.

Working Models of Endodormancy Induction in UABs of Leafy Spurge

A role for *DEHYDRATION-RESPONSIVE ELEMENT BINDING*/*C-REPEAT BINDING FACTOR* (*DREB*/*CBF*) family members has been proposed as central regulators of molecular networks involved in endodormancy induction (Doğramacı

et al. 2010). *DREBs* belong to the ETHYLENE RESPONSE FACTOR (ERF) family of transcription factors involved in abiotic and biotic stress signaling, which has been extensively reviewed (Khan 2011; Nakashima et al. 2009; Xu et al. 2011). The observation that overexpression of a peach *CBF1* in apple resulted in short photoperiod-induced dormancy and cold acclimation (Wisniewski et al. 2011) provides evidence for *DREB*/*CBFs* playing a role in photoperiod-induced processes leading to bud endodormancy. These results are also consistent with long photoperiod repression of *DREB*/*CBFs* and thus repression of cold acclimation, through interactions with PHYTOCHROME B (PHYB), PHYTOCHROME INTERACTING FACTOR (PIF)-4 (PIF4), and -7 (PIF7) in Arabidopsis under warm environments (Lee and Thomashow 2012). It is still unclear whether short photoperiods play a role in cold acclimation of herbaceous perennials under warm environments. However, a decrease in temperature was determined to be the main environmental factor driving expression of numerous *DREB*/*CBFs* in UABs of leafy spurge, although photoperiod was proposed to have, as yet, unknown synergistic effects to induce endodormancy (Doğramacı et al. 2013).

Interestingly, soil applied 1-aminocyclopropane-1-carboxylate (ACC), the precursor to ethylene, induced a dwarfed phenotype from paradormant crown buds of treated plants after decapitation of aerial tissue (Doğramacı et al. 2013). Numerous leafy spurge transcripts with putative homology to Arabidopsis *DREBs* were differentially expressed in response to the ACC treatment, consistent with the expression observed in endodormant buds (Doğramacı et al. 2013). These results support the hypothesis that a transient spike in ethylene is a prerequisite to induction of endodormancy (Horvath et al. 2003; Ruttink et al. 2007; Suttle 1998), likely through cross-talk with abscisic acid (ABA) signaling in perennials (Anderson et al. 2010). As illustrated in Fig. 12.1, environmental factors (photoperiod and temperature, or dehydration) leading to senescence of aerial tissues are proposed to shift the balance of physiological signals (sugar and auxin) that impact molecular processes in UABs to induce endodormancy. The increase in abundance of a transcript coding for ACC OXIDASE and decreased abundance of a transcript coding for LIGHT HARVESTING CHLOROPHYLL a/b BINDING 1 (LHCB1) in aerial tissues in response to cold, dehydration, and xenobiotic stress (Fig. 12.2) would be consistent with this previous hypothesis.

A model illustrating potential interaction between leafy spurge DREB/CBFs and *DAM* has been proposed, based on the fact that the promoter of a leafy spurge *DAM1* homolog contains a CCGAC *cis*-regulatory element (CRE) in its upstream promoter (Horvath et al. 2013). Although not yet functionally confirmed in leafy spurge, a similar mechanism for regulation of Japanese pear (*Pyrus pyrifolia*) *PpDAM13-1* by PpCBF2 was proposed (Saito et al. 2013) and later confirmed using a transient reporter assay, indicating that *PpMADS13-1* transcription is enhanced via interaction of PpCBF2 with the *PpMADS13-1* promoter (Saito et al. 2014). This interaction is proposed to impact vegetative growth responses through DAM's regulation of the floral integrator *FLOWERING LOCUS T* (*FT*) as shown in Fig. 12.3. In poplar, *FT1* and *FT2* are involved in regulating reproductive versus vegetative growth, respectively (Hsu et al. 2011).

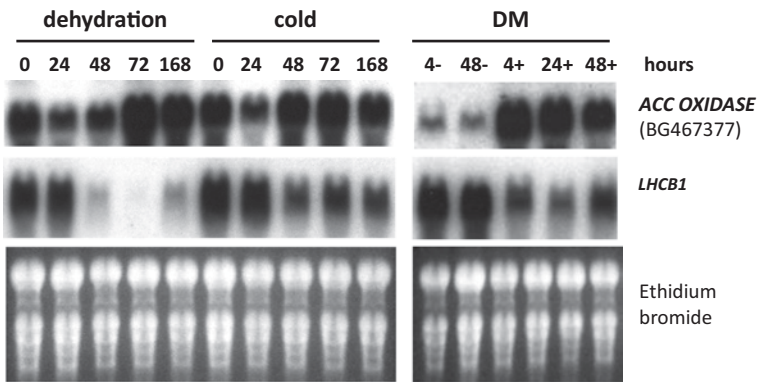

Fig. 12.2 Transcript abundance of *ACC OXIDASE* and *LIGHT HARVESTING CHLOROPHYLL B1* (*LHCB1*) in aerial tissue of leafy spurge in response to dehydration, cold, and xenobiotic stress. Methods and materials are the same as described in Anderson and Davis (2004). Briefly, soil applied water was withheld for dehydration, and plants were subjected to 4–7 °C in a cooling chamber under 16-h light, or sprayed with (+) or without (−) 5 mM technical grade diclofop-methyl (DM) in combination with an emulsified carrier

