TISSUE AND CELL-TYPE LOCALIZATION AND PARTIAL CHARACTERIZATION OF A XYLEM PAPAIN-TYPE CYSTEINE PROTEASE FROM *ARABIDOPSIS*

by

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(ABSTRACT)

Cysteine proteases are associated with xylem tracheary element differentiation. XCP1 was recently identified as a xylem-specific cysteine protease in *Arabidopsis* (Zhao, *et al*., 2000). For this study a recombinant polyhistidine-tagged XCP1 (XCP1H6) was expressed and purified from an *E. coli* expression system. A polyclonal anti-XCP1 antibody was produced using purified XCP1H6. Immunoblot analysis of a developmental time course of xylem and bark protein extracted from root-hypocotyl segments demonstrated that XCP1 was expressed in xylem only. Further analysis under optimized immunoblot conditions, however, revealed that anti-XCP1 antibody reacted with protein present in both xylem and bark. The vast majority of immunoreactivity, however, was restricted to xylem. Cell-type localization of *GUS* expression under the control of a putative *XCP1* promoter indicated that the *XCP1* promoter specifies expression of *XCP1* in tracheary elements in leaves, stems, roots and flowers. *XCP1* promoter-driven *GUS* activity was not associated with senescing tissues.

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TABLE OF CONTENTS

Abstract ii

Acknowledgements iii

List of Figures vii

Chapter 1: Literature Review 1

Chapter 2: Materials and Methods 9

2.1 Expression in *E. coli* and purification of XCP1 for antibody production 9

2.1.1 Construction of polyhistidine-tagged XCP1 9

2.1.2 Preparation of chemically competent *E. coli* 9

2.1.3 Transformation of competent M15 cells 11

2.1.4 Analysis of transformants 11

2.1.5 Expression of XCP1H6 and purification by metal-chelate chromatography 12

2.1.6 Activation of protease 13

2.1.7 Protease activity gel 14

2.2 Developmental time course and tissue-level localization of XCP1 protein in the root-hypocotyl of *Arabidopsis* 14

2.2.1 Plant growth and separation of xylem and bark 15

2.2.2 Preparation of tissue extracts for immunoblot analysis 15

2.2.3 Production and affinity purification of anti-XCP1 antibody 15

2.2.4 Immunoblot analysis 16

2.3 Tissue and cell-type localization of *GUS* expression under the control of a putative promoter for *XCP1* 17

2.3.1 Construction of *XCP1* promoter*::GUS*-binary vector for transformation of *Arabidopsis*..... 17

2.3.2 Transformation of competent *Agrobacterium* 20

2.3.3 Transformation of *Arabidopsis* by vacuum infiltration 20

2.3.4 Selection of *Arabidopsis* transformant..... 21

2.3.5 Histochemical detection of β-glucuronidase activity..... 21

Chapter 3: Results 22

3.1 Expression and purification of a recombinant polyhistidine-tagged XCP1 . 22

3.2 Activation of renatured recombinant protein..... 22

3.3 Developmental time course and tissue-level localization of XCP1 protein... 25

3.4 Cell-type localization of *GUS* expression directed by *XCP1* promoter..... 28

Chapter 4: Discussion 36

4.1 Expression and purification of a recombinant polyhistidine-tagged XCP1 . 36

4.2 Developmental time course and tissue-level localization of XCP1 protein... 37

4.3 Cell-type localization of *GUS* expression directed by *XCP1* promoter..... 39

References 41

Curriculum Vitae 48

LIST OF FIGURES

Figure 1 10

Construction of a recombinant, polyhistidine-tagged XCP1 (XCP1H6)

Figure 2 19

Construction of *XCP1* promoter*::GUS*-binary vector

Figure 3 23

Polyhistidine-tagged XCP1 is localized in the insoluble fraction of *E. coli*

Figure 4 24

Purified polyhistidine-tagged XCP1 remains bound to Ni^{+}-NTA agarose in the presence of 500 mM imidazole

Figure 5 26

Following renaturation, polyhistidine-tagged XCP1 is proteolytically processed and activated during incubation at pH 5.5

Figure 6 27

Anti-XCP1 antibody reacts with protein in xylem only

Figure 7 29

Optimized immunoblot conditions reveal that anti-XCP1 antibody reacts with several proteins in xylem and bark

Figure 8 32

The putative *XCP1* promoter directs *GUS* expression in leaf vascular tissue

Figure 9 33

The putative *XCP1* promoter directs *GUS* expression in stem vascular tissue

Figure 10 34

The putative *XCP1* promoter directs *GUS* expression in sepal vascular tissue

Figure 11 35

The putative *XCP1* promoter directs *GUS* expression in root vascular tissue

CHAPTER 1: LITERATURE REVIEW

Wood and related forest products are important as renewable resources available in tremendous quantities. Wood formation derives from the secondary xylem. The properties of wood depend on the composition and morphology of the cell wall of the mature xylem. Xylem has been recognized as a conduit for water and nutrient transport throughout the plant, as a source of mechanical support, and as a tissue for the periodic storage and mobilization of reserve materials (Esau, 1965). Development of xylem involves cell elongation, cell expansion, and formation of a secondary cell wall (involving cellulose, hemicellulose, and lignin synthesis), and programmed cell death (PCD). Cell types represented in the xylem include tracheids and vessel elements (collectively known as tracheary elements), parenchyma cells and sclerenchyma fibers. Tracheary elements (TEs) lose their cytoplasm at maturity and hence the terminal differentiation of TEs is recognized as an example of developmentally programmed cell death (Beers, 1997).

Programmed cell death involves three connected parts: 1) physiologic signals for inducing PCD, 2) signal transduction processes, and 3) effector or execution pathways (Roberts *et al*., 1999). Recent studies have shown that PCD in animals and plants is similar with respect to some morphological and biochemical changes (Greenberg, 1996b; Wang *et al*., 1996). Proteases play important roles during PCD. In animals, the most important proteases known to be involved directly in PCD are caspases (cysteinyl aspartate-specific proteases). It has been reported that the signal transduction mediator

protein kinase MEKK1 is a caspase substrate and is cleaved when caspase is activated during PCD. The cleavage of MEKK1 results in an irreversible commitment to cell death characterized by cell shrinkage, protein degradation, nuclear condensation and fragmentation (Widmann *et al.*, 1998). Caspases also appear to function as effectors of cell death by disabling cell repair processes thereby leading to a particular type of PCD, apoptosis. Although direct evidence for the functional significance of proteases during plant PCD has not been presented, it appears that caspase-like proteolytic activity is involved in regulating local lesion formation during the hypersensitive response to infection by tobacco mosaic virus (del Pozo and Lam, 1998).

During TE differentiation, cell death, including collapse of the vacuole and cessation of cytoplasmic streaming, is initiated by a signal that requires the movement of Ca^{2+} into the cell (Groover and Jones, 1999). It was shown that the production of a 40 kD serine protease during secondary cell wall synthesis is a primary trigger of cell death. Secretion of this protease leads to an increase in protease activity in the extracellular matrix and also activates Ca^{2+} influx. Groover and Jones (1999) suggested that this secreted serine protease may activate an influx of Ca^{2+} by releasing a signaling molecule from the extracellular matrix or by cleaving an associated receptor to activate a Ca^{2+} channel. Thus, extracellular proteolysis appears to play an important role during PCD which is coordinated with secondary cell wall synthesis during TE differentiation (Groover and Jones, 1999). Serine protease activity is also involved in signal transduction that results in the hypersensitive cell death of tobacco cells (Yano *et al.*, 1999). Plant proteases have also been implicated in the effector stage of PCD, in particular, as

essential catalysts of protoplast autolysis. Woffenden, *et al*. (1998) reported that a cysteine protease inhibitor could prevent the complete autolysis of differentiating TEs without preventing cell death.

Other examples of proteases associated with PCD include cysteine proteases active during flower senescence (Valpuesta *et al*., 1995), leaf senescence (Drake *et al.*, 1996) and anther dehiscence (Li *et al.,* 1995), and aspartic acid proteases expressed during phloem sieve element autolysis (Runeberg-Roos and Saarma, 1998) and nucellar cell degeneration (Chen and Foolad, 1997). These reported increases in protease gene expression during PCD considered together with the observation that animal PCD can be initiated by several mechanistically distinct proteases such as granzyme B, a serine protease (Greenberg, 1996a), cathepsin D, an aspartic protease and calpain, a cysteine protease (Squier and Cohen, 1996) suggest that proteolytic pathways may interact in plants to regulate the death and autolysis of cells.

Of particular interest to our laboratory is the role of proteases during PCD. TE differentiation *in vitro* has been studied as an example of PCD restricted to individual plant cells. The hormone-induced *in vitro* differentiation of cultured *Zinnia elegans* mesophyll cells into TEs (Fukuda and Komamine, 1980) has been a particularly valuable model system for investigations of TE proteases. For example, the activity of a cysteine protease increased transiently at the beginning of autolysis (Ye and Varner, 1996; Minami and Fukuda, 1995) and was specific to differentiating TEs in *Zinnia* (Beers and Freeman, 1997). In addition to the serine protease described by Groover and Jones (1999), the 26S proteasome (Woffenden *et al*., 1998) has been implicated as a regulator

of TE PCD. Despite its proven utility for biochemical and molecular characterizations of proteases active during TE formation, methods for stable transformation of *Zinnia* for reverse genetic studies of protease have not been described.

