Transgenic Analysis of a Hybrid Poplar Wound-Inducible Promoter Reveals Developmental Patterns of Expression Similar to That of Storage Protein Genes

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The wound-inducible win3 multigene family from hybrid poplars (Populus trichocarpa × Populus deltoides) encodes proteins with structural similarities with Kunitz-type protease inhibitors (H.D. Bradshaw Jr., J.B. Hollick, T.J. Parsons, H.R.G. Clarke, M.P. Gordon [1990] Plant Mol Biol 14: 51-59), and at least one member, win3.12, is transcribed de novo in the injured and uninjured leaves of wounded trees (J.B. Hollick, M.P. Gordon [1993] Plant Mol Biol 22: 561-572). A previous study demonstrated that 1352 bp of 5′ flanking DNA from the win3.12 gene confers local wound-regulated expression of the β-glucuronidase gene in transgenic tobacco (Nicotiana tabacum cv Xanthi n.c.) (J.B. Hollick, M.P. Gordon [1993] Plant Mol Biol 22: 561-572). We extend this transgenic analysis here by examining the developmental regulation and systemic wound induction conferred by the same transgene construct in tobacco. Biochemical and histochemical surveys of β-glucuronidase activity are described for four, independent transgenic lines. The observed spatial and temporal expression patterns coincide with dormant storage tissues and with previously described expression patterns for both seed and vegetative storage protein genes. Developmental northern blot analysis of win3 RNA levels in poplar seeds confirms that proper temporal expression of the reporter gene is maintained during tobacco seed maturation. These results demonstrate that a putative Kunitz-type protease inhibitor can be wound inducible in addition to being expressed in developing seeds.

The Kunitz-type Ser PIs are a class of plant PIs that have structural features similar to STI, one of the first well-studied PIs. STI was relatively easy to study because large quantities could be purified from mature soybean seeds, where trypsin inhibitors account for approximately 6% of the total protein (Rackis and Anderson, 1964). Due to its abundance in seeds, STI has been considered by some to be a storage protein (Ryan, 1973), a reserve source of nitrogen encountered in working with annual tree material. Molecular studies of reproductive biology are especially troublesome in Populus because the once-a-year production of floral structures does not occur until the trees are 5 to 7 years old. By that time, space requirements are enormous and the material is physically difficult to collect. Additionally, nucleic acid extractions are sometimes impossible due to protective resins and phenolic compounds. The physical architecture of stems, which places the living tissues

Kunitz-type Ser PIs are characteristically expressed in seeds, with the only clear exception being where induction by drought stress was observed (Downing et al., 1992). Seed expression of STI occurs during the latter stages of seed development in soybean (Walling et al., 1986) and at the same relative stage in transgenic tobacco (Jofuku and Goldberg, 1989). Unlike most other plant PIs, however, the seed-prevalent Kunitz-types have not been reported to be wound inducible. We show in this report that a Populus (Populus trichocarpa × Populus deltoides) STI gene is both wound inducible and expressed during seed development. Analogous to the STI example from soybean, regulation of the Populus STI in transgenic tobacco (Nicotiana tabacum cv Xanthi n.c.) seeds is maintained.

We have previously described a systemically wound-inducible multigene family (win3) from Populus whose gene products have structural features characteristic of PIs (Hollick and Gordon, 1993). Two of the win3 family members, win3.6 and win3.12, have the greatest amino acid identity with a wound-inducible trypsin inhibitor (swin1) from willow trees (Salix viminalis) (P. Saarikoski, personal communication), an aspartate PI from potato (Hildmann et al., 1992), legume Kunitz-type Ser PIs, and sweet potato storage proteins (sporamins) (Bradshaw et al., 1990; Hollick and Gordon, 1993). Nuclear run-on experiments demonstrate that de novo transcription of the win3.12 gene occurs in response to mechanical wounding, and transgenic analysis establishes that 1352 bp upstream of the translation initiation site of the win3.12 gene is sufficient to confer wound-regulated expression of the GUS reporter gene in tobacco (Hollick and Gordon, 1993).

We are using transgenic tobacco to study the regulation of Populus genes partly because of experimental difficulties encountered in working with annual tree material. Molecular studies of reproductive biology are especially troublesome in Populus because the once-a-year production of floral structures does not occur until the trees are 5 to 7 years old. By that time, space requirements are enormous and the material is physically difficult to collect. Additionally, nucleic acid extractions are sometimes impossible due to protective resins and phenolic compounds. The physical architecture of stems, which places the living tissues

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Abbreviations: CaMV, cauliflower mosaic virus; DAP, days after pollination; 4-MU, 4-methyl umbelliferone; NPT II, neomycin phosphotransferase; PI, proteinase inhibitor; STI, soybean trypsin inhibitor; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.
around a solid heartwood core, also prohibits the facile extraction of intercellular components. Most of all, the physical size of mature tree material makes a detailed tissue survey using molecular analyses an impractical proposition.

Here we show the environmentally and developmentally regulated expression patterns conferred by the poplar win3.12 promoter in several genetically defined transgenic tobacco lines. The win3.12-GUS (win3-GUS) promoter fusion is systemically responsive to wounding and is also expressed in various vegetative and reproductive storage tissues. Transgene expression is observed in mature pollen and mid- to late-maturation seeds. These transgenic results led us to discover that win3 is also temporally expressed at an analogous developmental stage during poplar seed maturation.

MATERIALS AND METHODS

win3-GUS Transgene

DNA construction details are found elsewhere (Hollick and Gordon, 1993). We have sequenced the entire win3.12 5′ DNA found in this transgene and now know that the win3.12 contribution extends from −1296 to +56.

Marker Segregation Analysis for win3-GUS Transgenic Tobacco

The win3-GUS families used in this report are derived from four unique transgenic tobacco (Nicotiana tabacum cv Xanthi n.c.) lines that, for simplicity, are referred to as lines 1 through 4. The transgene genetics of these families were assessed by scoring the seedlings of controlled crosses for kanamycin resistance (ability to survive on 100 mg/L kanamycin). Crosses were performed as detailed below and now know that the win3.12 contribution extends from −1296 to +56.

