ANALYSIS OF THE ROLE OF *LEAFY* AND *APETALA-1* GENES IN SOUTHERN HARDWOODS

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Abstract:--In order to determine whether floral production could be manipulated in forest trees, two floral meristem identity genes from *Arabidopsis thaliana (Leafy* [LFY] from D. Weigel and *Apetala-1* [AP1] from M. Yanofsky) were placed under control of either a constitutive promoter or a copper-inducible promoter element and used to transform yellow-poplar *(Liriodendron tulipifera).* Cell lines were transformed by microprojectile bombardment and selected on 100mg/ ml kanamycin plates. Resistant cells were assayed for transgene integration by genomic PCR and for transgene expression by RT- PCR. In order to determine copy number, Southern blots were done on cell lines that were positive according to genomic PCR and RT-PCR tests. *In vitro* plantlets were regenerated from transformed cell lines showing integration, expression, and low copy number. Plants were then potted and placed in a greenhouse for a period of approximately two years. RT-PCR tests revealed that some constitutive API lines lost expression during regeneration: *in vitro* plantlets (100%LFY, 80% AP1), and plants (100%LFY, 50% API). Phenotypic variations of the transgenic plants from wild type were seen in reduced size as well as a reduction in apical dominance. Thus far, none of the plants have shown an ability to flower precociously. RT- PCR tests showed that the copper-inducible promoter element was constitutively expressed under tissue culture conditions even with no exogenous copper added to the medium. In addition, a partial cDNA representing a putative homolog of the LFY gene was recovered from in immature flower buds of yellow-poplar.

INTRODUCTION

Increased understanding of the molecular genetics underlying flower development in the model species, *Arabadopsis thaliana* has led to successes in the control of flowering through gene manipulation (Ma, 1994). This has raised the possibility that the same genes might be used to modulate flowering in forest trees. Tree breeding has always been a slow process due to the long lag to sexual maturity in most tree species. Chemical applications, as well as girdling and other mechanical stresses have been used to induce some species of trees to flower precociously. However, these methods often induce trees to make a phase change to a mature phenotype characterized by slow growth (Meilan 1997). In addition, not all juvenile trees flower in response to these treatments. If trees could be manipulated to flower precociously in a reproducible manner, breeding cycles and generation time could reach levels comparable to that of agricultural crops.

Genes that control flower development also have potential uses in creating sterile trees. Many genes that have the potential to be very useful in forestry, such as insect resistance genes, carry with them the potential to be harmful to the ecosystem if planted stands of transgenic trees were allowed to interbreed with wild populations. These concerns have been heightened by recent observations that pollen from transgenic plants expressing the Bt toxin gene can be lethal to non-target species feeding on native plants proximal to transgenic stands (Losey et al. 1999). It will therefore be critical to develop techniques for creating trees that are sterile if we are to deploy genetically engineered trees. Note that testing sterility in such trees would be significantly less time consuming if flowering could reproducibly controlled.

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Two genes in *Arabidopsis thaliana* that have been shown to reduce the lag time to flowering when over-expressed are LEAFY (LFY)(Weigel et al., 1992) and APETELA I (AP1)(Mandel et al. 1992). These genes were identified by screening for meristem identity mutants disrupted in the transition between the inflorescence and floral meristem (Weigel et al., 1992; Mandel et al. 1992). The Arabidopsis LFY gene under control of the CaMV 35S promoter has been shown capable of inducing precocious flowering in transgenic hybrid aspen trees (Weigel and Nilsson,1995). The flowering time in the trees was shortened to only five months in the greenhouse from a period of 8-20 years in nature. Single flowers arose on the transgenic aspens at the axis where lateral shoots would normally occur in a wild-type seedling. In Arabadopsis, constitutive expression of AP I resulted in a similar pattern of flowering with the lateral, as well as apical meristems forming single flowers.