A role for a DAM/*FT* interaction to regulate vegetative growth and flowering, bud set, and dormancy in perennial fruit trees has been reported (Bielenberg et al. 2008; Yamane 2014). Thus, it is not surprising that chromatin immunoprecipitation (ChIP) assays indicate that leafy spurge DAM1 binds the promoter of a leafy spurge gene most similar to *FT2* of poplar, and increased expression of leafy spurge *DAM1* is inversely correlated with decreased expression of the putative *FT2* homolog (Hao and Horvath unpublished). Likewise, because *DAM* and *FT* are differentially regulated under short photoperiod conditions in perennials (Böhlenius et al. 2006; Cooke et al. 2012; Ruttink et al. 2007) and *DREBs* are known to be gated by the circadian clock (Dong et al. 2011; Fowler et al. 2005), it seems reasonable to assume that the impact of photoperiod and/or temperature on the circadian clock (see reviews by Cooke et al. 2012) could also affect DREB/DAM/FT interactions. Equally intriguing, a report by Chow et al. (2014) demonstrated that Arabidopsis DREB1B/CBF1 binds to a C-repeat (CRT)/dehydration-responsive element (DRE) in the promoter of *LUX ARRYHTHMO* (*LUX*) to mediate cold input into the circadian clock. Although not included in the proposed model (Fig. 12.3), this process may involve SUPPRESSOR OF CONSTANS 1 (SOC1) as part of a negative feedback loop to regulate *DREB1*/*CBFs* (Seo et al. 2009) and, thus, the cold response regulon. In this same context, cold-induced expression of *DREB1*/*CBF* impacts expression of *FLOWERING LOCUS C* (*FLC*), thereby providing a mechanism for repression of *FT* and *SOC1* in Arabidopsis (Thomashow 2010).

Objectives

The long-term goal of our research program is to provide insights into developing next-generation weed management strategies by identifying new targets for manipulation of plant growth and development. As part of this goal, our current objective

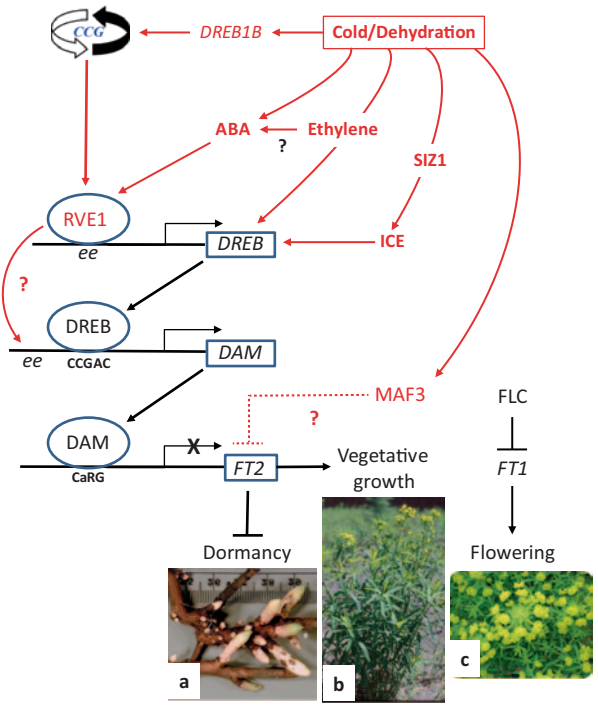

Fig. 12.3 A proposed model for regulation of endodormancy in UABs of leafy spurge. **a** Autumn-induced endodormancy in crown buds of leafy spurge, **b** vegetative regrowth of aerial tissues from UABs in spring and early summer, and **c** leafy spurge in full bloom spring and early summer. Abbreviations and rational for model are provided within the text. *Question marks* and *dashed lines* indicate hypothetical deductions as outlined in the text, and *orange lines* indicate updates on our previous existing models

is to identify CREs within promoters of putative endodormancy marker genes and determine the transcriptional machinery that interacts with these elements.

Materials and Methods

Plant Material and Experimental Designs

Leafy spurge plants were propagated as previously described by Anderson and Davis (2004), and standardized treatments for inducing well-defined phases of dormancy in UABs were employed (Foley et al. 2009). In brief, leafy spurge plants were propagated from a genetically uniform biotype (1984-ND001) and maintained in a greenhouse (~25–27 °C with 16h:8h light:dark photoperiod) for 3 months. Prior to the start of each experiment, plants were entrained in a growth chamber for 1 week at 27 °C, 16h:8h light:dark photoperiod. Each experiment was replicated three

or four times, and each replicate included 30–40 plants. Six to eight plants from each replicate were used to determine the dormancy status of buds by measuring the vegetative growth and flowering potential of crown buds after removal of existing aerial tissues by decapitation at soil level (see Fig. 12.1); the remaining plants were used to collect crown buds for studying transcriptome profiles (Doğramacı et al. 2010, 2011, 2013, 2014). All samples were collected between 1100 and 1300 Central Standard Times to avoid diurnal variation. Various environmental treatments (photoperiod, temperature, dehydration) were used to determine their impact on induction or release of endodormancy as summarized in Table 12.1.