To take advantage of the superior characteristics of *Arabidopsis,* relative to the *Zinnia*, as a model for genetic studies, our lab recently developed a method for research with secondary vascular tissue from *Arabidopsis*. *Arabidopsis thaliana* is an important model system for studies of plant development and molecular biology. It has a small genome (1.2×10^8 bp), a short life cycle (8-10 weeks), is easily transformed for foreign gene expression, and it produces large quantities of seed (up to 10,000 seeds/plant). Also available are detailed genetic maps and molecular resources (Somerville and Somerville, 1996, and references therein). *Arabidopsis* develops a true vascular cambium and significant amounts of secondary xylem and phloem when senescence is delayed (Lev-Yadun, 1994; Zhao *et al*., 2000). Xylem-forming tissues in *Arabidopsis* roots can be separated from other tissues easily. Dissection of root-hypocotyl segments under the dissecting microscope to obtain xylem and bark results in isolated secondary xylem which is not contaminated by secondary phloem and nonvascular tissue (Zhao *et al*., 2000). Since *Arabidopsis* is an excellent genetic model, and secondary xylem is a major tissue that will develop to be wood, to understand secondary xylem development of *Arabidopsis* will be very useful for studies of vascular development in other plants. Using this system we have identified three xylem proteases (Zhao *et al*., 2000). One of those proteases, XCP1, a papain-type cysteine protease, is the subject of this thesis.

The cysteine proteases are the most frequently characterized proteases from plants. Cysteine proteases can be classified into more than 20 different families including the papain family (Rawlings and Barrett, 1999). Papain-type proteases represent the largest family (C1 family) (Rawlings and Barrett, 1999) and papain was the first cysteine protease to have its three-dimensional structure solved (Drenth *et al*., 1968). Of all plant proteases studied thus far, papain-type proteases are most frequently found to be associated with PCD occurring during organ senescence (Kardailsky and Brewin, 1996), seed germination (Granell, 1998), stress and pathogen response (Solomon *et al*., 1999), and autolysis of differentiating TEs (Woffenden *et al*., 1998). Papain-type proteases are expressed during xylem differentiation *in vitro* and in planta (Ye and Varner, 1996; Beers and Freeman, 1997; Woffenden *et al*., 1998; Zhao *et al*., 2000). For example, p48h-17 encoding a papain-type cysteine protease was expressed in cultured *Zinnia* mesophyll cells induced to differentiate to TEs. p48h-17 mRNA accumulated after 36 h of culture. This gene expression is associated with the final events of TE differentiation, secondary cell wall thickening, lignification, and autolysis (Ye and Varner, 1993).

All papain-like proteases are produced as inactive precursors (zymogens) which consist of a predomain, a prodomain and a catalytic domain. Proteolytic processing of the predomain and prodomain to form the active enzyme is required (Cohen *et al.*, 1990; Vernet *et al.*, 1991; Taylor *et al.*, 1992; Cygler and Mort, 1997). This was demonstrated in transgenic tobacco expressing the *Zinnia* protease p48h-17, which was processed from a 38 kD preproprotein to a 20 kD active enzyme. Propapain, the pro-form of a recombinant cysteine protease, has been expressed in *E. coli* (Taylor *et al.*, 1992).

Insoluble recombinant propapain could be refolded to form soluble precursor protein and processed autocatalytically to yield active papain by incubating at pH 4.0. The maturation of propapain to papain is a cysteine protease-dependent process. The processing of propapain to papain was inhibited by the cysteine protease inhibitors, E-64 and leupeptin, but not by specific inhibitors of serine, metallo or acid proteases (Vernet *et al.*, 1991; Taylor *et al.*, 1992). It has been reported that the maximal proteolytic enzyme activity of human cathepsin K, papain-like cysteine protease, is obtained at assay conditions near pH 5.5 (Bossard, *et al.*, 1996).

Recent investigations of cysteine protease expression in plants have used the bacterial gene GUS (*β*-glucuronidase gene, *uidA*) as a reporter gene. The expression of senescence-associated genes (*SAGs*) during ozone exposure was studied using transgenic *Arabidopsis* (Miller, *et al.*, 1999 and references therein). SAG12 is a senescence-specific cysteine protease. It was shown that *SAG12* promoter-driven GUS activity was restricted to senescing leaves and was not detected in control or O_3-treated plants. Promoter*::GUS* fusions are very useful for examining gene expression specified by promoter regions, in that, if promoter*::GUS* fusions are introduced into the plant cell, GUS expression reports activity of that promoter. Expression of *β*-glucuronidase at the location of the gene product within organ, tissue and cellular locations can be measured by a histochemical assay (Gallagher, 1992). Hence, histochemical localization of GUS activity in transgenic plants is one method for studying the regulation of the gene expression and localization of gene products during plant development. With regard to secondary vascular tissues, a transgenic line of Poplar (*rolC::uidA)* in which the *rolC* promoter from *Agrobacterium*

rhizogenes directed the expression of *uidA* has been characterized. It was shown that GUS activity was detected in the phloem, cambium and developing xylem (Regan, *et al*., 1999). This study indicates that GUS is a useful reporter for gene expression in secondary tissues.

In conclusion, research on the roles of proteolysis in xylem differentiation and wood formation has recently intensified. Lately, our lab has made significant progress characterizing proteolytic pathways active during vascular development in *Zinnia* and *Arabidopsis* (Beers and Freeman, 1997; Woffenden, *et al*., 1998; Zhao *et al.,* 2000). This thesis focuses on the localization and partial characterization of a xylem papain-type cysteine protease, XCP1, from *Arabidopsis*. First, XCP1 was expressed as a C-terminal polyhistidine-tagged recombinant fusion protein in an *E. coli* expression system, and purified using metal-chelate chromatography to obtain a ready source of pure XCP1 for antibody production. The second objective was to attempt to confirm RNA gel blot data localizing XCP1 to the secondary xylem (Zhao *et al*., 2000) using antibodies directed against XCP1. The final objective was to determine cell-type localization of XCP1. To achieve the latter objective, an *XCP1* putative promoter*::GUS* fusion was constructed for *Arabidopsis* transformation and transgenic *Arabidopsis* plants were evaluated to determine whether a *XCP1* promoter*::GUS* fusion could be expressed.

The complex nature of xylem, which is composed of TEs, parenchyma cells, and fibers, dictates the use of cell-type localization experiments to predict roles for proteases. For example, a protease localized in differentiating TEs, as reported here for XCP1, is more likely to have a role in PCD than is one residing in xylem parenchyma. Even

though a loblolly pine system has been used for studying xylem differentiation (Loopstra and Sederoff, 1995), using *Arabidopsis* for studying secondary vascular development will be beneficial to genetic manipulation for the purpose of functional analysis of the xylem proteome.

CHAPTER 2: MATERIALS AND METHODS

2.1 Expression in *E. coli* and purification of XCP1 for antibody production

2.1.1 Construction of polyhistidine-tagged XCP1

XCP1 was cloned using a QIAGEN type III construct (QIAGEN, Valencia, CA) in which the 6xHis tag is placed at the C-terminus. The vector pQE-50 and pQE-16 were used to provide 5' and 3' acceptor arms, respectively (Figure 1). The vector pQE-50 was digested with HindIII/BglI to release a 1025 bp fragment, pQE-16 was digested with BglII/BglI to release a 2428 bp fragment. The insert was prepared as a HindIII/BamHI fragment from pXCP1 (Zhao *et al*., 2000). The ligation of the insert with the 2 prepared vector fragments created pXCP1H6.

2.1.2 Preparation of chemically competent *E. coli*

M15 [pREP4] cells were streaked on LB agar containing 25 μg/ml kanamycin and incubated overnight at 37°C. A single colony was picked, inoculated into 1 ml of LB-kanamycin (25 μg/ml) and grown overnight at 37°C. The 1 ml overnight culture was added to 100 ml prewarmed LB broth containing 25 μg/ml kanamycin, and shaken at 37°C until an OD_{600} of 0.5 was reached (about 3 h). The culture was cooled on ice for 5 min, and was transferred to a sterile tube. The cells were collected by centrifugation at low speed (5 min, 4000*g*, 4°C). The supernatant was discarded carefully. Cells were always kept on ice. Cells were resuspended gently in 30 ml cold (4°C) TFB1 buffer (100

