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UNE ANALYSE HISTOPATHOLOGIQUE ET GÉNOMIQUE D'UNE INTERACTION *IN VITRO* ENTRE *ULMUS AMERICANA* ET *OPHIOSTOMA NOVO-ULMI*

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Résumé

Les interactions entre les plantes et les agents pathogènes fongiques conduisent habituellement à la mise en place par l'hôte de différents mécanismes de défense. Des exemples de ces mécanismes sont le renforcement de la paroi cellulaire par la subérine, la lignine et les composés phénoliques pariétaux, ainsi que l'accumulation de protéines PR et la production de phytoalexines qui peuvent être toxiques pour l'agent pathogène. Chez les plantes susceptibles, ces mécanismes ne réussissent pas à empêcher le développement de la maladie. La maladie hollandaise de l'orme est une maladie à caractère épidémique qui a causé la mort de millions d'ormes en Europe et en Amérique du Nord. Les bases moléculaires de cette maladie sont encore peu connues. Afin d'identifier des gènes impliqués dans l'interaction entre l'espèce susceptible *Ulmus americana* L. et son champignon pathogène *Ophiostoma novo-ulmi* Brasier, un système in vitro a été mis au point, celui-ci utilisant des cultures de cals auxquelles des cellules levuriformes du champignon ont été inoculées. Afin de valider son utilisation pour l'analyse génomique, ce système a d'abord fait l'objet d'une analyse histopathologique en microscopie photonique et électronique. Le développement du champignon dans les cals et les réactions de défenses de ces derniers face à la présence du champignon ont été observés à 4, 24 48, 72 et 96 heures post-inoculation (hpi). Le champignon a été détecté sous forme de réseau d'hyphes dans toutes les parties du cal à partir de 48 hpi. Les tests histochimiques ont montré l'importance de certaines réactions telles que l'accumulation de phénols, de lignine et de subérine dans les cellules des cals. Le pourcentage de cellules subérisées était 4,6 fois plus élevé à 96 hpi dans les cals infectés que dans les cals témoins. Une banque d'ADN complémentaire a été construite à partir de cals infectés (72 hpi) en utilisant la technique des hybridations suppressives et soustractives (SSH). Un total de 535 étiquettes de séquences exprimées (EST) a été obtenu et déposé dans la banque de données Genbank suite au séquençage partiel des clones. Ces étiquettes ont été regroupées en 314 uniséquences dont la majorité correspondait à des gènes d'orme identifiés durant l'interaction. Cinquante-trois uniséquences représentant des gènes impliqués dans différentes voies métaboliques associées à la défense ont été sélectionnées par un criblage différentiel et considérées comme étant induites durant l'interaction. Les profils d'expression à différents temps après inoculation ont été établis par PCR quantitative chez

18 uniséquences provenant de cals infectés ainsi que de cals traités à l'eau stérile. Ils confirment l'induction ou l'expression constitutive des gènes correspondants durant le processus de l'infection. Cette étude fournit pour la première fois une ressource génomique pour l'orme et révèle des mécanismes moléculaires impliqués dans l'interaction entre l'orme américain et l'agent pathogène responsable de la maladie hollandaise de l'orme.

Abstract

Interactions between plants and fungal pathogens usually lead to the induction of different host defense mechanisms. Some of these mechanisms are the reinforcement of the plant cell wall by suberin, lignin and wall bound-phenolics, the accumulation of pathogenesis-related proteins, and the production of phytoalexins that could be toxic to the pathogen. Yet in susceptible plants, these mechanisms are not effective to produce resistance and disease develops. Dutch elm disease (DED) is a pandemic tree disease that killed millions of elm trees, especially in North America and Europe. The molecular bases of this disease are still poorly understood. With the objective of identifying genes involved in the interaction between the susceptible *Ulmus americana* L. and the pathogen *Ophiostoma novo-ulmi* Brasier, an in vitro system was developed using callus cultures inoculated with budding cells of the fungus. In order to validate the use of this system for genomic analyses of the interaction, a histopathological analysis was carried out using light and electron microscopy. Fungal colonization of the callus tissue and reactions of callus cells to the presence of the pathogen were observed at 4, 24, 48, 72 and 96 hours post-inoculation (hpi). The fungus was seen in its hyphal form by 48 hpi in all parts of the callus. Histochemical tests showed the importance of host reactions such as the accumulation of phenols, lignin, and suberin. The percentage of suberized cells was 4.6 times higher at 96 hpi in infected calli than in mock-inoculated control calli. A cDNA library using suppression subtractive hybridization (SSH) was constructed from infected elm callus tissue harvested at 72 hpi. A total of 535 expressed sequence tags were generated through partial sequencing and submitted to Genbank. These were grouped into 314 unisequences, the majority corresponding to elm genes identified during the interaction. Fifty-three unisequences representing genes involved in different pathways associated with plant defense were selected by differential screening and considered upregulated in the infected tissues. The expression profiles in mock and infected elm callus cultures of a subset of 18 elm genes were analyzed in more detail by quantitative reverse transcriptase polymerase chain reaction. These confirmed upregulation and constitutive expression of selected genes during the infection process. This study provides, for the first time, a genome-wide resource for the elm, and furthermore identifies molecular mechanisms likely involved during the interaction between *U. americana* and the DED pathogen.

Avant-Propos

Les cinq parties de cet ouvrage se proposent comme suit. Une introduction en français mettant la thèse dans le contexte des recherches sur la maladie hollandaise de l'orme et introduisant les chapitres suivants. Puis, un chapitre bibliographique rédigé en anglais donnant un contexte scientifique de ce travail dans le cadre des connaissances sur les interactions hôte-agent pathogène fongique. Ensuite, deux chapitres expérimentaux rapportent les résultats de la recherche. Ces chapitres prennent la forme d'articles scientifiques rédigés en anglais et pour lesquels l'auteur de cette thèse agit comme auteur principal. Les trois chapitres rédigés en anglais sont tous précédés de résumés en français. Enfin, une conclusion en français fait le point sur l'apport de cette thèse et les perspectives futures qu'ouvre le projet.

Cette thèse a vu le jour après plusieurs années de travail rendues fructueuses grâce à l'apport, le soutien et la participation de plusieurs personnes. Une page d'encre restera très courte pour les remercier.

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Introduction

Les interactions! Parmi plusieurs définitions, une interaction a été définie en psychologie comme étant un échange d'informations, d'affects ou d'énergie entre deux agents au sein d'un système. C'est une action réciproque qui suppose l'entrée en contact de sujets (Marc et Picard 2006). En biologie, une interaction désigne plutôt un processus impliquant des échanges et induisant des effets. Bien qu'on observe dans la nature des types d'interaction induisant des effets bénéfiques, tel que la symbiose et le mutualisme, l'on observe cependant d'autres qu'on a qualifié de parasitisme et de pathogénique, et dont l'effet est néfaste pour une des deux espèces impliquées qu'on dénomme l'hôte. Tel est le cas de l'orme américain, jadis espèce urbaine de choix en Amérique du Nord et espèce indigène des peuplements forestier nord-américains. Jadis, car depuis son association en tant qu'hôte, avec l'espèce fongique *Ophiostoma ulmi* sensu lato (s.l.), une interaction pathogènique s'est développée dont le résultat est la maladie hollandaise de l'orme (MHO), un événement marquant dans l'histoire de la pathologie des arbres (Karnosky 1979). Appelée ainsi suite à sa première description en Hollande en 1921 (Spierenburg 1921), la MHO qui s'est déployée en deux épidémies durant le XXème siècle a causé la quasi-disparition des ormes sensibles dans le paysage urbain partout dans les aires de répartition de l'orme en Europe et en Amérique du nord (Karnosky 1979). La première épidémie a été causée par l'espèce *O. ulmi*. La deuxième épidémie (au début des années 1970 à aujourd'hui) est la plus sévère et est causée par l'espèce la plus agressive du champignon, *O. novo-ulmi* Brasier (Brasier 1991).

Depuis son apparition, la MHO a fait l'objet de plusieurs recherches. C'est ainsi que depuis le premier isolement de l'agent pathogène par M.B. Schwarz en 1919 (Schwarz 1922), l'on a démontré sa transmission par un insecte vecteur (Middleton et al. 1934). Les études subséquentes sur l'agent pathogène ont permis de mieux connaître sa biologie, sa virulence et ont touché aussi à son bagage génétique. Par exemple, on a mis en évidence que le dimorphisme d'*O. ulmi* (*s. l.)* est sous contrôle nutritionnel (Kulkarni et Nickerson 1981). Les systèmes de compatibilité sexuelle et végétative de ce champignon ont fait l'objet de plusieurs études. Les locus correspondant à ces systèmes, tel que le locus MAT (locus de compatibilité sexuelle), sont associés à d'autres traits importants tels que la virulence (Brasier 1984, 1996). L'analyse génétique de la pathogénie d'*O. ulmi* a été

principalement basée sur la production de mutants et sur les croisements en laboratoire entre souches différentes quant à leur phénotype pathogène (Bernier et Hubbes 1990b; Kile et Brasier 1990; Plourde et al. 2008). Les données provenant des études génétiques sur cet organisme ont fortement suggéré que plusieurs gènes sont impliqués dans la pathogénie de ce champignon (Brasier et Gibbs 1976; Brasier 1988). Et-Touil et al. (1999) ont localisé un gène, dénommé Pat1, qui contrôle le taux de virulence chez des souches *O. novo-ulmi* différentes dans leur agressivité.

Les métabolites extracellulaires secrétés par ce champignon ont été particulièrement étudiés en vue de leur possible association à la pathogénie. *O. ulmi* (s.l.) produit un nombre de métabolites extracellulaires dont la production a été observée *in vitro* (Elgersma 1976) et in vivo (Scheffer et Elgersma 1982). Ces métabolites sont des enzymes de dégradation de la paroi végétale (Elgersma 1976), des polysaccharides (Strobel et al. 1978) et des métabolites phénoliques (Claydon et al. 1980), des glycoprotéines (Yang et al. 1994), ainsi qu'une hydrophobine (Takai 1974). Certains de ces métabolites, telle que la glycoprotéine isolée par Yang et al. (1994) ont été capables d'induire la production de phytoalexines de l'orme. Cependant, l'implication directe de ces métabolites dans la pathogénie du champignon n'est pas démontrée (Scheffer 1983; Temple et al. 1997).

D'autre part, les études sur l'orme ont permis d'identifier sur le plan anatomique certains facteurs qui peuvent déterminer partiellement la susceptibilité de l'orme à l'infection par la MHO. La longueur des vaisseaux du xylème (Elgersma 1970) et leur diamètre (Elgersma 1970; Sinclair et al. 1975; Solla et Gil 2002) ainsi que la taille moyenne des groupes de vaisseaux (McNabb et al. 1970) sont parmi ces facteurs. On a aussi mis en évidence que la réponse de l'orme à la MHO (Shigo et al. 1981) suit le modèle CODIT (*compartmentalization of decay in trees*), tel que proposé par Shigo et Marx (1977). Selon ce modèle, l'arbre utilise quatre murs pour limiter le développement des tissus endommagés. Selon ce modèle, l'arbre utilise quatre murs pour limiter l'extension des tissus endommagés dans le xylème et dans le phloème. La formation de ces murs implique des dépôts de subérine (Rioux et Ouellette 1991b; Rioux et al. 1995), de lignine et phénols (Rioux et Ouellette 1991a; 1991b), ainsi que l'accumulation de phytoalexines (Duchesne et al. 1985; Proctor et Smalley 1988).

Plusieurs auteurs ont décrit l'accumulation in vitro (avec des cals) et in vivo de phytoalexines de type mansonone, produites par des membres du genre *Ulmus* après leur infection par la maladie hollandaise de l'orme (Dumas et al. 1983; Duchesne et al. 1986; Yang et al. 1989). Les mansonones sont des phytoalexines qui appartiennent à une famille de sesquiterpénoïdes quinones. Sept mansonones (nommés A, C D, E, F, G, H) ont été isolés à ce jour. Probablement produits par les cellules du parenchyme, les mansonones interviennent dans l'inhibition de la croissance fongique chez les ormes résistants et exercent un ou plusieurs effets sur la physiologie et l'ultrastructure du champignon (Dumas et al. 1986; Wu et al. 1989). L'implication des mansonones dans la résistance chez l'orme n'est cependant pas clairement établie (Yang et al. 1993; Duchesne et al. 1994; Meier et Remphrey 1997).

En plus des mansonones, les composés phénoliques ont été associés à la réponse de l'orme à l'infection. La présence de composés phénoliques a été détectée in vivo (Rioux et Ouellette 1991a) et in vitro dans des cultures de cals auxquels le champignon a été inoculé (Pijut et al. 1990b, Krause et al. 1996). De plus, Corchete et al. (1993) ont montré l'induction de l'activité enzymatique de la phénylalanine ammonia-lyase (PAL), enzyme impliquée dans les premières étapes de synthèse des phénols, chez des cultures cellulaires de l'espèce resistante *U. pumila* auxquelles le champignon a été inoculé. Par la suite, Valle et al. (1997) ont isolé une hydroxycoumarine, la scopoletine, qui a été induite suite à l'infection d'une façon plus rapide et en une quantité plus élevée chez les cultures cellulaires d'*U. pumila* par rapport à celles de l'espèce sensible *U. campestris*.

Sur le plan génétique, Nasmith et al. (2008a, 2008b) ont cloné et étudié l'expression de trois gènes d'orme américain, soit les gènes encodant la chitinase, la phénylalanine ammonia-lyase (PAL) et la *polygalacturonase inhibiting protein* (PGIP). L'expression de ces gènes in vivo a été induite suite à l'inoculation de semis par le champignon.

Avec la venue de l'ère génomique, des technologies ont été développées afin de permettre l'identification d'une panoplie de gènes qui contrôlent un mécanisme donné. Des outils, tels que ceux permettant l'analyse transcriptionnelle, offrent une opportunité exceptionnelle pour l'étude de l'interaction entre l'orme sensible et son agent pathogène fongique, surtout que les mécanismes impliqués dans cette interaction sont peu connus, et ce particulièrement au niveau moléculaire.

Les hypothèses sous-tendant le travail décrit dans cette thèse peuvent être définies comme suit : lorsque l'orme sensible et son agent pathogène fongique entrent en interaction, l'expression de plusieurs gènes chez les deux organismes est affectée par le contact et l'interaction. Il est possible d'identifier de tels gènes dans un système in vitro qui permet le contact entre les cellules de l'orme et les cellules de l'agent pathogène.

À partir de ces hypothèses, un système fut établi pour permettre l'identification de gènes induits durant l'interaction entre une espèce hôte hautement sensible qu'est l'orme américain (*Ulmus americana* L.) et une espèce pathogène hautement virulente qu'est *O. novo*-ulmi Brasier. Puisque ce genre d'interaction est dynamique et peut évoluer rapidement lors des premiers stades de développement de la maladie, il était nécessaire de déterminer les meilleures conditions pour l'analyse de l'expression des gènes durant l'interaction. Pour ce faire, les objectifs de cette thèse étaient, dans un premier temps, d'établir et de valider, par des analyses histopathologiques, un système d'interaction in vitro permettant de confronter l'agent pathogène à son hôte et se prêtant facilement à la manipulation génomique; dans un deuxième temps, d'isoler l'ARN total à un point précis de l'interaction afin de construire une banque d'ADNc qui soit enrichie de gènes induits, et d'analyser le profil d'expression de certains gènes ainsi identifiés, afin de permettre de mieux comprendre leur implication dans l'interaction.

La thèse se divise en trois chapitres. Le premier chapitre est une revue bibliographique qui fait le point, chez les plantes, sur des mécanismes de défenses impliqués dans l'interaction avec des agents pathogènes fongiques. Ce chapitre permet de mieux situer le travail dans le cadre des connaissances acquises sur ces pathosystèmes. Ce chapitre tente également d'analyser les raisons pour lesquelles l'hôte perd la bataille dans une interaction compatible et devient un hôte sensible. Cette approche vise à proposer des hypothèses de travail pour de futurs travaux qui dépendent des résultats obtenus dans les deuxième et troisième chapitres, ces derniers constituant le corps expérimental de la thèse.

Le deuxième chapitre, qui a paru dans le numéro du mois de juin 2009 de la revue *Phytopathology*, consiste en une étude histopathologique faite sur des cals d'orme américain auxquels ont été inoculées des cellules levuriformes du champignon *O. novo-ulmi*. Étant donné les avantages que peut offrir l'utilisation d'un système in vitro en laboratoire, nous avons opté dans ce projet exploratoire, pour l'utilisation de cals (tissus

dédifférencié) notament pour faciliter le suivi de la colonisation par le champignon et l'extraction d'acides nucléiques. L'utilisation de cals pour étudier la MHO a fait l'objet de plusieurs études, et ce particulièrement sur le plan histopathologique (Pijut et al. 1990b, Krause et al. 1996). Dans ce chapitre, nous tentons de valider ce système pour l'analyse génomique en déterminant à quel moment le champignon a bien colonisé la surface du cal tout en ayant envahi celui-ci. En faisant ces examens microscopiques, nous avons aussi porté une attention spéciale aux mécanismes de défense de l'hôte pour contrer cette agression.

Le troisième chapitre, soumis à la revue *Tree Physiology*, décrit la construction et l'analyse d'une banque d'ADNc réalisée avec des cals inoculés par le champignon à un temps d'interaction choisi suite aux résultats du deuxième chapitre. L'étude génomique de l'interaction est effectuée à travers la génération et le criblage d'une banque d'interaction et à travers l'analyse bioinformatique de la banque. Cette étude est complétée par l'analyse de l'accumulation des transcrits de certains gènes représentatifs de la banque sur une échelle de temps qui s'étale de 4 à 144 heures après inoculation. L'assocation de gènes identifiés en particulier avec des mécanismes de défenses chez les arbres ou les plantes en général est discutée. Ce chapitre vise ainsi la description de certains mécanismes moléculaires qu'on peut associer à l'interaction entre l'orme et son agent pathogène fongique.

En conclusion, nous faisons le point sur l'apport de cette thèse quant à l'interaction étudiée ainsi que sur les débouchés sur des études complémentaires que permettront les résultats obtenus.

Des parties des résultats ou leur ensemble ont fait l'objet de publications ainsi que de communications scientifiques dans des conférences nationales et internationales (Aoun et al. 2004a; 2004b; 2005; 2006a; 2006b; 2007; 2008; 2009; Bernier et al. 2003; 2004).

1. Host defense mechanisms during fungal pathogenesis and how these were overcome in susceptible plants

Note: This chapter is a review of important aspects in host defense mechanisms related to fungal pathogenesis that are meant to give a larger and comprehensive context to the interaction studied in this thesis. One can find a detailed review of matters discussed here and other subjects in this reference:

Vidhyasekaran, P., 2008. Fungal pathogenesis in plants and crops: Molecular biology and host defense mechanisms. 509 pages. 2nd edition. CRC Press, Taylor and Francis Group, Boca Raton, FL, USA.

1.1 Résumé-Abstract

L'interaction entre les plantes et leurs agents pathogènes fongiques fait intervenir une variété de mécanismes qui déterminent la finalité de l'interaction, soit compatible qui mène à la susceptibilité chez l'hôte, soit incompatible qui mène à la résistance. Plusieurs mécanismes de défense chez l'hôte agissent autant chez les plantes sensibles que chez celles qui sont résistantes. Cependant, dans le cas d'une interaction compatible impliquant un hôte sensible et un agent pathogène virulent, ce dernier est capable de gagner la bataille et causer la maladie. Ce chapitre passe en revue certains des mécanismes utilisés par les plantes pour se défendre, tel que les modifications de la paroi cellulaire, l'accumulation des protéines PR et la production de métabolites secondaires. Sont décrits aussi des mécanismes par lesquels les agents pathogènes arrivent à contourner les défenses mises en place par la plante.

The interaction between plants and fungal pathogens comprises a range of mechanisms that determine the outcome of the interaction, i.e. compatible leading to susceptibility or incompatible leading to resistance. Several host defense mechanisms act both in susceptible and resistant plants. Yet, in the case of a compatible interaction involving a susceptible host and a virulent pathogen, the latter is able to win the battle and cause disease. This review describes some of the mechanisms which plants use for defense such as the reinforcement of the cell wall, and the accumulation of Pathogenesis-related (PR) proteins and of secondary metabolites, and ways by which pathogens overcome plant defense.

1.2 Introduction

Molecular communication between plant and pathogen starts almost immediately after the pathogen makes contact with the plant surface (Fujita et al. 2004). In this battle, susceptibility or resistance is determined by the winner. If the pathogen is able to overcome the toxic environment in the plant tissue, the disease develops, and if the plant is able to ward off the pathogen's toxic weapons, disease resistance develops. Plant cells recognize the presence of spores of fungal pathogens on the surface and initiate defense-associated responses even within 30 min of contact with pathogen spores (Fujita et al. 2004). Plant cells then launch diverse defense mechanisms. Here we describe three important aspects of host defense mechanisms during fungal pathogenesis: 1) reinforcement of the cell wall, the first barrier against fungal pathogens (Thordal-Christensen 2003); 2) the induction and accumulation of new readily detectable proteins, called pathogenesis-related (PR) proteins (Van Loon 1999); and 3) the accumulation of antifungal secondary metabolites (Dixon 2001). In this chapter, focus was placed on the way these defenses are counteracted in the compatible interaction leading to susceptibility.

1.3 Cell wall reinforcement during fungal pathogenesis

1.3.1 Reinforcement of plant cell wall by phenolics

Plant cell walls respond to invasion by fungal pathogens by accumulating phenolics and phenolic polymers such as lignin (Nicholson and Hammerschmidt 1992). Ferulic acid, *p*-coumaric acid, and sinapic acid are the predominant cell wall-bound phenolics, whereas lignins are the wall-bound polymerized phenolics (Bily et al. 2003). Bound forms of ferulic acid can be dimerized by peroxidases to form cross-links between arabinoxylan chains that strengthen the cell wall (Fry et al. 2000). Dehydroferulic acid may impede cell wall degradation caused by cell wall-degrading enzymes produced by pathogens (Grabber et al. 1998). Also, high concentrations of chlorogenic and caffeic acids were found to inhibit

cutinase production by the fungus, *Monilinia fructicola,* in the cell layers of resistant peach genotypes, suggesting that wall-bound phenolics may induce resistance by suppressing the activities of wall-degrading enzymes of the pathogen (Bostock et al. 1999).

The pathway of synthesis of major cell wall-bound phenolics is presented in Figure 1.1.

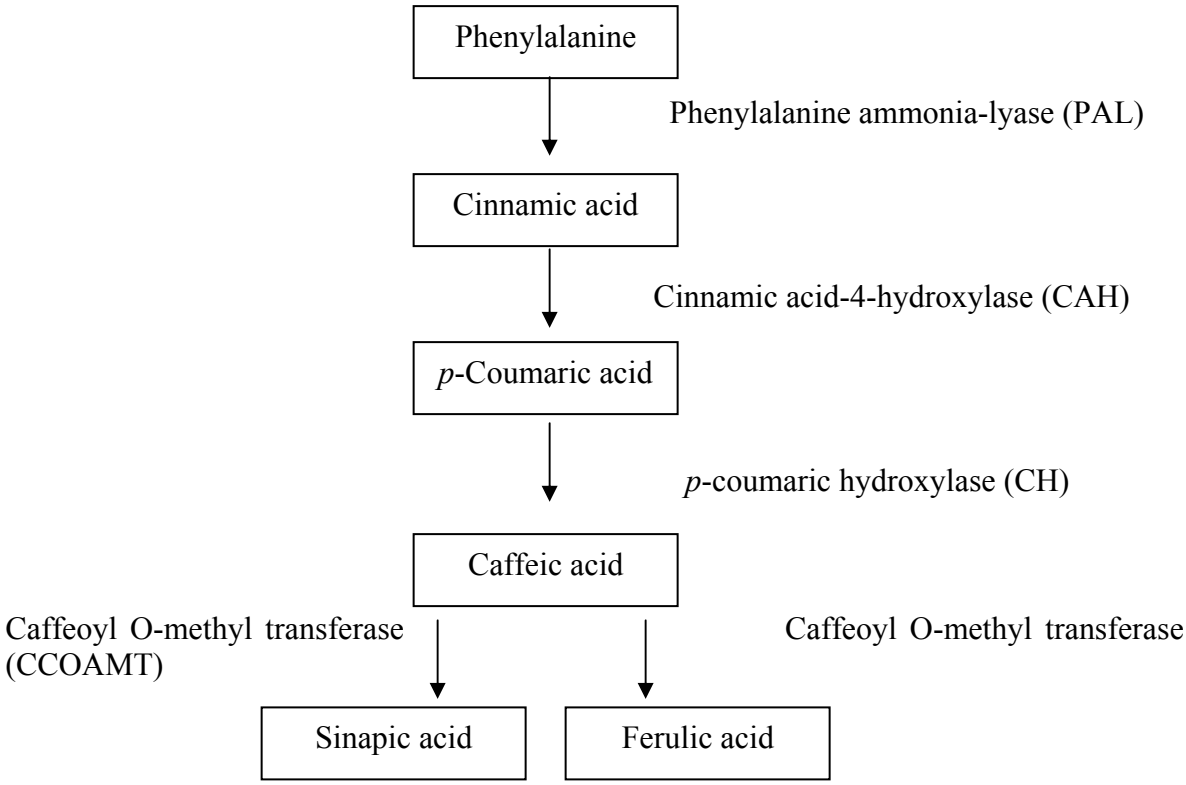

Figure1.1: Biosynthesis of wall-bound phenolics. (Adapted from Vidhyasekaran 2008a)

1.3.1.1 Deposition of phenolics in host cell wall in response to fungal invasion

The appearance of yellow autofluorescence (at 365 nm) and of autofluorescence under blue light excitation in diseased plant tissues is considered to be a result of the presence of phenolic compounds that accumulate in the tissues as the host attempts to limit the development of the pathogen (Cohen et al. 1990; Bennett et al. 1996). Tomato cell cultures inoculated with *Verticillium albo-atrum* accumulated up to fivefold more of wall-bound phenolics than were found in uninoculated control cultures (Bernards and Ellis

1991). The analysis of this cell wall-bound material revealed that two populations of phenolic material existed. The first comprised esterified compounds and the second comprised nonbase-labile polymeric material. An array of phenolic compounds was detected in wall preparations of inoculated cell cultures, whereas walls from uninoculated cells yielded only traces of esterified phenolics (Bernards and Ellis 1991).

1.3.1.2 Activation of enzymes involved in the biosynthesis of phenolics during fungal invasion

Several studies have shown that the increase by hosts of the levels of phenylalanine ammonia lyase (PAL), the first enzyme in the phenylpropanoid pathway, was a direct response to attempted penetration by the fungus. PAL was induced in tomato cell cultures both at the translational and transcriptional level by the inoculation with *Verticillium albo-atrum* (Bernards and Ellis 1991). Maximum enzyme activity occurred by 36-48 hours post-inoculation (hpi), concomitant with a marked accumulation of PAL messenger RNA (mRNA). Only a low level of PAL and its mRNA were detectable from uninoculated cell cultures (Bernards and Ellis 1991). The accumulation of wall-bound phenolics in inoculated tomato cell cultures was inhibited by the PAL inhibitor 2-amino-2-indanephosphate (AIP) (Bernards and Ellis 1991). Shiraishi et al. (1995) observed increases in PAL activity at two different times in barley cultivars inoculated with *Blumeria graminis* regardless of the resistance or susceptibility of the barley cultivar to the fungus. The first increase began at 3 hpi and was followed by a second increase between 12 and 15 hpi. The conidium produced a primary germ tube that attempted penetration beginning about 2 hpi and an appressorium that attempted to penetrate beginning 9-10 hpi. Clark et al. (1994) showed that an initial accumulation of PAL transcripts occurred in barley cultivars inoculated with *B. graminis* between 4 and 6 hpi. This accumulation declined to near constitutive levels by 8-10 hpi. A second peak was observed from 10-12 hpi and then declined until 15-18 hpi. The first increase occurred in response to contact with the fungal germ tube, whereas the second increase was because of appressorial contacts (Clark et al. 1994). Besides PAL, other enzymes implicated in synthesis of wall-bound phenolics, such as cinnamyl alcohol dehydrogenase (CAD) and Caffeoyl-COA-O methyltransferase

(CCOAMT), showed transient increase in transcript activity after treatment with fungal elicitors (Grand et al. 1987; Pakusch and Matern 1991).

The rapid synthesis of phenolics and the polymerization in the cell wall was generally regulated by a *p*-coumaric hydroxylase enzyme that is extremely pH dependent, and not by de novo enzyme synthesis. Membrane damage leads to a decrease in cytoplasmic pH, which activates the hydroxylase (Matern and Kneusel 1988).

1.3.1.3 How does the pathogen overcome the cell wall-bound phenolics to cause disease?

Suppression of accumulation of phenolics in the host cell wall and delay of synthesis of wall bound phenolics are two ways that help the pathogen overcome and cause disease.

Several studies have indicated that successful pathogens may be able to suppress accumulation of phenolics in the plant cell wall. Phenolics were observed in cell walls of epidermal cells of resistant muskmelon inoculated with the powdery mildew fungus *Sphaerotheca fuliginea*, *a* at 24-96 hpi. The infected epidermal cells emitted autofluorescence from the lumen. Penetrated cells of the susceptible variety did not show any autofluorescence, thus indicating absence of phenolics (Cohen et al. 1990). Suppression of PAL has been shown to induce susceptibility in resistant varieties. Inhibiton of PAL by α-aminooxy-β-phenylpropionic acid (AOPP) suppressed the accumulation of phenolic compounds in epidermal cell walls in barley and wheat, and made the resistant hosts susceptible to the pathogens *Blumeria graminis f. sp. hordei* and *B. graminis f. sp. tritici*, respectively (Carver et al. 1992).

Synthesis of cell wall-bound phenolics may be delayed at the fungal penetration site in compatible interactions. Yellow autofluoresence (indicating synthesis of phenolics) after excitation at 365 nm was emitted, by inoculation with *Plasmopara viticola* in the resistant *Vitis rotundifolia*, as early as two days after inoculation. In contrast, a few stomatal cells with yellow autofluorescence were detected only 8 days after inoculation in lesions of the susceptible *V. vinifera* (Dai et al. 1995a).

1.3.2 Reinforcement of plant cell wall by lignin

1.3.2.1 Biosynthesis of lignin

The pathway of lignin synthesis is not yet completely understood, and the lignin roadmap has been rewritten frequently (Humphreys and Chapple 2002). Monolignols are the precursor of lignin biosynthesis. Coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol are the most important monolignols. The important enzymes involved in the biosynthesis of lignin are: PAL, CCOAMT, CAD, 4-coumarate:CoA ligase (4CL), coumarate alcohol dehydrogenase, coniferyl-CoA reductase, coniferyl alcohol dehydrogenase, sinapoyl-CoA reductase, synapoyl alcohol dehydrogenase, and peroxidases (Humphreys and Chapple 2002).

1.3.2.2 Lignification in compatible and incompatible interactions

Lignification is a common response to plant infection (Carver et al. 1998). Phenolic polymers which accumulate in response to infection have been identified to be lignin- and suberin-like polymers (Graham and Graham 1991). PAL activity increased at 24 hpi by more than 10 fold in jack pine *(Pinus banksiana*) cell cultures after treatment with fungal elicitor, coinciding with the initiation of cell wall lignification (Campbell and Ellis 1992).