RNA Extraction and Transcript Analyses

At the end of each treatment (Table 12.1), crown bud samples were collected and flash-frozen in liquid N_2. RNA was extracted according to the pine tree RNA extraction protocol (Chang et al. 1993), and RNA quality and quantity was confirmed by spectrophotometry and agarose gel electrophoresis. Microarray hybridizations were performed as described in detail by Doğramacı et al. (2010). Various bioinformatics tools were utilized for analyses of transcriptome data reported in Doğramacı et al. (2010, 2011, 2013, 2014). Specifically, GeneMaths XT 5.1 (Applied Maths Inc., Austin, TX, USA) was used for normalization and statistical analyses, and Pathway Studio (Ariadne Genomics Inc., Rockville, MD, USA) was used for Gene Set Enrichment Analysis and Sub Network Enrichment Analysis. Expression data are deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) as GEO dataset queries GSE19217 (Doğramacı et al. 2010), GSE28047 (Doğramacı et al. 2011), GSE37477 (Doğramacı et al. 2013), and GSE55133 (Doğramacı et al. 2014).

Incorporation of Meta-Analysis to Detect Marker Genes for Endodormancy

Data from transcriptome studies (Table 12.1) were evaluated to detect genes showing consistent trends during endodormancy induction. Genes with increased transcript abundance in endodormant crown buds induced by the various environmental treatments but having opposite expression patterns during para- or ecodormancy were selected for further investigations.

Quantitative Real-Time PCR Analysis (qRT-PCR)

A leafy spurge EST database (Anderson et al. 2007) was used to design primer pairs (Table 12.2) employing the Primer Select program of DNASTAR Lasergene 11. cDNA synthesis and qRT-PCR were prepared as described in Doğramacı et al. (2010, 2011, 2013, 2014). Transcript abundance was measured from at least three biological replicates and three technical replicates using a LightCycler 480 II

Table 12.1 Summary of environmental treatments, treatment length (d: day, h: hour, wk: week), and associated publications demonstrating specific environmental signals impacting endodormancy induction or release in leafy spurge

Referred to as	Treatment	Temperature (°C)	Photoperiod (light)	Treatment period	Pretreatment bud status	Post-treatment bud status	Reference
Para	Plants entrained in growth chamber	27 °C	16 h	1 wk	Paradormant	Paradormant	Doğramacı et al. 2010
RDtp	Plants exposed to ramp down in temperature and photoperiod	27 °C → 10 °C	16 h → 8 h	12 wk	Paradormant	Endodormant	Foley et al. 2009; Doğramacı et al. 2010
RDp	Plants exposed to ramp down in photoperiod under constant temperature	27 °C	16 h → 8 h	12 wk	Paradormant	Paradormant	Foley et al. 2009
RDt	Plants exposed to ramp down in temperature under constant photoperiod	27 °C → 10 °C	16 h	12 wk	Paradormant	Paradormant	Doğramacı et al. 2013
FC Eco	Plants exposed to extended cold	7 °C	8 h	11 wk	Endodormant	Flower competent ecodormant	Doğramacı et al. 2010
NFC Eco	Plants exposed to extended cold	7 °C	8 h	11 wk	Paradormant	Non-flower competent ecodormant	Doğramacı et al. 2010
Endo- > 3-day dehydration stress	Plants exposed to dehydration stress	27 °C	16 h	3 d	Endodormant	Growth competent	Doğramacı et al. 2011
Three-day dehydration stress	Plants exposed to dehydration stress	27 °C	16 h	3 d	Paradormant	Paradormant	Doğramacı et al. 2014
Seven-day dehydration stress	Plants exposed to dehydration stress	27 °C	16 h	7 d	Paradormant	Para- to endodormant transition	Doğramacı et al. 2014
Fourteen-day dehydration stress	Plants exposed to dehydration stress	27 °C	16 h	14 d	Paradormant	Endodormant	Doğramacı et al. 2014

Table 12.2 Primer sequences for selected leafy spurge genes (Primer Sequence). Annotation of homologs of leafy spurge transcripts most similar to Arabidopsis were identified using the Arabidopsis Information Resource (TAIR) and to obtain gene identifications (TAIR ID) and abbreviations (Abv.)

