The Role of the Ubiquitin-Proteasome Pathway During Xylem Differentiation in *Zinnia elegans* Mesophyll Cells and *Arabidopsis thaliana*

by

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ABSTRACT

A biochemical characterization of ubiquitin (Ub)-proteasome pathway activity was conducted in *Zinnia* mesophyll cell cultures to examine potential differences between differentiating cells of tracheary element (TE) cultures and non-differentiating cells of control cultures. The pathway is highly active throughout development of differentiating TEs , a programmed cell death (PCD) process during which the majority of cellular proteins and biochemical processes are expected to be down-regulated in activity and/or expression. Addition of the proteasome inhibitors *clasto*-lactacystin β-lactone (LAC) and carbobenzoxy-leucinyl-leucinyl-leucinal (LLL) at culture initiation prevented TE differentiation in this system. Proteasome inhibition at 48h did not alter the final percentage of TEs compared to controls. However, proteasome inhibition at 48 h delayed the differentiation program by approximately 24 h, as indicated by examination of morphological markers and the expression of putative autolytic cysteine proteases. These results suggest that proteasome activity is required both for induction of TE differentiation and for progression of the TE program in committed cells. Treatment at 48 h with LLL resulted in partial uncoupling of

autolysis from differentiation. Results of protease activity gel analysis suggest that incomplete autolysis was due to the ability of LLL to inhibit TE cysteine proteases.

A characterization of phytohormone-stimulated growth of non-differentiating cultured *Zinnia* cells is also presented. Differential effects on radial cell expansion versus cell elongation were observed for the four plant growth regulators examined. Auxin (naphthaleneacetic acid, NAA) and a brassinosteroid (2,4-epibrassinolide, BI) stimulate only cell elongation. Cytokinin (N-6-benzyladenine, BA) has a greater effect on growth in cell girth rather than length. Gibberellic acid (GA_3) has equivalent effects on expansion in both dimensions. These results demonstrate that radial cell expansion and cell elongation can be uncoupled, and therefore, may be controlled by different mechanisms. Additionally, this study establishes the utility of *Zinnia* suspension cultures as a valuable model for studies of cell expansion.

Finally, we modified *Arabidopsis* plant growth conditions to promote proliferation of secondary tissues, permitting the separation of secondary xylem from bark (phloem plus nonvascular) tissues using hypocotyl-root segments. Dissected tissues were used for semi-quantitative and quantitative RT-PCR and for the construction of bark and xylem cDNA libraries for PCR-based screening of several Ub pathway components, including Ub-conjugating enzymes (*UBC*s), deubiquitinating enzymes (DUBs), and an α (*PAF1)* and β (*PBF1)* subunit of the proteasome. All targeted *UBC* families, candidate *UBC*s and DUBs, and proteasome subunits are expressed in secondary xylem and bark in this system.

DEDICATION

This dissertation is dedicated to my family, especially to my parents, Mary Ann and Robert William Woffenden, whose ever-present love and encouragement nourished my contemplation and sustained the completion of this undertaking.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

CHAPTER 1

Literature Review

In the vasculature of higher plants, xylem tissue is responsible for the transport of water and mineral solutes. The water-conducting elements of xylem tissue, TEs, are essentially cell corpses through which water and solutes flow. The development of TEs requires that nondifferentiated parenchyma cells of either the procambium (primary xylem) or the vascular cambium (secondary xylem) respond appropriately to differentiation signals and initiate vast morphological changes, including the deposition and lignification of cellulosic thickenings in the secondary cell wall. Differentiating cells then undergo PCD, involving the degradation of cytosolic materials followed by hydrolysis of unlignified cell wall components, permitting the release of any remaining cellular contents, referred to as autolysis. The differentiation of xylem tissue in higher plants is an example of genetically programmed cell death (PCD) as a prerequisite for proper eukaryotic development. The development of xylem within the vascular system thus serves as a model for the study of the mechanisms that regulate both PCD and differentiation in plants.

Zinnia elegans mesophyll cell tissue culture system as a model of TE differentiation

The process of TE differentiation can be reproduced in culture using mesophyll cells isolated from young leaves of *Zinnia elegans* (Church 1993; Fukuda 1996; Fukuda 1997; Fukuda and Komamine 1980). This process proceeds essentially in the same manner and at a similar rate in culture as in the intact leaf (Burgess and Linstead 1984). In this system, cells are isolated from young leaves and cultured in suspension in the presence of auxin (1-naphthaleneacetic acid)

and cytokinin (N-6-benzyladenine). These cells are initially homogenous, and differentiation into TEs occurs semi-synchronously in 40-60% of living cells in 72 h. Noninduced control cultures contain only nondifferentiated cells.

The *Zinnia* system has facilitated biochemical and molecular studies of xylem development that have discovered several markers for TE differentiation and autolysis; additionally, pharmacological studies involving application of specific protease inhibitors to *Zinnia* cultures have discovered several roles for proteases during TE formation. Proteins that are associated with TE differentiation include DNAses (Aoyagi, *et al.* 1998), the lignification enzyme phenylalanine ammonia-lyase (Fukuda and Komamine 1982; Lin and Northcote 1990), xylan synthase (Suzuki, *et al.* 1991), 4-coumarate:CoA ligase (Church and Galston 1988), and peroxidase (Church and Galston 1988; Fukuda and Komamine 1982). Markers for TE autolysis include an endonuclease, (Thelen and Northcote 1989), an RNAse (Ye and Droste 1996), and cysteine and serine proteases (Beers and Freeman 1997; Minami and Fukuda 1995; Ye and Varner 1993; Ye and Varner 1996). Woffenden, *et al.* (1998) demonstrated that specific inhibition of the proteasome (discussed in detail below, Chapter 3) completely blocked TE development, suggesting that proteasome activity is required for differentiation. Groover and Jones (1999) demonstrated that inhibition of serine protease activity in TE cultures prevents vacuolar lysis and TE PCD.

Hormonal control of xylem development

The phytohormones auxin and cytokinin have long been recognized as important in vascular development (Aloni 1987), and more recently, brassinosteroids have been shown to be involved in xylem differentiation (Clouse and Sasse 1998). However, the signal transduction pathways regulating the diverse possible plant responses to each of these hormones are just now

being unraveled. Recently, the components of auxin signaling have begun to be elucidated (reviewed by del Pozo and Estelle 1999). Transcription factors (encoded by the AUX/IAA family of genes) mediating the auxin response have been discovered (reviewed by del Pozo and Estelle 1999). Responses to auxin apparently involve protein degradation via the ubiquitin (Ub)-proteasome pathway (to be discussed in detail below). In *Arabidopsis* two genes that appear to specifically function in auxin signaling are *AXR1* and *TIR1*, both of which are components of a Ub-like pathway that has been recently discovered in plants and other eukaryotes (del Pozo and Estelle 1999). These studies indicate that the Ub pathway may mediate degradation of repressors of auxin signaling (del Pozo and Estelle 1999), though direct evidence of this is lacking. Brassinosteroid signal transduction is under active examination (Clouse and Sasse 1998), but few details about brassinosteroid signaling have yet emerged from these efforts.

Overview of plant proteolysis

Plant cells possess both ATP-dependent and ATP-independent proteolytic activities (Ryan and Walker-Simmons 1981; Vierstra 1996). The Ub-proteasome-mediated pathway (to be described in detail below) is the best-studied system of ATP-dependent proteolysis in eukaryotes. Proteases of the Ub-proteasome system (26S proteasome and deubiquitinating enzymes) are found in both the nucleus and cytoplasm (Callis 1997; Vierstra 1993). Chloroplasts harbor an ATP-dependent proteolytic system (Clp system) analogous to the bacterial ATP-dependent protease, Lon (Gottesman and Maurizi 1992).

Many other proteolytic activities exist in plant cells, and the subcellular localization of some of these has been reviewed by Vierstra (1993; 1996). Cysteine and serine proteases are present in the cell wall and also in the vacuole and peroxisomes. Metalloproteases are also present in peroxisomes. Aspartic

acid protease activity has been demonstrated in plants, but its subcellular localization is yet unknown. Plant mitochondria must possess processing proteases that act upon proteins imported into this organelle, but none has yet been described. The plant cell cytoplasm may contain calcium-dependent protease activity analogous to that found in animals, but neither the calcium-dependent protease, calpain, nor any other calcium-dependent proteases has been cloned from plants.

Involvement of proteases in PCD

Proteases have the capacity to participate in regulatory and/or nonregulatory functions of PCD. Proteolysis may be regulatory in PCD either through the removal of cell death inhibitors or the processing of inactive cell death promoters to an active state. As mentioned previously, proteolysis is emerging as important for auxin signal transduction (del Pozo and Estelle, 1999), and perhaps signaling by other plant growth regulators involves proteases. Nonregulatory functions of proteolysis in PCD include remobilization of nutrients from dying cells, modification of structures, and autolysis of cells.

The role of proteases in regulating PCD is currently an extremely active area of animal and plant research. Evidence for essential regulatory roles of protein degradation in PCD comes predominantly from animal systems, from which a family of cell death proteases has been identified. Principle among the proteases shown to be required for PCD in animal systems is the large and still-growing family of proteases known as caspases (cysteinyl aspartate-specific proteases) (recently reviewed by Nicholson and Thornberry 1997), which appear to be involved in disabling cellular repair processes essential for maintaining homeostasis, thereby leading to apoptosis. The members of the ICE/CED family are cysteine proteases with varying degrees of homology to the CED-3 protease.

CED-3 is essential for neuronal PCD during *C. elegans* development (reviewed by Kumar and Lavin 1996). Significantly, no relevant *in vivo* substrates have been unambiguously identified for these animal cell death proteases. Moreover, caspases are not the only proteases demonstrated to play a role in animal PCD. Animal apoptosis pathways can also be initiated by several other mechanistically distinct proteases including the serine protease granzyme B (Greenberg 1996), members of the cathepsin D family (Deiss, *et al.* 1996), and the cysteine protease calpain (Squier and Cohen 1996, 1997).

A growing body of evidence indicates that proteolysis is important for plant PCD processes, as well (reviewed by Beers, *et al.*, in press). However, although del Pozo and Lam (1998) implicated functional homologs of caspases as important in the plant hypersensitive response to pathogens, no proteases with significant sequence homology to the caspases have yet been identified from plants. Many other proteases, mostly non-ATP-dependent in their activity, have been implicated in PCD in plants (Beers *et al.*, in press). For example, increases in serine and cysteine proteases are associated with two developmentally-programmed suicide pathways, organ senescence (recently reviewed by Hadfield and Bennett 1997) and TE differentiation (recently reviewed by Beers 1997; Fukuda 1997; Pennell and Lamb 1997; Beers *et al.*, in press). It is generally assumed that these ATP-independent plant enzymes function in the autolysis of intracellular proteins rather than as components of regulatory proteolytic cascades; however, the apparent participation of multiple proteolytic pathways during animal PCD indicates that the ability of plant proteases to regulate PCD may be underestimated. The Ub-proteasome pathway is also broadly implicated as a regulator of cell fate in plants and animals, and evidence in support of a role of this proteolytic system in PCD will be presented in detail here. Identification

of plant PCD proteases and their *in vivo* targets remains a major challenge in basic plant research.

Overview of the ubiquitin-proteasome pathway of proteolysis

Both animals and plants possess nuclear and cytosolic proteolytic ability that is ATP-dependent (Ciechanover, *et al.* 1981; Vierstra 1987). In eukaryotes, the best-studied example of ATP-dependent protein degradation is the Ub-proteasome system (Hershko and Ciechanover 1998). The major biochemical function of this pathway is to mediate the degradation of cellular protein targets that have been modified by the covalent attachment of the highly conserved 76 amino acid protein Ub. Ub is attached through the carboxyl group of its C-terminal glycine residue to the ε-amino group of specific internal lysine residues in substrate proteins, forming an isopeptide linkage. The covalent attachment of Ub to cellular proteins is referred to as ubiquitination. Ub moieties may be attached to targets as monomers at one or multiple locations (monoubiquitination events) or as a chain of Ub molecules attached to one another through the carboxy-terminus of one Ub monomer and lysine 48 of another Ub molecule (polyubiquitination) (Chau, *et al.* 1989). Polyubiquitination of substrates has been shown to be sufficient for targeting proteins for degradation by the multicatalytic protease, the 26S proteasome (Chau, *et al.* 1989).

Ubiquitination of target proteins requires a series of reactions catalyzed by members of three families of enzymes, including Ub-activating enzyme (E1), Ub-conjugating enzymes (UBCs or E2s) and Ub-protein ligases (E3s). Another family of enzymes, deubiquitinating enzymes (DUBs), is involved in removing Ub from targeted proteins. The first step of the ubiquitination process is the ATP-dependent activation of a Ub monomer by E1, followed by transfer of

activated Ub to an essential cysteine residue of an E2 enzyme, forming a thioester linkage. Depending on the identity of the substrate, E2 may or may not cooperate with an E3 enzyme in its attachment of Ub to the target protein. Together, E2s and E3s impart substrate specificity to the ubiquitination process (reviewed by Haas and Siepman 1997; Scheffner, *et al*. 1998).

Ubiquitination may target proteins for degradation by the 26S proteasome (Jentsch 1992), while other substrates appear to become ubiquitinated without subsequent degradation (Hershko, *et al*. 1982; Paolini and Kinet 1993; Siegelman, *et al*. 1986; Wu, *et al*. 1981). Modification without degradation may affect the structure and/or function of the target. Thus, the Ub pathway can modulate cellular and developmental events at the level of protein turnover and also through effects on protein structure/function.

Cellular events that appear to involve the Ub pathway include heat shock response (Binet, *et al*. 1991; Christensen, *et al*. 1992; Seufert and Jentsch 1990), degradation of short-lived regulatory proteins (Chen, *et al*. 1993; Zhen, *et al*. 1993), cell cycle control in animals (Glotzer, *et al*. 1991; Ishida, *et al*. 1993; Mori, *et al*. 1993a) and plants (Genschik, *et al*. 1998), oogenesis and embryo development (Zhang, *et al*. 1993), mitogenic signaling (Mori, *et al*. 1993b), organelle biogenesis (Erdmann, *et al*. 1989), and PCD (Delic, *et al*. 1993; Schwartz, *et al*. 1990). Known *in vivo* substrates of Ub-dependent proteolysis from both animal and plant systems are many, and include short-lived regulatory proteins such as cyclins A and B and the yeast MAT α2 transcription repressor, growth factor receptors (platelet-derived growth factor and growth hormone receptors), the T lymphocyte antigen receptor, lymphocyte homing receptor, the tumor suppressor p53, and the plant photoreceptor phytochrome (reviewed by Hershko and Ciechanover, 1998). The diversity of regulatory functions in cell growth and differentiation of these Ub targets suggests that this degradative pathway may be

involved in the turnover of other important regulatory proteins in animals and plants.

Ubiquitin-conjugating enzymes (UBCs)

The function of Ub-conjugating enzymes (UBCs) in the Ub-proteasome pathway has been recently reviewed (Haas and Siepman 1997). UBCs have long been considered the principle regulators of substrate specificity of the pathway, in some cases in combination with cognate E3s. *UBC* genes encode 14-35 kD proteins defined functionally by their ability to accept Ub thioester from E1 to form a UBC-Ub thioester. These enzymes share little homology with one another outside a core catalytic region that contains an essential cysteine residue; insertions within this core domain as well as amino- and carboxy-terminal extensions are thought to confer additional substrate specificity.

Arabidopsis UBC genes comprise at least five families (*UBC1-3*, *UBC4-6*, *UBC8-12*, *UBC7/13/14*, *UBC15/16/17/18*) with more than 30 total members (Bartling, *et al.* 1993; Girod, *et al.* 1993; Sullivan, *et al.* 1994; van Nocker, *et al.* 1996b). Using probes that could not differentiate among the members of a given family, Sullivan, *et al.* (1994) detected expression of members of the *UBC1-3* and *UBC4-6* families in flowers, stems, leaves, and roots by Northern analysis. The expression patterns in different tissues for individual members of the *UBC8-12* family have not yet been thoroughly examined, but Girod, *et al.* (1993) demonstrated by Northern analysis that members of this family are expressed in leaves and roots. In addition, because cDNAs for *UBC8*, *9*, *10*, and *11* were isolated from a library prepared from leaves, these authors suggested that these genes are likely expressed simultaneously in leaf tissue (Girod, *et al.* 1993). Members of the *UBC1-3* and *UBC7/13/14* families appear to be concurrently expressed in multiple tissues (Thoma, *et al.* 1996; van Nocker, *et al.* 1996b).

Thoma, *et al.* (1996) generated transgenic *Arabidopsis* plants expressing fusions of the promoter regions of *UBC*s *1-3* and *4-6* with a β-glucoronidase (GUS) coding region reporter. Histochemical analysis of these plants suggested that at least one member of the *UBC1-3* and *UBC4-6* families is expressed in most cell types and at most stages of development (Thoma et al., 1996). Furthermore, Thoma, *et al.* (1996) observed coincident expression of *UBC1-3* genes, but nonredundant expression of members of the *UBC4-6* family. Therefore, these authors concluded that the combined activities of the *UBC1-3* gene products are likely important in *Arabidopsis* for numerous purposes in development and physiology, while the individual members of the *UBC4-6* family may be involved in more specialized functions.

Of interest to our studies on xylem differentiation, members of the *UBC4-6* family display vascular tissue localization (Thoma, *et al.* 1996). For example, *UBC4*-GUS expression was observed in the vascular tissue of hypocotyl and senescing antipodal cells in the embryo. *UBC5* also showed vascular localization, first observed in the seedling root and later in the hypocotyl. *UBC6* staining was observed in roots, hypocotyl, and cotyledonary vascular tissue (Thoma, *et al.* 1996). Watts, *et al.* (1994) reported floral expression of *UBC6* in *Arabidopsis*. The *Arabidopsis UBC1-6* genes do not appear to be heat shock responsive (Thoma, *et al.* 1996).

Although at least one animal *UBC*, the *Drosophila* gene, *Semushi*, has been shown to be required for normal body segmentation during embryogenesis (Epps and Tanda 1998), evidence suggesting regulatory functions of specific *UBC*s in plant development is largely lacking. Interestingly, though, a GA-stimulated UBC was shown to be important for α-amylase gene expression in rice aleurone during seed germination (Chen, *et al.* 1995). Additionally, the aforementioned participation of the Ub pathway in auxin signaling (del Pozo

and Lam, 1999) suggests the potential for individual *UBC*s to emerge as important in plant development via the contribution of target specificity of ubiquitination in response to auxin.

Deubiquitinating enzymes (DUBs)

Recent evidence implicates deubiquitinating enzymes (DUBs) as having regulatory functions in the Ub pathway (reviewed by D'Andrea and Pellman 1998; Wilkinson and Hochstrasser 1998). DUBs are cysteine proteases that share little overall sequence homology with each other or other cysteine proteases. These enzymes specifically cleave Ub conjugates at the C terminus of Ub. The two major families of DUBs are the UBPs (Ub processing proteases; Baker, *et al*. 1992; Papa and Hochstrasser 1993; Tobias and Varshavsky 1991) and UCHs (Ub C-terminal hydrolases; Larsen, *et al*. 1996; Wilkinson, *et al*. 1989). None of the 17 known yeast deubiquitinating enzymes is essential (Hochstrasser 1996; Papa and Hochstrasser 1993).

As a group, DUBs act at numerous points in the Ub pathway and help maintain cellular pools of free Ub (reviewed by D'Andrea and Pellman 1998). In summary, DUBs: 1) generate free Ub by processing the linear polyUb chains of precursor fusion proteins that are the primary Ub translation products; 2) rescue ubiquitinated proteins from degradation by the 26S proteasome; 3) cleave Ub from monoubiquitinated substrates, possibly to modulate their activity or localization; 4) recycle free Ub from branched polyUb chains; and finally, 5) maintain access of substrates to the 26S proteasome particle, by removing Ub chains from peptide remains that might occlude entrance to the proteasome's catalytic core. Among these biochemical roles for DUBs, the second and third activities mentioned above yield the greatest potential for DUBs to serve regulatory roles in the Ub pathway.

UCHs comprise a small family of DUB enzymes consisting of relatively small (approximately 30 kD) proteins (reviewed by D'Andrea and Pellman 1998). Less is known about these enzymes compared to UBPs; however, UCHs are thought to have less crucial roles in Ub pathway regulation. UCH enzymes show substrate specificity for small leaving groups, for example, peptides or amino acids. Although the 210 amino acid catalytic domain of UCH proteases contains the same active site residues as UBPs (cysteine, aspartate, and histidine) and four regions of conserved sequence , these enzymes do not have the six homology domains that characterize members of the UBP family (Larsen, *et al.* 1996) and only contain a single Ub binding site (Stein, *et al.* 1995).

UBPs are a larger family of DUB enzymes than the UCHs, at least in yeast, where 16 UBPs are known but only 1 UCH (D'Andrea and Pellman 1998). UBPs contain six homologous regions that span 200-500 amino acids. Three of these homology regions are known to contribute catalytic residues to the active site (Baker, *et al.* 1992; Papa and Hochstrasser 1993), and the others are thought to contribute Ub-binding and/or cellular localization functions. Many UBPs are known only by these signature motifs, while their cellular functions remain to be elucidated (Hochstrasser 1996; Wilkinson and Hochstrasser 1998). Amino- and carboxy-terminal extensions outside the catalytic region and insertions within this core likely contribute to UBP substrate specificity. Compared with UCHs, UBPs are thought to cleave Ub from larger substrates, for instance, proteins (D'Andrea and Pellman 1998). Individual UBPs are assumed to have unique target specificities and/or UBPs may be differentially distributed within cells to localize/restrict their functions. Interaction of the yeast UBP protein, DOA4, with the 26S proteasome has been recently demonstrated (Papa, *et al.* 1999)

UBPs have been implicated in the control of numerous biological processes including cell growth in mammals (Nakamura, *et al.* 1992; Papa and

Hochstrasser 1993; Zhu, *et al*. 1997; Zhu, *et al*. 1996), transcriptional silencing in yeast and an analogous process in *Drosophila* (Henchoz, *et al*. 1996; Moazed and Johnson 1996), control of Herpes viral infection in mammalian cells (Everett, *et al*. 1997), and developmental events in *Drosophila* (Huang, *et al*. 1995; Huang and Fischer-Vize 1996) and *Dictyostelium* (Lindsey, *et al*. 1998). Additionally, UBPs apparently function in the biochemistry of Ub-like proteins and their substrates as well. Recently, a UBP that shows activity on two Ub-like proteins has been shown to be required for cell cycle regulation in yeast (Li and Hochstrasser 1999). Unraveling the roles of individual UBPs will undoubtedly be complicated due to the fact that many UBPs are currently known, but their *in vivo* targets are yet unknown (Wilkinson and Hochstrasser 1998). Additionally, some UBPs promote the degradation of their substrates while others promote stabilization (Hochstrasser 1996; Wilkinson and Hochstrasser 1998).

To date, only a single characterization of *Arabidopsis* UBPs, *AtUBP3* and *AtUBP4*, has been published (Chandler, *et al*. 1997). Immunoblot analysis of AtUBP3 and AtUBP4 revealed expression of both proteins in all organs tested from 6-8 week-old plants, including juvenile-, young adult-, and adult-stage leaves, immature and mature flowers, green and yellow siliques, and roots and stems. The *UBP3* and *UBP4* gene products are 93% identical and are present in the nucleus. Because these two UBPs display ubiquitous expression throughout the plant, and also are expressed at multiple stages of development, these authors concluded that these enzymes may be important for numerous physiological functions in the plant.