Line 1

The T1 individual, JH9-37, of this line produced 100/100 resistant seedlings upon self-pollination, suggesting that the original transformant contained at least three independent transgene loci. The selected T2 individual, JH32-1, produced 72/100 resistant self-pollinated progeny, implying that JH32-1 contains a single hemizygous win3-GUS locus. Some of these T3 progeny were used in experiments that are presented in Figures 1 and 2. The selected T3 individual, JH50-72, appears to be homozygous for the single transgene locus, since 100/100 self-pollinated progeny were kanamycin resistant. These progeny are given the JH71 family designation and are the source material (unless otherwise indicated) used in this study.

Line 2

The T1 individual, JH9-16, of this line produced 21/100 resistant seedlings upon self-pollination, demonstrating non-Mendelian transmission of NPT II activity. Resistant seedlings grew much slower than resistant seedlings from the other lines, and they were also mildly chlorotic. A resistant T2 individual, JH16-3, was both self-pollinated and backcrossed to a wild-type female. The same weak kanamycin resistance appeared in 82/100 of the self-pollinated progeny and 46/100 of the backcrossed progeny, suggesting that JH16-3 contains a single hemizygous win3-GUS locus. Subsequent transmission of this resistance from the backcrossed progeny confirmed this zygosity prediction. The source material used in this study (line 2) is from the T2 family (JH16) derived from the self-pollination of JH9-16.

Line 3

The T1 progenitor, JH9-5, of this line produced 39/48 resistant seedlings upon self-pollination, suggesting that JH9-5 contains a single hemizygous win3-GUS locus. The JH37-1 T2 individual was both self-pollinated and backcrossed, as discussed for line 2. One hundred of one hundred resistant progeny were scored from both of these crosses, suggesting that JH37-1 is homozygous for the same single transgene locus. One of the backcrossed individuals, JH70-1, produced 78/100 resistant seedlings upon self-pollination, thus confirming our zygosity assessment. The source material used in this study (line 3) is from the JH70 backcross family.

Line 4

The T1 progenitor, JH9-63, produced 100/100 resistant seedlings upon self-pollination, suggesting that the original transformant contains at least three independent transgene loci. Both the selected T2 and T3 individuals, JH22-1 and JH51-5, also produced 100/100 resistant seedlings upon self-pollination. The source material for this study (line 4) is the T4, self-pollinated progeny of JH51-5.

Plant Material and Growth Conditions

Derivation of the original transgenic tobacco plants has been previously described (Hollick and Gordon, 1993). Plants were grown first in 2-inch pots until they were approximately 20 cm tall and then transplanted to 6-inch standard pots. Environmental stresses were minimized with twice-daily watering and weekly fertilizing. Plants were grown under a combination of halogen and fluorescent lighting with 16-h daylength. Average light and dark period temperatures were 23 and 21°C, respectively.

Twelve-year-old F1 hybrid poplar trees (Populus trichocarpa × Populus deltoides, clones 47-165 and 50-188) were used for controlled crosses. These trees belong to the University of Washington/Washington State University Poplar Research Program; clones of family 47 have been described elsewhere (Bradshaw and Stettler, 1994).

CROSSES AND SEED HARVESTING

Tobacco crosses were performed by hand. Unopened flowers were physically emasculated and then used 12 to 24 h later as receptive females. Flowers with dehisced anthers were removed and either used as pollen donors or
discarded. For the breeding experiments, seeds from mature seed capsules were bulked from single plants. For the temporal expression studies, independent seed capsules were removed at the appropriate days and the developing seeds were dissected out for GUS assays.

Dormant poplar floral branches were harvested with a pruning pole and subsequently allowed to flush out in a greenhouse. The crossing procedure has been detailed by Stettler and Bawa (1971). Developing seed capsules were harvested from the floral branches at the indicated DAP. Capsules were snap frozen in liquid nitrogen and subsequently stored at -80°C.

**Kanamycin-Resistance Screening**

Tobacco seeds were surface sterilized in 10% bleach for 10 min followed by generous rinses with sterilized H2O. Sterile seeds were placed in Petri plates on Murashige and Skoog media (Murashige and Skoog, 1962) containing 100 mg/L kanamycin sulfate (Boehringer Mannheim). Seedlings were grown under fluorescent lighting at 23°C. Plates were scored after 30 d for seedling viability. Seedlings with no visible Chl were scored as sensitive. Plates were again scored after 30 d for seedling viability. Seedlings with no visible Chl were scored as sensitive. Plates were again scored after 60 d. Resistant individuals were either fully green or, in the case of some JH9–16 derivatives, partially green after 60 d on kanamycin-containing media.

**Woundings**

Leaf designations are based on morphological criteria; the first apical leaf of greater than 2 cm is assigned as number 1 and then each successive basipetal leaf is assigned a consecutive number. Unless otherwise indicated, the number 8 leaves were wounded around their periphery with either a pair of pliers or, where indicated, hemostats (about 20–30 “bites” per wounding episode).

A paper punch was used to obtain tissue samples from the central portion on one side of the midrib from the indicated leaves. Unless otherwise indicated, the same leaf was resampled 8 h after a single wounding in the same relative location on the opposite side of the midrib from the original sample. Where further samples were taken, a region within 1 inch of the original sample was used.

Petiole response to wounding was ascertained by first measuring the GUS activity in the number 10 petiole (unwounded), wounding the number 11 leaf as above, and then measuring the GUS activity in the number 11 petiole 8 h later.

**GUS Measurements**

Protein extracts were prepared as described (Jefferson et al., 1990) and protein content was measured using a standardized Bradford assay (Bradford, 1976). GUS activity was measured as described (Jefferson et al., 1990; Hollick and Gordon, 1993).

