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Analysis And Localization of the Water-Deficit Stress-Induced Gene (*lp3*)

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ABSTRACT

LP3 is a water-deficit-induced protein, which is highly homologous to ASR (ABA, stress and ripening) proteins. Homology was found in the C-terminal region of the putative LP3 protein while lower homologies were found in the N-terminal region. The goal of this study was to investigate the function of the LP3 protein and the mechanism of the *lp3* promoter in response to water-deficit stress (WDS) and other stresses. In regenerated transgenic tobacco (T_0), expression of β-glucuronidase (GUS) from the *lp3* promoter-GUS construct was observed in polyethylene glycol (PEG), abscisic acid (ABA), methyl-jasmonate (MeJa), and fluridone (Flu) treatments. GUS expression was not observed following gibberellin (GA_3), 2-methyl-4-dichlorophenoxy acetic acid (2,4-D), silver nitrate, or ethephon (ethylene releasing agent) treatments. Germinated T_1 seedlings containing the *lp3* promoter-GUS construct exhibited GUS activity up to 40 days postgermination. Expression could be restored when 5-azacytidine was included in the culture media, indicative of a developmentally regulated silencing mechanism involving methylation. In transgenic tobacco, the LP3 protein localized in the cell nucleus was induced by WDS and appeared to be developmentally regulated.

Key words: Tobacco; Water-deficit stress (WDS); Abscisic acid (ABA); Polyethylene glycol (PEG); Fluridone; *lp3* gene; Methylation

INTRODUCTION

Water-deficit stress (WDS) is one of the major environmental factors that affect woody plant survival and growth. The *lp3* gene (Padmanabhan and others 1997) was isolated from a cDNA library from water-stressed loblolly pine roots and appears to be a member of a small multigene family (Padmanbhan 1996). Changes in mRNA levels of *lp3* observed during WDS reflect transcriptional activation and are common phenomena observed with most WDS-

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inducible genes (reviewed by Bray 1993; Ingram and Bartels 1996). The most abundant expression of the native gene appears in roots, to a lesser extent in stems, and with very little or no expression in needles (Padmanbhan 1996). The coding region of *lp3* has 85% homology with *Asr* (ABA, stress and ripening, Isuem and others 1993) genes in the C-terminus but has lower homology at the N-terminus region. Aligned sequences showed high similarity (60–72% at the amino acid level) to *Asr* proteins indicating that the *lp3* gene could be a member of the *Asr* family. The putative sequence of the LP3 protein contains a bipartite nuclear localization signal, KKESKEEEKEAEGKKHHH (Varagona and others 1992) in the C-terminal region.

The promoter region of *lp3* was sequenced (Padamanabhan 1996) and several putative responsive elements, such as ABA responsive element (Bray 1993), CaMV root-response element (Katagiri and others 1989), GA responsive element (Gubler and Jacobsen 1992), MeJa element (Rouster and others 1997), Opaque-2 responsive element (Varagona and others 1992), and *rd22* responsive element (Iwasaki and others 1995) have been found. Abscisic acid (ABA) is a plant hormone involved in the regulation of ripening and WDS conditions (Rossi and others 1996) and the plant hormone ethylene plays an important role in fruit ripening (Agar and others 2000). Fluridone is an inhibitor of ABA synthesis (Sprecher and others 1998) and silver nitrate ($AgNO_3$) is an inhibitor of ethylene action (Kong and Yeung 1995). Jasmonic acid (JA) and its methyl ester methyl-jasmonate (MeJa) influence many physiological and developmental processes affected by ABA including WDS, ripening and defense (Harms and others 1995) and may function as intermediates in the ABA signaling pathway (Chao and others 1999). Transgenic tobacco plants containing an *lp3* promoter-GUS construct were challenged with ABA, fluridone, ethylene, silver nitrate, MeJa, GA, kinetin and 2,4-D treatments in culture for gene expression test. In this study, gene silencing also could be a factor that affected gene expression (Prakash and Kumar 1997).