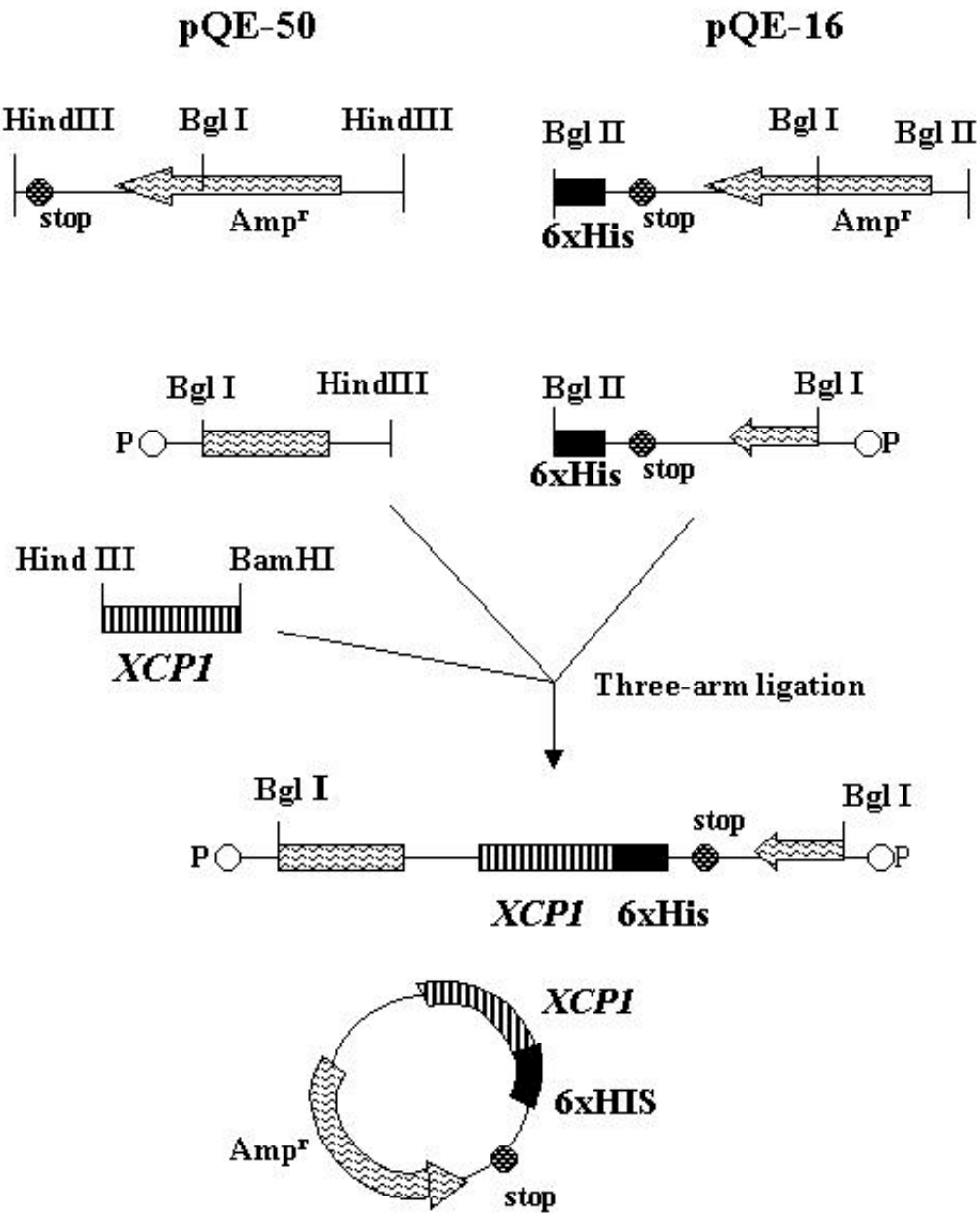

Figure 1. Construction of recombinant, polyhistidine-tagged XCP1 (XCP1H6)

mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol, pH 5.8, filter-sterile) and the suspension was kept on ice for an additional 90 min. The cells were collected by centrifugation (5 min, 4000*g*, 4°C). The supernatant was discarded carefully. The cells were resuspended carefully in 4 ml ice-cold TFB2 buffer (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, adjust to pH 8.0 with KOH; filter-sterile). Aliquots of 100-200 µl were frozen in liquid nitrogen and stored at –70 °C.

2.1.3 Transformation of competent M15 cells

An aliquot of frozen competent M15 [pREP4] cells was thawed on ice. The 50 µl ligation mix of pXCP1H6 was transferred into a microfuge tube containing competent M15 [pREP4] cells and mixed carefully. The cells were kept on ice for 10 min. The tube was transferred to 42°C for 2 min. After addition of SOC broth (1 ml), cells were incubated at 37°C for 20 min. Transformed cells were plated on LB-agar plates containing 25 µg/ml kanamycin and 100 µg/ml ampicillin and incubated at 37°C overnight.

2.1.4 Analysis of transformants

A clone containing an appropriate insert was identified by digestion of plasmid mini-prep DNA from 2 ml overnight cultures of 8 colonies grown in antibiotics. Cultured cells were spun at 4000*g*, 1 min. The supernatant was decanted. The pellet was resuspended in 200 µl GTE (50 mM glucose, 25 mM Tris, pH8, 0.5 M EDTA, pH 8) by vortexing. Fresh 0.2 M NaOH, 0.5% SDS (400 µl) was added. The tube was inverted several times to mix well and was incubated on ice for 5 min. Alkaline lysis solution III

300 µl (Sambrook *et al.*, 1989) was added. The tube was inverted gently but thoroughly and was spun immediately for 10 min. The supernatant 750 µl was removed and transferred into new tube with 450 µl isopropanol, then vortexed and spun at 10,000*g* for 10 min at 4°C. The supernatant was decanted and the pellet was dried in air. The pellet was resuspended in 100 µl TE, pH 8.0. The plasmids of 8 transformants were screened by the digestion of plasmid mini-prep with BglI/NsiI. NsiI is a unique restriction site of *XCP1*, and BglI is a unique restriction site of the pQE-50 and pQE-16 vectors. The DNA digestion was expected to release 2 fragments, 1133 bp and 3250 bp, of plasmid DNA if the insert was present. If no insert was present, the plasmid would be linearized but no fragment would be released.

2.1.5 Expression of XCP1H6 and purification by metal-chelate chromatography

Cells (100 ml) were cultured according to instructions provided by QIAGEN. Expression was induced by addition of IPTG to 1 mM. At 4 h post-induction, cells were harvested by centrifugation at 4,000*g* for 20 min. All purification steps were performed at 4°C. Cells were resuspended in 12 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 1 mg/ml lysozyme) and incubated on ice for 30 min prior to sonication. The sonicated cell lysate was pelleted at 10,000*g* for 30 min. The supernatant was discarded and the remaining pellet containing inclusion bodies was solubilized in 1 ml of denaturing buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 8.0). The solubilized sample was clarified by centrifugation at 14,000*g* for 10 min. The resulting supernatant was added to a microfuge tube containing a 300 ml bed-volume of Ni^+-NTA agarose beads equilibrated in denaturing buffer and the polyhistidine-tagged protein was allowed

to bind with periodic, gentle mixing over at least 30 min. After binding, beads were pelleted by centrifugation at 2,000*g* for 2 min. The supernatant containing unbound proteins was discarded. The following washes were used to remove background proteins from Ni^{+}-NTA beads: 2 ml denaturing buffer; 5 ml denaturing buffer, pH 6.5; 5 ml lysis buffer, without lysozyme; 2 ml lysis buffer, pH 4.5. Purified protein was released from beads into elution buffer (8M urea, 100 mM NaH_2PO_4, 10 mM tris, 500 mM imidazole, 10 mM β-mercaptoethanol, pH 8.0) by adding 1 ml of elution buffer, gently mixing beads, pelleting beads by centrifugation and removing the supernatant. Addition of β-mercaptoethanol was essential for efficient elution of XCP1H6 (E. Beers, personal communication). The elution was repeated once and supernatants containing purified protein were pooled for renaturation.

Purified, denatured XCP1H6 was renatured as described by Smith and Gottesman (1989). Purified protein in elution buffer was added slowly to 200 volumes of renaturation buffer (buffer R: 50 mM KH_2PO_4, pH 10.7, 5 mM EDTA, 10 mM β-mercaptoethanol) and stirred overnight at 4°C. The pH of the renaturation buffer was then adjusted to 8.0 with HCl followed by concentration using a YM10 membrane.

2.1.6 Activation of protease

Renatured inactive XCP1H6 was processed to active XCP1H6 by adjusting the pH to 5.5 with 1 M sodium acetate-phosphate buffer to a final concentration of 250 mM, followed by incubation for a further 1 h at 37°C. Enzyme activity was demonstrated by protease acitivity gel. For inhibition of activity of the processed XCP1H6, the irreversible cysteine protease inhibitor, E-64 (L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)

butanebutane) (Barrett *et al*., 1982) was added to processed XCP1H6 to a final concentration of 25 μM. Enzyme and inhibitor were incubated at room temperature for 10 min after addition of activation buffer. To prevent SDS-stimulated proteolysis in sample buffer prior to SDS-PAGE, the reversible cysteine protease inhibitor leupeptin was added to SDS-PAGE sample buffer (final concentration, 20 μM) (Beers and Freeman, 1997).

2.1.7 Protease activity gel

Following resolution of proteins by SDS-PAGE (Laemmli, 1970), the 12% gel was incubated in activity gel incubation buffer (1 M potassium acetate, pH 5.5, 14 mM β-mercaptoethanol) for 10 minutes, placed against a gelatin-impregnated (0.5% w/v gelatin) polyacrylamide (7% w/v), sandwiched between 3MM paper wetted with resolving gel incubation buffer, sealed in a plastic bag, and incubated in the dark at 27°C for 16 h. Following incubation, the substrate-impregnated gel was fixed in destain (40% methanol, 10% acetic acid) for 15 min, then stained with Coomassie brilliant blue for 1 h and destained until clear bands were visualized. The coomassie-stained gel was over-stained with amido black for 1 h and destained until clear bands were visualized. Clear bands in the substrate-impregnated gels, which result from hydrolysis of the substrate, indicate the positions of the proteolytic activity in resolving gels.

2.2 Developmental time course and tissue-level localization of XCP1 protein in the root-hypocotyl of *Arabidopsis*

2.2.1 Plant growth and separation of xylem and bark

Arabidopsis thaliana ecotype Columbia was grown in 4-inch-pot in Sunshine Mix 1 (Wetsel Seed Co., Harrisonburg, VA), under continuous fluorescent lighting. Plants were grown at a density of 6 to 8 plants per pot. Plants received nutrient solution (according to Somerville and Ogren, 1982) with each watering twice a week. Five-, 6-, 7-, 8- and 9-week-old plants were used to produce a developmental time course for immunoblot analysis. Roots were harvested and soil was removed from the roots by washing with tap water. Root-hypocotyl segments of primary roots were excised with a razor blade. Isolation of xylem and bark was performed under the dissecting microscope according to Zhao *et al*., 2000). Isolated xylem and bark were placed in liquid nitrogen or dry ice and stored at –70°C until use.

2.2.2 Preparation of tissue extracts for immunoblot analysis

The isolated xylem and bark were ground separately in a chilled mortar using liquid nitrogen. Protein was extracted in 100 mM sodium phosphate, pH 7.2, 20 μM leupeptin using a tissue:buffer ratio of 1:4 (w/v). The tissue homogenate was centrifuged at 12,000*g* at 4°C for 10 min. The supernatant was concentrated approximately 10-fold using YM10 concentrators (Millipore, Bedford, MA) and stored at -70°C until use.

