2005

BIOSYNTHESIS OF trans-2-HEXENAL IN RESPONSE TO WOUNDING IN STRAWBERRY FRUIT AND INTERACTION OF trans-2-HEXENAL WITH BOTRYTIS CINEREA

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By
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Co-Directors: Dr. Douglas D. Archbold, Professor of Horticulture
and Dr. Thomas R. Hamilton-Kemp, Professor of Horticulture

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Intact strawberry fruit did not produce detectable *t*-2-H which is derived from α-linolenic acid (18:3). However, in response to wounding by gentle bruising, strawberry fruit emitted *t*-2-H with the largest quantity produced within 10 min following injury. The level of total lipid 18:3 in the fruit increased two-fold in response to wounding whereas free 18:3 declined slightly (about 30%). At 10 min following wounding, fruit exhibited a 25% increase in 13-lipoxygenase (LOX) activity, which leads to the production of 13-hydroperoxyoctadecatrienoic acid (13-HPOT) from 18:3. The activity of hydroperoxide lyase (HPL), which catalyzes formation of *cis*-3-hexenal (*c*-3-H), the precursor of *t*-2-H, from 13-HPOT, increased two-fold at 10 min after wounding. Thus, within 15 min after wounding, free 18:3 substrate availability and the activity of two key enzymes, LOX and HPL, changed in a manner consistent with increased *t*-2-H biosynthesis.

The site and mode of interaction of C_6 aldehydes with *Botrytis cinerea*, a common pathogen of many plant species, was characterized using radiolabeled six carbon (C_6) aldehydes, including *c*-3-H and *t*-2-H. An approximately 25% molar conversion of 18:3

to C_6 aldehydes was obtained by enzymatic manipulation with LOX and HPL extracts. Following exposure of *Botrytis* cultures to radiolabeled aldehydes, radiolabeled aldehydes were recovered in protein fractions, but not in the lipid fraction. They were incorporated into conidia at a 20-fold higher level than mycelia (per mg fresh weight). About 95% of the radiolabeled aldehyde was recovered in proteins on the surface (wash protein) of the fungal tissue, while 5% was from protein in internal tissue (cell wall and membrane and cytosol). Supplementing radiolabeled aldehydes with nonradiolabled C_6 aldehydes to increase the vapor phase concentration affected distribution of radiolabel in each protein fraction.

The *t*-2-H at both 5.4 and 85.6 μmol affected protein expression patterns, changing the intensity of expression in over one third of all proteins. Both up- and down-regulation of specific proteins were observed. Though five proteins of interest were analyzed, their identities were not determined. However, the data indicate a clear effect of *t*-2-H on protein expression in *Botrytis cinerea*.

KEYWORDS: Strawberry, *Botrytis cinerea*, Wounding, C_6 aldehydes, *trans*-2-Hexenal

Kyung Myung

07/06/2005

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DEDICATION

This is dedicated to my parents, Jae Young Myung and Wha Sun Won, who supported me throughout the entire work

ACKNOWLEDGMENTS

I would like to extend my sincerest gratitude to my co-advisors, Drs. Douglas Archbold and Thomas Hamilton-Kemp, for their guidance, advice, and funding throughout the project. They provided critical insights, allowing me to complete the project. I would like to thank Dr. David Hildebrand for instructive discussions of the dissertation process. In addition, I would like to thank Dr. Robert Houtz for his suggestions and comments and thank Drs. Mark Williams to be on my committee and Chris Schardl to be on my outside examiner.

I would like to thank people, lab personnel and colleagues, who provided assistance in this project: Ms. Marta Nosarzewski, Ms. Frederica Galli, Ms. Valeria Sigal, Mr. Randall Collins, Dr. Hirotada Fukushige, Dr. Keshun Yu, Mr. Charles McCracken, Ms. Wipawan Siangdung, Dr. Tiina Alamae, Dr. Carol Beach, Dr. Kyungsoo Chang, Ms. Etta Nuckles, and Mr. Wangsun Choi.