**Histochemical Staining**

Tissues were hand sectioned where necessary and incubated in 1 mM X-gluc (Research Organics, Cleveland, OH), 50 mM NaPO4 (pH 7.2) at 37°C for various times (no longer than 12 h). Tissues were subsequently rinsed with 50 mM phosphate buffer, fixed in 20% ethanol, 5% glacial acetic acid, 5% formaldehyde for 10 min, and then washed in multiple changes of 70% ethanol.

**RNA Isolations and Northern Blot Analysis**

Poplar seeds from single capsules were ground to a fine powder with a pestle in a prechilled mortar and then directly thawed in phenol:chloroform (1:1, v/v). The organic slurry was transferred to microcentrifuge tubes containing an equal volume of RNA extraction buffer (Hollick and Gordon, 1993). RNA was precipitated from the aqueous phase with 1 m NH4OAC and 95% ethanol overnight. Resuspended RNA was re-extracted with phenol:chloroform and reprecipitated overnight. Resuspended RNA was quantitated by both spectrophotometry and ethidium bromide-staining samples subjected to agarose gel electrophoresis.

Five micrograms of total RNA from each indicated sample were separated in a 0.8% agarose:6% formaldehyde gel and transferred to a Hybond N+ membrane (Amersham) by capillary transfer in 10× SSC. Equal loading and RNA integrity were confirmed and ascertained by methylene blue staining (Khandjian, 1986) of the ribosomal RNA bands after transfer to the blotting membrane. RNA blots were probed with a win3.12 genomic fragment and subsequently processed as described (Hollick and Gordon, 1993).

**RESULTS**

**Genetic Description of win3-GUS Transgenic Lines**

A T-DNA construct containing both a win3.12 transcriptional fusion (~1296 to +56) to GUS and the selectable NPT II gene was used to generate transgenic tobacco plants as previously described (Hollick and Gordon, 1993). As shown previously, 37 of the 50 kanamycin-resistant transgenotes showed at least a 3-fold increase of measurable GUS activity after wounding of their leaves (Hollick and Gordon, 1993). Using resistance to kanamycin as a dominant transgene marker, transgene inheritance was determined for 24 of the wound-inducible transgenotes; in 25% (6/24) kanamycin resistance segregated as a single locus, in 33% (8/24) it segregated as two unlinked loci, in 37% (7/24) it segregated as three or more unlinked loci, and 2/24 showed non-Mendelian inheritance of the kanamycin resistance. The distribution of transgenotes with multiple insertion events and the observations of non-Mendelian inheritance are consistent with previous reports of Agrobacterium-mediated transformation events, especially those using octopine strains (Spielmann and Simpson, 1986; Topping et al., 1991).

Four wound-responsive primary transformants were bred to obtain genetically defined lines for further study (see "Materials and Methods" for details). One of the selected primary transformants, which gave rise to line 3, displayed a very weak wound response (GUS activity was only 3-fold greater after wounding), whereas the other three were among those with the largest responses (activ-
ities were 10- to 14-fold higher after wounding). On the basis of kanamycin-resistance segregation, we assessed the number and zygosity of unlinked transgene loci found in the four lines used as experimental material (see "Materials and Methods" for details).

Line 1: All of the individual plants in line 1 that are used in this study, unless otherwise indicated, appear to have a single homozygous win3-GUS transgene locus.

Line 2: The primary transgenote of this line transmits the kanamycin resistance in a non-Mendelian manner. Wound-inducible GUS activity is nonetheless transmitted to kanamycin-sensitive progeny, suggesting that NPT II expression (kanamycin resistance) is suppressed in many T2 individuals. Details of these observations can be found in "Materials and Methods." Such regulatory autonomy of linked genes appears to be common (Eckes et al., 1986; Heberle-Bors et al., 1988; Scheid et al., 1991; Bagga et al., 1992; Renckens et al., 1992), but the basis of this is unknown. Because the activities of these two genes are separable, kanamycin resistance is not an accurate indicator of transgene locus number and zygosity in this transgenic background.

Line 3: The individuals that are used appear to be hemizygous for a single transgene locus.

Line 4: These individuals appear to be homozygous for at least one transgene locus, although our analysis does not discriminate whether these individuals are hemizygous or homozygous at other unlinked transgene loci.

Since each locus could potentially have multiple transgenes, we estimated the relative dosage of win3-GUS transgenes in each line by DNA dot blot hybridization. Five micrograms of genomic DNA from five individuals of each win3-GUS line were spotted in equal volumes onto a nylon membrane and hybridized with a radiolabeled win3.12 genomic fragment. No hybridization was detected with nontransgenic tobacco DNA. Hybridization to the transgenic DNA samples was detected and quantified using a phosphoimage analyzer (Molecular Dynamics, Sunnyvale, CA). Using the average hybridization value for line 3 as a reference, we estimate that individuals in both line 2 and line 4 have, on average, eight times as many transgene copies as individuals in line 3, and that line 1 individuals have six times as many (Table I). The observed variations of hybridization values within each line are consistent with our zygosity estimations; variations of hybridization values within line 1 and line 3 are very low, and the variation within line 2 and line 4 is much greater, consistent with the possibility that multiple transgene loci are segregating in various zygosity states in lines 2 and 4.