TAIR ID	Abv.	5′F/R	Primer Sequence	Leafy spurge ID
CONSTITUTIVE PHOTOMORPHOGENIC 1 (AT2G32950)	*COP1*	5′F	TCTTGTTTTTCTTCCCCTCTATCT	DV130469
		5′R	AGCACGTTTTTCATGTTCTTCA	
ELONGATED HYPOCOTYL 5 (AT5G11260)	*HY5*	5′F	CTCAACAAGCAAGGGAAAGGAAGA	DV157454
		5′R	CTAGCCAACGAAGAAACGGAAAAT	
MADS AFFECTING FLOWERING 3 (AT5G65060)	*MAF3*	5′F	ATCGAAGAAAAGAGCATCCGTCAG	CV03083A2E08
		5′R	TCTTCAAGTTGCATGTCAGTAGTT	
RESPONSIVE TO DESSICATION 22 (AT5G25610)	*RD22*	5′F	AATCAAACCCCGAAGCAAAAGTAT	DV131779
		5′R	CCTGAGGAAGAAAATGGCAAACC	
REVEILLE 1 (AT5G17300)	*RVE1*	5′F	CGGATTGAAGAGCATGTAGGTAGC	DV113864
		5′R	AGTTGGGGATTGATTTTCGTGTTC	

(Roche). Transcript values were normalized using reference genes (*ARF2*, *MD-100*, *ORE9*, *PTB*, *SAND*) identified for leafy spurge (Chao et al. 2012).

Identification of Conserved Cis-Regulatory Elements

Assembly (*de novo*) of promoter sequence for candidate genes was accomplished as previously described (Doğramacı et al. 2014). Briefly, promoter sequence (~3000–7000 bases upstream of putative ATG start sites of CDS) was created using the program PriceTI (Ruby et al. 2013), which were used to identify the most conserved genes within the Malpighiales family (*Manihot esculenta*, cassava; *Ricinus communis,* castor bean; *Linum usitatissimum,* flax; *Populus trichocarpa,* poplar) using the program Phytozome (www.phytozome.net). The non-transcribed promoter regions for each family member were run in the MEME program (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) to identify conserved promoter sequences. Conserved motifs identified by MEME were entered into Plant Promoter Analysis Navigator (http://plantpan.mbc.nctu.edu.tw/) to determine the function of putative CREs.

Results and Discussion

Identification of Potential Marker Genes

Meta-analysis of microarray data (Doğramacı et al. 2010, 2011, 2013, 2014) highlighted five leafy spurge transcripts with putative sequence homology to Arabidopsis *COP1*, *HY5*, *MAF3*, *RD22*, and *RVE1* that consistently had increased abundance in endodormant crown buds (Table 12.3), but had opposite expression in para- and often ecodormant buds of leafy spurge. Based on these results, we propose that PCR-amplified cDNA (see Table 12.2) for these transcripts may be used as endodormancy markers.

COP1

COP1 encodes for an E3 ubiquitin ligase that can target up to 20% of the transcription factors in Arabidopsis (Moon et al. 2004), including HY5 and the floral promoter CONSTANS (CO) for degradation and stabilization of growth-promoting transcription factors such as PIF3 (Alabadí and Blázquez 2009; Henriques et al. 2009). Although COP1 targets HY5 for degradation in far-red and visible light-induced photomorphogenesis, it positively regulates HY5 in UV-B-induced photomorphogenesis (Favory et al. 2009). Further, in Arabidopsis, *COP1* expression is regulated by HY5 and FHY3 via a positive feedback loop (Huang et al. 2012). Thus, the simultaneous

Table 12.3 Transcript abundance of potential marker genes. Values (log2 scale) represent averages of four biological and three technical replicates relative to controls. Red indicates positive values, and blue indicates negative. Arabidopsis genes are used to annotate homologs of leafy spurge transcripts

Reference	Status of Buds	Treatment Summary	COP1	HY5	MAF3	RD22	RVE1
Doğramacı et al. 2010, 2013	Para	Para--Control	0	0	0	0	0
	Endo	RDtp	1.77	1.30	9.27	0.68	0.97
	FC Eco	RDtp + Cold	0.62	-0.24	17.35	-1.20	-0.35
	NFC Eco	Para + Cold	0.46	-0.43	17.51	-0.98	0.46
	RDt	RDt + Constant light	-0.23	-0.15	-1.38	-0.33	-0.55
Doğramacı et al. 2014	Para	Para--Control	0	0	0	0	0
	Day 3	Para--3 day dehydration	0.25	1.01	-0.67	0.65	0.11
	Day 7	Para--7 day dehydration	-0.13	0.96	1.08	2.35	0.62
	Day 14 (Endo)	Para--14 day dehydration	2.30	2.58	3.15	2.42	2.05
Doğramacı et al. 2011	Endo	Endo--Control	0	0	0	0	0
	Day-01	Endo--1 day dehydration	-2.11	-1.58	-4.53	-1.95	-5.17
	Day-03	Endo--3 day dehydration	-2.74	-1.55	-4.75	-2.30	-4.13
	FC Eco	Endo (RDtp) + Cold	-0.65	-1.38	7.44	-1.69	-0.74

increase in abundance of putative leafy spurge *HY5* and *COP1* transcripts by RDtp or dehydration alone indicates that normal light-mediated regulation of COP1–HY5 interactions is disrupted in endodormant UABs. This might be a consequence of RDtp or dehydration treatments on senescence of aerial tissue, suggesting that senescence-induced signaling could be a common factor leading to endodormancy in UABs.