We tested the LFY and API genes from Arabadopsis to determine their appropriateness for engineering two southern hardwoods yellow-poplar *(Liriodendron tulipifera)* and sweetgum *(Liquidambar styracifula).* Yellow-poplar is a commercial hardwood used for veneer, parts of furniture that are not visible, (i.e. the inside of drawers) and pallets (Leopold et al 1998). Yellow-poplar grows from Vermont to Florida with a western limit between Louisiana and Michigan. It is a tall straight tree that generally grows to heights between 100-200ft and has a primitive, perfect flower. Sweetgum is another large hardwood that grows to heights of 80-150ft, and is used for pulp as well as solid wood products such as furniture, flooring, veneer and pallets *(Ibid.).* It is monoecious with imperfect flowers. Sweetgum grows well in bottom lands and has a growing range from Connecticut to Florida with a western border in Texas. These two hardwoods were chosen for this study because efficient transformation and regeneration systems are available for both species and each is of some commercial importance.

LFY and AP1 were placed under control of either the nominally constitutive CAMV 35S or a copper-inducible promoter (Mett et al., 1993). Additional studies to test function of LFY and API promoters in Liriodendron were run using the GUS reporter gene. A partial clone of a yellow-poplar homolog to LFY was recovered.

MATERIALS AND METHODS

Tissue Culture and Transformation and Selection of Yellow-poplar and Sweetgum Cells

Protocols for tissue culture, transformation, selection, and regeneration have previously been described for yellow-poplar (Wilde et al.,1992; Merkle and Sommer, 1986) and sweetgum (Kim et al., 1997; Merkle et al., 1997).

Subcloning and Plasmid Manipulation

LFY and API promoters and genes were graciously shared with us by Drs. D. Weigel (Scripps) and M. Yanofsky (U.C. San Deigo), respectively. Both genes were sent to us in the agrobacterium-based transformation plasmids pDW-139/LFY(unpublished) and pAM563/AP I (unpublished). These plasmids both have the same general components such as a NPT-II gene for kanamycin selection and the 35S CAMV promoter. Using microprojectile bombardment, these constructs (pDW-139/LFY and pAM563/AP I) were used to transform embryogenic yellow-poplar cells for flower induction studies.

Fusions of the LFY and API promoters with the B-glucuronidase GUS reporter gene were prepared using appropriate restriction digests were ligated into pWLGUS. The pWLGUS plasmid was derived from the plasmid, pSLll80 (Pharmacia, Piscataway N.J.) by adding the GUS gene, 35S promoter, and the

hygromicin resistance gene hph, from PTRA-140 (unpublished). The constructs were used to transform sweetgum as well as yellow-poplar.

In an effort to introduce a level of variable control over the flowering process, a copper-inducible promoter was also fused to the LFY and API genes in a second set of vectors, pPMB7066 that has been previously in tobacco (Mett et al. 1993). The copper-inducible LFY and API constructs were only tested in yellow-poplar.

Nucleic Acid Extraction

RNA extraction was carried out via one of three methods: 1) Grinding in Trizol reagent (Life Technologies Rockville, MD), 2) a modified Trizol protocol; or 3) a RNA isolation procedure developed for pine tissues. For cultured yellow-poplar cells, the Trizol protocol was carried out according to the manufacturers' instructions was sufficient to yield good quality RNA. However, genomic DNA contamination required that a DNase treatment be added after the extraction procedure. Yellow-poplar bud tissue required a modification to the Trizol protocol in order to recover clean RNA. In the modified protocol a blocking agent containing 4.0 M Guanidine thiocyanate, 0.1M Tris-HCL (pH 7.5), and 1% B-mercaptoethanol was ground along with the tissue in a liquid nitrogen-cooled mortar. The blocking reagent was added in a ratio of lml of reagent to every 2 grams of ground tissue. In addition 0.05g of activated charcoal were added per gram of ground tissue to aid in the removal of phenolic materials. Otherwise, the procedure was preformed according to the original protocol. For isolation of RNA from yellow-poplar leaves RNA was isolated by the method of Hughes and Galau (1998). Integration of the transgene in transformed cell lines was verified by PCR using the DNA preparation protocol described by Klimyuk et al. (1993). DNA for southern analysis was prepared from tissue cultured cells using a modified CTAB protocol found by Lasser et al.

