ELEMENTS REQUIRED FOR XYLEM-SPECIFIC EXPRESSION ARE LOCATED DOWNSTREAM OF A LOBLOLLY PINE GENE

C. A. Loopstra and E.-G. No[2]

Genetic engineering is likely to be a component of tree improvement activities in the next century. In order for genetic engineering of forest trees to be successful, the isolation of regulatory elements controlling when and where the transgenes are expressed will be needed. Although many promoters isolated from various crop or model plant species or from plant viruses are available, they may not always suit the needs of the forest biotechnologist. For various reasons, it may be important for an introduced gene or sequence to be expressed only in specific tissues or cell types or only in response to specific environmental cues. Occasionally, expression in other locations may be detrimental or even deadly to the plant. At other times, expression of a transgene in tissues where it is not needed may not be harmful, but it may not be useful either, and expression is therefore, a waste of the plant's resources. In some cases, regulatory elements that are satisfactory have been isolated from another species. However, elements regulating gene expression in one species are not always correctly recognized in another, especially when isolated from distantly related species such as the use of a monocot promoter in a dicot or an angiosperm promoter in a gymnosperm. Certain "constitutive" promoters have frequently been used in our early attempts at transformation of tree species. Although these promoters have sometimes been found to be valuable, some are protected by patents and cannot be used for commercial operations. Several tissue-specific promoters isolated from crop species are also protected by patents. For the reasons discussed above and others not mentioned, it is sometimes necessary to isolate regulatory elements from forest trees. Here, we will present the results of our attempts to isolate elements capable of conferring xylem-specific expression in loblolly pine for use in genetic manipulation of wood properties.

We initiated our search for xylem-specific regulatory elements by isolating clones of two genes preferentially expressed in differentiating xylem of loblolly pine. The cloning and characterization of PtX3H6 (Pinus taeda xylem) and PtX14A9 have been previously described (Loopstra and Sederoff, 1995). Transcripts of both are extremely abundant in differentiating xylem, much less abundant in needles and embryos, and non-detectable in megagametophytes. Transcript levels are similar in xylem isolated from the stems of 1, 2, and 10 year old trees and in earlywood and latewood. Expression is slightly lower in stems of very small seedlings (less than 10cM in height). Expression of neither gene is induced within two hours of wounding. We believe these two genes are the% most abundant xylem-specific genes found in loblolly pine. PtX3H6 transcripts are more abundant than those of PtX14A9 and both are much more abundant than those of cinnamyl alcohol dehydrogenase and phenylalanine ammonia lyase.

Genomic clones were isolated for both PtX3H6 and PtX14A9 in order to obtain xylem-specific promoters. Sequence analyses of the PtX3H6 and PtX14A9 promoters have revealed some regions of interest. There are several short sequences (7 to 10 bp) found in both promoters. It is not known if this is due to chance or if they are conserved functional elements. Several 7 to 8 bp sequences are shared by the PtX3H6 or PtX14A9 promoter and that of GRP 1.8, a glycine-rich cell wall protein associated with vascular tissue in bean (Keller and Baumgartner, 1991). One 7 bp sequence is found in all three promoters as well as the promoter of GRP 1.0, another glycine-rich protein found in bean. In the GRP 1.8 promoter, the sequence is found at the 5' end of a negative regulatory element involved in vascular-specific expression. It is possible this sequence is involved in the xylem-specific

[1] Assistant Professor and 'Graduate Research Assistant, Dept. of Forest Science, Texas A&M University, College Station, TX 77843-2135 and TAMU Crop Biotechnology Center

expression of PtX3H6 and PtX14A9 but it is also likely that other elements are involved. Other sequences identified as regulatory elements in angiosperms are also present in the pine promoters. Primer extension analyses have been used to identify both transcription start sites. Both genes contain putative TATA boxes the appropriate distance upstream.

Fusions containing the PtX3H6 and PtX14A9 promoters and *uidA,* encoding GUS, have been tested in transient assays using microprojectile bombardments (Loopstra et al., 1995). A 3.6 kb PtX3H6 promoter and both 750 bp and 1250 bp PtX14A9 promoters were tested by bombardment of differentiating loblolly pine xylem, embryos, and megagametophytes. Promoters of both genes were found to be active in differentiating xylem. However, both the PtX3H6 and PtX14A9 promoter fusions resulted in stained foci on bombarded megagametophytes and embryos. The lack of xylem-specificity was originally attributed to the putative negative regulatory element. Fusions with both promoters were introduced into tobacco and hybrid poplar using *Agrobacterium tumefaciens*. The promoters were active in both systems although the PtX14A9 promoter was poor in tobacco. Again, we did not observe xylem-specific GUS expression. We attributed this to the fact that we were testing conifer promoters in angiosperms. A construct containing a 3.2kb PtX3H6 promoter was introduced into transgenic white spruce *(Picea glauca)* by Dr. David Ellis (U. Wisc. and BC Research) using microprojectile bombardment of somatic embryos and plantlets were produced. We believe this was the first time a pine sequence had ever been introduced into a conifer. Since spruce is much more closely related to pine than tobacco or poplar, we expected to see xylem-specific GUS expression but did not. All three lines produced had very different patterns of GUS expression. The combined results of the transient assays and the transgenic tobacco, poplar, and spruce led us to hypothesize that we were missing elements required for xylem-specificity and that they might be located in a position downstream of the transcription start site rather than in the promoter.