The proteasome

The proteasome is the major protease of the Ub pathway. Proteasome genes and proteasome functions are being actively researched. Several recent

reviews are available (Coux, *et al*. 1996; DeMartino and Slaughter 1999), including two that specifically describe the proteasome in plants (Callis 1997; Vierstra 1996). Intriguingly, Wei, *et al*. (1998) recently described a novel protein complex, COP9, that shows similarity in structural features to regulatory subunits of the proteasome and is conserved between plants and mammals.

The 20S proteasome particle consists of four stacked rings, each composed of seven subunits. Two adjacent interior rings are composed of β subunits, and each is flanked by an exterior ring of α subunits, yielding a 700 kD cylindrical particle with a "hollow" core having an overall arrangement $\alpha_7\beta_7\beta_7\alpha_7$. It has been proposed that polypeptide degradation substrates are unfolded, channeled into the interior of the particle, and cleaved internally to produce 4-10 amino acid residue degradation products (Akopian, *et al*. 1997; Dick, *et al*. 1991). In *Arabidopsis*, a superfamily of 23 homologous genes encodes the 14 proteasome subunits (Fu, *et al*. 1998; Genschik, *et al*. 1992; Shirley and Goodman 1993). Association of the 20S proteasome with another multisubunit protein, the 19S cap, creates the ATP-dependent, 2100 kD, 26S proteasome. At least one of the subunits of the 19S cap of the *Arabidopsis* proteasome recognizes polyubiquitin chains (van Nocker, *et al*. 1996a), and it is generally assumed that one function of the 19S complex is to recognize ubiquitinated proteins and present them to the 20S catalytic core for degradation. The 26S proteasome complex shows nuclear and cytoplasmic localization (reviewed by DeMartino and Slaughter 1999), and has even been demonstrated to translocate from the nucleus to apoptotic bodies during PCD induced in an immortalized animal cell line (Pitzer *et al*. 1996).

The catalytic mechanism of the proteasome was unknown for many years. Determining the mechanism was a challenging problem, particularly because the sensitivity of the proteasome to protease inhibitors was unlike known proteases, and its subunits did not share high levels of amino acid homology to any

previously studied proteases. Lowe *et al*. (1995) discovered that binding of the peptide aldehyde inhibitor Ac-LLnL-al occurred at the N-terminal threonine of each proteasome β subunit, suggesting that the hydroxyl group of this threonine is the reactive nucleophile catalyzing peptide bond cleavage. Groll *et al*. (1997) demonstrated that the reaction mechanism of the proteasome involves the N-terminal threonine residues of some β subunits, and also revealed a second active site within an α helix present in all seven β subunits that comprise each β ring of the particle. It is assumed that both active sites participate in catalysis. The α subunits are catalytically inactive. The mammalian proteasome has five known distinct catalytic activities: after basic residues (trypsin-like activity), after large hydrophobic residues (chymotrypsin-like activity), after acidic residues (peptidylglutamyl peptide hyrolyzing activity, PGPH), after branched-chain amino acids (BrAAP activity), and cleavage between small neutral amino acids (SNAAP activity) (reviewed by Grimm and Osborne 1999). This dissertation reports the use of the proteasome inhibitors *clasto*-lactacystin β-lactone (LAC), that inhibits the trytic-like, chymotryptic-like, and PGPH activities of the proteasome, and a peptide aldehyde inhibitor, carbobenzoxy-leucinyl-leucinyl-leucinal (LLL), that inhibits the proteasome's chymotryptic-like, PGPH, and BrAAP activities (reviewed by Grimm and Osborne 1999).

Through its major role in the degradation of ubiquitinated proteins, the proteasome affects many developmental and other important biological processes in plants and animals. Examples of significant cellular roles of the proteasome include the processing of antigens for cell surface expression by MHC (Major Histocompatibility Locus) Class I molecules in the mammalian immune system (Rock, *et al*. 1994). Appropriate processing of these antigens requires substitution of three β-subunits of the proteasome, a change that does alter the profile of proteasome-generated antigen degradation products. Using

homologous recombination to create a gene disruption, Fu, *et al.* (1999) demonstrated a requirement for RPN10/MBP1 during development in the moss *Physcomitrella*. Strains harboring the RPN10/MBP1 knockout were arrested developmentally and were unable to initiate gametophorogenesis to complete their life cycle. Genschik, *et al.* (1998) have implicated the proteasome in the regulation of the plant cell division cycle, as well. Support for a role of the proteasome in PCD phenomena is reviewed elsewhere in this chapter.

As previously mentioned, molecular characterization of the 20S proteasome in *Arabidopsis* (Fu, *et al.* 1998) suggests that a superfamily of 23-27 genes encode the α and β subunits of the 20S particle. It is not yet known whether *Arabidopsis* proteasome subunit genes are expressed concurrently or differentially regulated for environmental-, tissue-specific, and/or developmental consequences. It is possible that members of each α and β subunit family may impart distinct proteolytic specificities and/or functions to the proteasome (Fu, *et al.* 1998). Shirley and Goodman (1993) demonstrated that the α-subunit, *PAF1*, was expressed at high levels in seedlings younger than 9 days, and again at high levels in plants three weeks of age. However, plants harboring a deletion of *PAF1* were phenotypically normal with respect to seedling germination, growth, and fertility (Shirley and Goodman 1993), suggesting functional redundancy for this gene, which perhaps is not surprising considering that *PAF1* is encoded by two genes (Fu, *et al.* 1998). Genschik, *et al.* (1994b) demonstrated that mRNA of two proteasome subunits, one α (*PAD1*) and one β (*PBF1*), increased in proliferating cultured cells, suggesting a role for these genes in cell division. For *Arabidopsis*, Fu, *et al.* (1998) predicts the discovery of subtle effects of subunit changes in the proteasome because the members of a given α or β gene family in *Arabidopsis* display a high degree of amino acid sequence conservation. Further characterization of the plant

proteasome in various physiological and developmental states is necessary before a conclusive statement concerning the importance of differential regulation of plant proteasome subunits can be made.

The proteasome and PCD

The role of the proteasome in PCD in animals is well documented (recently reviewed by Orlowski 1999). One of the best-studied examples of animal PCD that involves changes in the proteasome and its activities is that of intersegmental muscle (ISM) degeneration during metamorphosis of the hawkmoth, *Manduca sexta*. Initial indication that the Ub pathway is important in this PCD process included increases in polyUb gene expression (Schwartz, *et al.* 1990), elevated levels of ubiquitinated proteins, and the induction of enzymes involved in Ub attachment to cellular proteins (Haas, *et al.* 1995) coincident with commitment of ISMs to death. Also using the hawkmoth model, Jones, *et al.* (1995) observed the appearance of four newly synthesized proteasome subunits, an eightfold increase in proteasome levels, and increases in two of the peptidase activities of the proteasome (caseinolytic, PGPH). Dawson, *et al.* (1995) reported a similar increase in proteasome abundance in ISM cells undergoing PCD, as well as a greater than two-fold increase in the mRNA levels of MS73, an ATPase regulatory subunit of the 19S cap of the 26S proteasome. These changes were correlated with dramatic increases in three of the peptidase activities (chymotrysin-like, trypsin-like, and PGPH) of the 26S proteasome (Dawson, *et al.* 1995). Together, these studies strongly support a role for the proteasome in the degeneration of hawkmoth ISMs.

The animal studies raise the question of whether plant PCD also depends on the Ub-proteasome pathway. Currently, results from plant studies are inconclusive on this issue because expression of genes encoding Ub-proteasome

pathway components is upregulated in some plant PCD models, but such increases have not been observed in other plant PCD systems. For example, increases in mRNA encoding Ub conjugating enzymes (UBCs) and Ub (Genschik, *et al.* 1994a) and increases in expression of a Ub promoter-driven GUS reporter (Garbarino and Belknap 1994) were observed in senescing leaves. However, no changes in the levels of several Ub pathway components, including the 26S proteasome component RPN10/MBP1 (van Nocker, *et al.* 1996a), were detected during senescence of daylily petals (Stephenson and Rubinstein 1998). Recently, Bahrami and Gray (1999) cloned an α-type proteasome subunit from tobacco and described its spatial and temporal expression pattern. High levels of expression in young, expanding tissues and decreasing levels during senescence of leaves and flowers led these authors to conclude that the proteasome does not degrade and recycle protein during senescence. The accumulation of Ub and/or ubiquitinated proteins (Li, *et al.* 1995) was observed in degenerating anthers. These collective studies appear contradictory, such that no general understanding concerning the role of the proteasome in plant PCD has been achieved. It is also possible that the Ub pathway participates in some, but not all, plant PCD processes.

The discovery and development of proteasome inhibitors, lactacystin (Omura, *et al.* 1991) and several peptide aldehydes, has led to a proliferation of investigations of the proteasome as a regulator of PCD in animal systems. Use of proteasome inhibitors has led to some confusion, however, as treatment may either promote or prevent PCD, depending on the system in question. For example, proteasome activity was shown to be required for PCD of primary mouse thymocytes in response to various cell death inducers (Grimm, *et al.* 1996), of sympathetic neurons following withdrawal of nerve growth factor (Sadoul, *et al.* 1996), and T cells activated by T-cell receptor cross-linking (Cui, *et al.* 1997). In

contrast, proteasome activity apparently prevents PCD in response to various PCD-eliciting stimuli in certain cancer cell lines, including multiple human leukemia cell lines (Dou, *et al*. 1999; Drexler 1997; Masdehors, *et al*. 1999; Shinohara, *et al*. 1996; Zhang, *et al*. 1999) human prostatic carcinoma cells (Herrmann, *et al*. 1998), and glioma cells (Kitagawa, *et al*. 1999).

This apparently contradictory data may be explained, in part, by a model summarized by Grimm and Osborne (1999) which suggests that proteasome inhibition may have different effects on proliferating versus nonproliferating cells due to cell cycle regulation by the Ub-proteasome pathway. In non-cycling (quiescent or terminally differentiated) cells, the proteasome may be responsible for directly or indirectly activating the caspase cascade to induce cell death. In this case, inhibition of the proteasome would prevent cell death. In cycling cells, on the other hand, proteasome inhibition results in the accumulation of cell cycle regulators normally degraded via the proteasome. Subsequent de-regulation of the cell cycle may drive cells into PCD, potentially involving participation of the caspase cascade. This model is consistent with much of the available data. It has also been suggested that prevention versus induction of PCD by proteasome inhibitors depends on the concentration of inhibitor (Lin, *et al*. 1998; Meriin, *et al*. 1998; Rock, *et al*. 1994). Obviously, the role of the proteasome in PCD is complex and experiments that do not rely entirely on inhibitors are needed before more detailed models can be proposed and tested.

Ub and xylem development

A growing body of evidence from our lab and others indicates that activity of the Ub pathway is important for normal xylem formation. In *Coleus*, regeneration of xylem vessel elements after wounding was accompanied by increased levels of Ub and/or Ub-protein conjugates (Stephenson, *et al*. 1996).

Furthermore, transgenic tobacco plants expressing a Ub molecule unable to form polyUb chains displayed abnormal vascular tissue (Bachmair, *et al.* 1990). Additionally, members of one *Arabidopsis UBC* family (*UBC4-6*) display vascular tissue-specific expression (Thoma, *et al.* 1996). Proteasome activity has been implicated in the development of TEs in cell suspension cultures of *Zinnia* (Woffenden *et al.*, 1998; Chapter 3). In the *Zinnia* TE study, application of proteasome inhibitors at culture initiation completely prevented TE differentiation, while inhibition of proteasome activity following commitment to differentiation did not prevent TE formation but resulted in a delay in development. That specific proteasome inhibition late during differentiation did not preclude autolytic clearing of TEs is consistent with the conclusion that the proteasome does not participate in bulk autolysis of differentiating TEs. These collective studies suggest that normal functioning of the Ub pathway is necessary for proper xylem development.

This dissertation presents a further examination of the role of the Ub pathway during xylem differentiation. Initially, we conducted a biochemical characterization of Ub pathway activity during TE development in *Zinnia* cell suspension cultures. Additionally, as described briefly above, we asked whether proteasome activity is required for TE differentiation in the *Zinnia* system. Finally, we investigated in *Arabidopsis* whether particular components of the Ub pathway machinery (*UBC*s, DUBs, or proteasome subunits) might contribute to secondary xylem differentiation via xylem-specific expression.

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CHAPTER 2

Biochemical Characterization of Ubiquitin Pathway Activity During Tracheary Element Differentiation in *Zinnia* Mesophyll Cell Cultures

Abstract

The *Zinnia* mesophyll cell culture TE system was used to examine the role of Ub-mediated proteolysis in a developmentally programmed cell death process. TEs are easily detected in induced cultures by 60 h and differentiation is complete by 96 h (Fukuda 1996). Noninduced control cultures contain only undifferentiated cells. Immunoblot analysis of extracts from noninduced cultures revealed steadily increasing levels of endogenous high molecular mass Ub-protein conjugates over 72 h in culture. Conjugate levels in induced cultures paralleled those in noninduced cultures through 48 h. However, by 72 h in culture extracts from induced cells showed a dramatic loss of high molecular mass conjugates, coupled with a gain in conjugates whose relative mobilities were consistent with those of Ub dimers, trimers, and tetramers. *In vitro* conjugation assays indicate that cells from noninduced and induced cultures possess similar abilities to produce high levels of Ub conjugates, with activity increasing between 0 and 24 h and remaining stable through at least 96 h. Regardless of the time point assayed, noninduced and induced cell extracts produced identical heat- and reducing agent-labile polyhistidine-tagged Ub-E2 thioesters (20, 22, 30 kD). Northern analysis of Ub gene expression revealed that the level of a 1.6 kb mRNA transcript increased during cell isolation and decreased during culture of both induced and noninduced cells. The observed differences in the profiles of endogenous conjugates in noninduced versus induced cells are probably not due to differences in Ub gene expression or in

overall conjugating activity. The reduction of high molecular mass conjugates between 48 and 72 h in induced cell cultures may be mediated at least in part by cysteine proteases, which increase in expression and activity during TE differentiation

Introduction

In order to function as the water-conducting tissue in higher plants, differentiating TEs undergo PCD, during which cellular contents are degraded. Using mesophyll cells isolated from young leaves of *Zinnia elegans*, TE differentiation can be reproduced in culture (for review, see Church 1993; Fukuda and Komamine 1980). Isolated cells are initially homogenous in culture and differentiation into TEs occurs semi-synchronously in 40-60% of living cells by 72 h.

The Ub pathway appears to be important for normal development of vascular tissue in plants. For example, expression in transgenic tobacco of a mutant Ub molecule incapable of forming polyUb chains via lysine-48 results in aberrant vascular development (Bachmair, *et al*. 1990). Additionally, in *Coleus*, regeneration of xylem vessel elements after wounding was accompanied by increased levels of Ub and/or Ub-protein conjugates (Stephenson, *et al*. 1996).

We have partially characterized Ub pathway activity during TE differentiation in the *Zinnia* mesophyll cell culture system. The pathway is highly active in both TE cultures and control cultures. Yet these similar levels of pathway activity result in the production of distinct profiles of endogenous Ub-protein conjugates. Protease inhibitors were used to demonstrate that the differences in endogenous conjugate profiles are in part due to the activity of cysteine proteases present during autolysis of TEs.

Materials and Methods

Plant Material and Growth Conditions: Seeds of *Zinnia elegans* cv. Envy (Grimes Seeds, Concord, OH) were sown in 4 inch pots containing Sunshine Mix 1 (Wetsel Seed Co., Harrisonburg, VA). Plants were grown at 27°C under a 16 h

photoperiod at 85 μmol photons $m^{-2}s^{-1}$ and watered as needed with distilled water.

Mesophyll Cell Isolation and Suspension Culture: The first true leaves were harvested from seven-day-old seedlings, and mesophyll cells were isolated and cultured in inductive (TE) medium containing 0.5 μM α-naphthaleneacetic acid and 0.89 μM N6-benzyladenine, according to Roberts, *et al.* 91992), except that cells were cultured in flasks in 12.5 ml of medium. Noninductive control medium was identical except that the cytokinin concentration was decreased to 4.5 nM. Cells were collected from cultures by centrifugation at 50*g* for 2 min at the times indicated in the figure legends. Cell pellets were stored at -70°C until extraction. Storage at -70°C was preceded by freezing in liquid nitrogen when harvesting cells for RNA extraction.

Analysis of endogenous Ub-protein conjugates: Extracts from noninduced and induced *Zinnia* mesophyll cell cultures were prepared by grinding cells in buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM PCMBS (Sigma; St. Louis, MO), 5 mM PMSF (Sigma; St. Louis, MO), 5 mM iodoacetamide, 1 mM Pefabloc (Boehringer Mannheim; Indianapolis, IN), 20 μM E64 (Sigma; St. Louis, MO), and 2 mM EDTA. Following boiling for 5 min in SDS-PAGE sample buffer, 40 μg of protein from each sample was resolved on a 13.5% polyacrylamide gel and electrophoretically transferred to PVDF membrane (Millipore-P; Millipore; Bedford, MA) for immunoblot analysis using a polyclonal, rabbit or chicken anti-Ub primary antibody and alkaline phosphatase-conjugated secondary antibody for colorimetric detection with the substrates nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (Sigma; St. Louis, MO).

Analysis of polyhistidine-tagged Ub protein conjugates generated *in vitro*: At the times indicated in the figures, cell extracts were prepared from induced and noninduced *Zinnia* cultures by grinding cells in buffer containing 50 mM Tris-HCl, pH 8.0, 100 μM leupeptin (Sigma; St. Louis, MO), 1 mM EDTA, 14 mM 2-mercaptoethanol, and 300 mM sucrose. Reactions contained 150 μg *Zinnia* protein, 13 μg polyhistidine-tagged Ub, 20 μM leupeptin and either an ATP-regenerating system or an ATP-depleting system in 20 μl total volume. The ATP-regenerating system consisted of 50 mM Tris-HCl, pH 8.0, 10 mM phosphocreatine, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and 1 unit phosphocreatine kinase (Sigma; St. Louis, MO). The control ATP-depleting system consisted of 50 mM Tris-HCl, pH 8.0, 10 mM deoxyglucose, 5 mM $MgCl_2$, 1 mM DTT, and 0.4 unit hexokinase (Sigma; St. Louis, MO). Conjugation reactions were incubated for 1 h at 30° C and stopped by boiling for 5 min in 2X SDS-PAGE sample buffer, followed by product resolution on a 12.5% polyacrylamide gel. Immunoblot processing was as for endogenous Ub-protein conjugate analysis described above except monoclonal, mouse anti-polyhistidine tag primary antibody (Qiagen; Chatsworth, CA) was used.

Analysis of polyhistidine-tagged Ub-protein thioesters generated *in vitro*: Cell extracts were prepared from noninduced or induced cultures at the indicated time points by grinding cells in buffer containing 50 mM Tris-HCl, pH 7.5, 0.2 mM DTT. Reactions consisting of 200 μg *Zinnia* protein, 13 μg polyhistidine-tagged Ub, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM ATP, and 0.1 mM DTT in a total volume of 20 μl were incubated for 2 min at 30°C. Thioester reactions were stopped by the addition of SDS-PAGE sample buffer containing the cysteine protease inhibitor E64 at 20 μM, without boiling. Control reactions were stopped by the addition of SDS-PAGE sample buffer containing

20 μM E64 and supplemented to 10% 2-mercaptoethanol followed by boiling. Reaction products (40 μg) were resolved on a 13.5% polyacrylamide gel at 4°C and processed for immunoblotting as described above for Ub-protein conjugates generated *in vitro*.

Northern analysis: Total RNA was isolated from *Zinnia* leaf and noninduced and induced cell cultures at 0, 24, 48, 60, and 72 h, resolved on a 1.2% formaldehyde-containing agarose gel, and transferred to a nylon membrane for Northern analysis. An antisense Ub coding region RNA probe was prepared by transcription from Sac I-digested p3002 using T3 RNA polymerase (Boehringer Mannheim; Indianapolis, IN) and biotin-UTP (Boehringer Mannheim; Indianapolis, IN). Hybridization was detected by alkaline phosphatase catalyzed chemiluminescence using Lumigen-PPD substrate (New England Biolabs; Beverly, MA).

Analysis of changes in endogenous Ub-protein conjugate profiles resulting from *in vivo* cysteine protease activity: The cysteine protease inhibitor, E64 (final concentration 58 μM), or leupeptin (final concentration 200 μM) was added at 48 h to *Zinnia* mesophyll cells cultured in inductive medium. Cells were collected at 72 h and protein was extracted and examined by immunoblot analysis as described above for analysis of endogenous Ub-protein conjugates.

Analysis of the ability of one cell extract to degrade Ub-protein conjugates generated by a second cell extract/Mixing experiment: Polyhistidine-tagged Ub-protein conjugates were prepared from cells collected from 48-h noninduced cultures for use as protease substrate as described above for *in vitro* generated polyhistidine-tagged Ub-protein conjugates , except that leupeptin was omitted

from the *in vitro* conjugation reaction. A 72-h induced cell extract was used as the source of protease. Additions and omissions for each proteolysis reaction are indicated at top of the figure. Reactions were incubated for 90 min at 27°C and stopped by the addition of E64 and sample buffer (if not already present) and boiling. Reaction products were resolved by 12.5% SDS-PAGE and detected by immunoblotting as described above for *in vitro* generated polyhistidine-tagged Ub-protein conjugates.

Results

As a first step in examining potential biochemical differences in the Ub pathway between differentiating and non-differentiating *Zinnia* cultures, immunoblot analysis of endogenous ubiquitin-protein conjugates was conducted. Differences were observed in the profiles of endogenous ubiquitin-protein conjugates present in extracts prepared from noninduced and induced cultured *Zinnia* mesophyll cells over a time course of 0 to 72 h (Figure 1). Both noninduced and induced cells yielded increasing levels of high molecular mass conjugates between 0 and 48 h. This trend continued through 72 h under noninductive conditions. In contrast, cultures containing cells induced to differentiate exhibited a marked decrease in high molecular mass conjugates between 48 and 72 h, concomitant with an increase in the levels of several immunoreactive species with SDS-PAGE mobilities consistent with those of Ub dimers, trimers, and tetramers.

To assess the overall Ub-conjugating ability of extracts, we performed *in vitro* conjugation reactions using polyhistidine-tagged Ub and endogenous substrates. Cell extracts from both noninduced (data not shown) and induced (Figure 2A) cultures produce high levels of high molecular mass Ub-protein conjugates *in vitro*, with activity increasing from 0 to 24 h and remaining stable

through at least 96 h. Control reactions, which contained an ATP-depleting system (described in Material and Methods), reproducibly failed to support Ub conjugate accumulation (Figure 2B).

A comparison of Figures 1 and 2A reveals that the products of *in vitro* conjugation reactions performed with induced 72 h *Zinnia* extracts are different from those produced *in vivo*. Since induced and noninduced cells appear to possess similar abilities at all time points to conjugate Ub to endogenous *Zinnia* proteins *in vitro*, alternative explanations for the differences in endogenous conjugate levels in 72 h cell extracts were explored, including: potential differences in gross expression levels or identities of particular Ub-conjugating enzymes (E2s), differences in Ub gene expression, and a potential role for proteases in the degradation of ubiquitinated proteins in 72 h induced cells.

E2-mediated ubiquitination of target proteins requires the formation of a transient heat- and reducing agent-labile thioester bond between the C-terminal glycine of Ub and an essential cysteine residue in the conjugating enzyme. *In vitro* thioester reactions were performed to determine whether differences in E2 activities could account for the differences in the profiles of endogenous conjugates from induced and noninduced cells. Immunoblot analysis of thioester complexes generated by cell extracts from 0, 24, and 72 h induced and noninduced cultures with polyhistidine-tagged Ub revealed no detectable differences in the profiles of E2 enzymes expressed (Figure 3). Three thioesters (20, 22, 30 kD) were evident in reactions from both induced and noninduced cells, regardless of the time point assayed. Control reactions, boiled in 2-mercaptoethanol-containing sample buffer before immunoblot analysis, confirmed the lability of the thioester bonds. Intervening time points (36, 48, and 60 h) were assayed in other experiments and produced identical results (data not shown). The data in Figure 2 indicate that the endogenous conjugate profile

differences between induced and noninduced 72 h cells cannot be explained by gross quantitative or qualitative differences in E2 activities in these cells.