### Table I. GUS activities from transgenic plant tissues

<table>
<thead>
<tr>
<th>Organ</th>
<th>Promoterless GUS</th>
<th>CaMV 35S GUS</th>
<th>win3-GUS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Line 1</td>
<td>Line 2</td>
<td>Line 3</td>
</tr>
<tr>
<td>Unwounded leaf</td>
<td>7.9 ± 2.1</td>
<td>8,200 ± 1,600</td>
<td>160 ± 60</td>
</tr>
<tr>
<td>Wounded leaf</td>
<td>1.8 ± 1.0</td>
<td>5,200 ± 1,300</td>
<td>560 ± 120</td>
</tr>
<tr>
<td>Unwounded leaf petiole</td>
<td>57b</td>
<td>8,200b</td>
<td>3,700 ± 1,200</td>
</tr>
<tr>
<td>Wounded leaf petiole</td>
<td>50b</td>
<td>6,700b</td>
<td>7,100 ± 1,300</td>
</tr>
<tr>
<td>Roots</td>
<td>110 ± 18</td>
<td>33,000 ± 8,000</td>
<td>3,900 ± 800</td>
</tr>
<tr>
<td>Mature pollen</td>
<td>180 ± 30</td>
<td>200 ± 20</td>
<td>12,000 ± 1,000</td>
</tr>
<tr>
<td>Mature selfed seed</td>
<td>110 ± 5</td>
<td>5,100 ± 500</td>
<td>33,000 ± 4,000</td>
</tr>
<tr>
<td>Relative copy No.</td>
<td>6</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

a GUS activity ± SE in pmol 4-MU mg⁻¹ protein min⁻¹ from five individuals of each line except where indicated. Values are based on measurable fluorescence after a 30-min incubation with methyl umbelliferyl glucuronide substrate. Tissue extracts from the promoterless GUS plants do not show a linear increase in fluorescence over time, suggesting that the reported values reflect endogenous fluorigenic compounds.
b Fluorogenic measurement from only one plant.  Relative transgene copy numbers based on genomic dot blot hybridization analysis (see text).

The Poplar win3.12 Promoter Is Locally and Systemically Responsive to Wounding

Plants from six transgenic lines were used in various experiments designed to measure the response of the win3-GUS reporter gene to wounding. Four of the six lines contained the win3-GUS transgene (lines 1-4), and the other two lines contained the control constructs pBI101 (promoterless GUS) and pBI121 (CaMV 35S-GUS) (Jefferson et al., 1987). In Table I we list the average GUS activities observed in these lines from various tissues.

Transgenic plants containing the CaMV 35S-GUS construct have decreased GUS activity after wounding. Decreased GUS expression measured from the CaMV 35S-GUS construct in response to elevated sugar levels has been reported (Tsukaya et al., 1991). In contrast, GUS activity was greater after wounding in plants harboring win3-GUS transgenes. The absolute increases in GUS activities (measurement of de novo transcription) upon wounding were roughly proportional to the relative transgene dosage (Table I). Histochemically stained wounded leaves (see Fig. 3D) indicated that nearly every identifiable cell type had detectable GUS activity. Staining was particularly prevalent in the vascular tissues, but we do not know whether this preference is due to substrate accessibility or selective promoter activity.

Using histochemical staining (see Fig. 3H), we also observed that GUS activity increased in petioles after wounding the attendant leaf. Internal and external phloem traces and their accompanying parenchymal cells stained for GUS activity only after wounding. We quantitated the changes in petiole GUS activity upon wounding their attendant leaves using the number 10 and 11 leaves from the same plants described above. The absolute increase of GUS ac-
activity in the petioles was even more dramatic than that seen in wounded leaves, but this increase was still roughly proportional to transgene dosage (Table I).

Members of line 1 were used to test for a systemic response after wounding single leaves. We first measured the GUS activities in a large number of upper leaves, wounded a single leaf once, or wounded a single leaf repeatedly over the course of several hours, and then re-measured GUS activities in the same leaves. We were initially concerned that the act of sampling alone (i.e. in the absence of a wounding episode) would elicit a local wound response. To address this concern, we punched out leaf discs from many leaves of several plants at time 0 and then subsequently resampled the same leaves at later times; GUS activities from the two samplings were then compared. The data obtained from one individual are presented in Figure 1 and show that after 5 h there was no change in GUS activities. GUS activities did not change in response to sampling in any of the other four plants that were tested. In contrast, data presented in Table I demonstrate that after only 5 h, a single wounding episode induced large amounts of GUS activity in the wounded leaf. Five hours after the initial sampling, leaf number 13 was wounded once to test for a systemic response. Twenty hours later the same leaves were resampled. Although there was an 8-fold increase of GUS activity in the wounded leaf, there was still no change in the unwounded leaves (Fig. 1). Thus, the unwounded leaves did not respond to our sampling technique or to a single wounding event. We then tested three line 1 plants for systemic induction by multiple woundings (Fig. 2). In contrast to single wounding events, multiple woundings on a single leaf correlated with increased GUS activities in the unwounded leaves. The most significant responses occurred above the wounded leaf, suggesting a primarily acropetal movement of a systemic wound signal. The same enhancement of systemic induction by multiple woundings has been previously mentioned for the pin2 genes of potato and tomato (Peña-Cortes et al., 1988).

The win3.12-GUS Transgene Is Developmentally Regulated in Transgenic Tobacco

In the absence of any apparent environmental influences, all of the transgenic plants we analyzed had significant levels of GUS activity in tissues that are, or are believed to be, sites of nutrient storage. A combination of fluorimetric quantitations and histochemical staining were used to describe these spatial and temporal expression patterns (Table I; Figs. 3 and 4).

Roots

Root tissue was assayed for GUS activity from five individuals of each experimental line (Table I). Although all of the win3-GUS plants had measurable levels of GUS activity, the win3-GUS lines did not display levels consistent with their relative estimated transgene numbers. Among the win3-GUS lines, line 2 individuals had the lowest GUS activity in the roots, yet in all other tissues line 2 individuals consistently had the highest levels of GUS activity. In contrast, and as previously documented, the CaMV 35S-GUS transgene was highly expressed in root tissue. Measurable activity was specific to the root tissues, since soil samples collected from the pots in which the win3-GUS plants were growing had no detectable GUS activity.

