

Promoter Deletion Analysis of Xylem Cysteine Protease 2 (XCP2) in *Arabidopsis thaliana*

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in
partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In

Horticulture

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May 2, 2007

Blacksburg, Virginia

Keywords: *Arabidopsis thaliana*, cysteine protease, promoter deletion, tracheary element, xylem

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(ABSTRACT)

The process of xylem tracheary element differentiation involves the coordination of vascular cambium activity, cell fate determination, cell expansion/elongation, secondary wall synthesis, programmed cell death, and cellular autolysis. The end result of tracheary element differentiation is a cellular corpse lacking a protoplast and consisting of a thickened cell wall composed mostly of lignin and cellulose. Little is known about the genetic mechanisms regulating the process of tracheary element differentiation. *XCP2* expression localizes to tracheary elements according to two independent methods of analysis: promoter reporter experiments and immunogold localization by electron microscopy. *XCP2* may be involved in catalyzing the degeneration of the protoplast during the final autolytic stages of tracheary element differentiation. To this date *XCP2* function has not been directly demonstrated. In principle, any tracheary element-specific markers can be linked to upstream regulatory genes with roles in tracheary element differentiation. To develop the *XCP2* promoter as a tool for identification of transacting factors, a promoter deletion analysis was carried out. Utilizing information from 5' and 3' deletion constructs, a 70-bp region upstream of the *XCP2* translational start site is both necessary and sufficient for TE-specific expression of the *Uida* reporter gene. Mutational analysis of the ACTTTA element at position -113-bp strongly suggests it is a *cis* element required for *XCP2* expression. *In silico* analysis of an 18-bp promoter region located within 200-bp of the translation start site and including the ACTTTA element revealed high identity shared between xylem-specific *XCP2* homologs from *Zinnia elegans*, *Populus trichocarpa*, and *XCP1* from *Arabidopsis thaliana*.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Eric P. Beers for his guidance in the pursuit of this project and for allowing me to pursue graduate studies in his laboratory. I would also like to thank my committee members Dr. Jerzy Novak, Dr. Gregory Welbaum, and Dr. James Tokuhsa for their advice and support in my graduate research.

I would also like express my gratitude to my friend and colleague Dr. Chengsong Zhao for always being there with excellent scientific advice in molecular biology and for his help in proofreading this document. I would like to thank Maura Wood for her valuable assistance in formatting this document. I would like to thank my colleagues Emily H. Grant and Jeff Stroud for their assistance.

I want to express my thanks to the National Science Foundation for supporting this research and for continuing to support basic scientific research in Agriculture.

The beginning of this project was significantly aided by the efforts of several undergraduate students. I would like to express my gratitude to Sara Anderson, Katie Schlesinger, Mark Levy, and Ben Sayler for their hard work.

I would like to express my thanks to my parents Earl Petzold Jr. and Diane Petzold for all their support towards my education throughout the years. I would like to thank Eileen Strahl for her constant support during my research and for her help in proofreading this document. I would like to thank Kayla and Jake Petzold for riding into work all those times and helping me take care of things.

I would like to dedicate this thesis to Eileen, Kayla, Jake, and Brayden.

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Chapter 1

Literature Review

Wood is a plant product of vast agro-economic importance as a renewable resource and potentially for the production of biofuels. Wood is produced as a result of growth of secondary xylem, one of the tissues that comprise the vascular system of plants. The plant vascular tissue system also includes cambium and phloem. Xylem and phloem arise from the procambium during primary growth and from the vascular cambium during secondary growth in stems and roots. The cambium (a lateral meristem) is located between the xylem and phloem and produces the xylem toward the center and phloem (inner bark) toward the periphery. An additional lateral meristem, the cork cambium, develops outside of the phloem and is responsible for producing the outer bark (periderm) cells, cork and phelloderm. The plant vasculature mediates the long distance conductance of water and solutes, and provides structural support to plant stems.

The phloem conducts sucrose, RNA, proteins and many types of small signaling molecules, whereas xylem is active primarily in the transport of water, minerals and amino acids. A variety of microorganisms are also known to move through the vascular system (Purcell and Hopkins, 1996). The xylem consists of vessels, fibers and parenchyma cells. Vessels are composed of vessel elements. Tracheids are another type of water-conducting cell in the xylem. Together, vessel elements and tracheids are known as tracheary elements (TEs). Tracheary elements lack a protoplast at maturity but retain their patterned, thickened secondary cell walls consisting largely of lignin and cellulose. (Mauseth, 1988) Essentially, the TEs of xylem are cellular corpses that serve the function of water and solute conductance throughout the plant. During TE differentiation the cells of the procambium (primary xylem) or the vascular cambium (secondary xylem) become highly active in cellulose and lignin biosynthesis and initiate a cell suicide program that culminates in complete cellular autolysis. Thus, differentiation of TEs is an example of programmed cell death (PCD). For this reason, the vascular system of plants is an excellent model for the study of developmentally programmed cell death.

Given the essential functions performed by the xylem, normal plant growth and development is expected to be dependant on proper differentiation of TEs. Indeed, plants harboring mutations resulting in loss of vascular tissues are severe dwarfs and do not survive past the seedling stage (Cano-Delgado et al., 2004; Zhao et al., 2005) Only a few such mutants have been isolated by forward genetics screens and characterized (reviewed by Ye, 2002). Hence the identification and characterization of rational candidates for reverse genetics of vascular tissue regulatory mechanisms is an important objective that can complement ongoing forward genetics screens. Reverse genetics experiments can begin with a gene known to be expressed in the tissue of interest or under specific conditions. Beers and colleagues have been working to identify genes that exhibit vascular tissue-restricted expression patterns in *Arabidopsis* (Zhao et al., 2000; Zhao et al., 2005). One such gene, the xylem TE-specific gene *XYLEM CYSTEINE PROTEASE2 (XCP2)* ([AT1G20850](#)), is the subject of this research.

XCP2 is a C1A family, papain-like cysteine protease (Beers et al., 2000). Papain is employed in a wide variety of industrial, home and medical applications (Begley et al., 1990; Yeh et al., 2002; Pieper and Caliri, 2003). In plants, cysteine proteases belonging to the C1A family have been implicated most frequently in protein remobilization in germinating seeds (Cercos et al., 1999; Taneyama et al., 2001; Sutoh and Yamauchi, 2003), senescencing leaves (Weaver and Amasino, 2001), petals (Wagstaff et al., 2002), and other plant organs (Xu and Chye, 1999). In the studies conducted by Woffenden et al. (1998) TE autolysis was inhibited when cysteine protease inhibitors were applied. More recently, the papaya (*Carica papaya*) C1A protease papain was found to function as part of a defense mechanism against predation by larvae from the order *Lepidoptera* (Konno et al., 2004). In support of this finding, the cysteine protease inhibitor E-64 was also found to abolish the toxicity of papaya latex against *Lepidoptera* sp. (Konno et al., 2004). Another papain family member, the maize cysteine protease *mir1*, has been shown to inhibit *Lepidoptera* larval feeding (Pechan et al., 2000). Cysteine proteases have also been implicated in the hypersensitive response (HR) to plant pathogens. The *Cladosporium* gene *Avr2* was shown to inhibit the C1A protease Rcr3 as a component of signaling leading to the HR in tomato plants (Rooney et al., 2005). The potential for cysteine proteases to mediate nutrient remobilization, plant-herbivore and plant-pathogen interactions and TE formation

indicate that these enzymes are of great fundamental and economic significance to fruit production, seed germination, plant stress response and defense and wood production.

The TE-specific protease *XCP2* and its paralogous, co-expressed partner *XCP1* (Pechan et al., 2000; Zhao et al., 2000; Zhao et al., 2005) are two members of the 30-member multigene family of C1A proteases in *Arabidopsis* (Beers et al., 2004). From studies with papain it is known that C1A proteases are synthesized as higher molecular weight precursors known as zymogens or prepropeptides (Vernet et al., 1991). Zymogens themselves are inactive consisting of a targeting prodomain, a prodomain and a catalytic domain. The prodomain and prodomain must be proteolytically cleaved in order for the enzyme to become active (Cygler and Mort 1997). Both *XCP1* and *XCP2* share 44% amino acid sequence identity with papain across the entire preproprotein. The *XCP1* and *XCP2* prepropeptides are 70% identical to each other at the amino acid level and exhibit even greater identity 80% (Funk et al., 2002) when the prodomain alone is considered. The high degree of identity shared by the prodomains of papain, *XCP1* and *XCP2* is important when considering the regulation of protein targeting (Ahmed et al., 2000) and enzyme activity and function (Taylor and Scheuring, 1994), and suggests similar functions for *XCP1*, *XCP2* and papain (Funk et al., 2002). While at least one of the functions for papain—insect feeding deterrence—is known, functions for *XCP1* and *XCP2* have not yet been determined. Ectopic expression of *XCP1* resulted in stunted growth, early senescence, and reduced fertility (Funk et al., 2002). Single-gene knockouts for either *XCP1* or *XCP2* and double knockouts for *XCP1/XCP2* exhibit normal growth and development under a variety of favorable and stress conditions in the laboratory. *XCP1/XCP2* double knockout are currently being evaluated at the ultrastructural level to determine whether TE differentiation is altered in more subtle ways not detectable at the whole-plant level (C. Haigler and E. Beers, unpublished).