Northern analysis of Ub gene expression (Figure 4) revealed that the level of a 1.6 kb mRNA transcript increased during cell isolation from leaf tissue and decreased during culture of both induced and noninduced cells. Two additional transcripts of lower abundance (2.8, 2.7 kb) showed a similar pattern of expression. This increase in Ub mRNA precedes the initial increase in levels of high molecular mass endogenous Ub conjugates in both induced and noninduced cultures between zero and 24 h.

It has been established that cysteine proteases present in differentiating TEs are capable of rapid, nonspecific degradation of cellular proteins (Beers and Freeman 1997). Hence, immunoblot analysis of endogenous conjugates extracted from cells cultured in the presence of cysteine protease inhibitors was performed to examine the potential role of proteases in creating the unique 72-h induced profile (Figure 5). Addition of either of two cysteine protease inhibitors, E64 or leupeptin, did not prevent differentiation of TEs (data not shown). However, the decrease in high molecular mass conjugates (those transferred from the stacking gel) occurring between 48 and 72 h in induced cultures was prevented by the inclusion of either inhibitor. Compared to control 72-h induced cultures, little change was detected in the profile of low molecular mass conjugates (≤ 45 kD) extracted from cells cultured in the presence of inhibitor. Preliminary data of cell counts performed at 96 h from inhibitor-containing cultures indicated that the inhibitors had no cytotoxic effects at the concentrations used in these experiments (data not shown).

Because cysteine proteases possess the ability to modulate the accumulation of conjugates in induced cells *in vivo*, we attempted to recreate the profile of 72-h induced cells using polyhistidine-tagged Ub-protein conjugates

produced by a 48-h noninduced cell extract as a protease substrate and a 72-h induced cell extract as a source of protease (Figure 6). Incubation of conjugates with the 72-h cell extract in this assay reduced levels of high molecular mass Ub-protein conjugates, thereby partially reproducing the typical 72-h induced cell extract profile. However, no accumulation of low molecular mass conjugates accompanied the disappearance of high molecular mass conjugates in the mixing experiment. Inclusion of SDS-PAGE sample buffer and/or E64 prevented the degradation of high molecular mass conjugates, indicating that degradation is due to thioprotease (cysteine protease) activity. In proteolysis reactions performed in the absence of both sample buffer and E64, free polyhistidine-tagged Ub appeared to be dramatically reduced.

Among the several biochemical activities we examined that have the potential to modulate levels of Ub-protein conjugates *in vivo*, few differences were observed between induced and noninduced cells. Both *in vitro* conjugation reactions and thioester reactions demonstrated that high levels of Ub-conjugating activity are maintained throughout differentiation and PCD of TEs, and similar high activity is found at all time points in noninduced cultures as well. Because these experiments revealed no unique aspects of attachment of Ub to cellular targets during TE differentiation and, furthermore, because these experiments were not designed to investigate time points between 0 and 24 h after culture initiation when key differentiation regulatory events might be expected to occur, future experiments will be aimed at defining the potential regulatory role of early Ub-proteasome proteolytic activity during TE differentiation.

Discussion

We have presented the first characterization of Ub pathway activity during the differentiation of TEs in the *Zinnia* mesophyll cell culture system. We

hypothesized that differences in Ub pathway activity may exist between differentiating and non-differentiating cells. *Zinnia* extracts prepared from cultures containing 60% of live cells as TEs (induced cultures) exhibited a dramatic reduction in levels of high molecular mass Ub-protein conjugates and an increase in low molecular mass conjugates compared to those present in control cultures containing no TEs. Assessment of overall conjugating ability of both induced and noninduced cell extracts revealed no gross differences throughout the culture period in the capacity of extracts to conjugate polyhistidine-tagged Ub to endogenous *Zinnia* proteins. Thus, an active Ub conjugating system is maintained by cells in induced cultures despite the progression of PCD in 60% of live cells. The initial increase in conjugating activity observed between 0 and 24 h in culture was preceded by a transient increase, during cell isolation, in Ub gene expression detected as a 1.6 kb Ub mRNA. The level of this transcript then decreased steadily throughout the culture period. Neither the early increase in conjugation activity nor its maintenance during inductive and noninductive cell culture was accompanied by detectable qualitative or quantitative changes in E2s. The addition of cysteine protease inhibitors to inductive culture medium at 48 h prevented neither TE differentiation nor the accumulation of low molecular mass Ub-protein conjugates. However, the reduction of high molecular mass conjugates typically observed in extracts from 72 h induced cells was prevented by proteinase inhibitors. A mixing experiment aimed at reproducing the typical 72 h induced Ub-protein conjugate profile from conjugates generated *in vitro* confirmed the above *in vivo* protease inhibitor experiment; i.e., cysteine protease activity may be responsible for the apparent enhanced degradation of high molecular mass conjugates during TE differentiation, but this degradation does not necessarily

result in detectable accumulation of the low molecular mass conjugates observed in extracts from TE cultures.

Among the several biochemical activities we examined that have the potential to modulate levels of Ub-protein conjugates *in vivo*, few differences were observed between induced and noninduced cells. Both *in vitro* conjugation reactions and thioester reactions demonstrated that high levels of conjugating activity are maintained throughout differentiation and PCD of TEs. One unresolved question is whether these *in vitro* assays are quantifying activities of nondifferentiating cells only. However, single-cell assays revealed that most TEs contain detectable levels of protease (Beers and Freeman 1997) and cellulase activities (data not shown) at 72 h. This, coupled with the unique profiles of Ub-protein conjugates extracted from cells in induced and noninduced cultures, indicates that TEs are biochemically active and hence may modulate Ub pathway activity until late in the differentiation process.

We concluded that the observed thioester complexes were E2-Ub rather than E1-Ub because E1-Ub is expected to resolve at greater than 100 kD by SDS-PAGE. Surprisingly, no Ub-E1 thioester was detected in our experiments. The Ub-E2 thioester profiles produced by extracts from noninduced and induced cells did not differ from one another, and did not vary over the time course assayed. These results contrast with findings in the tobacco hawkmoth PCD system, in which Haas, *et al.* (1995) observed increases in levels and activities of E1 (Ub-activating enzyme) and two unique, cell death-specific E2s just prior to the commitment of intersegmental muscles to degeneration during hawkmoth metamorphosis.

Changes in Ub gene expression in cells of induced cultures compared to cells in noninduced cultures might have been expected considering that dramatic increases in polyUb gene expression and Ub protein have been observed in

hawkmoth intersegmental muscles with commitment to cell death (Haas, *et al*. 1995; Schwartz, *et al*. 1990). Northern analysis, however, revealed that while a transient increase in Ub mRNA levels occurs during isolation of cells from leaf tissue and is followed by a steady decrease in transcript levels during culture, the patterns are similar in cells of both noninductive and inductive cultures. Thus, differential expression of Ub mRNA does not appear to explain the observed differences in endogenous conjugate profiles. Although an RNA loading control did reveal some disparity among the samples (Figure 4B), the RNA levels used were not inconsistent with the general conclusion that levels of Ub transcripts decrease over time in culture.

Proteases are emerging as important players in PCD through several biochemical mechanisms, including nonspecific clearing of cellular proteins (as must occur to form a mature, hollow TE), activation of cell death-promoting proteins by proteolytic processing (for review, see Yuan 1995), and potentially, specifically degrading cell death inhibitory proteins. Cysteine protease activity appears to have a role in degrading Ub-protein conjugates in cells of 72 h induced cultures, since the addition of cysteine protease inhibitors stabilized high molecular mass conjugates *in vivo* without preventing TE differentiation (Figure 5). The presence of cysteine protease inhibitors may also have inhibited 26S proteasome activity in these experiments (Ozaki, *et al*. 1992), suggesting that proteasome activity may also be responsible for the reduction of endogenous conjugates occurring between 48 and 72 h in culture.

Although the mixing experiment partially confirmed the results of the cell culture inhibitor experiment in that the degradation of high molecular mass conjugates was prevented by inhibition of thioproteases, complete reconstitution of the conjugate profile of 72 h induced cells was not achieved. That low molecular mass conjugates did not accumulate suggests that the smaller Ub

conjugates detected in TE culture extracts late during culture are not necessarily the degradation products of the high molecular mass Ub-protein conjugates. Surprisingly, reduction of detectable free polyhistidine-tagged Ub, possibly through degradation, accompanied the degradation of high molecular mass conjugates in this experiment. An alternative interpretation of these data is that the epitope tag (MRGS-6H) may be cleaved by an E64/SDS-sensitive endoprotease, thereby precluding recognition of the epitope during immunoblot analysis.

Interestingly, the profile of endogenous conjugates observed in cell extracts from 72 h induced cultures resembles the pattern of ubiquitinated proteins that accumulates in a yeast deubiquitinating enzyme mutant (Papa and Hochstrasser 1993), i.e., the absence of high molecular mass ubiquitinated proteins was accompanied by the appearance of a number of smaller ubiquitinated species. Perhaps the absence of a particular UBP in induced cells compared to noninduced cells is the cause of the differences in endogenous conjugates observed in the *Zinnia* system. Determination of cell fate by a deubiquitinating enzyme has been demonstrated during *Drosophila* eye development (Huang, *et al.* 1995). Chapter 3 describes work conducted to determine the contribution of the 26S proteasome to TE differentiation in the *Zinnia* mesophyll cell culture system.

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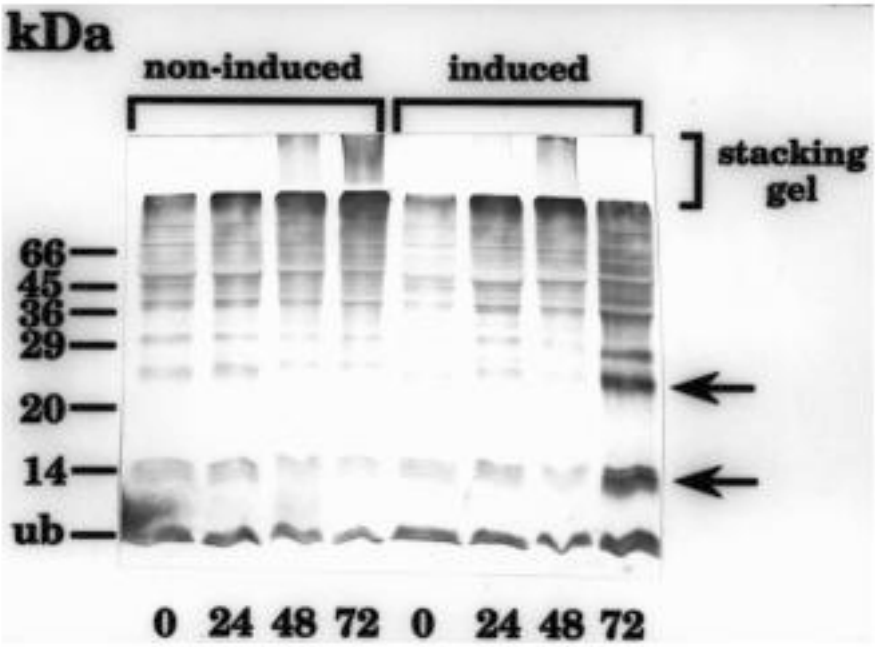

Figure 1. Analysis of endogenous ubiquitin-protein conjugates present in extracts from *Zinnia* mesophyll cells cultured under noninductive and inductive conditions. Extracts were prepared in the presence of a battery of protease inhibitors at the times indicated in hours at the bottom of the figure. Following boiling in SDS-PAGE sample buffer, 40 µg of protein from each sample was resolved on a 13.5% polyacrylamide gel and electrophoretically transferred to PVDF membrane for immunoblotting using a polyclonal, rabbit anti-ubiquitin primary antibody and alkaline phosphatase-conjugated secondary antibody for colorimetric detection with the substrates NBT/BCIP. Arrows mark prominent low molecular mass ubiquitin conjugates.

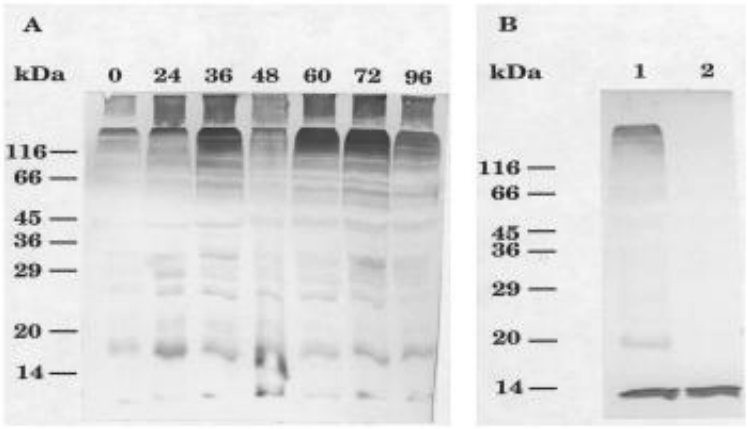

Figure 2. Analysis of polyhistidine-tagged ubiquitin-protein conjugates generated *in vitro* by extracts from *Zinnia* mesophyll cells cultured under inductive conditions. Cell extracts were prepared at the times indicated in hours. Reactions consisting of 150 µg *Zinnia* protein, 13 µg polyhistidine-tagged ubiquitin, and either an ATP-regenerating system (A and B, lane 1) or ATP-depleting system (B, lane 2) were incubated for 1 h at 30°C. Reaction products were resolved by 13.5% SDS-PAGE. Immunoblot processing was as described for Figure 1, except monoclonal, mouse anti-polyhistidine tag primary antibody (Qiagen) was used.

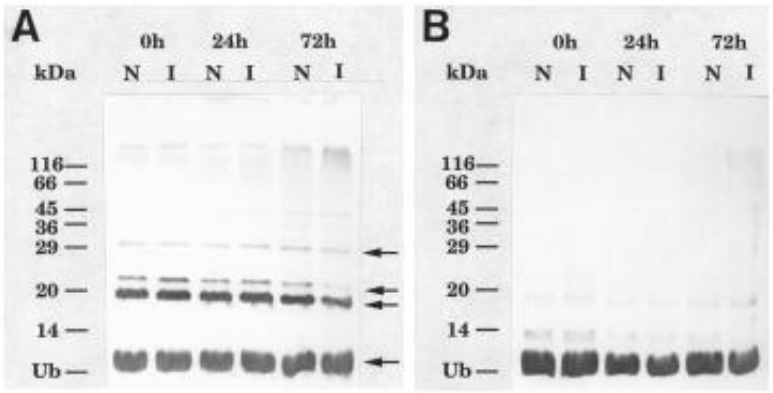

Figure 3. Analysis of polyhistidine-tagged ubiquitin-protein thioesters generated by cell extracts from noninduced and induced *Zinnia* cultures. Cell extracts were prepared from noninduced (N) or induced (I) cultures at the indicated timepoints in the presence of DTT. Reactions containing 200 µg *Zinnia* protein and 13 µg polyhistidine-tagged ubiquitin were incubated for 2 min at 30°C and subsequently stopped either by the addition of SDS-PAGE sample buffer containing E64 (A) or by the addition of SDS-PAGE sample buffer containing E64 and supplemented with 2-mercaptoethanol to 10%, followed by boiling (B). Reaction products were resolved on a 13.5% polyacrylamide gel at 4°C and processed for immunoblotting as described for Figure 2. Arrows indicate the positions of putative polyhistidine-tagged ubiquitin-E2 thioesters and HisUb.

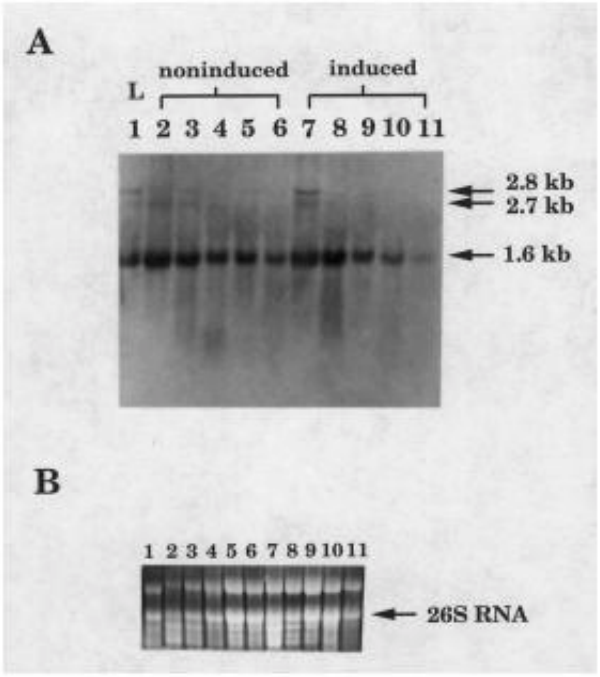

Figure 4. Analysis of ubiquitin gene expression in *Zinnia* leaf and cells cultured under noninductive and inductive conditions. Total RNA (21 μg per lane) (A) was isolated from *Zinnia* leaf (lane 1) and noninduced (lanes 2-6) and induced (lanes 7-10) cell cultures at 0 h (lanes 2 and 7), 24 h (lanes 3 and 8), 48 h (lanes 4 and 9), 60 h (lanes 5 and 10) and 72 h (lanes 6 and 11), resolved on a 1.2% formaldehyde-containing agarose gel, and transferred to a nylon membrane for northern analysis. A biotinlylated antisense ubiquitin coding region RNA probe was used and hybridization was detected by alkaline phosphatase catalyzed chemiluminescence using Lumigen-PPD substrate (New England Biolabs). Sizes (kb) and positions of ubiquitin mRNA are indicated at right. An ethidium bromide-stained agarose gel is also shown as a control for equivalent RNA loading (4 μg RNA per lane) (B).

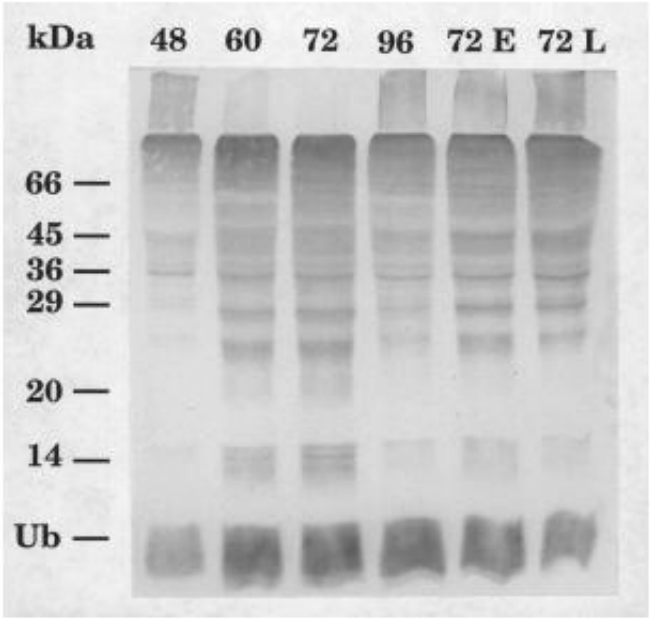

Figure 5. Analysis of changes in endogenous ubiquitin-protein conjugate profiles resulting from *in vivo* cysteine proteinase activity. E64 (final concentration 58 μM) or leupeptin (final concentration 200 μM) was added at 48 h to *Zinnia* mesophyll cells cultured in inductive medium. Cells were collected and protein extracted at 72 h. Endogenous ubiquitin-protein conjugates extracted from cells cultured in the absence (lanes 48, 60, 72, 96) or presence of cysteine protease inhibitors E64 (lane 72E) or leupeptin (lane 72L) were detected by immunoblotting as described for Figure 1.

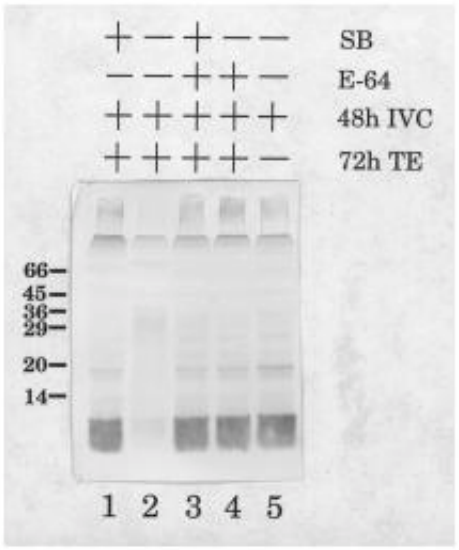

Figure 6. Analysis of the ability of cell extracts from 72 h induced *Zinnia* cultures to degrade conjugates generated *in vitro* by a cell extract from a noninduced *Zinnia* culture. Polyhistidine-tagged ubiquitin-protein conjugates for use as protease substrate were prepared from cells collected from 48 h noninduced cultures as described for Figure 2, except that leupeptin was omitted from the *in vitro* conjugation reaction. A 72 h induced cell extract was used as the source of protease. The components of each proteolysis reaction are indicated at top of figure. Reactions were incubated for 90 min at 27°C and stopped by the addition of E64 and sample buffer (SB; if not already present) and boiling.

CHAPTER 3

Proteasome Inhibitors Prevent Tracheary Element Differentiation in *Zinnia* Mesophyll Cell Cultures

The work presented in this chapter has been published: Woffenden, B.J., Freeman, T.B., and Beers, E.P. (1998) Proteasome inhibitors prevent tracheary element differentiation in *Zinnia* mesophyll cell cultures. Plant Physiol. 118:419-431.

Abstract

To determine whether proteasome activity is required for TE differentiation, the proteasome inhibitors *clasto*-lactacystin β-lactone and carbobenzoxy-leucinyl-leucinyl-leucinal (LLL) were used in a *Zinnia* (*Zinnia elegans*) mesophyll cell culture system. The addition of proteasome inhibitors at the time of culture initiation prevented differentiation otherwise detectable at 96 h. Inhibition of the proteasome at 48 h, after cellular commitment to differentiation, did not alter the final percentage of TEs compared with controls. However, proteasome inhibition at 48 h delayed the differentiation program by approximately 24 h, as indicated by examination of both morphological markers and the expression of putative autolytic proteases. These results indicate that proteasome function is required both for induction of TE differentiation and for progression of the TE program in committed cells. Treatment at 48 h with LLL but not *clasto*-lactacystin β-lactone resulted in partial uncoupling of autolysis from differentiation. Results of gel analysis of protease activity suggest that the observed incomplete autolysis was due to the ability of LLL to inhibit TE cysteine proteases.

Introduction

The role of proteases in regulating programmed cell death (PCD) is currently an extremely active area of research. Principle among the proteases shown to be required for PCD in animal systems is the large and still-growing family of proteases known as caspases (cysteinyl aspartate-specific proteases) (recently reviewed by Nicholson and Thornberry 1997), which appear to be involved in disabling cellular repair processes essential for maintaining homeostasis, thereby leading to apoptosis. However, caspases are not the only proteases demonstrated to play a role in PCD. Animal apoptosis pathways can also be initiated by several other mechanistically distinct proteases including the serine protease granzyme B (Greenberg 1996), members of the cathepsin family (Deiss, *et al.* 1996), and the cysteine protease calpain (Squier and Cohen 1996).