2.2.3 Production and affinity purification of anti-XCP1 antibody

XCP1H6 that had been purified by metal-chelate chromatography was used to immunize a rabbit for polyclonal antibody production at Cocalico Biologicals (Reamstown, PA). For affinity purification of anti-XCP1 antibody, XCP1 purified by metal-chelate chromatography and gel filtration (Sephacryl S-200) was added to affigel-

10 (Biorad) and rotated overnight at 4 °C in 6 M urea, 3 mM EDTA and 25 mM HEPES, at pH 8.0. The affinity resin was then blocked with 100 mM Tris HCl pH 7.5 and washed with phosphate-buffered saline (PBS), pH 7.2. The terminal bleed (75 ml) from the immunized rabbit was passed through the column. The column was then washed with 30 ml PBS. Bound antibody was eluted with 3 ml of acid elution buffer (200 mM glycine, 20 mM sodium citrate, pH 2.8) and also with base elution (100 mM diethylamine, pH 11.5). The fractions exhibiting maximum A_{280} were pooled, the pH was adjusted to pH 8 and samples were concentrated using YM10 concentrators (Millipore, Bedford, MA). To desalt samples using YM10 concentrators, PBS was used as a wash.

2.2.4 Immunoblot analysis

The protein concentration of concentrated tissue extract supernatant was determined by the bicinchoninic (BCA) assay using bovine serum albumin as the standard. Following SDS-PAGE and electrophoresis transfer of root-hypocotyl protein to PVDF, immunoblot analysis was conducted according to Beers and Freeman (1997). The membranes were blocked with Blotto (Johnson, 1984). Blots were incubated using anti-XCP1 as the primary antibody (1:1000 dilution) for 2 h at room temperature. The membranes were washed in TBS (50mM Tris, pH 7.4 and 200mM NaCl) for 15 min, followed by incubation with secondary antibody (1:1000 dilution) for 1 h at room temperature. Secondary antibodies used were affinity purified, alkaline phosphatase-labeled, goat anti-rabbit IgG (H+L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The blots were then washed for 30 min with 3 changes of TBS. To visualize bands, the substrates BCIP (5-bromo-4-chloro-3-indoxyl-phosphate) and NBT (nitroblue

tetrazolium) (Sigma, St.Louis, MO) in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5) were used.

2.3 Tissue and cell-type localization of *GUS* expression under the control of a putative promoter for *XCP1*

2.3.1 Construction of *XCP1* promoter*::GUS*-binary vector for transformation of *Arabidopsis*

XCP1 promoter was obtained from the genomic DNA of *Arabidopsis* by polymerase chain reaction using two synthetic primers (sense primer, 5'-GCACTAGTGTGTTTGCACTTTGCAGG-3';
antisense primer, 5'- GCCATGGCCAAATTTGTTCACTGAG-3'). The sense primer was designed to include an SpeI site, and the antisense primer was designed to include an NcoI site for subcloning of the promoter fragment. The resulting 591-bp PCR product was ligated to a pGEM-T Easy vector (Promega, Madison, WI) creating pPXCP1.

The *XCP1* promoter was isolated from pPXCP1 as an NotI-NcoI fragment and used to replace the NotI-NcoI fragment of *SAG12* promoter of pSG506 vector (gift of S. Gan) in fusion with the 2680-bp *GUS* coding region and the Mas 3' terminator fragment creating pPXCP1GUS. Ampicillin (100 mg/l) was used for the selection of pPXCP1GUS plasmid DNA in both liquid and solid LB medium.

The 3271-bp insert from pPXCP1GUS, was then cloned as SpeI-BamHI fragments between SpeI-BamHI multiple cloning site region of 5000-bp binary vector, pCB302 (gift of C. Xiang) between the borders of the transferred DNA (T-DNA) (Xiang,

et al., 1999) creating pPXCP1GUSB as shown in Figure 2. Kanamycin (50 m/l) was used for the selection of pPXCP1GUSB plasmid DNA in both liquid and solid LB medium.

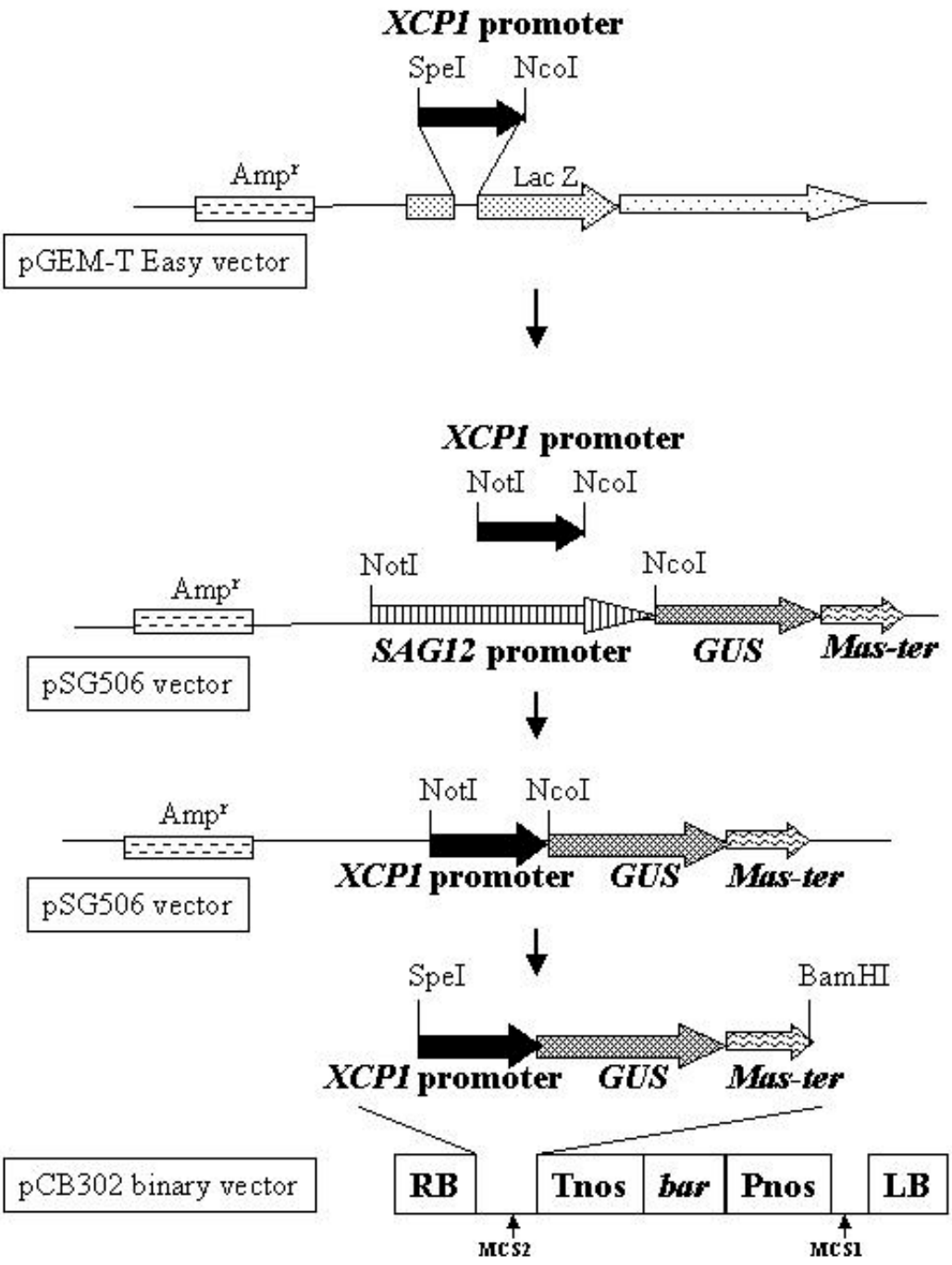

Figure 2. Construction of *XCP1* promoter::*GUS*-binary vector

2.3.2 Transformation of competent *Agrobacterium*

For transformation of *Agrobacterium* by the freeze-thaw method, an aliquot of *Agrobacterium* GV3101 competent cells was thawed on ice. The plasmid DNA of pPXCP1GUSB (1 μg) was added. The mixture was incubated on ice for 30 min. The mixture was fast-frozen by dipping into liquid N_2 until the microfuge tube was totally frozen, about 30 sec. The tube was thawed in a 37°C water bath, about 2 min. 0.8 ml 2XTY medium was added to the tube. The microfuge tube was incubated at 28°C for 2 h with vigorous shaking at 200 rpm. 100 μl of the mixture was spread on LB containing 34 mg/l rifampicin, 25 mg/l gentamycin and 50 mg/l kanamycin. The remainder was pelleted at 5000 rpm. The pellet was resuspended in 100 μl 2XTY medium (16 g tryptone, 10 g yeast extract, 5 g NaCl, 1 L total, pH 7) and was spread on another plate. Both plates were incubated at 28°C for 2 days.

2.3.3 Transformation of *Arabidopsis* by vacuum infiltration

The pPXCP1GUSB-transformed *Agrobacterium* were transferred to *Arabidopsis* plant cells by vacuum infiltration (Bechtold *et al.*, 1993). The *Agrobacterium* carrying the pPXCP1GUSB was grown in 6 ml 2XTY medium containing 34 mg/l rifampicin, 25 mg/l gentamycin and 50 mg/l kanamycin at 28°C for 2 days. The *Agrobacterium* culture was pelleted and resuspended to OD_{600} in 5% sucrose, 0.05% Silwet L-77. The solution was poured into a plastic dish on which the plants had been placed in an inverted position, with their inflorescences spread on the dish surface. Vacuum was applied for 15 min and then was released. Dipped plants were placed under a dome of plastic film for 24 hours to

maintain high humidity. The plants were watered and grown normally the next day. Siliques were harvested when they turned brown.

2.3.4 Selection of *Arabidopsis* transformant

Approximately 3,000 T_1 seeds were germinated in flats for about 1 week until the cotyledon stage. The seedlings were sprayed with 0.5% Liberty herbicide (glufosinate-ammonium;butanoic acid, 2-amino-4-(hydroxymethylphosphinyl)-monoammonium salt). Putative transformants (plants resistant to herbicide) were transplanted to pots, grown and watered normally until screening for GUS activity.