Finally, I would like to acknowledge my wife, Seyong, and my daughter, Sarah, for their love, support and patience. I also thank to my brother, Sung and Korean friends at University of Kentucky for their encouragement. I specially thank to my parents-in-law (the Parks family) for the desire they showed me.

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CHAPTER ONE

LITERATURE REVIEW

1. Postharvest characteristics of strawberry fruit

Strawberry (*Fragaria* x *ananassa* Duch.) is cultivated worldwide. Often used as a model system, the strawberry fruit is defined as a modified receptacle with achenes or the true ovaries, on the surface (Perkins-Veazie, 1995). Strawberry fruit ripening is generally categorized into four stages based on its color: green, white, pink, and red. As strawberry fruit reaches the red stage where anthocyanins, such as pelargonidin-3-glucoside, pelargonidin-3-rutinoside, and cyanidin-3-glucoside, contribute to red color, distinct changes occur. These include fruit softening, mainly due to degradation of cell wall components in the middle lamella composed of hemicellulose and cellulose, and increased release of pectins. The typical aroma of strawberry fruit develops, which is composed of a broad range of volatile compounds (Zabetakis and Holden, 1997). Among them, methyl and ethyl butanoates, methyl and ethyl hexanoates, hexyl and 3-hexenyl acetates, and ethyl propionate provide much of the aroma of ripe strawberry fruit. However, little is known about the biosynthesis of these compounds (Perkins-Veazie, 1995; Ménager *et al*., 2004). Other characteristic events include increased fructose and glucose content, limited respiration rate change and low ethylene production (non-climacteric ripening).

Because it is a very soft fruit when ripe strawberry fruit is subject to a variety of types of physical injury during handling and transport. The injuries may disrupt the tissue surface and are potential sites for pathogen attack. A number of compounds are released from damaged plant tissues including terpenoids, phenylpropanoids, and lipoxygenase (LOX)-derived volatiles (Paré and Tumlinson, 1999). Tomato and strawberry fruit produce a group of wound-induced volatile compounds, such as aldehydes, especially *trans*-2-hexenal (*t*-2-H), alcohols, and esters, derived from the LOX pathway (Moretti *et al*., 2002; Hamilton-Kemp *et al*., 2003). The level of *t*-2-H has been observed to stimulate growth of *Botrytis cinerea* Pers., a destructive pathogen of grapes, strawberries, and other fruits throughout the world (Fallik *et al*., 1998; Hamilton-Kemp *et al*., 2003). *B. cinerea* infects senescent floral parts, stigma and style, early in the growing season, becomes latent in the necrotic tissues, and resumes growth and causes rot upon maturation of fruit (McClellan and Hewitt, 1973; Boff *et al*., 2001). However, neither *t*-2-H biosynthesis upon wounding strawberry fruit nor the possible role of *t*-2-H on the development of *B. cinerea* has been elucidated. The ease of wounding strawberry fruit provides a unique opportunity to explore the biosynthesis of *t*-2-H in response to wounding and the interaction of *t*-2-H with *B. cinerea*.

2. Biosynthesis of *trans*-2-hexenal and jasmonic acid

Oxylipins are a group of biologically active compounds derived from polyunsaturated fatty acids in oxylipin metabolism (Howe and Schilmiller, 2002). Plants synthesize many fatty acid derivatives from α-linolenic acid (LNA), an 18 carbon compound. Through the

reactions of cytochrome P450 epoxygenase (CE), LOX, and cyclooxygenase-like activity, LNA is metabolized to various oxylipins, including jasmonates, aldehydes, ketols, and epoxy-, hydroxy-, and divinyl-ether derivatives (Blée, 2002; Howe and Schilmiller, 2002; Weber, 2002). Even though it has been known that oxylipins are generally synthesized *de novo* in response to stress, much remains to be learned about the biosynthesis and physiological roles of oxylipins other than jasmonates, a collective name for free jasmonic acid (JA) and its methyl ester.