Materials and Methods

Construction of Promoter-GUS Fusion

The *lp3* promoter (1 kb) was ligated within the plasmid pBI221 (Clonetech, Polo Alto, CA). The resulting plasmid (p221LP3GUS) was digested with *Hind*III and *Xba*I and subcloned into the *Hind*III-*Xba*I site of the binary plasmid vector pBI101.3 (Clonetech, Polo Alto, CA), which contains the *E. coli uidA* gene, encoding β-glucuronidase (GUS) (Jefferson 1987) and *nptII* conferring kanamycin resistance. The resulting plasmid, pGILP3(P), contained the promoter sequence of *lp3* transcriptionally fused to *uidA* (GUS), was used to study *lp3* expression. For localization of the LP3 protein, pBI121(I), containing the CaMV35S promoter and GUS, was digested with *Hind*III. *uidA* was subcloned into the *Hind*III site of p221LP3GUS, containing *lp3* cDNA and CaMV35S promoter from previous research (Padmanabhan 1996). The resulting plasmid pB1LP3 contained the CaMV35S promoter, *lp3* cDNA and *uidA* (GUS), as well as the selectable marker *npt*II. *Agrobacterium tumefaciens* strain LBA4404 (Horsch and others 1985) was used for plant transformation.

Plant Material and Culture Conditions

Tobacco (*Nicotiana tabacum* L. ''Havana'') seeds were surface sterilized and germinated in MS media (Murashige and Skoog 1962) for 2 two weeks at 16 h/day and 27°C. After the tobacco seedlings were 2 weeks old, they were transferred into MS media in 400 ml Kerr jars. When seedlings were 20 cm in height, leaves were harvested and leaf discs were excised and co-cultured 48 h with *Agrobacterium tumefaciens* strain LBA4404, harboring pGILP3(P), in MS medium (Horsch and others 1985). Leaf disk explants were then cultured in regeneration medium containing MS salts and vitamins, 30 g/l sucrose, 8 g/l agar, pH 5.7 plus 0.1 mg/l 2-methyl-4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l benzyl aminopurine (BA), 50 mg/l kanamycin; and 250 mg/l Clavamox (amoxicillin + clavenate Smith Kline Beecham Veterinary) to remove *Agrobacterium* infection (Gould and Magallanes-Cedeno 1999). Explants were transferred to fresh medium weekly until distinct shoots appeared, then shoots were transferred to a hormone-free medium (MS salts, 30 g/l sucrose, 8 g/l agar, pH 5.7, supplemented with kanamycin and Clavamox, as described above) to regenerate rooted plants.

Embryogenic calli of Slash pine (*Pinus elliottii* Engelm) were initiated from zygotic embryos (seeds obtained from the Texas Forest Service) cultured in DCR media (Gupta and Durzan 1987) which included 10 μM BA, 10 μM 2-methyl-dichlorophenoxyacetic acid (2,4-D) and agar 8 g/l, pH 5.7 (Sarjala and others 1997). Embryogenic calli were cultured and maintained as a suspension culture in liquid DCR medium cultured in flasks and agitated

at 50 rpm (Lab-Line shaker) (Gupta and others 1985). After continuous multiplication in liquid DCR for 2 weeks, aliquots of the cell suspension were poured onto sterilized filter paper disks placed in a Buchner funnel attached to a vacuum. The mass of cells from the suspension on the filter paper disks was transferred onto a MS media plate one day prior to biolistic bombardment.

Onion bulbs were cut into 2-inch pieces and sterilized in 20% Clorox for 10 min then rinsed 3X in sterile water. The inner epidermis was peeled off and placed onto MS medium overnight prior to bombardment.

Developmental Assays

Transgenic regenerated tobacco plants (T_0) and seeds (T_1) carrying pGILP3(P) (J-T Wang and others unpublished) were germinated in MS/kanamycin media and harvested 10, 20, 30, 40, 60, 80, and 100 days after germination. Plants of the T_0 were allowed to flower and set seed. Harvested seedlings and plant tissues were immediately assayed for GUS activity using the GUS histochemical stain (Jefferson 1997).

Stress and Hormone Treatments

Transgenic tobacco plants (T_0) and seedlings (T_1) harboring pGILP3(P) (J-T Wang and others unpublished), containing the *lp3* promoter and *uidA* gene, were maintained in MS media with kanamycin, 50 mg/l. Regenerated tobacco plants (T_0) were allowed to flower and set seed (T_1). Surviving tobacco seedlings (T_1) 5 cm tall were transferred into MS/kanamycin media in 400 ml Kerr jars. After 3 weeks, seedlings approximately 15–20 cm tall, were transferred to the MS/kanamycin media with the following treatments: 10% PEG (molecular wt. 6000) added into the media before autoclaving; 50 μM ABA; 100 μg/l fluridone, 30 μM each of gibberellin (GA_3), kinetin, and 2,4-D. Silver nitrate ($AgNO_3$) was used at 100 μM (Kong and Yeung 1995). Tobacco plants were transferred from culture to soil, and to greenhouse culture after 2 weeks of acclimatization. Ethephon-treated plants were sprayed with 35 mM ethephon (Carolina Biological Supply Co., Burlington, NC), an ethylene-releasing agent (Shatters and others 1998). Nontreated plants were saturated with a spray (total volume = 250 ml) of water. Applications were performed once daily for 2 weeks, the plants were harvested and GUS assays were performed.