In plants, it is well documented that increases in serine and cysteine proteases are associated with two developmentally programmed suicide pathways, organ senescence (recently reviewed by Hadfield and Bennett 1997) and TE differentiation (recently reviewed by Beers 1997; Fukuda 1997; Pennell and Lamb 1997). Although it is generally assumed that these plant enzymes function in the autolysis of intracellular proteins rather than as components of regulatory proteolytic cascades, the apparent participation of multiple proteolytic pathways during animal PCD indicates that the ability of plant proteases to regulate PCD may be underestimated.

An additional proteolytic system, the ATP-dependent Ub-proteasome pathway of proteolysis, is known to regulate numerous cellular processes via degradation of short-lived regulatory proteins in mammals, yeast, and plants (Glotzer, *et al.* 1991; Hochstrasser, *et al.* 1991; Shanklin, *et al.* 1987). The importance of the Ub-proteasome pathway in degrading long-lived proteins is

well-established for mammals, but its function in this capacity in lower eukaryotes is uncertain (see recent review by Goldberg, *et al*. 1997). Ub is a 76-amino acid protein which becomes covalently ligated to cellular proteins via isopeptide bonds between the carboxyl-terminal glycine of Ub molecules and the ε-amino group of lysine residues of the target protein (reviewed by Ciechanover and Schwartz 1994; Varshavsky 1997; Vierstra 1996). Attachment of Ub to protein targets requires the activity of multiple enzymes, including an ATP-dependent Ub-activating enzyme (E1), one of a family of Ub-conjugating enzymes (E2s), and in some cases, one of a number of substrate recognition proteins (E3s). Ub may be attached to protein substrates as a monomer, or alternatively, Ub may be ligated to a lysine residue of another Ub molecule, forming polyUb chains. Polyubiquitination of proteins is sufficient to target them for degradation by a large (26S), ATP-dependent multicatalytic protease, the proteasome. Additionally, proteasome-mediated degradation of a few proteins has been shown to occur without ubiquitination (Jariel-Encontre, *et al*. 1995; Murakami, *et al*. 1992).

Recent evidence indicates that the Ub-proteasome pathway may regulate PCD in some systems. Levels of Ub-proteasome pathway components were observed to increase during animal PCD events, including molt-induced claw muscle atrophy in lobster (Shean and Mykles 1995) and intersegmental muscle degeneration during metamorphosis of the hawkmoth, (Haas, *et al*. 1995; Jones, *et al*. 1995). Depending on the experimental system under investigation, proteasome activity may promote PCD (Cui, *et al*. 1997; Grimm, *et al*. 1996; Sadoul, *et al*. 1996) or prevent it (Drexler 1997; Monney, *et al*. 1998; Shinohara, *et al*. 1996). Requirements for proteasome activity during differentiation events not involving PCD have also been documented from animal systems, including maturation of starfish oocytes (Sawada, *et al*. 1997) and differentiation of

photoreceptor cells in the *Drosophila melanogaster* eye (Li, *et al*. 1997). In plants, upregulation of components of the Ub pathway has been detected during diverse developmental PCD events, including leaf senescence (Garbarino and Belknap 1994), anther dehiscence (Li, *et al*. 1995), and fruit ripening (Picton, *et al*. 1993). Thus, the Ub-proteasome pathway is broadly implicated as a regulator of cell fate.

Using the *Zinnia* (*Zinnia elegans* L.) mesophyll cell culture system for TE differentiation (Church 1993; Fukuda 1997; Fukuda and Komamine 1980b), we investigated the role of proteolysis during differentiation and PCD. Mature TEs function *in planta* in water and solute transport and are characterized by a patterned deposition of lignified, cellulosic, secondary cell wall thickenings and the absence of a protoplast within the cell corpse. The *Zinnia* mesophyll cell system permits the study of plant cell differentiation, death, and autolysis during the semi-synchronous TE differentiation of 40 to 60% of the cultured cells. Although no genes or proteins controlling cell death or the initiation of the autolytic phase in differentiating TEs have been identified, hydrolytic enzyme activity increases dramatically late in the differentiation process.

Markers for TE autolysis include an endonuclease (Thelen and Northcote 1989), an RNAse (Ye and Droste 1996), and proteases (Beers and Freeman 1997; Minami and Fukuda 1995; Ye and Varner 1996). The Ub-proteasome pathway also appears to be required for proper vascular tissue development. Transgenic tobacco expressing a mutant Ub unable to form polyUb chains exhibited aberrant vasculature (Bachmair, *et al*. 1990), and in *Coleus*, regeneration of xylem vessel elements after wounding was accompanied by increased levels of Ub and/or Ub-protein conjugates (Stephenson, *et al*. 1996).

Ub-protein conjugating activity is detectable in *Zinnia* TE culture extracts (B.J. Woffenden and E.P. Beers, unpublished data; Chapter 2). Using inhibitors

of the proteasome, we asked whether the proteasome has a regulatory role during TE differentiation. Proteasome inhibitors used included LAC (Dick, *et al.* 1997), which was derived from the microbial metabolite, lactacystin (Omura, *et al.* 1991), and the synthetic tripeptide aldehyde inhibitors, LLnL, LLM (Sasaki, *et al.* 1990) and LLL (Tsubuki, *et al.* 1996). LAC irreversibly inhibits the tryptic, chymotryptic, and peptidylglutamic cleavage activities of the proteasome by covalently binding to the amino-terminal Thr residue of the ß-subunits (Fenteany, *et al.* 1995). Radiographic, crystallographic, and mutagenesis studies have demonstrated that this residue provides the active-site nucleophile (Lowe, *et al.* 1995; Seemuller, *et al.* 1995). Specificity of LAC for the proteasome has been established, to the exclusion of the Cys proteases calpain, papain, and cathepsin B, and the Ser proteases chymotrypsin, and trypsin (Fenteany, *et al.* 1995). Conversely, peptide aldehydes are reversible, competitive inhibitors that act as transition state analogs (Löwe, *et al.* 1995) and inhibit both calpain and the proteasome (Rock, *et al.* 1994; Tsubuki, *et al.* 1996).

The data presented here implicate the proteasome as a regulator in both early and late stages of TE differentiation in *Zinnia* mesophyll cell cultures but do not support a direct role for the proteasome in TE autolysis. Rather, autolysis appears to depend at least in part on LLL-sensitive Cys proteases.

Materials and Methods

Plant Material and Growth Conditions: Seeds of *Zinnia* (*Zinnia elegans* cv. Envy) (Grimes Seeds, Concord, OH) were sown in 4-inch pots containing Sunshine Mix 1 (Wetsel Seed Co., Harrisonburg, VA). Plants were grown at 27°C under a 16-h photoperiod at 85 µmol photons $m^{-2}s^{-1}$ and watered as needed with distilled water.

Mesophyll Cell Isolation and Culture: The first true leaves were harvested from 7- to 9-day-old seedlings, and mesophyll cells were isolated and cultured in TE inductive medium according to the method of Roberts, *et al.* (1992), except that cells were cultured in scintillation vials in 2.4 ml of medium. Cells were collected from suspension cultures by centrifugation at 50*g* for 2 min at the times indicated in the figure legends. Pellets were stored at -70°C until extraction. When harvesting cells for RNA extraction, this step was preceded by freezing in liquid nitrogen.

Proteasome Inhibitor Treatments: The proteasome inhibitors LLnL, LLM (Sigma), LLL (sold as MG132, Calbiochem; San Diego, CA)), or LAC (Calbiochem; San Diego, CA) were added to TE cultures as follows: Because the level of solubility of LLL in aqueous solution is approximately 50 μM, LLL was added to cultures as 20 μM pulses every 6 h either between 0 and 18 h or between 48 and 66 h, to a final concentration as indicated in the figure legends. LAC was added to a final concentration as indicated in the figure legends. DMSO solvent controls were evaluated for all treatments. Regardless of the method of inhibitor addition (i.e., pulsed multiple times or single additions), inhibitor additions begun at culture initiation and at 48 h are referred to as t_0 and t_{48}, respectively.

Cell Counts: For counting, an aliquot of cells was mixed with an equal volume of 1% Evans blue (Sigma), which is excluded from live cells (Roberts and Haigler 1989), in culture medium. Values for three populations were recorded using a hemacytometer : live nondifferentiated cells, live TEs, and dead TEs. To score incomplete autolysis, dead TEs were counted as either autolytically cleared or retaining cellular contents.

Hoechst 33342 Staining of Cultured Cells: Paraformaldehyde-fixed (Planchais, *et al.* 1997) cells stained with 1 μg ml^{-1} Hoechst 33342 in Galbraith's buffer (20 mM MOPS, pH 7, 45 mM $MgCl_2$, 30 mM sodium-citrate, and 1% [w/v] Triton X-100; Galbraith, *et al.* 1983) were viewed and photographed using a fluorescence microscope (model MC 63; Zeiss) .

Protein Extraction: In each of two independent experiments for each inhibitor, four replicates (scintillation vials) were pooled at each harvest (72 and 96 h) and scored for TE differentiation. Extracts were prepared by four freeze-thaw cycles in 100 mM $NaPO_4$ buffer, pH 7.2, containing 20 μM leupeptin and 14 mM 2-mercaptoethanol. Lysed cells were pelleted by centrifugation at 12,000*g* for 10 min at 4°C. The supernatant was concentrated approximately 25-fold using YM10 concentrators (Millipore, Bedford, MA) and stored at -70°C for subsequent use in either activity gels or immunoblots.

Antibody Production and Purification and Immunoblot Analysis of Ub-Protein Conjugates: Antibodies to denatured, cross-linked bovine Ub (Sigma; St. Louis, MO) were prepared in chickens at Cocalico Biologicals (Reamstown, PA). The immunoglobulin fraction was purified from egg yolk using the caprylic acid extraction protocol of McLaren et al. (1994) and was then subjected to affinity purification (Haas and Bright 1985; Hershko, *et al.* 1982). After resolution by SDS-PAGE (13.5% [w/v] acrylamide) using the buffer system of (Laemmli 1970), *Zinnia* proteins were electrophoretically transferred to PVDF membranes (Immobilon-P, Millipore; Bedford, MA) using a semidry transfer apparatus (Amersham Pharmacia Biotech; Arlington Heights, IL) according to the manufacturer's recommendations. The transfer buffer was 48 mM Tris, 39 mM

glycine, pH 8.4, 1.3 mM SDS, 20% methanol. Blocking and incubation in primary and secondary antibodies were performed with "Blotto" made according to the method of Johnson, *et al.* (1984). Blots were incubated in antibody diluted 1:1000 in Blotto at room temperature, with rotation for 2 h (primary) or 1 h (secondary). Blots were washed between steps using 200 mM NaCl buffered with 50 mM Tris-HCl, pH 7.4. Colorimetric detection with the substrates nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (both from Sigma) was catalyzed by alkaline phosphatase-conjugated, goat anti-chicken antibody (KPL, Gaithersburg, MD).

Protease Activity Gels: Protease activity gels were prepared essentially according to the method of Beers and Freeman (1997). Aliquots from each extract, representing an equal number of cells (1×10^5 cells), were resolved by SDS-PAGE (12% [w/v] acrylamide). Samples were not boiled prior to electrophoresis. Hydrolysis of the gelatin substrate (0.5% [w/v]) resulted in unstained bands in the substrate-impregnated gels, indicating the position of the proteolytic activity in resolving gels. For *in vitro* inhibitor studies, polyacrylamide gel lanes were excised and incubated at room temperature for 15 min either in 4 μM LAC, 20 μM LLL, or 0.1% DMSO as solvent control prior to exposure to the substrate gels, as described above.

RNA Isolation: Following each of two independent experiments for LLL and one experiment for LAC, 11 replicates were pooled, an aliquot was removed and scored for TE differentiation, and the balance was frozen in liquid nitrogen and stored at -70°C until extraction. Total RNA was prepared by the method of Chirgwin, *et al.* (1979). Immediately after the addition of 2 ml of a guanidine thiocyanate stock solution (4M guanidine thiocyanate, 0.5% N-lauroylsarcosine, 25 mM sodium citrate, pH 7.0, 100 mM 2-mercaptoethanol, and 0.1% antifoam A)

the sample was homogenized on ice for a total of 3 min and clarified by centrifugation. RNA was precipitated overnight at -20°C by the addition of 0.025 volume of 1 N acetic acid and 0.75 volume of ethanol. The pellet was resuspended at one-half the original volume in guanidine-HCl solution (7.5 M guanidine-HCl, 25 mM sodium citrate, pH 7.0, and 50 mM 2-mercaptoethanol) and precipitated (0.025 volume of 1 N acetic acid and 0.5 volume of ethanol) at -20°C for at least 3 h. This was repeated twice, reducing the volume of guanidine-HCl by one-half each time. The pellet was washed by suspension in absolute ethanol (-20°C), extracted twice in 100 µl diethyl-pyrocarbonate-treated water, and precipitated (0.1 volume of 2 M potassium acetate, pH 5, and 2 volumes of ethanol) overnight at -20°C. The pellet was washed twice with 95% ethanol, dried, and resuspended in diethyl-pyrocarbonate-treated water.

RNA Probe Synthesis: For template preparation, 1 µg of plasmid p48h-17 in pBluescript K/S (Stratagene; La Jolla, CA) was linearized with *Bgl* II, purified by electrophoresis through low-melting point agarose (FMC, Rockland, ME), recovered, and rendered free of RNase by phenol extraction and precipitation. Biotinylated antisense p48h-17 was prepared using T7 polymerase and biotin RNA-labeling reagents (Boehringer Mannheim Biochemicals) according to the manufacturer's directions. The probe was checked for integrity by RNA gel electrophoresis as described below.

RNA gel-blot analysis: Samples and biotinylated RNA molecular mass markers (New England Biolabs; Beverly, MA) were separated on 1.2% agarose gels containing formaldehyde as described in Sambrook, *et al.* (1989), except that the formaldehyde gel-running buffer contained 5 mM sodium acetate. After electrophoresis, gels were photographed and washed three times for 10 min each

in 2xSSC and transferred to positively charged nylon membranes (Boehringer Mannheim) by overnight capillary transfer in 2xSSC. The membranes were UV cross-linked and then dried for 2 h at 80°C.

The membranes were hydrated for 2 min in 5xSSC and prehybridized in prehybridization/hybridization buffer (NorthernMAX, Ambion, Austin, TX) in a bag at 68°C for at least 1 h. The bag was drained, refilled with fresh buffer containing biotinylated antisense probe, and hybridized overnight at 68°C. Membranes were washed twice for 5 min each time at room temperature in 2xSSC/0.1% SDS, at 0.2xSSC/0.1% SDS and then twice for 15 min at 68°C in 0.1xSSC/0.1% SDS. Bands were visualized by chemiluminescent detection (Phototope K6 kit, New England Biolabs) following the manufacturer's directions except that one or two additional washes were added following incubation in streptavidin.

Results

Lactacystin and LLL Prevent TE Formation when Applied at Culture Initiation and Delay Differentiation when Applied after Cell Fate Determination

Preliminary experiments using the proteasome inhibitors, LLnL, LLM (data not shown), LAC, and LLL revealed that only the latter two effectively inhibited TE formation when added at culture initiation (t_0). Therefore, subsequent experiments were conducted using only LAC and LLL. Figure 1 shows the effect of a range of concentrations of LAC (Fig. 1 A) and LLL (Fig. 1 B) on the inhibition of TE differentiation. The observed effective doses for nearly complete inhibition of TE development that were used in subsequent experiments were 4 μM LAC and 80 μM LLL.

The concentrations of LAC and LLL used in these studies are comparable to the non-toxic levels reported previously from research in animal systems

(Grimm, *et al*. 1996; Palombella, *et al*. 1994), and several parameters indicate that these concentrations of inhibitors are nontoxic in the *Zinnia* system. Most significantly, cell growth was not affected. The mean length of non-differentiating cells present in cultures treated at culture initiation with either LAC or LLL increased 1.8-fold (from 48 μm to 88 μm) over 150 h in culture, as did the cells of control cultures. Additionally, these levels of proteasome inhibitors did not increase cell mortality above that seen in the solvent controls (data not shown), as determined from the ability of cells to exclude the nonpermeant vital stain Evans blue. Cells in inhibitor-treated cultures also continued to exhibit obvious cytoplasmic streaming and secrete characteristic protoplast fragments, as observed by Groover, *et al*. (1997) to be normal behavior of cells in healthy *Zinnia* mesophyll cell cultures. Finally, a washout experiment demonstrated that a high percentage of cells (near control levels for LAC) scored at 96 h could respond to signals leading to TE differentiation even after a 24-h exposure to inhibitor (Fig. 1, A and B).

In three independent experiments, the addition of either 4 μM LAC (Fig. 1A) or 80 μM LLL (Fig. 1B) at t_0 of culture resulted in virtually complete inhibition of TE differentiation over the 96-h culture period. Although new TEs did develop in inhibitor-treated and control cultures over an additional two days of culture beyond 96 h, LAC-treated cultures reached only 35% of control levels and LLL-treated cultures attained only 22%.

Delaying addition by as little as 6 h after culture initiation resulted in decreased efficacy, and inhibition was no longer detected following additions at and beyond 48 h of culture (Fig. 1C). At, or soon after 48 h in TE cultures, differentiation was evident as secondary cell wall thickenings became visible. A similar time course of LLL addition was not conducted because the method of

application (4 pulses over 18 h) required to achieve the effective dose of 80 μM in solution precluded single-time-point additions.

To determine whether the proteasome plays a role late in the differentiation process, for example, during the cell death or autolytic programs, TE cultures were treated with 4 μM LAC or 80 μM LLL at t_{48}. Table I shows that following LAC or LLL addition at t_{48} the percentage of TEs visible by 72 h in treated cultures was reduced to 13% and 70%, respectively, of the levels in control cultures. That these lower levels of TEs represented a delay and not a prohibition of differentiation was evident by 96 h, when the percentage of differentiated cells in inhibitor-treated cultures was nearly identical to the control levels.

Despite the ultimate attainment of control-level numbers of TEs in t_{48} LAC-treated cultures, Table I illustrates that the LAC-induced delay in TE differentiation apparent by 72 h was still evident at 96 h of culture, as reflected by a 5-fold higher level of live TEs in treated cultures compared with controls. The observed level of live TEs in LAC-treated cultures at 96 h was intermediate between the levels observed in control cultures at 72 h (39%) and 96 h (4%). The stages of differentiation represented among live TEs in LAC-treated cultures at 96 h ranged from cells with barely detectable cell wall thickenings to those with extensive thickenings (data not shown). This observed higher level of live TEs at 96 h in LAC-treated cultures was apparently not the result of an uncoupling of secondary cell wall thickening from cell death, as was revealed by culturing cells an additional 24 h, by which time equivalent numbers of mature, dead TEs were detected in inhibitor-treated cultures and controls (data not shown). In contrast to these results with LAC, no such disparity in the number of live TEs in control versus treated cultures was evident at late times in culture in the LLL experiments (Table I).

LLL Prevents Completion of TE Autolysis

Despite equivalent numbers of dead TEs present at 96 h in t_{48} LLL-treated and control cultures (Table I), we observed that LLL treatment induced an approximately 6-fold increase (from 15% to 85%) in the percentage of TEs that had not yet completed autolytic clearing by 96 h and retained some portion of intracellular contents. In contrast, t_{48} LAC treatment did not result in retention of protoplasmic material by dead TEs above the levels observed in control cultures (data not shown). Bright-field micrographs shown in Figures 2, A and B, depict cells harvested at 96 h from control and LLL-treated cultures. All TEs visible in Figure 2A are mature TEs of the control culture, lacking any detectable contents (representative of 85% of control TEs). All TEs exhibiting protoplasmic retention in LLL-treated cultures appeared plasmolyzed, with the collapsed protoplasm most often localized to one or two tight masses within the cell (Fig. 2B, arrowhead) but sometimes dispersed throughout the cell (Fig. 2B, arrow).

Three other cytological characteristics did not distinguish dead TEs exhibiting incomplete autolysis in LLL-treated cultures from mature TEs in control cultures. First, secondary cell wall thickenings of LLL-treated and control dead TEs appeared equivalent under bright-field microscopy (Fig. 2, compare A and B). Second, phloroglucinol staining of cells harvested at 96 h revealed no detectable differences in the extent of cell wall lignification between dead TEs in LLL-treated and those in control cultures (data not shown). Finally, Calcofluor white staining of cells at 96 h did not distinguish TEs of LLL-treated cultures from those in control cultures with respect to the degree of cell wall cellulose deposition (data not shown).

It has been reported that degradation of the nucleus and other organelles occurs late during the autolysis of differentiating TEs, just before or after

tonoplast disruption (Groover, *et al*. 1997). To characterize the intracellular material retained by TEs in LLL-treated cultures, we determined whether TEs present at 96 h in LLL-treated cultures contained a nucleus (or at least, dye-binding DNA) by staining with a fluorescent DNA-binding dye (Hoechst 33342). Figures 2, C and D, are bright-field and fluorescence micrographs, respectively, of paraformaldehyde-fixed cells demonstrating two important features of TEs and cells present at 96 h in LLL-treated cultures. First, live TEs exhibiting a diffuse and uniformly distributed protoplasm similar to that observed in live, undifferentiated cells contained intact nuclei (Fig. 2, C and D). Second, we did not observe TEs exhibiting both plasmolysis (i.e., incomplete autolysis) and dye-binding DNA. These results indicate that the apparent ability of LLL to stabilize intracellular contents against autolysis does not include the preservation of nDNA.

LAC and LLL Have Different Effects on the Activity and mRNA Levels of Cysteine Proteases Associated with Late Stages of TE Differentiation

Data presented thus far consist of a characterization of morphological markers associated with TE development as indicators of apparent inhibitor-induced disruptions in the differentiation program. To evaluate potential LAC- and LLL-induced changes in biochemical and molecular markers of TE differentiation, the activities and mRNA levels of Cys proteases putatively involved in autolysis were examined. TE-specific proteases are well-documented markers for the late stages of TE differentiation (Beers and Freeman 1997; Minami and Fukuda 1995; Ye and Varner 1996). Examining the expression and activity levels of these proteases may therefore indicate the extent to which the TE differentiation program is affected by proteasome inhibition and provide clues as to the apparent inability of LLL-treated cells to complete autolysis.

Because the level of intracellular components decreases during TE autolysis, it was decided that comparison of TE markers on an equal-cell-number basis would best represent relative effects of proteasome inhibitors on the progression of differentiation. Proteins were protected from degradation during and following isolation by inclusion of leupeptin in the extraction buffer (Beers and Freeman 1997). Protease activity gels were prepared using extracts from 1.5 x 10^5 cells harvested at 72 and 96 h to determine the effects of t_{48} application of proteasome inhibitor on the activity of two Cys proteases (28 and 24 kD) postulated to participate in autolysis of TEs (Beers and Freeman 1997).

The activities of the 28- and 24-kD proteases were detectable at very low levels in extracts from LAC-treated cells harvested at 72 h compared with the control (Fig. 3A). By 96 h, however, protease activities in extracts from LAC-treated cultures had increased to levels similar to those observed in 72-h control extracts. During the same period in control cultures, activity of the 24-kD protease decreased to a barely detectable level, whereas activity of the 28-kD enzyme was undetectable by 96 h. In contrast, LLL treatment resulted in recovery of a slightly higher level of the 24-kD protease at 72 h compared with controls (Fig. 3B). By 96 h, as was observed in extracts from LAC experiments, activity of the 24-kD protease decreased to a barely detectable level in control samples, whereas the level of activity of this enzyme remained relatively high in extracts from LLL-treated cells. Despite the apparent overall lower level of protease activity in LLL versus LAC experiments (Fig. 3, compare A and B, 72-h DMSO controls), identical results concerning the relative levels of protease activity (treated versus control) were obtained in a second independent experiment for each inhibitor. These protease activity profiles indicate that addition of proteasome inhibitors results in altered regulation of expression and/or activity of TE-associated Cys proteases, and in the case of the LAC

experiments, the results are consistent with an inhibitor-induced delay in the progression of the differentiation program of approximately 24 h.