Lignification is common in healthy plants also. However, the increased lignification observed in resistant varieties appears to involve a new type of lignin. A number of histochemical tests, such as phloroglucinol-HCL, toluidine blue O, chlorine-sulfite, and the modified chlorine-sulfite are being employed to detect the presence of lignin. When these tests were used to detect the deposition of lignin in infected leaf tissues, additional lignin was observed (Southerton and Deverall 1989, 1990). The additional lignin observed in walls of wheat leaves because of the incompatible interaction with the leaf rust fungus, *Puccinia recondita* f. sp. t*ritici*, was considered different from that in uninfected leaves because of its green rather than blue-green response to toluidine blue. The failure to react to phloroglucinol may indicate an absence of cinnamaldehyde groups. The additional lignin formed in the incompatible interaction may not be rich with syringic groups because of its failure to stain with chlorine-sulfite. This additional lignin was not detected in the susceptible interaction (Southerton and Deverall 1989, 1990).

Lignification is often suppressed in several compatible interactions while in incompatible interactions lignification is often predominant. It has been observed that the lignin content in the cell walls of susceptible wheat cultivar tissues infected by *Fusarium culmorum* increased slightly, whereas lignin accumulated intensely in the host cell walls from infected wheat spikes of resistant cultivars (Kang and Buchenauer 2000). The important lignin biosynthesis enzymes PAL, 4CL, CAD, and peroxidases appear to be suppressed in compatible interactions by the pathogen. The first three enzymes exhibited increased activities at very early stages of infection (8-16 hpi) by *P. graminis f. sp. tritici* in both susceptible and resistant wheat lines. Subsequently both PAL and 4CL activities decreased to the level of uninoculated controls in the compatible interaction. In the incompatible interaction however, the activities continued to increase from 32 hpi onward until 6-7 days after inoculation (Moerschbacher et al. 1988).

1.3.2.3 How does the pathogen suppress lignification in host cell wall?

Oligogalacturonides with more than eight galacturonosyl residues are endogenous elicitors of lignification in both susceptible and resistant plants (Ridley et al. 2001). The elicitor active pectic fragments are produced because of partial digestion of the host polygalacturonic acid by fungal enzymes (Robertsen 1986). In the compatible interaction, more rapid degradation of pectic substances by higher concentration of the pectolytic enzymes produced by the pathogen results in complete degradation of the endogenous elicitors, and accumulation of pectic polymer of less than eight galacturonosyl residues which do not have elicitor activities and hence no lignification occurs (Robertsen 1986; Lorenzo et al. 1990). Fungal cell walls also contain molecules that elicit lignification. These molecules are released by host enzymes (such as chitinase) and activation of the host enzymes is under the control of fungal cell wall components (Kurosaki et al. 1986). Chitinase activity was induced in cultures of carrot cells incubated with fungal walls of *Chaetomium globosum*, and the soluble fragments liberated from the fungal walls stimulated the biosynthesis of phenolic acids which are precursors of the lignin synthesized in cells (Kurosaki et al.1986). Thus, fungal cell wall extracts can induce lignification. However, the release of elicitor containing fungal cell wall components would have been suppressed in compatible interactions (Biggs and Peterson 1990).

1.3.3 Reinforcement of plant cell walls by suberin

1.3.3.1 Host cell walls respond to fungal invasion by suberization

Suberin, a complex biopolyester organized in a characteristic lamellar structure, comprises a phenolic (aromatic) domain attached to the cell wall and an aliphatic (lipid, hydrophobic) domain attached to the phenolic domain (Lulai and Corsini 1998). Historically, the phenolic domain has been likened to lignin and the aliphatic domain was represented as a random network of polyesterified modified fatty acids and alcohols. Recently, however, a new model for suberin has emerged in which a hydroxycinnamic acid-monolignol polyphenolic domain embedded in the primary cell wall was covalently linked to a glycerol based polyaliphatic domain located between the primary cell wall and the plasma membrane (Bernards 2002, Gandini et al. 2006). The production of suberin coatings was dependent on PAL activity (Street et al. 1986). PAL induces the synthesis of many phenolic acids, which are required for synthesis of suberin (Niemann et al. 1991). Cross-linking of such phenolics forms a polymeric matrix which is made hydrophobic by attachment of aliphatic polyester domains and by deposition of highly non-polar waxes into the layer. The formation of this layer is called suberization (Pearce and Rutherford 1981). The formation of the aromatic matrix is the first step in suberization. The polymerization of the aromatic components of suberin involves an isoperoxidase in a manner similar to that involved in lignin biosynthesis (Espelie and Kolattukudy 1985).

Suberization is responsible for the reinforcement of cell walls limiting ingress of pathogens in the host (Biggs and Miles 1988). Suberization was observed in both susceptible and resistant interactions in tomato (Chen et al. 2004). Suberin was deposited on cell walls of various plant tissues when the pathogen invaded host tissues (Kolattukudy 1981). Suberin is highly resistant to enzymatic degradation by pathogens and hence it is considered as an effective barrier to penetration by many fungal pathogens (Kolattukudy 1981; Kamula et al. 1995).

1.3.3.2 How does the pathogen overcome suberization of host cell walls?

During pathogenesis, suberization appears to be delayed in compatible interactions; this delay would help the pathogen penetrate host tissues. In tomato, one of the earliest defense responses against *Verticillium albo-atrum* was the coating of xylem vessels and pit membranes with suberin (Street et al. 1986). In resistant tomato varieties suberization was found to be very rapid, beginning at 8-10 hpi. In sharp contrast, almost no suberization was visible in susceptible varieties at that same time. After 24 hpi, suberization was visible in susceptible plants, but was much less pronounced as compared to resistant plants (Lee et al. 1992).

The delay in suberin accumulation in compatible interactions appears to be a result of suppression of suberin-synthesizing enzymes by the pathogen. Accumulation of PAL mRNA, the first enzyme involved in suberin synthesis, increased in resistant tomato plants infected with *Verticillium albo-atrum* to about 30% above the constitutive normal level. In contrast, the level did not increase in susceptible plants, but proceeded to drop until it was only 30% of the constitutive level after 15 hpi. These results suggest that fungal components may suppress PAL mRNA levels in susceptible plants (Lee et al. 1992).

Some fungal pathogens have been reported to penetrate suberized cell walls (Peterson et al. 1980; Chen et al. 2004). This can be a result of the action of degrading enzymes. Esterases able to degrade the aliphatic and aromatic domains of suberin have been isolated in several fungal species (Fernando et al. 1984; Ofong and Pearce 1994; Schultz et al. 1996). Suberinase activity was also observed in some cases, as shown by GarciaLepe et al. (1997).

The ability of plants to accumulate suberin also appears to determine susceptibility or resistance (Biggs 1989). Total resistance to fungal infection was attained after completion of deposition of the suberin aliphatic domain within the first layer of suberized cells (Lulai and Corsini 1998).

1.4 Induction of pathogenesis-related proteins

1.4.1 Variety of pathogenesis-related (PR) proteins

PR proteins may be defined as proteins encoded by the host plants and induced specifically in response to pathogen-attack. These were readily detected in infected, but not in uninfected tissues (Van Loon 1999). Proteins that were constitutively expressed were considered as PR proteins when the expression was induced in specific organs of a plant or in specific varieties during infection (Van Loon 1999). A variety of PR proteins were present in infected plant tissues. For example, more than 30 PR proteins have been identified in Norway spruce (*Picea abies*) (Sharma et al. 1993). PR proteins have been classified into 17 families based on structure and sequence similarity, rather than on biological activities (Van Loon et al. 2006; Vidhyasekaran 2008b). Table 1 lists recognized PR-protein families and the functions.

Table 1: Pathogenesis-related protein families and associated functions

PR-family	Functions or Properties	Reference
PR-1	Plant cell wall thickening, some inhibitory function on growth of the pathogen	Santén et al. 2005
PR-2	Show β-1,3-glucanase activity	Ménard et al. 2004
PR-3	Chitinases (classes I, II, IV, V, VI and VII) and β-1,4-glucosaminidases	Van Loon et al. 1994; Neuhaus 1999
PR-4	• hevein and wound induced (WIN) proteins • acidic chitinases	Van Loon 1999; Pierpoint 1986
PR-5	Thaumatin-like (TL) proteins	Koiwa et al. 1994
PR-6	Serine proteinases inhibitors, cysteine proteinases inhibitors, aspartic proteinases inhibitors and metalloproteinases inhibitors	Koiwa et al. 1997; Sels et al. 2008
PR-7	show endoproteinase activity	Vera and Conejero 1988
PR-8	Class III chitinases	Van Loon et al. 1994
PR-9	Peroxidases induced during pathogenesis	Lagrimini et al. 1987; Vale et al. 1994
PR-10	Intracellular acidic proteins. Possess ribonuclease activity	Warner et al. 1992; Zhou et al. 2002
PR-11	Endochitinases	Melchers et al. 1994
PR-12	Defensins induced during pathogenesis	Penninckx et al. 1996; Sels et al. 2008
PR-13	Thionins	Sels et al. 2008

PR-14	Lipid transfer proteins (LTPs)	Sels et al.2008
PR-15	Germin-like oxalate oxidase	Zhang et al. 1995; Schweizer et al. 1999.
PR-16	Germin-like proteins without oxalate oxidase activity	Wei et al. 1998
PR-17	Peptidases with similarity to the active site and peptide-binding groove of the exopeptidase aminopeptidase N from eukaryotes	Christensen et al. 2002

Besides these 17 families, some unclassified proteins have also been described. Grenier and Asselin (1990) have identified chitosanases as pathogenesis related proteins. Chitosanases act on chitosan, but have no activity on chitin. These were distinguished from chitinases that act on chitin without activity on chitosan.

1.4.2 Induction of PR proteins during fungal pathogenesis

When the fungal pathogen invades host tissues, several PR proteins accumulate both locally and systemically (Sharma et al. 1993; Lawrence et al. 1996). Accumulation of PR proteins in response to pathogenesis has been detected in compatible as well as in incompatible interactions (Benhamou et al. 1991; Van Kan et al. 1992). Genes encoding PR proteins belong to multigene families (Sharma et al. 1992). These have been identified in different plants but were almost silent in healthy plants (Sharma et al. 1992). The PR genes that were quiescent in a healthy plant may be induced either at the level of transcription or the translation stage. Van Loon (1985) provides evidence that induction of PR protein synthesis was observed only at the translational stage, suggesting that mRNAs of the PR proteins exist constitutively in healthy plant tissues and that were translated into proteins during pathogenesis. In contrast, Matsuoka et al. (1988) showed that translatable mRNAs for PR-1 proteins in tobacco leaves were present only in infected leaves, but not in healthy leaves, suggesting that synthesis of PR-1 proteins was not regulated at the translational, but at the transcriptional step. Chitinase and β-1,3-glucanase were shown to be regulated coordinately at the level of mRNA (Vogeli et al. 1988). Also, accumulation of proteinase inhibitors I and II was accompanied by the appearance of the respective translatable mRNAs (Nelson and Ryan 1980). All these studies suggest that PR proteins are transcriptionally regulated.

1.4.3 Signals involved in transcriptional induction of PR genes

1.4.3.1 Induction of PR genes by elicitors

Elicitors are compounds capable of inducing any type of plant defense (Boller 1995). Fungal pathogens produce several elicitors and these elicitors may serve as primary signal molecules in triggering PR genes. *Cis*-acting elicitor responsive elements (EREs) have been identified in the promoters of many PR genes. These elements modulate expression and responsiveness to the fungal elicitor (Fukuda 1997). Several secondary messengers may be involved in the process of binding of the elicitor message to the *cis*-acting elements in the promoter of PR genes. The activation of the potato PR gene *PR-10a* was positively controlled by a protein kinase that affected the binding of the nuclear factors PBF-1 and PBF-2 to an elicitor response element in the promoter of the gene (Subramaniam et al. 1997).

1.4.3.2 Induction of PR genes by salicylic acid

Salicylic acid has been reported as one of the important signal molecules which act locally in intracellular signal transduction and systemically in intercellular signal transduction (Raskin 1992). Salicylic acid accumulates during fungal pathogenesis and its accumulation induces expression of PR genes (Ryals et al. 1996), whereas inhibition of salicylic acid accumulation inhibits expression of PR genes (Takahashi et al. 1997). Salicylic acid binds to catalase, inhibits its activity, and thereby increases the intracellular concentration of H_2O_2, which may then serve as a secondary messenger for the induction of a defense response (Chen et al. 1993). Although salicylic acid induces synthesis of several PR proteins, some of these such as tobacco class I β-1,3-glucanase and chitinase were not induced in response to salicylic acid (Niki et al. 1998)

1.4.3.3 Induction of PR genes by ethylene

Ethylene is a phytohormone that regulated a wide range of plant processes, from growth and development to defense responses (Zhao et al. 2005). However, the role of ethylene signaling can be dramatically different depending on the plant species and the

invading pathogen. For example, soybean mutants with reduced ethylene sensitivity produced less severe chlorotic symptoms when challenged with virulent *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae*, whereas virulent strains of the fungi *Septoria glycines* and *Rhizoctonia solani* caused more severe symptoms (Hoffman et al. 1999).

Ethylene induces basic rather than acidic PR proteins (Knoester et al. 1998). The sequence motif AGCCGCC is highly conserved in the promoters of several PR genes and may constitute ethylene-responsive *cis* elements (EREs) (Hart et al. 1993). Also, several *trans*-acting factors called ethylene-responsive element binding proteins (EREBPs), recently referred to as ethylene-responsive factors (ERFs) (Jiménez et al. 2005), which regulate the ethylene-induced expression of PR genes, have been identified (Ohme-Takagai and Shinshi 1995). AtEBF, a homolog of the tobacco EREBP, has been detected in *Arabidopsis thaliana*. It binds specifically to an AGC box-containing sequence and confers ethylene responsiveness to promoters of genes encoding PR proteins (Buttner and Singh 1997). Ethylene pretreatment in tomato inoculated with *Botrytis cinerea* induced expression of several PR genes (Diaz et al. 2002).

1.4.3.4 Induction of PR genes by jasmonic acid

Jasmonic acid and its cyclic precursors and derivatives produced by the catabolism of fatty acids are commonly refered to as JA signals (Zhao et al. 2005). These are a major group of signaling compounds, and are another signal that systemically induces accumulation of PR proteins in plants during fungal pathogenesis (Schweizer et al. 1997; Zhou et al. 2002). Jasmonic acid induced class I and class IV, but not class II, chitinase in members of the genus *Pinus* (Davis et al. 2002). Methyl jasmonate induced expression of a 23 KDa PR-5 protein in white pine (*Pinus monticola*), whereas salycilic acid treatment did not (Piggott et al. 2004). The *cis*-acting element in promoting jasmonate-induced PR protein synthesis was identified as a G box sequence (CACGTGG) in the promoter of the potato protease inhibitor *pin2* gene (Kim et al. 1992). Ethylene response factors also appear to play important roles in regulating jasmonate-responsive gene expression via interaction with the GCC box (Brown et al. 2003).

1.4.3.5 Induction of PR proteins require different signal transduction systems

Distinctly different signal transduction pathways may exist in triggering induction of PR proteins in plants. The PR protein genes *PR-1*, *PR-2* and *PR-5* were induced by salicylic acid, whereas the *PR-3*, *PR-4*, and *PR-12* genes were induced by jasmonic acid in *Arabidopsis thaliana* (Kunkel and Brooks 2002). Ethylene, but not salicylic acid, induces class I chitinase in tobacco (Penninckx et al. 1996). It has been shown that in some cases there may be a synergistic effect of different signals in triggering PR synthesis (Xu et al. 1994). But, there may also be antagonistic effects between different signals in inducing PR proteins. Jasmonic acid induced PmPR-10 protein accumulation in western white pine (*Pinus monticola*), whereas its induction was suppressed by salicylic acid and abscisic acid (Liu et al. 2003).

1.4.4 Role of PR proteins in inhibiting fungal disease development

The role of PR proteins in inhibiting fungal disease development has been demonstrated both in vitro and in vivo. PR proteins accumulate in both compatible and incompatible interactions (Punja 2001). In many instances, these accumulate more in incompatible interactions (Rasmussen et al. 1992). However, there were also reports indicating that PR proteins accumulate more in compatible interactions. In fact, some proteins were exclusively induced during disease development, and such proteins were not induced in incompatible interactions (Baga et al. 1995).

1.4.4.1 Inhibition of fungal growth by PR proteins in vitro

Purified chitinases were shown to be effective inhibitors of spore germination and hyphal growth. Swelling of the hyphal tips and hyphal distortion was also observed (Benhamou et al. 1993). Disruption of chitin macromolecules in the fungal cell wall preceded cell wall breakdown and protoplasm alteration (Benhamou et al. 1993). PR-4 class I protein from tobacco exhibits antifungal activity toward *Trichoderma viride* and *Fusarium solani* by causing lysis of the germ tubes and growth inhibition (Ponstein et al. 1994b). The PR-5 group of proteins contains many antifungal proteins (Salzman et al. 2004). Defensins (PR-12 proteins) isolated from white spruce (*Picea glauca*) were found to

cause extensive growth inhibition of *Cylindrocladium floridanum*, *Fusarium oxysporum*, and *Neonectria galligena* at 2.5 μM (Pervieux et al. 2004). Thionins (PR-13 proteins) have been shown to be toxic to fungal pathogens; these penetrate fungal cell membranes and inhibit DNA, RNA, and protein synthesis (García-Olmedo et al. 1989). PR-14 proteins (LTPs) have also been shown to be fungitoxic. These may insert into the fungal cell membrane and the central hydrophobic cavity may form a pore, allowing efflux of intracellular ions, thus leading to fungal cell death (Selitrennikoff 2001). Some of the PR proteins act synergistically with other PR proteins in inhibiting the growth of fungi. Different chitinases show more antifungal activity when combined with other proteins such as β-1,3-glucanase and PR-4 protein (Ponstein et al. 1994a, 1994b; Anand et al. 2003).

1.4.4.2 Inhibition of fungal growth by PR proteins in vivo

Inhibitory action of some PR proteins against fungal pathogens has been demonstrated in the infected tissue itself. PR-1 proteins were found to be associated with host cell wall outgrowths and papillae in infected tobacco. These proteins increase mechanical strength of these defense-related structures and inhibit the development of the fungus (Tahiri-Alaoui et al. 1993). Transgenic plants overexpressing PR proteins showed enhanced resistance to fungal pathogenesis and reduction in fungal growth with growth anomalies in hyphae (Epple et al. 1997; Oldach et al. 2001).

1.4.4.3 Indirect action of PR proteins in the defense response

In some cases, PR proteins act indirectly in the defense response and not directly on the pathogen. The role of PR proteins involves the release of elicitor molecules in planta and the reinforcement of cell wall structure. Several chitinases release specific oligosaccharides from the plant cell walls, which act as signal molecules in triggering host defense mechanisms (Van Loon 1999). The PR-9 (peroxidases) proteins were involved in biosynthesis of lignin and suberin (Bernards 2002) which act as a cell wall barrier against fungal pathogens. Petioles of carrot plants overexpressing a rice cationic peroxidase had higher levels of constitutive lignin accumulation compared to control plants and symptoms reduced by up to 90% when infected with *Botrytis cinerea* (Wally et al. 2009). The PR-15

and PR-16 proteins have been suggested to release H_2O_2 necessary for cross-linking of cell wall components during formation of papillae (Wei et al. 1998).

1.4.4.4 PR proteins involved in triggering disease resistance

The role of PR proteins in disease resistance has been demonstrated through inducing mutations resulting in the overexpression of PR proteins that conferred resistance to fungal disease (Penninckx et al. 2003) and by developing transgenic plants in many pathosystems involving fungus infection (Xiong and Yang 2003; Thordal-Christensen et al. 2004). Transgenic hybrid poplar leaves overexpressing a wheat PR-15 gene showed increased resistance against *Septoria musiva* (Liang et al. 2001). The role of PR proteins in disease resistance has also been demonstrated using chemical or biological elicitors. Thiamine treatment induced three rice PR genes, *PR-1*, *PR-9* (*Pox 22.3*, a gene encoding peroxidases) and *PR-10* (*PB 21*). Induction of these PR genes resulted in disease resistance against *Magnaporthe oryzae* (Ahn et al. 2005).

However, not all PR proteins are involved in disease resistance since there were reports that transgenic plants overexpressing some PR proteins do not show resistance to the pathogen (Wang et al. 1999; Moravčikava et al. 2004). There was no guarantee that a protein that was effective in one host against one pathogen would be effective in a different host against a different pathogen (Wang et al. 1999).

1.4.5 How do pathogens overcome PR proteins of the host?

1.4.5.1 Slower accumulation of PR proteins enable pathogens to escape the antifungal action of PR proteins

The virulent pathogen delays accumulation of PR proteins in the host. Histological observations using antiserum and gold antibodies against the tomato PR-1 (PRP14) protein allowed for detection of PRP14 in the roots of a resistant and a susceptible variety at 48 hpi and 72 hpi, respectively (Benhamou et al. 1991). At 72 hpi, the pathogen had already colonized the root tissues in the susceptible variety (Benhamou et al. 1991). The expression of the Osmotin-like protein gene, *CAOSMI*, was higher and occured earlier (12 hpi) in the incompatible than in the compatible interaction (24 hpi) in pepper leaves inoculated with

Colletotrichum coccodes (Hong et al. 2004). The major causes for slower accumulation of PR proteins in the susceptible hosts may be because of the delayed release of elicitors from the cell wall of fungal pathogens into host tissues (Roby et al. 1988; Lawrence et al. 2000). Other mechanisms involved include the absence or reduced action of some elicitors to induce accumulation of PR proteins in susceptible varieties. This was thought to be a result of the absence or reduced presence of receptor molecules for binding the available elicitor molecules in those susceptible varieties (Montesano et al. 2003).

1.4.5.2 Pathogens shed away from the cell wall the substrate for enzymatic PR proteins and avoid the lytic enzyme action

Chitin is an important structural component in the cell walls of plant pathogenic fungi. It is the substrate for PR proteins with chitinase enzymatic activities which cause lysis of hyphal tips (Benhamou et al. 1993). By excluding chitin from its wall, the fungus may not only resist lysis by host chitinases, but also avoid triggering other host defense mechanisms as chitin also acts as an elicitor of plant defense reaction (O'Connell and Ride 1990). Several observations have shown the absence of chitin in specialized infection fungal structures (Chong et al. 1986; Nicole and Benhamou 1991).

1.4.5.3 Pathogens produce enzymes that aid in the protection them from the fungitoxic action of PR proteins

Chitosan is present along with chitin in the cell wall of fungal pathogens. It arises mainly by deacetylation of nascent chitin which was formed by chitin synthase before the polymer chain aggregates to form fibrils (Davis and Bartnicki-Garcia 1984). The chitin deacetylation activity in the infected plant was correlated with hyphal growth in cucumber plants inoculated with *Colletotrichum lindemuthianum* (Siegrist and Kauss 1990). When deacetylation occurs rapidly, the polymer chains in the form of partially N-acetylated chitosan polymer would become less accessible to chitinase and remain bound to the hyphae sustaining the rigidity of the fungal wall (Siegrist and Kauss 1990). N-acetylation of chitin might therefore be a way by which the fungal wall could be partially protected against the fungitoxic action of plant disease.

1.4.5.4 Pathogens produce enzymes to inhibit the activity of some PR proteins

It has been suggested that virulent pathogens may inhibit plant apoplastic proteases and cause disease in susceptible plants (Tian et al. 2004, 2005). PR-7 proteins show serine protease activity that confers disease resistance (Tian et al. 2005). It has been shown that a protease inhibitor EPI10 secreted by the oomycete *Phytophthora infestans* completely inhibited the protease activity of P69b, a PR-7 protein of *Nicotiana benthamiana* (Tian et al. 2005).

1.4.5.5 Less elicitor released from the pathogen's cell wall to activate synthesis of PR proteins

Results obtained by Krebs and Grumet (1993) suggest that the ability of the virulent pathogen to invade and infect the host may reside in its cell wall structure being less accessible to chitinase, avoiding the mechanism through which elicitor is released. Thus less elicitor is released in the case of a virulent pathogen. When a large amount of extracellular matrix (ECM) was released from the conidia of the non-pathogens *Blumeria graminis* f. sp. *tritici* and *Erysiphe pisi*, these induced more resistance in barley against the pathogen *B. graminis* f. sp. *hordei* than when a small amount of ECM was released from the conidia in barley leaves (Fujita et al. 2004)

1.4.5.6 PR proteins degraded quickly in the susceptible host tissues

Acidification of the apoplast appears to be important in degradation of PR proteins. Fungal infection can lead to acidification of the apoplast and activation of host aspartyl proteinase enzyme activity that degrades PR proteins (Rodrigo et al. 1989). Tomato PR proteins were degraded by an aspartyl proteinase that was constitutively present in healthy and infected leaves at similar levels. However, an acidic pH, attained upon fungal inoculation, was required for its activity (Rodrigo et al. 1989). Tobacco leaves were also found to contain an extracellular aspartyl proteinase that endoproteotically cleaves tobacco PR-1a, Pr-1b and PR-1c at an acidic pH (Rodrigo et al. 1991).

1.4.5.7 Site of accumulation of some PR proteins may determine susceptibility or resistance

Intracellular (i.e. vacuolar) PR proteins generally show antifungal activity. Basic chitinases and glucanases that are found intracellularly show antifungal activity, whereas those occurring extracellularly (acidic forms) do not have appreciable antifungal activity (Woloshuk et al. 1991b). For fungal pathogens that grow exclusively in the intercellular space without penetrating plant cells, chitinase or β-1,3-glucanase may not interact with fungal hyphae (Wubben 1992). Woloshuk et al. (1991a) used transgenic tobacco plants to demonstrate that if the vacuolar basic proteins were targeted into the apoplast, these might induce resistance. Although secretion of intracellular PR proteins to the intercellular spaces was possible, delay in PR protein accumulation would nevertheless favor the escape of the pathogen from the plant defense arsenal (Ohashi and Matsuoka 1987).

1.4.5.8 Adaptation of pathogens to PR proteins

It could be possible that the expression of chitinases does not lead to resistance against fungal pathogens because the fungus has adapted to the defense mechanisms of its host. Basic chitinases and β-1,3-glucanases from tomato were overcome by the pathogen *Cladosporium fulvum*, which was insensitive to these PR proteins (Joosten et al. 1995). It has been shown that constitutive overexpression of a basic vacuolar chitinase gene in tobacco did not lead to increased resistance of transgenic plants against *Cercospora nicotianae*. However, chitinases from unrelated species in transgenic plants could not be overcome by the invading fungus (Lamb et al. 1992).

1.4.5.9 Some PR proteins may not be involved in disease resistance

Some PR proteins may not have any inhibitory action against fungal proteins. For instance, class III chitinases (PR-8 proteins) seem to lack antifungal activity (Vogelsang and Barz 1993). Also, there were many reports indicating that PR proteins were only stress-induced because of infection, and that these may not be involved in host defense mechanisms. In tomato, expression levels of genes encoding PR proteins were correlated with the severity of gray mold disease (*Botrytis cinerea*) (Diaz et al. 2002). Thus, PR proteins in this case acted as truly pathogenesis-related and not as defense-related proteins

(Diaz et al. 2002). A transgenic wheat line coexpressing a chitinase and a β-1,3-glucanase gene combination, and another wheat line expressing a *PR-5* gene were developed. Though these lines showed enhanced resistance in the greenhouse in response to a single application of inoculum, none of these showed resistance under field conditions which provided a continuous inoculum of *Fusarium graminearum* (Anand et al. 2003).

1.5 Accumulation of secondary metabolites

1.5.1 Types of secondary metabolites

Plants produce several secondary metabolites that are distinct from the components of intermediary (primary) metabolism, in that these are generally non essential for the basic metabolic process of the plant (Dixon 2001). There are two types of antifungal secondary metabolites: phytoalexins (inducible secondary metabolites) and phytoanticipins (constitutive secondary metabolites) (VanEtten et al. 1994). In general, phytoalexins are defined as the compounds that are synthesized de novo in response to infection, accumulating to antimicrobial concentrations in the area of infection (VanEtten et al. 1995). Whereas phytoanticipins are defined as the compounds that are preformed infectional inhibitors (VanEtten et al. 1994). Phytoalexins and phytoanticipins may belong to the same chemical classes (Dixon 2001), and both accumulate because of infection almost in a similar way (Vidhyasekaran 2007). Both of these have been detected in compatible and incompatible interactions (Dixon 2001; Brader et al. 2006).

1.5.2 Phytoalexins

1.5.2.1. Chemical structure classes of phytoalexins

More than 300 phytoalexins have been identified and characterized (Vidhyasekaran 2007). Phytoalexins constitute chemically heterogeneous groups of substances (Vidhyasekaran 2008c). Several phytoalexins belong to the phenylpropanoid structural class such as isoflavonoids. Another major group of phytoalexins belongs to the terpenoid class such as sesquiterpenoids. Some of the phytoalexins are alkaloids, whereas others are nitrogen- and sulfur-containing compounds. Some phytoalexins belong to fatty acid derivative compounds (Vidhyasekaran 2008c).

In elm, mansonones belonging to a family of sesquiterpenoid quinones have been described as phytoalexins. Seven mansonones (named A, C, D, E, F, G, and H) have been isolated from American elm (Dumas et al. 1983; Duchesne et al. 1986; Yang et al. 1989).

1.5.2.2 Site of synthesis of phytoalexins

Phytoalexins may be released toward the infection sites by living cells of the host undergoing attack by the pathogen (Snyder and Nicholson 1990). Subcellular vesicle-like inclusions appear in the host cell and these inclusions were directed to the fungal penetration sites (Nielsen et al. 2004). Nuclear migration, cytoplasmic streaming, and intracellular pH provide an environment for inclusion trafficking and release of the phytoalexins to the fungal penetration sites (Nielsen et al. 2004). The phytoalexins synthesized in healthy living cells may be secreted from the cells to accumulate in the adjoining necrotic tissue (Brindle et al. 1983).

In elm, high concentrations of mansonones were found only in the immediate vicinity of the *Ophiostoma ulmi* (s.l.) infection (Duchesne et al. 1985; Proctor and Smalley 1988). Parenchyma cells surrounding the xylem were thought to be responsible for the production of these phytoalexins (Yang et al. 1993)

1.5.2.3 Phytoalexins are fungitoxic

Phytoalexins are recognized only based on the antimicrobial activity (VanEtten et al. 1994). Most of these have been reported to be highly fungitoxic (Hrazdina et al. 1997). These were found to be inhibitory to fungal spore germination and hyphal growth (Hrazdina et al. 1997). Phytoalexins may also suppress toxin production by the pathogen (Desjardins et al. 1989).

In elm, in addition to a general inhibition of fungal growth, mansonones have been reported to exhibit several effects on *O. ulmi* (s.l.) physiology and ultrastructure, i.e. ion leakage, respiration rate reduction, cell wall disruption, aggregation of ribosomes, and the accumulation of electron-dense material in the mitochondria (Dumas et al. 1986; Wu et al. 1989).

1.5.2.4 How do pathogens overcome antifungal phytoalexins?

1.5.2.4.1 Pathogens detoxify phytoalexins or suppress their accumulation in compatible interactions

Potential pathogens have been reported to detoxify phytoalexins of the host (Delserone et al. 1999). These produce specialized enzymes to degrade phytoalexins, such as pisatin demethylase encoded by cytochrome P450 in the pea pathogen *Nectria haematococca* (Matthews and VanEtten 1983), and the hydroxystilbene-degrading enzyme of *Botrytis cinerea* (Pezet et al. 1991). These enzymes appear to be pathogenicity factors and were shown to be essential for fungal pathogenesis (Hoos and Blaich 1990; Oeser and Yoder 1994).