5-Azacytidine (5-azaC) Treatment

The demethylation agent, 5-azacytidine (5-azaC), is an analog of cytosine that is used to decrease methyltransferase activity in animal and plant systems (McInerney and others 2000), and inhibits DNA methylation (Prakash and Kumar 1997). Sterile 5-azaC was added to MS media after autoclaving and before cooling. Seeds of transgenic tobacco (T_1) containing the *lp3* promoter: GUS construct (pGILP3(P) were germinated on MS/kanamycin media or MS/kanamycin media containing 25 μM 5-azaC. After 1 month, seedlings were recultured on MS media +/− 5-azaC in 400-ml Kerr jars, without added kanamycin. After 1 month (80 days postgermination), tissues were assayed for GUS activity.

Transient Transformation: Nuclear Localization

Onion epidermal cells and cell suspensions of slash pine were transformed with the binary *lp3* cDNA and GUS (*uidA*) gene driven by the CaMV35S promoter using the biolistic method of Oard and others (1990). For transient expression studies, gold particles were prepared according to manufacture's instructions (Biolistic delivery system, PDS-1000, E. I. du Pont de Nemours & Co. Biotechnology Systems Division, Wilmington, DE). Particles coated with DNA (pBILP3) were driven into onion epidermal tissue and slash pine cell suspensions by helium gas. The vacuum chamber pressure was down to 25 Hg in vacuum, the helium pressure was 1000 psi, and the small membrane was 650 psi. After bombardment, the plant cells were incubated at room temperature overnight and prepared for GUS assay. β-glucuronidase (GUS) expression was assayed using 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) as described by Jefferson (1987).

Results

Subcellular Localization of LP3 Protein

Transient expression of GUS from pBILP3, containing the *lp3* cDNA and GUS (*uidA*) gene driven by a CaMV35S promoter, was found localized in the cell nucleus in onion epidermis (Figure 1A, B), in slash pine cell suspension (Figure 1C, D), and in tobacco seedling root hair cells (Figure 1E, F) but not in controls.

WDS, Plant Growth Regulator Treatments and Expression

The developmental course of expression from the *lp3* promoter (pGILP3) was studied in two genera-