Expression of p48h-17, a Cys protease that is upregulated in the late stages of TE differentiation (Ye and Varner 1993, 1996), was examined following inhibitor treatment at t_{48}. During our efforts to prepare total RNA from treated and control cells for p48h-17 RNA gel-blot analysis, we discovered that equivalent numbers of cells yielded markedly different quantities of RNA. Cells harvested from LAC- and LLL-treated cultures yielded 1.7- and 3.4-fold higher levels of RNA, respectively, compared with controls at 72 h, and less than 0.5 μg total RNA per 10^6 cells at 96 h. This low level of RNA recovery at 96 h from inhibitor-treated cultures occurred despite our efforts to denature RNAses throughout RNA extraction (Chirgwin, *et al.* 1979). Back-extraction of cell pellets yielded no additional RNA (data not shown), revealing that the observed differences in RNA yield apparently were not due to treatment-induced differences in retention of RNA by the cells. Since it was not possible to isolate useful quantities of RNA at 96 h following LAC or LLL treatments, we have presented RNA gel-blot data (equal cell number and equal RNA comparisons) for the 72-h time point only.

Cells treated with LLL and harvested at 72 h yielded a higher level of p48h-17 mRNA compared to the control, whether analysis was conducted on an equal-cell-number or equal-RNA basis (Fig. 4A). Similarly, but to a lesser extent, p48h-17 mRNA was more abundant in cultured cells following LAC treatment than in controls when compared on equal-cell-number or equal RNA basis (Fig. 4B).

LAC is More Effective than LLL at Stabilizing Endogenous Ubiquitinated Proteins in Cultured *Zinnia* cells

To confirm that the proteasome inhibitors used in this study were capable of exerting their effects via inhibition of the proteasome, we examined the effects of LAC and LLL on levels of endogenous Ub-protein conjugates. Immunoblots of total *Zinnia* protein probed with anti-Ub antibody reveal that treatment of cells with either LAC or LLL resulted in stabilization of conjugates extracted from 72 h cultures (Fig. 5). The apparent stabilization by the reversible inhibitor LLL was no longer evident by 96 h in culture (data not shown). In contrast, treatment of cells at t_{48} with the irreversible inhibitor LAC resulted in a high degree of Ub-protein conjugate stabilization through 96 h of culture (Fig. 5B).

LLL Inhibits the Activity of Cys Proteases of Potential Importance to TE Autolysis

LLL has been reported to inhibit the Cys protease calpain in addition to the proteasome (Tsubuki, *et al*. 1996). To address the possibility that LLL inhibition of Cys proteases putatively involved in TE autolysis might explain the failure of TEs in LLL-treated cultures to complete autolysis, protease activity gels were prepared in the presence of LLL and LAC. LLL treatment resulted in complete inhibition of the 28- and 24-kD TE-specific proteases (Fig. 6). As expected, LAC treatment had no effect on the activity of these proteases (Fig. 6).

Discussion

We have demonstrated that, when added at the time of culture initiation, inhibitors of the proteasome can prevent TE differentiation, apparently without significantly affecting the health of cultured *Zinnia* cells. In addition, proteasome inhibition following the appearance of cell wall thickenings resulted in an approximately 2-fold increase in the time required to complete differentiation. This delay was demonstrated by characterization of morphological markers and

with evaluation of the expression of Cys proteases putatively involved in autolysis. Although LLL is capable of inhibiting both the proteasome and cysteine proteases, the observation that application of the specific proteasome inhibitor LAC prevents TE differentiation indicates that proteasome inhibition by LLL is sufficient for prevention of TE differentiation. In contrast, the ability of LLL to partially uncouple autolysis from the TE differentiation program is probably due to its inhibition of autolytic cysteine proteases.

The Proteasome as Mediator of Early Signals Leading to TE Differentiation

The Ub-proteasome pathway is known to regulate the cell cycle (for review see Pagano, *et al.* 1995), and although cell division can occur in up to 40% of cells undergoing TE differentiation, the remaining 60% of TEs form without intervening mitosis (Fukuda and Komamine 1980a). Therefore, disruption of cell cycling by proteasome inhibitors is not likely to account for the nearly complete prevention of TE formation reported here. Rather, our results indicate that TE differentiation may require the proteolytic removal of endogenous differentiation inhibitor(s), perhaps due to a role for the proteasome in transducing differentiation signals initiated by the phytohormones auxin and/or cytokinin which are required for TE development (Church and Galston 1988). It has been proposed that the turnover of short-lived repressor proteins is a requirement for auxin response (for review see Abel and Theologis 1996), and recent work by Estelle and colleagues (Ruegger, *et al.* 1998) specifically implicates the Ub-proteasome pathway by showing that two *Arabidopsis* genes, *TIR1* and *AXR1*, which encode proteins related to Ub pathway components, are required for normal auxin signal transduction.

The Proteasome as a Regulator of the Time Course of TE Differentiation

That application of LAC after cellular commitment to TE development causes a strong delay in the overall program is corroborated by the 24-h delay in the peak of Cys protease activity noted in extracts from LAC-treated cells compared with controls (Fig. 3A). One interpretation of the apparently contrasting 72-h profile of protease activity extractable from LLL-treated cultures is that LLL does not result in a similar delay in peak activity of TE proteases. However, the higher level of 24-kD protease activity detectable at 72 h following LLL treatment compared with controls (Fig. 3B) may have resulted from upregulation of the expression of genes encoding LLL-sensitive proteases in response to LLL-mediated inhibition of Cys proteases (Fig. 6). Increases in mRNA levels of enzymes following application of competitive inhibitors has been documented, as in the case of 3-hydroxy-3-methylglutaryl CoA reductase (Cohen and Griffioen 1988). Alternatively, the higher level of protease activity observed at 72 h following LLL treatment may have resulted from inhibition by LLL of an unknown protease(s) that normally functions to degrade the 24-kD enzyme as part of a post-translational mechanism for regulating its activity prior to autolysis. Such a posttranslational mechanism would not be expected to be affected by LAC treatment, which has no activity against cysteine proteases.

Our inability to harvest useful quantities of RNA from inhibitor-treated TE cultures at 96 h may indicate that RNA was degraded prior to or during isolation from inhibitor-treated cells. RNAse activity levels have been shown to increase after 48 h in normal TE cultures (Thelen and Northcote 1989; Ye and Droste 1996), with the highest levels detected at 84 h (Thelen and Northcote 1989; Ye and Droste 1996). A proteasome inhibitor-induced delay in the overall differentiation program would be expected to include a delay in peak RNAse activity relative to the controls. Thus, recovery of low levels of RNA at 96 h from proteasome inhibitor-treated cultures, despite the presence of 40%

nondifferentiated cells, may represent indirect evidence of a delay in the differentiation process relative to control cultures, in which mostly empty, mature TEs contain little or no RNAse to degrade RNA released from nondifferentiating cells during RNA isolation. Similarly, the recovery at 72 h of higher levels of RNA from inhibitor-treated cells compared with controls may reflect the presence of higher levels of early-stage (preautolysis) TEs in treated cultures at this time.

The apparent higher level of p48h-17 mRNA observed at 72 h in both LLL- and LAC-treated cultures is also consistent with an inhibitor-induced delay in the TE differentiation program, assuming that p48h-17 expression peaks prior to 72 h in normal TE cultures. (Ye and Varner 1996) reported the highest level of p48h-17 mRNA expression at 60 h in TE cultures, although data from later times in culture were not presented. Whether p48h-17 encodes the 24-kD protease detected in this study is not known. However, when expressed in transgenic tobacco, p48h-17 yielded a 20-kD mature Cys protease, close to both its predicted size (22.7 kD; Ye and Droste 1996) and to the 24-kD cysteine protease detected here and previously (Beers and Freeman 1997). Therefore, it seems reasonable to speculate on the significance of p48h-17 expression relative to the activity of the 24-kD Cys protease.

The inverse relationship evident between 72-h p48h-17 mRNA and 72-h protease activity levels following LAC treatment may indicate that 72-h p48h-17 mRNA levels predict the much higher 96-h 24-kD protease activity levels. Alternatively, as discussed above, protease levels may normally be kept low, despite high transcript levels, by some as yet undescribed post-translational proteolytic mechanism that is inhibited by LLL and not by LAC. Thus, uncoupling of this regulatory mechanism might be expected in LLL-treated cells,

revealing an apparent direct correlation between p48h-17 transcript level and TE protease levels.

Ub-protein Conjugate Stabilization by LLL and LAC

It is possible that the endogenous conjugate profiles evident in cell extracts from inhibitor-treated cultures were not the direct result of proteasome inhibition but rather, represent the profile of a delayed culture relative to that of a normally progressing culture. However, if this were the case, we would expect the levels of conjugates extracted at 96 h from LAC-treated cells to appear more similar to those from 72-h control cells, thereby reflecting the approximately 24-h delay observed at both the morphological (Table I) and biochemical (Fig. 3) levels. Instead, conjugate stabilization in LAC-treated cultures clearly persisted through 96 h.

The Role of Cys Proteases in TE Autolysis

In contrast to LAC treatment, LLL treatment resulted in the partial uncoupling of autolysis from differentiation of what otherwise appeared to be normal TEs (Fig. 2). We have presented evidence that LLL but not LAC inhibits TE-associated Cys proteases (Fig. 6), suggesting that the apparent prohibition of TE autolysis in LLL-treated cultures is due to inhibition of the activity of the 28- and 24-kD Cys proteases or other LLL-sensitive proteases not detected by our activity gels and not caused by inhibition of the proteasome.

Hoechst 33342 staining of cells following LLL treatment demonstrated that the cellular material retained by 85% of the dead TEs in these cultures does not include nDNA, indicating that nuclear integrity is no longer maintained in TEs that otherwise exhibit incomplete autolysis. This absence of DNA is consistent with the proposal that endonucleases expressed during TE

differentiation function to degrade DNA and RNA during autolysis (Thelen and Northcote 1989) independently of Cys proteases. Although tonoplast rupture is known to be associated with autolysis of intracellular components in developing TEs (Groover, *et al*. 1997), the results presented here represent the first demonstration to our knowledge that application of a Cys protease inhibitor prevents the complete autolysis *Zinnia* TEs.

Additionally, while the activity of the Ub-proteasome pathway has been previously implicated as a necessary component of vascular differentiation (Bachmair, *et al*. 1990; Stephenson, *et al*. 1996), to our knowledge this is the first demonstration that a specific inhibitor of the proteasome can reversibly prevent TE differentiation and that proteasome function is also required for regulating the time course of TE differentiation in *Zinnia* mesophyll cell cultures. The proteasome, however, does not appear to participate directly in TE autolysis.

As used in this study, LLL and LAC were not able to uncouple the differentiation process from cell death. If TE death is dependent on the proteasome or on LLL-sensitive proteases, perhaps the window of opportunity to uncouple death from TE differentiation is narrow, occurring at a time not tested in this report. Although it is possible that proteases do not play an important role in the regulation of plant PCD, further investigation using inhibitors against proteases other than the proteasome and Cys proteases may lead to the identification of proteases specifically involved in the regulation of cell death during TE differentiation.

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Table 1. *Effect of t_{48} treatment with proteasome inhibitors on the percentage of TE differentiation*

Numbers within parentheses indicate the total number of replicates from a minimum of two independent experiments.

	total TEs[a]		live TEs[b]
Treatment	72 h	96 h	96 h
LLL	35 ± 16 (10)	60 ± 6 (10)	4 ± 1 (7)
DMSO	50 ± 5 (10)	58 ± 7 (10)	4 ± 4 (7)
LAC	5 ± 4 (7)	59 ± 7 (11)	20 ± 8 (11)
DMSO	38 ± 14 (7)	53 ± 6 (11)	4 ± 3 (11)

[a]Mean ± SD percentages of TEs in total cells (TEs plus live, undifferentiated cells) .

[b]Mean percentages of live TEs in total TEs ± SD.

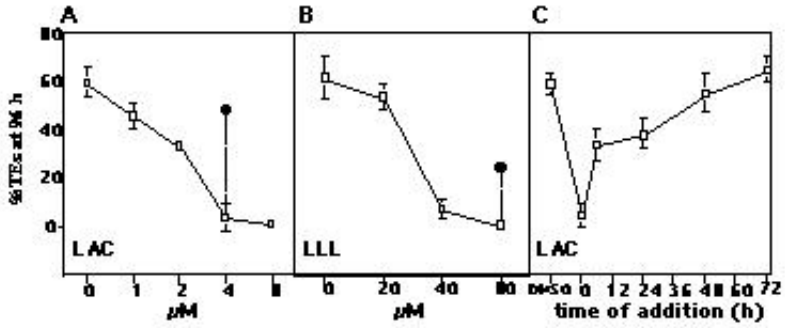

Figure 1. Effects of inhibitor dosage and time of addition on TE differentiation. Shown (open squares) are the percentages of TEs in total cells (live, undifferentiated cells plus TEs) detectable at 96 h following treatment at t_0 with the indicated concentrations of LAC (A) or LLL (B). Also shown are the percentages of TEs detectable at 96 h following removal of inhibitors at 24 h of culture (filled circle in A and B). C, Percentages of TEs in total cells detectable at 96 h following addition of 4 µM LAC to TE cultures at the times indicated. Values are the mean percentages ± SD of a minimum of four replicates.

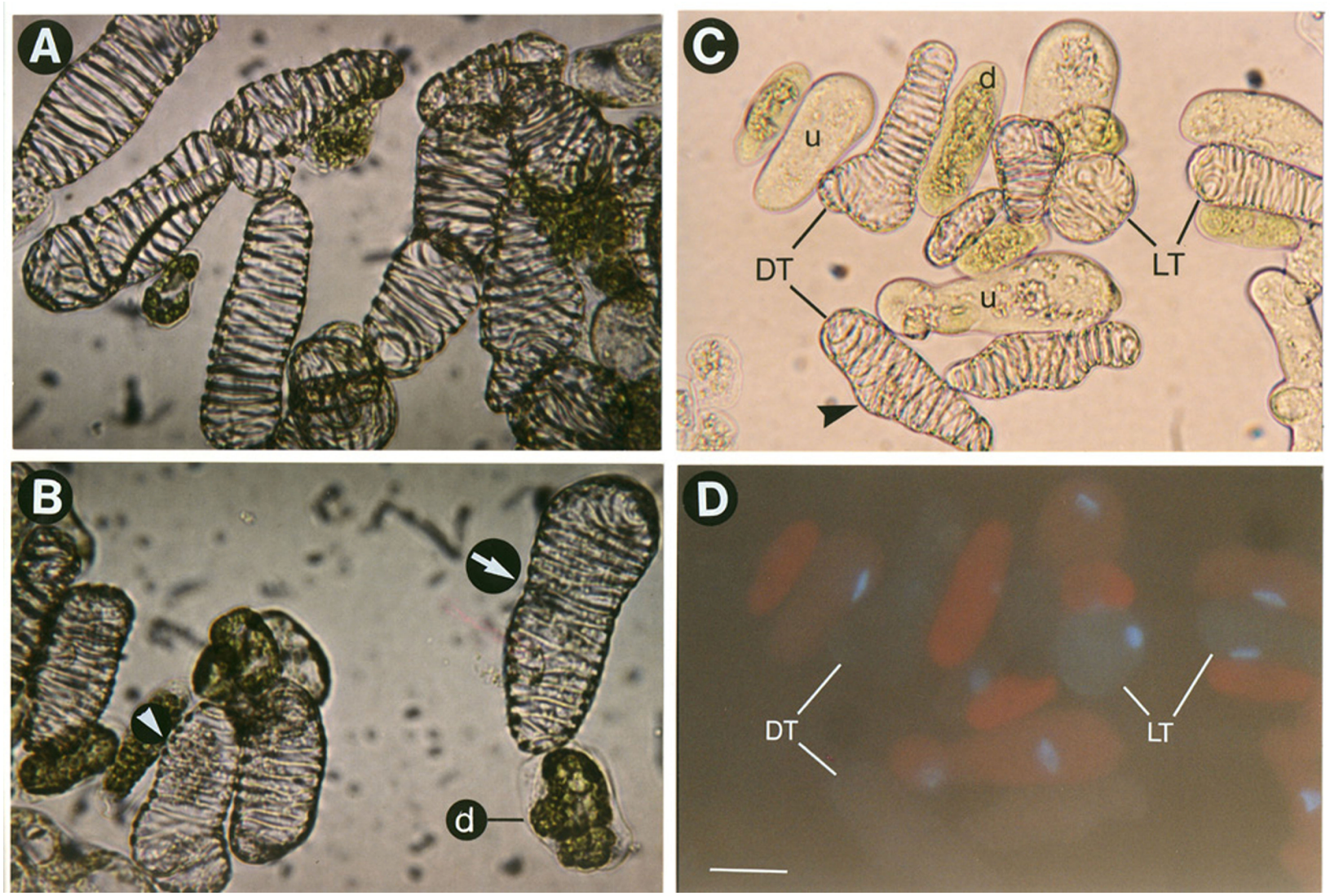
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Figure 2. Characterization of LLL-induced, incomplete TE autolysis.
For all panels, samples were taken from TE cultures at 96 h for staining and microscopy. C and D show the same field of view.
A, Bright-field microscopy of unstained control culture TEs after autolysis. B, Bright-field microscopy of unstained TEs from the t_{48} LLL-treated culture. The two forms of protoplasmic retention by dead TEs are indicated: condensed to a single or few locations (arrowhead); distributed throughout the cell (arrow). C, Bright-field microscopy of paraformaldehyde-fixed cells from the t_{48} LLL-treated culture. Arrowhead indicates protoplasm retained by dead TE that failed to complete autolysis. Live TEs showed diffuse protoplasm similar to that of live, undifferentiated cells. D, Fluorescence microscopy of cells from the t_{48} LLL-treated culture following DNA staining with Hoechst 33342. Blue fluorescence indicates presence of nDNA; red fluorescence is due to chlorophyll autofluorescence. LT, live TE; DT, dead TE; u, live undifferentiated cell; d, dead undifferentiated cell (likely killed during isolation of mesophyll cells from leaves). Bar = 10 μm.

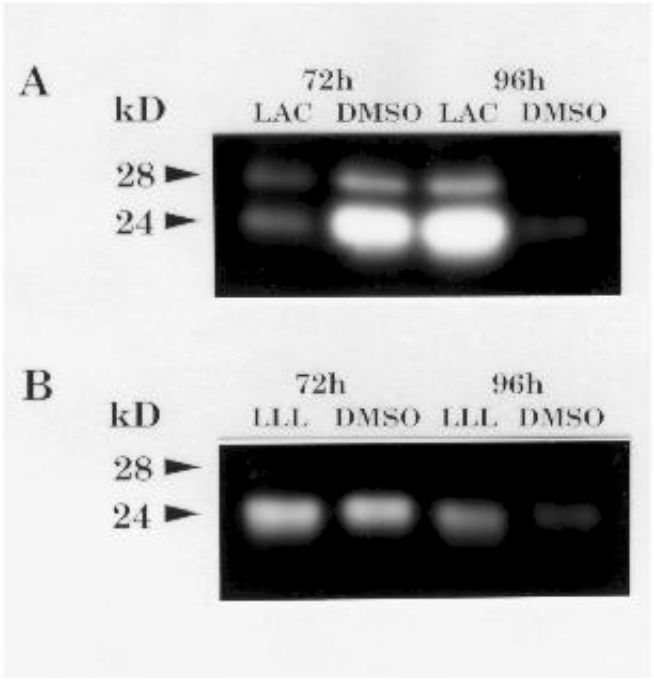

Figure 3. LAC or LLL treatment at 48 h alters the activity of TE Cys proteases. LAC (A) or LLL (B) was applied to TE cultures at t_{48}, and cells were harvested at 72 and 96 h. Shown are activity gels prepared following SDS-PAGE of total protein extracted from 1.5×10^6 zinnia cells per lane (see "Materials and Methods"). Bands represent regions of proteolytic activity in gelatin-impregnated substrate gels. Molecular masses of protein standards are indicated on the left (in kD).

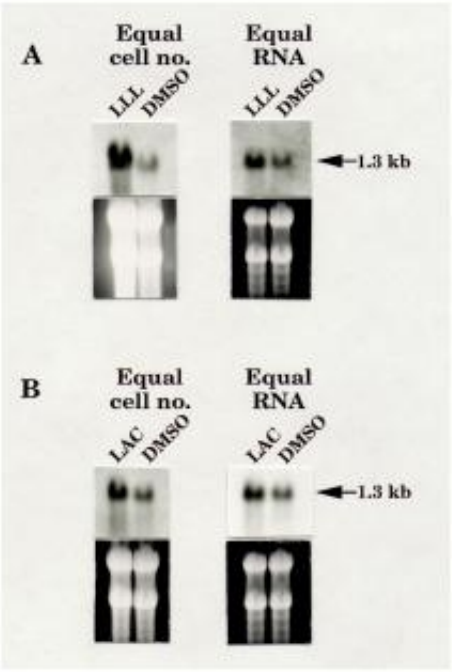

Figure 4. LLL and LAC treatments at 48 h result in elevated levels of p48h-17 expression. LLL (A) or LAC (B) was added to t_{48} TE cultures, and cells were harvested at 72 h. Shown are RNA gel blots of total RNA performed on both equal-cell-number (1θ) and an equal-RNA (3.5 μg per lane) basis, probed with biotinylated antisense p48h-17. RNA levels loaded for equal-cell-number analyses are as follows: LLL, 13.8 μg, and corresponding DMSO, 4.1 μg; LAC, 8.1 μg, and corresponding DMSO, 4.8 μg. Corresponding ethidium bromide-stained agarose gels are shown below each blot.

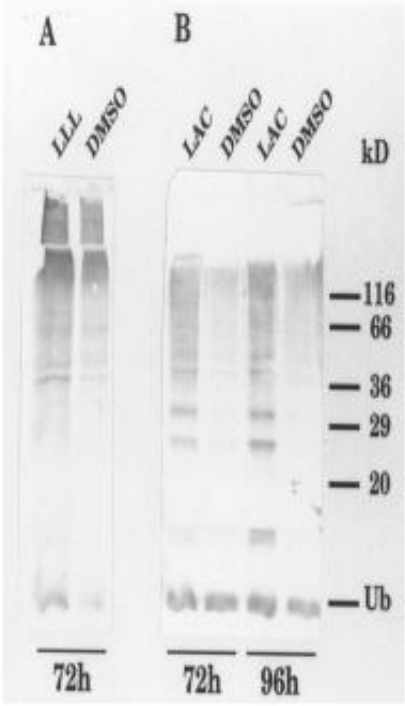

Figure 5. Endogenous ubiquitin-protein conjugates are stabilized by LLL and LAC treatments at 48 h. LLL (A) or LAC (B) was added to t_{48} TE cultures, cells were harvested at 72 h (LLL and LAC) and 96 h (LAC only), and protein was extracted for immunoblot analysis as for Figure 3. Extracts from 1.5×10^6 cells (LAC) and 2×10^5 cells (LLL) were loaded per lane. Blots were probed with anti-ubiquitin antibody. Molecular masses of protein standards and the position of free ubiquitin (Ub) are indicated on the right (in kD).