Spores from a virulent race of *Magnaporthe grisea* were able to suppress accumulation of phytoalexins in rice leaves, whereas its elicitors induced phytoalexins faster suggesting that a suppressor or a suppressing system may exist in living cells (Iwakuma et al. 1990). Yoshioka et al. (1990) found that the effect of a suppressor isolated from *Mycosphaerella pinoides* on host defense reactions seemed to result from its inhibition of the ATPase in the host plasma membrane.

1.5.2.4.2. Phytoalexins accumulate less and their induction may be delayed in susceptible hosts

The level of accumulation of Phytoalexins may be less in susceptible hosts. In broad bean (*Vicia faba*) leaves infected with *Botrytis fabae*, rapid accumulation of the phytoalexin wyerone acid was observed in resistant cultivars that reached a level greater than twofold of that in the susceptible cultivars (Nawar and Kuti 2003). In elm, inoculation of *U. pumila* with aggressive species *O. novo-ulmi* led to the accumulation of 90 μg/g of mansonones E and F. In contrast accumulation reached only 28 μg/g in inoculated susceptible *U. americana* (Duchesne et al. 1985, 1986).

A delay in the induction of phytoalexins was common in various compatible interactions, and the delay helped the pathogen escape from the toxic environment created by the accumulation of phytoalexins at the infection site (Lo et al. 1999). In susceptible cultivars of sorghum seedlings, notable amounts of phytoalexins accumulated only 72 hpi, but the primary hyphae of the *Colletotrichum sublineolum* pathogen had emerged from infection vesicles by 48 hpi. In contrast, phytoalexins accumulated in considerable amount at about 36 hpi in the resistant cultivar (Lo et al. 1999).

In elm, Duchesne et al. (1985, 1986) showed that resistant *U. pumila* and susceptible *U. americana* differ in the rapidity at which these accumulate mansonones upon infection with *O. ulmi* (*s. l.*). Mansonone E and F accumulation peaked 2 weeks after infection in resistant *U. pumila*. In contrast, it peaked 4 weeks after infection in susceptible *U. americana.*

1.5.2.4.3 Some phytoalexins may not have any roles in disease resistance of plants and the highly toxic phytoalexins may not accumulate in susceptible hosts

In some host-pathogen interactions, phytoalexins may not have any role in disease resistance. Accumulation of phytoalexins may be only a metabolic process activated by stress. In pea, the pathogen *Fusarium solani* f.sp. *pisi* induced more pisatin than the non-pathogen *F. solani* f. sp. *phaseoli* (Kendra and Hadwiger 1987). In both sorghum mesocotyls and leaves, the deoxyanthocyanidin phytoalexin accumulates rapidly following attempted fungal infection by pathogens as well as non-pathogens (Nicholson et al. 1988).

In vitro bioassays showed that luteolinidin and 5-methoxyluteolinidin phytoalexins that accumulate in resistant sorghum cultivars exhibit higher toxicity than other phytoalexin components that accumulate in both resistant and susceptible sorghum cultivars and do not have roles in disease resistance (Lo et al. 1999).

1.5.3 Phytoanticipins

1.5.3.1 Chemical structural classes of phytoanticipins

Phytoanticipins are low-molecular weight, antifungal compounds that are present in plants before challenge by fungal pathogens or are produced after infection solely from preexisting constituents (VanEtten et al. 1994). Numerous antifungal phytoanticipins have been detected in plants. These belong to several chemical classes including: phenolics, flavonoids, terpenoids and steroids (Vidhyasekaran 2008c).

1.5.3.2 Phenolics as phytoanticipins and the toxicity

Several phenolics and phenylpropanoids that may act as phytoanticipins have been detected in plants (Vidhyasekaran 1988). Phenolics such as isoflavones and isoflavans were highly toxic to fungal pathogens (Weidenborner et al. 1990). However, both the toxicity of different phenolics and the sensitivity of pathogens to the phenolics vary (Cahill and McComb 1992). The toxicity of phenolics has been demonstrated by artificially increasing the synthesis of phenolics in some plants. The flavonoid epicatechin plays an important role as phytoanticipin in avocado fruits (Guetsky et al. 2005). Inoculation of freshly harvested avocado fruits with a mutant strain of *Colletotrichum magna* inhibited subsequent decay development by the pathogen *C. gloeosporioides*. The mutant strains induced higher levels of the phenolic epicatechin (Prusky et al. 1994).

1.5.3.3 How do pathogens overcome the anti-fungal phenolics?

1.5.3.3.1 Pathogens degrade phenolics to nontoxic products

Nicholson et al. (1989) showed that the proline-rich proteins found in the mucilage of spores of some foliar fungal pathogens may protect conidia from toxic phenols that accumulate in the water which is necessary for conidium dispersal and secondary spread of the fungus. The enzymes found in the mucilage (i.e. β-glucosidase and non specific esterase) may cleave the phenolic esters and glycosides, freeing the aglycones and making these more available for binding to the extracellular proline-rich proteins of the mucilage (Nicholson et al. 1986). Guetsky et al. (2005) showed that *Colletotrichum gloeosporioides* produced a laccase that may be a pathogenicity factor. This laccase degrades epicatechin in culture and infected avocado fruit tissues. Isolates of the fungus with reduced laccase activity and no capability to metabolize epicatechin showed reduced pathogenicity on ripening fruits (Guetsky et al. 2005).

1.5.3.3.2 Pathogens suppress production of phenolics in plants

The pathogen may suppress phenolics by: 1) preventing their accumulation, 2) suppressing enzymes involved in biosynthesis, and 3) using a suppressor molecule.

A correlation between accumulation of phenolic compounds and resistance in alfalfa stems inoculated with *Colletotrichum trifolii* was reported by Baker et al. (1989). In resistant plants, phenolics accumulated to higher levels, whereas in susceptible plants, phenolic synthesis did not seem to be highly induced (Baker et al. 1989). Thus, reduced induction of phenolic synthesis may occur in compatible interactions.

Cahill and McComb (1992) showed that suppression of PAL using aminooxyacetic acid, a PAL inhibitor, led to a reduction in the synthesis of phenolics which rendered the resistant *Eucalyptus calophylla* susceptible to the oomycete *Phytophthora cinnamomi*. In the susceptible *E. marginata,* the activity of PAL increased only slightly in the first 24 hpi and declined by up to 77% of control level at 96 hpi (Cahill and McComb 1992). Thus, the pathogen may suppress phenol biosynthetic enzymes in compatible interactions.

A glycoprotein isolated from the chickpea pathogen *Ascochyta rabiei* was found to cause a concomitant decrease in the accumulation of all phenolic constituents of chickpea. The glycoprotein was called a suppressor (Kessmann and Barz 1986). Vidyasekaran et al. (1992) showed that a toxin produced by *Helminthosporium oryzae* was able to suppress the phenolic content and PAL activity in rice leaves resulting in severe incidence of the disease. Oxidized phenolics were more toxic than phenolics, and polyphenol oxidase (PPO) was involved in the oxidation of phenolics (Retig 1974). Some pathogens, such as *Alternaria alternata,* possess a tentoxin and were able to suppress oxidation of phenolics by inhibiting PPO with the tentoxin (Vaughn and Duke 1984). Thus, in compatible interactions, the pathogen may suppress phenolics metabolism by a suppressor molecule or a toxin.

1.6 Conclusion

Walls are the first barrier of plant cells against fungal pathogen attack, and appear to be important to prevent penetration by the pathogen. While the pathogen tries to penetrate the plant cell wall, the plant tries to reinforce its cell wall with compounds such as lignin, suberin, and wall-bound phenolics. In the compatible interaction, however, this reinforcement process seems to be delayed. The mechanisms causing the delay are still not

fully understood. Yet this process, along with plant cell wall degradation, appear to be key events in the interaction.

Another important event occurring during fungal pathogenesis in plants is the induction and accumulation of new readily detectable proteins called PR proteins. At least 17 structurally different families of PR proteins have been reported in the plant kingdom. Different signal transduction systems are involved in induction of PR proteins. The time and amount of accumulation of PR proteins differ in compatible and incompatible interactions. Several PR proteins have been shown to have antifungal activity in vitro and many transgenic plants overexpressing individual or combinations of PR proteins showed enhanced disease resistance. Some PR proteins are involved in reinforcement of host plant cell walls and in the induction of disease resistance mechanisms. However, some PR proteins did not directly enhance disease resistance and some accumulated more in susceptible than in resistant tissues. More studies are needed to uncover the function and the implication of PR proteins in disease susceptibility or resistance.

Several antifungal secondary metabolites have been detected in plants. Some of these are synthesized de novo in response to infection, and labelled phytoalexins. Others are preformed infectional inhibitors and labelled phytoanticipins. The secreted phytoalexins inhibit fungal spore germination and hyphal growth. However, virulent pathogens are able to detoxify phytoalexins using specialized degrading enzymes. Induction of phytoalexin synthesis was delayed in the compatible interaction, where phytoalexins seem to accumulate at a time when the pathogen had already invaded the host tissues. The amount of phytoalexins produced may not be sufficient to inhibit the pathogen growth in compatible interactions, whereas only less toxic phytoalexins accumulate in some susceptible plants. Several phenolics act as phytoanticipins and most of these are highly toxic to fungal pathogens. Nevertheless, virulent pathogens were able to degrade these phenolics into non-toxic or less toxic ones. Specific phenolic degrading enzymes have been identified in spore germination fluid. Virulent pathogens were also able to suppress production of phenolics in a compatible interaction, and some phenolics may not accumulate to fungitoxic level in susceptible plants.

It seems that susceptible plants do not lack defense mechanisms to defend against pathogen attack. Rather, it seems that an anomaly has occurred in the interaction with the

microbe. This anomaly is reflected at the molecular level, by a delay, a lack of coordination, or an insufficiency in responses, that render the plant susceptible. Thus, gaining a better understanding of the biochemical processes leading to susceptibility would be important and could help develop better control strategies for a given plant disease.

2. Fungal colonization and host defense reactions in *Ulmus americana* callus cultures inoculated with *Ophiostoma novo-ulmi*

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2.1 Résumé-Abstract

L'interaction hôte-agent pathogène conduisant à la maladie hollandaise de l'orme a été analysée à l'aide d'un système in vitro en utilisant des tests histo- et cyto-chimiques. On a inoculé des cellules levuriformes de l'agent pathogène agressif *Ophiostoma novo-ulmi* à des cultures de cals durs et mous de l'espèce sensible *Ulmus americana*. Les tissus de cals inoculés ont été comparés à des tissus de cals traités à l'eau en utilisant la microscopie photonique et électronique (MET) à transmission et à balayage (MEB). De nouveaux aspects de l'interaction sont décrits. Ceux-ci incluent l'observation histologique de la subérine et, pour la première fois dans des cultures de cals de plantes, de la mise en évidence en MET de sa structure lamellaire, de même que la présence intracellulaire d'*O. novo-ulmi*. L'expression du gène de la phénylalanine ammonia-lyase (PAL), analysée en PCR quantitative (qRT-PCR), a été corrélée à la formation de subérine, de phénols et de lignine dans les cals infectés. Cette étude valide l'utilisation potentielle du système in vitro pour des analyses génomiques afin d'identifier les gènes induits au sein du pathosystème de la maladie hollandaise de l'orme.

The host-pathogen interaction leading to Dutch elm disease was analyzed using histo- and cyto-chemical tests in an in vitro system. Friable and hard susceptible *Ulmus americana* callus cultures were inoculated with the highly aggressive pathogen *Ophiostoma novo-ulmi*. Inoculated callus tissues were compared with water-treated callus tissues and studied with light, transmission- (TEM) and scanning- electron microscopy (SEM). New aspects of this interaction are described. These include the histological observation, for the first time in plant callus cultures, of suberin with its typical lamellar structure in TEM and the intracellular presence of *O. novo-ulmi*. Expression of the phenylalanine ammonia lyase (PAL) gene, monitored by real-time quantitative PCR, was correlated with the accumulation of suberin, phenols, and lignin in infected callus cultures. This study validates the potential use of the in vitro system for genomic analyses aimed at identifying genes expressed during the interaction in the Dutch elm disease pathosystem.

2.2 Introduction

During the last century, elm populations worldwide were decimated by two successive pandemics of Dutch elm disease (DED) caused by the ascomycete fungi *Ophiostoma ulmi* (Buism.) Nannf. and *O. novo-ulmi* Brasier, respectively. In North America, the highly susceptible *Ulmus americana* L., one of the most widely planted shade trees in urban areas, suffered heavy damage, with local populations often seriously reduced, for example by up to 80% in Toronto (Huntley 1982).

Research on the host-pathogen interaction leading to DED and efforts in developing more resistant plant material have been made mostly on elm saplings and trees. For example, the accumulation of mansonone phytolaexins (Duchesne et al. 1985, 1986) and the formation of barrier zones (Shigo and Tippet 1981) impregnated with suberin, phenolics, and lignin (Shigo and Tippet 1981) have been proposed as induced mechanisms by which infected elms attempt to stop the spread of the pathogen within their vascular system. Disease-tolerant *U. americana* clones (Townsend et al. 1995) and interspecific hybrids (Smalley and Guries 1993) have been obtained through conventional selection and breeding programs (Mittempergher and Santini 2004).

In vitro systems based on callus and suspension cultures have also been used for the study of DED. These provide a more controlled environment, make it possible to analyze one factor at a time all year long, and facilitate manipulation of both host and pathogen. Investigators have relied on elm callus culture for rapid screening of resistant host genotypes or aggressive isolates of the pathogen (Pijut et al. 1990a; Domir et al. 1991; Schreiber et al. 1994; Diez and Gil 1998). The use of elm tissue culture has also brought insights into some aspects of the host-pathogen interaction. Microscopic observations of fungal growth in inoculated callus culture showed the occurrence of a dense hyphal network and intensive conidia formation in susceptible *U. americana* callus tissue (Domir et al. 1992; Krause et al. 1996). The latter exhibited mitochondrial and cytoplasmic disruptions as well as extensive vacuolation and plasmolysis, whereas resistant callus tissue were characterized by heavy deposition of starch grains and electron-opaque material thought to be phenolic-like (Pijut et al. 1990b, Krause et al. 1996). Mansonone phytoalexins were also shown to accumulate in elm callus tissue treated with either fungal

inoculum (Duchesne et al. 1994) or culture filtrates (Yang et al. 1989). Analysis of the latter led to the identification of a glycoprotein mansonone-inducing elicitor in both *O. ulmi* and *O. novo-ulmi* (Yang et al. 1994). Using elm suspension cultures, Corchete et al. (1993) also showed that inoculation of the DED-resistant *U. pumila* with spores of *O. ulmi* induced an increase in phenylalanine ammonia-lyase (PAL) activity, whereas no stimulation was observed in the susceptible *U. campestris*.

A large-scale genomic analysis of *O. novo-ulmi* is currently under way (Bernier et al. 2004). The objective of this study was to establish the suitability of callus cultures as an experimental system for facilitating the genomic study of the interaction between the susceptible *U. americana* and yeast-like cells of the aggressive pathogen *O. novo-ulmi*. Analyses included microscopic observations of fungal colonization of callus tissues, histochemical characterization of elm cell responses to fungal infection, and transcript profiling of the *PAL* gene, which was selected as a molecular marker for defense reactions.

2.3 Materials and methods

2.3.1 Plant tissue culture

Stable hard and friable callus lines were grown from the DED- susceptible species *U. americana*. Hard callus cultures were initiated from buds collected in May 2003 from a single tree on the Université Laval campus (Québec, QC, Canada). Explants were treated according to Yang and Bernier (1996). Friable callus cultures were initiated from mature seed collected in June 2001 near trees also located on the campus. After seed germination, cotyledons and hypocotyls were soaked for 20 min in a solution of 0.5% sodium hypochlorite and 0.01% Tween 20, and rinsed three times with sterile, distilled water. Explants were cultured on modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (Sigma-Aldrich, St. Louis MO, USA) (catalog no. M5519, 4.4 g/L) supplemented with 3% (w/v) sucrose, 5.4 µM naphthaleneacetic acid (Sigma-Aldrich), 4.4 µM benzyladenine (Sigma-Aldrich), and 0.48% (w/v) Agargel (Sigma-Aldrich), as described by Eshita et al. (2000). Cultures were incubated in the dark at 23 °C and subcultured to fresh medium every 4 (friable callus cultures) or 6 weeks (hard callus cultures).

2.3.2 Inoculations

The aggressive *O. novo-ulmi* subsp. *novo-ulmi* isolate H327 (Et-Touil et al. 1999) was used in this study. Budding cells were produced by incubating a 2 mm mycelium disk in *Ophiostoma* liquid minimal medium (Bernier and Hubbes 1990a) supplemented with L-proline (1.15 g/L). After 3 days of incubation at 23-24 °C with shaking (120-170 rpm), the cells were filtered through cheese cloth, centrifuged at 4,500 × g for 5 min, and resuspended in sterile, distilled water at a concentration of 2×10^6 budding cells/mL (Domir et al. 1991). A final transfer of 40 mm^2 callus pieces on 5 cm diameter Petri dishes containing 10 ml of fresh MS medium was made 11 days prior to inoculations (Krause et al. 1996). Callus inoculation with either sterile water or fungal cells were made as follows: a 6 mm diameter blank paper disk (Becton Dickinson, Sparks, MD, USA) was cut in half, sterilized by autoclaving, soaked with 20 µl of either fungal inoculum or water, air-dried in a laminar flow cabinet, and placed at the center of the top portion of the callus. Additional

controls included inoculum or water disks placed on sterile *Ophiostoma* complete medium (Bernier and Hubbes 1990a) and modified MS medium (Eshita et al. 2000) over a cellophane membrane.

2.3.3 Tissue processing for microscopy

Three friable callus lines and one hard callus line were used for microscopy. Samples from each line were taken at 24, 48, 72, and 96 h post-inoculation (hpi). For each time, one infected callus from each line was fixed and controls were taken 96 h following inoculation with sterile water. Different parts of a callus were fixed to estimate the extent of colonization by the fungus. Samples were fixed with 3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer (SCB) (pH 7.4) under vacuum for 2 h, rinsed in the same buffer, and post-fixed with 1% osmium tetroxide (OsO_4) in 0.1 M SCB for 1 h under vacuum. After dehydration in ethanol series, samples were embedded in JEMBED 812 resin (Canemco Inc., Montréal, Québec). For light microscopy (LM), sections (1 µm) were obtained with an Ultracut E microtome (Reichert-Jung, Vienna, Austria), stained with 2% toluidine blue O followed by 1% safranin O (Rioux and Ouellette 1989), and observed with a Polyvar light microscope (Reichert-Jung, Vienna, Austria). For transmission electron microscopy (TEM), ultrathin sections (90 nm) were contrasted with uranyl acetate and lead citrate (Reynolds 1963) (Fisher Scientific International, Fairlawn, NJ, USA) and examined with a Philips 300 (Eindhoven, The Netherlands) electron microscope operating at 80 kV. For scanning electron microscopy (SEM), samples were processed as for TEM. In addition, samples were dehydrated in hexamethyldisilazane (Sigma-Aldrich), and sputter-coated with gold before examination with a JEOL JSM6360LV microscope (JEOL USA Inc., Peabody, MA) at 10 kV.

For histochemical tests, samples were fixed in 4% formalin diluted in 0.1 M phosphate buffer (pH 7.4) and dehydrated in ethanol series. Ethanol was gradually replaced by Protocol® Safeclear (Fisher Scientific, Middletown, VA, USA) and the samples were thereafter placed in pure Paraplast (Oxford Labware, St. Louis, MO, USA) for 3 days at 65 °C, with replacement every 24 h. Longitudinal sections (5 µm) were obtained with a Jung 2035 rotary microtome (Heidelberg, Germany), deparaffined, and processed as described below. For detection of suberin, sections were treated with phloroglucinol-HCl, which

stains lignin red and masks its autofluorescence, and examined under ultraviolet light (UV) excitation (Biggs 1984) using a BP 330-380 exciter filter with a DS 420 separator mirror and a LP 418 barrier filter. Sudan black staining (Jensen 1962) was carried out on some sections as additional evidence of suberin deposition. The number of suberized cells was calculated under UV light after phloroglucinol-HCl staining. The total number of cells and the number of suberized cells were counted in ten optical fields taken randomly at 40 X magnification; hence, for each section, at least 1000 cells were counted. For detection of condensed tannins, sections were immersed in 0.1% 4-dimethylaminocinnamaldehyde (DMCA) and 0.5 M sulfuric acid in butanol for 2 min (Valette et al. 1998). Tannins such as pro-anthocyanidins, appeared dark blue or blue-green. Less condensed tannins (oligomers) and monomers, such as catechins, were stained red after immersion for 5 min in a solution of 10% vanillin in one volume of absolute ethanol and one volume of concentrated HCl (Valette et al. 1998).

For TEM, the lamellar structure of suberized layers was made more obvious by contrasting the sections with 1% OsO_4 followed by a mixture of one volume of 2% uranyl acetate (UO_2Ac) and one volume of 1% potassium permanganate ($KMnO_4$) (Wattendorff 1974). Cellulose molecules were labeled with an exoglucanase (EC 3.2.1.91, kindly provided by Dr. C. Breuil, University of British Columbia, Vancouver, BC, Canada) having an affinity with ß-(1,4)-D-glucans. The enzyme was gold-complexed and used according to Nicole et al. (1992). Ultrathin sections were floated on a drop of 0.01 M phosphate buffered saline (PBS) (pH 6.5) containing 0.02% polyethylene glycol 20,000 for 10 min, and then incubated with the exoglucanase gold complex for 30 min. Before contrasting with uranyl acetate and lead citrate for examination in TEM, sections were rinsed with PBS, and thereafter with filtered distilled water. Control tests were carried out using the enzyme gold complex previously adsorbed with ß-(1,4)-D-glucans.

2.3.4 Quantitative RT-PCR

Elm translation initiation factor 5A (EIF5A) was used as a reference gene to monitor the expression of the elm PAL gene. An EIF5A expressed sequence tag (EST) designated C2-F8 was obtained from a subtractive cDNA library constructed in our laboratory. The nucleotide sequence is available in the GenBank EST database under the

accession number FC325680. A partial cDNA sequence from an *Ulmus americana* phenylalanine ammonia-lyase (PAL) mRNA was obtained from the GenBank nucleotide database under the accession number DQ078279. These two cDNAs sequences (FC325680 and DQ078279) were used to develop qRT-PCR primers (EIF5AF: 5' GTTTTGCTGAGGGAAAAGACTTGGT 3';
EIF5AR: 5'AACGCTACCAAAGGCATACTTGTGA 3'; product size: 217 bp;
PALF: 5' TGCCCAAAGAAGTTGAGAGTTCAAG 3';
PALR: 5'AGTCCCCAATTCCTCTCTCACAAAC 3'; product size: 122 bp).

Hard callus cultures were inoculated with either fungal cells or water as described previously, and harvested in liquid nitrogen at 4, 24, 48, 72, 96, and 144 hpi. There were three replicates per treatment and each treatment was composed of three callus weighting between 130 and 170 mg each and harvested together. In addition, three healthy callus were harvested at the beginning of the experiment (0 hpi) as an additional control. Five hundred nanograms of total RNA from each sample were extracted according to Chang et al. (1993) and used for first strand cDNA synthesis with SuperScript™ II Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo $(dT)_{12\text{-}18}$ (500 µg/ml) according to the manufacturer's instructions. These cDNAs were diluted 1:5 in sterile water prior to real-time PCR quantification.

The absence of genomic DNA contamination was verified by running all RNA samples in the same run with cDNA samples. No amplification was detected with RNA samples. Quantitative RT-PCR was performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the QuantiTect SYBR PCR kit (Qiagen Inc., Mississauga, ON, Canada). The three biological replicates were run on the same plate. All qRT-PCR reactions contained cDNA equivalent to 10 ng of total RNA. Amplifications were performed in a 15 µL reaction volume in 2X Quantitect SYBR Green mixture (Qiagen) with 0.3 µM of 5' and 3' primers. After an initial 15-min activation step at 95 °C, 40 cycles (94 °C for 10 s, 62 °C for 2 min) were performed, and a single fluorescent reading was obtained after each cycle immediately following the annealing and elongation step at 62 °C. A melting curve was analyzed at the end of cycling to ensure that there was a single amplification product. Threshold cycle (C_T) values were determined with the software supplied with the instrument. Standard curves generated using cDNA clones as

templates were used to transform C_T values obtained from total RNA samples into transcript numbers. PAL samples were normalized with EIF5A samples.

2.3.5. Statistical analysis

Statistical analyses were done with SAS version 9.1 Software (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) according to general linear models procedure (GLM) procedure and Tukey's tests were used to analyze qRT-PCR data. For all analyses, when normality or homogeneity of residues was not respected, data were first transformed by rank.

2.4 Results

2.4.1 Fungal development

When plated directly onto MS medium, budding cells of *O. novo-ulmi* H327 developed into mycelial colonies that reached only half of the 8.5 cm diameter of Petri dishes, even when left for up to 50 days in culture. In contrast, it took the fungus only 4 days to fill dishes containing *Ophiostoma* complete medium. When examined in SEM, few or no conidia were observed on hyphae from MS media. The fungus developed more extensively on elm callus than on MS medium. Budding cells germinated into hyphae with typical morphology, such as those bearing conidia. Mycelium and spores were seen in all parts of the inoculated callus by 48 hpi, for both types of callus tissue. By 96 hpi, the fungus had reached the medium in the plate, the color of the callus had changed to yellow and necrosis was seen under the inoculation point. In the water-treated hard and friable callus tissues, all cells kept a healthy appearance except occasionally for an area just under the disk, indicating cell damage or a reaction to the presence of the disk.

Fungal colonization (Fig.2.1A-C) of the callus took place mostly in intercellular spaces, the fungus being more abundant in friable callus where empty spaces were larger and more numerous (Fig.2.1B). However, intracellular presence of the fungus was also observed in both types of callus tissues by light (Fig.2.1A,B) and electron microscopy (Fig.2.1C). Direct penetration of the host cell wall was occasionally observed (Fig.2.1D-F).

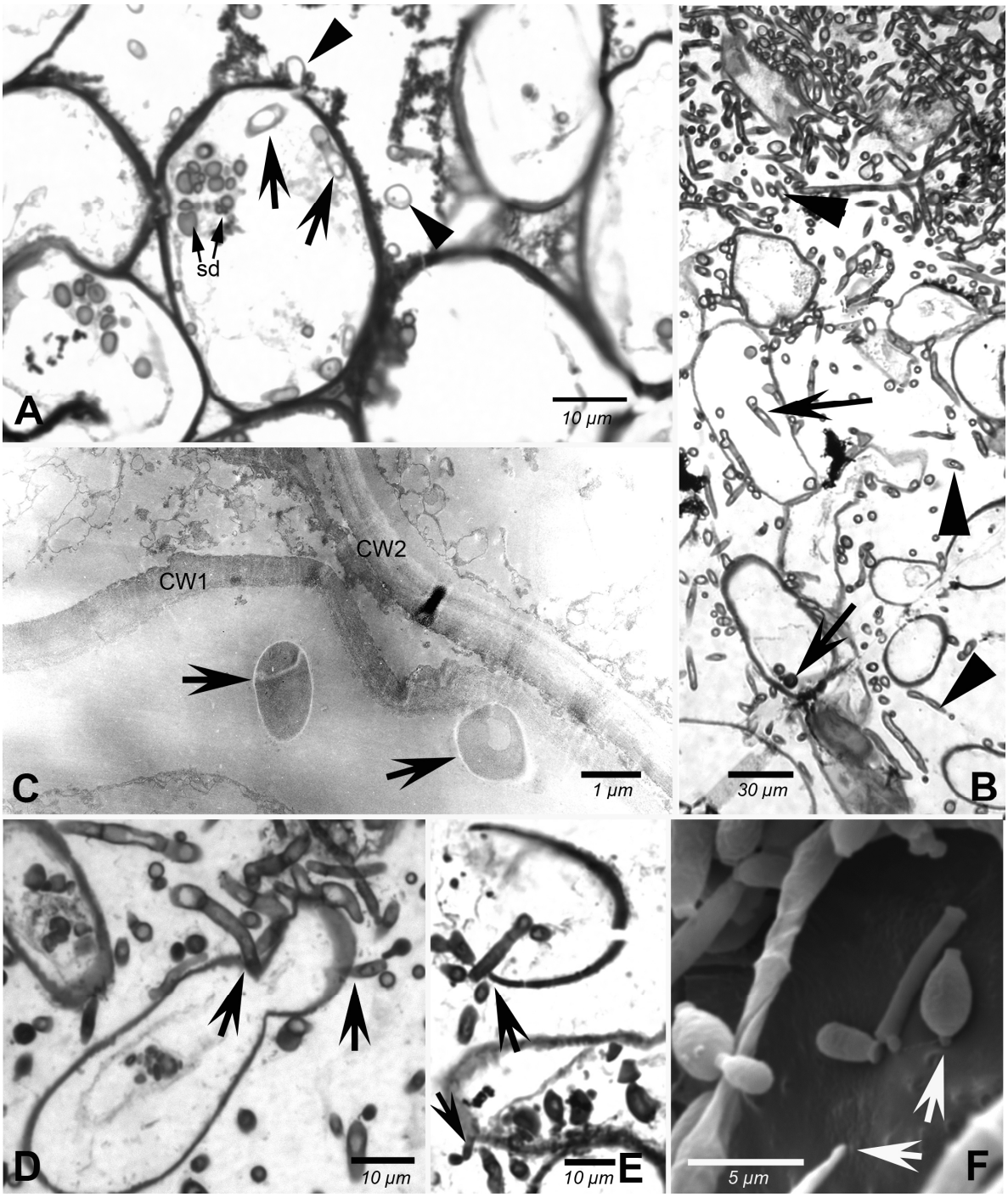

Figure 2.1: Fungal development in *U. americana* callus cultures inoculated with *O. novo-ulmi*.

A-B, Presence of pathogen cells at the center of friable callus samples at 48 h post-inoculation (hpi) and 96 hpi, respectively. The fungus is mainly observed in intercellular spaces (arrowheads), but its intracellular presence (arrows) is also shown; sd: starch deposits. **C**, In TEM, two fungal cells (arrows) are clearly visible in a host cell. Sections from the center of a hard callus at 48 hpi. CW1 and CW2: walls of two callus cells. **D-F**, Penetration of elm callus cell walls by the pathogen (arrows) as observed in friable callus

samples in LM at 72 hpi (**D**) and 96 hpi (**E**) and in SEM on the surface of a hard callus at 48 hpi (**F**).

2.4.2 Callus morphology and reactions

The main structural difference observed between healthy friable and hard callus tissues was the organization of the cells. In friable callus tissue, cells were dissociated and loose with many intercellular spaces, whereas in hard callus tissue cells were grouped in closely packed layers leaving almost no space between the cells. No important differences were noted in the histopathology of the three friable callus lines used in the study, and the cell reactions described in this section were found in both types of callus tissue, friable and hard, unless otherwise stated. Phenolic and starch deposits were more abundant in inoculated callus samples than in controls. This deposition was seen by 48 hpi, particularly in layers of cells located just beneath the front line of dead cells in direct contact with the fungus (Figs. 2.2A-C and 2.3A). Positive vanillin tests confirmed the phenolic nature of these opaque deposits and showed the abundance of less condensed tannins, such as catechins, in infected callus tissues (Fig. 2.3B). More condensed tannins detected through the DMCA test became evident only at 96 hpi (results not shown).