The decreased abundance for a transcript with putative homology to an Arabidopsis *CO-like* (At2g33500) in endodormant crown buds of leafy spurge (Doğramacı et al. 2010) could imply that COP1 is targeting some members of this transcription factor family in endodormant crown buds. Although CO is a positive regulator of *FT*, and the *CO-FT* module has been associated with growth cessation and bud set in poplar (Böhlenius et al. 2006), data for leafy spurge *FT-like* transcripts were not available for all the samples included in our meta-analysis. However, transcript abundance for a putative leafy spurge *FT1-like* homolog in endodormant buds was reported to be induced by RDtp (Doğramacı et al. 2013) and 14-day dehydration stress treatments (Doğramacı et al. 2014). Because leafy spurge DAM1 has been shown to bind the promoter of a gene with putative homology to poplar *FT2* (Hao and Horvath unpublished), we propose that increased transcript abundance of leafy spurge *DAM1* and *DAM2* (Doğramacı et al. 2010) does not lead to repression of *FT1-like* transcripts in endodormant buds. Instead, it is proposed to repress transcripts with functional similarity to poplar *FT2* (see Fig. 12.3).

HY5

HY5 encodes for a bZIP transcription factor involved in the positive regulation of photomorphogenesis and the PHYA-mediated inhibition of hypocotyl elongation in Arabidopsis (Jiao et al. 2007; Saijo et al. 2003). Studies using *hy5* mutants indicate that HY5 promotes the expression of negative regulators of auxin signaling, thus linking hormone and light signaling pathways (Cluis et al. 2004). Because HY5 can bind to targets involved in regulating circadian rhythms, flowering, and hormone signaling in Arabidopsis, it has been proposed that HY5 likely has other roles in plant growth and development beyond light regulation (Lee et al. 2007). Indeed, Catalá et al. (2011) reported that HY5 levels in Arabidopsis are regulated by low temperature transcriptionally, via a CBF- and ABA-independent pathway, and post-translationally, via protein stabilization through nuclear depletion of COP1. Thus, increased expression of a leafy spurge transcript with putative homology to *HY5* could be acting as one of the central modulators of gene expression that helps coordinate light and cold signaling to promote endodormancy by the RDtp treatment.

Interestingly, induction of endodormancy in crown buds by 14-day dehydration stress, where plants were under ambient greenhouse conditions (~25–27 °C with 16-h photoperiod) prior to and during the dehydration stress treatment, also caused an increase in abundance of the putative leafy spurge *HY5* transcript (Table 12.3). As previously mentioned above, perhaps senescence-associated signaling (e.g., sugars, auxin) of aerial tissues by 14-day dehydration stress also causes an effect similar to that occurring under cold temperature–short photoperiod conditions

(RDtp). Therefore, the increase in putative leafy spurge *HY5* and *COP1* transcripts in response to 14-day dehydration demonstrates that this process also can occur independent from changes in cold and light signaling. If the product of this putative *HY5* transcript has similar functions in leafy spurge as in Arabidopsis, HY5 could be involved in negatively modulating auxin signaling in endodormant crown buds. These results also suggest that endodormancy induction by dehydration or RDtp likely involves overlapping mechanisms.

MAF3

In Arabidopsis, *MAF3* encodes for a MADS-box domain protein and flowering regulator that is closely related to the floral repressor *FLC* (Caicedo et al. 2009; Ratcliffe et al. 2003). Induction of endodormancy by RDtp treatment and 14-day dehydration stress increased abundance of this putative leafy spurge *MAF3* transcript relative to paradormant controls (Table 12.3). Transcript abundance of this *MAF3-like* was even greater in ecodormant buds (FC Eco), and it was also increased in NFC Eco buds even though these buds did not go through the endodormant phase. Thus, this putative *MAF3* transcript appears to be a marker for both endo- and ecodormant crown buds. As shown in Table 12.3, this putative *MAF3* transcript was not induced by a ramp down in temperature under a constant 16-h photoperiod (RDt), but was induced by ramp down in temperature and photoperiod (RDtp) or extended cold treatment under 8 h of short photoperiod (NFC Eco). These results suggest that expression of this putative *MAF3* transcript is induced by either short photoperiod alone or an interaction between short photoperiod and cold temperature signaling. However, no transcript data are currently available for short photoperiods under constant temperature to confirm this hypothesis in leafy spurge. Interestingly, abundance of this *MAF3-like* transcript was also induced by 14-day dehydration stress (Table 12.3), which further supports the hypothesis that senescence of aerial tissues by RDtp and dehydration induces an overlapping response that impacts the transition from para- to endodormancy in UABs.

RD22

RD22 is generally associated with abiotic stress responses (such as dehydration and salt stress) mediated by ABA in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki 1993). Induction of endodormancy in crown buds of leafy spurge by RDtp treatment or 14-day dehydration stress caused an increase in transcript abundance of *RD22* (Table 12.3). Further, release of endodormancy by 3-day dehydration or extended cold treatment (FC Eco) caused a decrease in transcript abundance of *RD22*. These results are consistent with increased abundance of RD22 class proteins (along with other ABA-inducible transcripts) in dormant potato tuber meristems, which were decreased when meristem dormancy was terminated (Campbell et al. 2008).