2.3.5 Histochemical detection of β-glucuronidase activity

To study the tissue and cell-type localization of *GUS* expression driven by putative *XCP1* promoter, the herbicide-resistant plants were assayed for GUS activity as described by Campisi *et al.*, 1999. Excised organs from 3- to 6-week-old putative transformants were transferred to a microfuge tube containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) in 50 mM sodium phosphate (pH 7.2), and 0.5% Triton X-100. Vacuum was applied and released repeatedly over 5 min to infiltrate the GUS substrate solution. The tube was incubated at 37 °C for 48 hours. The GUS substrate solution was removed and replaced with 70% ethanol. The organs remained in ethanol until visible chlorophyll had been removed. The tissue and cell-type localization of GUS staining were detected by a light microscope and photomicrographs were prepared using Tungsten 160 slide film.

CHAPTER 3: RESULTS

3.1 Expression and purification of a recombinant polyhistidine-tagged XCP1

Expression of a recombinant polyhistidine-tagged XCP1 (XCP1H6) is documented in Figure 3. A comparison of extracts prepared from pre- versus post-induction reveals that the addition of IPTG resulted in the expression of a novel polypeptide at approximately 40 kD, the expected molecular mass for XCP1H6. After lysis by sonication, the bacterial cell components were separated by centrifugation. The 40 kD protein was detected only in the pellet (inclusion bodies).

Purification of XCP1H6 from inclusion bodies required denaturing conditions and is documented in Figure 4. XCP1H6 remained bound to Ni^{+}-NTA beads throughout several washes. In fact, the protein remained bound to Ni^{+}-NTA beads even under conditions of low pH and high imidazole expected to release XCP1H6. Ultimately, it was determined that the addition of β-mercaptoethanol to 10 mM final concentration was necessary for effective elution (data not shown).

3.2 Activation of renatured recombinant protein

Purified denatured XCP1H6 in elution buffer was renatured by using buffer R to dilute urea, which is a denaturing agent, and by incubating the diluted protein overnight at 4 °C (Smith and Gottesman, 1989). After desalting to remove remaining urea, the renatured recombinant protein was not capable of degrading gelatin in an activity gel

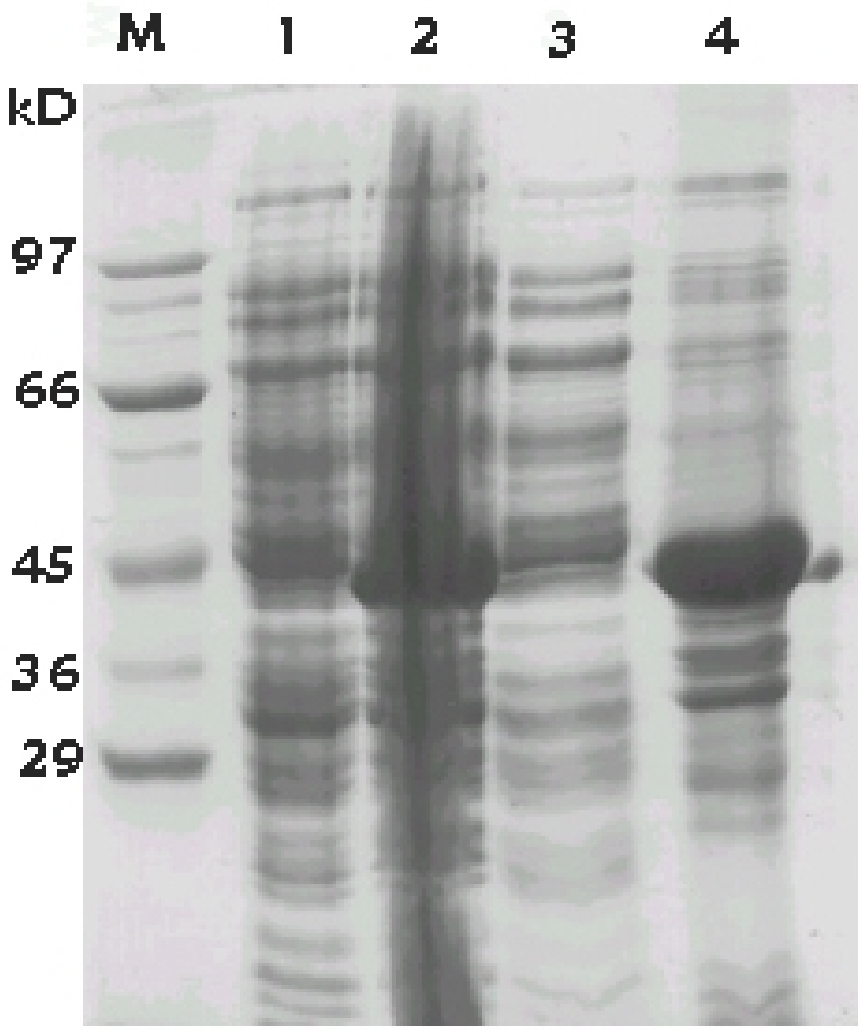

Figure 3. Polyhistidine-tagged XCP1 is localized in the insoluble fraction of *E. coli*. The samples were analyzed by 12% SDS-PAGE and the resolved proteins were stained with Coomassie brilliant blue. Lane M, molecular mass markers; lanes 1, whole cell lysate (prior to induction with IPTG); lane 2, whole cell lysate (post-induction with IPTG); lane 3, lysate supernatant (post induction with IPTG); lane 4, lysate pellet (post induction with IPTG). Molecular masses of protein standards (kD) are shown at left.

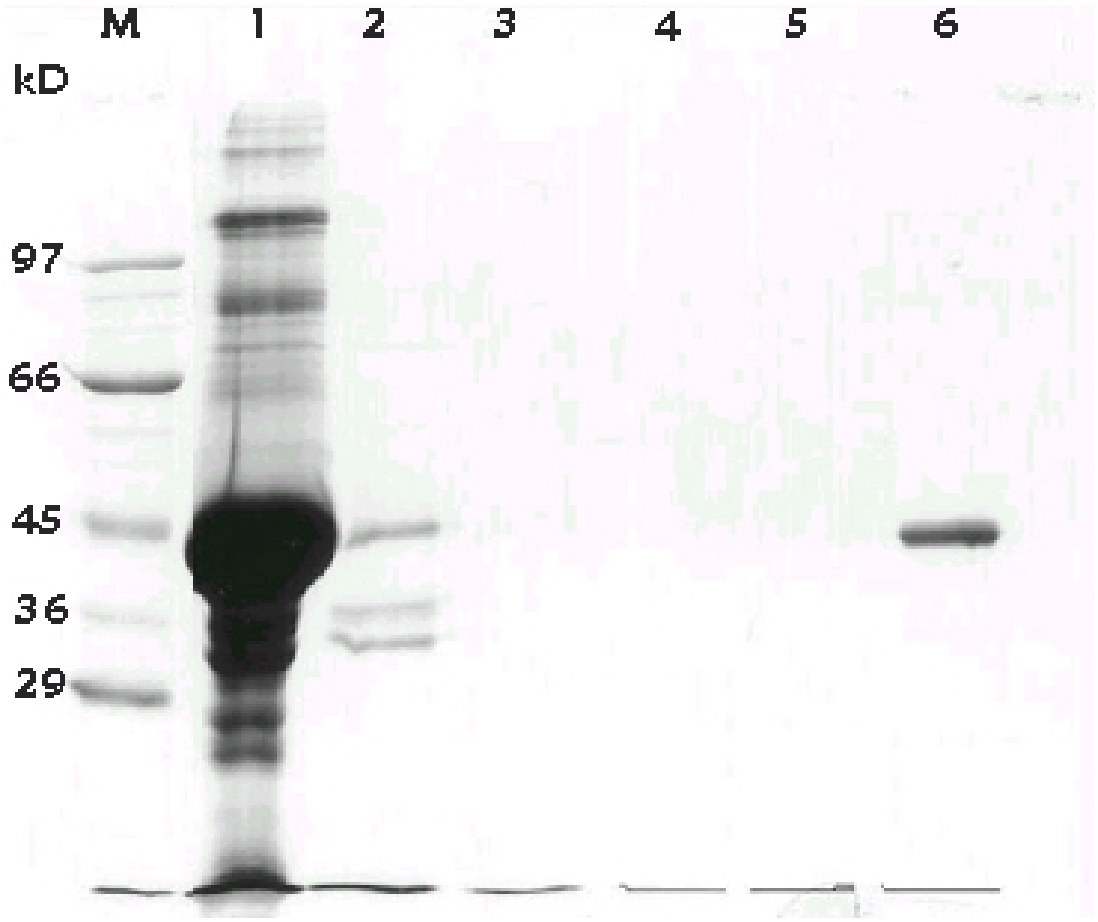

Figure 4. Purified polyhistidine-tagged XCP1 remains bound to Ni^{+}-NTA agarose in the presence of 500 mM imidazole. The samples were analyzed by 12% SDS-PAGE and the resolved proteins were stained with Coomassie brilliant blue. Lane M, molecular mass markers; lane 1, cell lysate; lane 2, flow-through; lanes 3 & 4, wash; lane 5, attempted elution with 500 mM imidazole; lane 6, purified XCP1bound to Ni^{+}-NTA beads. Molecular masses (kD) of protein standards are shown at left.

assay (data is not shown). Renatured XCP1H6 was activated by incubating at pH 5.5 (Figure 5). Concomitant with activation, XCP1H6 was processed to a smaller protein during incubation at pH 5.5. Processing to the smaller form was inhibited by the papain-type protease inhibitors E-64 and leupeptin (data not shown). The 26 kD band detected on a Coomassie-stained gel following activation was proteolytically active as observed in the gelatin-impregnated substrate gel (Figure 5 b). An additional activity at 22 kD was also visible on the activity gel but was not detectable by Coomassie staining. Proteolytic activity of both forms of processed XCP1H6 was inhibited by E-64 (data not shown).