Suberization and lignification of cell walls were especially apparent in hard callus. The light-blue autofluorescence emitted by suberin was conspicuous under UV illumination, particularly after quenching lignin autofluorescence with phloroglucinol-HCl (Fig. 2.3C). Staining with Sudan black B and phloroglucinol-HCl confirmed the presence of suberin in these cells as well as that of lignin in the walls of many adjacent cells (Fig. 2.3D). In electron microscopy, cell wall reinforcements and alterations were also evident, mainly in the vicinity of fungal cells (Fig. 2.4). In TEM, the exoglucanase gold labeling was a means to help localize suberized layers as most of these were nearly devoid of gold particles while adjacent layers were often strongly labeled (Figs. 2.2C and 2.4A). After contrasting the sections with OsO_4 and $UO_2Ac/KMnO_4$, the typical presence of electron-opaque and electron-translucent lamellae within suberized layers became particularly evident (Fig. 2.4B). The proportion of suberized cells, as determined under UV light microscopy, was higher in callus samples inoculated with *O. novo-ulmi* than in water-

treated callus samples (Fig. 2.5). At 96 hpi, the former showed a 4.6-fold increase over the latter.

Cell wall degradation was especially obvious in callus cells in close contact with fungal hyphae (Fig.2.4C-E). Even when *O. novo-ulmi* cells were not detected close to the wall, in those callus areas where degradation was prominent, the exoglucanase gold labeling in TEM frequently revealed that cellulose was apparently degraded within certain host wall areas (Fig. 2.4F). The impact of the pathogen on cell walls was also obvious in SEM (Fig. 2.4G) with collapsed cells in infected callus when compared with control callus (Fig. 2.4H) in which cells were turgescent.

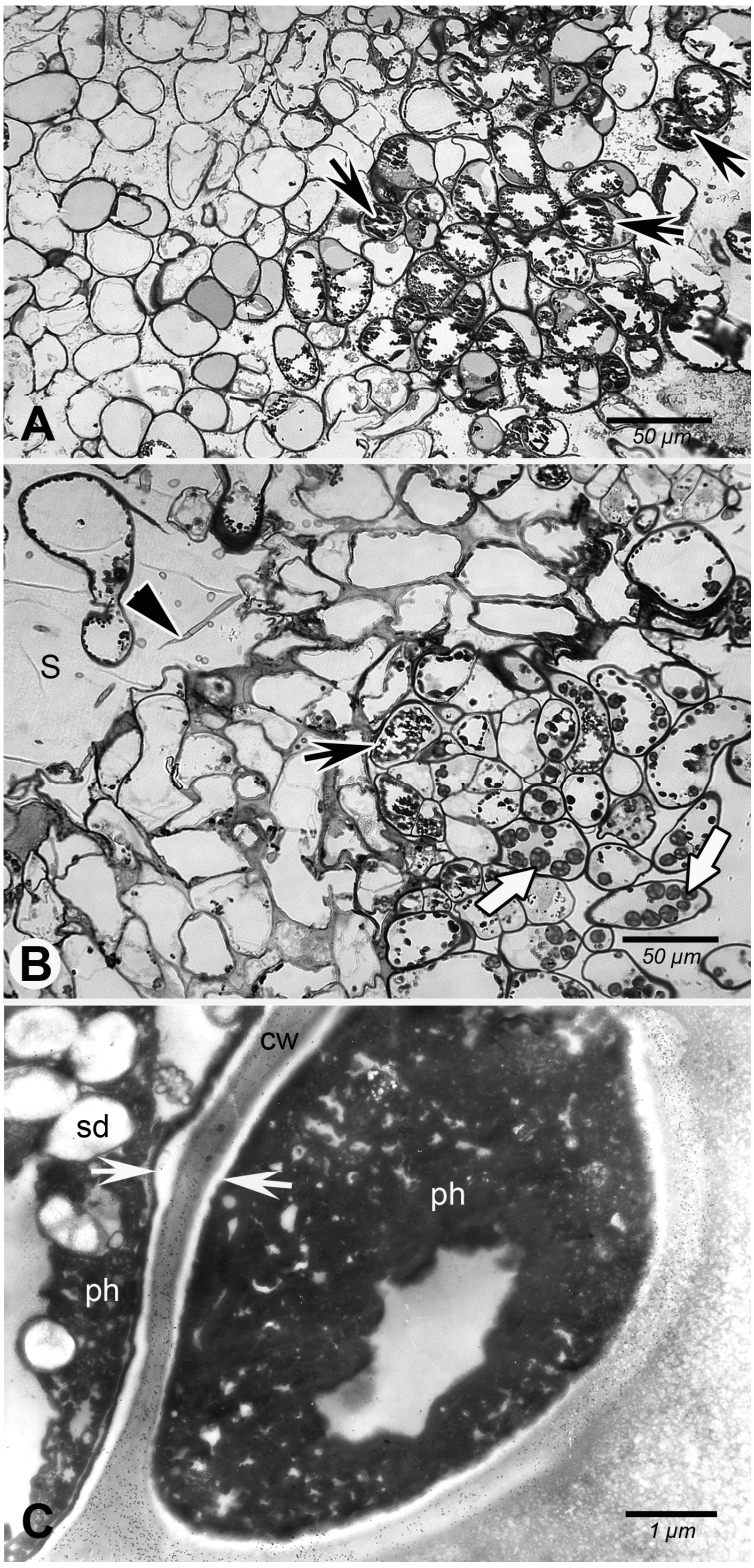

Figure 2.2: Phenol accumulation and starch deposits in *U. americana* callus cells after inoculation with *O. novo-ulmi*.

Figure 2.2: A-B, Friable callus samples at 48 and 72 h post-inoculation (hpi), respectively. Phenols (black arrows) appear dark-stained with toluidine blue. Starch grains (**B**, white arrows) are obvious in host cells located just beneath the front line of dead cells in direct contact with the fungus (arrowhead) at the surface (S) of the callus. **C**, Electron-opaque phenolic-like material (ph) adjacent to translucent suberized wall layers (arrows) in host cells of a hard callus at 72 hpi. The exoglucanase-gold labeling is intense over the host cell wall (cw) while it is virtually absent over the suberized layers; sd: starch deposit.

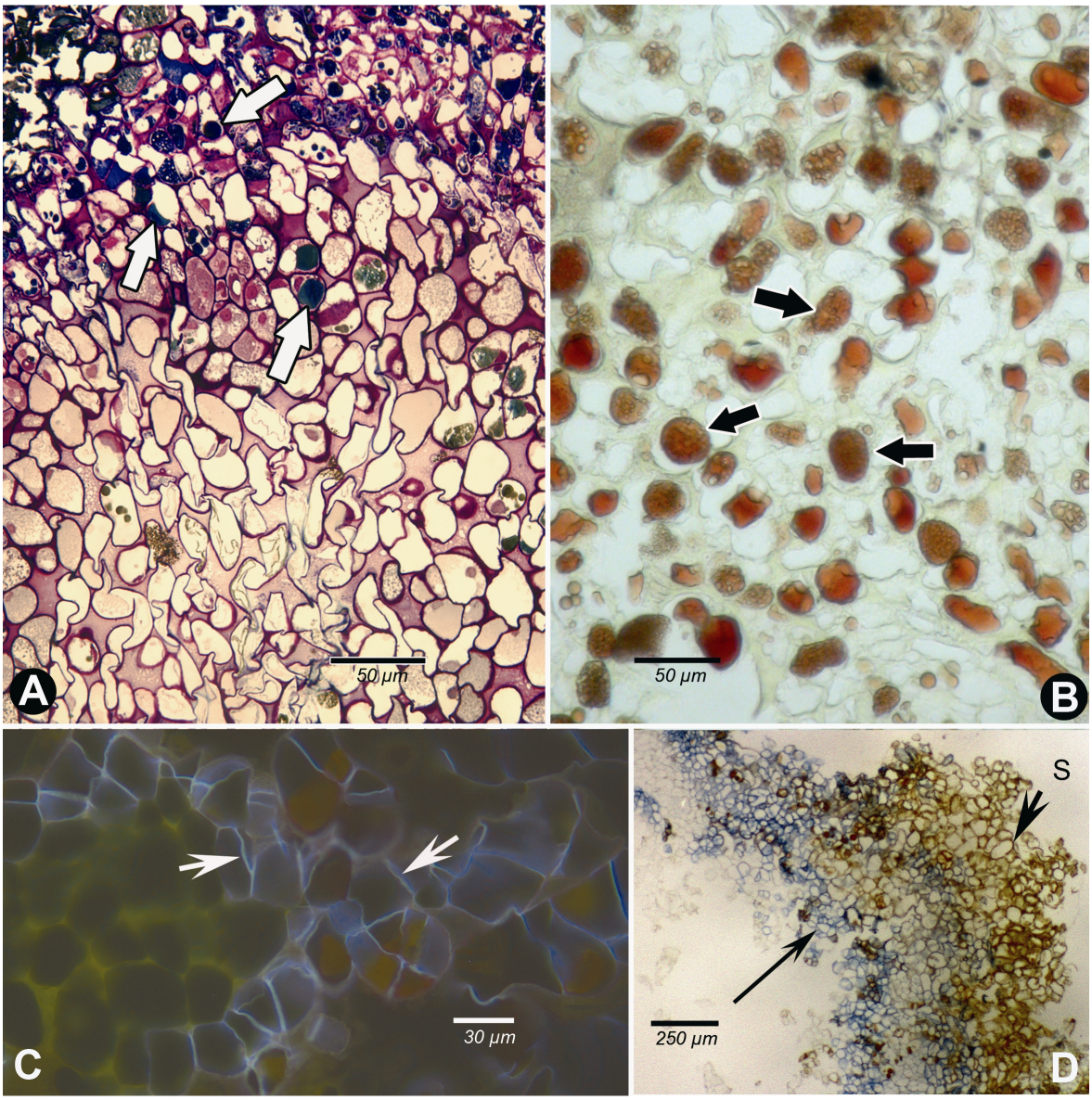

Figure 2.3: Defense reactions in hard *U. americana* callus cultures inoculated with *O. novo-ulmi*.

A, Cells rich in phenols (arrows) stained blue with toluidine in this sample collected at 72 h post-inoculation (hpi) on the surface of this callus. **B-D**, Histochemical tests revealing the chemical nature of some of the cell defense reactions. **B**, Heavy accumulation of tannin oligomers and monomers (e.g. catechins) stained in red (arrows) using the vanillin test at 96 hpi. **C**, Suberin autofluorescence (arrows) revealed under UV illumination in a section previously stained with phloroglucinol-HCl at 48 hpi. **D**, Lignified cells (short arrow) at the surface (S) of this callus at 48 hpi appear brownish-red after staining with phloroglucinol-HCl, whereas suberized cells (long arrow) formed more internally are stained dark blue with Sudan black B.

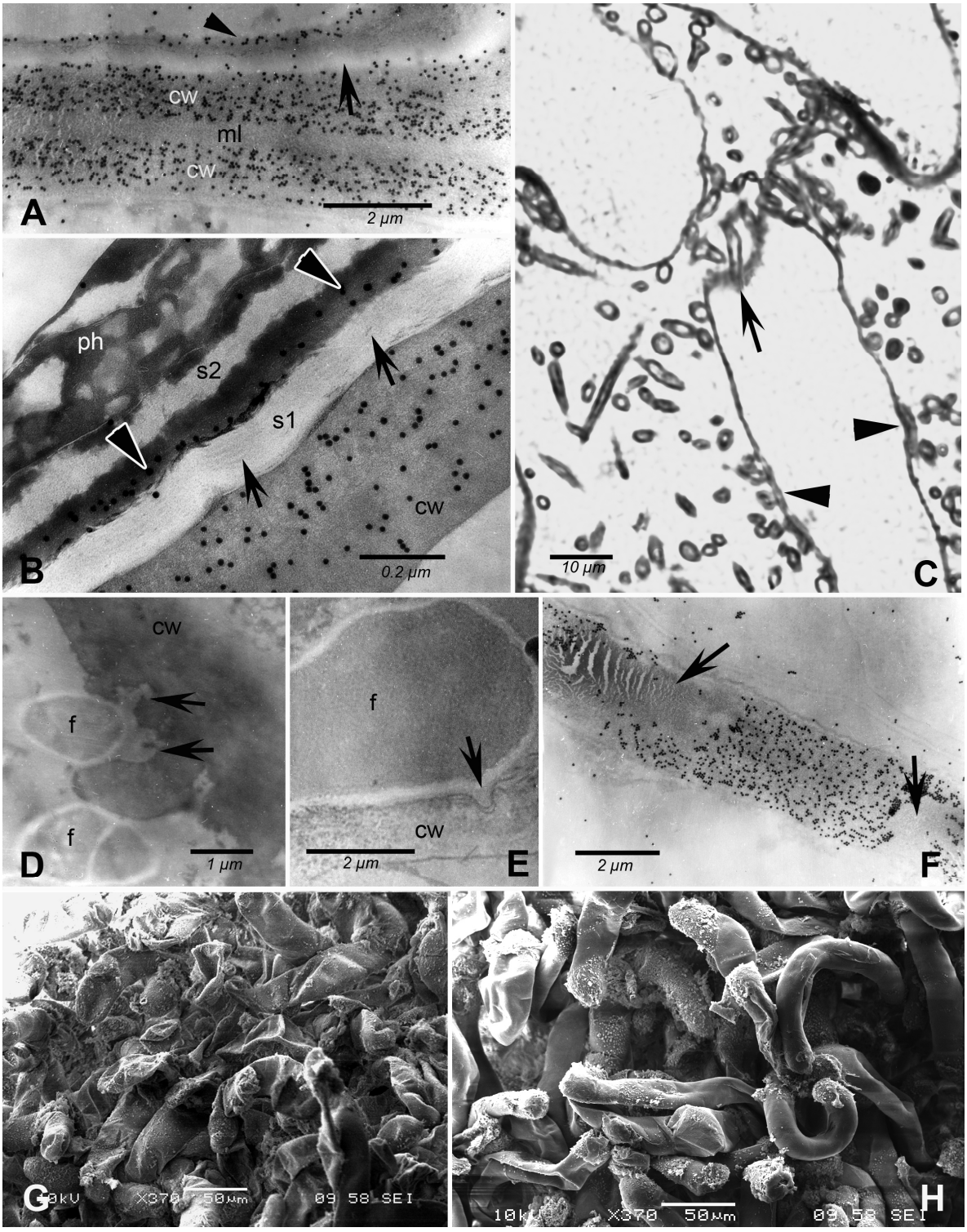

Figure 2.4: Cell wall modifications in *U. americana* callus cultures inoculated with *O. novo-ulmi*.

A-B, Suberized cell wall layers (arrows) are shown next to primary cell walls (cw) intensely labeled with the exoglucanase-gold complex in these hard callus cells at 48 h

post-inoculation (hpi). Note the formation of another wall layer (arrowheads) labeled with the exoglucanase-gold complex internally to the first suberized layer; ml: middle lamella. **B**, A second suberized layer is also seen (s2). The typical lamellar structure of suberin is evident (arrows) in the thicker layer after contrasting with OsO_4 and $UO_2Ac/KMnO_4$. Phenol-like (ph) material is present in the cytoplasm. **C-E**, Degradation of host cell walls in contact with fungal cells. **C**, In light microscopy, the wall appears eroded (arrow) or deteriorated (arrowheads) close to pathogen cells in this friable callus at 96 hpi. **D-E**, In TEM, the host cell wall (cw) appears strongly degraded (arrows) next to fungal cells (f); (**D**) at the center of a friable callus at 96 hpi and (**E**) on the surface of a friable callus at 48 hpi. **F**, In TEM, the absence of labeling with the exoglucanase-gold complex (arrows) suggests that cellulose was degraded in certain areas of the host cell wall even though no pathogen cells were seen contiguous to the wall in this hard callus at 72 hpi. **G-H,** In SEM, the cells of the infected friable callus (**G**) are collapsed and the walls appear degraded and show a rough appearance while cells of the control (**H**) appear turgescent and the walls present a smoother appearance. Samples collected in both callus at 96 hpi.

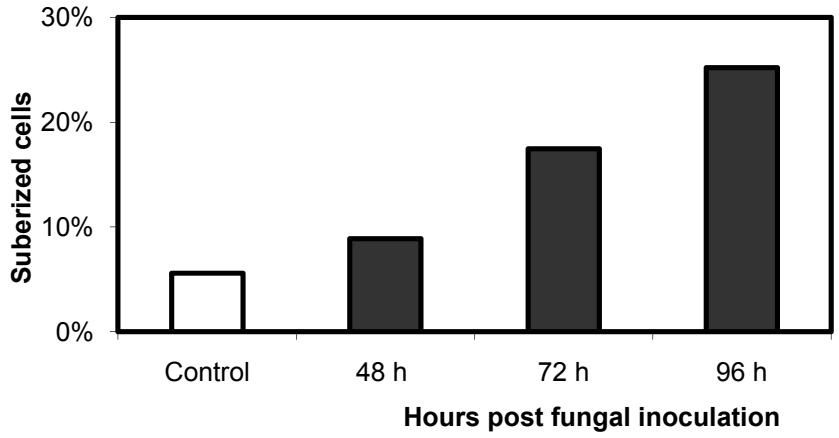

Figure 2.5: Proportion of suberized cells in *Ulmus americana* callus tissues inoculated with *Ophiostoma novo-ulmi.*

Cells were counted in infected callus samples at 48, 72 and 96 h post-inoculation (hpi), and in a water-treated control callus at 96 hpi.

2.4.3 PAL gene expression

Transcript accumulation of the PAL gene was monitored over time in water-treated and fungal-inoculated callus cultures (Fig. 2.6). This monitoring covered 4 to 144 hpi and included the time points chosen for microscopic observations. Healthy callus samples harvested at the start of the experiment (0 hpi) were chosen as controls. PAL expression was relatively stable in water-treated callus samples and it did not exceed 1.5 times (48 hpi) the level observed in healthy callus sample, whereas it varied from 1.3 (24 hpi) to 7.2 (144 hpi) times in callus samples inoculated with *O. novo-ulmi*. Absolute quantification enabled us to calculate the number of transcripts in each sample. In healthy callus sample, the average number of PAL mRNA molecules/ng of total RNA was 536. At 96 hpi, the latest time point of microscopic observations, this number was 785 in water-treated callus sample, whereas it had reached 1950 molecules in inoculated callus sample. Significant difference in PAL gene expression between water-treated and fungal-inoculated callus samples started at 72 hpi according to statistical analysis (F was significant at $P = 0.033$).

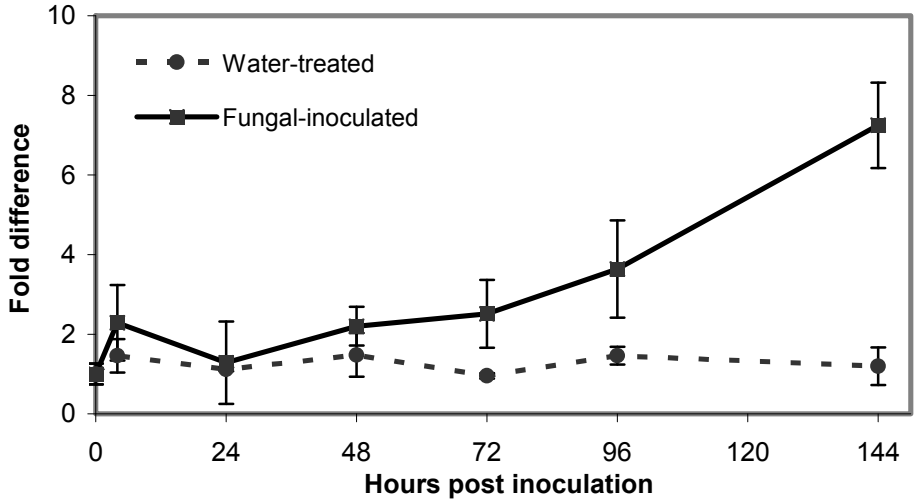

Figure 2.6: Fold difference in PAL gene expression in water-treated and fungal-inoculated *Ulmus americana* callus cultures.

The number of PAL mRNA molecules in water-treated and fungal-inoculated (*O. novo-ulmi*) callus samples was calculated at six time points after inoculation (4, 24, 48, 72, 96, 144 h post-inoculation [hpi]) and compared with that in healthy callus sample collected at 0 hpi. PAL samples were normalized with Elm translation initiation factor 5A (EIF5A) samples. Error bars were drawn based on the calculation of the standard deviation for the three biological replicates used in the study for each treatment. To calculate the number of molecules, absolute quantification was used and the trendline equation and R-squared value for each gene were as follows: EIF5A: $y = -0.298x + 9.61$; $R^2 = 0.99$; amplification efficiency: 99.3%; PAL: $y = -0.3001x + 10.249$; $R^2 = 1$; amplification efficiency: 99.8%.

2.5 Discussion

The purpose of this study was to analyze and validate an in vitro system that would reflect the interaction leading to DED in order to identify genes involved in this interaction using genomic tools. Histopathological observations showed the accumulation of suberin, phenols, lignin, and starch in *U. americana* callus cultures inoculated with *O. novo-ulmi*. Results also shed more light on the colonization of elm tissue by the fungus, particularly the direct penetration of cell walls accompanied by partial degradation of cellulose. These observations likely reflect changes in the levels of gene expression in both host and pathogen. We detected significant changes in PAL transcript levels in association with the defense responses. Overall, our results show that this in vitro system is a valuable tool for future genomic studies, and revealed the importance of newly observed mechanisms in the development of the DED pathogenic interaction.

Ophiostoma novo-ulmi H327 exhibited the same pattern of development on fungal growth medium and on elm callus. By 48 h, yeast-like cells had germinated into hyphae that were sporulating into conidia, and normal mycelial growth continued with time. This was not the case on the MS medium alone, where fungal growth was slow and conidia were absent. We can therefore conclude that the development of the fungus in infected callus tissues reflects the susceptibility of the latter rather than the contribution of the growth medium.

Ophiostoma novo-ulmi is known to colonize the vascular system mainly through direct penetration of pit membranes and intercellular spaces in the xylem of elm trees during its pathogenic phase (Campana 1978). While this pattern of development is also observed in callus tissue (i.e. development in different directions with a tendency to occupy mainly empty spaces), direct penetration of elm cell walls did occur. Based on SEM observations, Krause et al. (1996) suggested that hyphae of *O. novo-ulmi* were able to penetrate cells of susceptible American elm callus tissues. Our micrographs provide the first evidence of the presence of *O. novo-ulmi* hyphae inside *U. americana* callus cells. In elm trees infected with DED, the presence of *O. novo-ulmi* cells inside living cells has only been reported in rare instances (Ouellette 1982). We also showed that *O. novo-ulmi* was able to directly penetrate host cell walls and that the area in close contact with the fungus

was clearly degraded. This suggests that the pathogen possesses and secretes cell wall degrading enzymes that facilitate the colonization of host tissue, as already hypothesized by others for the in vivo interaction (Woods and Holmes 1974; Scheffer and Elgersma 1982).

This study compared different lines of callus cultures and is the first to consider and compare the responses of two types of elm callus tissues, i.e. friable and hard. An important finding was that the response of elm callus to inoculation included the production of suberin which, in DED literature, had only been reported in saplings (Et-Touil et al. 2005) and trees (Rioux and Ouellette 1991a, 1991b; Rioux et al. 1995). Furthermore, observations in TEM allowed us to distinguish the typical lamellar structure of suberin. To our knowledge, this is the first time that suberin lamellae are described in a plant callus tissue. The presence of suberin was previously suggested in callus tissues from Sitka spruce challenged with wood decay fungi (Woodward and Pearce 1988), anthracnose-resistant alfalfa (Mould and Robb 1992), and grapevine resistant to downy mildew (Dai et al. 1995b). These reports, however, were only based on the observation in resistant tissues of a positive reaction with Sudan dyes, which are specific to lipid-containing molecules, such as cutin and suberin, and did not include ultrastructure and fluorescence micrographs. Besides revealing the typical structure of suberin in callus tissues, our results also showed that susceptible callus tissues are able to produce suberin as a defense mechanism and that non-infected tissues contain suberin as well. But, while suberin was detected in both non-infected and infected susceptible callus tissues, it accumulated to a larger extent in the latter. From the time the fungus had germinated in infected callus (48 hpi) to the final sampling time (96 hpi), the proportion of suberized cells was almost five times higher in inoculated than in water-treated callus . This shows that suberin accumulation is stimulated by the presence of the fungus, and that this biotic stress is greater than the abiotic stress resulting from subculturing, wounding, or water application.

Suberin has previously been associated with defense reactions in adult *U. americana* trees affected by DED. Rioux and Ouellette (1991a, 1991b) showed that barrier zones of annual shoots and small branches of *U. americana* infected with *O. novo-ulmi* frequently contained suberized parenchyma cells, and at times suberized fibers. In addition, Rioux et al. (1995) observed that tyloses formed in the xylem of infected *U. americana* trees had internal suberized layers. The formation of more than one suberized layer, close to

phenols and cellulosic wall layers, as shown in Fig. 2.4B, has also been reported in trees (Rioux and Ouellette 1991b; Rioux et al. 1995). Thus it appears that callus and parenchyma tissues, as well as cells in a differentiating mode (e.g. cambial derivatives), share the capacity to produce suberin. Although the lack of structure in callus makes suberin deposition less organized than in whole plants, the finality of the process is most likely the same (i.e. to restrict exchanges with the medium outside of healthy cells that are not in the front line or in direct contact with the fungus). For instance, in Fig. 2.3D, the presence of lignified cells adjacent to a suberized layer is somewhat reminiscent of what happens in trees. When the bark is injured, it is well known that a lignified impervious tissue is formed just before the differentiation of a suberized necrophylactic periderm (Mullick 1975). This fact was confirmed many times, even though it has been shown that the lignified cells also contain a thin internal suberized layer (e.g. see Biggs 1984 and 1985). The presence of lignified cells contiguous to that of suberized tissues has also been reported in defensive xylem tissues of broad-leaved trees and conifers (Rioux and Ouellette 1991a; Simard et al. 2001).

Phenylalanine ammonia-lyase is a key enzyme controlling the biosynthesis of phenolics in plants (Strack 1997). It is induced in response to wounding and infection by plant pathogens (Jones 1984) and is associated with the accumulation of suberin in plants (Lee et al 1992; Oosterhaven et al. 1995; Bernards et al. 2000; Ghanati et al. 2002). Our measurements of PAL gene expression confirmed this association in elm callus inoculated with *O. novo-ulmi*, as patterns for suberization and PAL expression followed similar trends. PAL seems to be constitutively expressed (536 RNA transcripts per ng of total RNA) in healthy callus sample, yet its association with fungal infection was also apparent. The significant increase in PAL expression observed at 72 hpi, and thereafter in inoculated but not in water-treated callus samples was evidence of its induction by the infection and seems to be directly associated with the development of the fungus inside the callus. This result confirms a recent observation by Nasmith et al. (2008b) who, based on RNA dot blot analysis, reported an increase in PAL expression in leaf midribs of *U. americana* saplings inoculated with *O. novo-ulmi*. Corchete et al. (1993) had reported that the DED pathogen induced a large increase in PAL enzyme activity in DED-resistant *U. pumila*, but not in susceptible *U. campestris* suspension cultures. The increase observed in the resistant *U.*

pumila reached its maximum at 24 hpi, which is earlier than the first significant increase we detected at 72 hpi in the susceptible *U. americana*. Direct comparison of PAL gene expression and translation in susceptible and resistant elm cell cultures grown under similar conditions is needed in order to confirm the importance of an early PAL response in resistance.

In addition to its association with suberization, PAL participates in the production of phenylpropanoid-derived phytoalexins produced in plants in response to infection (Dixon et al. 1983). PAL is also essential to the formation of cinnamic acid, which is involved in the biosynthetic pathway of flavonols, catechols, anthocyanidins, tannins, and lignin (Dixon and Paiva 1995). Flavonoids, tannins, and lignin were characterized in infected elm callus in this study. Pijut et al. (1990b) observed deposits of phenolic-like structures in resistant, but not in susceptible *U. americana* callus tissues treated with culture filtrates of *O. novo-ulmi*. Krause et al. (1996) later reported that electron-opaque phenolic-like material accumulated in both resistant and susceptible American elm callus tissue, but without identifying its nature. Our results confirmed that electron-opaque deposits accumulated in susceptible elm callus tissues inoculated with *O. novo-ulmi*. The use of histochemical tests with DMCA and vanillin enabled us to conclude that these deposits are indeed phenolics and contain a high proportion of less condensed tannins, such as catechins, and a certain proportion of condensed tannins, such as pro-anthocyanidins, that start accumulating at the later stages of infection. Some of the phenols shown in the present study possibly served as building blocks in the synthesis of lignin-like molecules, also observed here for the first time in elm callus tissues, as an additional barrier against the pathogen (Bird 1988). The energy required for these biochemical transformations was likely derived from starch grains, which were abundant in numerous cells of inoculated callus. The large number of cells accumulating phenols and starch deposits as well as those showing walls enriched with lignin or suberin that are grouped together resembles, at a rudimentary stage, the compartmentalization walls which are frequently observed in infected trees (see reviews by Shigo 1984 and Pearce 1996).

In summary, new aspects of the DED interaction were observed in vitro using susceptible American elm callus cultures inoculated with *O. novo-ulmi*. The homogeneity of the results obtained for the different callus lines used in the study shows the importance

of these mechanisms and the reproducibility of the results. The pathogen was able to penetrate into host cells, likely through the secretion of cell-wall degrading enzymes. The occurrence of defense reactions based on the accumulation of suberin, phenolics, and lignin was demonstrated unequivocally for the first time in elm callus tissues, and it was found to be associated with the upregulation of the PAL gene. Based on our findings, we expect elm callus cultures to be a useful system for identification and transcript profiling of other genes and pathways involved in the DED plant-pathogen interaction.

2.6 Acknowledgements

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3. In vitro identification and monitoring of *Ulmus americana* genes induced during the interaction with the Dutch elm disease pathogen *Ophiostoma novo-ulmi*

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3.1 Résumé-Abstract

Les mécanismes moléculaires à la base de l'interaction conduisant à la maladie hollandaise de l'orme ont été étudiés dans un système in vitro en utilisant des cultures de cals de l'espèce *Ulmus americana* auxquelles on a inoculé des cellules levuriformes du champignon pathogène *Ophiostoma novo-ulmi*. Une banque d'ADN complémentaires issue de l'interaction a été construite à partir de tissus de cals d'orme infectés au temps 72 heures post-inoculation en employant la technique d'hybridations suppressives et soustractives. Cinq cents trente-cinq étiquettes de séquences exprimées provenant majoritairement de l'hôte ont été regroupées en 314 uniséquences et distribuées au sein de catégories fonctionnelles. Suite au criblage différentiel, 53 uniséquences d'*U. americana* ont été considérées induites durant l'interaction. Le profil d'expression de 18 transcrits d'orme a été analysé en détail à 6 temps différents par PCR quantitative. Les séquences induites incluent des gènes encodant différentes classes de protéines PR et des enzymes appartenant à des branches de la voie métabolique des phénylpropanoïdes. Leur association possible avec des composés chimiques liés au phénomène de compartimentation et à la production de phytoalexines est discutée. Cette étude fournit pour la première fois un aperçu des mécanismes moléculaires impliqués dans l'interaction compatible entre *U. americana* et *O. novo-ulmi*. Elle fournit également une première ressource génomique, notamment les données générées par la banque d'interaction, pour l'orme américain qui représente une essence urbaine de grande valeur.