Matsui *et al*. (2000) suggested that a lipase or a lipase-like activity specific to galactolipids, especially monogalactosyldiacylglycerol (MGDG), is involved in the formation of six carbon (C_6) aldehydes like *t*-2-H and *cis*-3-hexenal (*c*-3-H). LNA released from membrane lipids by lipases is converted to 13-HPOT [(13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid] by LOX, a non-heme iron-containing dioxygenase (Fig. 1.1). When hydrogen is abstracted at the 11 position of LNA, molecular oxygen can be introduced, leading to dioxygen insertion at the 9 or 13 position of LNA (Feussner and Waternack, 2002). The oxygenation at the 13 position of LNA by 13-LOX produces 13-HPOT. The majority of plant LOXs prefer free fatty acids as substrates, even though they can oxygenate polyunsaturated fatty acids esterified to membrane lipids (Hildebrand, 1989; Fuller *et al*., 2001).

The 13-HPOT formed by LOX is immediately metabolized by hydroperoxide lyase (HPL) (Noordermeer *et al*., 2002). HPL cleaves 13-HPOT into 12-oxo-(9*Z*)-dodecenoic acid and *c*-3-H. In the action of HPL from guava fruit on 13-HPOT, the actual end product was a short-lived hemiacetal, which spontaneously dissociated into *c*-3-H and an unstable enol form of a C_{12} oxo-acid (Grechkin and Hamberg, 2004). The *c*-3-H