3.3 Developmental time course and tissue-level localization of XCP1 protein

XCP1 was previously shown by RNA gel blot analysis to be expressed in xylem (Zhao *et al.*, 2000). To determine the tissue distribution of XCP1, xylem and bark were isolated from root-hypocotyl segments and protein extracts from these tissues were resolved by SDS-PAGE for immunoblot analysis using affinity-purified anti-XCP1 antibody. To evaluate changes in XCP1 levels during development of secondary vascular tissue, extracts from 5-, 6-, 7-, 8-, and 9-week-old root-hypocotyl segments were probed with anti-XCP1 antibody. The resulting immunoblot shows that two bands at 36 and 50 kD were detected in xylem extracts by anti-XCP1 antibody (Fig. 6). No bands were detected in bark extracts.

These immunoblot results indicate that XCP1 was expressed in xylem and not in bark. The most striking difference among the time points tested was that 9-week-old roots

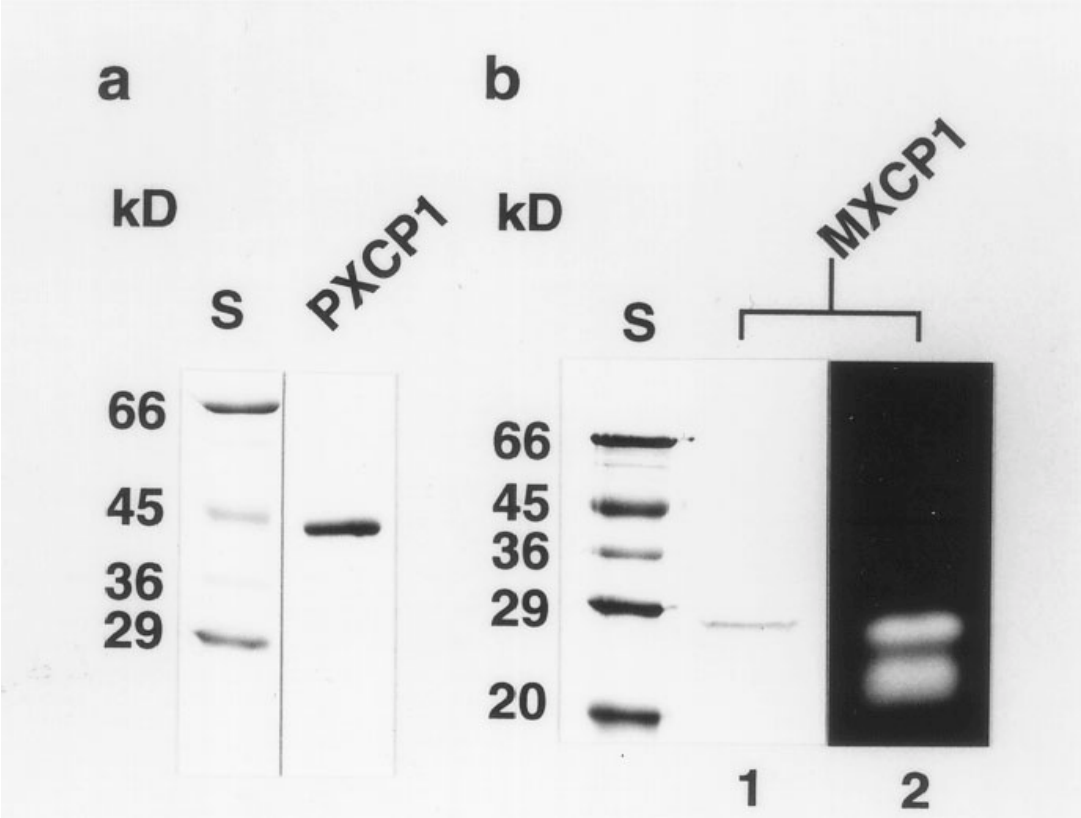

Figure 5. Following renaturation, polyhistidine-tagged XCP1 is proteolytically processed and activated during incubation at pH 5.5. Renatured polyhistidine-tagged XCP1 is a stable but inactive, 40 kD propeptide (PXCP1) when stored at pH 8.0 **(a)**. Incubation at pH 5.5 results in the replacement of the 40 kD polypeptide with a proteolytically active 26 kD protease (MXCP1) **(b)**. For **b**, the smaller version of XCP1 (MXCP1) is detectable by Coomassie staining (lane 1) and proteolytic activity is detected by activity gel analysis (lane 2). An additional activity at 22 kD is detectable on the activity gel but not on the Coomassie-stained gel. Molecular masses (kD) of protein standards are shown to the left of each Coomassie-stained gel.

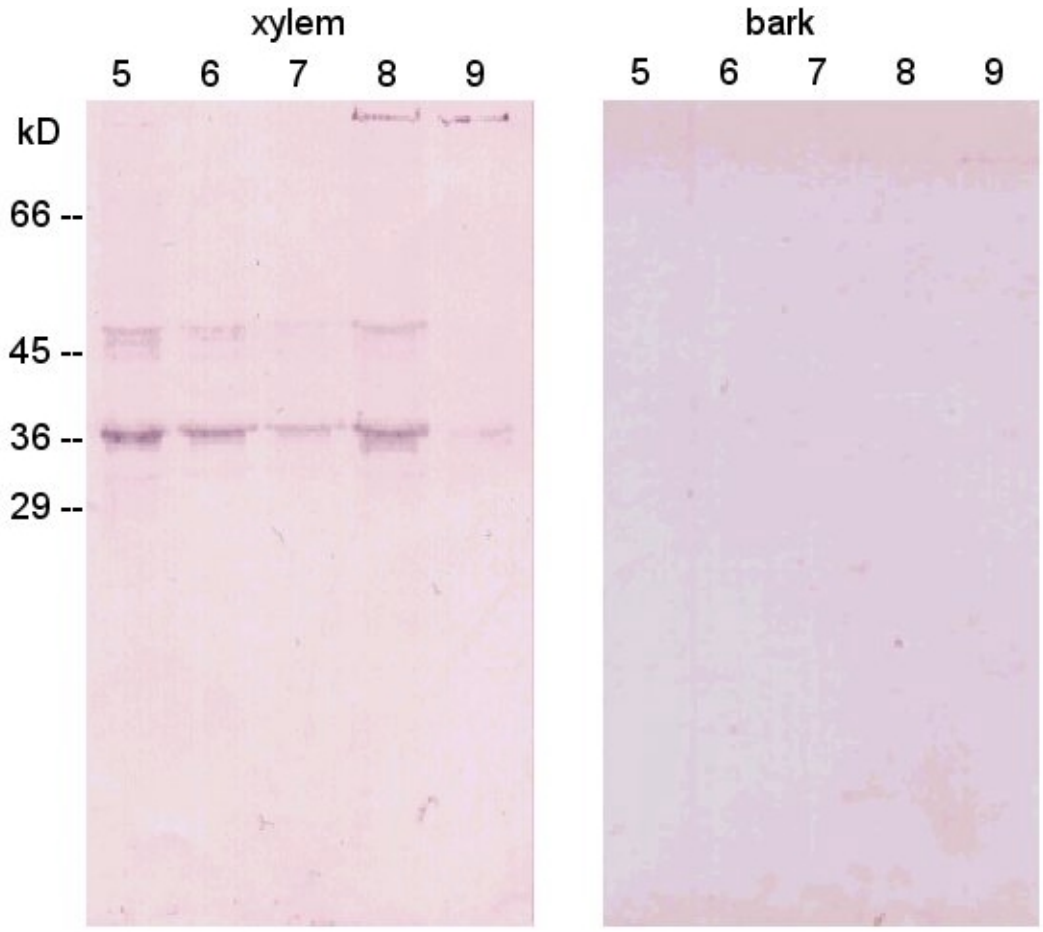

Figure 6. Anti-XCP1 antibody reacts with protein in xylem only. Xylem and bark extracts (15 μg total protein in each lane) from 5-, 6-, 7-, 8- and 9-week-old root-hypocotyl segments of primary roots were separated by 12% SDS-PAGE and transferred to a PVDF membrane. The highest level of immunoreactivity was detected at 36 kD at all time points. Faint bands between 45 and 50 kD were also visible. Molecular masses (kD) of protein standards are shown at left.

yielded less immunoreactive protein than all other time points, with the possible exception of 7-week-old roots. Assuming that the 36 kD band is the XCP1 propeptide, these results are consistent with the previous observation that *XCP1* mRNA is restricted to xylem (Zhao *et al*, 2000). However, the ability of anti-XCP1 antibody to detect a higher molecular mass species (50 kD) suggests that anti-XCP1 antibody may react with other proteins. To explore the possible non-specific nature of anti-XCP1 antibodies, an additional immunoblot was produced using extracts from 8-week-old roots and a higher protein level.

The Immunoblot prepared with 25 μg (versus 15 μg for developmental time course) total protein and long exposure (45 min) to alkaline phosphatase substrates shows that anti-XCP1 antibody can detect multiple proteins in both xylem and bark (Fig. 7). As expected for this antibody which was raised against a putative xylem-specific protease, a higher level of immunoreactive protein at 36 kD was detected in xylem versus bark. The presence of faint 50, 32, and 18 kD bands was also noted in extracts from xylem. Additionally, two poorly resolved bands between 45 and 50 kD were detected in xylem extracts. In bark extracts, the 36 kD band was also detected, but was very faint. Two other polypeptides (45 and 32 kD) were also detected at low levels in bark extracts.

3.4 Cell-type localization of *GUS* expression directed by the *XCP1* promoter

To begin to explore both cell-type localization of XCP1 and to characterize the *XCP1* promoter, *XCP1* promoter::*GUS* fusion transgenic plants were evaluated. Transgenic plants were selected by 0.5% Liberty spraying and 0.25% Liberty painting.