RVE1

RVE1 encodes a clock-regulated MYB-like transcription factor that, in Arabidopsis, is homologous to *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL 1* (*LHY1*), but inactivation of RVE1 does not affect the circadian rhythm (Rawat et al. 2009). More specifically, RVE1 is an output component of the circadian clock and has been shown to regulate hypocotyl growth by modulating free auxin levels in a time-of-day-specific manner in Arabidopsis (Rawat et al. 2009). Because RVE1 appears to modulate plant growth through regulation of auxin levels, while CCA1 and LHY1 likely control growth via different mechanisms, *RVE1* is considered an important node connecting circadian- and auxin-signaling pathways (Rawat et al. 2009). In leafy spurge crown buds, induction of endodormancy by the RDtp treatment or 14-day dehydration stress (Table 12.3) caused increased abundance of a transcript for this putative *RVE1* homolog. Further, release of endodormancy by 3-day dehydration or extended cold treatment (FC Eco) caused a decrease in transcript abundance of this *RVE1* transcript. In endodormant buds of leafy spurge, increased abundance of *RVE1* (Table 12.3) and a moderate increase in auxin levels (unpublished data), compared to paradormant controls, are consistent with RVE1-modulated free auxin levels in Arabidopsis. Because exogenous auxin treatment to Arabidopsis enhances hypocotyl elongation, while higher concentrations inhibit hypocotyl growth (Rawat et al. 2009), perhaps the putative leafy spurge RVE1 homolog plays a role in modulating auxin levels in endodormant UABs of leafy spurge, assuming that the product of the putative leafy spurge *RVE1* homolog performs the same function as in Arabidopsis.

Characterization of Proposed Marker Genes

Identification of promoter sequence for putative leafy spurge endodormancy marker genes (Table 12.4) was accomplished through *de novo* assembly as previously described in Doğramacı et al. (2014). Currently, sufficient promoter sequence is only available for *RVE1* and *HY5* among the five proposed endodormancy markers for leafy spurge. The validity of *de novo* assembly for *RVE1* was confirmed by comparing it to the sequence of leafy spurge genomic clones for *RVE1*; comparison of the 5′ upstream promoter sequences was >95 % conserved (Doğramacı et al. 2014). The promoter of the leafy spurge genomic clone for *RVE1* contains a conserved ABA-responsive element (ABRE)-like sequence that in other plant systems is involved in early response to dehydration and calcium (Whalley et al. 2011). The *RVE1* promoter also contained a putative MYC consensus sequence, common to dehydration-responsive genes, and a PIF3 binding element (Table 12.4). Because the circadian clock is disrupted in perennials by cold temperatures (Ramos et al. 2005; Ibáñez et al. 2008), uncoupling of the circadian clock by dehydration and/or temperature in UABs of leafy spurge may be compensated through ABA signaling involving ABREs to regulate circadian clock outputs.

Table 12.4 Conserved *cis*-acting elements (colored and underlined) within the promoters of putative leafy spurge homologs (genes) in the Malpighiales family of sequenced genomes. Element location (position) within promoters is upstream of proposed ATG start of coding sequence

Genes	Element	Strand	Position	Sequences		
RVE1	ABRE-Like	+	2178	GAAGGGCGGC	CTGACGTGGC	TAAAAGCGCA
RVE1	MYC Cons	+	3620	GACAAACTAA	CGCCACGTGTCT	GACTTGTCTT
RVE1	PIF3	+	3620	GACAAACTAA	CGCCACGTGTCT	GACTTGTCTT
HY5	ARR1	+	2662	ACCATCATAC	CACGATTCTCTC	CCACTATCCT
HY5	GT1 Cons	+	2820	AATTAATATC	CCGCTCTTTTTC	TCTCCCCAAA
ABR1	GCC Core	+	2658	TCTTGTAATA	AGGCGGC	GTTTACTTGT
ABR1	LTRE Core	+	2411	TCCAAAAAAA	CTCAGCACCGAC	TGAATGTGCA
ABR1	LTRE Core	+	2371	ATATCTAAAT	GGGCCGACC	TGGTTGTCGG
ABR1	LTRE Core	+	2187	CTCGATTAGA	CCCGACCC	AATCCACGAA
ERF1	ABRE-Like	-	126	CAGATGTTTA	GCTGACGTGGC	AGATTTTTAG
ERF1	ASF-1	-	126	CAGATGTTTA	GCTGACGTGGC	AGATTTTTAG
ERF1	GCC Core	+	2432	TATAAATGAA	GGCCGCC	TGATTGTATA
ERF1	MYC Cons	+	1782	TTATAAACAA	GTGGAGCAACC	AAACCAATAC
PIF3	ABRERATCAL	+	919	TATTAAATAT	GTCCACGTGGAC	CAATGAGATA
PIF3	CBF2	+	919	TATTAAATAT	GTCCACGTGGAC	CAATGAGATA
PIF3	MYC Cons	+	919	TATTAAATAT	GTCCACGTGGAC	CAATGAGATA

GCCGCC = GCC CORE - ethylene-responsive element.
CAAGTG = MYC recognition sequence in CBF3 promoter.
LTRE = Low temperature responsive element core.
ASF-1 binds to TGACG motifs - involved in transcriptional activation by auxin/salicylic acid.
GT-1 is a binding site in many light-regulated genes.
ARR1 is a typeB cytokinin response element.