Figure 2. The win3.12 promoter is systemically responsive to wounding. Average GUS activities ± se (n = 3) in pmol 4-MU mg⁻¹ min⁻¹ are plotted for each leaf at time 0 (open bars) and 26 h after the initial wounding of leaf No. 11 (filled bars). Leaf No. 11 was repeatedly wounded at 0, 3, 6, 9, and 24 h. Asterisks denote statistically significant (*, P < 0.05; **, P < 0.01) differences between unwounded and wounded measurements. Averages were compared using the two-sample Z test (Freedman et al., 1980).

Figure 1. Multiple samplings do not elicit a wound response. Data from one representative line 1 individual (JH50-5) are presented. GUS activities in pmol 4-MU mg⁻¹ min⁻¹ are plotted for each leaf at time 0 (open bars), 5 h after the initial sampling (hatched bars), and 20 h after leaf No. 13 was wounded once (filled bars). Duration of the entire experiment was 25 h. Leaf No. 1 is the most apical leaf where the lamina length is greater than 2 cm.
Figure 3. Temporal and spatial patterns of win3-GUS expression. X-gluc staining of various transgenic tobacco tissues. A, Longitudinal section of a vegetative apex. B, Exterior of an apical stem segment with the attendant petiole obliquely sectioned. C, Longitudinal section of a floral apex. D, Portion of a wounded leaf with translucent wound marks. E, Transverse stem section with emerging petiole. F, Transverse stem and petiole section immediately below an axillary bud. G, Transverse stem section through an axillary bud. H, Petiole from a wounded leaf in transverse section. I, Pollen from a hemizygous plant (JH70-1). J and K, Developing seed capsules at 12 and 20 DAP, respectively. L, Section of seed capsule at 21 DAP.
Figure 4. The win3.12 promoter confers expression during tobacco seed development. GUS activities in pmol 4-MU mg⁻¹ min⁻¹ ± se are plotted for seeds between 10 and 22 DAP from six transgenic lines. Filled circles represent the average value from 10 seed capsules (2 capsules per five individually self-pollinated plants of each line). Open circles represent the average value from three seed capsules where one individual from each line was used as the only pollen parent to wild-type flowers. Seeds were fully mature (detached from the placenta) by approximately 21 DAP.

Stems

Stem staining is shown for line 3 individuals in Figure 3. Identical patterns of associated axillary meristem staining were observed in the other five win3-GUS lines that we sampled (lines 1-4 and one other unrelated line). The stem region immediately surrounding the vegetative axillary buds and the axillary meristems themselves had the most intensive staining. Panels A through C and E through G in Figure 3 present a three-dimensional view of this region. In sharp contrast, this pattern was not observed in plants transformed with the CaMV 35s-GUS construct, and no staining was detectable in the promoterless GUS plants.

Stems of those plants that have many more transgenes (lines 1, 2, and 4) also showed additional staining in the epidermis, pith parenchyma, and cortex parenchyma throughout the stem (data not shown). Staining in the pith parenchyma was often very mottled. Also notable was the observation that epidermal and subepidermal parenchymal cells stained in those areas where the stem segments were physically squeezed in the process of cutting tissue slices (note bottom of stem in Fig. 3B).

Pollen

Freshly dehisced pollen was assayed for GUS activity. The observed values presented in Table 1 demonstrate that the win3.12 promoter conferred strong pollen expression to the GUS reporter. GUS activities were again roughly proportional to the estimated transgene numbers except that the line 1 individuals had two to three times as much activity as expected. Half of the pollen grains (69/145 counted) from the hemizygous line 3 plants stained positively with the X-gluc substrate (Fig. 3I), confirming that reporter gene expression was specific to gametophytic pollen cells. Maximal GUS activity appeared very late in pollen maturation, since predehisced anthers had very little to nondetectable levels of GUS activity. In contrast to the win3-GUS expression, pollen from the promoterless GUS and CaMV 35S-GUS plants had no enzymatic GUS activity (Table 1).

Developing Seed Capsules

At every day postpollination, whole seed capsules from line 2 individuals were sliced in half and incubated in
X-gluc. Stained capsules from 12 and 20 DAP are displayed in Figure 3, J and K. Most notably, the receptacle portion of the seed capsules stained for GUS activity early on but not later when the seeds were nearly mature. This region was dissected out of developing seed capsules from line 2 plants and GUS activity was quantified; the temporal decreases of GUS activity in this region confirm the histochemical trend (activity decreases from 22,000 to 3,000 pmol 4-MU mg\(^{-1}\) min\(^{-1}\) between 7 and 21 DAP). The soybean vegetative storage protein B promoter has also been reported to confer receptacle expression in transgenic tobacco (Mason et al., 1993). In wild-type receptacles, non-enzymatic fluorogenic compounds increase during seed maturation (0–200 pmol 4-MU mg\(^{-1}\) min\(^{-1}\) equivalents).

**Seeds**

The highest level of GUS activity within each win3-GUS line was observed in seeds (Table I). Again, GUS values among the win3-GUS lines were roughly correlated with relative transgene numbers. Fluorimetric quantitations with dissected embryos and endosperms indicated that the bulk of the GUS activity was localized in the embryo. Dissected embryos and endosperms from line 3 seeds had 1300 and 600 pmol 4-MU mg\(^{-1}\) min\(^{-1}\) GUS activities, respectively. Staining of postimbibition seedlings further localized the majority of embryonic GUS activity to the cotyledons (data not shown).