Hormonal control of TE differentiation

Several plant hormones have been implicated in the development of TEs. Auxin and cytokinin are required for the induction and differentiation of TEs in the zinnia mesophyll cell suspension culture system (Sato et al., 1997). Endogenous auxin levels have also been shown to affect the number of TEs present in xylem (Sachs, 2000). The elongation of TE's also depends on gibberellic acid (GA); application of compounds which block GA biosynthesis were found to

inhibit TE elongation (Inada and Shimmen, 2000). Inhibition experiments utilizing brassinosteroid biosynthesis inhibitors also resulted in the inhibition of TE elongation (Yamamoto et al., 1997).

The most widely studied plant hormone is auxin. The promoters of many genes upregulated by auxin possess auxin-response elements (AuxREs) that bind transcription factors known as auxin response factors (ARFs). ARFs are encoded by a multi-gene family consisting of 23 members in *Arabidopsis* (Hagen and Guilfoyle, 2002; Remington et al., 2004). A few ARFs have been characterized and shown to bind to the *cis* element motif TGTCTC (Guilfoyle et al., 1998; Hagen and Guilfoyle, 2002). The action of ARFs depends on their ability to dimerize, through their conserved domains III and IV, with members of the family of Aux/IAA response proteins. There are currently 29 Aux/IAA family members in *Arabidopsis* (Remington et al., 2004). The large family sizes of ARFs and Aux/IAAs is apparently exploited by plants for regulation of a very large number of processes mediated through unique dimerizations between ARFs and Aux/IAA proteins (Ulmasov et al., 1999; Liscum and Reed, 2002). The gene *IAA8* is one member of the AUX/IAA family known to interact with ARFs (Guilfoyle et al., 1998). *IAA8* activity has been linked to early transcriptional regulation of the differentiation of TE's in both *Zinnia* tissue culture, and in *Arabidopsis* (Groover et al., 2003; Zhao et al., 2005). Zhao et al. (2005) found that *IAA28* and *IAA19* also exhibited xylem-biased expression. Hence, at least three Aux/IAA genes may have roles in regulation of xylem differentiation. Xylem-biased ARFs were not noted by Zhao et al., (2005), however, indicating that xylem specificity of ARF/IAA interactions may be conferred by IAA proteins. In addition to its well documented role as an ARE, the *cis* element TGTCTC and its inverted repeat GAGACA have also been linked to auxin-independent sulfate deficiency response (Maruyama-Nakashita et al., 2005). *XCP1* and *XCP2* possess TGTCTC or a nearly identical sequence AGTCTC, in the case of *XCP2*, within 13-bp upstream of the predicted TATA box. Previous work identified a 30-bp region of the *XCP1* promoter containing this TGTCTC element to be necessary for TE-specific expression of GUS in *Arabidopsis* (Ismail, 2004).

GUS as a reporter system

Numerous studies have utilized the bacterial *uidA* gene that codes for β -glucuronidase (GUS) for reporting tissue specific gene expression *in planta* (Miller et al 1999; Regan 1999; Fukuda 2004, and LI et al., 2006). The promoters under investigation are cloned as transcriptional fusions with GUS, these transgenes are used to transform plants and the resulting GUS activity patterns are indicative of promoter activity. The data revealed by promoter::GUS fusion deletion analysis has the potential not only to reveal important *cis* elements but also to result in the development of useful tissue-specific promoters for plant genetic engineering. *XCP2* expression occurs in TEs, thus offering a unique opportunity to develop the *XCP2* promoter as a tool for both understanding the process of PCD and targeted gene expression in the developing xylem.

Yeast one-hybrid system

The yeast one-hybrid system (Y1H) is a variation on the yeast two-hybrid system (Y2H). Y1H, like Y2H, is based on functional domains of transcription factors arising in eukaryotic organisms. Both systems utilize the activation domain (AD) and DNA binding domain (BD) from the GAL4 protein in *Saccharomyces cerevisiae*. GAL80 represses transcription of GAL4 when glucose is present. The GAL4 protein is involved in galactose metabolism in yeast, when galactose is present GAL4-response elements (REs) are bound by the protein upstream of genes involved in galactose metabolism referred to as the upstream activating system (UAS). Since GAL4 is under tight regulation it is useful as a tool for the utilization of reporter gene constructs in yeast strains containing *gal4* and *gal80* genes.

Y1H in principle enables researchers to identify and characterize proteins that bind to specific *cis* DNA sequences located in the promoter regions upstream of the transcription site leading to enhanced transcription of the target gene. Usually the potential *cis* elements are cloned into the vector pHis2 as a multiple tandem repeat. The vector pHis2 contains the nutritional reporter *HIS3* allowing for the yeast strain 187, a His auxotroph, to grow on media deficient in histidine. The Y1H assay also contains a second component which is a cDNA library inserted into a vector containing a constitutive activation domain to create a fusion protein. The cDNA library is then screened for proteins that contain the DNA binding domain of

a potential transcription factor that can interact with the target DNA located in the vector pHis2 (Clontech Laboratories, 2001; Lopato et al., 2006). Y1H has been used successfully to identify several transcription factors in plants, among these are the bZIP transcription factor (CpbZ1P1) located by using the CpC2 promoter from the resurrection plant *Craterostigma plantagineum* (Ditzer and Bartels, 2006) and two drought responsive proteins in maize that are both members of the AP2/DREBP transcription factor family from the abscisic acid responsive gene *RAB17* (Kizis and Pages, 2002).

Chapter 2: Materials and Methods

2.1 Overview of cloning and transformation

Standard recombinant DNA techniques were utilized to amplify and manipulate DNA by first utilizing the pGEM–T Easy Vector system, Promega Corp. Madison, WI as a shuttle vector, then cloning the recombinant DNA into the appropriate binary vector followed by transformation of *Agrobacterium tumefaciens* strain GV3101.

Transformations of *Escherichia coli* strain DH5 cells were conducted as outlined in the technical manual for the pGEM–T Easy Vector system. *Agrobacterium tumefaciens* strain GV3101 was used for transformation with recombinant plasmid DNA according to the method outlined by (Chen et al., 1994), except the liquid nitrogen freezing step was replaced by a heat shock method that consisted of placing the cells in a 37°C water bath for 1 minute, then immediately placing them on ice for 2 minutes. 0.8 ml of 2XTY was then added to the tubes containing transformed cells. Cells were incubated at 28°C for 2 hours with shaking at 250 rpm. *Agrobacterium* cells were then pelleted by centrifugation at 5,000 rpm for 30 sec and re-suspended in 100µl of 2XTY prior to spreading on a LB media with antibiotics kanamycin, rifampicin, and gentamicin.

Arabidopsis thaliana ecotype Columbia was used for all experiments.

Transformation of *Arabidopsis* was conducted by the floral dip method (Clough and Bent, 1998). Transformed plants were selected by spraying 7-day-old plants with Finale (Basta) Herbicide (active ingredient, Glufosinate ammonium 0.03%) followed by re-application one week later.

2.1.1 Vectors

The vector used for construction of 5' deletions of *XCP2p* fused with GUS was pBI121.3 constructed by C. Zhao by modifying pBI121 (Jefferson et al., 1987) to include a greater variety of restriction endonuclease sites (Zhao, 2005a).

The vector used for construction of 3' deletions of *XCP2p* was modified from vector p1060 (Ismail, 2004). Vector p1060 contains the 35S minimal promoter fused to the GUS reporter gene.

The new vector (p2060) was constructed by digesting vector p1060 with *Hind III*, blunting with Klenow and digesting with *BamHI*. Next, the multi-cloning site from pFGC5941 (<http://www.arabidopsis.org/servlets/TairObject?type=vector&id=500300075>) was ligated into vector p1060 by digesting with *Sma I* and *BamHI*, thus creating p2060.

2.1.2 Promoter Deletion 5' constructs:

Constructs for the 5' unidirectional deletions were inserted into vector pBI121.3. The promoter regions of *XCP2* (*XCP2p*) were amplified from genomic DNA using the primers listed in Table 2 via the polymerase chain reaction (PCR). The amplified promoter regions were cloned into pGEM-T easy then sequenced at the Virginia Bioinformatics Institute (VBI) (Blacksburg, VA), for confirmation that mutations had not been introduced during PCR. Primers used contained an *Xba I* site at the 5' end and a *BamHI* (underlined) site at the 3' end allowing for digestion and subsequent cloning into pBI121.3 (Fig 1). The antisense primer used for all of the 5' deletions is XCP2P2 (5' - GGATCCAAAGAGCCGTTTGAG-3') this primer contains a 1 nucleotide mutation to change the initiator methionine codon from ATG to ACG.

2.1.3 Promoter Deletion 3' constructs:

Constructs for the 3' unidirectional deletions were inserted into P2060 by first cloning the promoter region into pGEM-T easy then sequencing at VBI. Primers used contained a 5' *Pac I* site and a *BamHI* site at the 3' end allowing for digestion and subsequent cloning into P2060 (Figure 2). The primers used were PacI-D12 at the 5' end and the 3' deletion primers listed in Table 3. PacI-D12 primer is 300-bp upstream from the transcription start site, and is used as the 5' end for all the 3' deletions.