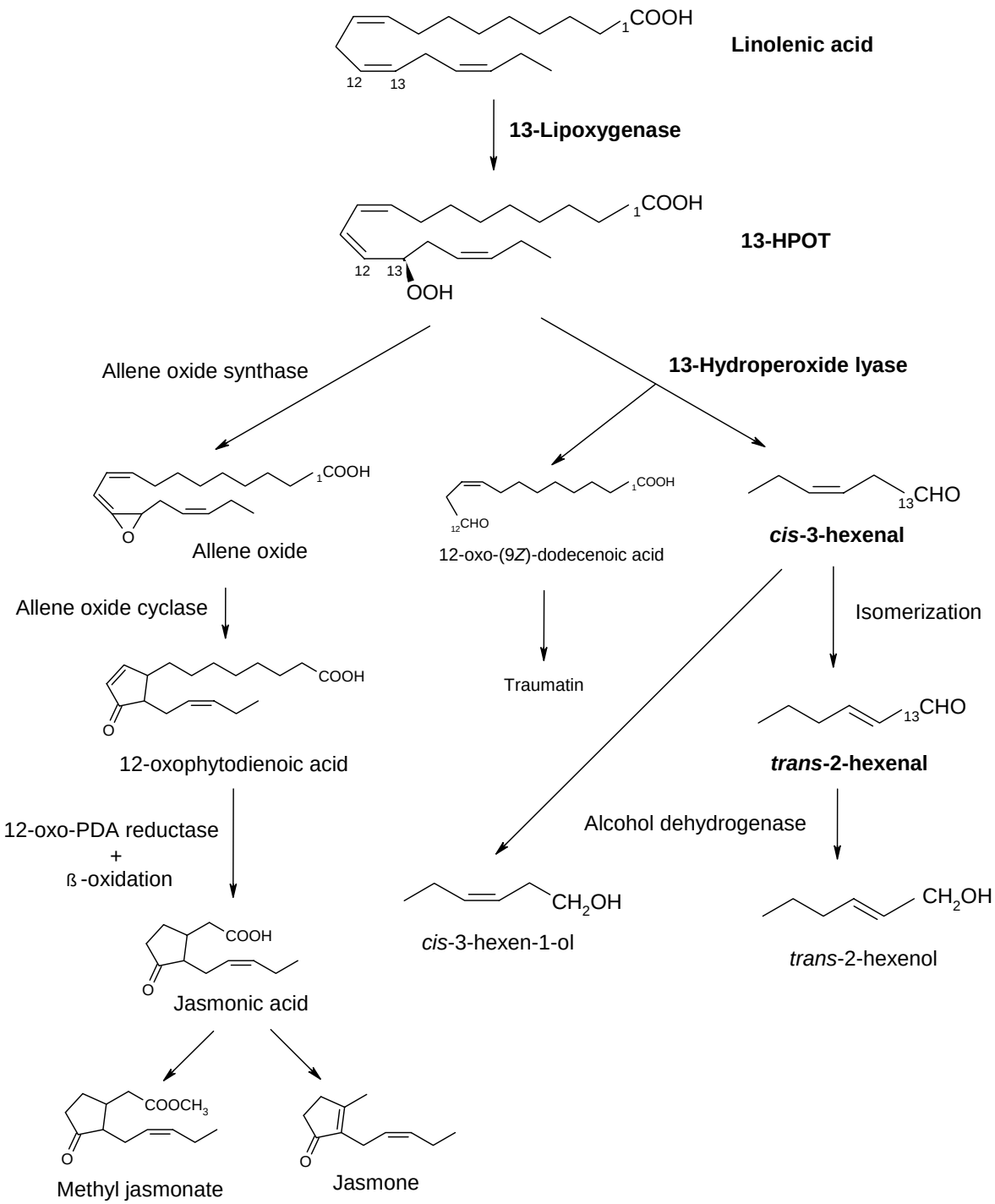


Figure 1.1 Biosynthesis of *trans*-2-hexenal and jasmonates in the oxylipin pathway in plants with key components for the former in bold letters

isomerizes spontaneously or enzymatically into more stable *t*-2-H (Hildebrand, 1989). The C_6 aldehydes are further reduced to alcohols by alcohol dehydrogenase. The C_{12} oxo-acid is the precursor of traumatin, previously known as 'wound signal' (Zimmerman and Coudron, 1979).

The *t*-2-H, a member of a class of volatiles produced by all green plant tissue, is released from macerated leaves and fruits (Hatanaka and Harada, 1973; Gray *et al*., 1999). The C_6 aldehydes can be detected within as little as 20 seconds after tissue disruption (Matsui *et al*., 2000), and most C_6 aldehydes are emitted from strawberry fruit within 15 minutes following injury (Hamilton-Kemp *et al*., 2003). Most of the *t*-2-H has been found in the cytosolic fraction in carnation petal and ripe tomato fruit when the amount of *t*-2-H was analyzed from microsomes, cytosol, and the lipid fraction (Hudak and Thompson, 1997; Riley and Thompson, 1997). Blée and Joyard (1996), Riley *at al*. (1996), and Froehlich *et al*. (2001) suggested that *t*-2-H formation appeared to occur on a membrane where LOX and HPL activities were exhibited. Chen *et al*. (2004) showed that 13-LOX involved in C_6 aldehyde synthesis was localized in a chloroplast. In strawberry fruit, LOX was associated with lipid body structures of the endoplasmic reticulum (Leone *et al*., unpublished data). However, it is still unclear whether *t*-2-H is synthesized at a membrane, is removed from the membrane bilayer, and is partitioned into cytosol. The hydrophobic, volatile nature and rapid metabolism of *t*-2-H have made it difficult to characterize the biosynthesis *in vivo*.

JA shares part of its biosynthetic pathway with *t*-2-H (Fig. 1.1). In JA biosynthesis, incubation of 13-HPOT with allene oxide synthase (AOS) led to the formation of an unstable allene oxide (Hamberg, 1988; Schaller, 2001; Turner *et al*., 2002). Further