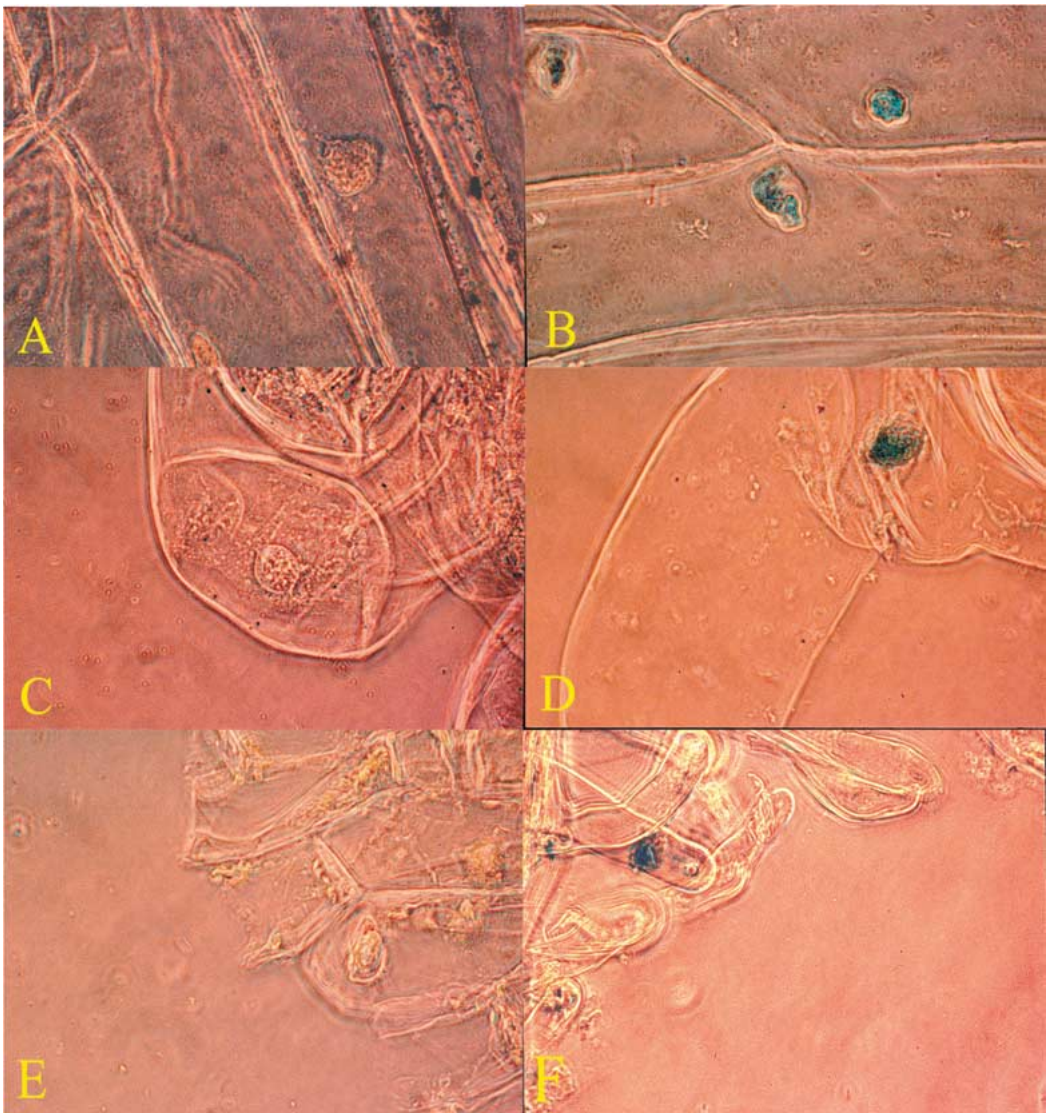

Figure 1. Histochemical localization of CaMV35S driven *lp3* cDNA fused to GUS in onion epidermal cells, slash pine suspension cells, and tobacco seedling root hairs. (**A**) Onion epidermal cells without transformation; (**B**) onion epidermal cells with transformation; (**C**) slash pine suspension cells without transformation; (**D**) slash pine suspension cells with transformation; (**E**) root hairs of tobacco seedling without transformation; and (**F**) root hairs of tobacco seedlings with transformation.

tions of transgenic tobacco: primary transformants (T_0) and the first generation progeny of these plants (T_1). Five to 10 independent transgenic plant lines were regenerated (T_0). Expression from the *lp3* promoter in the T_0 population under nonstressed conditions was intense and observed in root meristematic regions, shoot meristematic regions, most cell types in leaves except the petiole, trichomes, root hairs, stems, pistils of developing floral buds (Figure 2A), developing ovary and embryos (seeds) (Figure 2B), placenta, and developing seeds (Figures 2C). In the T_1 population, expression was observed only during seed germination and early seedling establishment up through 40–60 days post germination.

Transgenic tobacco plants (T_0) containing the *lp3* promoter-GUS construct (pGILP3) were grown in culture and growth room. Both treated and control plants were used. Tobacco plants were subjected to WDS stimulated by PEG, and also treatments of ABA, fluridone, MeJa, GA_3, paclobutrazol, an inhibitor of GA synthesis, 2,4-D, kinetin, and an inhibitor of ethylene action, $AgNO_3$. Members of the same clone were challenged with ethephon, an ethylene generator, in the greenhouse. Expression of GUS was observed in WDS (Figure 2D), fluridone