2.1.4 Analysis of GUS Activity

Newly emerged true leaves from transformed plants were analyzed for GUS activity by placing them in GUS histochemical staining buffer (100 mM Na²HPO₄, pH 7.0, 10 mM EDTA, 0.5 mM K₄Fe(CN)₆, 0.5 mM K₃Fe(CN)₆, 0.1% Triton X-100, 1mM filter-sterilized X-Glu (Oono et al., 1998). The staining buffer was vacuum infiltrated for 5 minutes at room temperature and incubated overnight at 37°C. Samples were de-greened by two brief 70% ethanol washes

followed by overnight incubation in 95% ethanol. The tissue and cell-type specific GUS localization were detected by using a light microscope.

2.1.5 Construction of Deletion 8 (D8)::XCP2GFP

D8::GUS vector construct was digested with *Bam*HI and *Sma*I allowing for XCP2 open-reading-frame and GFP to be ligated into the vector. The new vector was named *D8XCP2::GFP*. XCP2 was amplified by PCR from a xylem cDNA library and inserted into pGEM-T Easy vector using 5' primer GGATCCATGGCTCTTTCTTCACCTTCA with *Bam*HI added and 3' primer CCCGGGCTTAGTTTGGTGGGGAAAGA with *Sma*I added. GFP was digested from vector pBI121.2 GFP (Zhao, 2005b).

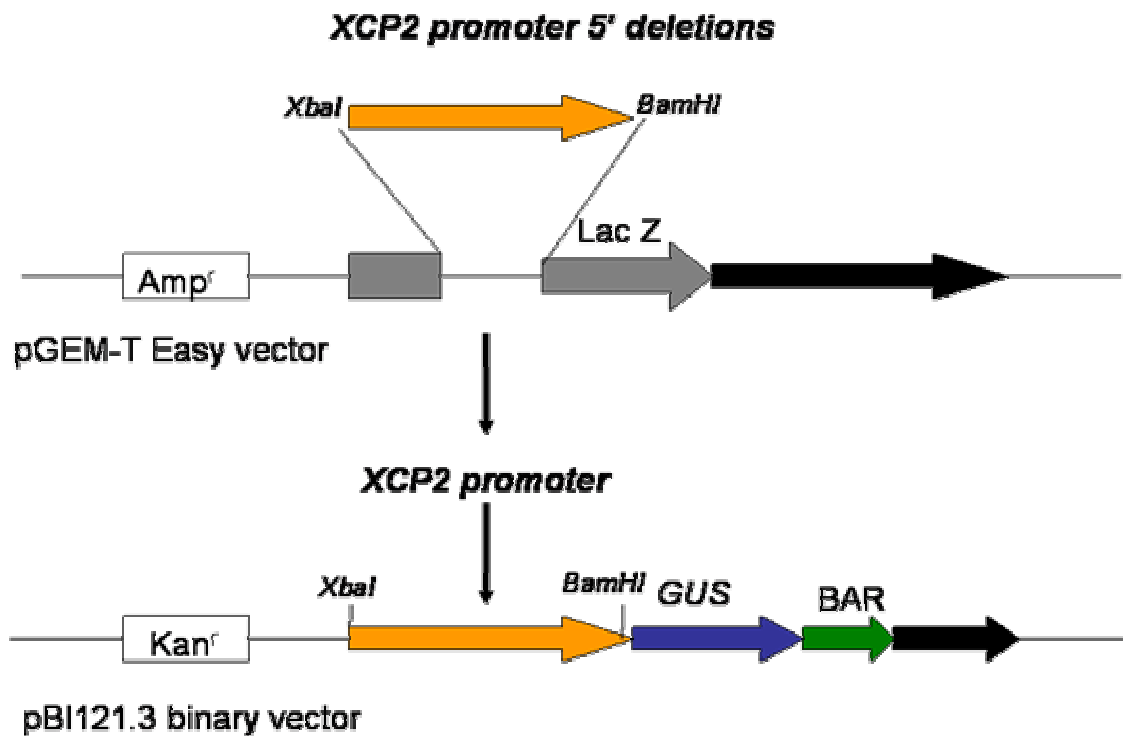


Figure 1. Strategy used to construct the *XCP2* 5' deletions into vector pBI121.3. Grey, regions flanking multi-cloning site in pGEM-T-easy vector. Black, vector DNA. Orange, *XCP2* promoter regions. Green, Basta resistance (*Pnos-Bar-Tnos*). Blue, *GUS*.

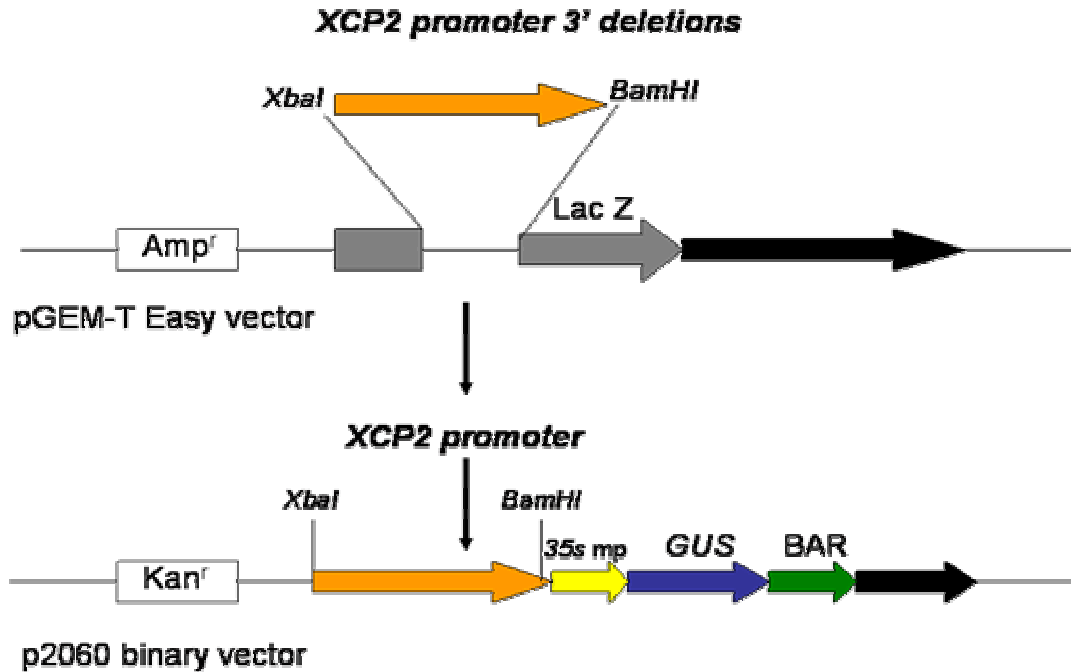


Figure 2. Strategy used to construct the *XCP2* 3' deletions into vector p2060. Grey, regions flanking multi-cloning site in pGEM-T Easy vector. Black, vector DNA. Orange, *XCP2* promoter regions. Green, Basta resistance (*Pnos-Bar-Tnos*). Blue, GUS. Yellow, 35S minimal promoter for basal transcription machinery.

2.2 Yeast one-hybrid-system:

Matchmaker yeast-one hybrid screen was used according to the user manual for BD Matchmaker™ Library Construction and Screening Kits (BD Biosciences, Palo Alto, CA). Proteins that act in *trans* can be identified by screening a cDNA library for proteins capable of interacting with putative cis elements present in the bait vector. The 42-bp fragment (Fig. 6) from -146 bp through -104 bp upstream of the transcription start site for *XCP2* (the bait) was cloned into the region upstream of the minimal promoter for *HIS3* in vector pHIS2, as a tetrameric repeat. The tetrameric repeat was created as follows. Double restriction sites were introduced to the ends of the primers X2-CisS and X2-CisAS used for amplification of the bait region (*Hind III* with *Bgl II* at the 5' end, and *BamH I* with *Sma I* at the 3' end) (Table 3). Digestion of vector containing a single copy of the bait sequence using *Sca I* (vector single cutter

that disrupts the amp^r gene) and *BamHI* yields a large (2000bp) DNA fragment consisting of the majority of the vector including the bait sequence but disrupted in the amp^r gene. *Sca I/Bgl II* digestion of the same vector yields a smaller (1200bp) fragment containing only the bait fragment and the complementary piece of the amp^r gene. Ligation of these two fragments via the common *Sca I* sites and compatible *Bgl II/BamHI* overhang doubles the copy number of the bait sequence and restores ampicillin resistance and ensures that only correctly ligated vectors are recovered on ampicillin-containing plates. Additionally, as the *Bgl II/BamHI* site is destroyed upon ligation, additional rounds of doubling the bait fragment can be carried out by repeating the above steps. Yeast cells transformed with pHis2 can grow on media lacking tryptophan by utilizing the *TRP1* cassette on the vector. Vector pGADT7-Rec2 is the vector utilized for insertion of the cDNA library (the prey) and it also complements leucine auxotrophy via the *LEU2* expression cassette. Hence, selection for transformation by pGADT7-Rec2 is accomplished on leucine drop-out media plates. If a yeast cell is co-transformed with the bait and the prey protein encoded by the prey cDNA is an in-frame fusion with the Gal4 activation domain, the prey fusion protein can bind the putative *cis* element for *XCP2p*, thus activating the reporter (*His3* gene) Yeast cells will then grow on media lacking histidine, leucine, and tryptophan. 3-amino-1,2,4-triazole (3-AT) is used in some cases with yeast cells transformed with the bait pHis2 vector containing the *cis* element(s). 3-AT is a competitive inhibitor of the yeast *HIS3* protein (His3p) and is used to inhibit low levels of leaky His3p expression, thereby suppressing background growth on SD medium lacking histidine. The prey DNA can then be amplified by PCR using vector-specific primer pairs and the PCR products can be sequenced using the T7 primer. Alternatively, and perhaps more ideally, plasmid is isolated from yeast for sequencing and retesting in yeast with appropriate controls.