conversion of the unstable allene oxide yields 12-oxophytodienoic acid (OPDA) through enzyme-catalyzed cyclization (Hamberg, 1988). Allene oxide cyclase (AOC) may be of major importance, since it determines the stereochemistry of the cyclopentanones. 12-Oxophytodienoic acid reductase (OPR) catalyzes the reduction of the C=C double bond of the conjugated enone moiety (Schaller, 2001; Turner *et al*., 2002). From the analysis of the metabolism of ^{18}O-labelled OPDA in several plant tissues, 3-oxo-2-[2'(*Z*)-pentenyl]-cyclopentane-1-octanoic acid (OPC-8:0) was identified as one of the labelled metabolites. Two OPRs from *Corydalis sempervirens*, three OPR isoforms from Arabidopsis, and three OPR isoforms from tomato have been identified. The functions of OPR1 and OPR2, found in Arabidopsis, are not yet understood, but it is likely that they are not involved in JA biosynthesis because their protein products did not catalyze the reduction of OPDA (Schaller *et al*., 2000). However, OPR3 effectively reduced OPDA. Taken together, even though all the isoforms are wound-induced, OPR3 is the only one related to biosynthesis of JA. This has been supported by the work of Stintzi and Browse (2000) who showed that OPR3 was the only isoform of OPR capable of reducing the correct stereoisomer of OPDA to produce JA. In addition, a peroxisomal targeting sequence has not been found for OPR1 and OPR2 but has been for OPR3, indicating that the peroxisome is the site where OPDA is reduced.

OPC-8:0 undergoes three rounds of β-oxidation to form JA (Schaller, 2001). Jasmonates consist of a cyclopentanone ring where an acetic acid and a pentenyl side chain are attached (Fig. 1.1). Since enzymes for β-oxidation are known to be located in peroxisomes, the reaction may occur in the peroxisome. However, the compartmentalization of these β-oxidation steps has not been investigated yet. JA is

converted into a number of derivatives (Turner *et al*., 2002). Methylation of JA to methyl jasmonate (MeJA) is catalyzed by an S-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase (JMT). Jasmone, a volatile compound, is formed by an additional round of β-oxidation of JA.

3. Initiation of wounding responses in plants

Plants have developed defensive and protective responses to various external threats such as environmental stress, pathogen and insect attacks, and mechanical damage. Unlike animals, plants cannot move around, resulting in development of a sophisticated mechanism to survive. Plants use an integrated defense mechanism, including cell wall and cuticle as a physical barrier, release of toxic compounds, and induction of defense-related genes.

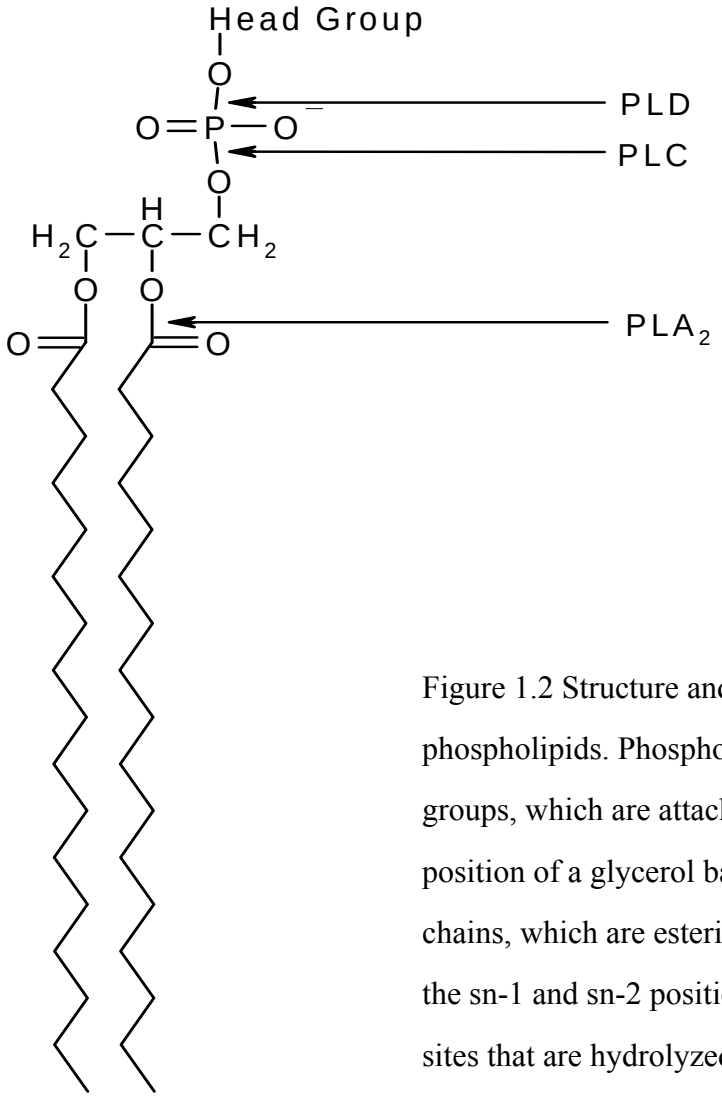
Systemin, an 18-amino acid peptide, has been proposed as a systemic signal produced upon wounding, while oligosaccharide fragments have been regarded as localized signal molecules (Doares *et al*., 1995). Systemin and oligosaccharide fragments elicit expression of defensive genes via a lipid-derived pathway. In the oxylipin pathway, induced by wounding, linoleic acid (LA) and LNA are liberated from membrane lipids and converted into various oxylipins via several enzymatic steps as described above. However, the mechanism of systemin-triggered LNA release is unknown.