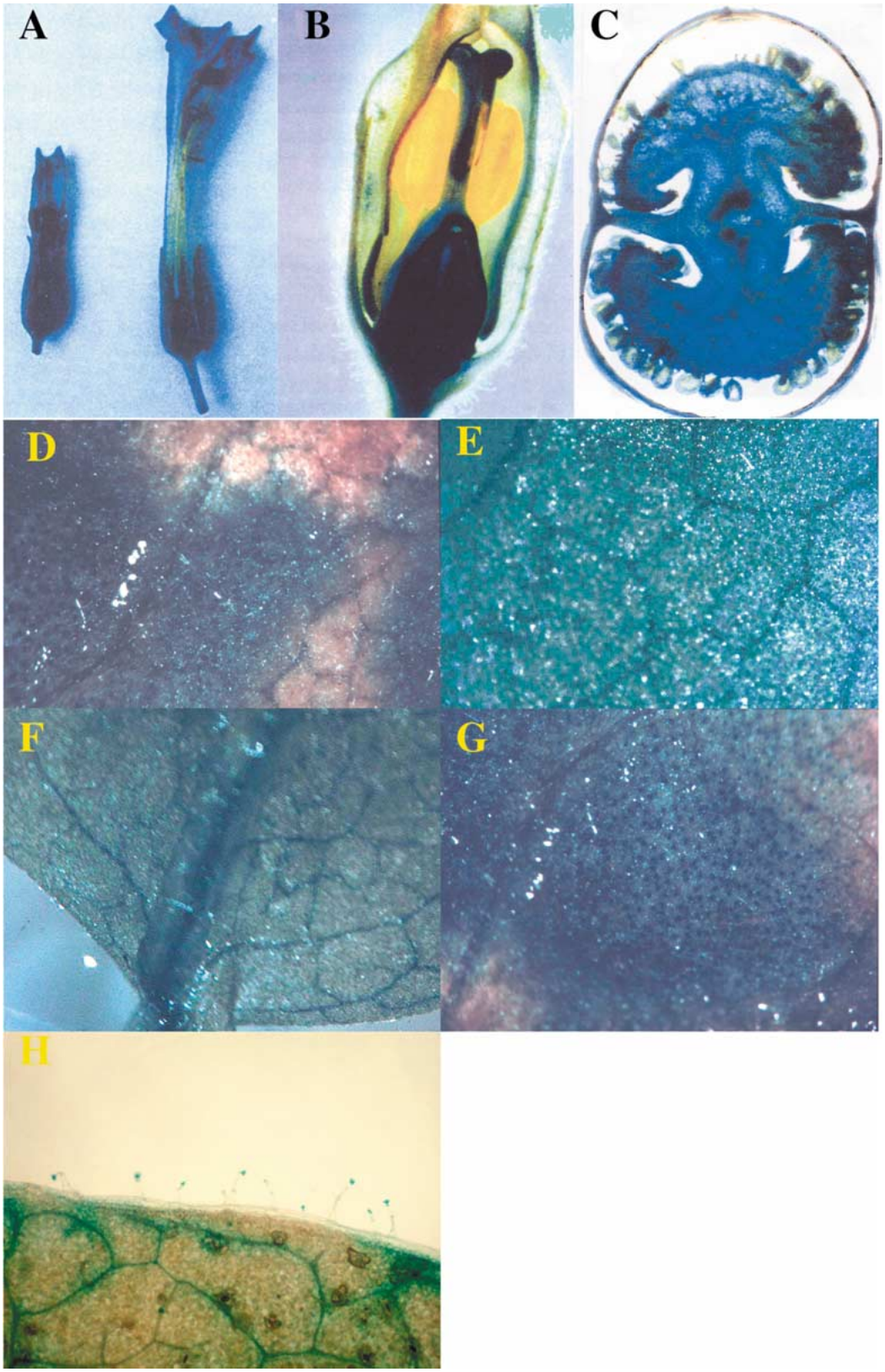

Figure 2. Histochemical localization of *lp3* promoter-driven GUS expression in transgenic tobacco (T_0). (**A**, **B**, **C**) Floral organs of the T_0 population. (**D**, **E**, **F**, **G**, **H**) GUS expression in transgenic tobacco leaves (T_0) 100+ days postgermination following treatment with (**D**) WDS, (**E**) fluridone, (**F**) ABA, (**G**) MeJa, and (**H**) control plant.