Polymerase Chain Reaction

Reverse transcription-PCR was used to monitor expression of LFY and API cell lines that displayed transgene integration. Two-step RT-PCR used Superscript (Life Technologies) reverse transcriptase and the appropriate gene specific primer for LFY (5337n) or API (829n) lines in a separate step to prepare template. This allowed for independent assessment of the degree of contaminating genomic DNA present in the samples. Two-step RT-PCR using an oligo-dT primer was used in homolog cloning experiments. Reverse transcriptase reactions utilizing Superscript enzyme were carried out according to the supplier's protocol with the reaction proceeding for lhr at 48 ^{0}C in a thermocycler. One-step RT-PCR was used to screen API and LFY expression in some cases, because it was faster that the two-step method. One-step RT-PCR was preformed using the Access RT-PCR system (Promega Madison, WI) according to the protocol provided and reactions were primed with the oligonucleotides mentioned above.

Primers for screening transformed lines with the LFY and API transgenes were developed from sequences deposited in Gene Bank. The screening primers for the LFY gene transformants were: (5004s) 5'-GGTACGCGAAGAAATCAGGAG-3' and (5337n) 5'-CAGCTAATACCGCCAACTAAAG-3'. The primers for AP1 screening were: (349s) 5'-GCCGAAAGACAGCTTATTGCAC-3' and (829n) 5'-TCCTCATTGCCATTGGATCATC-3'.

In experiments to amplify a LFY homolog from yellow-poplar using two-step RT-PCR the cDNA was primed with oligodT and subsequently amplified with the degenerate primers (LFY 490n) 5'-GTIGGIACRTACCADAT-3' and (LFY 250s) 5'-GARMGICARMGIGARCAYCCITTYAT-3'. The

product was then reamplified with two nested primers (LFY 275s) 5'-G A YTAY Y TI T T Y CA Y Y TITA Y GA -3 ' and (LFY 360n) 5 '-ARIGGYTTRTARCAIGCYTGICKCCAY-3. The product was cloned directly into the PCR 2.1 using the TA-Cloning Kit (Invitrogen) and colonies were screened by PCR using universal primers for the Ml3 reverse and T7 sites. The clone was subsequently sequenced in both directions and homology analyses were done using the BLAST program available through the NCBI web site (http/www.ncbi.nlm.nih.gov/).

All PCR experiments included plasmid controls and a water blank to control for contamination.

Southern Analysis

Yellow-poplar lines transformed with the LFY and AP 1 constructs that showed transgene integration and expression by genomic PCR and RT-PCR screens were subsequently analyzed by Southern Blotting (Southern, 1975). Southern blots were probed using the AlkPhos kit (Amersham) to label full-length versions of either the LFY or API genes. Gels were run overnight and blotted to a charged nylon membrane. All wash and probe steps were preformed according to the supplied protocol from Amersham. The Southern blots were developed using CDPstar (Amersham) as described in supplier's protocol with an exposure time of 2.5 hours.

GUS Staining

The GUS reporter gene was utilized to determine whether the LFY and AP1 promoters retained floral-specific expression patterns in yellow-poplar and sweetgum. GUS staining was carried out using the protocol described by Jefferson et al. (1992).

Greenhouse Care

Plants regenerated from transformed and control cell lines were maintained in a greenhouse under natural light for a period of two years. The plants were watered and fertilized regularly.

RESULTS AND DISSCUSION

Two different genetic lines of embryogenic yellow-poplar cells were transformed with the over-expression constructs (35S:LFY and 35S:AP1). The kanamycin-resistant lines were tested first for gene integration using genomic PCR. Lines which showed stable integration were retained for further study, all other lines were discarded. Relatively few kanamycin-resistant lines were discarded suggesting that rearrangement events leading to loss of the Arabadopsis genes during integration were infrequent. In fact, only five resistant lines, all having received the API construct, were shown not to contain the transgene and had to be discarded.

RT-PCR was used to test whether the integrated transgene was expressed properly in the transformed cell lines and the results of RT-PCR tests are summarized in Table l. All of the cell lines harboring the 35S:LFY construct expressed the transgene. Sixteen of the 35S:AP1 lines lost transgene expression at the cell culture stage, and fifteen of those sixteen lines had the same genetic background. Instability of the API transgene expression was further demonstrated by loss of expression when plants were regenerated from two lines of the second genetic background.