2.2.1 Construction of first strand cDNA library and synthesis of double-stranded cDNA

The Qiagen Plant RNA minikit was used as per directions for isolation of 100 mg of total RNA isolated from 4-6 week old *Arabidopsis* plants grown as described by Zhao et al., (2005). First strand cDNA synthesis was performed by using 2 µg of total RNA utilizing the Advantage 2 PCR kit using the Oligo (dT) primer (Clontech). RNA was isolated by the RNA easy kit (Qiagen), and the first strand cDNA was synthesized prior to amplification by Long Distance PCR utilizing the CDIII and BD SMART III anchors for the ds cDNA library. The CDIII and BD SMARTIII anchors allow for the facilitation of cloning of a single ds cDNA molecule into vector pGADT7-Rec2 by *in vivo* homologous recombination. Double stranded cDNA was synthesized by using the Long distance PCR primers provide by Clontech and 10% of first strand cDNA as the template. The Long Distance PCR protocol utilized the Advantage 2 reagents and the program followed was (94°C 1' 1x; (94°C ° 30'' 68°C 6'*) 25x; 68° 5' (* 5-sec extension per cycle). Post synthesis the double stranded cDNA was purified with BD CHROMASPIN™ TE-400 columns for removal of small fragments of ds cDNA. The quality of the ds cDNA was then analyzed by running an aliquot equal to 8% of the cDNA on a ethidium-bromide 1% agarose gel.

2.2.2 Isolation of plasmid DNA from yeast

Plasmid isolation from yeast was based on the method by (Robzyk and Kassir, 1992). Four mL of overnight yeast culture grown in SD drop out media was centrifuged at 13,000 rpm/14,500 rcf for 2 minutes. The pellet was resuspended in 100 µL STET (8% sucrose, 50 mM Tris pH 8, 50 mM EDTA, 5% Triton X-100). 200 mg glass beads were added to the mixture and vortexed vigorously for 5 min, another 100 µL of STET was then added. Samples were boiled for 3 minutes, followed by cooling on ice prior to centrifuging for 10 minutes at high speed. Next, 100µl of supernatant was transferred to a sterile centrifuge tube followed by addition of 50 µl of 7.5 M NH₄OAc. Samples were then incubated at -20 degrees C for a minimum of 1 hour. After incubation samples were centrifuged for 10 minutes to remove impurities that may hinder transformations. 200 µl of ice cold EtOH was added to 100 µl of supernatant to precipitate the plasmid DNA, followed by centrifugation for 10 minutes. The supernatant was discarded and the remaining pellet was washed with 70% EtOH. Following the wash step the pellet was dried

overnight at room temperature and resuspended in 20 µl of sterile water. Plasmid DNA thus prepared can be used to transform *Escherichia coli*.

Chapter 3: Results

3.1 Deletion analysis of *XCP2* promoter

3.1.1 Deletion analysis of the 5' end of *XCP2p*

The *XCP2* (AT1G20850) promoter (*XCP2p*) regions were cloned from genomic DNA isolated from wild-type *Arabidopsis thaliana* ecotype Columbia. Expression of *GUS* driven by the *XCP2* promoter should result in a blue precipitate forming in the cells that express the authentic *XCP2* gene, when the *GUS* substrate X-Gluc is present (Figure 3). *XCP2* is known to be expressed in the developing xylem of roots, stems, and floral organs (Funk et al., 2002). All transgenic plants that tested positive exhibited *GUS* expression localized to TEs.

Transcriptional fusions with *GUS* coding sequence were made using several deletion constructs beginning with deletion 1 (D1). D1 consist of a region 1967-bp upstream of the of the translational start site for *XCP2* (Fig. 4). Each 5' promoter deletion utilizes a common 3' anti-sense primer that ends at the mutated initiator methionine for *XCP2*. The locations of upstream deletion primers for D3 – D12 were chosen to isolate known *cis* elements identified by ATHAMAP, (<http://www.athamap.de/>) (Fig. 5). The 5' deletions were all positive from D1 – D15 and the level of *GUS* expression appeared unchanged for all deletions except D15. D15 resulted in *GUS* expression at a reduced level compared to the other deletions, although TE localization was preserved (Table 1).



Figure 3. Typical TE-specific GUS activity due to expression of the uidA gene driven by the full-length promoter of *XCP2* in 5-day-old *Arabidopsis thaliana* seedling. The same expression pattern is seen for all promoter deletion experiments that result in GUS-positive plants. Black arrows GUS expression in xylem.

aaatacagaggatccacgtataaaatcacccgtcgtcgagggttaataacgtgttcattgttttttccgtaactctattttgcttactc
atctcatttttgcgaagtcatgataggtcagacactttgaatcatagattggtgattggtggggataaaaaatacgtggaatctctta
caggaattcacatgtaatcatgtggcttaaacctcagctataaacataaaaacacacacacacacaaatagtggaatcactataaattt
gCGGCTAAAAATattatagattttggaaggaattacaaagattactatacaataataacaaataaaaactactatttaaaacaaaaatt
aaaaattaaagagacagatgggtttgatgcaataggagacgaaatggcgtcaatgagagggcaatgtgattataaatattagtgctttg
tcactttctcagctaccttttatgcccaccatttctctctactagtatttttataatataaacacacacaaatcaatttaga
aaaatgaacattgatagcatatagctatagttttcaattctgtagcaagttagatgtttttctttctcagcacaaggctatttgaga
atatataaaaaatctaacgtcctctcatatttttaacgctggaatgagattttatgtgaaaaagaaaactttggatagatatac
atagtcagcagtagcacctagaaaaataagcaatggcttttcttgatagagttcgtgtcccaaatcatttgagcattccgacca
cgtggctccacctcaaacggatcttatgatagttttattatcattttatttttctcttataatatttggttgatttttccctgcata
agaaataacaatcatttatcttttggacagcagtagatggtcctgtaaagttagaaccaagtgagtttcactgtaacctt
aacatgatctatgttttaaatgaatccactttatgaaaaatagggttaatttagatcactcgtagtgggtgggtgggtcatcatt
ataaaaatcaagttttacgtagtttcaaattttataatctttatcaacaatagctgattaaaaactaagtaaaacggaat
ccttatctcctgacattgttatcagaaatctgggtggattgacaggtcaaaccttgtctttacagaataaaatcttataaaaaaaa
acagaaatatacttcaccgtaatctcatgctttgtgtaatttataatgtctcattaagaatattggttaaaactttgaaacagtgaa
aaaagaagagtaattataactcaactaaactcaacatctcataccataaaaaggtagtgattatgggcccgaatccatgatg
tatattgttcaatagagttggtgatacaaaaattttcatcagtttaacatagttggcaccctttgggtcttctattgttgagg
ttggcattttatcattgaggccttctttaaataatctctgaccgagacagaggttctagttctgcacataatataaacacacata
taccgcaagggcggctcagaataataaaggaccttagagctggaaaaaaatagttctcattttgtgtttggtatatacaaaatt
ttggaccctaaactataatagatttacttttaaaaaaaagaaattttgggtagaccctgtgcccagctcctccagcacatgtgtaa
gcccggctgcataacgatttagtacttggagtagcaaatcaacctcatggaaagacaaaaactaagcttaagaacacagacttacaacaag
tacagattcagactacttactctaaagcaaaaagagcagcctctacagagtctccagcctataaaaacaggctctctctccatctc
ctcttatcaagtgacaagaacacaaacgtactcaaa**ATC**GCTCTTCTTCACTTCAAGAAATCCTCTGTTTGTCTTTGGCTTATCC
GCTGCTTCTCTCTCCCTCTCTTTCGCTTCTTCCCACGATTACTCCATCGTTGGATACTCCCCCGAGGATTTGGAATCTCATGACAACT
CATAGAATCTTTCGAAAATGGATCTCAAATTTTGAGAAAAGCTTATGAACCGTTGAAGAGAAGTTTCTTAGGTTCCGAAGTTTCAAGG
ATAATCTAAAGCACATCGATGAGACTAACAGAAAAGGGAAAAGCTACTGGCTCGGGCTCAACGAGTTTGGCGGATTTGAGCCATGAGGAG
TTCAAGAAAATGTATTAGGGCTCAAGACTGATATAGTGAGACGCGATGAAGAAAAGATCTTACGAGAGTTTCGCTTACAGGGACGTCGA
AGCTGTTCTAAGTCTGTTGACTGGAGAAGAAAGGAGCTGTGGCGGAAGTTAAGAACCAGGGCTCTTGtgaatatacaaatcttgg
ctctgatctgttaatggaagtttgactcttaactaaaccatttaactttaaaaaaactggttcgcagGAAGTTGTTGGGCGTTTTCGA
CAGTAGCAGCTGTGAAGGTATAAAACAGATTTGTGACAGGAACTTGACAACATTTGTCAGACAAGAAGCTCATAGACTGTGACACGACC
TACAACAATGGCTGCAACGGTGGCTCATGGACTATGCCTTTGAGTACATTTAAGAACGGAGGCTACGCAAGGAAGGATTATCC
TTACTCTATGGGAAGGAACTTGGCAGATGCAAAAGgtcccattcaatatacaactcttggctctctgatgtgtaaatgaaaggtggtc
ccattctaattatctctgtttggaaaacagGATGAATCTGAAACAGTAAACCTTAATGGACACCAAGACGTACTACTAATGATGAGAA
GAGTCTCTTGAAGGCATTGGCTCATCAGCCTCTCAGTGTGCCATTGATGCATCTGGTAGAGAGTTCCAGTTCTATAGCGGCgtaagta
cattataaacctataaaataaagaaatgggtttagtaattgctctgtttgatgttgaatcattttgattttgggtatgatgaagGGCGT
GTTTGTGAGGGCGGTGCGGGTTGATCTTGACCACGGTGTGGCTGCGGTGGGTATGGATCAAGCAAGGGTTTCAGATTACATCATTGTGA
AGAATTTGGGGACCACAAAATGGGGAGAAAAGGTTACATCAGGCTGAAGAGGAACACTGGGAAACCAGAGGGTCTCTGTGGAATCAAC
AAGATGGCTTCTTTCCCAACAAAATAAGTGAcatcagatcattctctcttatctgcatttgatcagcatcttacagtgtcttcggt
ttttctcttttgaattatcaacggaagcttgttaacaactatgtgcaataatccctatataaagaagcagagaattgacaaaattctg
gtaccaaccatgaagaatataagcagagaaatataaaaataatg