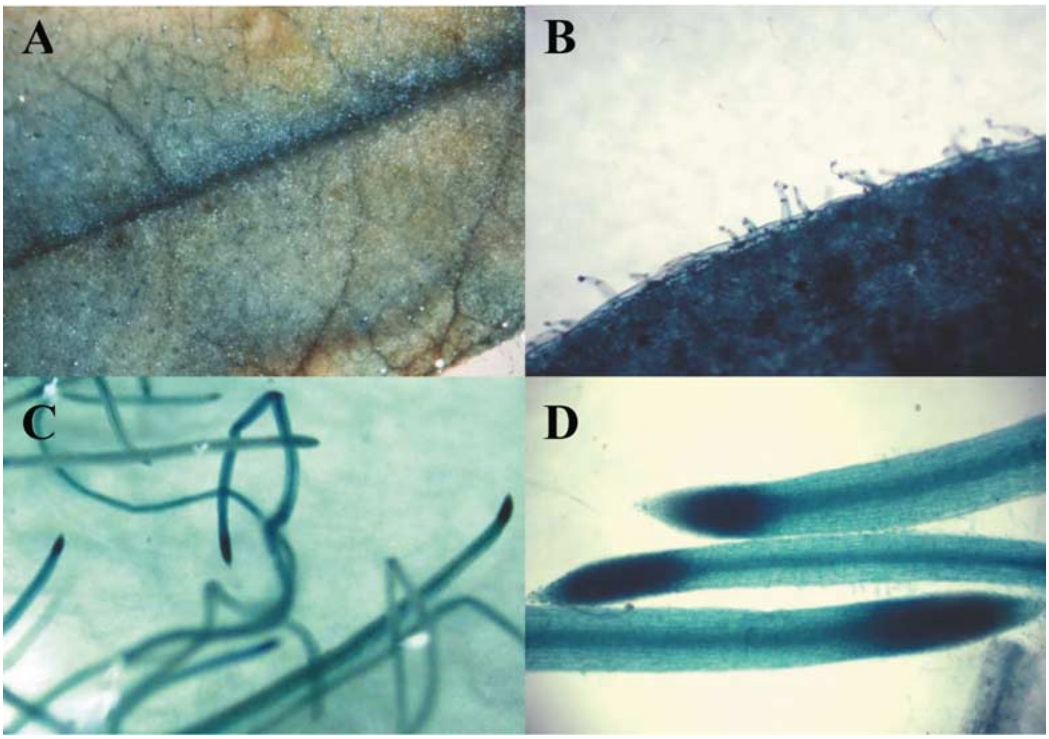

Figure 3. Histochemical localization of *lp3* promoter-driven GUS expression in transgenic tobacco. **A**, **B**, **C**, **D**, represent vegetative organs of the T_1 population 20 days postgermination. (**A**) Leaf showing vascular staining, (**B**) leaf trichomes, (**C**) vascular and tip staining in roots, and (**D**) higher magnification of root.

(Figure 2E), ABA (Figure 2F) and MeJa (Figure 2G) treatment only. However, control leaves only showed GUS expression in trichomes (Figures 2H). Expression with exogenous ABA and MeJa was lower than expression observed with PEG or fluridone. ABA and MeJa gave moderate to low levels of expression in leaf trichomes, leaf, root vascular systems, and in root tips.

Developmental Expression Pattern

In a 20-day postgermination trial using the T_1 tobacco population, expression was associated with leaf midrib (Figure 3A), trichomes (Figure 3B), root vascular tissue system and root tips (Figure 3C), (Figure 3D). Expression was strong at 10 and 20 days postgermination but became weaker at 30 and 40 days. Expression was completely shut down by 60 days postgermination and disappeared. Expression was not observed in floral or reproductive structures. This silencing pattern observed in the T_1 was not observed in the T_0, and in general, expression in the T_1 generation was less intense than that observed in the T_0 population. Methylation of the *lp3* promoter and/or the GUS gene was suspected in this silencing.

Gene Silencing

To test the hypothesis that methylation contributed to the developmental silencing observed in the T_1 population, seeds (T_1) were germinated on media containing 5-azaC, a compound that can block methylation. Seedlings that were germinated for 80 days in the presence of 5-azaC continued to demonstrate GUS expression in roots, stems and leaves, whereas expression in non-treated seedlings had subsided (Figure 4). These results implicate methylation of the *lp3* promoter, or the GUS gene, in transgene silencing observed in these plants.

Discussion

Localization of LP3 in the Nucleus

The *lp3* cDNA sequence was aligned in GenBank, and the putative LP3 protein was highly hydrophilic with a pI of 5.88 (Padmanabhan 1996). The protein also was considered to be nuclear targeted because it has a putative bipartite nuclear localization signal and an alpha-helix at the carboxy terminal. Due to the difficulty of transforming and regenerating loblolly pine, expression of the *lp3* promoter and pro-