In addition to loss of expression, lines harboring the API construct also demonstrated what appeared to be reduced expression compared to lines transformed with the LFY transgene. They did not yeild a strong signal in RT-PCR (faint bands Fig. I) used to test for transgenes expression preformed under identical conditions for both LFY and API, even though the AP 1 primers worked very well on the plasmid construct. Although suspect as a quantitative method, bands of similar intensity should have been apparent since both transgenes were driven by the same promoter. The observed difference in band intensity might have resulted from mispriming of the API primers within the genome, or from the API transgene being silenced in some way by the host cells. AP1 is a member of the MADS-box family of genes and

Table l. Summary of RT-PCR experiments on35S:LFY and 35S:AP1 yellow poplar. LFY expression was detected in all of the 35S:LFY lines tested in cell culture and regenerated plants. API expression was detected in 80% of cell cultures and 50% of regenerated plants. Letters designate uniquely transformed cell lines.

Construct	Kanamycin-R	In vitro Expression	No Expression in Vitro	Lines and # of Regenerated Plants	Expression in Plants
35S:AP1;91	11	A,C,E,F,G, J,H,K	B	A(4),B(10),E(15), F(15),C(6)	None in A
35S:AP1;92 (1)	20	B,D,R	C,H,K,P	C(15),R(16)	None in C 80% in R
35S:AP1;92 (2)	15	D,K,O,S	A,B,C,E,G,H,I, L,M,N,Q	NONE	NONE
35S:LFY; 91	13	A,B,C,E,F,I, J,L,M,N,O	NONE	C(16),E(4),A(13), J(15),F(15)	100% Expression in A,J,F,C

mispriming could have occurred because of the strong sequence homology that the genes in this family display. However, mispriming does not satisfy our finding that expression from API transgenes in some cell lines was in several cases turned off by an unknown mechanism (Table 1). We suspect that in addition to mispriming constitutive expression of the API transgene product is deleterious to yellow-poplar cells, and as a consequence the gene tends to be silenced over time in transformed cells.

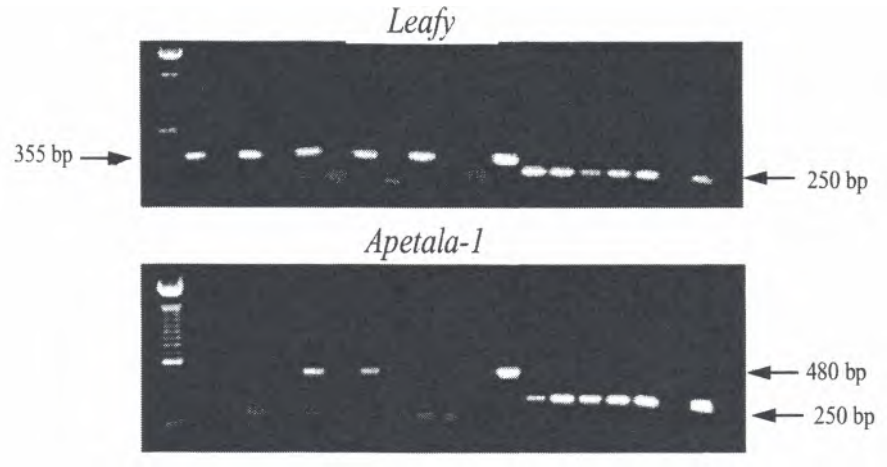

Figure l. RT-PCR of LFY and API genes in transgenic yellow-poplar. Both gels were loaded with alternating +RT and -RT. The 250bp band corresponds to the GAP-DH control used for each cDNA tested.

Southern analyses were used to determine whether cell lines contained multiple copies of the LFY or AP I transgene and lines having more than three repeating copies of the genes were not regenerated into plants (Fig 2). In general about 20% of the kanamycin resistant cell lines contained only I or 2 copies of the repetitive transgene

Yellow-poplar plants regenerated from cell lines harboring the 35S:GUS reporter gene fusion are indistinguishable from wild-type plants. Although such plants might have arguably served as the best controls for this study, we opted to use untransformed, regenerated plants for comparison with regenerated transgenic plants.