The *de novo*-assembled promoter of leafy spurge *HY5* contains a conserved CRE that, in Arabidopsis, interacts with B-type ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1) (Sharma et al. 2011). Because B-type ARR1 regulates transcription of target genes in response to cytokinin (Heyl et al. 2008; Sharma et al. 2011), our results suggest that cytokinin signaling could be playing some role in the increased abundance of *HY5* in endodormant crown buds of leafy spurge. However, because silencing of *ARR1* in Arabidopsis induced over a 4-fold increase in the expression of *COP1* (Heyl et al. 2008), it appears that repression of cytokinin signaling is likely required to induce *COP1* and *HY5* expression in endodormant buds, at least in UABs of leafy spurge.

The assembled promoter sequences for several other genes of interest included putative homologs of *APETALA2*/*ERF* family members (*ABR1* and *ERF1*) and *PIF3*. The promoters of putative leafy spurge *ERF1* and *PIF3* genes also contained ABRE-like and MYC CREs, whereas these same binding elements do not appear to be conserved in the promoters of putative leafy spurge *HY5* and *ABR1* genes (Table 12.4). However, *ABR1* and *ERF1* do contain ethylene-responsive GCC core elements, which have been reported to play important roles in regulating jasmonate-responsive gene expression (Ohme-Tagaki et al. 2000). In addition, the putative leafy spurge *ABR1* promoter sequence contained low temperature-responsive elements (LTRE), which also includes the core C-repeat/dehydration-responsive element (C/DRE; -CCGAC-), and light signaling mediated by phytochrome is necessary for cold- or drought-induced gene expression through the C/DRE in Arabidopsis (Kim

et al. 2002). Collectively, these results suggest that timing of transcriptional activation or repression of target genes involved in the regulation of seasonal dormancy is likely modulated by a complex set of binding interactions that are responsive to environmental cues and phytohormones.

Updated Hypothetical Model for Endodormancy Induction

A hypothetical model for endodormancy induction (Fig. 12.3) shows how cold or dehydration stress impacts circadian clock genes, as previously reported in leafy spurge (Doğramacı et al. 2010, 2013, 2014) and other species (Ibáñez et al. 2008; Ramos et al. 2005; Rios et al. 2014). Indeed, in the case of cold, *DREB1B*/*CBF1* has been shown to bind to a C/DRE in the promoter of Arabidopsis *LUX* to regulate oscillation and mediate cold input into the circadian clock (Chow et al. 2014). Cold uncoupling of the circadian clock in perennials (Ibáñez et al. 2008; Ramos et al. 2005) would be expected to impact circadian clock output genes such as *RVE1*. Thus, regulation of a homolog of *RVE1* in endodormant UABs of leafy spurge in response to cold and dehydration could involve regulation through ABA signaling (Doğramacı et al. 2014). Indeed, RVE1 can bind *cis*-acting evening elements (*ee*) in genes (Franco-Zorrilla et al. 2014; Mizoi et al. 2012), and these *ee* have been linked to circadian-regulated and cold-induced expression, and coupling with ABRE-like can enhance the cold-induced expression in Arabidopsis (Mikkelsen and Thomashow 2009).

Because some *AP2*/*ERF*s are gated by the circadian clock as previously described, it is possible that the impact of cold or dehydration on circadian clock components in leafy spurge also affects abundance of these transcription factors. We have considered DREBs/CBFs as a central component that impact GA catabolism, sugar signaling, and other processes associated with various transitional phases of dormancy in leafy spurge (Doğramacı et al. 2010, 2014; Horvath et al. 2013). Indeed, DREBs can affect GA catabolism and signaling in Arabidopsis (Magome et al. 2009), and GA catabolism would be expected to impact downstream GA signaling of DELLAs, which, in turn, could affect vegetative growth through its repression of growth-promoting transcription factors such as PIFs (see review by Hirsch and Oldroyd 2009). We hypothesize that DREBs could also be playing a role in dormancy processes as shown in Fig. 12.3, based on their known involvement in expression of genes similar to *FLC*, which in turn leads to repression of *FT* in Arabidopsis (Seo et al. 2009).