Molecular mechanisms underlying the interaction leading to Dutch elm disease were studied in vitro using *Ulmus americana* L. callus culture inoculated with budding cells of the fungal pathogen *Ophiostoma novo-ulmi* Brasier. An interaction cDNA library employing suppression subtractive hybridization was constructed from infected elm callus tissue 72 hours post-inoculation. Five hundred and thirty-five expressed sequence tags, mostly from the host, were grouped into 314 unisequences and distributed into functional categories. After differential screening, 53 *U. americana* unisequences were considered upregulated during the interaction. The expression profiles at six time points of a subset of

18 elm transcripts were analyzed in more detail by quantitative reverse transcriptase polymerase chain reaction. Upregulated sequences included genes coding for different classes of pathogenesis-related proteins and enzymes belonging to different branches of the phenylpropanoid pathway. The possible association with compartmentalization-related compounds and phytoalexin production is discussed. This study provides, for the first time, snapshots of molecular mechanisms involved in the compatible interaction between *U. americana* and *O. novo-ulmi*. It also represents a valuable genomic resource, namely the interaction library dataset, for the highly appreciated urban tree *U. americana*.

3.2 Introduction

Since its detection early in the twentieth century, Dutch elm disease (DED) has developed into one of the most devastating tree diseases. Two successive pandemics have killed over 1 billion elm trees in North America and Europe (Paoletti et al. 2005). While the first epidemic was caused by the Ascomycete fungus *Ophiostoma* ulmi (Buisman) Nannf., the second and more devastating epidemic was caused by a more aggressive species, *O. novo-ulmi* Brasier. Among the hosts, American elm (*Ulmus americana* L.) is known to be particularly susceptible to DED (Ouellet and Pomerleau 1965).

When elm bark beetles introduce fungal spores into the xylem vessels of healthy American elm trees, *O. novo-ulmi* moves throughout the xylem and reaches leaves and roots eventually causing death of the tree. Induced defense mechanisms against DED which have been documented in *U. americana* include compartmentalization and production of phytoalexins known as mansonones. During compartmentalization, the strongest barrier, wall 4 (barrier zone), is formed by the cambium in response to infection, and usually consists of cells filled with phenolic compounds and cell walls reinforced with lignin and suberin (Shigo and Tippett 1981; Rioux and Ouellette 1991a; 1991b).

Mansonones belong to a family of sesquiterpenoid quinones and were synthesized in vitro as well as in vivo in elms in response to infection by *O. novo-ulmi* (Duchesne et al. 1985; 1994; Yang et al. 1993; 1994). These secondary metabolites exhibit several effects on the fungus including inhibition of growth, ion leakage, respiration rate reduction, cell wall disruption, aggregation of ribosomes, and the accumulation of electron-dense material in the mitochondria (Dumas et al. 1986; Wu et al. 1989).

Little is known about elm genes involved in defense mechanisms. A class I chitinase (*hs2*) gene from a resistant individual of *U. americana* was cloned and was used to produce transgenic lines of creeping bentgrass (*Agrostis palustris* Huds) resistant against the brown patch fungus *Rhizoctonia solani* (J.G. Kuhn) (Chai et al. 2002). Transcripts of chitinase, phenylalanine ammonia-lyase (PAL), and polygalacturonase inhibiting protein (PGIP) genes were shown to be induced in leaf midrib tissues of *U. americana* upon colonization by *O. novo-ulmi* (Nasmith et al. 2008a; 2008b). Yet during host-pathogen interactions in model systems, panoplies of genes involved in different metabolic pathways

were reported to be induced, representing up to 20% of *Arabidopsis thaliana* (L.) Heynh. genes (Nimchuk et al. 2003). These encode pathogenesis-related (PR) proteins (Van Loon et al. 2006), or are involved in the production of secondary metabolites such as phytoalexins which can be derived from the phenylpropanoid or terpenoid pathways (Dixon 2001). These can also be involved in signalling and activation of regulatory genes and proteins such as transcription factors and protein kinases (Zhao et al. 2005). Tools for transcriptome analysis have proven useful for revealing pronounced alterations in transcriptional activity in large scale plant-pathogen interaction studies. Suppression subtractive hybridization (SSH) (Diatchenko et al. 1996) was one of the techniques that have been successfully used in different pathosystems to identify host genes coding for defense related proteins in compatible and incompatible interactions with fungal pathogens (Birch et al. 1999; Xiong et al. 2001; Jansen et al. 2005; Han et al. 2005; Bogacki et al. 2008). Also, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) used in combination with SSH proved to be a powerful tool for analyzing and validating gene expression, and has been used as such in plant-fungus interaction studies (Kong et al. 2005; Bogacki et al. 2008).

Tissue culture systems have been successfully used to study molecular aspects of tree-pathogen interactions, for example between Norway spruce (Picea abies (L.) Karst.) and *Heterobasidion annosum* (Fr.) Bref. sensu lato (Hietala et al. 2003; Karlsson et al. 2007). Concerning the study of DED, elm calli have been shown to display defense responses that are typically observed in parenchyma cells of the xylem of American elm trees upon infection with *O. novo-ulmi*, such as production of phytoalexins (Yang et al. 1993; 1994) and accumulation of phenolic compounds (Pijut et al. 1990b; Krause et al. 1996; Aoun et al. 2009).

The objective of this study was to identify genes upregulated during the *U. americana – O. novo-ulmi* compatible interaction. To achieve this objective we: 1) constructed an *U. americana – O. novo-ulmi* interaction cDNA library from fungus inoculated elm callus tissue 72 hours post-inoculation (hpi); 2) sequenced cDNA clones to obtain expressed sequence tags (ESTs); 3) assembled ESTs into unisequences prior to their annotation; 4) selected unisequences upregulated during the interaction by differential

screening and 5) validated upregulation of a subset of American elm unisequences by qRT-PCR.

The in vitro system used in this study was previously subjected to a histopathological analysis in which defense reactions have been revealed using histochemical tests and microscopic observations (Aoun et al. 2009). Here, molecular aspects were being analyzed. The interaction library consisted of 638 cDNA clones, yielding 535 GenBank quality ESTs which were assembled into 314 unisequences with the majority of these being of host origin. After differential screening of the library, 155 ESTs representing 53 unisequences were selected as being upregulated in the presence of *O. novo-ulmi*. Analysis by qRT-PCR of 18 selected constitutive and induced genes validated differential screening results. Induced transcripts identified in this study coded for proteins that are involved in different metabolic pathways of the host, in particular representing different branches of the phenylpropanoid metabolism and different families of PR proteins.

3.3 Materials and methods

3.3.1 Biological material and callus inoculation

The aggressive *O. novo-ulmi* subsp. *novo-ulmi* isolate H327 (Et-Touil et al. 1999) was used in this study. Hard calli of American elm (*U. americana*) were initiated from buds, cultured on modified Murashige and Skoog (MS) medium and inoculated as described by Aoun et al. (2009). Water and fungus inoculated calli, along with mycelium grown on solid medium, were harvested 72 hpi, ground to a fine powder in liquid nitrogen and stored at -80°C for use in SSH cDNA library construction.

For qRT-PCR experiments, calli were either mock-inoculated with sterile water or inoculated with *O. novo-ulmi* budding cells and harvested at 4, 24, 48, 72, 96, and 144 hpi. Mycelium grown on solid medium was harvested at 48, 72, 96, and 144 hpi. Three biological replicates were prepared for each type of tissue at each time point. As for *U. americana* callus cultures, a biological replicate consisted of three calli that were combined prior to grinding. In addition, for each biological replicate, three non-inoculated healthy calli were harvested at the start of the experiment, i.e. 0 hpi. Thus, a total of 117 (108 fungus- or mock-inoculated and nine non-inoculated) calli were used for qRT-PCR experiments.

3.3.2 RNA extraction and cDNA library construction

Total RNA from fungus inoculated and water treated American elm calli, as well as from *O. novo-ulmi* mycelium, was isolated using a small scale version (Bouvet et al. 2008) of the method originally described by Chang et al. (1993). RNA quantity and quality were determined using a Multiskan spectrophotometer (Thermo Fisher Scientific Inc, Nepean, ON, Canada) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples were treated with DNaseI (Invitrogen, Burlington, ON, Canada). For qRT-PCR, total RNA samples were treated with amplification grade DNaseI (Sigma Aldrich Canada Ltd, Oakville, ON, Canada).

The host-pathogen interaction cDNA library was constructed using the Super SMART PCR cDNA Synthesis Kit and PCR Select™ cDNA Subtraction Kit (Clontech Laboratories Inc., Takara Bio Group, Mountain View, CA, USA). Forward subtraction was

done with the tester being a cDNA population from 72 hpi infected calli, and the driver being an equal mix of cDNA populations from 72 hpi water inoculated calli and 72 h axenic *O. novo-ulmi* mycelium grown on complete medium. Subtracted PCR products were ligated into the pDRIVE plasmid vector (Qiagen PCR Cloning plus Kit, Qiagen Inc., Mississauga, ON, Canada) for a total of 638 cDNA clones. Presence and size of inserts were determined by direct amplification from crude bacterial lysates using primers M13F (-20) and T7 Promoter on the pDRIVE vector.

3.3.3 Differential screening

Differential screening of all 638 cDNA clones was performed using the PCR Select™ Differential Screening Kit (Clontech Laboratories Inc.). Inserts were amplified from each clone with the nested PCR primers 1 and 2R provided with the kit and spotted onto duplicate Hybond-N nylon membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Membranes were screened with three probes labelled with *Redivue*™ [α-^{32}P]dCTP (GE Healthcare Bio-Sciences): (1) unsubtracted driver, (2) unsubtracted tester, and (3) forward subtracted tester. This third probe was used to identify clones enriched by the subtraction process. In addition to the negative controls provided with the differential screening kit, we amplified part of the constitutively expressed *O. novo-ulmi* H327 elongation factor (*ef1*α) from the three probes for use as a positive control on all membranes. Intensities of hybridization signals were visualized by phosphor autoradiography using an Amersham Biosciences Typhoon 9400 Variable Mode Imager (GE Healthcare Bio-Sciences).

3.3.4 Sequence analysis

PCR products of the 638 cDNA clones were purified and sequenced at the Centre de recherche du CHUL (Plateforme de séquençage et de génotypage des génomes, Québec City, QC, Canada). High quality ESTs (N-threshold = 10%, cut-off high quality contiguous length = 250 bp) were then submitted to GenBank's EST database (dbEST). Sequences were assembled into contigs using GCG's Seqmerge (Accelrys Software Inc., San Diego, CA, USA) with default criteria (word size=7; stringency=0.80; minimum overlap=14; minimum identity=14). The resulting unisequences were then annotated by comparison

against the UniProt database (v9.3) (Wu et al. 2006) using the BLASTX algorithm (Altschul et al. 1997) with a cut off E value $<e^{-5}$. Additional homology searches were done using the NCBI BLASTN algorithm (E value $<e^{-5}$) to the following specific databases: NCBI non-human, non-mouse ESTs (est_others), and a manually constructed database consisting of the *Magnaporthe oryzae* (B.C.Couch) genome from the Broad Institute (MIT) and the *Populus trichocarpa* (A.Gray) Brayshaw genome (US Department of Energy Joint Genome Institute, DOE JGI) and used to distinguish plant and fungus sequences (Hsiang and Goodwin 2003). Functional annotation of the unisequences based on the MAt DB1 *A. thaliana* database (MIPS) was done according to the Functional Catalogue (FunCat) Scheme (Ruepp et al. 2004).

3.3.5 Primer design and PCR experiments

Primers were designed to amplify products between 120 and 220 bp with an optimal primer size of 25 bp and optimal primer Tm of 65°C using Primer3 software (Rozen and Skaletsky 2000) and Oligo Calc software (Kibbe 2007). Table 2 lists all primers successfully used in this study.

To assign a more precise annotation to a group of 41 ‘No-hit’ sequences, conventional PCRs were run on cDNAs from *O. novo-ulmi* mycelium as well as healthy and *O. novo-ulmi* inoculated *U. americana* calli with sequence specific primer pairs. Amplifications were performed in a 25 µl reaction volume containing 20 ng cDNA. The conditions were 94°C for 2 min, 35 cycles at 94°C for 15 sec, 65°C for 30 sec, and 72°C for 1 min, followed by an extension cycle at 72°C for 5 min. The *U. americana* PAL amplicon and *O. novo-ulmi* elongation factor (*ef1*α) amplicon were used as positive controls with all runs. A subset of amplicons was then sequenced to verify amplification specificity.

Table 2: Primers used in this study.

Target sequence	5'oligonucleotide	3'oligonucleotide	Amplicon size (bp)
No-hit sequences			
FC325638	GGGAGGTTGATTCCAAGTACCGTAG	GCATAAACCATTCTTCTCGGCATTTC	128
FC325653	CGGGAGATTTTAGGGTTTGGTTTTAG	TTTTCATTTCTCAAGAGCCGACAAG	192
FC325656	CGGGATACACTAAACACATAGGGCTAG	CCCCTCAAATAGTGCCTCCAGAG	168
FC325659	GGCGATCAGCTTATTGAACTTTCCA	CTTCGCGACAAATATCAATTCAAATG	164
FC325575	ACCGAGAAAGAGAGTCCCATTTGAG	AAAAACCACGGGCAAATTGTAGAAG	216
FC325586	ACATCACTTGATGCAAGAACAGCAG	CAGCAATCTGTGCTAATACATGGTGA	138
FC325587	CATCTTCGCTGTGATTCTTTCTCCA	GAGATCAAATAGTCTCGGTCTGCGT	176
FC325705	TTGCTTGCTTTGGTGGAAGTAGAAC	CAATGCCATCTATTCCCAAATTCAC	197
FC325590	CTGTTGTGCCTTGGATATTTGCTTC	TGCCAAACATAAACAACATCAGGTG	181
FC325210	AAGAGGAAGGAGAGCATGAGGAGAG	GATACCAGATGCCAGATAGCGTCAC	152
FC325262	TTCACATCCGAGAGACGCAACTTAG	CATCGCCTCTTTAATCCCATACCTC	154
FC325289	GGTGCTCACCTGTTCTCCTTATTTG	ATCAAGGATCTGCTTCGGTTTTCTC	166
FC325305	CACTTCTCCAGCCAGTTATGCTTTG	CCAAGCAACGAAAGCGTAGATTAAAG	152
FC325553	AAATCTTCGGAGGTGTAATCGTCGT	TTATCTCGCGACTCCGATTTAGTCA	125
FC325327	GTTGCTGGATTGAGATTTTTCGACA	GATAAAACGCCACATCCTCAAAATCA	123
FC325316	GGGGCATTTTTGATTAACCCTGA	TTGCTCAAAGAGGTTCAAACACTTCA	221
FC325392	CGCCGATGCTAATTTGGTGTAATAATC	GCAAAAGGCCATTACCGTCTAACTC	188
FC325411	GTTTTGGAGAGATGGGTTTGGGA	TCTCATATACCACCGGAAGCGAGTAG	171
FC325432	GGAAGCCAAAAACACCATTCTCTCT	GCGAGCGACGAAACTGTAGATCA	213
FC325434	GGAGTCAATTTTGAAGGAGGAGGAG	CGGCTGATCGTGGTGTTAATCTATC	151
FC325514	AGGGTCACTGTAACGGTTTCCATCT	TTAGAATGTGTGGCAAAACGGTCA	170

Fungus sequences

FC325617	AGAGAATGTCCAGATGGTCGAGTTG	CTTGCCAGGGTTTTCCTCAATAAAG	169
FC325271	GCCTCGTTTTACAACTCGACAACTG	TGTGGTACACAGCAAACCCTGAAG	178
FC325418	GACCAAGGGTCTGGACTTTATCGAC	CATCAACGCTCGTGTCAACTAGAGA	176
FC325410	AGATCATCTTCATCACCTCCACCAC	GCTGACGCCAGCTAGAAGAAGAAAG	151

Sequences used in qRT-PCR analysis

FC325256	GTCTCCGTTGTTCGTACGTGTCTCT	CACTTCTGGCAGCATTCACAATATG	153
FC325680	GTTTTGCTGAGGGAAAAGACTTGGT	AACGCTACCAAAGGCATACTTGTGA	217
FC325436	ATGCAGGCCAGTGATAGGTTTAACA	CAAAGTAAGCCAAAAGGGGGTAGTG	166
FC325344	TGACTTCTTACGCAGCATACACGAG	TTCAATGATCAAACCAGCAAGTCAG	176
FC325614	AGAGCAACAATAGCCCTCACACTTG	TCCGGTTATAGTTGCACACAAGGTT	161
FC325348	TTGATTTTTGCGAGGAAGTGAAGTC	GTGCACCTTGTTTAGCATCTGGAAG	183
FC325537	TTGGTTTAAGACCTCCTGCCAACA	AGCAACTCCTTTCACAAACATTCCA	191
FC325312	GCTTGGCTCATCTATAACAGCAGGA	CTTTCTGCTGACCAAACCACTGTCT	165
FC325322	ACGGGAAGCTTTGGAATAATGAATG	TCCAAATTAAACACATGCCCTCAAC	164
FC325422	GTGCTATTGAGCTTGGGATAGCTGA	CGCGAAATAGGGGTTTGAGAATAAG	193
FC325592	TGGACAAGTTTAAGGTGGGAGATGA	TCATAGGCAGCCTCAATAGCAAGAG	187
FC325589	TAGCAACGATGAGTTCATGTGAGGA	ACCCAAACCCTTGAGCAGTCATAGT	175
FC325238	AATGCTGTGAGACTTTGGGTAGCTG	AGGATCATAGTTGCAGCCAATGAAG	180
FC325621	CTCGGTGACCTTTGATCCTTATGTG	CGGTCACAATCAATCTCCTCTGAGT	161
FC325284	GAATGTCCATGGTGTGATAAGCCAG	TCCAGGAGAAACACCGAGTTGATTAC	151
FC325206	CGGGGATATCTCAAATAACCGAAAAG	GCACGGAGATAGTCATAGCCAATGT	179
FC325445	TTGTGACCTTCTCTTTCGCTTCATC	ATCTTACCCAACTGGTCCAAAAGGT	120
FC325532	TCCCAGGGACAAAGTGAACATTCTAG	ATTGTAGCTTTTGATGTTGGCAGGA	160

Additional sequences[a]			
FC325415	ACTCAGTCGGAGGTAGGGTCCAG	CTTCCCTTGCCTACATTGTTCCATC	185
U. americana PAL	TGCCCAAAGAAGTTGAGAGTTCAAG	AGTCCCCAATTCCTCTCTCACAAAC	122
O. novo-ulmi ef1α	ATCGACAAGCAGCCCAAACAGTTC	ATTGTCAAGCTGACTCCCTCCAAG	434

[a] PAL = Phenylalanine ammonia lyase; ef1α = Elongation factor 1α.

3.3.6 qRT-PCR analysis

Eighteen cDNAs representing singlets and contigs were selected for further analysis by qRT-PCR based on differential screening results. Five-hundred ng of DNaseI treated total RNA from water inoculated, fungal inoculated, and non-inoculated American elm calli as well as from *O. novo-ulmi* mycelium grown on complete medium were used for first strand cDNA synthesis using SuperScript™ II Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with an oligo $(dT)_{12-18}$ primer according to the manufacturer's instructions. A 10X buffer (1 M Tris-HCl (pH 8.4), 2 M KCl, 0.1 M $MgCl_2$, 1M DTT) was used instead of the 5X first-strand buffer provided with the enzyme, and 4 µL of 25 mM $MgCl_2$ were added to the reaction. The cDNA sample was diluted 1:5 in water prior to real-time PCR quantification. All qRT-PCR reactions contained a cDNA equivalent of 10 ng of total RNA. Amplifications were performed in a 15 µL reaction volume in 2X QuantiTect SYBR Green mixture (Qiagen Inc.) with 0.3 µM of 5' and 3' primers. Primer design was performed as described above. Specific amplification of elm cDNAs by the primers was verified by having the mycelium cDNA samples run in parallel. Absence of contaminating genomic DNA was confirmed in reactions with DNAseI-treated RNA as template. Amplifications were carried out in a LightCycler 480 (Roche, Basel, Switzerland). After an initial 15 min activation step at 95°C, 45 cycles (94°C for 10 s, 62°C for 2 min) were performed, and a single fluorescent reading was obtained after each cycle immediately following the annealing-elongation step at 62°C. A melting curve analysis was performed at the end of cycling to ensure single product amplification. Crossing point (Cp) values were determined with the software supplied with the instrument. Standard curves were used to transform Cp values obtained from total RNA samples into transcript numbers. Samples were normalized according to Vandesompele et al. (2002) by determining the average expression stability value of five control genes and calculating normalization factors using geometric averages. Three biological and two technical replicates were performed per treatment.

3.3.7 Statistical analysis

Statistical analyses were done with SAS version 9.1 Software (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) according to general linear models

procedure and Tukey test were used to analyze qPCR data. For all analyses, when normality or homogeneity of residues was not respected, data were first transformed by rank.

3.3.8 Accession numbers

The 535 ESTs sequences reported in this paper were submitted to GenBank's dbEST (http://www.ncbi.nlm.nih.gov/dbEST/index.html) under accession nos. FC325180 to FC325714.

3.4 Results

3.4.1 Characterization and differential screening of an *U. americana* - *O. novo-ulmi* interaction cDNA library

An SSH interaction library was constructed from *U. americana* hard calli 72 hpi with *O. novo-ulmi* budding cells. In order to enrich for sequences upregulated during the interaction, we used cDNAs from infected calli harvested at 72 hpi (Fig 3.1A) as tester, whereas the driver consisted of an equal mix of cDNA populations derived from a 72 hpi axenic culture of *O. novo-ulmi* mycelium (Fig. 3.1B) and mock- (water) treated calli (Fig. 3.1C) harvested at 72 hpi.

A total of 638 cDNA clones with inserts ranging from 300 bp to 1500 bp constituted the interaction library. The subtraction efficiency was verified by amplifying a portion of the constitutive *O. novo-ulmi* H327 elongation factor (*ef1*α), which amplified after 23 and 38 cycles from the unsubtracted and subtracted tester cDNA populations, respectively (data not shown). Differential screening of the 638 clones identified 178 clones (28%) hybridizing more strongly with the unsubtracted tester probe as compared to the unsubtracted driver probe, and thus indicating upregulated sequences during the interaction. In addition, 196 clones (31%) hybridized more strongly with the forward subtracted tester probe as compared to the forward unsubtracted tester probe indicating sequences enriched by the subtraction process. Sixty-seven of the 178 upregulated cDNA clones were also enriched by the subtraction process.

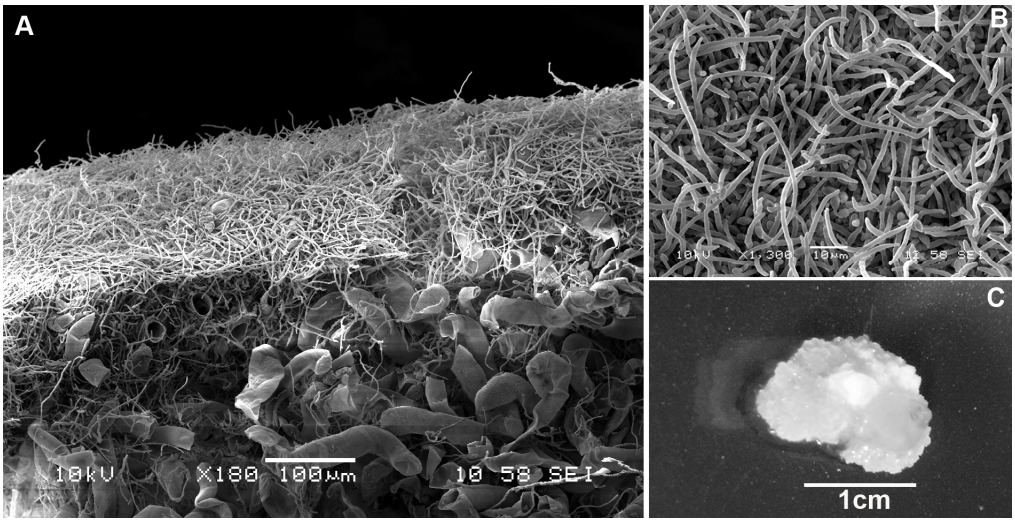


Figure 3.1: Composition of tester and driver used for cDNA library construction.

A: Scanning electron micrograph of *Ophiostoma novo-ulmi* hyphae on the surface of an *Ulmus americana* callus 72 hpi. B: Axenic *O. novo-ulmi* mycelium grown on complete medium. . C: Mock-inoculated *U. americana* callus at 72 hpi. The cDNA interaction library was constructed by using (A) as tester and an equal mix of (B) and (C) as driver.

3.4.2. Annotation and functional classification of ESTs

Since little sequence information was available for the two organisms investigated in this study, especially for American elm, we chose to sequence and annotate all 638 interaction library clones. Five-hundred and thirty-five high quality ESTs were obtained and submitted to GenBank's EST database (dbEST). The ESTs were assembled into 314 unisequences (247 singlets and 67 contigs) and were annotated using the sequential strategy described in Table 3.

Table 3: Databases and algorithms used to annotate the 314 unisequences of the *Ophiostoma novo-ulmi – Ulmus americana* interaction library.

Database	**Method or Algorithm**	**No. (%) of unisequences per category**			**Sum of unisequences**
		U. americana	***O. novo-ulmi***	**Unknown**	
Magnaporthe oryzae – Populus trichocarpa	BLASTN	193	2	119	314
UniProt v9.3	BLASTX	268	5	41	314
-	PCR analysis on the five '*O. novo-ulmi*' and the 41 'Unknown' sequences	290	4	20	314
est_other	BLASTN analysis of the remaining 20 'Unknown' sequences	**296 (94.3)**	**4 (1.2)**	**14 (4.5)**	**314 (100)**

According to BLASTX homology searches against entries in the UniProt v9.3 database, 207 ESTs (66%) showed similarity to plant proteins with known function. Five singlets had similarity to fungal proteins, but only four of these were confirmed by PCR to be of fungal origin. These included three hypothetical proteins and a hydrolase from the isochorismatase family. EST FC325415 turned out to be of plant origin according to PCR and a BLASTN search against a *M. oryzae* - *P. trichocarpa* database [strong homology (e0.0) with a *Populus* sequence (scaffold_21038, genomic sequence JGI)]. Overall, BLASTN homology searches against the *M. oryzae* - *P. trichocarpa* database identified 193 hits of 314 unisequences to be of plant origin and two sequences of fungus origin. The latter corresponded to fungus sequences identified with BLASTX.

Forty-one of the 314 unisequences (13%) did not have homologies with known proteins in the UniProt database and were considered as orphans. PCR analysis confirmed a plant origin for 21 of these 41 unisequences. BLASTN homology searches against

GenBank's est_other database identified an additional six sequences as being of plant origin. In summary, of 314 unisequences analyzed, 296 (94.3%) were classified as being of plant origin, four (1.2%) were classified as being of fungus origin, and 14 (4.5%) could not be assigned to a particular organism. A complete list of the 314 unisequences and their putative functions is available in the Appendix.

A comparison to the *A. thaliana* MAt DB1 database classified 288 of the 296 American elm unisequences into 20 functional categories (Fig. 3.2). The categories with the highest proportion of sequences included Subcellular localization (18%); Metabolism (16%); Protein with binding function or cofactor requirement (14%); Unclassified proteins (9%); and Cell rescue, defense, and virulence (7%). It is noteworthy that 4% (10 entries) of the matches belonged to the subcategory Plant-fungal specific systemic sensing and response (36.20); 4% (nine entries) to the subcategory Disease, virulence, and defense (32.05); and 4% to the subcategory Response to biotic stimulus (34.11).

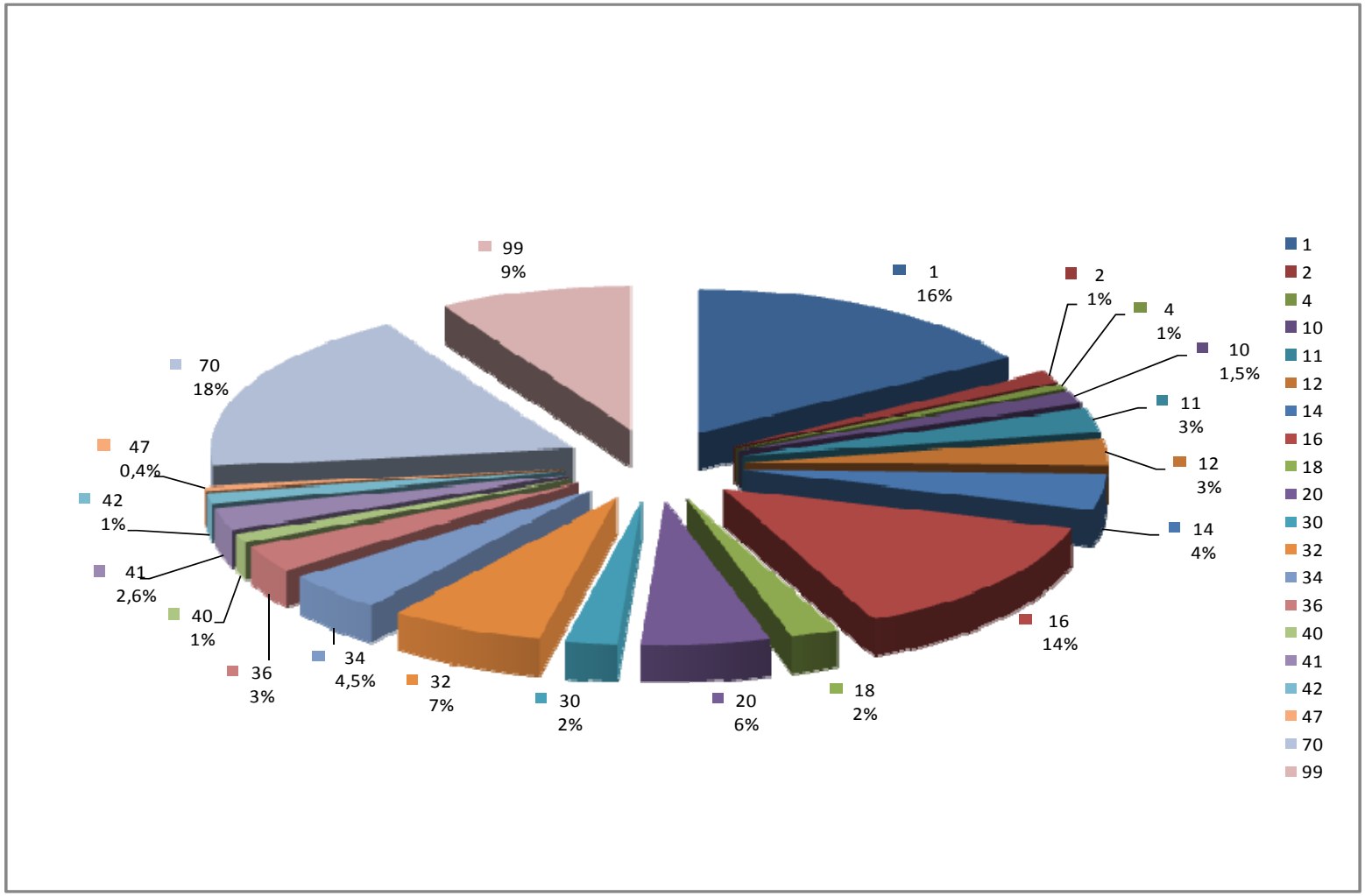


Figure 3.2: Functional classification of American elm unisequences of the interaction library according to the Functional Catalogue (FunCat, v2.1) by comparison (BLASTN) to the MAt DB1 *Arabidopsis thaliana* database.