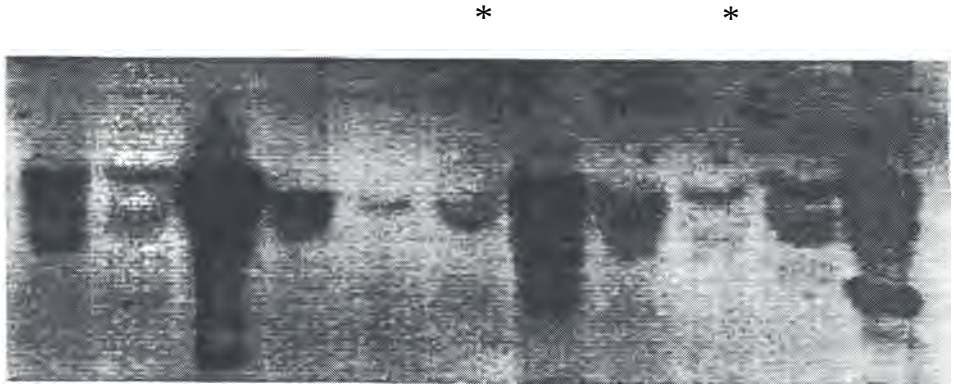

Figure 2. Southern analysis of the AP 1 gene in 35S:AP1 yellow-poplar. To determine copy number, Southern blots were probed with the full length Arabadopsis LFY or API gene. **ASTERISKS denote** transformants harboring only one or two copies of the transgene.

Plants harboring the 35S:LFY and 35S:AP1 transgenes were compared to plants regenerated from untransformed lines of the appropriate host genotype. The plants have been maintained in the greenhouse for a period of over two years, and thus far, no flowers have been seen. However, the plants do have a distinctive phenotype (Fig 3). The 35S:LFY and 35S:AP1 transformants exhibit a reduced growth rate and reduced apical dominance compared to wild-type plants. Also, the transformants appeared "bushy" due to shortened internode lengths. The "bushy" phenotype found in these transformed yellow-poplar trees is similar to what is seen in the Arabadopsis LEAFY mutant (Weigel et al., 1992). Therefore, it is apparent from the phenotype that the transgenes are playing a role in yellow-poplar meristem identity, as expected, and that the pathway affected is similar to the one in Arabadopsis. Phenotypic differences were not apparent in comparisons between plants harboring the two different transgenes, suggesting that both transgenes had similar effects. This finding supports the assumption that the overlapping roles played by the LFY and API genes in Arabadopsis is reflected in yellow-poplar. Unfortunately, the transformed plants did not display any phenotypic characteristics desired by the forest products industry.

Because of the difficulties we had in maintaining constitutive expression of the API gene in transformed yellow-poplar, we attempted to used an inducible expression vector based on elements of the copper-response system from yeast. (Mett et al., 1993). Kanamycin-resistant cell lines containing copper-inducible versions of the transgenes were tested using RT-PCR to determine under what conditions the transgene would be expressed. LFY and API transcripts were both expressed constitutively under normal tissue culture conditions (data not shown). In addition, plants regenerated from all lines harboring the copper-inducible AP 1 transgene also demonstrated constitutive expression in soil under normal growing

conditions (data not shown). Thus, the copper-inducible expression system appears to be too leaky for our desired uses in tissue culture or whole tree situations.

To identify promoter elements that might be useful for the development of sterile trees, we tested tissue-specific actin promoters, as well as the LFY and API promoters. The ACT2 promoter had been shown to express constitutively in Arabadopsis (An et al., I 996) while expression of the ACT 11 promoter was]imited to reproductive tissues (Huang et al.,1997). ACT2:GUS and ACT11:GUS fusions were used to transform yellow-poplar cells, and kanamycin-resistant colonies were assayed for GUS expression. Staining showed that the ACT1 1 promoter was very active in embryogenic yellow-poplar cells while the ACT2 promoter showed no expression in cultured cells. This finding would appear to highlight the difficulty in finding promoters active in reproductive tissues, but not in embryogenic cultures. Since most forest tree transformation systems utilize embryogenic cultures such promoters will be critical to the development of sterile trees.

35sAP1

35sLFY

Figure 3. Comparison of 35S:AP1 and 35s:LFY yellow-poplar to a wild-type regnerat. Plants are all of the same age and have been grown in a greenhouse for a period of one and a half years.

LFY and AP 1 promoter/GUS fusions were also examined for floral-specific expression in yellow-poplar and sweetgum. Both the LFY and API promoters were active in embryogenic cells of yellow-poplar and sweetgum cells; thus, neither promoter would be useful for cell ablation strategies for sterility in forest trees.