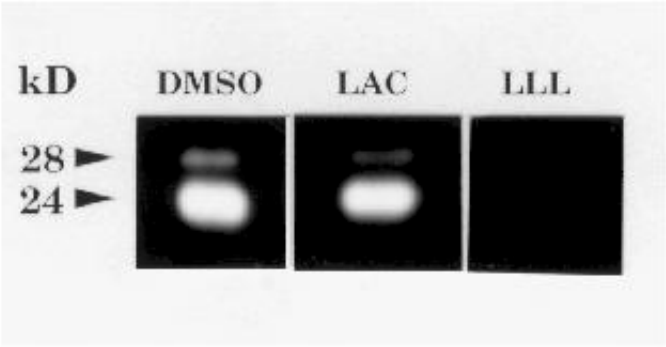

Figure 6. LLL but not LAC inhibits the activity of TE Cys proteases. Following SDS-PAGE of total zinnia protein, excised gel lanes were incubated in 4 μM LAC, 20 μM LLL, or 0.1% DMSO prior to exposure to gelatin-impregnated substrate gels. Bands represent regions of proteolytic activity in substrate gels. Molecular masses of protein standards are indicated on the left (in kD).

CHAPTER 4

Optimization of Growth of *Arabidopsis* Secondary Tissues for the Study of Ubiquitin-Proteasome Pathway Components Expressed During Xylem Differentiation

Abstract

Developing vasculature typically represents a small percentage of tissue used to construct cDNA libraries from herbaceous plants. We utilized a system that promotes proliferation of secondary tissues in *Arabidopsis*, permitting the separation of secondary xylem from bark (phloem plus nonvascular tissues) from 8-week-old hypocotyl-root segments. Dissected hypocotyl-root segments were used to prepare RNA for RT-PCR and for the construction of bark and xylem cDNA libraries. We investigated the expression of Ub pathway components in the developing secondary tissues by PCR-based screening of the two cDNA libraries. Using sequences for *Arabidopsis* Ub-conjugating enzyme genes (*UBC*s), three degenerate primers were designed to target three *UBC* families (*UBC1-3*, *UBC4-6*, *UBC8-12*). Seven additional primers were designed to specifically target ten candidate *UBC*s identified from *Arabidopsis* GenBank entries. Similarly, eight primers were designed to target eleven candidate deubiquitinating enzymes (DUBs). Gene-specific primer pairs for an α (*PAF1*) and a β(*PBF1*) subunit of the proteasome were also tested. Library screening revealed that all targeted *UBC* families and candidate genes, and both proteasome subunits are expressed in xylem and bark. Partial cDNA clones of *UBC*s from xylem (*UBC 2*, *4*, *5*, *8*,*10*) and bark (*UBC 4*, *5*) show length heterogeneity in the 3'UTR. Library screening suggested xylem-specific expression of two DUBs (AC002409 and AL021889);

however, RT-PCR analysis demonstrated that both DUBs are expressed in xylem and bark. Semi-quantitative RT-PCR analyses suggested that one DUB (AC002409) and *PAF1* may be expressed at higher levels in xylem than bark, but competitive, quantitative RT-PCR revealed that *PAF1* is likely expressed at equivalent levels in bark and xylem in this system.

Introduction

Evidence suggests that the Ub pathway is important for normal vascular tissue development in plants. For example, transgenic tobacco expressing a mutant Ub molecule displayed aberrant vascular development (Bachmair, *et al*. 1990). In *Coleus*, xylem vessel element regeneration after wounding was accompanied by increased levels of Ub and/or Ub-protein conjugates (Stephenson, *et al*. 1996). Additionally, using proteasome-specific inhibitors in the *Zinnia elegans* model for TE differentiation, we demonstrated that proteasome activity is required for TE development (Chapter 3, Woffenden, *et al*. 1998).

However, interesting questions remain about the involvement of the Ub pathway in xylem differentiation that cannot be addressed using the *Zinnia* model. Limitations of the *Zinnia* system include the lack of available genomic sequence information and the lack of facile methodology for stable *Zinnia* transformation; thus the potential for research progress in this system is severely constrained, particularly with respect to reverse genetics approaches.

The machinery of the Ub pathway is largely encoded by multigene families, and thus it is possible that individual family members may display xylem-specific expression to regulate xylem formation. Because of the noted limitations of the *Zinnia* model system, the multigene nature of the pathway necessitated the development of a tractable model for investigating the roles of particular members of each family in xylem differentiation. Therefore, we

optimized the proliferation of secondary tissues in *Arabidopsis* first described by Lev-Yadun (1994) to facilitate isolation of vascular tissues. In our lab, *Arabidopsis* plants are grown at low population density and inflorescences are removed during growth over 8 weeks, resulting in larger plants than observed from growth at high population density. This modified growth condition results in a remarkable proliferation of secondary xylem and phloem, especially in the root and hypocotyl. This vascular tissue enrichment permits the separation of secondary xylem, phloem, and nonvascular (cortex and epidermis) tissues from root-hypocotyl segments. Alternatively, dissections into two fractions may be performed, yielding secondary xylem and bark (phloem plus nonvascular tissue).

We constructed *Arabidopsis* secondary xylem and bark cDNA libraries for PCR-based library screening of several Ub pathway gene families, including *UBC*s, DUBs, and 26S proteasome α and β subunits. It is well established that UBCs function with Ub-protein ligases (E3s) in regulating the specificity of Ub-mediated proteolysis via recognition of target cellular proteins. Recently, DUBs and proteasome subunits have been implicated as having a regulatory function as well, and some display differential regulation of expression during development (Bahrami and Gray 1999; Coux, *et al*. 1996; D'Andrea and Pellman 1998; Jones, *et al*. 1995). The *Arabidopsis* model for secondary growth, optimized in our lab, provides a useful herbaceous model for the study of vascular development to complement the existing wood development models in poplar (Sterky, *et al*. 1998) and pine (Allona, *et al*. 1998).

Materials and Methods

Plant growth and isolation of xylem and bark tissues: *Arabidopsis thaliana* ecotype Columbia was grown in Sunshine Mix 1 (Wetsel Seed Co., Harrisonburg,

VA) under continuous light at a planting density of 4 to 6 plants per 4 inch pot. Plants were watered with nutrient solution according to Somerville and Ogren (1982). Under these conditions inflorescences were typically visible three weeks after germination. For the next five weeks, inflorescences were routinely removed as they emerged. Eight-week-old plants were harvesting and potting medium was washed from the roots with a strong stream of cold tap water. Approximately 1 cm of root-hypocotyl was excised just below the cotyledons and lateral roots were trimmed from the primary root with a razor blade. Prior to dissection, root-hypocotyl segments were washed with ddH_2O, blotted dry and placed on ice. Separation of root-hypocotyl segments into xylem, and bark fractions was done under a dissecting microscope. Briefly, using a double-edged razor blade, a longitudinal cut was made along the entire length of the root-hypocotyl segment, passing through the nonvascular tissue and secondary phloem but not into the xylem. Using a dissecting probe and forceps, the nonvascular tissue and phloem tissues (bark) were peeled from the xylem. Bark and xylem tissues were immediately frozen in liquid N_2 and stored at -80°C until RNA isolation.

RNA isolation: RNA was isolated from root-hypocotyl segments by lithium chloride extraction, as follows (modified from Sharrock and Quail 1989). Tissue was powdered in liquid N_2 and ground further in 1:1 extraction buffer (50 mM Tris pH 8.3, 150 mM NaCl, 10 mM EDTA, 1% lauryl sarcosine) and phenol:chloroform (1:1). Samples were centrifuged at 11,750g for 5 min at 4°C. The aqueous phase was transferred to new tubes containing 0.3 volume phenol:chloroform, vortexed, and centrifuged at 11,750g for 5 min at 4°C. The aqueous phase was transferred to a new tube, and 0.1 volume 3 M sodium acetate and 1 volume isopropanol were added. Samples were precipitated at -

20°C for at least 1 h. Samples were centrifuged at 11,750g for 15 min at 4°C. Pellets were resuspended in 1125 µl 10 mM Tris pH 8.3, 10 mM EDTA (10:10), and 375 µl 8 M LiCl was added. Samples were incubated on ice at 4°C overnight. Samples were centrifuged at 11,750g for 15 min at 4°C. Pellets were washed with 300 µl cold 80% ethanol, centrifuged briefly, and the supernatant was removed using a micropipet tip. RNA pellets were resuspended in 10 mM Tris pH 8.3, 1 mM EDTA. RNA was stored at -80°C. RNA samples were evaluated qualitatively by agarose gel electrophoresis in 1.2% agarose containing formaldehyde as described by (Sambrook, *et al*. 1989).

Construction of cDNA libraries: Total RNA was isolated as described above from xylem and bark tissue dissections of approximately 500 *Arabidopsis* root-hypocotyl segments. mRNA was isolated from total RNA using PolyAtract mRNA Isolation System III (Promega; Madison, WI) according to the manufacturer's instructions. Xylem and bark cDNA libraries were constructed using the ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene; La Jolla, CA) according to the manufacturer's instructions.

cDNA library screening by PCR: cDNA libraries were screened by PCR in the Robocycler Gradient 40 Temperature Cycler (Stratagene; La Jolla, CA) using custom ordered PCR primers (GibcoBRL; Grand Island, NY). Approximately 1 x 10^6 phage particles from either the bark or xylem library were incubated with 0.25 µM appropriate primers in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, and 1.5 units RedTaq (Sigma; St. Louis, MO) in a total volume of 50 µl. PCR cycling parameters were: denaturation by 5 min at 94 °C; 32 cycles of amplification: 1 minute at 94 °C, 1 minute at an annealing temperature indicated in figure legends, 1 minute at 72 °C; completion of

product extension by 10 min at 72 °C. A 10 µl aliquot of each PCR was fractionated on an agarose gel (composition indicated in figure legends), and products were visualized by ethidium bromide staining (0.5 mg/ml).

Quality of the PCR was controlled for in most experiments by preparation of a PCR containing a pair of control primers [Indicated in the figure legends as targeting either a cysteine protease associated with TE development in *Zinnia* (p48h-17; Ye and Varner 1993, 1996], or aspartic acid protease sequences obtained from GenBank. The gene-specific primers for p48h-17 were used at 0.125 µM and contained the following sequences: TGTGGTAGTTGTTGGGC and AAACCCTTTCTCTCCCC. The primers for aspartic acid proteases were used at 0.25 µM and contained the following sequences: TTTTTGAYACYGGRAGCTCTAACC and CCAGAATCTGCTATCGCAGAACA.

Reverse transcription: One to two µl polyA RNA [isolated from 170 root-hypocotyl segments as described above under "Construction of cDNA libraries"] was incubated with 2 mM dNTPs and 5 µM first strand primer (oligo-dT) and heated to 70-85 °C for 3 min. Samples were snap-cooled on ice, spun briefly, and returned to ice. To this mixture was added 2 µl 10X buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 10 units Placental RNAse-inhibitor (Ambion; Austin, TX), and 100 units MMLV reverse transcriptase (Ambion; Austin, TX) in a final volume of 20 µl. Samples were mixed gently, spun briefly, and incubated at 42 °C for 1 h. Reactions were stored at –20 °C. One or 2 µl of the RT reactions was used in subsequent PCR amplification, which was conducted as described under the section on cDNA library screening.

***UBC* PCR primers:** Three degenerate sense *UBC* primers were designed to target the active site region of three *Arabidopsis UBC* families. These primers were

used in PCR-based cDNA library screening with an antisense vector primer (T7) at 0.25 μM , predicted to yield active site through 3′-terminus partial cDNAs. These *UBC* primers were used at 0.25 μM and contained the following sequences: GGGAGTATMTGCTTGGACAT (*UBC1-3* family), GGTTCTGTTTGTYTAGATGT (*UBC4-6* family), GGAAGCATYTGCCTYGACAT (*UBC8-12* family). Seven additional gene-specific sense primers were designed to target ten candidate *UBC*s identified from GenBank entries. These primers were predicted to yield partial cDNAs containing the active site region through 3′-terminus when used in PCR-based cDNA library screening with an antisense vector primer (T7). These *UBC* primers were used at 0.25 μM and contained the following sequences: 1) CCATCTGGAMCTGTCTGT (N65743 and U44976), 2) GACATTCTGAAAGAGCAG (R30069), 3) GACAAGCTTGGAAGAATC (T22122 and T43710), 4) GACATCTTGAAGGAGCAG (N65609), 5) GGTGCTGTTTGCTTAGATG (R65499 and N38533), 6) CATTCTCAAAGACCAATGG (AA394327), 7) GGAAGCATTTGCCTTGAC (Z17692). The T7 primer was used at 0.25 μM and contained the sequence TAATACGACTCACTATAGGG.

DUB PCR primers: Seven gene-specific primers antisense were designed to target the putative Cys box of eleven candidate deubiquitinating enzymes identified from GenBank entries. These primers were used in combination with a sense vector primer (T3) for PCR-based cDNA library screening, and were predicted to yield partial cDNAs containing 5′-terminal through Cys box sequences. These DUB primers were used at 0.25 μM and contained the following sequence: 1) CCCAAGGTTAACTAAACC (Z17750 and AC004808), 2) CCCAAGATTTAGCAGAAA (AC002409), 3) CATGAAGCATGTATTTCC (AC006424), 4) CATGTAACACGTGTTGCC (Z25610), 5)

GGCGAAACAACTATTTCC (AL031004), 6) AAGGAAACAACTGTTGCC (AL021889), 7) CATAAAGCAGGTATTTCC (AF118222 A and B), 8) GTAACAGGTGTTKCCRAG (AB02220 and AB023045). The T3 primer was used at 0.25 μM and contained the sequence AATTAACCCTCACTAAAGGG. Gene-specific primer pairs were designed to target the 3'-terminal region of two of the candidate DUBs (AC002409 and AL021889); these DUB primers were used at 0.25 μM and contained the following sequences: CTCCGACTTGGGTGCTTCTAA and ACTAAGACCTCAGGAAGCCTC (AC002409), and CACCTGACTTCCAGCGTTATC and CCTCATATATGGCTGCAAGTC (AL021889).

Proteasome subunit PCR primers: Gene-specific primer pairs were designed to target two proteasome subunits and were used in PCR at 0.25 μM. *PAF1*, α subunit, primers had the following sequences: AGTCTCCTCTTCCTGTTG and GCCTTCTCCTTCCTCTTC. *PBF1*, β subunit, primers had the following sequences: GCTGGATCGGATTACTGT and TCCCTCTCTGTGGCAGAT.

Cloning and sequence analysis of partial cDNAs: Partial cDNAs obtained from the bark cDNA library using the degenerate primers targeting three *UBC* families were cloned into pCR2.1 according to manufacturer's instructions (Invitrogen; Carlsbad, CA). cDNAs obtained from xylem cDNA library screening with these same primers, all DUB partial cDNAs, and the proteasome α subunit PCR product from pAtPSM.R2B [gift from Brenda Winkel-Shirley; Shirley and Goodman 1993] were all cloned into pGEMT-Easy according to manufacturer's instructions (Promega; Madison, WI). Cloned cDNAs were sequenced at the Virginia Tech DNA Sequencing Facility.

Semi-quantitative RT-PCR of DUBs and proteasome subunits: RT reactions were prepared as described above using an oligo-dT primer at 5 μM and polyA RNA isolated from xylem and bark tissue dissections from 170 root-hypocotyl segments. RT reactions were serially diluted four times (1:10) in either TE (proteasome α and β subunits) or water (DUBs). For each gene tested, a series of PCRs was prepared using a 10 ul (DUBs) or 2 ul (proteasome subunits) aliquot of each RT dilution as template for PCR amplification as described above (under "cDNA Library Screening by PCR"), using the annealing temperatures indicated in the figure legends. PCR primers were used at 0.25 μM. A 10 μl aliquot of each PCR was loaded onto an agarose gel. The gene-specific primers for the *ACT7* control had the following sequences: GGCCGATGGTGAGGATATTC, CTGACTCATCGTACTCACTC.

Results

Ub-conjugating enzymes

PCR-based screening with degenerate primers directed to the *UBC* active site generated multiple PCR products for each of the three targeted *UBC* families from both the xylem and bark cDNA libraries (Fig. 1, A and B), suggesting that multiple members of each family are represented in both tissues. The products obtained were within the expected size range, as predicted from known *Arabidopsis UBC* gene sequences. The banding pattern for a given family was remarkably similar between xylem and bark, with minor differences mostly in the *UBC4-6* family. Members of the *UBC4-6* family have been shown to be expressed in *Arabidopsis* root, hypocotyl, and the vascular tissues of these organs (Thoma, *et al.* 1996).

Partial cDNAs obtained from both libraries were selected for cloning and sequencing as follows. To investigate whether members of the *UBC4-6* family

are differentially expressed between the two vascular tissues, PCR products from this family were cloned from bark and xylem. To determine if novel *UBC*s might be expressed in developing xylem, products from the other two targeted families (*UBC1-3* and *UBC8-12*) were cloned from xylem as well. Sequence analyses revealed that none of the cloned partial cDNAs are novel *UBC* enzymes; rather, they represent known *UBC* genes. Table I lists the *UBC* partial cDNAs obtained from xylem and bark, and illustrates that while a number of these clones were identical or nearly identical in length to the published *Arabidopsis UBC* sequences, others showed length heterogeneity in the 3'UTR.

Library screening using a battery of additional primers to target the active site of candidate *UBCs* identified from GenBank entries suggested that all ten targeted sequences were expressed in both secondary xylem and bark (Fig. 2). Because of our previous experience cloning partial cDNAs from the 3' ends of *UBC*s only to discover multiple 3'UTR length variants rather than novel genes or tissue-specific expression patterns, none of these PCR products was cloned.

Deubiquitinating enzymes

Initial PCR-based screening of the cDNA libraries with a battery of eight primers directed to the Cys box of putative DUBs suggested potential xylem-specific expression for two of eleven candidates (GenBank sequences AC002409 and AL021889) (indicated by arrows, Fig. 3). These two partial cDNAs were cloned from the xylem library, sequenced, and confirmed to be GenBank sequences AC002409 and AL021889.

For subsequent analyses, gene-specific primer pairs for each candidate DUB were designed to target the 3' end of each gene, in case the 5' ends of these cDNAs were not intact in the libraries. The expected size fragments for each candidate DUB were obtained from xylem library screening using these primer pairs (Fig. 4). The AC002409 product of RT-PCR analysis using whole root total

RNA was cloned and sequenced (data not shown). Northern analysis of AC002409 using this 372 bp fragment as a probe produced no signal from xylem or bark (data not shown).

Contrary to expectations following the library screening results, RT-PCR analysis using polyA RNA isolated from whole root, xylem and bark demonstrated that mRNAs of both candidate DUBs are expressed in all three samples (Fig. 5). Subsequent semi-quantitative RT-PCR analysis suggested that AC002409 mRNA is expressed at a slightly higher level in xylem than in bark (Fig. 6, A), while AL021889 appears to be expressed at equivalent levels in both tissues. The control for semi-quantitative RT-PCR, one of the *Arabidopsis* actin genes, *ACT7*, appeared to be expressed at equivalent levels in bark and xylem (Fig. 6, B).

Proteasome subunits

cDNA library screening revealed that *PAF1* and *PBF1*, α and β proteasome subunits, respectively, are expressed in both secondary xylem and bark of *Arabidopsis* (Fig. 7). Semi-quantitative RT-PCR suggested that *PAF1* is expressed at a slightly higher level in xylem than bark (Fig. 8, A and B). The *ACT7* control was not included in this experiment because we had observed equivalent expression in xylem and bark in three previous experiments (Fig. 6, B and data not shown). In contrast to the semi-quantitative results, preliminary indications from competitive RT-PCR analysis for *PAF1* are that this proteasome subunit is expressed at equivalent levels in xylem and bark (data not shown).

Discussion

Ub-conjugating enzymes

Although no novel *UBC* genes were identified in secondary xylem or bark by PCR-based screening of the cDNA libraries, our results indicate that the genes

UBC 1, *2*, *4*, *5*, *8*, and *10* are expressed in secondary xylem, and *UBC4* and *UBC5* are expressed in secondary bark in *Arabidopsis*. It should be considered, however, that although numerous PCR products were generated from all three targeted *UBC* families by bark library screening, only cDNAs from the *UBC4-6* family were cloned from bark. Thus, it is formally possible that novel members of the *UBC1-3* and *UBC8-12* families are expressed in bark, and also possible that these families are differentially regulated between xylem and bark. Moreover, considering the large number of PCR products generated for all three *UBC* families (Figure 1, A and B) compared to the number of clones analyzed from each family, an even greater diversity of family members than reported here may actually be expressed in both secondary xylem and bark.

While members of most *Arabidopsis UBC* families have been demonstrated to be expressed concurrently in most tissues examined (Sullivan, *et al*. 1994; Thoma, *et al*. 1996; van Nocker, *et al*. 1996), tissue-specific expression patterns have been reported for members of the *UBC4-6* family (Thoma, *et al*. 1996; Watts, *et al*. 1994). In our study, for the single *UBC* family for which cDNAs were cloned from both libraries (*UBC4-6*), tissue-specific expression was not observed; i.e., *UBC4* and *UBC5* are represented in both libraries. Additionally, more than a single *UBC* family member may be expressed in a given tissue, as also observed in *Arabidopsis* by others (Thoma, *et al*. 1996).

Though no differences were apparent between secondary xylem and bark in the identity of expressed *UBC* genes, many *UBC* partial clones contained 3'UTRs either longer or shorter than the *UBC*s sequences available in GenBank (Table I). Additionally, for a given gene, differences in 3'UTR length were observed both between and within tissues (Table I). Finally, although screening of these libraries in our lab has generated cDNAs for members of several other gene families (including cysteine proteases, an aspartic acid protease, serine

proteases, and two MADS box transcription factors), *UBC*s and MADS box proteins are the only two gene families for which members cloned from these libraries exhibit such a diversity in the length of the 3'UTR.

Differences in 3'UTR length reflect usage of alternative polyadenylation sites during transcription. Heterogeneity in 3'-end processing sites has commonly been observed in plant mRNAs (Rothnie 1996). Functional importance of 3'UTRs in mRNA stability (Chan and Yu 1998; Ohme-Takagi, *et al.* 1993; Rott, *et al.* 1998; Sullivan and Green 1996) and translational efficiency (Tanguay and Gallie 1996) have also been reported from several plant systems. Apparently, both the length of the 3'UTR and the nucleotide sequence may have significant consequences for mRNA accumulation (Rott, *et al.* 1998; Tanguay and Gallie 1996). Recently, Chen, *et al.* (1998) reported a 3'UTR determinant for nodule parenchyma-specific expression in *Sesbania*. Considering these findings for other plant genes, it is possible that the 3'UTR heterogeneity observed here plays a role in regulating expression of these *UBC* genes in *Arabidopsis*.

Deubiquitinating enzymes and proteasome subunits

PCR-based library screening and semi-quantitative RT-PCR suggested that one DUB (GenBank AC002409) and the α proteasome subunit, *PAF1*, may be expressed at higher levels in xylem than in bark, while another putative DUB (GenBank AL021889) and *PBF1* appear to be expressed at equivalent levels in the two tissues. However, semi-quantitative RT-PCR analysis is not a conclusive method for transcript quantitation, and either Northern analysis or competitive RT-PCR must be used to more definitively quantify transcript levels.

To this end, we have conducted competitive RT-PCR analysis for *PAF1*, and preliminary results indicate that this gene is expressed at similar levels in xylem and bark rather than more abundantly in xylem (data not shown). Although examples of differential regulation of proteasome subunits have been

reported from various systems (see Chapter 1 and Introduction to this chapter), that *PAF1* may not display such regulation perhaps is not surprising considering that Fu, *et al.* (1999) demonstrated that no *Arabidopsis* proteasome subunits were differentially regulated out of six genes examined, though *PAF1* was not tested by these authors.

In summary, all targeted *UBC* families and candidate *UBC*s, DUBs, and proteasome subunits are expressed in secondary xylem and bark in this system. While this study did not reveal tissue-specific expression of Ub pathway genes in developing secondary tissues of *Arabidopsis*, another report from our lab concerning the expression in these tissues of non-ATP-dependent proteases demonstrated vascular tissue-specific expression of several genes, thus illustrating the value of this secondary xylem-specific cDNA library.