Two-hundred and eighty-eight out of 296 unisequences were classified into 20 functional categories. 1: Metabolism; 2: Energy; 4: Storage protein; 10: Cell cycle and DNA processing; 11: Transcription; 12: Protein synthesis; 14 : Protein fate; 16 : Protein with binding function or cofactor requirement; 18: Regulation of metabolism and protein function; 20: Cellular transport, transport facilities and transport routes; 30: Cellular communication-signal transduction mechanism; 32: Cell rescue, defense and virulence; 34: Interaction with the environment; 36: Systemic interaction with the environment; 40: Cell fate; 41: Development (Systemic); 42: Biogenesis of cellular components; 47: Organ differentiation; 70: Subcellular localization; 99: Unclassified proteins.

From the 178 cDNA clones identified as upregulated by the differential screening procedure, 155 gave high quality EST sequences and were distributed among 53 unisequences. A list of the 53 unisequences and the potential implication in plant defense mechanisms and pathways is presented in Table 4.

Table 4: Fifty-three *Ulmus americana* unisequences upregulated during the interaction with *Ophiostoma novo-ulmi* based on differential screening[a].

U. americana EST acc. no.[b]	No. of ESTs in unisequence		Closest database match			Implication in defense
	Total number[c]	D.E.[d]	UniProt acc. no.[e]	Putative function - taxon	E value	
Signalling						
FC325614	1	1	Q1PDI2	Diacylglycerol kinase - *Arabidopsis thaliana*	E-110	Elicitor triggered signalling ; Van der Luit et al. 2000
FC325561	1	1	Q70AB2	Ethylene transcription factor - *Fagus sylvatica*	5E-74	Ethylene-responsive factor ERF, ethylene signalling; Xu et al. 2008.
FC325332	1	1	A2Q1X9	TIR - *Medicago truncatula*	2E-06	Cell death signaling pathway; Swiderski et al. 2009
FC325341	5	3	Q1SEJ9	SAM dependent carboxyl methyltransferase - *M. truncatula*	1E-75	Acts on the defense signalling molecules of jasmonic and salycilic acid; Zulak et al. 2009
FC325658	3	2	Q9LRJ9	Similarity to receptor kinase - *A. thaliana*	1E-45	Transmembrane receptors; Morris and Walker 2003
FC325306	10	1	Q9LRJ9	Similarity to receptor kinase - *A. thaliana*	2E-45	Same as above
Phenylpropanoid metabolism						
FC325532	34	27	Q3KN67	Isoflavone reductase-like protein 6 - *Vitis vinifera*	E-106	Isoflavonoid phytoalexins biosynthesis; Dixon 2001
FC325182	4	2	Q3KN67	Isoflavone reductase-like protein 6 - *V. vinifera*	2E-35	Same as above
FC325263	1	1	Q9SDZ1	Isoflavone reductase homolog 1 - *Glycine max*	1E-36	Pterocarpan phytoalexin synthesis; Dixon 2001
FC325537	1	1	Q9M528	Phenylcoumaran benzylic ether reductase - *Forsythia intermedia*	7E-37	Lignan biosynthesis; Van der Mijnsbrugge et al. 2000
FC325422	2	1	Q1S8W2	O-methyltransferase, family 2 - *M. truncatula*	3E-68	Anthocyanin biosynthesis; Winkel-Shirley 2002
FC325322	1	1	Q1SJP3	E-class P450, group I - *M. truncatula*	1E-38	Monolignols biosynthesis; Werck-Reichhart 1995
FC325406	5	1	Q1SJP3	E-class P450, group I – *M. truncatula*	1E-35	Same as above
FC325648	4	3	Q96560	NADPH-ferrihemoprotein reductase - *Helianthus tuberosus*	3E-21	NADPH-cytochrome P450 reductase on which cyt b5 involved in anthocyanin biosynthesis is dependent; De Vetten et al. 1999.

Terpenoid phytoalexins biosynthesis						
FC325369	1	1	Q64K29	(-)-germacrene D synthase - *Populus jackii*	8E-55	Sesquiterpenoid synthesis; Arimura et al, 2004
FC325692	1	1	Q5Q1I3	3-beta hydroxysteroid dehydrogenase - *Digitalis thapsi*	6E-24	Similarity to (-)-isopiperitenol dehydrogenase; Herl et al. 2007
FC325551	3	2	Q6Q3H2	Terpenoid synthetase - *V. vinifera*	3E-80	Terpenoid classes of phytoalexins; Dixon 2001
FC325381	6	1	Q5C9I9	(-)-isopiperitenol dehydrogenase - *Mentha piperita*	1E-69	Monoterpene and lignan biosynthesis; Ringer et al. 2005
Pathogenesis related proteins						
FC325238	6	6	Q00MX6	Pathogenesis-related protein 1a - *Malus domestica*	2E-62	PR-1 family protein; Santén et al. 2005
FC325338	6	5	Q9M2U5	Class IV chitinase (CHIV) (At3g54420) - *A. thaliana*	5E-83	PR-3 family protein; Selitrennikof 2001
FC325284	10	7	Q6RV28	Class IV chitinase - *M. truncatula*	5E-73	Same as above
FC325581	2	2	Q9M2U5	Class IV chitinase (CHIV) (At3g54420) - *A. thaliana*	E-110	Same as above
FC325206	10	8	Q6KF83	Pseudo-hevein (Fragment) - *Hevea brasiliensis*	7E-52	Chitinase activity; Koo et al. 2002
FC325261	4	2	Q1RYK4	Glycoside hydrolase, family 18 - *M. truncatula*	E-119	Chitinases; Hamel et al. 1997
FC325240	4	2	P29063	Pathogenesis-related protein PR-4B - *Nicotiana tabacum*	1E-58	PR-4 family protein; Van Loon and Van Strien 1999
FC325601	2	1	Q2HPG3	Osmotin-like protein I - *Gossypium hirsutum*	1E-98	PR-5 family protein; antifungal protein; Monteiro et al. 2003
FC325266	9	7	Q9FSG7	Thaumatin-like protein 1a - *M. domestica*	7E-90	PR-5 family protein; antifungal protein; Breiteneder 2004
FC325545	2	2	Q1SV12	Kunitz inhibitor ST1-like – *M. truncatula*	1E-29	PR-6 family protein; Heitz et al.1999
FC325621	9	7	Q1SV12	Kunitz inhibitor ST1-like – *M. truncatula*	1E-26	Same as above
FC325534	1	1	Q41361	Pathogenesis-related protein PR-6 type - *Sambucus nigra*	7E-21	Same as above
FC325446	1	1	Q8LNY1	Protease inhibitor 1 (Fragment) - *Zinnia elegans*	2E-17	Same as above
FC325440	6	2	Q6YEY6	Protease inhibitor – *V. vinifera*	5E-16	Same as above
FC325589	4	3	Q1S278	Proteinase inhibitor I13 - *M. truncatula*	6E-18	Same as above
FC325660	5	1	Q1S278	Proteinase inhibitor I13 - *M. truncatula*	5E-14	Same as above
FC325445	22	17	Q4QTJ1	S-norcoclaurine synthase 2 - *Papaver somniferum*	3E-20	PR-10 family protein; benzylisoquinoline alkoloids secondary metabolites synthesis; Samanani et al. 2004

FC325451	2	1	Q9LCZ9	Photoassimilate-responsive protein - *A. thaliana*	3E-32	Disease inducible gene; Takemoto et al. 2003
Other antifungal proteins						
FC325317	2	1	Q9FE98	Alliinase, putative; 28821-30567 – *A. thaliana*	1E-81	Synthesis of antifungal Allicin ; Luo et al. 2009
FC325281	3	2	Q9M7D7	Non-specific lipid-transfer protein - *Pisum sativum*	1E-29	Antifungal activity and surface wax formation; Subhankar et al. 2006
Other stress related proteins						
FC325502	1	1	P52706	(R)-mandelonitrile lyase 1 precursor - *Prunus serotina*	5E-50	Cyanogenesis; Hickel et al. 1996
FC325407	2	1	Q9C938	Putative oxidoreductase; 33116-34434 – *A. thaliana*	3E-35	Oxidative stress; Babiychuk et al. 1995
FC325592	3	3	O23939	Ripening-induced protein - *Fragaria vesca*	E-109	Ethylene response; Nam et al. ,1999
Proteins not associated with defense						
FC325542	1	1	O24465	Thymidine diphospho-glucose 4-6-dehydratase homolog - *P. armeniaca*	6E-72	
FC325346	1	1	Q9ZRF1	Probable mannitol dehydrogenase - *Fragaria ananassa*	1E-67	
FC325482	2	1	Q9ZTT3	Subtilisin-like protease C1 - *Glycine max*	4E-24	
FC325315	3	3	Q1H8M8	B12D-like protein - *Beta vulgaris*	4E-39	
Hypothetical proteins						
FC325183	1	1	Q2PFW4	Hypothetical protein - *Macaca fascicularis*	1E-13	
FC325212	4	1	Q0WYB7	Hypothetical protein – *N. benthamiana*	1E-28	
FC325412	4	1	Q1S5R4	Hypothetical protein – *M. truncatula*	4E-30	
FC325579	10	7	Q7F9L9	OSJNBa0006A01.5 protein - *Oryza sativa*	6E-27	
No homology Hit						
FC325556	1	1	*****	No significant hits	*****	
FC325653	1	1	*****	No significant hits	*****	
FC325690	1	1	*****	No significant hits	*****	
FC325705	1	1	*****	No significant hits	*****	
Total	230	155				

[a] The 638 cDNA library clones were screened with unsubtracted tester (cDNA population prepared from RNA extracted from *O. novo-ulmi*-inoculated *U. americana* callus tissues at 72 hpi) and unsubtracted driver (1:1 ratio of cDNA populations prepared from RNA extracted from mock-inoculated *U. americana* callus tissues at 72 hpi and from 72 h old axenic cultures of *O. novo-ulmi*) probes. cDNAs hybridizing more strongly to the unsubtracted tester as compared to the unsubtracted driver probe were considered upregulated.

[b] Singlets: GenBank EST accession number; contigs: GenBank accession number of EST with the longest overlap within a contig.

[c] Total number of ESTs in a unisequence

[d] Number of differentially expressed (upregulated) ESTs within a unisequence.

[e] Same homology hits were homologous sequences and not different parts of same transcript.

3.4.3 Expression profiles of selected American elm unisequences by qRT-PCR

Six cDNAs that showed constitutive expression and 12 that showed upregulation in infected tissues at 72 hpi according to the differential screening procedure were selected for further study using qRT-PCR. The corresponding unisequences represented singlets and contigs and belonged to different biochemical pathways (Table 4 and 5). The plant origin of all 18 sequences was confirmed by PCR with specific primers.

We monitored the expression profile of each gene in mock- (water) and fungus-inoculated calli at 4, 24, 48, 72, 96, and 144 hpi and compared it to the expression in healthy calli harvested at the start of the experiments, i.e. at 0 hpi. After confirmation of constitutive expression by qRT-PCR, GeNorm was used to identify the most stably expressed control genes (Vandesompele et al. 2002). Of the original six constitutively expressed genes, the following four were retained to calculate a reliable normalization factor: FC325256, FC325680, FC325436, and FC325344. The expression was stable over time, i.e. varying less than two fold in qRT-PCR experiments (Fig. 3.3). Of the 12 genes selected by the differential screening procedure as upregulated in infected tissues at 72 hpi, only one sequence (diacylglycerol kinase [FC325614]) showed constitutive expression over the time course studied and was considered as a reference gene (Fig. 3.3). The remaining 11 unisequences were upregulated in infected calli according to qRT-PCR analysis (Fig. 3.4). Statistically significant differences between expression in mock- and fungus-inoculated calli were observed starting at 48 hpi and continued on with the exception of three unisequences: FC325592 (Ripening-induced protein) and FC325621 (Kunitz inhibtor ST-1 like) whose differences in expression became statistically significant at 72 hpi, and FC325422 (O-methyltransferase family 2) which was highly induced only at 144 hpi. FC325422 exhibited the highest level of upregulation (a 1326-fold increase in fungus-infected calli) among the unisequences tested. The results of qRT-PCR assays were summarized and contrasted with differential screening results in Table 5. Although most unisequences were expressed in mock-inoculated calli and upregulated during the infection, some had a very low expression level (FC325322 and FC325206) or were barely detectable (FC325422) in mock-inoculated callus and still showed induction during infection (Table 5).

Table 5: Expression analysis of 18 *Ulmus americana* unisequences by qRT-PCR and differential screening.

***U. americana* EST acc. no.**[a]	**Putative function**	**Expression analysis**			
		DS[b]	**qRT-PCR**		
		Expression level[c]		**Initial no. of transcripts**[d]	**Maximum no. of transcripts**[e]
FC325256	Vacuolar ATP synthase subunit G 1	-	-	759±128	892±170 at 24 hpi (i)
FC325680	EIF5A	-	-	2637±518	2796±325 at 24 hpi (i)
FC325436	Splicing factor 3B subunit 5-like protein	-	-	1176±80	1404±147 at 96hpi (m)
FC325344	NAD(H) kinase 1	-	-	124±2.5	245±36 at 144 hpi (i)
FC325614	Diacylglycerol kinase	+	-	102±31	209±41 at 144 hpi (i)
FC325348	Ascorbate peroxidase	-	-	372±58	478±105 at 24 hpi (i)
FC325312	CCHC-type integrase	-	-	3±1	7±1 at 144 hpi (i)
FC325537	Phenylcoumaran benzylic ether reductase	+	+	263±46	13063±1893 at 144 hpi (i)
FC325322	E-class P450, group I	+	+	15±9	1446±203 at 144 hpi (i)
FC325422	O-methyltransferase, family 2	+	+	0±0.1	92±25 at 144 hpi (i)
FC325592	Ripening-induced protein	+	+	912±158	3131±457 at 144 hpi (i)
FC325589	Proteinase inhibitor I13	+	+	192±312	10925±2506 at 144 hpi (i)
FC325238	Pathogenesis-related protein 1a	+	+	697±348	11570±3973 at 144 hpi (i)
FC325621	Kunitz inhibitor ST1-like	+	+	1432±581	15114±1420 at 144 hpi (i)
FC325284	Class IV chitinase precursor	+	+	418±42	5457±1371 at 144 hpi (i)
FC325206	Pseudo-hevein	+	+	32±18	1280±508 at 144 hpi (i)
FC325445	S-norcoclaurine synthase 2	+	+	809±169	16915±3091 at 144 hpi (i)
FC325532	Isoflavone reductase-like protein 6	+	+	155±31	10430±1003 at 144 hpi (i)

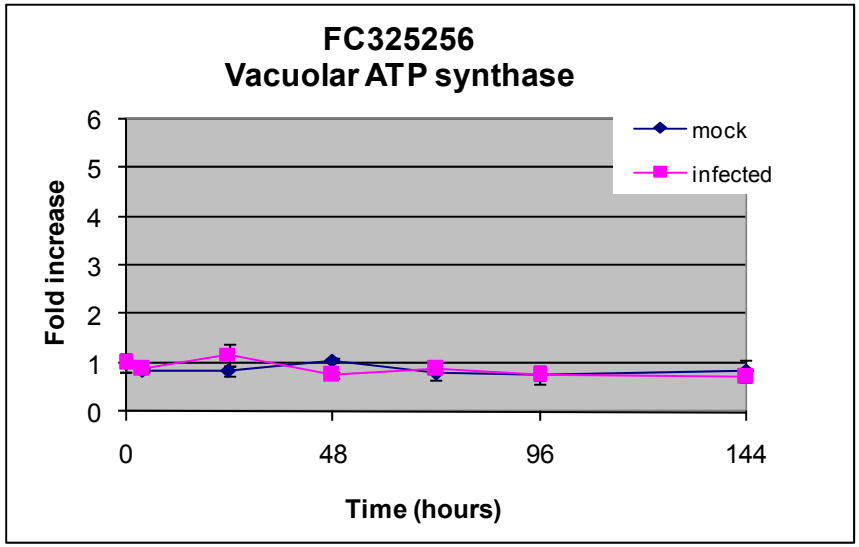
[a] Genbank accession number of EST. Accession numbers corresponding to reference genes were indicated in bold type.

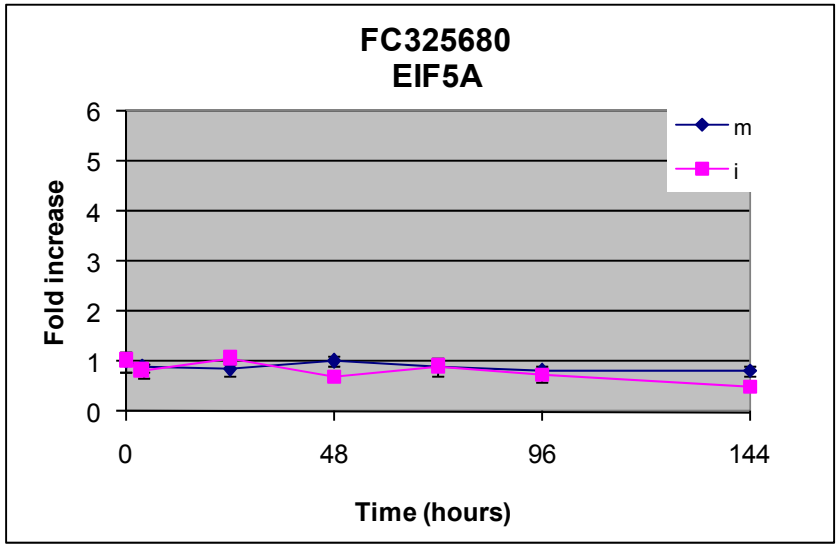
[b] DS = differential screening.

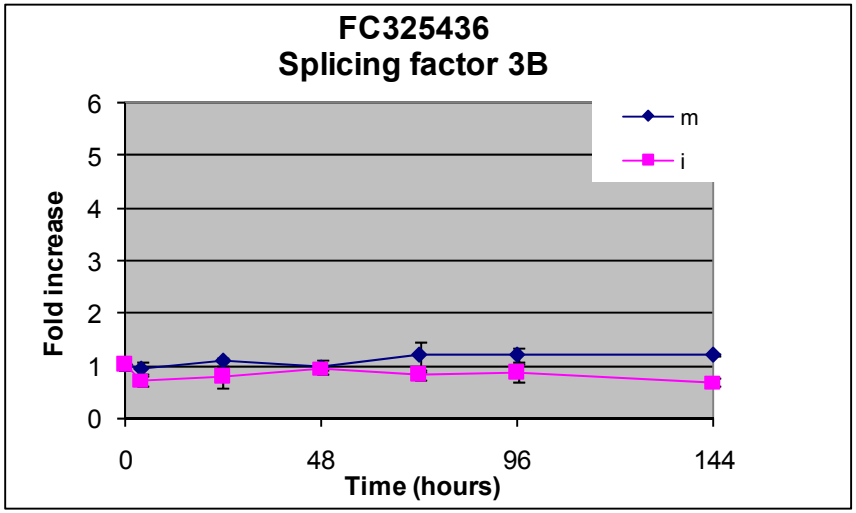
[c] - = no upregulation; + = upregulation.

[d] Number of transcript molecules/ng total RNA calculated after normalization at 0 h in healthy callus samples used for qRT-PCR tests (n = three biological replicates).

[e] Maximum number of transcript molecules/ng total RNA calculated after normalization at the specified time point (numbers are means ± standard deviations; n = three biological replicates; m = mock-inoculated elm callus tissue, i = *O. novo-ulmi* inoculated elm callus tissue).


FC325256
Vacuolar ATP synthase
Fold increase
Time (hours)
mock
infected
0
1
2
3
4
5
6
0
48
96
144


FC325680
EIF5A
Fold increase
Time (hours)
m
i
0
1
2
3
4
5
6
0
48
96
144


FC325436
Splicing factor 3B
Fold increase
Time (hours)
m
i
0
1
2
3
4
5
6
0
48
96
144

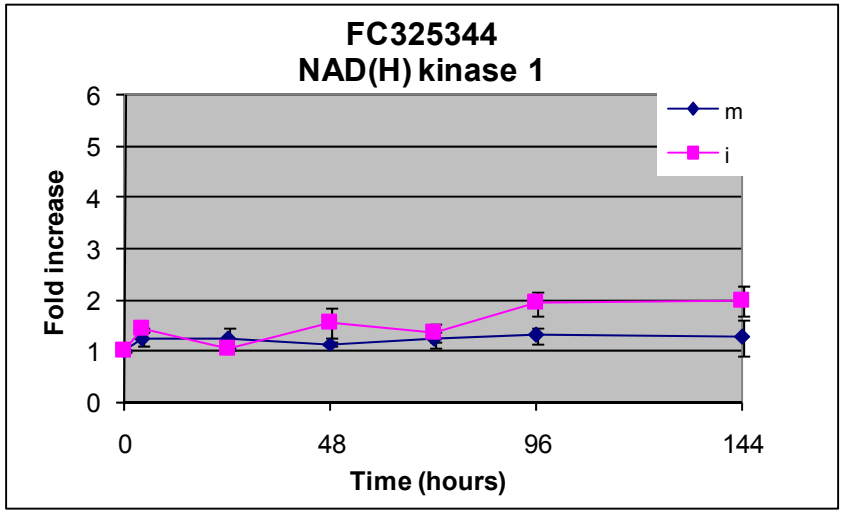
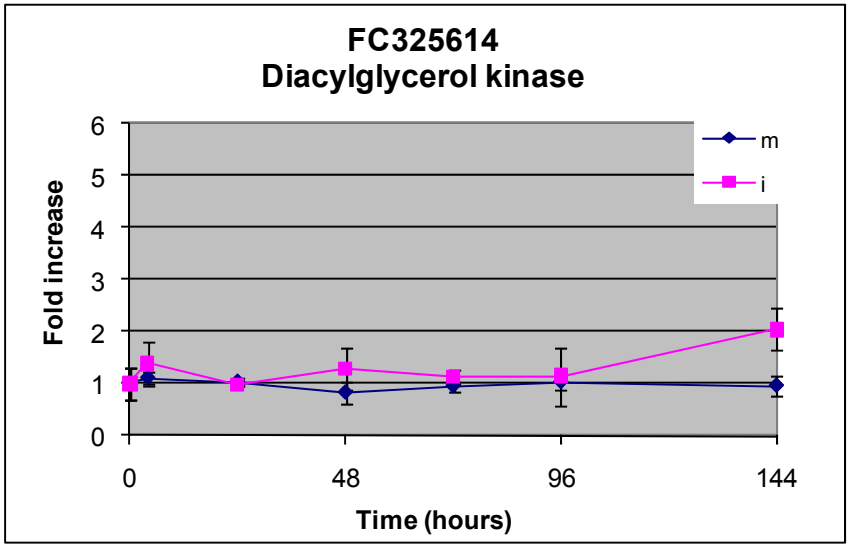
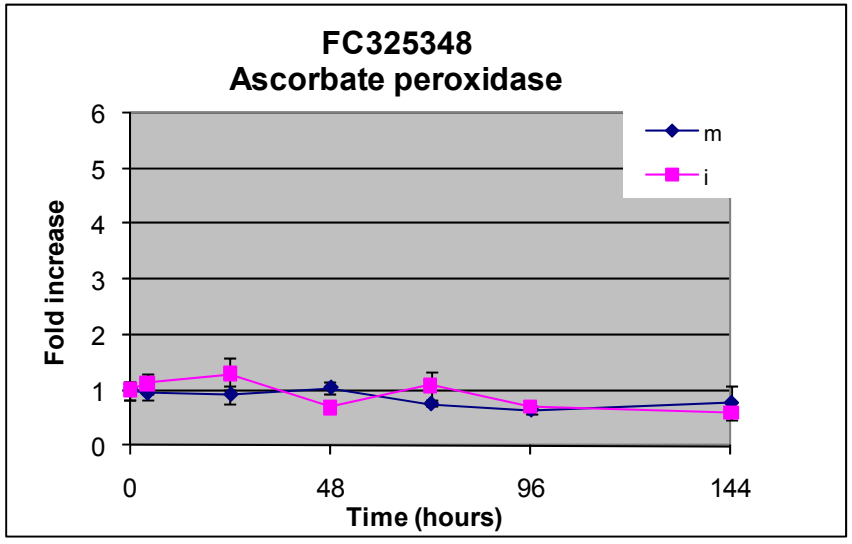

FC325344
NAD(H) kinase 1
Fold increase
Time (hours)
m
i
0
1
2
3
4
5
6
0
48
96
144

Figure 3.3


FC325614
Diacylglycerol kinase
Fold increase
Time (hours)
m
i
0
1
2
3
4
5
6
0
48
96
144


FC325348
Ascorbate peroxidase
Fold increase
Time (hours)
m
i
0
1
2
3
4
5
6
0
48
96
144

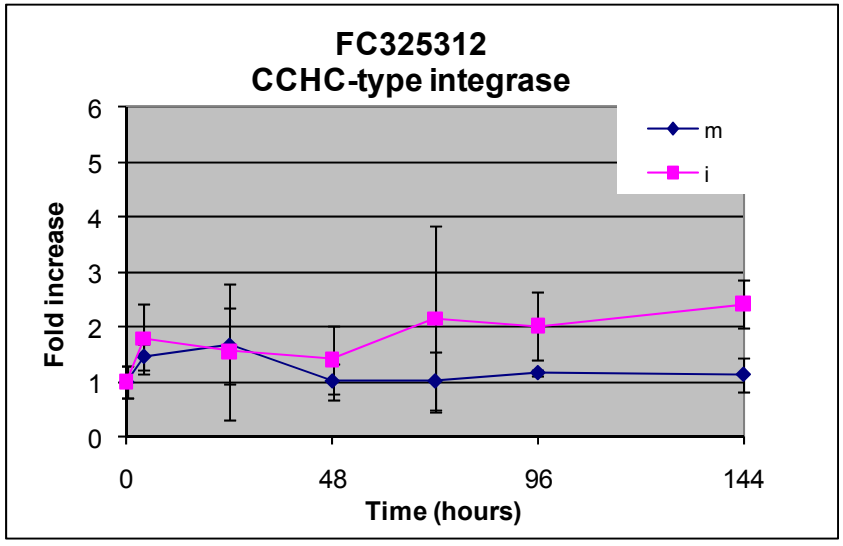
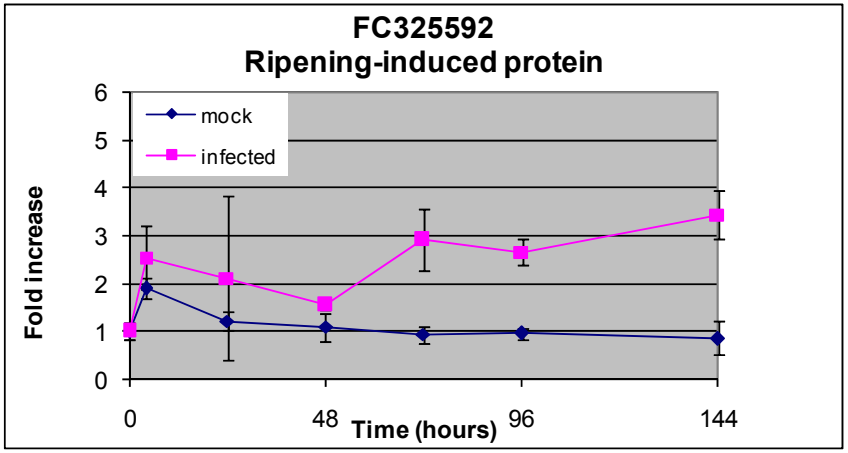
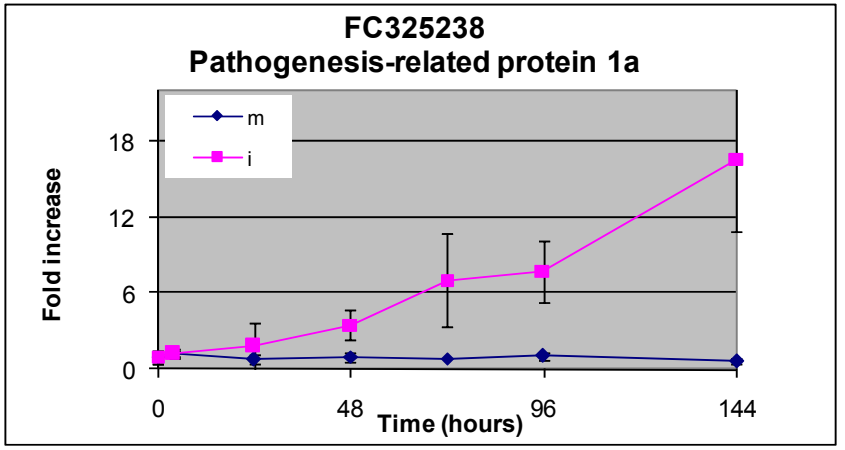

FC325312
CCHC-type integrase
Fold increase
Time (hours)
m
i
0
1
2
3
4
5
6
0
48
96
144

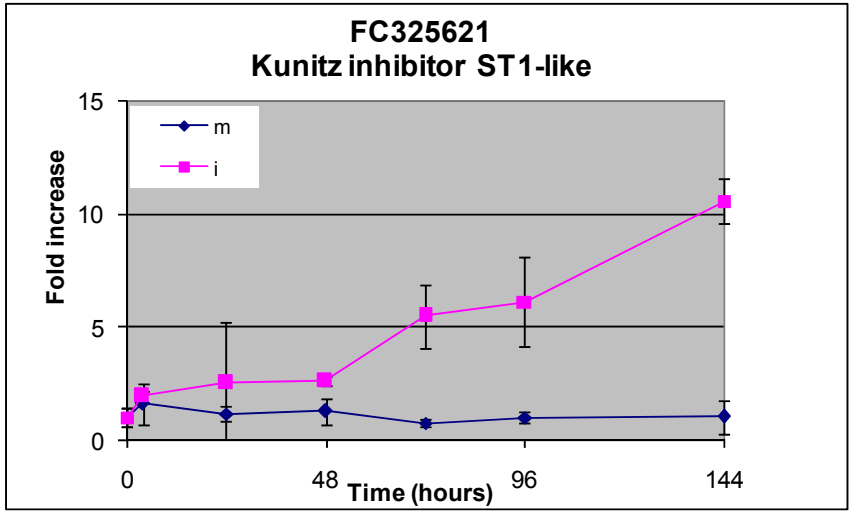
Figure 3.3

Figure 3.3: Seven *Ulmus americana* unisequences that showed constitutive expression profiles in American elm callus tissue inoculated with the fungus *Ophiostoma novo-ulmi* or water-treated (mock inoculation). The monitoring of gene expression profiles was done by quantitative reverse-transcriptase polymerase chain reaction[a] at 4, 24, 48, 72, 96, and 144 hours post-inoculation (hpi).