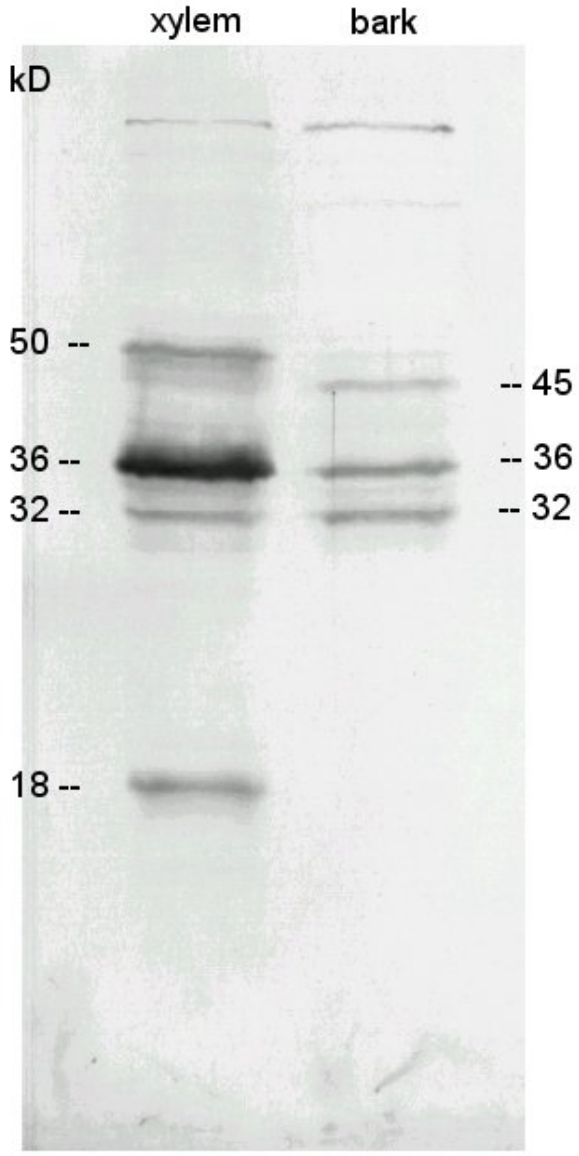

Figure 7. Optimized immunoblot conditions reveal that anti-XCP1 antibodies react with several proteins in xylem and bark. The immunoblot was prepared using extracts from 8-week-old roots as for Figure 6 except that 25 µg of protein was loaded in each lane. Incubation in NBT/BCIP alkaline phosphatase substrate was for 45 min versus 30 min for Figure 6. Molecular masses (kD) of immunoreactive proteins are indicated.

Approximately 1% of plants (32 transgenic plants from 3,000 progeny seed) were fully resistant to these applications, while non-transgenic plants were killed. The rate of transformants obtained by vacuum infiltration was within the expected range (0.5-3% of all progeny seed) as reported by Clough and Bent (1998).

The localization of *GUS* expression under the control of a putative promoter for *XCP1* was determined by histochemical staining for GUS activity. If the *XCP1* promoter can direct *GUS* expression, a blue precipitate resulting from β-glucuronidase activity in the presence of X-Gluc should be detected in the cells that also express the authentic *XCP1* gene. A preliminary survey of putative transgenic plants revealed that GUS activity was detectable in leaves (Fig. 8), stems (Fig. 9) and flowers (Fig. 10) of 3 individual transformants. GUS activity was also detected in roots of two additional transformants (Fig. 11). Among those organs testing positive for GUS activity, staining was present only in the vascular tissue of leaves, stems, roots and flowers. Senescent leaves were also taken from 3 plants which exhibited GUS activity. Although, no GUS activity was visible in senescing (yellow) portions of these leaves, GUS activity was detected in a few isolated vascular tissue branch-points in the youngest, basal portion of one leaf (data not shown). GUS activity was not detected in senescing siliques (data not shown).

For all organs tested, GUS staining was observed in the vascular tissue cells that possess the thick secondary wall patterning common to TEs (Fig. 8, 9 and 10). These results from the histochemical analysis for GUS activity are consistent with our observation that *XCP1* is expressed in xylem and allow us to extend tentatively our tissue

level localization to cell-type. Specifically, the putative *XCP1* promoter evaluated in this study is capable of directing gene expression that appears to be limited to TEs.

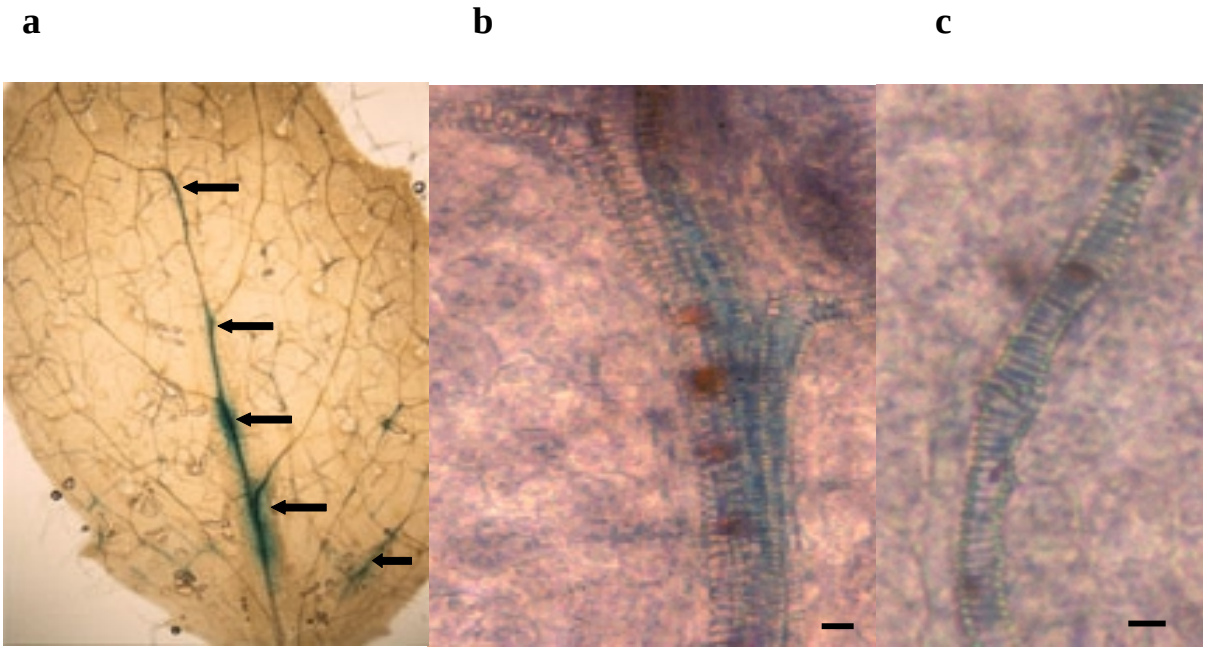

Figure 8. The putative *XCP1* promoter directs *GUS* expression in leaf vascular tissue. GUS activity (arrows) was detected in vascular tissue **(a)**. Higher magnification from individual transformants **(b** and **c)** reveals vascular GUS activity limited to tracheary elements. Bar = 10 μm

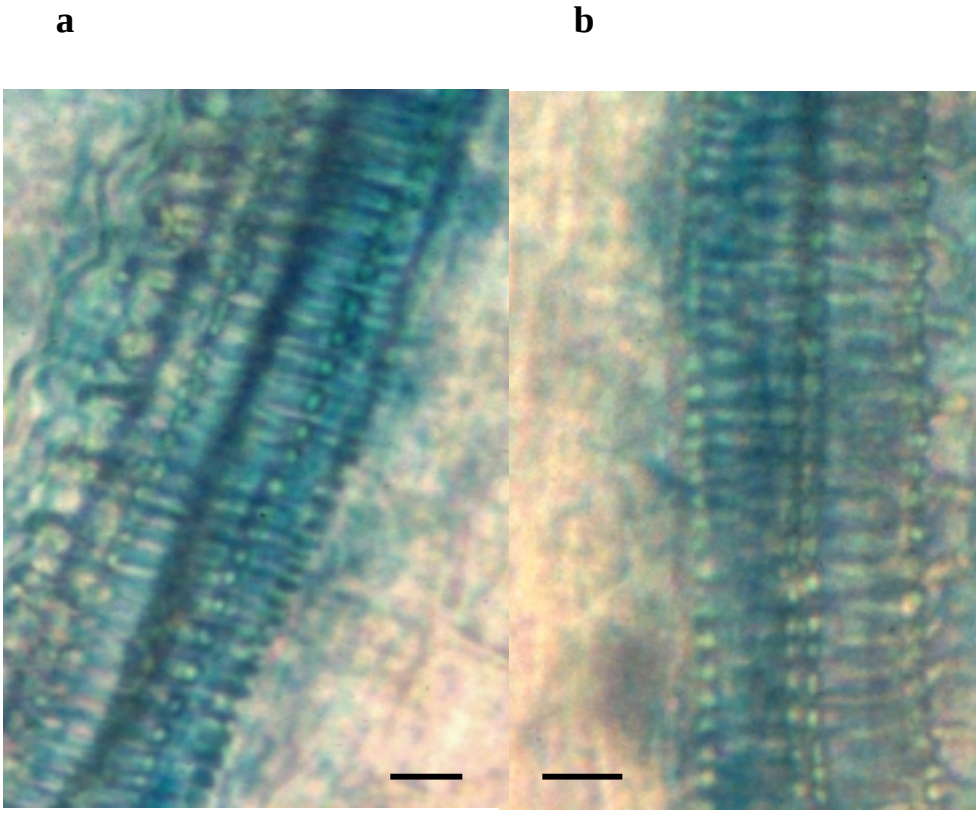

Figure 9. The putative *XCP1* promoter directs *GUS* expression in stem vascular tissue. GUS activity was detected in tracheary elements of stems from individual transformants **(a** and **b)**. Bar = 10 μm