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Table I. 3'UTR length heterogeneity observed in ubiquitin conjugating enzyme (*UBC)* partial clones obtained from xylem (X) and bark (B) cDNA libraries. Numbers are the length of the 3'UTR (in nucleotides) from the stop codon to the polyA recognition sequence. [a]Number in parentheses indicates the 3'UTR length from known *UBC* cDNA clones obtained from GenBank.

Clone	Library of origin	3'UTR length
UBC1	X	180 (178)[a]
UBC2	X	154 (678)
	X	229
	X	227
UBC4	X	150 (137)
	B	150
	B	135
UBC5	X	391 (270)
	X	283
	X	229
	B	283
UBC8	X	275 (172)
	X	153
UBC10	X	275 (243)
	X	239
	X	233
	X	187
	X	169

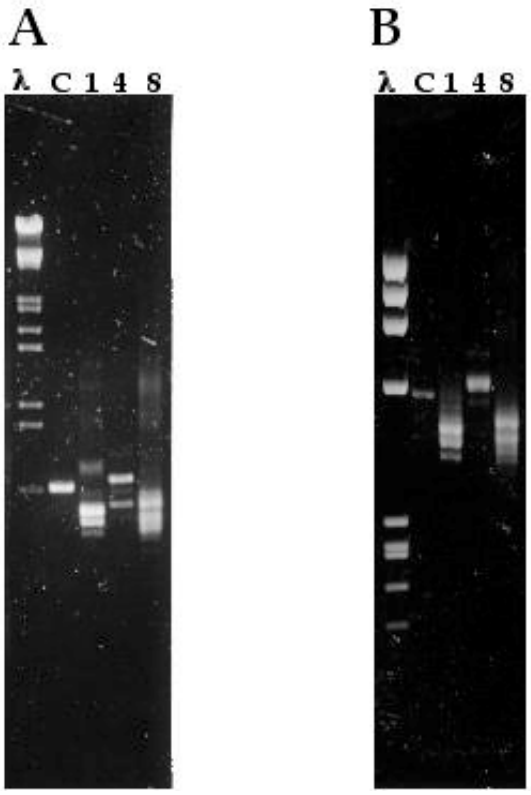

Figure 1 PCR-based screening of bark (A) and xylem (B) cDNA libraries for *UBCs*. Degenerate sense primers targeting the active site of known *Arabidopsis UBC* gene families were used in PCR with a T7 antisense vector primer at 55°C annealing temperature. Ethidium bromide-stained, agarose (A = 1.5%; B = 2%) gels are shown. Lane numbers indicate primer family targeted: 1= *UBC1-3*; 4 = *UBC4-6*; 8 = *UBC8-12;* C = PCR control, aspartic acid protease primers (see "Materials and Methods, cDNA library screening by PCR"); λ = λ HindIII/EcoRI.

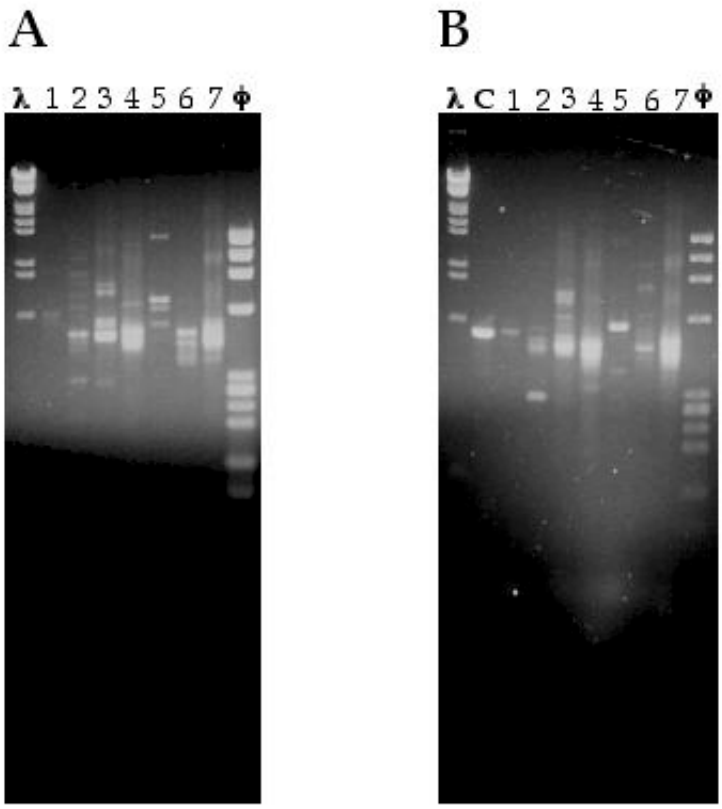

Figure 2. PCR-based screening of bark (A) and xylem (B) cDNA libraries for *UBC*s. Antisense primers targeting the predicted active site of candidate genes were used in PCR with a T3 sense primer at 50°C annealing temperature. Lane numbers indicate the primer used (see "Materials and Methods, UBC PCR primers" for GenBank accession targeted). Shown are ethidium bromide-stained, 2% agarose gels. C = PCR control, p48h-17 primers (see "Materials and Methods, cDNA library screening by PCR"); λ = λ HindIII/EcoRI; ϕ = ϕX174 HaeIII.

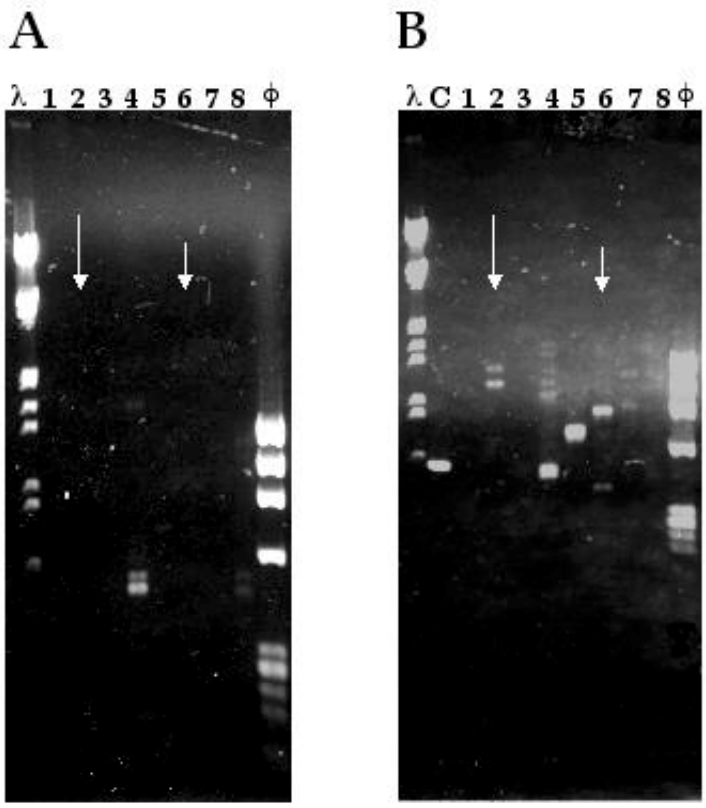

Figure 3. PCR-based screening of bark (A) and xylem (B) cDNA libraries for de-ubiquitinating enzymes. Antisense primers targeting the predicted Cys box of candidate genes were used in PCR with a sense T7 vector primer at 50°C annealing temperature. Lane numers indicate the primer used (see "Materials and Methods, DUB PCR primers" for GenBank accession targeted). Shown are ethidium bromide-stained, 1% agarose gels. C = PCR control, p48h-17 primers. λ = λ HindIII/EcoRI; ϕ = ϕX174 HaeIII.

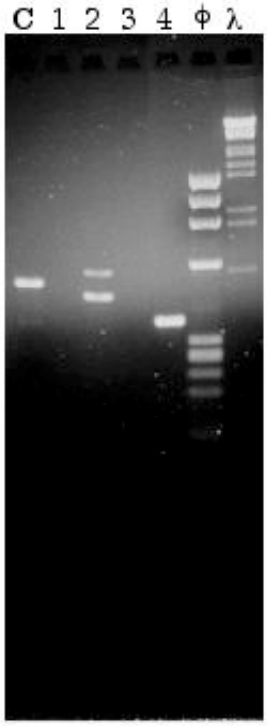

Figure 4. PCR-based screening of bark (Lanes 1 and 3) and xylem (Lanes 2 and 4) cDNA libraries with gene-specific primer pairs for candidate de-ubiquitinating enzymes, AL021889 (Lanes 1 and 2) and AC002409 (Lanes 3 and 4). Annealing temperature = 55°C. Shown is an ethidium bromide-stained, 2% agarose gel. C = PCR control, p48h-17 primers; ϕ = ϕX174 HaeIII; λ = λ HindIII/EcoRI.

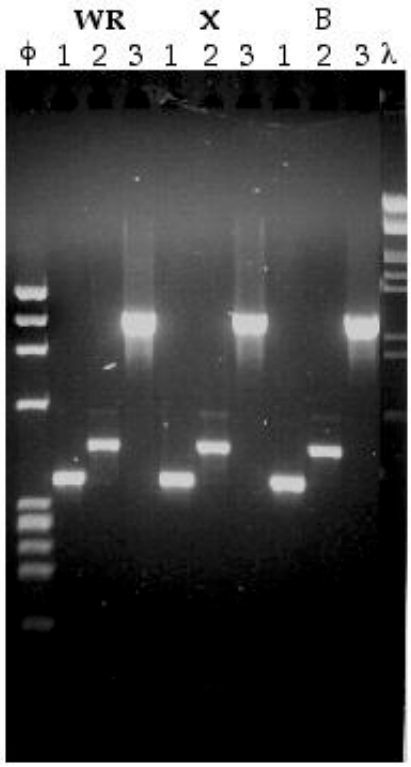

Figure 5. RT-PCR analysis of candidate de-ubiquitinating enzymes using polyA RNA isolated from whole root (WR), xylem (X), and bark (B). Gene-specific primer pairs were used in PCR at 55°C annealing temperature. Shown is an ethidium bromide-stained, 2% agarose gel. 1 = AC002409; 2 = AL021889; 3 = *ACT7*, RT-PCR control; ϕ = ϕX174 HaeIII; λ = λ HindIII/EcoRI.

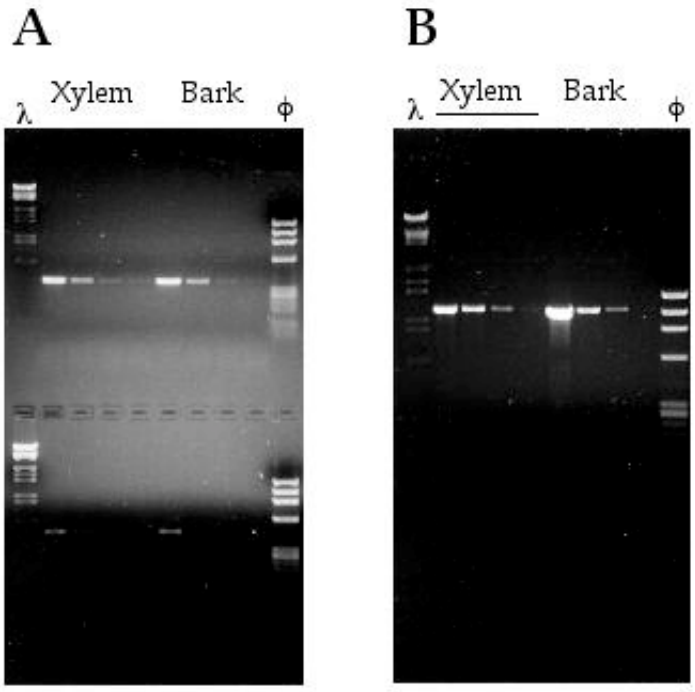

Figure 6. Semi-quantitative RT-PCR analysis of the expression of two candidate de-ubiquitinating enzymes, AC002409 and AL021889 (A) and *ACT7* (B) in xylem and bark. In (A), top row = AC002409, bottom row = AL021889. An oligo-dT-primed RT reaction was prepared from xylem and bark polyA RNA. The RT reaction was serially diluted (1:10) four times, and a 10 µl aliquot of each dilution was used as template for PCR using gene-specific primer pairs at annealing temperature 55°C. A 10 ul aliquot of each PCR was resolved on the ethidium bromide-stained agarose gels shown. Each group of lanes labeled Xylem or Bark shows the PCR product obtained with increasing dilution of the RT reaction. A = 1.5% agarose; B = 2% agarose. λ = λ HindIII/EcoRI; ϕ = ϕX174 HaeIII.

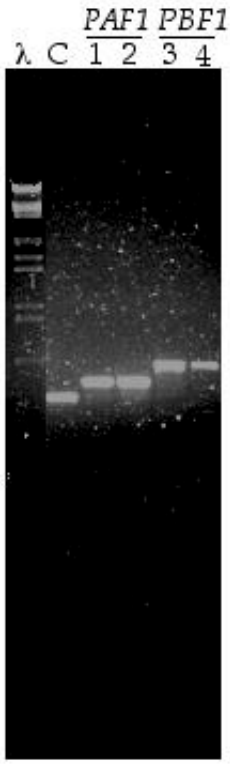

Figure 7. PCR-based screening of xylem (lanes 1 and 3) and bark (lanes 2 and 4) cDNA libraries for proteasome subunits, *PAF1* and *PBF1*. Gene-specific primer pairs were used in PCR at 50°C annealing temperature. Shown is an ethidium bromide-stained, 1.5% agarose gel. C = PCR control, gene-specific primer pair for DUB AC002409. λ = λ HindIII/EcoRI.

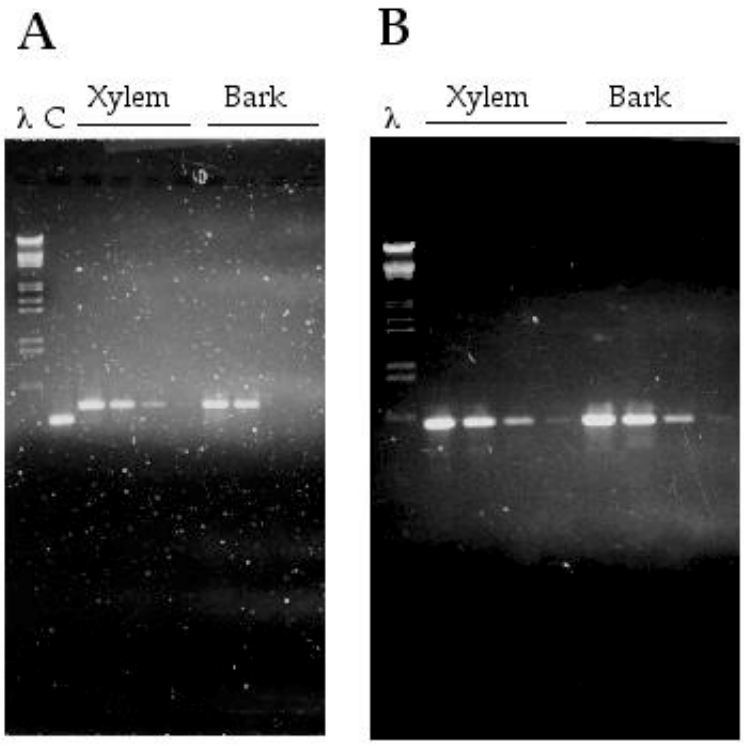

Figure 8. Semi-quantitative RT-PCR analysis of the expression of *PAF1* (A) and *PBF1* (B) proteasome subunits in xylem and bark. Xylem and bark RT reactions were prepared, serially diluted, and used for PCR template as described for Figure 6, except that a 2 µl aliquot of each RT dilution was used as PCR template and the annealing temperature was 50°C. A 10 ul aliquot of each PCR was resolved on the ethidium bromide-stained, 1.5% agarose gels shown. Each group of lanes labeled Xylem or Bark shows the PCR product obtained with increasing dilution of the RT reaction. C = PCR control, gene-specific primer pair for DUB AC002409. λ = λ HindIII/EcoRI.

CHAPTER 5

Plant Growth Regulators Promote Expansion of Cultured *Zinnia* Primary Mesophyll Cells

Abstract

Conditions for culturing *Zinnia* mesophyll cell suspensions have been modified to inhibit TE differentiation and cell division, while promoting cell expansion stimulated by the phytohormones auxin (α-naphthaleneacetic acid, NAA), cytokinin (N-6-benzyladenine, BA), gibberellic acid (GA_3) and 2,4-epibrassinolide (BI). The degree of cell expansion was determined by measurement of cell length and width using an ocular micrometer under the light microscope. Growth regulator treatments differed in their relative effects on cell elongation and radial cell expansion. Auxin and epibrassinolide stimulated only cell elongation, while cytokinin had a greater effect on radial cell expansion, and gibberellic acid had equivalent effects on expansion in both directions. These results demonstrate that cell expansion can be induced by plant growth regulators in *Zinnia* primary mesophyll cell cultures and suggest that *Zinnia* cell suspensions may serve as a new model for investigating how phytohormones promote growth in higher plants.

Introduction

Plant development and form depend not only on cell differentiation but also on cell expansion, the direction and extent of which determine cell shape and the resultant shape of plant organs and the adult plant body. Cell growth is controlled by the nature of the primary cell wall, principally by the direction in which cellulose microfibrils are deposited and the amount of microfibril cross-linking contributed by extracellular matrix polysaccharides and proteins (Carpita

and Gibeaut 1993; Cosgrove 1997; Taiz 1984). Phytohormones, light, and extracellular pH are known to affect cell expansion, demonstrating that both the environment and endogenous factors regulate growth. However, a mechanistic understanding of how these elements control growth by affecting the orientation of cellulose microfibrils and the chemical properties of the cell wall matrix polymers has yet to be achieved.

A number of methods and model systems have been employed to examine cell expansion *in vivo*. For example, auxin and acid have been shown to stimulate cell growth using coleoptiles, leaf disks, and hypocotyls bathed in various solutions. However, the cuticle of these organs may preclude effective infiltration by the test solution and thus confound interpretation of experimental results (Luthen, *et al.* 1990). A second limitation of cell growth studies that utilize whole organs or portions thereof is that plant organs contain multiple tissues, and the contribution of each to the control of organ expansion may not be obvious (Cleland 1991; Kutschera 1992; Rayle and Cleland 1992).

To circumvent the limitations organ segments impose on studies of cell growth, plant suspension cultures have been developed for examining cell expansion. Cultured cells derived from a variety of higher plants can elongate substantially (Hasezawa and Syono 1983; Henshaw, *et al.* 1966; Kant and Hildebrandt 1969; Nishi, *et al.* 1977; Trolinder, *et al.* 1987). This cell elongation frequently follows termination of cell division (Lloyd and Barlow 1982) resulting from nutrient limitation (Henshaw, *et al.* 1966), inhibition of DNA synthesis (Lloyd, *et al.* 1980), or auxin depletion (Nishi, *et al.* 1977). Plant hormones also promote cell growth in cell suspension cultures of higher plants. Cultured rose and spinach cells expanded in response to gibberellin (Fry and Street 1980) and protoplasts derived from tobacco BY2 cells were stimulated to expand in response to cytokinin (Hasezawa and Syono 1983).

Serially-transferred suspension cultures are commonly auxin-dependent, however, such that auxin deprivation promotes cell expansion. Tobacco BY-2 protoplasts expanded upon reduction of the auxin concentration in culture (Hasezawa and Syono 1983), for example, and growth of cultured carrot cells was stimulated following transfer to auxin-free medium (Lloyd, *et al*. 1980). Thus, the auxin-dependency of these serially-cultured cell suspension models obviously precludes their use in studies that aim to examine auxin-stimulated cell expansion (Fry 1988).

Additionally, since cultured cells tend to clump, researchers have increasingly experimented with protoplasts in order to obtain single cells for studies of cell growth control (Hasezawa and Syono 1983). However, as previously mentioned, the cell wall largely controls the direction and the magnitude of cell expansion. Therefore, the removal of the cell wall during protoplast production is likely to alter growth control in the resulting cell lines. Furthermore, even the walls of cells in serially-transferred suspension cultures not derived from protoplasts are likely to be substantially altered as a result of long-term cell culture. Thus, multiple limitations of the cell suspension cultures currently available for the study of cell growth control underscore the need for the development of additional models for the investigation of the mechanisms that regulate cell expansion.

Zinnia elegans mesophyll cell suspension cultures have served as a model for TE differentiation for twenty years (Fukuda and Komamine 1980). A number of characteristics make these primary cell suspension cultures amenable to studies of cell expansion as well. The *Zinnia* leaf mesophyll is extremely friable, allowing facile production of cell suspensions by gentle maceration of leaves with a mortar and pestle and without the use of hydrolytic enzymes. Therefore, the cell wall composition and organization in cells of *Zinnia* suspension cultures

are initially identical to those of mesophyll cells in the plant. Furthermore, Knight and Roberts (1994) have characterized the time course of mesophyll cell expansion in developing leaves of *Zinnia*, thus allowing culture of cells at different stages of expansion as well as comparison of the responses of unexpanded and fully expanded cells to conditions that modulate growth. Additionally, responses of *Zinnia* primary mesophyll cells in suspension culture to factors that regulate growth can be compared with the responses of mesophyll cells *in situ*. This comparison should provide an assessment of the suitability of the *Zinnia* cell suspension cultures for modeling the expansion of mesophyll cells within leaves.

The data presented in this chapter are part of a body of work which has been submitted for publication (Lee, *et al*. 2000), conducted in collaboration with the lab of Alison Roberts (University of Rhode Island; Kingston, RI). Certain differences exist between the details of the experimental methods and results obtained for some of the work reported here and that contained in the manuscript, for reasons to be discussed. The data presented herein demonstrate that auxin, cytokinin, brassinosteroid, and gibberellic acid stimulate growth of cultured *Zinnia* mesophyll cells. We suggest that *Zinnia* mesophyll cell suspension cultures may serve as a new model for the investigation of mechanisms by which various plant growth regulators promote cell expansion.

Materials and Methods

Plant Materials and Growth Conditions: Seedlings of *Zinnia elegans* cv. Envy (Grimes Seeds, Concord, OH) were grown under conditions described previously in this dissertation (Chapter 2).

Preparation of primary mesophyll cell suspension cultures: Cell suspensions were prepared and cultured as described previously in this dissertation (Chapter 1 and 2), except that the medium contained various concentrations of N-6-benzyladenine (BA), α-naphthaleneacetic acid (NAA), gibberellic acid (GA_3) or 2,4-epibrassinolide (BI) as described in the figure legends. Stock solutions of BA and NAA were aqueous, BI was dissolved in DMSO, and GA_3 and Ethephon were dissolved in ethanol. Control cultures contained appropriate solvents. Cultures were incubated on rotary shakers at 100 rpm in a 27 °C growth chamber in the dark.

Measurement of cell expansion: Cell length and width of cultured cells were measured at time points indicated in the figure legends using an ocular micrometer in a light microscope (model MC 63, Zeiss). Stimulated increase in cell length and width was calculated by subtracting the final value for the control from the final value for the treatment. Percent increase was calculated by dividing the final cell length or width of the treatments by the final cell length or width of the solvent control.