[a] Transcript levels were normalized with five control genes (FC325256; FC325680; FC325436; FC325344; FC325614; first five graphs of this Figure) that had the most stable expression and were used to calculate a reliable normalization factor according to Vandesompele et al. (2002). Results are expressed as fold increase in expression level compared to healthy calli harvested at the start of the experiment (0 hpi). Means ± standard deviations of three biological replicates are presented. Two technical replicates were run for each gene.


FC325592
Ripening-induced protein
mock
infected
Fold increase
Time (hours)
0
1
2
3
4
5
6
0
48
96
144


FC325238
Pathogenesis-related protein 1a
m
i
Fold increase
Time (hours)
0
6
12
18
0
48
96
144


FC325621
Kunitz inhibitor ST1-like
m
i
Fold increase
Time (hours)
0
5
10
15
0
48
96
144

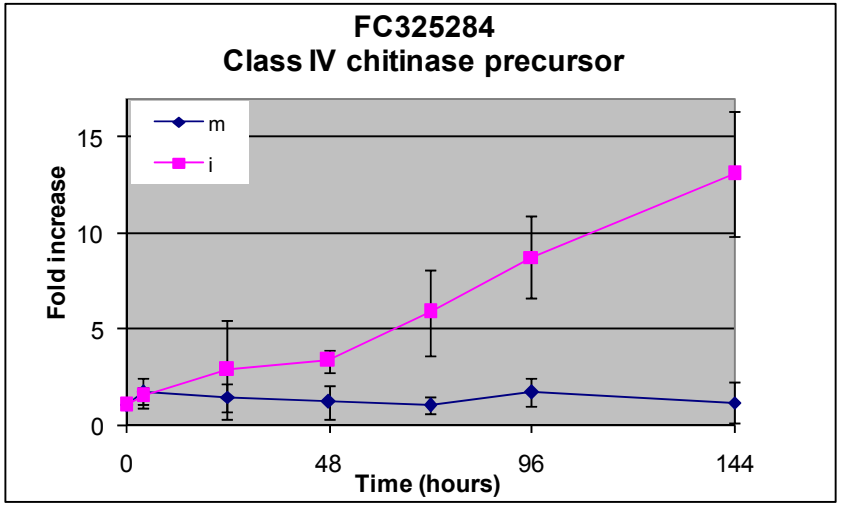
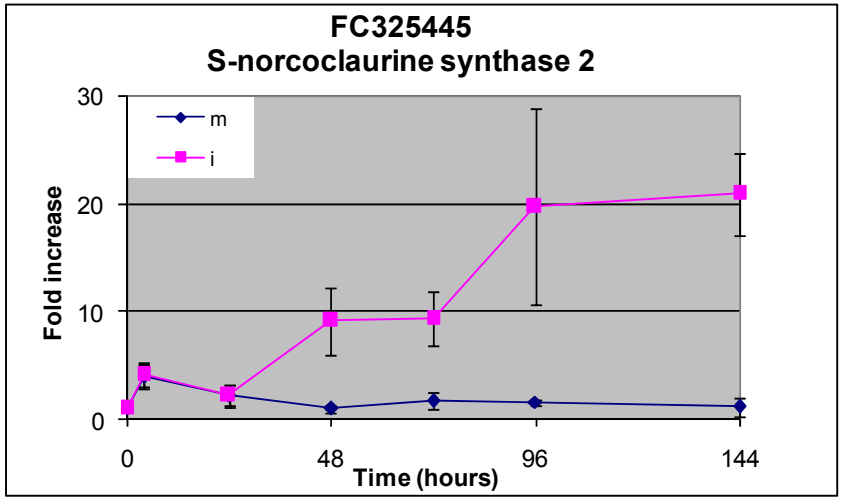
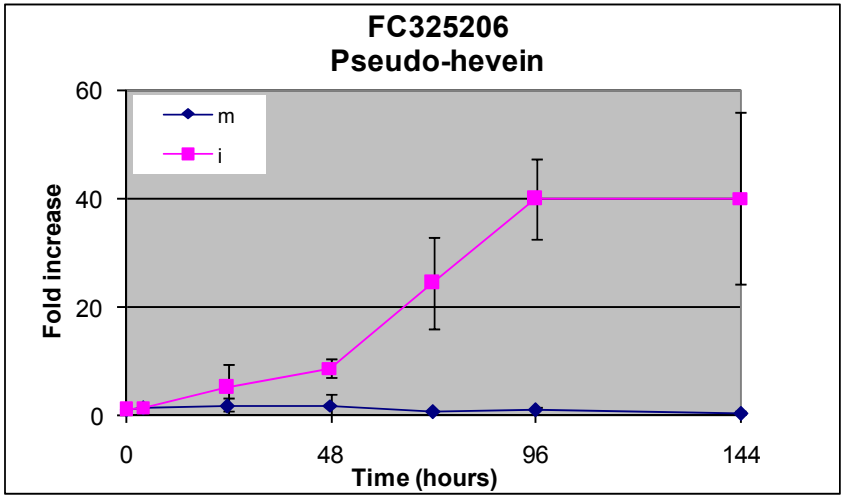
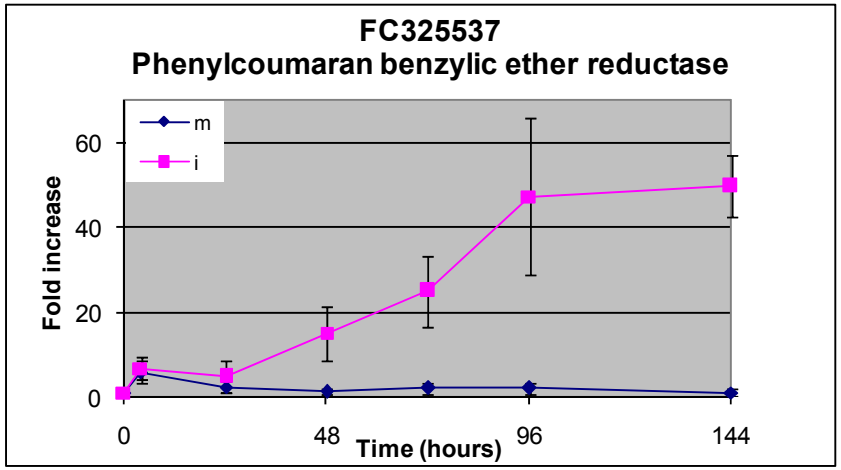

FC325284
Class IV chitinase precursor
m
i
Fold increase
Time (hours)
0
5
10
15
0
48
96
144

Figure 3.4


FC325445
S-norcoclaurine synthase 2
Fold increase
Time (hours)
m
i
0
10
20
30
0
48
96
144


FC325206
Pseudo-hevein
Fold increase
Time (hours)
m
i
0
20
40
60
0
48
96
144


FC325537
Phenylcoumaran benzylic ether reductase
Fold increase
Time (hours)
m
i
0
20
40
60
0
48
96
144

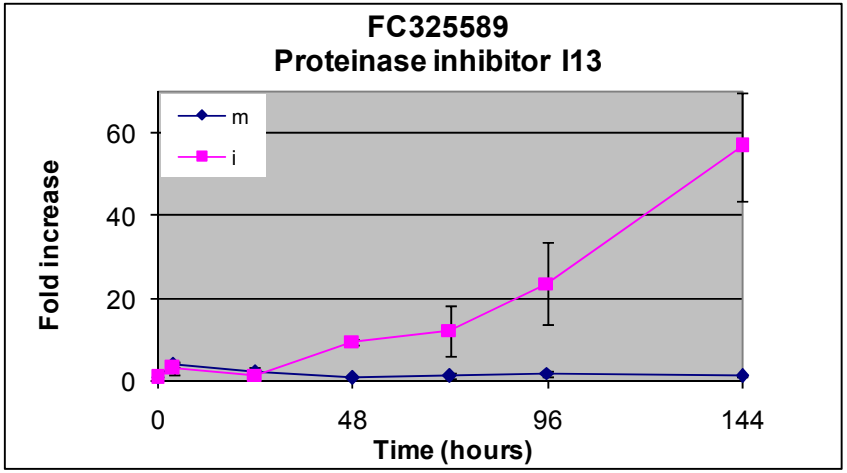

FC325589
Proteinase inhibitor I13
Fold increase
Time (hours)
m
i
0
20
40
60
0
48
96
144

Figure 3.4

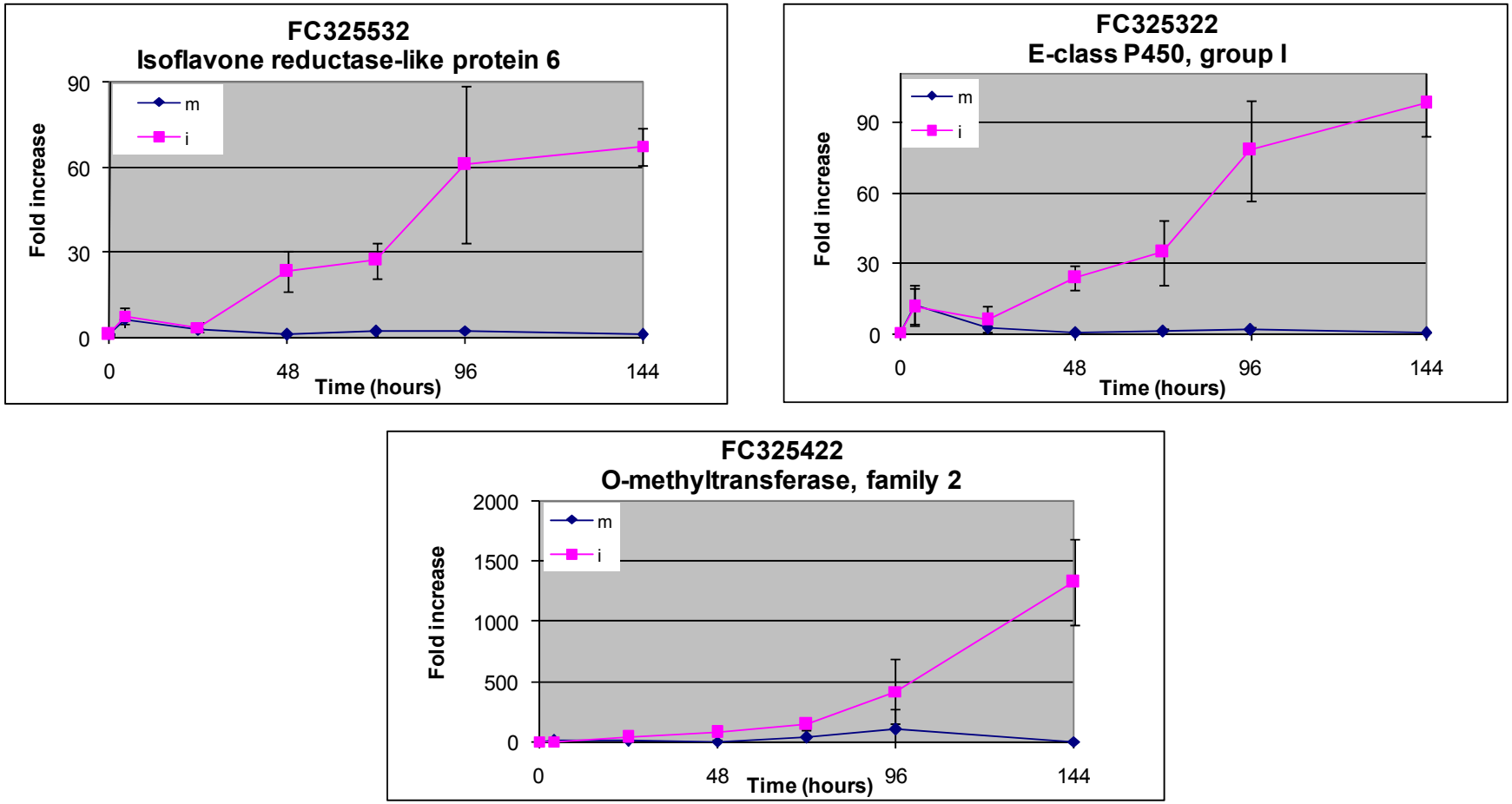


Figure 3.4: Eleven *Ulmus americana* unisequences that were upregulated in *Ophiostoma novo-ulmi* infected American elm callus tissue.

Callus tissues were either mock-inoculated with water or inoculated with *O. novo-ulmi* budding cells. The monitoring of gene expression profiles was done by quantitative reverse-transcriptase polymerase chain reaction[a] at 4, 24, 48, 72, 96, and 144 hours post-inoculation (hpi).

[a] Transcripts levels were normalized by comparison with five control genes (FC325256; FC325680; FC325436; FC325344; FC325614) that had the most stable expression and were used to calculate a reliable normalization factor according to Vandesompele et al. (2002). Results are expressed as fold increase in expression level compared to healthy calli harvested at the start of the experiment (0 hpi). Means ± standard deviations of three biological replicates are presented. Two technical replicates were run for each transcript.

3.5 Discussion

Dutch elm disease (DED) is by far the most important disease of Amercican elm. In stark contrast, very little is known about the molecular mechanisms underlying the host-pathogen interaction. Genomic resources are being developed for *O. novo-ulmi* (Bernier et al. 2004; Bouvet et al. 2007; Plourde et al. 2008). Lesser resources, however, are available for *U. americana* which was a common forest canopy and popular urban tree before the onset of DED. Recently, Newhouse et al. (2007) were able to genetically transform American elm with a synthetic antimicrobial peptide. Nasmith et al. (2008a, b) cloned and analysed three *U. americana* genes. In contrast, this study provides for the first time a more comprehensive analysis at the molecular level of the compatible interaction between the two protagonists of DED.

An in vitro system consisting of *U. americana* callus tissue cultures inoculated with *O. novo-ulmi* budding cells was used to study the interaction. Previously we analysed and validated this system histopathologically using different types of microscopy (Aoun et al. 2009). This approach enabled us to identify an appropriate time point for tissue harvest, namely 72 hpi, at which the fungus had germinated and spread throughout the callus tissue such that most host cells were in close contact with the pathogen and exhibited reactions against the presence of the fungus (Aoun et al. 2009).We then used a combination of SSH and qRT-PCR techniques to identify and study American elm transcripts involved in the interaction. Our results show that *O. novo-ulmi* infection of elm callus culture has a pronounced effect on gene expression of the host and that the in vitro system is a useful tool to examine molecular aspects of the interaction.

The library consisted mostly of *U. americana* sequences (94%). We believe this to be due to an over-representation of host cDNAs during the SSH process. Using *O. novo-ulmi* elongation factor (*ef1*α) primers in qRT-PCR, we were able to estimate at 2% the proportion of fungal RNA in the total RNA from the inoculated sample that served for the construction of the library (data not shown). Low number of pathogen mRNAs isolated during SSH had been previously reported (Beyer et al. 2001). Since the driver consisted of an equal mix of cDNA populations from both organisms, and an excess of driver was used during the two hybridization steps in the SSH procedure, the excess of cDNAs of fungus origin may have removed a large number of fungus tester cDNAs during the SSH

hybridization steps. A driver that represents the real percentage of mRNAs could possibly circumvent at least part of this problem and allow isolation of more fungus sequences. In fact, by constructing another cDNA library subtracted only with cDNAs synthesized from RNA extracted from healthy elm callus, we were able to raise the total number of all fungus sequences obtained from 1 to 18 % (data not shown). Considering that the overwhelming majority of the library unisequences was of plant origin, the results of the present study benefit the DED community in at least two ways: 1) identification of *U. americana* sequences that were upregulated in the presence of *O. novo-ulmi* and thus may be considered involved in the tree response to pathogen attack; and 2) identify a starting point for the development of a valuable genome resource for American elm which will allow, among other things, a more focused approach to management and improvement of disease resistance.

The qRT-PCR tests confirmed the differential expression of 11 of the 12 genes retained after differential screening. Furthermore, by using qRT-PCR, we were able to trace gene expression profiles during a time course experiment, and define and validate reference elm genes with stable expression in mock and infected callus tissues. These reference genes could be tested and used in future work in transcription analysis related to DED (Gutierrez et al. 2008). Expression profiles generated by qRT-PCR showed that differential expression started at 48 hpi for eight of the 11 genes and that it continued without decreasing for all upregulated genes at the considered time points. Interestingly, 48 hpi was the time point at which *O. novo-ulmi* mycelium was seen in infected *U. americana* callus cultures (Aoun et al. 2009). Since none of the genes analyzed in this study were induced prior to 48 hpi, we hypothesize that defense reactions against *O. novo-ulmi* may not be completely expressed in susceptible *U. americana* callus cells before fungal germination and spread take place. In poplar, a model system in forest biology, Rinaldi et al. (2007) showed that a striking alteration in steady-state RNA populations took place at or after 48 hpi when the biotrophic rust fungus pathogen *Melampsora larici-populina* (Kleb.) already penetrated host leaf cells to form haustorial infection structures, whereas few genes were induced at earlier stages of interaction.

The 53 unisequences containing upregulated cDNAs of the interaction library represented genes coding for proteins with diverse functions from signalling to secondary

metabolite production. In general, genes encoding enzymes or proteins leading to the accumulation of phytoalexins as well as genes encoding proteins belonging to different classes of PR proteins were abundant among the 53 selected unisequences. Proteins involved in the phenylpropanoid pathway which leads to biosynthesis of phenolic compounds such as flavonoids, pigments, lignan, and lignin (Zhao et al. 2005) were particularly well represented. High amounts of phenolic compounds accumulated in infected calli (Aoun et al. 2009). The present study links these observations to events at the molecular level, i.e. the upregulation of American elm transcripts involved in the production of isoflavonoids (i.e. FC 325532), anthocyanin pigments (i.e.FC325422), and lignan (i.e.FC325537). In addition, genes encoding P450s were differentially expressed. It has been shown that a class of P450s in plants, called biosynthetic P450s, play a paramount role in the biosynthesis of cell wall constituents (particularly lignin) and of secondary metabolites in plant defense (particularly isoflavonoids and coumarins) (Werck-Reichhart 1995). Thus, American elm genes associated with the biosynthesis of products related to compartmentalization mechanisms in infected trees were identified in callus tissue. This was not surprising considering that *O. novo-ulmi* spreads in the elm xylem and that reaction zones induced by its presence have been observed in xylem tissues, particularly in parenchyma cells (Rioux et al. 1991a, b), which resemble structurally the de-differentiated callus tissue. Other genes directly involved in lignin and suberin biosynthesis such as caffeoyl-CoA O-methyltransferase (FC325352) and peroxidases (FC325230, FC325247, FC325468) were found in the library (see Appendix) but not among the upregulated sequences. These genes may be differentially expressed, but at levels below the detection limit of the assays used in this study.

Several authors have described the accumulation in vivo, as well as in vitro, of mansonones in elms after *O. novo-ulmi* infection (Duchesne et al. 1985; 1994; Yang et al. 1993; 1994). These phytoalexins belong to a family of sesquiterpenoid quinones within the terpenoid classes of phytoalexins. Genes coding for enzymes involved in the terpenoid pathway were found in the library and among the 53 upregulated unisequences (Table 4). To our knowledge, this was the first report of genes coding for enzymes possibly leading to mansonone biosynthesis in elm. In addition, we observed a significant accumulation of transcripts of genes involved in the synthesis of isoflavonoid phytoalexins which belong to

the phenylpropanoid class of phytoalexins. Induction of this type of phytoalexins in elm in response to the DED pathogen was not previously reported. Flavonoid pathway genes were also found to be strongly upregulated in the compatible poplar-*M. medusae* (Thüm.) interaction (Miranda et al. 2007).

Our results also suggest the potential importance of de novo PR protein expression by *U. americana* in response to attack by *O. novo-ulmi*. PR proteins are coded for by the host plant, but induced specifically in pathological or related situations. It has been suggested that the collective set of PR proteins may be effective in inhibiting the multiplication and/or spread of a pathogen (Ryals et al. 1996, Van Loon and Van Strien 1999). Genes encoding proteins belonging to different PR protein families (PR-1, PR-3, PR-4, PR-5, PR-6, and PR-10) were shown to be differentially expressed upon *U. americana* callus infection with *O. novo-ulmi*.

Clone FC325238 represents a gene encoding a protein from the PR1 family. PR-1 proteins were proposed to have antifungal activity and may have a role in strengthening host cell walls to prevent spread of the pathogen (Santén et al. 2005). Clones FC325589 and FC325621 represent genes encoding a proteinase inhibitor I and a Kunitz ST1-like inhibitor, respectively. Both of these PR-6 proteins belong to the serine proteinase inhibitors induced in response to infection by pathogens (Heitz et al. 1999). Kunitz ST1-like inhibitor was induced up to 10.5 fold in this study, thus confirming the results obtained by Azaiez et al. (2009) who reported upregulation of some Kunitz inhibitors in response to rust infection of hybrid poplar.

Clone FC325284 represents a gene encoding a class IV chitinase precursor. In fact, several differentially expressed clones representing class IV chitinases belonging to the PR-3 family were found in our library. Nasmith et al. (2008b) showed the induction of a class I chitinase in *U. americana* leaf midribs in response to infection by *O. novo-ulmi*. In this study, we identified different classes of chitinases in elm that were induced upon infection by the DED fungus. Clone FC325206, which represents a gene encoding a pseudo-hevein, could belong to another class of chitinases, as hevein-like peptides usually contain a chitin-binding domain and exhibit an antifungal activity against a broad spectrum of phytopathogenic fungi in vitro (Parijs et al. 1991; Koo et al. 1998). These have been shown to enhance resistance to fungal pathogens in transgenic plants (Koo et al. 2002). In tree-

pathogen interactions, chitinases appeared to be a prominent feature of the inducible defense profile of pine (Davis et al. 2002) and poplar trees (Clarke et al. 1998). Increased chitinase expression was observed in tissue cultures from susceptible and resistant Norway spruce in response to infection by the basidiomycete fungus *H. annosum* sensu lato and was confirmed in mature trees (Fossdal et al. 2006; Karlsson et al. 2007). Induction of chitinase was often coordinated with induction of specific β-1,3-glucanases (Collinge et al. 1993), and both enzymes have been confirmed to act synergistically in vitro and in planta. (Mauch et al.1988). Genes encoding β-1, 3-glucanases were not identified in this study. Whether this reflects a technical bias or a biological fact leading to susceptibility still needs to be determined.

FC325445 represents a trancript encoding (S)-norcoclaurine synthase, an enzyme within the PR-10 and the Betv1 tree allergen families. (S)-norcoclaurine is the ultimate precursor to all benzylisoquinoline alkaloids (BIAs), a large and diverse group of secondary metabolites found in several related plant families (Samanani et al. 2004, Liscombe et al. 2005). BIAs are structurally diverse, as a result of (S)-norcoclaurine being elaborated by a highly branched metabolic pathway consisting of several enzyme types including O-methyltransferases and cytochrome P450, which were both found to be upregulated in this study .

It was interesting to note that some enzymes involved in elicitor triggered signal cascade such as diacylglycerol kinase and NAD(H) kinase (Van der Luit et al. 2000) were expressed in a constitutive manner in *U. americana* callus during the infection period studied. It has been documented that successful plant defenses against pathogens were dependent on early recognition of the invader and the initiation of appropriate signalling processes that activate multi-cascade defense responses (Van Loon and Van Strien 1999). We hypothesize that lack of induction of several of these molecules, especially at early time points of the interaction, could reflect a lack of coordination of different defense strategies in susceptible *U. americana* and thus failure to produce resistance.

This study provides, for the first time, snapshots of molecular mechanisms involved in the interaction between *U. americana* and *O. novo-ulmi*. More specifically, we have identified American elm transcripts upregulated in callus tissue 72 hpi with the pathogen. Predominant within this group of sequences were transcripts coding for phenylpropanoid

pathway and PR proteins. However, significant increases in the expression of these genes were not recorded prior to 48 hpi. By this time the pathogen had alread germinated and colonized the callus tissue (Aoun et al. 2009). Thus this work suggests that a delay in host responses at the molecular level could lead to susceptibility in pathogen-challenged American elm. Spectroscopy coupled with chemometric methods showed that differential changes in lignin composition were detected in elm clones (*U. minor* Mill. and *U.minor* x *U. pumila* L.) that differed in susceptibility to DED. Increased levels of lignin in inoculated xylem tissues were observed earlier in a clone with lower susceptibility to the pathogen (Martin et al. 2007). Although the basis for resistance in elm species toward *O. novo-ulmi* is not clear, the rapidity (time) and level (amount) of the overall responses have been reported to determine the level of host resistance in other pathosystems (Daube 1986, Katagiri 2004, Van Loon et al. 2006). In the compatible interaction of hybrid poplar with the leaf rust fungus *M. medusae*, the largest number of changes in gene expression was observed late in the infection at 6 to 9 days post-inoculation (Miranda et al. 2007). In susceptible cultivars of sorghum seedlings, notable amounts of phytoalexins accumulated only 72 hpi. In contrast, phytoalexins accumulated to a considerable amount at about 36 hpi in the resistant cultivar (Lo et al. 1999). To further verify our hypothesis on the delay of host responses at the molecular level in susceptible *U. americana*, the genes identified in this study will provide a useful genomic resource for carrying out comparisons between DED-resistant and susceptible-elm species and varieties in the *Ulmus-Ophiostoma* pathosystem in vitro and eventually in planta.

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Conclusion

Dans cette thèse, nous avons décrit par deux approches, histopathologique et génomique, une interaction in vitro entre des cultures de cals de l'espèce sensible *Ulmus americana* et des spores du champignon pathogène *Ophiostoma novo-ulmi,* responsable de la maladie hollandaise de l'orme. Le choix d'un système in vitro s'est fait en considérant surtout sa facilité d'utilisation en laboratoire. L'existence d'études ayant utilisé avec succès de tels cals pour analyser certains aspects de l'interaction avec *O. ulmi* (s.l.) a aussi contribué à adopter ce système.

L'objectif de ce travail était d'utiliser des technologies génomiques de pointe qui permettent l'étude de plusieurs gènes à la fois afin d'aborder les mécanismes moléculaires impliqués dans cette interaction, ceux-ci ayant été peu étudiés pour ce pathosystème. Il a d'abord fallu bien définir ce système in vitro, d'où l'approche histopathologique utilisée dans le deuxième chapitre de cette thèse. Les observations dans le temps ont mis en évidence une interaction dynamique entre les deux organismes en contact. D'une part, le champignon a germé et formé un réseau d'hyphes qui a atteint tout le cal dans un délai de 48 h et qui a même été observé à l'intérieur de certaines cellules du cal. D'autre part, une accumulation de composés liés à la défense chez l'arbre, tels que les phénols, la subérine et la lignine, a été détectée chez les cellules du cal. Au fur et à mesure que l'infection progressait, certains composés semblaient être présents en plus grande quantité, comme rapporté par exemple au chapitre 2 pour la subérine avec un pourcentage de cellules subérisées 4.6 fois plus élevé à 96 heures post-inoculation (hpi) dans le cal inoculé par rapport au cal traité à l'eau.

Ces observations ont confirmé les résultats d'études précédentes qui, par exemple, ont montré la colonisation du cal par le champignon (Krause et al. 1996) et la détection des composés phénoliques chez les cellules du cal (Pijut et al. 1990b; Krause et al. 1996). En plus, d'autres aspects de l'interaction in vitro ont été mis en évidence pour la première fois dans la présente étude, tel que la détection et l'accumulation de la subérine qui rappelle des mécanismes étudiés chez l'arbre, par exemple dans le cadre de la compartimentation (Rioux et Ouellette 1991a; 1991b). Nous avons aussi observé en microscopie électronique à transmission pour la première fois dans des cals de plantes la structure lamellaire typique de la subérine. Il nous a alors été permis de conclure à l'existence, dans le système in vitro

utilisé, d'une interaction pathogénique montrant beaucoup de similarités avec l'interaction in planta. Ce système peut ainsi servir à l'indentification de gènes impliqués dans des mécanismes de pathogenèse ou de défense qui ont fait l'objet des observations microscopiques.

L'analyse génomique de l'interaction entre les cals d'*U. americana* et *O. novo-ulmi* a été réalisée suite à l'obtention d'une banque d'ADN complémentaires (ADNc) enrichie en séquences différemment exprimées par la technique des hybridations suppressives et soustractives (SSH) et sur la validation de l'induction de gènes par criblage différentiel et avec la PCR quantitative en temps réel (qRT-PCR). L'analyse bioinformatique de cette banque et l'analyse PCR des séquences sans homologie (No-hit) ont permis de constater l'origine végétale de 94% des étiquettes de séquences exprimées (EST). Cette analyse génomique, décrite dans le chapitre 3 de la thèse, a permis la production de plus de 500 EST d'orme américain, espèce méconnue du point de vue génomique. Un total de 535 séquences EST ont été déposées dans la banque de données Genbank. De plus, le criblage différentiel de la banque a permis l'identification des gènes dont l'expression est induite en présence du champignon. Ces gènes encodent différentes classes de protéines PR ainsi que des enzymes dont plusieures sont impliquées dans la voie de synthèse des phénylpropanoïdes. Les produits issus du métabolisme des phénylpropanoïdes sont des phytoalexines, des monolignols et des composés phénoliques qui servent entre autres à la production de subérine, de lignine et de lignan (cf. chapitre 1 de la thèse). Le profil d'expression en qRT-PCR de certains gènes encodant des enzymes dans la voie métabolique des phénylpropanoïdes telles que la PAL, l'isoflavone reductase et la phénylcoumarane reductase montre que ces gènes sont induits d'une façon significative dans les cals infectés du moins à partir de 48 hpi et que leurs transcrits deviennent plus abondants lors du développement de l'infection. Une corrélation est alors possible entre l'induction de ces gènes de la voie de synthèse des phénylpropanoïdes et l'accumulation des composés dérivés observés en microscopie. Alors que le champignon tente de coloniser le cal, ce dernier tente de se défendre en induisant des gènes dont les produits sont impliqués dans la synthèse de composés de défense. Néanmoins, dans cette interaction compatible, le champignon avait déjà colonisé le cal à 48 hpi alors que l'induction dans les cals infectés des gènes analysés par qRT-PCR n'était pas significative avant 48 hpi. Nous

pouvons alors nous demander si ce profil d'expression est particulier au cal sensible. En d'autres termes, quel sera le profil d'expression de ces gènes dans un cal d'orme résistant ? Dans le premier chapitre de cette thèse, nous avons examiné chez d'autres pathosystèmes comment les mécanismes de défense peuvent être contournés dans une interaction compatible et comment leurs produits semblent être inefficaces à produire la résistance chez les plantes sensibles. Une des hypothèses les plus marquantes semble être le délai de l'activation d'une défense efficace. Il serait alors nécessaire de comparer la réponse de cultures de cals d'ormes résistants dans le cadre d'une interaction incompatible avec le même agent pathogène (*O. novo-ulmi*), afin de tenter de percevoir l'expression différentielle des mécanismes de défenses entre un cal sensible et un cal résistant, et la contribution de ceux-ci à la résistance.

Un des apports de cette thèse est la démonstration que l'orme américain, espèce sensible, possède plusieurs mécanismes moléculaires de défense et que ces derniers sont exprimés lors de son interaction avec l'agent pathogène *O. novo-ulmi*. Cependant, la spécificité de la réponse de l'orme américain face à l'agent pathogène n'a pas été particulièrement examinée dans cette thèse. Une comparaison de la réponse du cal à l'agent pathogène avec celle à d'autres agents pathogènes (ex. *O. ulmi* ou certains *Verticillium*) ou à d'autres champignons (ex. des espèces saprophytes [telle *O. picea*] et mycorhiziennes) permettrait de préciser la spécificité de la réponse face à *O. novo-ulmi*. Celle-ci peut être qualitative ou peut dépendre de l'amplitude et du délai d'activation de la réponse.