Ethylene's impact on some *AP2*/*ERF* family members in leafy spurge has been proposed to have a role in regulating the transition from para- to endodormancy (Doğramacı et al. 2013). Indeed, overexpression of several *AP2*/*ERFs*, similar to those induced by ethylene in leafy spurge (Doğramacı et al. 2013), causes dwarfed phenotypes or induces endodormancy in other plant systems (Khan 2011; Wisniewski et al. 2011; Xu et al. 2011). Because DREBs are known to bind CREs similar to those identified in leafy spurge *DAM* genes (Horvath et al. 2013), and leafy spurge DAM1 has been shown to bind the promoter of a putative leafy spurge *FT2-like* gene (Hao and Horvath unpublished), we propose that RVE1 may func-

tion through binding to *ee* of *DREB/CBFs* or directly to the promoter of *DAM1* (see Fig. 12.3). Although ACC synthase is the rate-limiting step of ethylene biosynthesis in Arabidopsis (Wang et al. 2004), the last step to ethylene biosynthesis involving ACC oxidase might also play a role in the senescence-induced spikes in ethylene that has been proposed to induce endodormancy in UABs (Anderson et al. 2010; Horvath et al. 2003). The observed increase in abundance of transcript coding for ACC oxidase in response to abiotic and xenobiotic stress in leafy spurge leaves (Fig. 12.2) would be consistent with this concept. In Arabidopsis, ethylene production also induces the nuclear transcription factor *ETHYLENE INSENSITIVE 2*, which impacts ABA signaling (Wang et al. 2007). Thus, we propose that senescence-induced ethylene signaling impacts mobile auxin and sugar signaling from the aerial tissues (see Fig. 12.1) and could impact cross-talk with ABA signaling pathways in leafy spurge (see Fig. 12.3).

A previous study (Doğramacı et al. 2014) also suggested a potential role for post-translational modification through interactions between SIZ1 (an E3 SUMO ligase) and INDUCER OF CBF EXPRESSION1 (ICE1) to impact *DREB* expression in endodormant UABs of leafy spurge. Because SIZ1 can stabilize ICE1 through sumoylation (Mizoi et al. 2013) and ICE1 binds the promoter of *DREBs* (Chinnusamy et al. 2006), it is also possible that cold- and dehydration-induced expression of *DREBs* in UABs of leafy spurge involves similar post-translational modification mechanisms as illustrated in Fig. 12.3.

Another major outcome from this meta-analysis was identification of a putative leafy spurge *MAF3-like* transcript, one of the several *FLC-like* genes in Arabidopsis (Ratcliffe et al. 2003), as a molecular marker for endo- and ecodormancy in crown buds of leafy spurge. In Arabidopsis, FLC is known to inhibit *FT* to block flowering (Ratcliffe et al. 2003; Reeves et al. 2007), and in perennial tree species, several members of DAMs (also members of the MADS-box domain family of proteins) are known to block *FT2* and induce growth cessation and bud set (Cooke et al. 2012; Rios et al. 2014). Based on the strong increase in abundance of a leafy spurge transcript with putative homology to Arabidopsis *MAF3* in endo- and ecodormant UABs (Table 12.3), we propose that the product of leafy spurge *MAF3-like* could inhibit *FT2-like* expression as part of a mechanism involved in maintaining endo- and ecodormancy (Fig. 12.3). However, leafy spurge *MAF3-like* transcript in endo- and ecodormant UABs appears to display alternative splicing (unpublished). Since the product of spliced variants of *MAFs* have been reported to interact with SHORT VEGETATIVE PHASE to regulate flowering in Arabidopsis in a temperature dependent manner (Posé et al. 2013; Severing et al. 2012), it is also plausible that spliced variants of the leafy spurge *MAF3-like* transcript could potentially interact with DAM-like MADS-box proteins to affect dormancy in UABs.

Future Direction

Conceptual models, such as proposed in Fig. 12.3, provide a starting point to test the functionality of these putative leafy spurge homologs and to determine their potential role in endodormancy maintenance in leafy spurge or other model perennial

systems. Here, we update a working hypothetical model to include new components for regulation of endodormancy in UABs of leafy spurge that involves (1) the potential interaction of MAF3 with *FT2* to inhibit vegetative growth and (2) an ABA-dependent signaling mechanism to regulate a putative homolog of the circadian clock output gene *RVE1* that may impact downstream genes containing evening elements, similar to that described in other systems, or to modulate auxin levels. The overlap between dehydration- and photoperiod/temperature-induced endodormancy in UABs of leafy spurge may involve senescence-induced ethylene signaling. Further research into well-defined phase of dormancy in UABs of leafy spurge would certainly benefit from studies that determine the impact of molecular and physiological signaling mechanisms associated with aerial tissues, for example, determining whether FT is produced in aerial tissues and whether it is mobile and transported to the underground adventitious buds. Likewise, further studies are needed to determine if spliced variants of *MAF3-like*, or other MAF family members, affect *FT2* directly or function through interaction with DAM-like MADS-box proteins.

However, relying on orthologous genomes to annotate genomes of weedy species has pitfalls associated with proposing biological interactions and processes. Spurious assumptions that transcripts with the best sequence homology to genes of other plant species have conserved functionality may lead to confounded models. Thus, meta-analysis of the leafy spurge transcripts based on annotation to other genomes only provides for the first step in building testable hypotheses. Future research will be needed to functionally characterize these leafy spurge marker genes and determine the upstream binding complexes that drive their expression.

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