Results

At the time the initial experiments reported in this dissertation for the study of cell growth were conducted, our cell culture conditions were not identical to the conditions later used for some experiments included in Lee, *et al.* (2000). The major difference is that the culture medium used in our lab included mannitol. Mannitol was absent from the culture medium for experiments conducted by our collaborators because the presence of mannitol decreases overall expansion of cultured *Zinnia* cells (Lee, *et al.*, 2000). However, although overall cell expansion was reduced by mannitol in experiments conducted in our laboratory, the overall effects of the phytohormones and the major conclusions were unchanged. A second difference between the preliminary experiments conducted in our lab and those of our colleagues is due to the fact that dose-response curves for NAA- and BA-stimulated cell growth generated by our collaborators yielded effective doses that were different (1 μM BA and 0.038-0.05 μM NAA) than the concentrations we had used (4.5 nM BA and 0.537 μM NAA). This report will show the data collected in our laboratory for the preliminary study of NAA and BA, as well as our data for BI and GA treatment effects that were included in the manuscript. Additionally, I will reference the results for NAA and BA experiments presented in the manuscript where there are differences from the data presented herein.

Figure 1 shows the stimulated increase in cell size by NAA and BA treatments alone as well as in combination, comparing the final size of cells in growth regulator treated cultures to the final size of cells from cultures containing no growth regulators. NAA alone (537 nM) stimulated both cell elongation and growth in cell girth, while BA alone (4.5 nM) had no effect on growth in either dimension. However, when NAA and BA are applied together, the growth-stimulating effects on both elongation and radial cell expansion are

greater than the effects of NAA alone. This suggests that BA potentially does stimulate growth but that this enhancement was not detected in the two experiments testing BA alone reported here.

Follow-up experiments with NAA and BA were conducted by our collaborators using the aforementioned effective doses determined in that lab rather than the levels previously tested in our lab; the results of these experiments are presented in detail elsewhere (Lee, *et al*. 2000). In summary, 0.038 μM NAA stimulated cell elongation in two of two independent experiments but, unlike the effect observed in our early study (Fig. 1), NAA did not result in growth in cell girth in either of two experiments (Lee, *et al*. 2000). In contrast to the lack of growth stimulation by 4.5 nM BA in our experiments (Fig. 1), 1 μM BA did promote growth, stimulating cell elongation in one of two experiments and radial expansion in two of two experiments (Lee, *et al*. 2000). This induction of cell growth by BA was likely due to the absence of mannitol in the culture medium and/or the higher concentration of BA used in their experiments.

We also examined the potential of the growth regulators Ethephon, BI, and GA to promote expansion of cultured *Zinnia* cells. No stimulation of growth by Ethephon over a wide range of concentrations (≤ 0.5 μM – 100 μM) was observed in three independent experiments (data not shown). Dose-response curves depict the ability of both GA (Fig. 2) and BI (Fig. 3) to stimulate cell expansion over a range of concentrations. The effective doses for promotion of cell elongation were determined to be 0.5 μM GA (Fig. 2) and 0.1 μM BI (Fig. 3). At these levels, GA also appears to stimulate radial cell expansion (Fig. 2) while BI does not (Fig. 3). Our findings were corroborated by our colleagues (Lee, *et al*. 2000).

Figure 1 shows the stimulated increase in cell size by BI and GA treatment, comparing the final size of growth regulator-treated cells to the final size of solvent-treated control cells. These data confirm that BI stimulates only cell elongation, while GA promotes both cell elongation and growth in cell girth in this system.

Discussion

Cell suspension cultures provide numerous experimental advantages over tissue segments for studies of cell expansion, including the absence of a cuticle, cell type homogeneity, opportunity for facile radioactive labeling and assay of biochemical activities, and the ability to quantitate growth in individual cells. Serially-transferred suspension cultures, however, often respond abnormally to plant growth regulators (Fry 1988). Protoplasts derived from tobacco leaves expand in response to auxin (Tao and Verbelen. 1996), but protoplast formation involves destruction of the native cell wall which may alter cellular responses to growth regulators.

We demonstrate here that *Zinnia* mesophyll cells isolated without the use of wall-hydrolyzing enzymes expand in response to auxin, cytokinin, gibberellin and brassinosteroids. Furthermore, the observed effective doses (0.038-0.05 μM NAA, 1 μM BA, 0.5 μM GA, and 0.1 μM BI) are within the ranges expected from studies conducted with leaf and other organ segments, assuming that suspension cultures have greater sensitivity due to more direct application. For example, GA_3 and BA both promoted leaf cell expansion at 10 μM (Brock and Cleland 1989). Brassinosteroids at nm-μm levels have been shown to stimulate expansion of stem segments, peduncles, and coleoptiles (see Clouse and Sasse 1998 for references). Based on these observations, we suggest that primary *Zinnia* mesophyll suspension cultures are unique in providing the advantages of a homogeneous cell suspension, along with retention of the native cell wall and growth regulator responses that are likely more normal than those of protoplasts or organ segments.

Previous observations in the *Zinnia* mesophyll system that TE differentiation is inhibited in cultures undergoing rapid cell expansion (Roberts, *et al.* 1997; Roberts and Haigler 1994) spawned our goal of developing *Zinnia*

suspension cultures for studies of cell growth. The osmotic potential of xylogenic culture medium is reduced by adding mannitol to inhibit cell expansion and promote differentiation (Fukuda and Komamine 1980; Roberts, *et al*. 1992). Our collaborators in the present study completely inhibited TE differentiation by eliminating mannitol from the culture medium, even in the presence of inductive levels of BA and NAA (Lee, *et al*. 2000). The level of NAA required to promote maximal cell expansion (0.05 μM) is 10-fold lower than the inductive concentration for TE differentiation (0.5 μM). This is interesting considering the suggestion by Aloni and Zimmermann (1983) that high auxin levels promote rapid differentiation, which results in cessation of cell expansion and small TEs.

The four plant growth regulators tested differed in their relative effects on cell elongation and radial cell expansion. The conclusions from our collaborative study are that BA has a greater effect on radial cell expansion, NAA and BI promote only cell elongation, and GA promotes expansion nearly equally in both dimensions (Lee, *et al*. 2000). In contrast to our expectations following our preliminary study (Fig. 1), the effects of BA and NAA are apparently additive (Lee, et *al*. 2000).

The four growth regulators tested also differed in their effectiveness relative to one another. The proportional size of treated cells to control cells (Fig. 1) is the most useful parameter by which to compare the growth-modulating effects of one growth regulator to another because the absolute amount of growth observed varied from experiment to experiment. These proportions suggest that NAA alone (at 0.537 μM) does not promote cell elongation as effectively as BI or GA, whereas BI and GA stimulate cell elongation equally well.

Knight and Roberts (1994) suggested previously that *Zinnia* mesophyll suspension cultures could serve as a model for investigating the regulation of mesophyll cell expansion within the leaf. These authors proposed using cultures from either unexpanded or fully expanded leaves to investigate the factors that halt cell expansion as leaves mature. However, results reported in Lee *et al*. (2000) and elsewhere (Frost and Roberts 1996; Uhnak and Roberts 1995) indicate that these regulatory mechanisms are substantially altered when cells are isolated and cultured. For example, *Zinnia* mesophyll cells expand more rapidly in culture (more than 300 $\mu m^2\ d^{-1}$) than in the leaf (160 $\mu m^2\ d^{-1}$, Knight and Roberts 1994). Whereas mesophyll cells within leaves stop expanding at a mean projected cell area of 2000 μm^2, they continue to expand in culture reaching a mean projected cell area of over 4000 μm^2 after 10 d. It has been demonstrated that microtubules (Uhnak and Roberts 1995) and microfilaments (Frost and Roberts 1996) are rapidly and drastically altered upon cell isolation. Given the important role of the cytoskeleton in directing cell wall synthesis, this disruption probably alters the mechanical properties of the cell wall. The loss of positional information and changes in the physical environment may also affect cell expansion in culture. The apparent uncoupling of the expansion response of *Zinnia* suspension cultures from factors that restrict cell expansion *in planta* limits the use of these cultures for investigating factors involved in cessation of cell expansion.

Finally, our results suggest that light and chemical plant growth regulators modulate cell expansion by different mechanisms (Lee, *et al*. 2000). Additionally, pH (Roberts and Haigler 1994) and substances secreted by expanding cells (Roberts, *et al*. 1997) also modulate cell expansion, possibly by yet other mechanisms. Although we do not yet understand how these mechanisms of action differ, *Zinnia* primary mesophyll cell suspension cultures

provide an opportunity to investigate these differences. In summary, primary *Zinnia* mesophyll cultures retain their ability to expand in response to growth regulators in a way that is unique among known cell suspension models for expansion. We suggest that primary *Zinnia* mesophyll cultures can provide a system for characterizing changes in biochemistry, signal transduction and gene expression associated with response to autonomous and nonautonomous growth-promoting factors.

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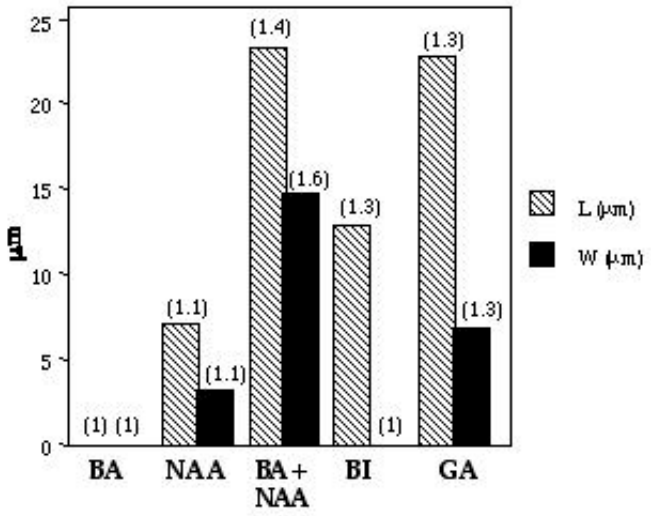

Figure 1. Stimulated increase in cell size promoted by various growth regulators over 4 days of culture. Data are the mean final size [length (L) and width (W)] of treated cells minus the mean final size (L and W) of cells in the solvent control. Numbers in parentheses are the proportion of the final size of treated cells to the final size of control cells. Means were calculated from six replicates from three independent experiments (BA, BI, and GA), 15 replicates from four independent experiments (NAA), or 16 replicates from four independent experiments (NAA + BA). The sample size of replicates was minimally 40 cells for NAA and BA experiments, except one experiment with a sample size of 25 cells. The sample size of replicates for GA and BI experiments was 25 cells. Concentrations of growth regulators used were: 4.5 nM BA, 537 nM NAA, 100 nM BI, 500 nM GA.

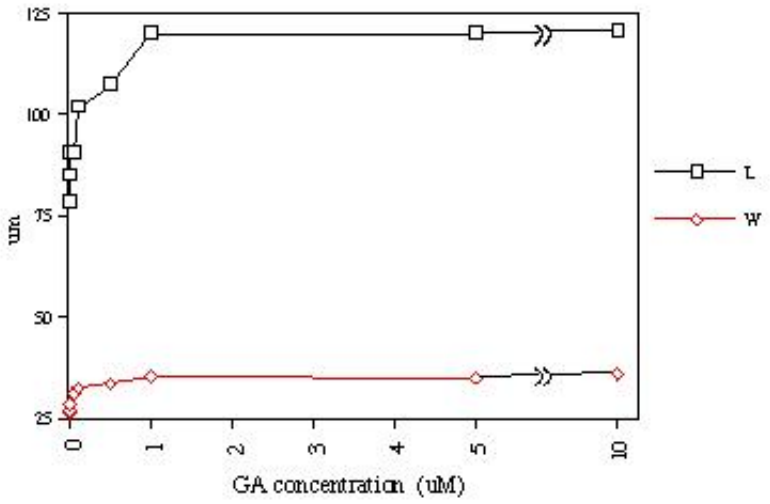

Figure 2. Effect of GA concentration on *Zinnia* cell elongation and radial cell expansion. Shown are the length (L) and width (W) of cells cultured for four days at the indicated concentrations of GA. Values are means of a minimum of three replicates (sample size 25 cells) from one experiment, except for the 0.1 and 0.5 μM doses, which were tested by six replicates from two experiments.

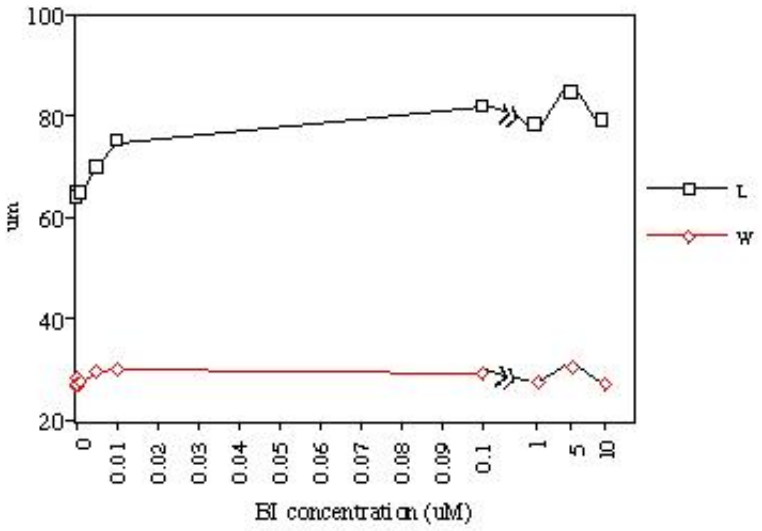

Figure 3. Effect of BI concentration on *Zinnia* cell elongation and radial cell expansion. Shown are the length (L) and width (W) of cells cultured for five days at the indicated concentrations of BI. Values are means of three replicates (sample size 25 cells) in one experiment, except for the 10 nM dose which was tested by six replicates from two independent experiments.

CONCLUSIONS

This dissertation has examined the role of the Ub-proteasome pathway during formation of TEs in *Zinnia* suspension cultures as well as during secondary xylem development in *Arabidopsis*. Although biochemical characterization of the pathway (Chapter 2) revealed no obvious differences in overall activity between control and differentiating *Zinnia* cultures, we determined that the pathway is very active in developing TEs. Such high overall levels of activity might not have been expected since the biochemistry of the Ub system requires great expenditure of cellular energy at a time when the cell is undergoing genetically programmed death. Therefore, such high activity levels corroborate our expectation that the Ub pathway plays a regulatory role during TE terminal differentiation, a cellular suicide process.

Importantly, our study (Woffenden, *et al*. 1998; Chapter 3) establishes a requirement for proteasome activity early during TE cell fate determination and further suggests that activity of this protease is essential for the timely progression of differentiation. Proteasome activity , however, is not required for completion of autolysis, a process that requires cysteine protease activity (Woffenden, *et al*. 1998). That TE autolysis can be uncoupled from other differentiation events is significant because, to our knowledge, this is first documentation of such uncoupling during an autolytic cell suicide program in plants.

The full complement of signals required for TE differentiation including PCD has not yet been described, but does include auxin signaling, which is known to depend on Ub-like pathway components. Thus, our results suggest that proteasome inhibition in *Zinnia* TE cultures may result in a block of an auxin signal required for TE differentiation. The hypothesis that proteasome activity is

required for certain auxin signals could be tested in a model system amenable to transformation (transient expression in cell cultures and/or protoplasts, for example) by expressing a reporter gene under an auxin-inducible promoter. If proteasome activity participates in the specific auxin signaling pathway that activates the given promoter, application of proteasome inhibitors with exogenous auxin might result in reduced induction of reporter gene transcription compared to induction in the absence of proteasome inhibitor. The promoter of an AUX/IAA transcription factor gene might prove useful for driving expression of these reporter gene constructs, since certain AUX/IAA family member genes have been demonstrated to be transcriptionally upregulated in response to auxin. Such promoter-reporter fusion experiments may not yield useful information about the auxin-dependency of TE development if cell-type-specific auxin signaling exists. Micro-array analysis of differentiating *Zinnia* TEs is currently being pursued in other laboratories. These micro-array studies will be instrumental in detailing gene expression patterns in developing TEs, and may ultimately reveal that the Ub system is involved in transducing the signals of other plant growth regulators in addition to auxin.

This dissertation also reports that *Arabidopsis* plant growth at low density combined with inflorescence removal remarkably augments the potential of the plants for secondary growth (Chapter 4). Secondary tissue proliferation was adequate for the isolation of sufficient polyA RNA for xylem and bark cDNA library construction. PCR-based screening of these cDNA libraries and subsequent RT-PCR experiments did not reveal differential expression of any of the ubiquitin pathway components surveyed, including multiple *UBC*s and UBPs, and two proteasome subunits. These results do not, however, preclude the possibility that differential regulation of Ub pathway components may occur during vascular development. Considering that particular *UBC*s and UBPs have

demonstrated roles in differentiation processes in animal and fungal systems, perhaps future research in this field will discover a *UBC* or UBP that regulates xylem formation. Additionally, the methodology developed herein for enhancement of *Arabidopsis* secondary growth and the existence of these bark and secondary xylem cDNA libraries can aid other researchers studying vascular development.

The identification of the *in vivo* targets of the Ub-proteasome system during TE development is necessary to make real progress in understanding the role(s) of the pathway in the differentiation of TEs. Compared to other models of xylem differentiation, the *Arabidopsis* system described herein has a greater potential to facilitate the discovery of mutants defective in secondary xylem differentiation. For example, T-DNA insertional mutagenesis could be used to identify plants harboring mutations in *UBC*s or UBPs. When grown under our modified plant growth conditions, these mutants could be screened for aberrant secondary xylem formation. Such mutants may accumulate a stabilized, ubiquitinated substrate in xylem tissue, e.g., if the mutation inactivated a UBP that normally functioned to potentiate degradation of the target. This target might be an important *in vivo* substrate of the Ub pathway whose degradation is essential for xylem differentiation.

This dissertation also further expands the utility of the *Zinnia* system from studies of TE development into investigations of cell growth (Lee, *et al.* 2000; Chapter 5). We report that four plant growth regulators differ in their relative abilities to promote cell girth expansion versus cell elongation. To our knowledge, this is the first such distinction of differential induction of radial cell growth and cell elongation by various plant growth regulators. Micro-array analysis of gene expression in this modified *Zinnia* system in response to various

growth regulators would be informative concerning events leading to cell expansion.

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Born September 23, 1968

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EDUCATION:

Virginia Polytechnic Institute and State University, Blacksburg, VA

Ph.D., September 1999, Horticulture; Molecular Cell Biology and Biotechnology Program; Advisor: Dr. Eric P. Beers

Duke University, Durham, NC

M.A., 1993, Microbiology and Immunology; Cell and Molecular Biology Program; Advisor: Dr. Michael C. Ostrowski

Characterization of the ras-responsiveness of the human c-myb promoter.

Roanoke College, Salem, VA

B.S., 1990, Biology, Magna cum laude, Honors Program Graduate

RESEARCH/TEACHING EXPERIENCE:

Virginia Polytechnic Institute and State University, Department of Horticulture, Blacksburg, VA (August 1995--present).

Investigated the role of the ubiquitin-proteasome proteolytic pathway in regulating tracheary element differentiation.

Roanoke College, Department of Biology, Salem, VA

- **Visiting Assistant Professor of Biology**. (August 1994--June 1995)

Courses taught: Life Science I Laboratory--Introductory freshman biology laboratory.

Microbiology and accompanying laboratory--junior/senior level course.

Cell Physiology and accompanying laboratory--junior/senior level

course.

- **Adjunct Professor of Biology**. (September 1993--May 1994)

  Courses taught: Life Science I Laboratory; Life Science II and accompanying laboratory-- freshman Five Kingdoms survey course; Microbiology Laboratory.

Duke University, Department of Microbiology and Immunology, Durham, NC (August 1990--July 1993).

Investigated the ability of the *ras* oncogene to stimulate the human *c-myb* promoter by transient transfection analysis in cultured mammalian fibroblasts.

Roanoke College, Department of Biology, Salem, VA

- **Departmental Laboratory Assistant**. (September 1986--April 1990)

  Prepared and organized lab materials weekly for undergraduate microbiology, cell physiology, and introductory biology courses, including bacterial culture media, solutions, and lab equipment.

•**Editorial Assistant**. (January 1987--October 1989)

Assistant to Dr. R. E. Jenkins in his writing of *The Freshwater Fishes of Virginia* book (1994). Performed specimen dissections, and word processing and proofing of text. Work commissioned by the VA Department of Game and Inland Fisheries.

TECHNICAL SKILLS:

Plasmid DNA construction via standard cloning strategies. Bacterial transformation; plasmid DNA isolation and analysis by restriction enzyme digestion.

Recombinant protein expression in bacterial system.

Protein purification by column chromatography.

Immunoblot analysis. SDS-polyacrylamide gel electrophoresis.

Genomic DNA and RNA isolation from plant cells/tissues. Quantitation of nucleic acids and protein by spectrophotometry. Quantitation of DNA by fluorometry.

RT-PCR. Northern analysis. Southern analysis. Agarose gel electrophoresis.

cDNA library screening by PCR. DNA sequencing.

Phage display library screening; phage growth and purification.

Plant cell suspension culture; *Arabidopsis* and *Zinnia* plant propagation and care.

Photomicroscopy of live and fixed/stained plant cells.

Extraction of chlorophyll from plants; quantitation by spectrophotometry.

Use of oxygen electrode to measure rates of plant photosynthesis and respiration.

Mammalian cell culture; transient transfection; analysis of reporter gene activity.

Metabolic testing of bacteria, species/strain identification. Aseptic technique, bacterial growth on solid and liquid media; culture maintenance. Bacterial staining.

Computer/Information Management skills: Microsoft Word, Powerpoint, Microsoft Excel, Wordperfect, JMP-In statistics software; use of genomics databases for DNA and protein sequence analyses.

AWARDS OF SUPPORT:

Graduate Research Assistantship, Virginia Polytechnic Institute and State University (August 1995--May 1999)

Travel Award from the American Society of Plant Physiologists (1998)

NIH National Research Service Award, Duke University (August 1991--July 1993)

Duke University Graduate Fellowship (August 1990--July 1991)

Fintel Senior Scholarship, Roanoke College (August 1989--April 1990)

Davis Honors Scholarship, Roanoke College (August 1988--May 1990)

Kinsey Honors Scholarship, Roanoke College (August 1987--May 1988)

Bittle Scholarship, Roanoke College (August 1986--April 1990)

HONORS:

Graduate student representative for Virginia Tech Horticulture Department Graduate Committee; participated in review of applicants to graduate program, choice of departmental funding recipients, and design of departmental Web page (August 1997-July 1999)

Roanoke College Honors Program (August 1987--April 1990)

Gary Wesley Leonard Memorial Award in Biology, Roanoke College (1990)

ACADEMIC ASSOCIATIONS:

American Society of Plant Physiologists (1995--present)

Southern Section of the American Society of Plant Physiologists (1998--present)

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ABSTRACTS FROM POSTERS AND TALKS AT NATIONAL MEETINGS:

Woffenden B.J., C. Zhao, and E.P. Beers (1999) Using *Arabidopsis* to evaluate gene expression in differentiating xylem: ubiquitin pathway enzymes as a case study. Annual Meeting of the American Society of Plant Physiologists.

Woffenden B.J., and E.P. Beers. (1998) Proteasome inhibitors prevent tracheary element differentiation in *Zinnia* mesophyll cell cultures. Annual Meeting of the American Society of Plant Physiologists.

Error! No table of contents entries found. Woffenden B.J., Freeman T.B., and E.P. Beers. (1997) Multiple, distinct

Requirements for proteasome activity during the differentiation and programmed cell death of tracheary elements. FASEB Summer Research Conference: Ubiquitin and Protein Degradation.

Woffenden B.J., and M.C. Ostrowski. (1993) The *ras* oncogene signals through the *c-myb* transcription factor. 9th Annual Meeting on Oncogenes.

PUBLICATIONS:

Lee S., Woffenden B.J., Beers E.P., and A.W. Roberts (expected 2000) Expansion of cultured *Zinnia* mesophyll cells in response to hormones and light. Physiol. Plant.

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