Bien que cette thèse ait permis l'identification de certains gènes impliqués dans la défense, l'obtention d'un plus grand nombre d'ESTs pour l'orme américain faciliterait l'analyse à grande échelle des mécanismes de défense, et permettra de mieux viser les voies métaboliques impliquées. La banque d'interaction générée dans le cadre de cette thèse est un bon pas dans cette direction. Celle-ci offrira l'avantage de profiter, des technologies transcriptionnelles et aussi de celles protéomiques pour une meilleure compréhension de la nature des protéines produites et de leur implication dans la défense.

Le système étudié dans cette thèse a permis l'identification de gènes impliqués dans l'interaction in vitro. Les gènes identifiés par cette analyse pourront faire l'objet d'une autre analyse in planta. Cette dernière permettra de valider l'implication de gènes dans des conditions plus proches des conditions naturelles. De plus, le développement et

l'optimisation de protocoles de transformation efficaces chez l'orme d'Amérique conduirait à une analyse fonctionnelle mieux ciblée des gènes associés à la défense.

Concernant l'agent pathogène, l'analyse génomique effectuée sur des cals durs n'a pas permis l'identification significative de gènes fongiques. Cependant deux types de lignées de cals ont été développés dans le cadre de cette thèse, soit les cals durs et les cals mous. Le choix de cals durs pour l'analyse génomique était basé sur l'intensité de production de composés liés à la défense observée en microscopie. Néanmoins, les cals mous sont plus riches en espaces intercellulaires et pourront servir à la production d'une plus grande biomasse fongique lors de l'inoculation avec le champignon. On pourra donc isoler une plus grande quantité d'ARN fongique. Une analyse génomique pourra ainsi aboutir à l'identification de gènes associés à la pathogénie du champignon.

Enfin, cette thèse se veut une porte d'entrée sur la génomique de l'orme américain et de son interaction avec son agent pathogène le plus important à présent, *O-novo-ulmi*. Le développement d'une ressource génomique de l'orme et la poursuite de l'étude moléculaire de l'interaction pourront déboucher sur de meilleures stratégies pour la protection de l'orme et aussi sur une meilleure compréhension des maladies qui se traduisent en épidémies.

Et en mémoire des millions d'ormes qui ont succombé à la maladie hollandaise de l'orme partout en Europe et en Amérique du Nord, je ne peux que constater l'effet de la soumission de la nature au pouvoir de la fragilité….

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Annexe-Appendix

List of 314 *Ulmus americana – Ophiostoma novo-ulmi* interaction library unisequences and the putative functions based on comparison with entries to the UniProt (v9.3) database.

EST acc. no.[a]	No. of ESTs in unisequence	Closest database match		
		UniProt accession number	Putative function and taxon	E value
FC325181	1	Q3ED77	Protein At1g21610 - *Arabidopsis thaliana*	4E-17
FC325182	4	Q3KN67	Isoflavone reductase-like protein 6 - Vitis vinifera	2E-35
FC325183	1	Q2PFW4	Hypothetical protein - *Macaca fascicularis*	1E-13
FC325184	1	Q3EAI8	Protein At3g56310 - *A. thaliana*	7E-11
FC325186	1	P49351	Farnesyl pyrophosphate synthetase 1- *Lupinus albus*	2E-10
FC325189	3	Q05929	EDGP precursor (Fragment) - *Daucus carota*	3E-58
FC325197	1	Q6Q3H3	Terpene synthase - *V. vinifera*	8E-83
FC325201	1	Q0GPH4	BZIP transcription factor bZIP68 - *Glycine max*	7E-14
FC325203	1	Q0WLP3	Hypothetical protein - *A. thaliana*	7E-58
FC325205	1	P28188	Ras-related protein ARA-5 - *A. thaliana*	6E-47
FC325206	10	Q6KF83	Pseudo-hevein (Fragment) - *Hevea brasiliensis*	7E-52
FC325208	1	Q69F92	B12D-like protein - *Phaseolus vulgaris*	6E-34
FC325211	1	P52427	Proteasome subunit alpha type 4 - *Spinacia oleracea*	2E-74
FC325212	4	Q0WYB7	Hypothetical protein - *Nicotiana benthamiana*	1E-28
FC325214	2	Q0GBZ6	Chitinase - *H. brasiliensis*	4E-39
FC325216	3	Q8L8G1	Lipase SIL1 - *Brassica rapa*	E-107
FC325217	1	Q8L5Y4	Hypothetical protein At2g38770 - *A. thaliana*	2E-21
FC325218	1	Q1T417	Orn/DAP/Arg decarboxylase 2 - *Medicago truncatula*	2E-57
FC325219	1	Q1M0P0	UDP-glucuronic acid decarboxylase 3 - *Populus tomentosa*	E-109
FC325223	1	P43281	S-adenosylmethionine synthetase 2 - *Solanum lycopersicum*	9E-83
FC325224	1	Q6PUG0	3-dehydroquinate dehydratase - *Nicotiana tabacum*	3E-74
FC325225	1	Q5ZF89	Hypothetical protein - *Plantago major*	4E-17
FC325228	1	Q5V9L2	Cytochrome b5 isoform Cb5-C - *Vernicia fordii*	1E-58

FC325230	1	Q94IQ1	Peroxidase - *N. tabacum*	6E-71
FC325231	1	Q8LD56	Hypothetical protein - *A. thaliana*	4E-33
FC325232	1	Q6IM91	DVL10 - *A. thaliana*	8E-12
FC325234	1	Q6WNQ8	CYP81E8 - *M. truncatula*	1E-54
FC325235	1	Q9AYA9	Hypothetical protein OSJNBa0004B24.3 - *Oriza sativa*	9E-45
FC325236	1	Q5YJN3	Diadenosine tetraphosphate hydrolase - *Hyacinthus orientalis*	2E-54
FC325237	1	Q8LG43	Hypothetical protein - *A. thaliana*	7E-80
FC325238	6	Q00MX6	Pathogenesis-related protein 1a - *Malus domestica*	2E-62
FC325239	1	Q84R09	Hypothetical protein At4g18230 - *A. thaliana*	1E-64
FC325240	4	P29063	Pathogenesis-related protein PR-4B precursor - *N. tabacum*	1E-58
FC325245	1	Q9M1R2	Multifunctional aminoacyl-tRNA ligase-like protein - *A. thaliana*	1E-97
FC325247	1	Q08671	Peroxidase precursor (EC 1.11.1.7) - *Gossypium hirsutum*	1E-78
FC325248	2	Q1RYZ6	Glycoside hydrolase, family 1 - *M. truncatula*	4E-13
FC325249	1	Q40287	Anthocyanidin 3-O-glucosyltransferase - *Manihot esculenta*	6E-19
FC325251	1	Q9LVA0	*Dbj|BAA90612.1Hypothetical protein - A. thaliana*	8E-57
FC325253	1	Q39836	Guanine nucleotide-binding protein subunit beta- like protein - *G. max*	1E-94
FC325256	1	O82628	Vacuolar ATP synthase subunit G 1 - *A. thaliana*	2E-23
FC325257	1	Q84XQ4	NtPRp27-like protein - *Solanum tuberosum*	9E-54
FC325261	4	Q1RYK4	Glycoside hydrolase, family 18 - M. truncatula	E-119
FC325263	1	Q9SDZ1	Isoflavone reductase homolog 1 - *G. max*	1E-36
FC325264	2	Q8VWZ7	Geraniol 10-hydroxylase - *Catharanthus roseus*	7E-56
FC325266	9	Q9FSG7	Thaumatin-like protein 1a precursor - *M. domestica*	7E-90
FC325271	1	A1CST5	Isochorismatase family hydrolase, putative - *Aspergillus clavatus*	1E-64
FC325275	1	Q93YH3	ATP citrate lyase b-subunit - *Lupinus albus*	9E-83
FC325277	1	Q3E8B3	Hypothetical Protein At5g54840 - *A. thaliana*	4E-47
FC325278	1	O22860	60 S ribosomal protein L38 - *A. thaliana*	4E-27
FC325279	1	Q40090	SPF1 protein - *Ipomoea batatas*	4E-92
FC325281	3	Q9M7D7	Lipid transfer protein - *P. sativum*	1E-29
FC325284	10	Q6RV28	Class IV chitinase precursor - *M. truncatula*	5E-73
FC325285	1	Q45Q23	PHB2 - *N. benthamiana*	3E-70
FC325290	1	Q84JX5	Hypothetical protein (Fragment) - *A. thaliana*	2E-10
FC325292	1	Q76CU1	PDR-type ABC transporter 2 - *N. tabacum*	2E-75
FC325293	1	Q9FSG7	Thaumatin-like protein 1a precursor - *M. domestica*	1E-67
FC325294	1	O81872	Hypothetical protein T16L1.40 - *A. thaliana*	2E-06

FC325295	1	Q7F9L9	OSJNBa0006A01.5 protein - *O. sativa*	5E-18
FC325298	1	Q70CE9	Glucan endo-1,3-beta-glucosidase - *Fagus sylvatica*	7E-64
FC325299	2	Q9FIC9	Germin-like protein subfamily 1- *A. thaliana*	5E-79
FC325300	1	A0MEV7	Hypothetical protein (Fragment) - *A. thaliana*	4E-73
FC325302	1	Q84MB7	At3g62970 - *A. thaliana*	1E-91
FC325303	2	Q39224	SRG1 protein (At1g17020/F6I1.30) - *A. thaliana*	2E-54
FC325306	10	Q9LRJ9	Similarity to receptor kinase (At3g22060) - *A. thaliana*	2E-45
FC325307	1	Q1S8D6	Hypothetical protein - *M. truncatula*	6E-07
FC325311	1	Q9AWG7	Zinc transporter (Zinc/iron permease) - *M. truncatula*	1E-46
FC325312	1	Q0ZCC5	CCHC-type integrase - *Populus trichocarpa*	1E-28
FC325314	1	Q9ZRF1	Probable mannitol dehydrogenase - *Fragaria ananassa*	1E-93
FC325315	3	Q1H8M8	B12D-like protein - *Beta vulgaris*	4E-39
FC325317	2	Q9FE98	Alliinase, putative; 28821-30567- *A. thaliana*	1E-81
FC325318	1	O24035	Pantoate--beta-alanine ligase precursor - *Lotus japonicus*	5E-61
FC325319	1	Q5VNP5	Hydrolase-like (Os01g0636400 protein) - *O. sativa*	4E-60
FC325321	1	Q1S9Z6	Thioredoxin-related - *M. truncatula*	1E-54
FC325322	1	Q1SJP3	E-class P450, group I - *M. truncatula*	1E-38
FC325324	1	Q76KT6	Hypothetical protein - *Nicotiana tabacum*	1E-16
FC325328	1	P93736	Valyl-tRNA synthetase - *A. thaliana*	2E-35
FC325330	1	Q6Q3H4	Fructokinase - *Citrus unshiu*	1E-38
FC325331	3	Q1S8W2	O-methyltransferase, family 2 - *M. truncatula*	8E-63
FC325332	1	A2Q1X9	TIR - *M. truncatula*	2E-06
FC325333	1	Q2I307	Pollen-specific protein - *V. pseudoreticulata*	1E-42
FC325338	6	Q9M2U5	Class IV chitinase (CHIV) (At3g54420) - *A. thaliana*	5E-83
FC325339	1	Q69WY4	Zinc finger A20 and AN1 domain - *O. sativa*	7E-46
FC325340	2	P19446	Malate dehydrogenase, glyoxysomal precursor - *Citrullus lanatus*	5E-86
FC325341	5	Q1SEJ9	SAM dependent carboxyl methyltransferase - *M. truncatula*	1E-75
FC325344	1	Q56YN3	AD(H) kinase 1- *A. thaliana*	5E-79
FC325346	1	Q9ZRF1	Probable mannitol dehydrogenase- *F. ananassa*	1E-67
FC325347	1	Q9ZRU5	Protein phosphatase - *Cicer arietinum*	E-114
FC325348	1	Q4JKA4	Ascorbate peroxidase - *Rheum australe*	4E-88
FC325352	1	Q6J524	Caffeoyl-CoA-O-methyltransferase - *Broussonetia papyrifera*	6E-71
FC325355	1	Q41140	Pyrophosphate--fructose 6-phosphate 1 - *Ricinus communis*	E-103
FC325360	1	Q8VXV4	Chaperone protein dnaJ 16 - *A. thaliana*	1E-99

FC325361	1	Q8LFP1	Hypothetical protein (At4g27540) - *A. thaliana*	1E-60
FC325362	1	Q949Z8	Hypothetical protein At1g36980 - *A. thaliana*	2E-23
FC325364	1	Q9SSF9	F25A4.28 protein - *A. thaliana*	2E-93
FC325365	1	Q6NKQ5	At2g01275 - *A. thaliana*	3E-40
FC325366	3	Q1T4Y2	VQ - *M. truncatula*	4E-05
FC325367	1	P25317	Probable glutathione S-transferase parA - *N. tabacum*	4E-67
FC325368	1	Q3HRZ8	Hypothetical protein - *S. tuberosum*	1E-16
FC325369	1	Q64K29	(-)-germacrene D synthase - *Populus jackii*	8E-55
FC325370	1	Q945U3	Acyl-CoA oxidase - *G. max*	2E-79
FC325371	1	Q9FXC5	Hypothetical protein F12A21.27 (At1g67600)- *A. thaliana*	1E-64
FC325372	1	Q45W78	Ubiquitin fusion protein - *Arachis hypogaea*	4E-67
FC325373	1	Q39833	Alfa-carboxyltransferase precursor - *G. max*	4E-46
FC325374	1	Q8GTE6	Transportin-like protein - *C. arietinum*	1E-30
FC325378	1	Q8H2A6	Germin-like protein - *Ananas comosus*	6E-64
FC325379	1	Q52QR2	NAC domain protein NAC4 - *G. max*	2E-90
FC325380	1	O49876	Class III chitinase precursor - *L. albus*	2E-23
FC325381	6	Q5C9I9	(-)-isopiperitenol dehydrogenase - *Mentha piperita*	1E-69
FC325382	1	O81862	Putative chitinase (At4g19810) - *A. thaliana*	5E-25
FC325383	1	A0FKR1	Vacuolar citrate/H+ symporter - *Citrus sinensis*	E-106
FC325384	1	O80407	Chalcone synthase - *V. vinifera*	3E-40
FC325386	1	Q6Q3H3	Terpene synthase - *V. vinifera*	2E-62
FC325388	1	Q6PML6	Metallothionein 1a - *P. jackii*	3E-12
FC325389	1	Q0WVL7	Hypothetical protein At1g79830 - *A. thaliana*	4E-21
FC325390	1	Q1AFF5	Aldehyde dehydrogenase - *V. pseudoreticulata*	3E-65
FC325393	1	Q7XJE7	Putative xyloglucanase inhibitor - *S. tuberosum*	1E-54
FC325394	1	Q84TK8	Hypothetical protein - *Populus tremuloides*	9E-33
FC325395	1	Q9ZUH1	Hypothetical protein At2g24240 - *A. thaliana*	2E-63
FC325397	2	O48646	Probable phospholipid hydroperoxide glutathione peroxidase 6 - *A. thaliana*	4E-08
FC325399	1	Q6Z8F0	Hypothetical protein P0459B01.18 - *O. sativa*	2E-19
FC325401	1	Q2VCJ2	Ribosomal protein L3-like - *S. tuberosum*	4E-49
FC325406	5	Q1SJP3	E-class P450, group I - *M. truncatula*	1E-35
FC325407	2	Q9C938	Putative oxidoreductase - *A. thaliana*	3E-35
FC325408	1	Q0GA85	Glycoside hydrolase family 1 protein - *Leucaena glauca*	4E-43
FC325409	2	Q5M9R1	Hypothetical protein orf138c - *N. tabacum*	3E-35

FC325410	1	Q2HHE5	Hypothetical protein - *Chaetomium globosum*	4E-32
FC325412	4	Q1S5R4	Hypothetical protein - *M. truncatula*	4E-30
FC325413	1	Q4VPK7	Major allergen Mal d 1.0501- *M. domestica*	5E-52
FC325415	1	Q6CQE6	strain NRRL Y-1140 chromosome - *Kluyveromyces lactis*	2E-13
FC325417	1	Q0J0U0	Os09g0483500 protein - *O. sativa*	1E-40
FC325418	1	Q4P9C9	Hypothetical protein - *Ustilago maydis*	4E-23
FC325419	1	Q9SDZ1	Isoflavone reductase homolog 1 - *G. max*	1E-51
FC325420	1	Q9ZRT5	Glutathione transferase AtGST 10 - *A. thaliana*	2E-46
FC325422	2	Q1S8W2	O-methyltransferase, family 2 - *M. truncatula*	3E-68
FC325423	3	Q9M2U5	Class IV chitinase (CHIV) (At3g54420) - *A. thaliana*	4E-83
FC325428	1	Q9AV65	Hypothetical protein OSJNBa0006L06.21 - *O. sativa*	1E-33
FC325429	1	Q41695	Pectinacetylesterase precursor - *Phaseolus aureus*	5E-09
FC325430	2	Q1T6M3	Hypothetical protein - *M. truncatula*	6E-71
FC325431	1	A0ELU9	Non-S-locus F-box protein 23 (Fragment) - *Petunia integrifolia*	2E-63
FC325435	1	Q0INB8	Os12g0484700 protein - *O. sativa*	2E-32
FC325436	1	Q6Z8Q6	Splicing factor 3B subunit 5-like protein- *O. sativa*	1E-43
FC325440	6	Q6YEY6	Protease inhibitor - *V. vinifera*	5E-16
FC325441	1	P46287	60S ribosomal protein L11 (L5) - *M. sativa*	2E-83
FC325442	1	Q42948	Dihydrodipicolinate synthase, chloroplast precursor- *N. tabacum*	2E-89
FC325444	1	Q9LVP2	Emb|CAB43660.1 (At3g29170)- *A. thaliana*	4E-34
FC325445	22	Q7F9L9	OSJNBa0006A01.5 protein - *O. sativa*	8E-23
FC325446	1	Q8LNY1	Protease inhibitor 1 (Fragment) - *Zinnia elegans*	2E-17
FC325450	1	O65387	F12F1.21 protein - *A. thaliana*	6E-21
FC325451	2	Q9LCZ9	Photoassimilate-responsive protein PAR-1b-like protein - *A. thaliana*	3E-32
FC325452	1	Q29VI1	Patatin-like protein - *G. hirsutum*	5E-21
FC325454	1	Q94F43	Hypothetical protein AT3g47831/T23J7 - *A. thaliana*	1E-16
FC325455	1	O23547	Expansin-like B1 precursor (AtEXLB1) - *A. thaliana*	2E-61
FC325457	1	Q6IDA4	At2g16510 - *A. thaliana*	4E-13
FC325459	1	A2Q4V6	Ribosomal protein S10 - *M. truncatula*	6E-59
FC325460	2	Q1SL98	Ribosomal protein L10E - *M. truncatula*	E-116
FC325462	1	Q8LGH7	Hypothetical protein - *A. thaliana*	6E-75
FC325463	1	Q947H4	Non-cell-autonomous protein pathway2 - *N. tabacum*	2E-62
FC325466	1	Q84W04	Hypothetical protein At1g12390 - *A. thaliana*	6E-37
FC325468	1	Q7XYR7	Class III peroxidase - *G. hirsutum*	4E-25

FC325469	1	Q8H2J8	Putative 40S ribosomal protein S12 - *O. sativa*	2E-55
FC325474	1	O65327	Superoxide dismutase - *Raphanus sativus*	5E-63
FC325477	1	Q2QQS1	Prefoldin subunit - *O. sativa*	8E-50
FC325478	1	Q589Y2	Glycosyltransferase NTGT5a - *N. tabacum*	8E-76
FC325479	2	A2TD22	GH3 protein - *Rhodiola sachalinensis*	3E-41
FC325482	2	Q9ZTT3	Subtilisin-like protease C1 - *G. max*	4E-24
FC325485	1	Q0DKA8	Os05g0178900 protein - *O. sativa*	4E-76
FC325486	1	Q1SRR5	Hypothetical protein - *M. truncatula*	4E-29
FC325487	1	Q7F9L9	OSJNBa0006A01.5 protein - *O. sativa*	4E-19
FC325488	1	Q9FYB3	SRPK4 - *A. thaliana*	2E-74
FC325489	1	Q9LRJ9	Similarity to receptor kinase (At3g22060)- *A. thaliana*	5E-48
FC325496	1	Q6YYA7	Putative ribosomal protein S17- *O. sativa*	3E-30
FC325497	1	Q8H9D7	WRKY-type DNA binding protein - *S. tuberosum*	3E-14
FC325499	1	A0AUB7	Mal d 1.0406 - *M. domestica*	2E-43
FC325502	1	P52706	(R)-mandelonitrile lyase 1 precursor- *Prunus serotina*	5E-50
FC325503	1	O24301	Sucrose synthase 2- *Pisum sativum*	E-110
FC325504	1	O48925	CYP82C1p - *G. max*	1E-05
FC325506	2	O22086	ZPT2-14 - *Petunia hybrida*	4E-31
FC325507	1	Q1SFJ9	Leucine zipper, homeobox-associated - *M. truncatula*	2E-49
FC325509	1	Q76DL0	LEDI-5c protein - *Lithospermum erythrorhizon*	E-104
FC325513	4	Q1SJP3	E-class P450, group I - *M. truncatula*	2E-96
FC325515	1	O04486	Ras-related protein Rab11C - *A. thaliana*	5E-75
FC325519	1	Q9FQE4	Glutathione S-transferase GST 14 - *G. max*	2E-24
FC325523	3	Q9FHR8	Enoyl CoA hydratase-like protein - *A. thaliana*	2E-91
FC325525	1	Q93Z27	AT5g39670/MIJ24_140 - *A. thaliana*	1E-37
FC325529	1	O65896	Cytidine deaminase (At2g19570) - *A. thaliana*	2E-44
FC325530	2	O65755	Putative deoxycytidylate deaminase - *C. arietinum*	5E-50
FC325532	34	Q3KN67	Isoflavone reductase-like protein 6 - *V. vinifera*	E-106
FC325533	1	Q56VS3	Glutathione peroxidase GSH-PX3 - *L. japonicus*	4E-77
FC325534	1	Q41361	Pathogenesis-related protein PR-6 type - *Sambucus nigra*	7E-21
FC325536	1	Q1S104	Embryo-specific 3 - *M. truncatula*	2E-53
FC325537	1	Q9M528	Phenylcoumaran benzylic ether reductase homolog Fi1- *Forsythia intermedia*	7E-37
FC325539	1	Q8VX72	Putative cysteine proteinase inhibitor - *Rumex obtusifolius*	4E-29
FC325541	1	Q6L429	Protein kinase APK1B, chloroplast - *Solanum demissum*	E-102

FC325542	1	O24465	Thymidine diphospho-glucose 4-6-dehydratase - *Prunus armeniaca*	6E-72
FC325543	1	Q9CA32	Hypothetical protein T26J14.6 - *A. thaliana*	4E-18
FC325544	1	Q9M602	O-methyltransferase - *F. ananassa*	1E-54
FC325545	2	Q1SV12	Kunitz inhibitor ST1-like - *M. truncatula*	1E-29
FC325551	3	Q6Q3H2	Terpenoid synthetase - *V. vinifera*	3E-80
FC325554	2	Q1SAA8	DSBA oxidoreductase - *M. truncatula*	3E-72
FC325555	1	Q7XAB2	10-hydroxygeraniol oxidoreductase - *Camptotheca acuminata*	5E-52
FC325557	1	Q8LRL5	Nam-like protein 10 - *P. hybrida*	2E-96
FC325558	1	Q40467	Eukaryotic initiation factor 4A-14 - *N. tabacum*	6E-12
FC325560	1	O48723	Similar to latex allergen from Hevea brasiliens - *A. thaliana*	6E-41
FC325561	1	Q70AB2	Ethylene transcription factor - *F. sylvatica*	5E-74
FC325565	1	Q84WG2	Putative VAMP protein SEC22 - *A. thaliana*	2E-16
FC325566	1	Q2XTB8	Putative translation initiation factor eIF-1- *S. tuberosum*	5E-38
FC325567	1	Q9XFX0	Cytochrome P450 monooxygenase - *C. arietinum*	1E-74
FC325568	1	O81970	Cytochrome P450 71A9 - *G. max*	2E-45
FC325569	3	Q9FSG7	Thaumatin-like protein 1a precursor - *M. domestica*	1E-64
FC325570	2	Q3L7K5	Cell wall apoplastic invertase - *V. vinifera*	2E-49
FC325571	1	O48939	Polyphosphoinositide binding protein Ssh1p - *G. max*	4E-22
FC325572	1	Q9FPK6	Aldehyde dehydrogenase - *O. sativa*	8E-52
FC325574	1	Q9FDV7	Protein kinase (PK) (Fragment) - *F. sylvatica*	1E-38
FC325576	1	Q9ZTT3	Subtilisin-like protease C1 - *G. max*	3E-75
FC325579	10	Q7F9L9	OSJNBa0006A01.5 protein - *O. sativa*	6E-27
FC325581	2	Q9M2U5	Class IV chitinase (CHIV) (At3g54420) - *A. thaliana*	E-110
FC325582	1	Q84VH0	Glutathione S-transferase Z1 - *Malva pusilla*	5E-86
FC325588	1	Q6XL72	Cytochrome P-450-like protein - *V. vinifera*	3E-61
FC325589	4	Q1S278	Proteinase inhibitor I13 - *M. truncatula*	6E-18
FC325592	3	O23939	Ripening-induced protein - *F. vesca*	E-109
FC325596	1	Q6BE25	Cycloartenol synthase - *Cucurbita pepo*	2E-70
FC325597	1	Q0GA75	Glutathione reductase - *R. australe*	7E-79
FC325598	3	Q9C899	Leucoanthocyanidin dioxygenase 2, putative - *A. thaliana*	4E-90
FC325601	2	Q2HPG3	Osmotin-like protein I - *G. hirsutum*	1E-98
FC325604	1	O48618	Cytochome b5 (Fragment) - *Olea europaea*	3E-46
FC325607	1	Q3HLY8	U-box protein - *Capsicum annuum*	8E-36
FC325608	1	P49299	Citrate synthase, glyoxysomal precursor - *Cucurbita maxima*	9E-35

FC325609	1	Q9LS43	Genomic DNA, chromosome 3, P1 clone: MYF24 - *A. thaliana*	1E-18
FC325610	1	Q9XHX3	Putative pathogenesis related protein - *O. sativa*	3E-65
FC325612	1	Q1SGY3	Hypothetical protein - *M. truncatula*	2E-15
FC325614	1	Q1PDI2	Diacylglycerol kinase - *A. thaliana*	E-110
FC325616	1	Q42768	Acetohydroxyacid synthase (EC 4.1.3.18) - *G. hirsutum*	E-145
FC325617	1	Q2GQH3	Hypothetical protein - *C. globosum*	E-130
FC325618	1	Q9FMS3	Hypothetical protein (At5g22280) - *A. thaliana*	3E-17
FC325621	9	Q1SV12	Kunitz inhibitor ST1-like - *M. truncatula*	1E-26
FC325623	1	Q8H0Y0	Hypothetical protein At4g02890 - *A. thaliana*	3E-99
FC325624	1	Q4KYL1	Pathogenesis-related protein 10 - *Solanum virginianum*	1E-41
FC325625	1	Q56YH5	Hypothetical protein (Fragment) - *A. thaliana*	5E-24
FC325627	1	Q39264	Zinc finger protein 5 - *A. thaliana*	4E-23
FC325628	1	Q1ZZ69	Secoisolariciresinol dehydrogenase - *Dysosma tsayuensis*	3E-24
FC325633	1	O48548	Aspartate aminotransferase - *G. max*	E-126
FC325636	1	Q39065	Copper transporter 1 - *A. thaliana*	9E-27
FC325637	1	Q2HTW4	Protein kinase - *M. truncatula*	6E-60
FC325639	1	Q4TZ01	Ubiquitinating enzyme - *A. thaliana*	3E-81
FC325643	1	O82201	Putative clathrin assembly protein - *A. thaliana*	7E-72
FC325644	3	Q9M7R2	Iron-superoxide dismutase precursor - *Vigna unguiculata*	7E-77
FC325645	1	Q8GWY9	Hypothetical protein At2g22475/F14M13.2 - *A. thaliana*	6E-14
FC325648	4	Q96560	NADPH-ferrihemoprotein reductase - *Helianthus tuberosus*	3E-21
FC325658	3	Q9LRJ9	Similarity to receptor kinase (At3g22060) - *A. thaliana*	1E-45
FC325660	5	Q1S278	Proteinase inhibitor I13 - *M. truncatula*	5E-14
FC325661	2	P93257	Probable mannitol dehydrogenase - *Mesembryanthemum crystallinum*	3E-46
FC325662	2	Q2L8A7	Acetoacetyl-CoA thiolase - *Picrorhiza kurrooa*	8E-19
FC325663	1	Q9M9M6	Hypothetical protein T17B22.24 - *A. thaliana*	2E-38
FC325664	1	Q8LBH2	Putative clathrin assembly protein At2g01600 - *A. thaliana*	5E-47
FC325665	1	Q64EX4	N19-like protein - *P. sativum*	5E-29
FC325669	1	Q1SV92	Hypothetical protein - *M. truncatula*	8E-12
FC325671	1	Q6R2K3	Strubbelig receptor family 3 - *A. thaliana*	2E-35
FC325675	1	Q1H8M8	B12D-like protein - *B. vulgaris*	1E-35
FC325676	1	Q9C7B3	Protein phosphatase 2C, putative - *A. thaliana*	E-123
FC325679	1	Q6UCJ2	Pyridine nucleotide-disulphide oxidoreductase - *Cucumis sativus*	3E-58
FC325680	1	A1Z3W7	EIF5A - *Rosa chinensis*	2E-10

FC325681	1	Q9LP65	T1N15.21 - *A. thaliana*	1E-50
FC325683	2	Q9M0B6	Nucleotide sugar epimerase-like protein - *A. thaliana*	2E-24
FC325685	1	Q8LG37	AtPH1-like protein - *A. thaliana*	2E-65
FC325686	1	Q27JA2	Dirigent-like protein pDIR10 - *Picea glauca*	1E-19
FC325689	1	Q1T5P6	Curculin-like (Mannose-binding) lectin - *M. truncatula*	1E-62
FC325691	1	Q9LS48	Hypothetical protein Gb|AAC98059.1, At3g18420 - *A. thaliana*	8E-49
FC325692	1	Q5Q1I3	3-beta hydroxysteroid dehydrogenase - *Digitalis thapsi*	6E-24
FC325696	1	Q7XJ83	Beta-1,3-glucanase - *Hevea brasiliensis*	2E-20
FC325698	3	Q96403	Stellacyanin - *C. sativus*	2E-17
FC325704	1	Q9SWS9	Ribosomal protein S26 - *P. sativum*	2E-27
FC325708	1	Q10AP7	Pirin, putative, expressed - *O. sativa*	9E-29
FC325714	1	Q9C939	Putative oxidoreductase; 32373-31266 - *A. thaliana*	5E-09
FC325192	1	No hits		
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FC325706	1	No hits

a Singlets: GenBank EST accession number; contigs: GenBank accession number of EST with the longest overlap within a contig.