Figure 4. Deletion 1 (D1) 1967-bp upstream promoter region of the *XCP2* gene. Black indicates the promoter region. Red indicates 5' and 3' UTR. Orange indicates exons. Blue indicates introns. Blue box indicates initiator methionine and stop codon.
<http://www.arabidopsis.org/servlets/sv?action=nucleft&pid=0&option=0&option2=0&on=>

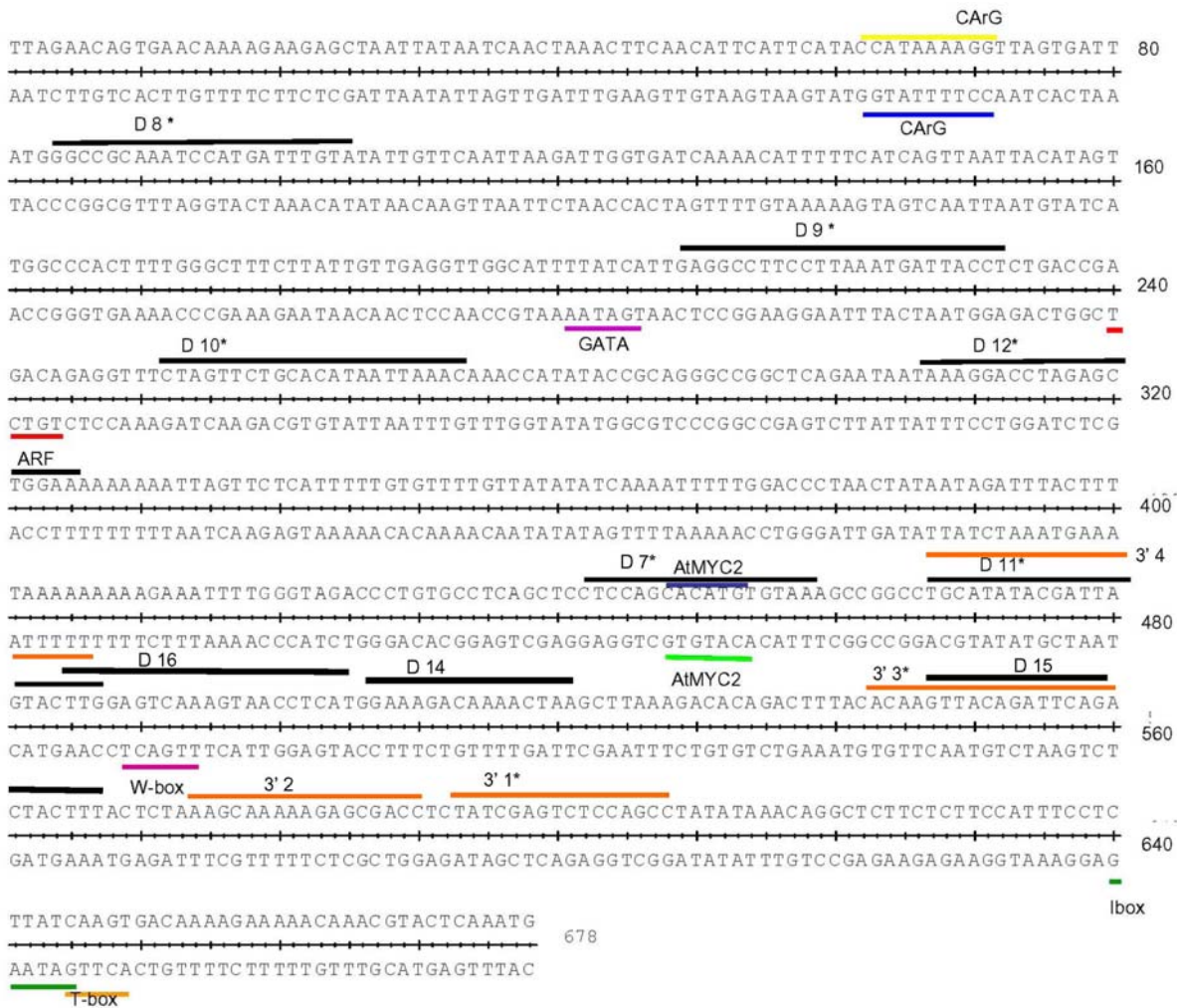


Figure 5. The 678-bp promoter region of *XCP2*, with known or predicted cis elements and primer locations for deletions 8-15 and 3' deletions 1-4. Deletions 7-10 were designed to isolate predicted cis elements, while subsequent deletions 11-15, are aimed at increasing resolution of a region between deletion 11 and 3'3 that according to *GUS* expression data is essential for xylem specific expression. Black solid line indicates primers used in construction of 5' deletions, * identifies deletions that yielded positive *XCP2p GUS* expression. Orange solid line indicates 3' deletions, the 3' deletions constructs use D12 as the 5' primer. *Cis* elements were identified using ATHAMAP, (<http://www.athamap.de/>)

Table 1. *XCP2* promoter deletion constructs showing relative strength of GUS expression. +, low-level, tissue-specific expression; +++, high-level tissue-specific expression ; - GUS expression not detected.

Deletion #	XCP2p size in bp	GUS expression	Direction of deletion
D1	1967	Positive +++	5'
D3	1613	Positive +++	5'
D4	1288	Positive +++	5'
D5	1076	Positive +++	5'
D6	672	Positive +++	5'
D7	238	Positive +++	5'
D8	593	Positive +++	5'
D9	466	Positive +++	5'
D10	425	Positive +++	5'
D11	209	Positive +++	5'
D12	371	Positive +++	5'
D13	305	Positive +++	5'
D14	170	Positive +++	5'
D15	130	Positive +	5'
D16	192	Positive +++	5'
D 3'1	300	Positive +++	3'
D 3'2	281	Positive +++	3'
D 3'3	253	Positive +	3'
D 3'4	178	negative -	3'

3.1.2 Promoter deletion analysis of the 3' end of *XCP2*

Promoter *XCP2* deletions were constructed using D12 primer as a sense primer and four different anti-sense 3' deletions beginning with D3'1, 1-bp upstream of the putative TATA box. D12 primer is located 350-bp upstream of the initiator methionine for *XCP2*. The 3' deletions were all placed into vector p2060 (Fig. 2) this vector contains a 35S minimal promoter sequence for activation of the basal transcription machinery. The use of the 35S minimal promoter in the vector p2060 establishes whether any sequence located downstream from the 3' deletion is necessary for TE specific expression of *XCP2*. Deletion 3'1 and D 3'2 were both strongly GUS positive, whereas D3'3 showed tissue specific GUS expression but at a reduced level compared to D3'1 and D3'2. Deletion 3'4 was negative for all plants assayed, indicating a potential *cis* element is located between D3'4 and D 3'2 (Fig 5). Since D3'3 showed GUS expression at a reduced amount compared to D3'2 a *cis* element possibly is also located between these to deletions, (Fig 5; Table 1).

3.1.3 Analysis of the region containing a putative *cis* element

Based upon the data generated from both the 5' and 3' deletion series, a region located between D14 and D3'2 was seen as being necessary for normal xylem specific GUS expression. The region between D14 and D3'2 is 84-bp in length and is located between -170-bp and -85-bp downstream from the initiator methionine for *XCP2*. The 84-bp region was further analyzed by using the PLACE *cis* element motif analysis tool (<http://www.dna.affrc.go.jp/PLACE/>).