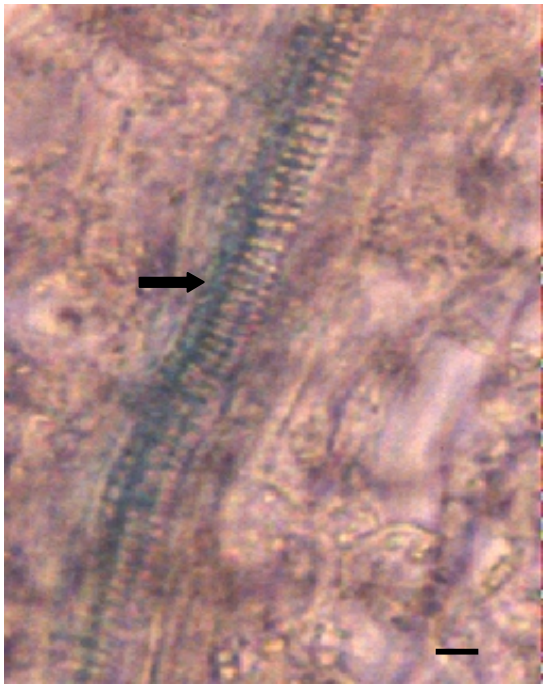

Figure 10. The putative *XCP1* promoter directs *GUS* expression in sepal vascular tissue. GUS activity (arrow) was detected in tracheary elements of sepal. Bar = 10 μm

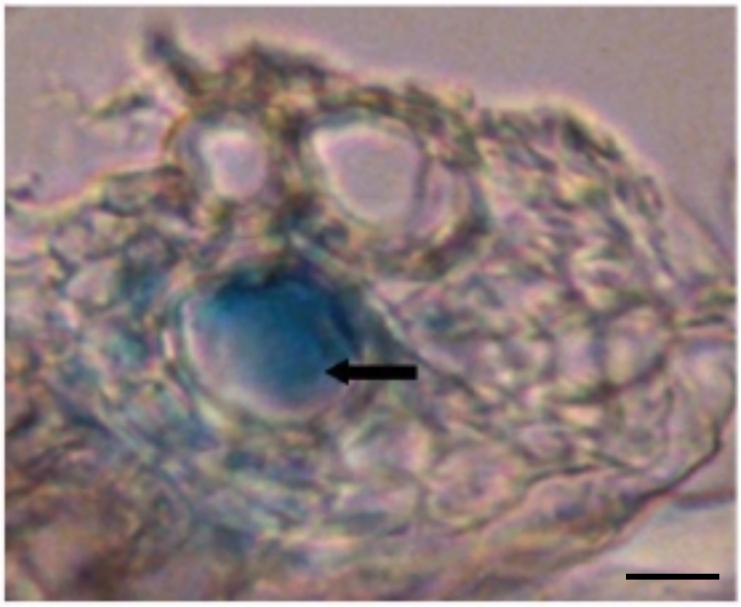

Figure 11. The putative *XCP1* promoter directs *GUS* expression in root vascular tissue. GUS activity (arrow) was detected in a tracheary element visible in this cross-section of a 5-week-old root. Bar = 10 μm

CHAPTER 4: DISCUSSION

XCP1 was cloned into an expression vector creating polyhistidine-tagged XCP1 (XCP1H6). This protein was then expressed in *E. coli* and purified with Ni^{+}-chelating chromatography. The purified XCP1H6 was used to raise polyclonal antibodies. Incubation of XCP1H6 at pH 5.5 was required for enzyme activation. Immunoblot analysis revealed high levels of a 36 kD protein in xylem which is most likely proXCP1. However, due to the high degree of sequence identity among papain-type cysteine protease, several other proteins were detected in both xylem and bark. To evaluate cell-type localized of XCP1, an *XCP1* promoter fused to *GUS* was stably expressed in *Arabidopsis* and demonstrated that XCP1 expression appears to be limited to TEs.

4.1 Expression and purification of a recombinant polyhistidine-tagged XCP1

In the design of XCP1H6 we wanted to ensure both high levels of expression and the retention of the polyhistidine purification tag. Consequently, the signal sequence of XCP1 was omitted from XCP1H6 as some research has shown that removing the signal sequence can lead the to higher expression levels of cysteine proteases in *E. coli* (Smith and Gottesman, 1989). The polyhistidine tag was placed at the C-terminus because extensive posttranslational processing of papain-type proteases results in the loss of the amino terminus.

The low pH (5.5) condition that was used in this study of XCP1H6 is similar to conditions in other investigations of cysteine protease activation. Processing of the papain precursor is stimulated by acidic pH environment (Bossard *et al*., 1996) and elevated

temperature (Vernet *et al.*, 1990). Low pH (pH 4.0-5.5) may induce a structural transition required for intramolecular cleavage (Vernet *et al.*, 1991). Processing to the active protease and activity of the two smaller processed proteins were both prevented by the papain-type inhibitor E-64. The protease inhibitor experiments indicate that 1) contaminating non-cysteine proteases are not likely to be responsible for XCP1 activation/processing *in vitro*, 2) E-64-sensitive, protease activity is required for activation and processing of XCP1H6, and 3) as predicted by the cDNA sequence, XCP1 is a cysteine protease.

4.2 Developmental time course and tissue-level localization of XCP1 protein

XCP1 was previously localized to xylem in *Arabidopsis* (Zhao et al., 2000). To address by independent methodology xylem localization for XCP1, immunoblot analyses using anti-XCP1 antibodies were performed. The results from a developmental time course experiment indicated that anti-XCP1 antibody immunoreactivity was present only in xylem, not in bark. This result was consistent with RNA gel blot result (Zhao *et al*., 2000). A very low level of immunoreactivity was detected in extracts from xylem of 9-week-old plants compared to other time points, perhaps because these older plants were not as actively engaged in the production of secondary vascular tissue as were younger plants.

Further analysis of xylem and bark protein revealed that anti-XCP1 antibodies could detect multiple bands in both xylem and in bark. By considering the predicted size of the translation products from genes *XCP1*, *XCP2* and *Atcp132*, and by recognizing the

high degree of sequence identity among these three cysteine proteases cloned by our lab, it is possible to speculate about the multiple bands detected by anti-XCP1 antibody. XCP1 and XCP2 are 70% identical at the amino acid level and appear to be xylem-specific. Molecular masses of these proteins are similar. Atcp132 is expressed in bark and xylem and while it is 48 % identical to XCP1 and XCP2, it possesses an 11 kD C-terminal extension, resulting in a higher molecular mass relative to XCP1 and XCP2. For XCP1, the full length is predicted to be 39.6 kD, the proprotein is 36.8 kD and the mature protein is 23.8 kD. For XCP2, the full length is predicted to be 39.7 kD, the proprotein is 37.1 kD and the mature protein is 23.8 kD. For Atcp132, the predicted full length is 48.1 kD, the proprotein is 45.5 kD, and the mature protein, including the C-terminal extension is 35.2 kD. It is possible that the 36 kD band in xylem extracts is the proprotein of XCP1. The 18 kD band detected in xylem extracts may be the mature protein of XCP1. Interestingly, proteolytic activity at 18 kD unique to xylem has been reported (Zhao *et al*., 2000). The 32 kD band of xylem extract might be the intermediate during the processing of the proprotein of XCP1. All of the bands in bark may be processing intermediates of Atcp132 or another cysteine protease. Another papain-type cysteine protease, rd21A (Koizumi *et al*., 1993) is also expressed in both bark and xylem (Zhao *et al*., 2000). Although it appears that anti-XCP1 antibody is capable of recognizing other related proteases or perhaps intermediate processing products of the XCP1 zymogen, the vast majority of immunoreactivity was restricted to the xylem. This abundance of antigen in xylem is consistent with the localization of XCP1 (or very closely related proteases) to

xylem. Consequently, the anti-XCP1 antibody used in this study may be a useful reagent for immunolocalization experiments aimed at localizing XCP1 to a xylem cell type.

4.3 Cell-type localization of *GUS* expression directed by the *XCP1* promoter

Out of the 32 plants which were resistant to the herbicide, only five plants showed any *GUS* expression. This may be due to the loss of the *XCP1* promoter*::GUS* gene during transformation. Another explanation for the lack of GUS activity in 27 of the 32 plants is that the *XCP1* promoter directs only a low level of expression. Low expression of *XCP1* is also consistent with the absence of *XCP1* from the reported *Arabidopsis* ESTs (Zhao *et al.*, 2000). It has been reported that XCP1 expression was nearly 20-fold less than that of the closely related xylem cysteine protease, XCP2. Consequently, only low levels of GUS may be expressed even in plants exhibiting high levels of *bar* gene expression which is driven by the *NOS* (nopaline synthase) promoter (Valvekens *et al*., 1988). As the GUS activity survey was not comprehensive, it is also possible that GUS-expressing organs were not evaluated from all plants. Finally, X-Gluc substrate solution may not have infiltrated all parts of the organs tested.

No GUS activity was visible in senescing, mesophyll cells of leaves or siliques. Such results are consistent with the extremely low to non-detectable levels of *XCP1* mRNA in mature and senescent leaves (Zhao *et al*., 2000) and indicate that *XCP1* is not expressed at this stage of leaf development. XCP1 therefore is a cysteine protease that appears to function during TE differentiation unlike many other plant cysteine proteases (Granell, 1998; Drake *et al*., 1996; Kardailsky and Brewin, 1996; Miller *et al*., 1999;

Valpuesta *et al*., 1995), XCP1 does not appear to play a role during plant senescence. Several reverse genetics experiments are currently underway in our lab to firmly establish the role of XCP1 during TE differentiation.

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