The importance of ω3 fatty acid desaturase has been suggested (Martin *et al*., 1999; Li *et al*., 2003). The ω3 desaturase acts on membrane lipids to catalyze the synthesis of LNA, which is a precursor of oxylipins, from LA. In a study by Martin *et al*. (1999),

depletion of ω3 desaturase lowered LNA content and reduced wound-inducible activation of proteinase inhibitor II (PI II), one of the defensive genes known to be induced by JA in response to wounding. Li *et al*. (2003) indicated the significance of ω3 desaturase in JA biosynthesis and JA-related defense response by showing inhibition of PI II expression in a mutant deficient in ω3 desaturase.

Activation of lipases is an initial step in generating lipid and lipid-derived second messengers (Wang, 2001; Meijer and Munnik, 2003). Paré and Tumlinson (1997) showed that the lipoxygenase metabolites jasmone, *c*-3-H, and *t*-2-H were produced from breakdown of stored lipids, not from *de novo* biosynthesis after wounding. Multiple forms of phospholipases A_2, C, and D (PLA_2, PLC, and PLD) have been characterized in plants according to their sites of hydrolysis (Fig. 1.2). Phospholipids are hydrolyzed into free fatty acids and 1-acyl-2-lysophospholipids by PLA_2. PLC hydrolyzes phospholipids into diacylglycerol and a phosphorylated head group. PLD hydrolyzes phospholipids into a water-soluble head group and phosphatidic acid.

In a study by Conconi *et al*. (1996a), one hour after wounding, a 15-fold excess of LNA was found and the increased level of LNA was related to the increased level of newly synthesized JA. The total lipid contents of the leaves remained relatively unchanged up to 8 hours after wounding but a gradual decrease in polar lipids was observed, mainly in MGDG of chloroplast lipids. Dhondt *et al*. (2000) supported the finding by demonstrating that a large increase in soluble PLA_2 activation, in response to tobacco mosaic virus, led to the accumulation of OPDA and JA. Wounding also induced PLA_2 and multiple PLDs in tomato and Arabidopsis leaves, leading to LOX induction and JA accumulation (Narváez-Vásquez *et al*., 1999; Wang *et al*., 2000). Ishiguro *et al*.



Head Group
Choline
Serine
Inositol
Ethanolamine
Glycerol

Figure 1.2 Structure and hydrolysis of the common phospholipids. Phospholipids consist of various head groups, which are attached to a phosphate at the sn-3 position of a glycerol backbone, and two fatty acyl chains, which are esterified to a glycerol backbone at the sn-1 and sn-2 positions. The arrows indicate the sites that are hydrolyzed by a series of phospholipases.