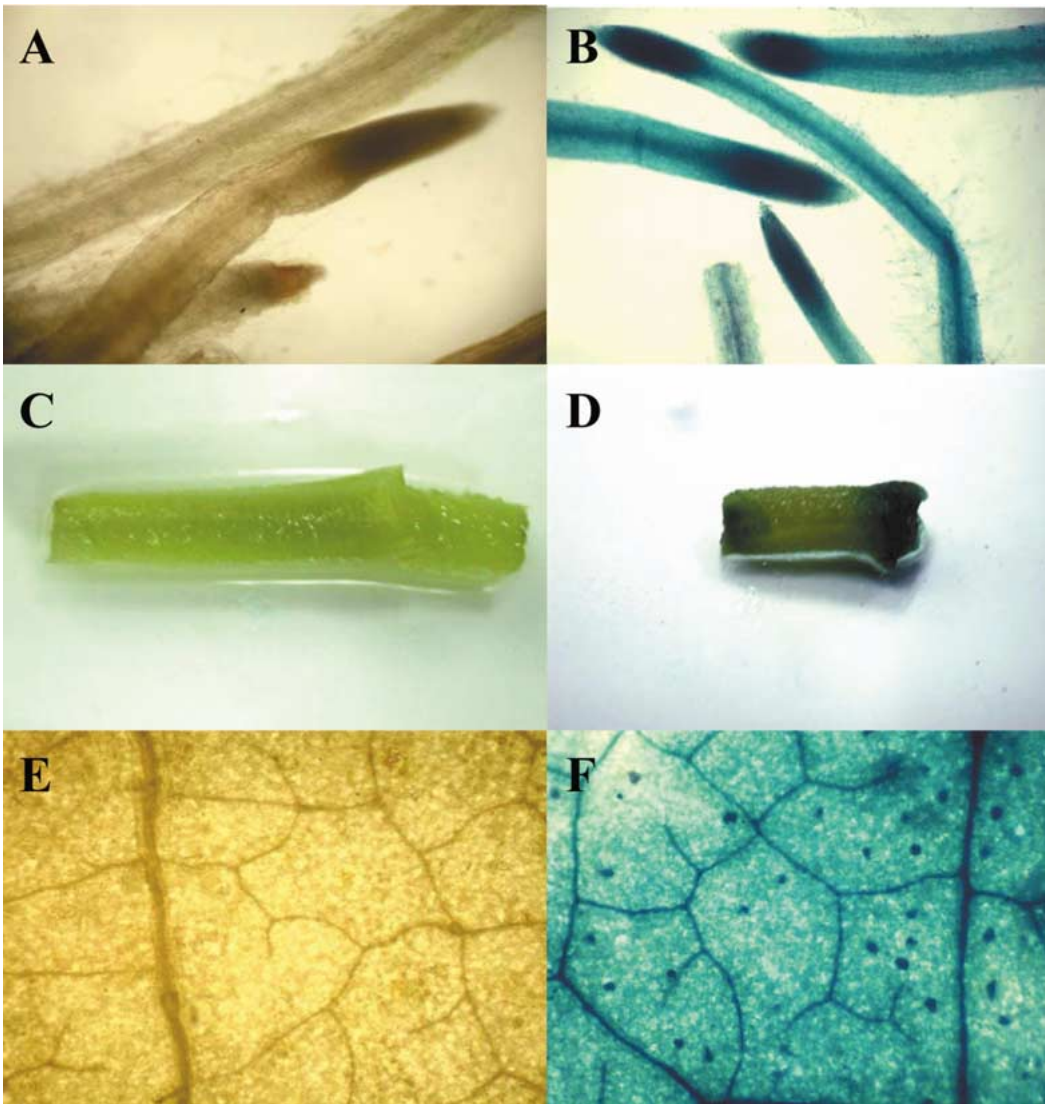

Figure 4. 5-azacytidine (5-azaC) treatment of tobacco plantlets transformed with *lp3* promoter and *uidA* gene and assayed with X-Glucuronide. (**A**) Controlroots; (**B**) 5azaC-treated roots; (**C**) Control stem; (**D**) 5azaC-treated stem; (**E**) Control leaf; (**F**) 5azaC-treated leaf.

tein was studied in transient and stable transformation settings. A homologous pine culture suspension system and a heterologous onion epidermal cell layer were used in transient expression studies. The heterologous transgenic tobacco was used to study expression from stably incorporated genes. Localization of the LP3 protein in onion epidermal cells (Figure 1A, 1B) in the cell nucleus was observed in transient expression studies in pine embryogenic callus (Figure 1C, D), in onion epidermal cells, and in transgenic tobacco (Figure 1E, F).

ASR Homology

In tomato, ASR1, a similar gene to LP3, is localized in the nucleus and is induced by ABA, WDS, and the ripening process (Rossi and others 1996). In this study, LP3 localized to the nucleus and the *lp3* promoter was induced by ABA, WDS, MeJa, and fluridone.

Putative Regulatory Elements In the *lp3* Promoter

The promoter region of *lp3* was sequenced (Padamanabhan 1996) and contains a number of putative regulatory elements, such as ABA (Bray 1993), CaMV (Katagiri and others 1989), GA (Gubler and Jacobsen 1992), Opaque-2 (Varagona and others 1992; Vincentz and others 1997), *rd22* (Iwasaki and others 1995), and MeJa (Rouster and others 1997). Based on this information, expression would be

expected in ABA, WDS, GA, and MeJa treatments; however, the function of these putative elements within the loblolly *lp3* promoter is not known. Ethylene is one of a stress-related plant hormone. In this study, transgenic tobacco seedlings were treated with ethephon, however, expression was not detected. Treatments of GA_3, 2,4-D, kinetin, or silver nitrate were also without effect. These results were expected, because responsive elements for ethylene and auxin were not found along the *lp3* promoter. Although a putative GA responsive element was found, GA treatment did not support expression in this system.