In many cases, a plant promoter and 1 kb of 5' flanking sequence is sufficient to confer correct expression of a reporter gene in transgenic plants. There are however, exceptions to this. Leader introns, 5' flanking sequences further upstream than 1 kb, internal elements, and 3' sequences have been shown to play important roles in controlling quantitative levels of expression, tissue-specificity, and inducibility. Sequences downstream of the translation stop codon have been shown to have regulatory properties in a few angiosperms. We now have evidence that sequences required for xylem-specific expression are found within the 3' untranslated or 3' flanking sequences of PtX3H6. Two different PtX3H6 promoter - *uidA* fusions were tested in transgenic tobacco. Both contained approximately 3.7kb of 5' flanking sequence. One construct contained the NOS (nopaline synthetase) terminator (pBI3H6GUS-5/N) and the other contained the pine PtX3H6 3 ' untranslated region including the terminator and approximately 1 kb of 3' flanking sequence (pBI3H6GUS-5/3). GUS expression in transgenic tobacco was compared. In stems of plants transformed with the pBI3H6GUS-5/N construct (NOS terminator), GUS expression was high in all tissues. In stems of plants transformed with the pBI3H6GUS-5/3 construct (pine terminator and 3' flanking sequences), total GUS expression was greatly reduced and primarily restricted to the vascular tissues. Similar expression patterns were seen in petioles.

Further experiments are needed to determine if the 3' elements required for xylem-specific expression are located in the 3' untranslated or 3' flanking region. There are 7 sequences from 8 to 10 base pairs in length that are found within the 3' untranslated portions of both PtX3H6 and PtX14A9 that are candidates for a 3' silencer. The translated portions of the two genes are not particularly similar. We have not yet sequenced the PtX14A9 3 ' flanking region to determine if any conserved elements are found there also.

Based on the transgenic tobacco results and the conserved 5' and 3' sequences found, it is likely that sequences required for vascular-specific expression are probably located

upstream and downstream of the translated portion of these genes. In order to determine if 5' sequences are also required for xylem-specificity and to examine PtX14A9 expression, the following constructs are currently being tested in transgenic tobacco and poplar. Comparison of plants containing constructs with the same promoter but different terminators or the same terminator but different promoters will help determine the location of essential elements.

Construct	Promoter	Reporter gene	Terminator
pBI3H6GUS-5/N	PtX3H6	GUS	NOS
pBI3H6GUS-5/3	PtX3H6	GUS	PtX3H6
pBI3H6GUS-35S/3	CaMV35S	GUS	PtX3H6
pBI121	CaMV35S	GUS	NOS
pB pBI14A9GUS-5/N	PtX 14A9	GUS	NOS
pBI14A9GUS-5/3	PtX 14A9	GUS	PtX3H6
pB114A9GUS-35S/3	CaMV35S	GUS	PtX14A9

It is possible that 3' elements controlling xylem-specificity do so not by controlling transcription but by affecting RNA stability. Run-on transcription assays are needed to determine the level of gene regulation. Within the 3' flanking region, there are two and one-half repeats of a 30 bp GT rich sequence and two repeats of a 28 bp sequence. Sequences within the repeats may be involved in RNA cleavage and processing.

Since the pBI3H6GUS-5/N construct resulted in higher levels of expression than the pBI3H6GUS-5/3 construct, it is expected that the sequences required for the high levels of expression observed are probably in the 5' flanking region. The PtX3H6 5' flanking region contains two pairs of repeats between approximately -830 and -570, one 63 bp in length and the other 36 bp. It will be interesting to determine if the 5' or 3' repeats contain regulatory elements. Once the required regions are identified, deletions will be used to more precisely locate elements required for xylem-specificity and high expression.

Certain patterns of GUS expression observed in transgenic tobacco containing either the pBI3H6GUS-5/N or the pBI3H6GUS-5/3 construct suggested that auxin may play a role in the regulation of PtX3H6. Plants containing both constructs had high levels of GUS expression in shoot apices and at nodes. Inhibitors of the plant growth regulators auxin, ethylene, and gibberellins were applied to loblolly pine seedlings. RNA was extracted from untreated seedlings and seedlings exposed to 1, 3 and 7 days of the inhibitors. Northern blot analyses were used to examine transcript levels. PtX3H6 transcripts decreased greatly following treatment with TIBA, an auxin transport inhibitor. Uniconazol, a gibberellin inhibitor, had no effect on transcript levels. Silver nitrate, an ethylene inhibitor, caused only a very slight decrease in PtX3H6 transcripts. PtX 14A9 transcripts decreased moderately in response to the TIBA and greatly in response to the uniconazol and silver nitrate treatments. These experiments need to be replicated but it appears that the two genes are regulated by these growth regulators but to different extents.

LITERATURE CITED

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