Analysis by PLACE revealed the 84-bp region contained 2 copies of the known *cis* element ACTTTA located at position -73 and -46 from the TATA box in *XCP2p* (Fig 6). The ACTTTA motif functions as a meristematic and vascular specific *cis* element in Tobacco (Baumann et al.,

1999). Interestingly, the work by (Baumann et al., 1999) found this *cis* element in the promoter of *ROLB*, a plant oncogene from *Agrobacterium rhizogenes* that effects the auxin signal transduction pathway. The trans-activating protein is NtBBF1, which belongs to the DOF family of widely distributed plant proteins. DOF proteins contain a 52-amino acid consensus domain and these proteins have been identified as transcription factors in maize (Yanagisawa and Sheen, 1998). In Arabidopsis the DOF protein OBP1 binds to the promoter region of Glutathione S-transferase (Chen et al., 1994). Arabidopsis expresses several DOF genes in a xylem-biased manner including At5g62430 and At1g51700 (Zhao et al., 2005). These results suggest the ACTTTA element is a *cis* element for the *XCP2* promoter and potentially a DOF protein is the trans-activating protein.

Factor or Site Name SITE #	Loc.(Str.)	Signal Sequence
-300CORE S000001	site	31 (-) TGTAAG
-300ELEMENT S000122	site	30 (-) TGHAARK
ARR1AT S000454	site	46 (+) NGATT
CACTFTPPCA1 S000449	site	56 (+) YACT
CACTFTPPCA1 S000449	site	61 (+) YACT
DOFCOREZM S000265	site	2 (+) AAAG
DOFCOREZM S000265	site	20 (+) AAAG
DOFCOREZM S000265	site	67 (+) AAAG
DOFCOREZM S000265	site	74 (+) AAAG
DOFCOREZM S000265	site	31 (-) AAAG
DOFCOREZM S000265	site	58 (-) AAAG
DPBFCOREDCDC3 S000292	site	35 (+) ACACNNG
EMHVCHORD S000452	site	30 (-) TGTAAGT
NODCON2GM S000462	site	75 (-) CTCTT
NTBBF1ARROLB S000273	site	30 (+) ACTTTA
NTBBF1ARROLB S000273	site	57 (+) ACTTTA
OSE2ROOTNODULE S000468	site	75 (-) CTCTT
TAAAGSTKST1 S000387	site	19 (+) TAAAG
TAAAGSTKST1 S000387	site	66 (+) TAAAG
TAAAGSTKST1 S000387	site	31 (-) TAAAG
TAAAGSTKST1 S000387	site	58 (-) TAAAG

Figure 6. PLACE analysis of the 84-bp region between D14 and D3'2 for XCP2 promoter. Red rectangle the ACTTTA *cis* element.
(<http://www.dna.affrc.go.jp/PLACE/>)

3.1.4 Analysis of the 84-bp region containing the ACTTTA elements

Based on the results from both 5' and 3' unidirectional deletion data and from the relevant published information further analysis of the 84-bp region was warranted. Figure 7 shows an alignment of constructs that define the necessary promoter region in relation to both the TATA box and the 35S minimal promoter.

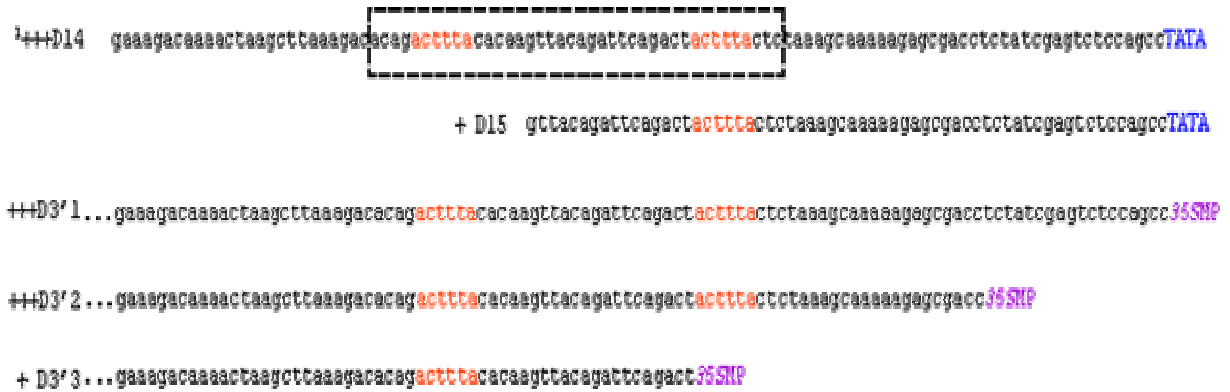


Figure 7. GUS-positive constructs that define the necessary promoter region. Red, indicates putative *cis* element location. Purple, 35S minimal promoter location. Blue, location of TATA box in D14 and D15, both D14 and D15 continue until the initiator methionine for XCP2. 3' deletions continue for an additional 220-bp upstream. Dashed line rectangle shows the location of the region ligated into vector pHIS2 as a tetrameric repeat for the yeast one-hybrid assay. ¹Relative GUS activity indicated as described for Figure 6.

3.1.5 Mutational analysis of the ACTTTA element

The region containing the ACTTTA element is necessary for normal *XCP2* expression based on the data from both 5' and 3' unidirectional deletions. PCR-based mutations of the ACTTTA element to TGATTA made it possible to design a series of deletions to investigate the

dependence of normal *XCP2* expression on these sites (Fig. 8). Deletion 18 is a 70-bp region spanning from -155-bp to -85-bp from the initiator methionine. D18 was positive for the native promoter region and positive for a mutation in the ACTTTA element located -73 from the TATA box. D18 was negative when the ACTTTA element at -43bp from the TATA box was mutated. Data from the mutational analysis was repeatable by two independent transformations of plants for D18, D18M1, and D18M2. D19 consisted of a 42-bp region containing the ACTTTA element located at -73-bp and included the region from -155-bp to -103-bp fused to the 35S minimal promoter, D19 was negative for GUS expression in all plants tested (Table 2).

```

+++ D17  agcttaaagacacagactttacacaagttacagattcagactactttactctaaagcaaaaagagcgacctctatcgagtctccagccTATA
          |-----|
+++ D17M agcttaaagacacagtgattacacaagttacagattcagactactttactctaaagcaaaaagagcgacctctatcgagtctccagccTATA
          |-----|
+++ D18  agcttaaagacacagactttacacaagttacagattcagactactttactctaaagcaaaaagagcgacc35SMP
          |-----|
++ D18M1 agcttaaagacacagtgattacacaagttacagattcagactactttactctaaagcaaaaagagcgacc35SMP
          |-----|
- D18M2  agcttaaagacacagactttacacaagttacagattcagacttgattactctaaagcaaaaagagcgacc35SMP
          |-----|
- D19    agcttaaagacacagactttacacaagttacagattcagact35SMP
          |-----|

```

Figure 8. Mutational analysis of ACTTTA *cis* elements. Red, putative *cis* element location. Purple, 35S minimal promoter location. Green, PCR based mutation incorporated into the sequence. Blue, location of TATA box in D17 and D17M, both constructs continue until the initiator methionine for *XCP2*. Relative GUS activities indicated as for Figure 6.

Table 2. Results for *XCP2p* ACTTTA mutation analysis. * indicates results were compiled from at least 2 independent transformations. +++, normal TE-specific GUS activity.

Deletion #	Promoter Size	GUS activity level	Total No. of GUS-positive plants	Total No. of plants assayed
D17	155-bp	+++	22	22
D17M	155-bp	+++	20	23
D18	70-bp	+++	25	25
D18M1	70-bp	+++	26*	28*
D18M2	70-bp	Negative	0*	20*
D19	42-bp	Negative	0*	30*

3.1.6 Comparison of *XCP2p* necessary region to promoter regions from other plant xylem specific cysteine protease promoters

Other plant species exhibit TE-restricted expression of cysteine proteases, such as the *Zinnia* cysteine protease *ZCP4* (Pyo et al., 2004). GUS expression driven by 429-bp of the *ZCP4* promoter was restricted to xylem; this 429-bp region contains two copies of the ACTTTA motif within 200-bp of the translational start site. *XCP1p* from *Arabidopsis* does not contain an exact match to the ACTTTA motif but it does contain ACTTTG in the region shown to be necessary (Ismail, 2004). The xylem-biased (A. Brunner, personal communication) poplar ortholog of *XCP2*, identified by BLASTp from the recently sequenced Poplar genome,

https://webmail.vt.edu/horde/util/go.php?url=http%3A%2F%2Fgenome.jgi-psf.org%2FPoptr1_1%2FPoptr1_1.home.html&Horde=bb41d9228b2944c33c5d0cf24312b76a

also contains the ACTTTA element within 150-bp from the translational start site (Fig. 9). The high degree of similarity between these promoter regions indicates the ACTTTA element is either a *cis* element in these promoters, or part of a larger conserved *cis* element necessary for xylem specific expression.