To define the point in seed development when the win3-GUS transgene is expressed, we fluorimetrically measured the GUS activities from seeds during their maturation. Figure 4 shows the results of this analysis with our six experimental lines. Individual flowers were self-pollinated or crossed as pollen parents to wild-type tobacco flowers. The two different crosses allowed us to compare reporter gene expression based on the dosage of transgenes in embryos versus endosperm tissues. A more limited data set was also generated with reciprocally backcrossed seed (win3-GUS females pollinated by wild-type males). Between 10 and 22 DAP, individual seed capsules were harvested and the seeds were assayed for GUS activity. Most seeds had fully matured (detached from the placenta) by 21 DAP. Seeds harboring the promoterless GUS transgene had endogenous fluorogenic compounds whose levels rose slightly during the mid-maturation period (Fig. 4A). When the CaMV 35S-GUS transgene was provided by the male alone, there was a notable dosage effect as expected (Fig. 4B). According to our measurements, however, these self-pollinated seeds had high levels of GUS activity prior to the mid-maturation stage, but this was not observed with backcrossed seed. In every line, the temporal induction of both the 35S-GUS transgene and the win3-GUS transgenes occurred during the mid- to late-maturation transition (Fig. 4, B–F). Previously reported temporal induction of the CaMV 35S-GUS transgene took place slightly earlier (Fujiwara et al., 1992). The differences between GUS activity levels seen in selfed versus backcrossed seeds were presumably due to differences in transgene dosage. Consistent with this idea, GUS activities of mature seeds derived from reciprocal backcrosses (wild-type pollen donor) were always intermediate values.

The relatively large SE values for the backcrossed line 1 seeds were due to abnormal transmission of transgene activity. Most wild-type flowers pollinated with this particular individual produced seed populations with high levels of GUS activity, yet a few flowers yielded seed lots with unexpectedly low GUS activity. Self-pollinated flowers from this same plant also gave rise to seed batches with significantly reduced GUS activities (four seed samples had values that were greater than 2 sds below the mean values of samples between 16 and 21 DAP). We do not know whether the pollen-donating flowers that produced these peculiar seeds were on the same floral branches. This observation of apparent transgene inactivation was not seen in any of our other crosses. Differential transmission of transgene activity from a single plant has also been reported for the NPT II gene (Deroles and Gardner, 1988).

Upon imbibition, there was a progressive decline in GUS activity in the win3-GUS seedlings, suggesting that the win3 promoter was not utilized subsequent to seed maturation (Fig. 5). In contrast, GUS activities continued to increase in the CaMV 35S-GUS seedlings upon imbibition (Fig. 5).

**Figure 5.** The win3-GUS and CaMV 35S-GUS promoter fusions are differentially regulated during seedling germination. GUS activities in pmol 4-MU mg\(^{-1}\) min\(^{-1}\) ± se are given for 20 pooled seedlings at the indicated days following imbibition for self-pollinated seeds from a line 2 individual (JH9–16) (A) and self-pollinated seeds from a CaMV 35S-GUS transgene (B).
**win3 Is Expressed during Seed Development in Hybrid Poplar**

Based on the above transgenic results, we tested whether *win3* RNA was expressed during poplar seed development. Two poplar hybrids were crossed in the greenhouse during the spring of 1992 and developing seeds were assayed for *win3* RNA by northern blot hybridization. We harvested seeds during most of the mid to late phases of seed maturation, 29 to 49 DAP. Poplar seed from this cross was rapidly released from seed capsules between 55 and 60 DAP, with capsules on the largest branches dehiscing prior to those on smaller branches. Seeds from single-seed capsules were used for most RNA preparations, so developmental identities among the different seed capsules may differ slightly at any one point (i.e. seeds from a capsule 47 DAP found on a large branch might actually be more mature than seeds from another capsule on a smaller branch 48 DAP). The northern blot hybridization in Figure 6 shows that at least one *win3* RNA transiently accumulated during the latter stages of seed maturation. Previous northern blot analysis of samples collected during the spring of 1991 showed *win3* RNA accumulation at 44 and 45 DAP. The relative time when *win3* RNA first appeared in developing poplar seeds (about 70% maturation) was similar to the time when GUS activity dramatically increased in the *win3*-GUS transgenic tobacco seeds (about 68% maturation). We did not detect any *win3* RNA in mature poplar seeds, suggesting that the protein product was synthesized prior to seed desiccation.

**DISCUSSION**

Due to the large physical size and extended juvenile stage of poplar trees, an exhaustive tissue survey by RNA analysis alone is a daunting proposition. As a surrogate, we used transgenic tobacco to determine the spatial and temporal expression patterns conferred by a poplar tree wound-inducible promoter. In a previous communication, we reported that the *win3.12* promoter directs wound-inducible expression of the GUS reporter gene in tobacco primary transgenotes (Hollick and Gordon, 1993). The results presented in this report show that the same promoter is responsive to systemic wound signals in tobacco and developmental cues in both transgenic tobacco and poplar trees. Despite observations of non-Mendelian inheritance, multiple transgene loci, and high transgene copy numbers, we find that transgenic tobacco is a suitable model for examining both environmentally and developmentally regulated woody tree gene expression.

Several vegetative storage proteins are wound inducible, but none of these are highly expressed in seeds (Ryan and Huisman, 1967; Staswick, 1989b). In contrast, most of the seed storage protein genes are not wound inducible (Leah and Mundy, 1989), or at least they have not been tested for such a response. Our transgenic analysis demonstrates that the wound-inducible *win3.12* promoter also confers reporter gene expression patterns that are similar to those of both vegetative and seed storage protein genes. Thus, *win3.12* represents the first reported case of a wound-inducible gene that is also regulated like a storage protein gene in both vegetative and reproductive tissues.