Part of a putative LEAFY homolog was amplified from yellow-poplar using PCR. The clone was 288 base pairs in length and had high homology to the 3' end of the Arabadopsis LFY gene (Fig 4). BLAST analysis showed that the isolated fragment was also very similar to a LFY homolog from *balsamifera,* although results appeared somewhat skewed because a full-length gene from yellow-poplar was unavailable for analysis. Efforts to extend the clone to full length are continuing.

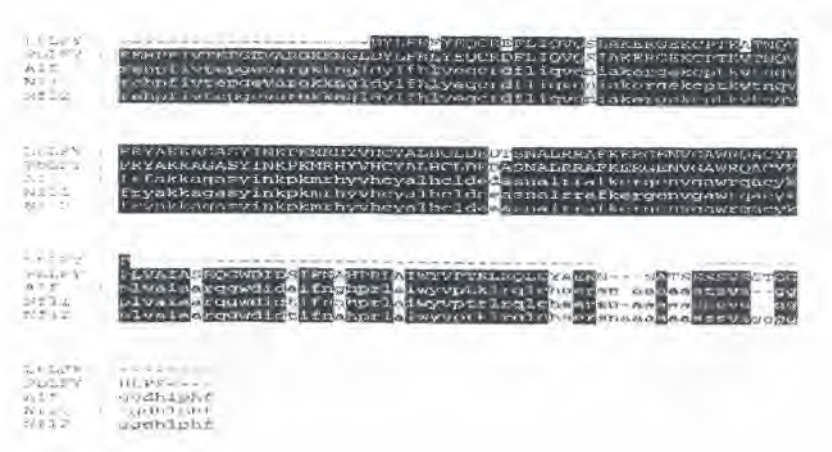

Figure 4. Alignment of putative LFY homolog from arabadopsis with homologs from Populus (PbLFY), Anterinum (Alf), and two LFY homologs from tobacco (Nfll and Nfl2).

CONCLUSIONS

This study showed that the Arabadopsis LFY and API promoters are expressed under normal tissue-culture conditions in embryogenic cells of yellow-poplar and sweetgum. Therefore, neither of these promoters is of much use for cellular ablation strategies in engineering sterility in forest trees. Similarly, the ACT11 promoter was also expressed in embryogenic cell lines. Expression of the ACT1 1 promoter in embryogenic yellow-poplar cells might be useful in situations where a gene needs to be expressed in tissue cultured cells, but not in regenerated plants. However, transgenic plants need to be analyzed in greater detail to determine whether the promoter is active in any other tissues. Studies of a copper-inducible promoter system found that the system was leaky throughout yellow-poplar tissue culture and regeneration. Thus, there is still a need to identify an inducible –promoter system that can routinely be used under the conditions required for genetic engineering of forest trees.

This study also showed that over-expression of the floral meristem identity genes, LFY, and AP l, from Arabadopsis was not sufficient to cause precocious flowering in yellow-poplar within a reasonable time frame. Yellow-poplar transformed with 35S:LFY and 35S:AP1 plants have yet to produce floral organs of any kind after more than two years of growth in a greenhouse. This is in contrast to work done in hybrid aspen which showed that expression of the LFY transgene could lead to flowering in a few months. The transformed yellow -poplar did exhibit a phenotype of reduced apical dominance, slow growth and shortened internodes. This phenotype seems consistent with the action of genes responsible for controlling meristem identity. Thus far, hybrid aspen transformed with 35S:LFY has been the only tree to exhibit precocious flowering in response to a floral development transgene. Precocious flowering is a must for researchers to properly test sterility in a reasonable amount of time. The finding that the meristem identity genes were insufficient to promote precocious flowering demonstrates the lack of knowledge in this area and that more research is needed on the genes and pathways that control flowering in trees. More studies are also needed to determine whether other transgenes, chemical applications, or mechanical stresses are required to determine whether precocious flowering can be achieved in forest trees.

Population increases will demand higher outputs from the forest products industry from reduced planted acreage and biotechnology is one of the most promising avenues for meeting the demand predicted in the future. Increased research into sterility and other aspects of flowering will be imperative if we are to deploy the genetically engineered trees necessary to meet the demand of future forestry.

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