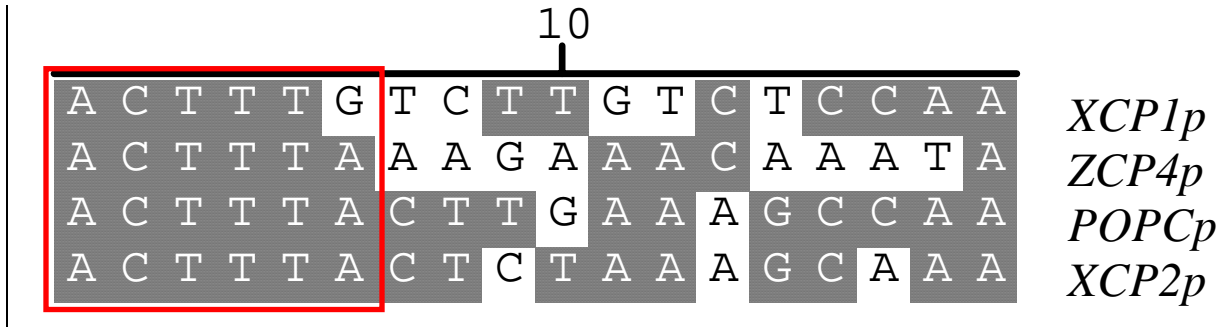


Figure 9. Alignment of a 18-bp region within 200-bp of the translational start site of four cysteine proteases. *XCP1p*, *XCP2p*, and *ZCP4p* are known xylem markers. A high degree of identity is evident between the promoters of these four cysteine proteases as indicated by the regions where the sequences are shaded in black. *POPCp* is known to be expressed in poplar xylem and is most similar to *XCP2p*. Red box, the ACTTTA *cis* element. DNASTAR, Madison, WI was used to produce this alignment.

3.1.7 Use of D8XCP2p::*GFP* as a tool for targeted gene expression in xylem

The use of a promoter for specifically targeting proteins to xylem will be of benefit as a genetic engineering tool for altering wood structure and content. The construct D8XCP2p::*GUS* was converted into D8XCP2p::*XCP2-GFP* for use as a tool to drive expression of XCP2 for the purpose of subcellular localization and trafficking. In order to study the trafficking of XCP2 the entire open reading frame was inserted into the D8XCP2p::*GFP* vector as an in-frame translational fusion with GFP. The XCP2-GFP fusion protein should follow the same subcellular localization as XCP2 and thus provide insight into XCP2 trafficking. The construct D8XCP2p::*XCP2-GFP* was successfully constructed and confocal microscopy showed the fusion protein was targeted to TEs (Fig. 10). XCP2-GFP is localized to TEs and occurs in varying

concentrations in the TE examined in Figure 10 suggesting XCP2-GFP is present in different organelles potentially the vacuole. The vacuole is known to collapse during TE differentiation, thus XCP2 may play a role in TE differentiation.

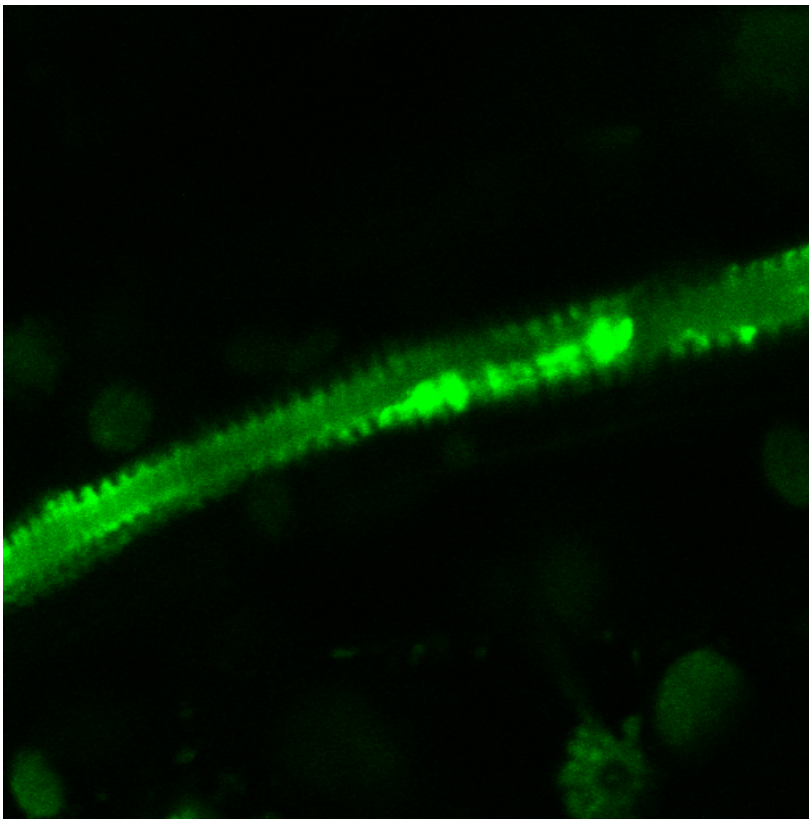


Figure 10. Confocal microscopy of XCP2-GFP showed XCP2-GFP was localized to a TE.

3.2 Yeast One-Hybrid analysis

3.2.1 Construction of *XCP2p* Bait sequence for Y1H analysis

The 42-bp region containing both ACTTTA motifs was selected to serve as the bait sequence for Y1H screen (Fig 7). The 42-bp region was amplified and integrated into vector pHis2 as a tetrameric repeat this construct was named *XCP2p*-pHis2. Tetrameric repeats are known to provide the proper spacing potentially necessary for the protein to DNA interaction in the Y1H assay.

3.2.2 Yeast strain Y187/*XCP2p*-pHis2 nutritional requirements

Y187 was transformed with *XCP2p*::pHis2 construct independently of the prey was able to grow on SD media/-trp. The vector pHis2 contains 2 TATA boxes one of these is constitutively expressed, thus inducing expression of *HIS3* resulting in background growth. In order for the background growth “leaky *HIS3*” to be suppressed, 3-AT was added to the media . The background growth for the vector *XCP2p*-pHis2 was significantly suppressed by supplementing the media with 40 mM 3-AT (data not shown).

3.2.3 Construction of Arabidopsis xylem double strand cDNA library (the prey)

Construction of the first strand cDNA library was done using RNA isolated from Arabidopsis stem tissue, which contains a relatively high proportion of vascular tissue compared to leaves. Since *XCP2* is expressed in xylem, transcription factors that activate *XCP2* expression should also be present in stem tissue. The quality of the ds cDNA was then analyzed by running a 7 μ l aliquot on a ethidium-bromide 1% agarose gel and by performing PCR using primers specific for

both actin and *XCP2*. Based on the size of the PCR products obtained from these primers, it was confirmed that the cDNA library was not contaminated with genomic DNA.

A second cDNA library for Y1H was prepared using RNA from *Arabidopsis* hypocotyls. *XCP2* is expressed at the highest level in roots and hypocotyls and thus *XCP2* transcription factors are expected to be found at the highest levels in the same tissue. By growing *Arabidopsis* under low population densities, 4-6 plants per pot, and pruning the inflorescences increased secondary growth and thus larger amounts of xylem tissue are produced by the root-hypocotyl. (Zhao et al., 2000).

3.2.4 Yeast One-Hybrid analysis using *XCP2p*-pHIS2

The bait and prey were both co-transformed in Y187 using *XCP2p*-pHIS2, pGADT7-rec2, and ds cDNA. The BD MATCHMAKER Library Construction and Screening Kit was used for conducting the library scale analysis. Approximately 200 colonies grew over a 7 day period, of these approximately 50 were larger than the others suggesting a true positive reaction. Colony PCR utilizing primers PR71 and PR72 should result in amplification of 180-bp (5' region) flanking the T7 site, and about 80-bp of the 3' region of the pGADT7-Rec2 vector. The products from amplification of the 20 colonies selected for PCR that gave single-banded products were placed into pGEM-t-easy and sent for sequencing to Virginia Bioinformatics Institute using primer T7. From these, 20 colonies were screened. From these 20 colonies, 5 unique sequences were obtained and the remaining sequences were either identical or impossible to BLAST search due 2 or more products being present (Table 2).

The background and the number of false positives was considered high so another cDNA library was constructed using dissected roots to enrich for xylem. The second yeast one-hybrid assay was conducted using 100 mM 3-AT in an attempt to further reduce the background. Yeast colonies in excess of 100 were still present after 7 days growth. Plasmid was isolated from the

largest 6 colonies and sequenced at Virginia Bioinformatics Institute using the T7 primer. Results for the second Y1H assay are also listed in Table 3. Based on the sequencing data and the number of background colonies present on the plates, the Y1H assay failed to show an interaction with the transcription factor that binds to the bait construct.

Table 3. BLASTn results for 13 sequenced yeast colonies compared to the Arabidopsis transcript database. * colonies isolated from the second Yeast one-hybrid screen.