**Transgene Genetics**

Our assessments of loci number and zygosity status were based on the kanamycin resistance conferred by the NPT II gene. Together with other published accounts of non-Mendelian transmission (Budar et al., 1986; Eckes et al., 1986; Deroles and Gardner, 1988; Heberle-Bors et al., 1988; Renckens et al., 1992) and regulatory autonomy of linked genes (Eckes et al., 1986; Heberle-Bors et al., 1988; Scheid et al., 1991; Bagga et al., 1992; Renckens et al., 1992), our transmission results with line 2 demonstrate the limited predictive power of such analyses (see “Materials and Methods” for details). Despite these inherent uncertainties, the variability in GUS expression seen within each line is consistent with our zygosity estimates (see “Results” and Table I). Although other transgenic studies have reported a negative correlation (reviewed by Finnegan and McElroy, 1994), no correlation (Eckes et al., 1986; Shirsat et al., 1989; Peach and Velten, 1991), or a positive correlation (Higgins et al., 1988; Gendloff et al., 1990) between transgene copy number and transgene expression, we find that GUS expression is directly proportional to transgene copy number. Expression in the root tissue was the only exception to this correlation (Table I). A possible chromosomal position effect explanation for this comes from an enhancer-trap experiment that showed that 91% of transgene integrations exhibit expression in roots (Topping et al., 1991). If insertions preferentially occur near root-specific enhancers, then transgene expression in root tissue may be misleading (Twell et al., 1991). Absence of proportionally abundant expression in root tissue of our *win3*-GUS individuals suggests that the *win3.12* promoter used in this analysis does not autonomously confer expression to root tissue.

**The win3.12 Promoter Confers Local and Systemic Wound-Inducible Reporter Gene Expression in Tobacco**

The *win3.12* gene is tightly regulated in poplar trees (Bradshaw et al., 1990; Hollick and Gordon, 1993); there is no detectable RNA in unwounded leaves. The background levels of GUS activity seen in the unwounded leaves of the plants used in this study suggest that either regulation of this gene is weak, or that the promoter is leaky in tobacco or that...
additional regulatory elements are missing in the −1296 to +58 region of \( \text{win3.12} \). However, even residual expression of the \( \text{uidA} \) (GUS) gene may lead to significant levels of GUS activity due to the stability of the UidA protein (Jefferson et al., 1987).

Our observations illustrate that tobacco plants systemically respond to wounding. These data support and add to previous observations that tobacco, like \( \text{Populus} \) and other solanaceous species, can systemically elicit gene expression in response to severe wounding (Peña-Cortes et al., 1988; Pearce et al., 1993; Clarke et al., 1994). Even if the actual extracellular signaling molecules or pathways are divergent between the Salicaceae and Solanaceae families, they must both share a similar intracellular genetic regulatory mechanism that responds to remote signals.

### The \( \text{win3.12} \) Promoter Is Responsive to Developmental Signals

Our survey of a wide range of tissues by fluorimetric quantitation and histochemical staining demonstrates that the \( \text{win3.12} \) promoter confers reporter gene expression in the absence of wound stimuli. Four wound-responsive transgenotes, chosen without any other a priori knowledge, gave rise to lines that displayed identical developmental expression patterns (except root), which argues against chromosomal position effect influences (Breyne et al., 1992). In general, the \( \text{win3-GUS} \) transgene is expressed in tissues known or believed to be involved with nutrient storage: pollen, seedling cotyledons, seed capsule receptacles, and lateral meristematic regions. Expression of storage protein genes in some of these tissues has been shown or proposed to be regulated by increased levels of carbohydrates (Mason et al., 1992; Ohta et al., 1992), nitrogen sources (Ryan and Huisman, 1967; Staswick, 1989a), and ABA (Leah and Mundy, 1989; Ohta et al., 1992). This type of regulation is also analogous to models proposed to explain the response to mechanical wounding. Elevated levels of starch or Suc are often correlated with enhanced wound-inducible gene expression (Wenzler et al., 1989; Johnson and Ryan, 1990; Mason et al., 1992; Ohta et al., 1992), and ABA can directly induce the expression of wound-regulated genes (Dowing et al., 1992; Hildmann et al., 1992). The observations that storage protein genes can also be wound inducible (Jefferson et al., 1990; Mason et al., 1993) suggests that both their developmental and environmental regulation are influenced by common molecular signals.

The \( \text{win3-GUS} \) transgene is strongly expressed in haploid pollen grains. Our observation of 50% GUS-positive pollen from a hemizygous plant rules out sporophytic contamination by intra-locule GUS protein. The observed pollen expression of GUS using the \( \text{win3.12} \) promoter was also much greater than that from the CaMV 35S promoter. Other promoters conferring expression during pollen development in transgenic plants include those of the late anther tomato genes (Twell et al., 1990), an asparagus pathogenesis-related gene (Warner et al., 1993), a maize polygalacturonase gene (Allen and Lonsdale, 1993), and a poplar chitinase gene (Clarke et al., 1994). The LAT52 gene may be distantly related to the \( \text{win3} \) gene, since its protein product also shares similarity to STI (22% amino acid identity). Similar to our \( \text{win3-GUS} \) construct, reporter gene fusions with the LAT52 promoter are also expressed in seeds (Twell et al., 1991).

Both the capsule receptacles and the stem regions immediately surrounding the axillary buds are believed to be sites of transient nutrient storage (Kursanov, 1984; Jefferson et al., 1990; Mason et al., 1993). Axillary meristems, like the apical and intercalary meristems, are nutrient sinks during the vegetative phase. However, unlike the latter, the axillary meristems remain relatively dormant until apical dominance is relieved. This dormancy is thought to be due to the elevated ABA levels seen in this region (Matthysse and Scott, 1984). Expression of the \( \text{win3-GUS} \) transgene in vegetative axillary meristems but not in the vegetative apical meristem or floral meristems implies that strong sink status alone is not sufficient to trigger \( \text{win3-GUS} \) expression. Inhibition of apical meristem expression may result from elevated auxin levels that are known to inhibit Suc modulation of soybean \( \text{vspB} \) expression (DeWald et al., 1994). Of the other six known promoters that confer reporter gene expression in the axillary bud regions, three of them derive from storage protein genes (Jefferson et al., 1990; Ohta et al., 1992; Mason et al., 1993). Since the axillary bud region and the seed-capsule receptacle presumably perform analogous storage roles, the receptacle may be the floral stem equivalent of the vegetative stem axillary bud region. Expression in these two regions conferred by both the poplar \( \text{win3.12} \) (our results) and soybean \( \text{vspB} \) promoters (Mason et al., 1993) further support a functional identity between these two tissues.