ABA and MeJa

ABA and MeJa treatments induced expression in transgenic tobacco (T_0). The expression pattern seen with MeJa treatments was similar to that observed with ABA. MeJa and jasmonate (JA) are thought to be components of signaling pathways involved in the regulation of plant responses to WDS and defense. These hormones are also involved in fruit ripening (Harms and others 1997; Wang 1999). JA and MeJa are derived from plant lipids, synthesized from alpha-linolenic acid by a lipoxygenase. MeJa has been found to effect changes in oxygen-scavenging activity and in membrane lipid composition under WDS (Wang 1999), enabling tissues to withstand water stress.

WDS and Fluridone

In transient expression and in transgenic tobacco, the *lp3* promoter was induced by WDS and exogenous ABA. Interestingly, fluridone also induced expression. This result was unexpected because other ABA-inducible genes have not been reported to respond to fluridone. Fluridone is an inhibitor of the ABA response. PEG-treated tobacco plants exhibited bleaching in the leaves that was similar to the bleaching observed in the fluridone treatment. Both treatments induced the highest levels of expression within bleached regions of leaves. The reason for this response is not known; however, photo-oxidation in leaves may produce a signal to which *lp3* is responsive. It is possible that MeJa may play a role in this response. WDS will induce the production of endogenous ABA, however, fluridone inhibits biosynthesis of ABA by way of the carotenoid pathway (Doong and others 1993). If ABA synthesis occurred by an alternate pathway in these plants, it could be argued that ABA was responsible for the expression that occurred during fluridone treatments. However, fluridone-treated plants were not drought-stressed and fluridone has not been reported to promote expression of ABA-induced genes in tobacco.

Silencing

In our study, the T_1 generation of tobacco transformed with the *lp3* promoter-GUS construct exhibited a silencing pattern that was tied to seedling maturation. Virtually no expression was detected 40 days postgermination (Figure 2); however, expression was maintained through 80 days postgermination in seedlings germinated in media containing 5-azaC, suggesting that silencing was due to DNA methylation. The 5-azaC is an analog of cytosine that is used to decrease methyltransferase activity in animal and plant systems (McInerney and others 2000) and inhibits DNA methylation (Prakash and Kumar 1997).

Gene silencing in transgenic plants has many origins, but one of the most common forms is transcriptional silencing produced by methylation of the transferred promoter (Fagard and Vaucheret 2000). Methylation of the coding region, here GUS, also occurs and is most often associated with posttranscriptional silencing. Unfortunately, transcriptional silencing is heritable, whereas posttranscriptional silencing can be reversed in the next generation (Fagard and Vaucheret 2000). When methylation is the silencing mechanism, the agent 5-azaC can be used to prevent silencing. Kovarik and others (2000) applied 5-azaC to transgenic tobacco, and observed approximately 30% reduction in methylated cytosine located in a nonsymmetrical context in the 3′ untranslated region of *npt*II transgenes. The hypomethylation was accompanied by a 12-fold increase in NPTII protein levels, suggesting that methylation may account for an increased degree of posttranscriptional gene silencing. In transgenic lettuce, McCabe and others (1999) also found a 50-fold variation in GUS activity and a 16-fold variation in NPTII protein content between T_1 plants derived from different T_0 parents. Reactivation of transgene expression with 5-azaC in partially silenced lines indicated that low levels of expression were associated with DNA methylation.

Summary

In conclusion, the LP3 protein appears to be targeted to the cell nucleus. The physiological func-

tion of LP3 during WDS may be the protection of the nuclear contents from desiccation, but it is possible that it may also be involved in regulation. The protein is structurally similar to members of the ASR gene family and it is active during seed development, maturation, and germination. The *lp3* gene was induced by PEG, MeJa, ABA, and fluridone, but was not induced by kinetin, GA_3, 2,4-D, ethylene, or silver nitrate. Fluridone induced *lp3* gene expression but the reason for this is unknown. Expression of the promoter-GUS construct in the T_1 of transgenic tobacco was silenced 40–60 days postgermination and expression was never recovered. Germination of T_1 seeds on media containing 5-azaC prevented silencing, suggesting that silencing was most likely due to methylation.

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