ID	Product	Gene	Identity	Description/TAIR database	Function/TAIR database
7LX	500	At4g03280	183/189	Cytochrome B6 Fe-S complex	Photosynthetic electron transport
8LX	550	At4g03281	240/250	Cytochrome B6 Fe-S complex	Photosynthetic electron transport
17LX	600	At4g03282	371/373	Cytochrome B6 Fe-S complex	Photosynthetic electron transport
13LX	500	At1g66239	396/397	Copper Homeostasis factor	Heavy metal detoxification
14LX	600	At1g66240	444/445	Copper Homeostasis factor	Heavy metal detoxification
15LX	700	At1g27320	568/571	AHK3- cytokinin receptor	Histidine Kinase
18LX	500	At1g07590	339/340	Pentatricopeptide (PPR) repeat	Similarity to DNA binding protein
20LX	400	At1g08450	124/124	CRT3/ calreticulin 3	unknown
2X1*	500	At3g44010	290/290	40s Ribosomal protein	Protein Biosynthesis
2X2*	600	At3g44011	342/345	40s Ribosomal protein	Protein Biosynthesis
2X3*	550	At3g44012	276/283	40s Ribosomal protein	Protein Biosynthesis
2X4*	400	At2g42530	172/185	COR15a- cold responsive protein	cold acclimation
2X5*	450	At2g42531	220/223	COR15a- cold responsive protein	cold acclimation

Chapter 4: Discussion

4.1 XCP2p deletion analysis

Data derived from both the 5' and 3' deletion analysis of XCP2 promoter showed a region of 84-bp to be necessary for TE-specific GUS expression. The data from the 3' deletion results indicate the sequence from the TATA box downstream is not necessary for TE specific expression of XCP2. The 3' deletion data showed the region from D3'3 – D12 was sufficient for tissue specific GUS expression although levels were reduced significantly when compared to D3'2 and D3'1. Since in D3'4 GUS expression is completely abolished, a *cis* element must lie in the region between -188-bp and -106-bp from the translational start site. The decrease in level of expression between D3'1 and D3'3 also indicates the presence of another *cis* element between -113-bp and -85-bp. The 5' deletion series indicates the loss of a potential *cis* element in the region between D17 and D15. The D15 construct expressed GUS at a reduced level compared to other 5' deletions indicating an enhancer region required for normal level expression had been lost. The bi-directional deletion data results are in agreement and indicate an 84-bp region between -170-bp and -85-bp is necessary and sufficient for normal xylem-specific expression of XCP2.

The *in silico* analysis for the 84-bp region using the PLACE website

(<http://www.dna.affrc.go.jp/PLACE/>) indicates the presence of several potential *cis* element sites in the region between D14 and D3'2. The potential *cis* elements include 2 copies of the ACTTTA element, which contains the DOF binding site AAAG in reverse orientation. DOF proteins contain a 52-amino acid consensus domain and these proteins have been identified as

transcription factors in maize (Yanagisawa and Sheen, 1998). In Arabidopsis the DOF protein OBPI binds to the promoter region of Glutathione S-transferase (Chen et al., 1994). Arabidopsis expresses several DOF genes in a xylem-biased manner including At5g62430 and At1g51700 (Zhao et al., 2005). These results suggest the ACTTTA motif is a *cis* element for the *XCP2* promoter and potentially a DOF protein is the trans-activating protein. Since the ACTTTA motif is present twice in the region of interest and there is literature existing on the importance of this element for xylem specific expression in tobacco a mutational series was designed. Deletion constructs that contained a PCR based mutation in the ACTTTA at -140-bp to TGATTA did not alter the level or the localization of GUS expression compared to the wild type region for the 70-bp construct when fused to the *35S* minimal promoter. In contrast the ACTTTA region at -113-bp when mutated completely abolished GUS expression in all transgenic plants examined. The analysis was repeated in triplicate and the results were reproducible indicating the ACTTTA element at position -113-bp is necessary for normal xylem-specific expression of *XCP2*. The ACTTTA motif at -140-bp when mutated did not alter GUS expression. It may be lacking a necessary upstream region required for the *cis* element to function. Since D3'3 is missing the ACTTTA element at -113-bp but contains the ACTTTA element at -140-bp and includes upstream regions not present in the mutational analysis it is possible the element at -140-bp was non-functional before it was mutated. Comparisons with other xylem-specific cysteine proteases from Zinnia, Poplar and *XCP1* from Arabidopsis show the ACTTTA element or a nearly identical region to be present in the promoter regions for these genes within -200-bp of the initiator methionine. Indeed, as a result of this analysis a region of 70-bp (D18) can be used to specifically target transgenes for the purpose of genetically altering the content or the formation of wood. The 70-bp promoter region could also be used to create a multimer aimed at allowing

regulation of expression level for a transgene. The size of the multimer could be fine tuned for the desired expression level of the transgene by using 1, 2 or even 4 copies of the 70-bp region. Previous attempts at using a multimer consisting of 4 copies of the 42-bp bait construct inserted into p2060 failed to drive GUS expression in Arabidopsis. The failure of the 42-bp construct to drive GUS expression potentially was due to the lack of distal and proximal regions present in the 70-bp (D18) construct necessary for the ACTTTA *cis* element to function.

4.2 Yeast one-hybrid analysis

Since the 84-bp region between D14 and D3'2 contained a 42-bp region containing both ACTTTA motifs this region was made into a tetrameric repeat and used as the bait in a yeast one-hybrid screen. The analysis resulted in numerous colonies both times it was performed indicating potentially many false positives were occurring. In principle, in the Y1H true positive interactions should result in numerous colonies harboring independent clones having a sequence from the same prey protein binding to the bait, thus indicating the interaction is real. The largest colonies were selected both times and these colonies were sequenced, results are listed in Table 3. Stress related proteins are possibly interacting with the bait sequence, indicating these reactions were false positives due to the yeast being stressed as a result of the inhibitory compound 3-AT being present in the media.

The analysis of the largest colonies did yield one interaction with a potential DNA binding protein At1g07590 but this protein resulted in only one positive colony raising the possibility that it was a false positive. Potential reasons for the Y1H failing to yield interacting proteins for these assays are the Y1H is known to result in many false positives, transcription factors that are heterodimers will most likely not be detected by the Y1H, and the Y1H does not allow for the detection of proteins that are post-translationally modified (Deplancke et al., 2006). The bait construct was placed into vector p2060 and the construct failed to drive GUS expression in all transformed plants. The 42-bp multimer used in the Y1H assay differed from the D18 (70-bp) construct expressing GUS tissue and cell specific localization by missing regions located both at the proximal and distal end of D18. The regions missing in the bait construct are potentially necessary for transcription factor binding to the DNA.

Table 4. Primer List

Primer	Sequence 5'-3'	Orientation	5' site	Purpose
XCP2P1 (D1)	TCTAGAACCGTCGCAGGTTAAT	Sense	Xba I	5' Deletion XCP2
XCP2P2 (D2)	GGATCCAAAGAGCCGTTTGAG	Antisense	Bam HI	3' end of 5' deletions XCP2
XCP2P3 (D3)	TCTAGATGCAATAGGAGACGAACATG	Sense	Xba I	5' Deletion XCP2
XCP2P4 (D4)	TCTAGACATATGTACGAGTACGACCT	Sense	Xba I	5' Deletion XCP2
XCP2P5 (D5)	TCTAGAGCAGTCAAGTAGATGTCCTG	Sense	Xba I	5' Deletion XCP2
XCP2P6 (D6)	TCTAGACAGTGAACAAAAGAAGAGCTA	Sense	Xba I	5' Deletion XCP2
XCP2P7 (D7)	TCTAGACTCCTCCAGCACATGTGTAA	Sense	Xba I	5' Deletion XCP2
XCP2P8 (D8)	TCTAGAGGCCGCAAATCCATGATTTGTA	Sense	Xba I	5' Deletion XCP2
XCP2P9 (D9)	TCTAGAGCCTTCCTTAAATGATTACCTCT	Sense	Xba I	5' Deletion XCP2
X2P-D10	TCTAGACTAGTTCTGCACATAATTAAC	Sense	Xba I	5' Deletion XCP2
X2P-D11	TCTAGATGCATATACGATTAGTACTTG	Sense	Xba I	5' Deletion XCP2
X2P-D12	TCTAGATAAAGACCTAGAGCTGGAA	Sense	Xba I	5' Deletion XCP2
X2P-D13	TCTAGATTGGACCCTAACTATAATAGA	Sense	Xba I	5' Deletion XCP2
D14N-XCP2	TCTAGAGAAAGACAAAACCTAAGCTTAA	Sense	Xba I	5' Deletion XCP2
D15-XCP2	TCTAGAGTTACAGATTCAGACTACTTT	Sense	Xba I	5' Deletion XCP2
D16-XCP2	TCTAGATTGGAGTCAAAGTAACCTCATG	Sense	Xba I	5' Deletion XCP2
D17-XCP2	TCTAGAAGCTTAAAGACACAGACTTTA	Sense	Xba I	5' Deletion XCP3
D17M-XCP2	TCTAGAAGCTTAAAGACACAGTGATTA	Sense	Xba I	5' Deletion XCP4
3'2M-AS	CCTAGGGGTCGCTCTTTTTGCTTTAGAGTAATCA	Antisense	AvrII	D18 M2
D12-Pac I	TTAATTAATAAAGGACCTAGAGCTGGAA	Sense	Pac I	3' Deletion XCP2 (5' end)
D3'-1AS	CCTAGGGGCTGGAGACTCGATAGAG	Antisense	AvrII	3' Deletion XCP2 (3' end)
D3'-2AS	CCTAGGGGCGCTCTTTTTGCTTTAGA	Antisense	AvrII	3' Deletion XCP2 (3' end)
D3'-3AS	CCTAGGAGTCTGAATCTGTAACCTGTG	Antisense	AvrII	3' Deletion XCP2 (3' end)
D3'-4AS	CCTAGGCAAGTACTAATCGTATATGCA	Antisense	AvrII	3' Deletion XCP2 (3' end)
X2-CisS	AAGCTTAGATCTCACAGACTTTACAAGTTA	Sense	Hind III, Bgl II	construction of bait for Y1H
X2-CisAS	CCCGGGGATCCAGAGTAAAGTAGTCTGAATCT	Antisense	Bam HI, Sma I	construction of bait for Y1H
PR06	ATCCAGACTGAATGCCACA	Antisense		sequencing 121.3 & 2060

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