The \( \text{win3-GUS} \) transgene was also expressed directly beneath the stipules. Although the functional role of the stipule remains obscure, it is a photosynthetic organ with the potential to store starch reserves (Dry et al., 1992). In \( \text{Populus} \), the stipule is confined to a lateral bulging at the base of the petiole and does not continue into the attendant stem region. The contiguous \( \text{win3-GUS} \) expression between this region under the stipule and the axillary bud may imply that these two regions are both involved in providing nourishment to the lateral meristem.

The most dramatic \( \text{win3-GUS} \) expression is seen in developing seeds. Controlled crosses demonstrate that the expression occurs post-zygotically at the mid-to late-maturation transition. Temporal induction of the \( \text{win3-GUS} \) transgene coincides with both the abundant expression of the major tobacco seed storage proteins (Sano and Kawashima, 1983) and the spike of ABA prevalence (Bustos et al., 1989). Bean \( \text{β}-\text{phaseolin} \) (Sengupta-Gopalan et al., 1985), soybean \( \text{α}1 \) subunit of \( \text{β}-\text{conglycinin} \) (Beachy et al., 1985), pea vicilin (Higgins et al., 1984), barley \( \text{a}-\text{amylase/subtilisin inhibitor} \) (Leah and Mundy, 1989), and STI (Jofuku and Goldberg, 1989) genes, or reporter genes driven by their promoters, are also expressed at a similar developmental time. Like the \( \text{win3-GUS} \) transgene, these seed storage genes are primarily expressed in the developing cotyledons, which provide the bulk of the primary storage reserves for the seedling (Higgins, 1984; Ho et al.,
Poplar seeds like legume seeds absorb their endosperm prior to desiccation, so the entire metabolic reserves are stored in the embryo. Even so, endosperm expression in transgenic tobacco seeds is reported when the promoters for some of these genes are used (Sengupta-Gopalan et al., 1985; Higgins et al., 1988; Bustos et al., 1991; our results), suggesting that factors other than tissue specificity alone (embryos versus endosperm) are responsible for storage protein gene activation.

The Functional Significance of Poplar win3 Gene Products

Structural evidence strongly suggests that the win3 multigene family encodes a set of PIs with highly divergent reactive sites (Hollick and Gordon, 1993). Our expression studies with win3 suggest that they may be involved in nutrient storage in addition to performing a defensive function. Some PIs have anti-herbivory activity (Ryan, 1990), and their frequently cited accumulation upon wounding and their prevalence in nutritive tissues (Richardson, 1977; Ryan, 1973) are consistent with their proposed defensive function. Equally attractive is the theory that PIs regulate endogenous proteases (Richardson, 1977; Peña-Cortes et al., 1988). This theory is especially well supported in the case of seed development (Ambe and Sohonie, 1956; Shain and Mayer, 1968; Kirsi and Mikola, 1971; Royer et al., 1974; Collins and Sanders, 1976). The stable sequestration and subsequent liberation of amino acids from storage proteins require that endogenous proteolysis be tightly regulated. Expression of PIs by the same signals that regulate storage proteins may ensure that endogenous vacuolar proteases (Boller and Kende, 1979) do not degrade newly synthesized storage proteins. These reserves might be subsequently mobilized by the de novo production of more of the same protease, or of another protease with a different substrate specificity. This theory predicts that some storage proteins themselves might have proteinase inhibitor activities. Indeed, sporamins, the major soluble protein of sweet potatoes, are defined as storage proteins (Hattori et al., 1985), but they share sequence identity with known PIs (Hattori et al., 1989) and with win3 (Bradshaw et al., 1990). A large fraction of the soluble proteins in many seeds have been shown or are thought to be Kunitz-type PIs (Richardson et al., 1986; Leah and Mundy, 1991). From an evolutionary standpoint it seems most parsimonious to suppose that regulation of endogenous proteolysis developed prior to defensive mechanisms. This is not to say that PIs are not utilized or recruited for defensive functions. The accurate functional role of plant PIs awaits characterization of their relevant endogenous and/or exogenous proteases.

Herbaceous Species as a Model for Gene Expression in Woody Trees

Using transgenic tobacco as a model system, we now know that win3 is expressed in tissues other than wounded tree leaves. At least one member of the win3 multigene family is expressed during poplar seed maturation (Fig. 6) at an analogous temporal stage to that of our win3-GUS transgene in tobacco. Without an indication of Win3 protein concentration in poplar seeds, it is premature to call it a seed storage protein. However, the expression in seeds and other storage tissues suggests that Win3 plays a role in the storage of nutrients in the seeds.

In addition to environmental influences, we find that there is an analogous response to at least some tobacco developmental signals. Therefore, 1296 bp of the win3.12 5’ region together with 56 bp of 5’ untranslated sequence is sufficient for both developmental and environmental regulation in tobacco. Thus, the transgenic analysis of the poplar win3.12 promoter in an annual herbaceous species has illuminated at least one of the complex regulatory patterns found in a perennial woody species. This finding has practical significance for tree biologists. By examining reporter gene expression in a more experimentally amenable herbaceous species, we can focus our attention in the tree species on particular regions or temporal events in which the gene in question may be expressed. For example, if Win3 is associated with other storage tissues in poplar, then we expect win3 to be expressed in lateral ray cell parenchyma, bark, and over-wintering buds. We further predict that win3 expression might be seasonally regulated like other poplar storage proteins (Langheinrich and Tischer, 1991; Coleman et al., 1993). Using the transgenic herbaceous model, we can reasonably study the gene regulation of individual multigene family members throughout an entire reproductive life span as well as through successive generations. Obviously, some aspects of tree gene regulation cannot be adequately modeled in herbaceous transgenic analyses, due to the peculiarities of the woody perennial growth habit. Even so, these studies validate the usefulness of heterologous reporter gene fusions in herbaceous species as a first approach to studying tree gene expression.

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