(2001) identified DAD1 gene from *dad1* mutant, which is defective in anther dehiscence and pollen maturation, and found that DAD1 protein induced by wounding was a chloroplastic PLA_1, which catalyzes the initial step of JA biosynthesis, at least in flowers. Therefore, the results from these studies support the hypothesis that wounding induces systemin and lipase-mediated release of LNA, which is converted by enzymes in the oxylipin pathway to form oxylipins, leading to defense responses.

4. Lipoxygenase

The oxylipin pathway is regulated by several stresses and developmental signals (Howe and Schilmiller, 2002). Expression of several genes involved in the biosynthesis or metabolism of oxylipins and subsquent increased level of oxylipins followed mechanical wounding (Doares *et al*., 1995; Reymond *et al*., 2000), herbivore and pathogen attacks (Paré and Tumlinson, 1999; Ongena *et al*., 2004), UV light (Conconi *et al*., 1996b), osmotic stress (Kramell *et al*., 2000; Dombrowski, 2003), and oligosaccharides (Doares *et al*., 1995). The oxylipin pathway starts with the oxygenation of LNA at the 9 or 13 position of LNA by 9- or 13-LOXs, producing either 9- or 13-fatty acid hydroperoxides. Most plants have numerous genes coding LOX, different isoforms of LOX have different enzyme specificities, and LOX is present in more than one compartment in the plant cell (Brash, 1999; Creelman and Mullet, 1997; Schaller, 2001; Turner *et al*., 2002). LOXs are widely distributed in animals, including humans, and plants and consist of a single polypeptide chain with a molecular mass of about 94–104

kDa. At least eight different LOX forms exist in soybean (Feussner and Waternack, 2002). At present, LOX has not been cloned from the strawberry plant.

According to sequence similarity, the LOX isoforms can be grouped into two gene subfamilies, type 1-LOXs and type 2-LOXs (Blée, 1998; Feussner and Waternack, 2002). The type 1-LOXs do not have a transit peptide but the type 2-LOXs carry a putative chloroplast transit sequence. However, various plant LOXs differ by their positional specificity, either 9 or 13. Several factors affecting the specificity include 1) 'head (carboxy group first, 9-LOX)-tail (methyl end first, 13-LOX)' orientation of substrate within the active site, 2) conserved substrate binding pocket residue (valine for 9-LOX; histidine or phenylalanine for 13-LOX), 3) pH, or 4) temperature (Blée, 1998; Feussner and Waternack, 2002).

There have been studies which address physiological functions of LOXs. León *et al*. (2002) found a marked reduction in the production of C_6 aldehydes when 13-LOX was silenced in potato leaves. In tomato fruit, when TomloxC (13-LOX), the closest homolog to potato LOX, was depleted, C_6 aldehyde production was significantly reduced (Chen *et al*., 2004). In contrast, Kessler *et al*. (2004) could not detect any change of *c*-3-H production in LOX-3 knockout tobacco plants. The results imply the flux through the oxylipin pathway is regulated, depending on expression of specific LOX isoforms. In addition, Hause *et al*. (2000) detected slightly increased amounts of *t*-2-H and JA levels when LOX was expressed in tobacco leaves which do not have LOXs, suggesting that substrate (LNA) availability may limit the formation of LOX-derived products and is a critical factor in determining *t*-2-H and JA levels.

Bell *et al*. (1995) showed that stroma-localized plastidial 13-LOX appears to be responsible for the wound-induced biosynthesis of JA in *Arabidopsis* by using transgenic plants lacking LOX2. In addition, Fornaroli *et al*. (1999) purified a plasma membrane-bound LOX from soybean, similar to soluble LOX1 from soybean cotyledon, suggesting that the LOX may be transferred from cytosol to plasma membrane by vesicles. The speculation is acceptable because LNA dissolves poorly in aqueous media at typical physiological pH values of 7.0–7.4 (Brash, 1999). Therefore, the studies indicate the possible release of LNA from plasma membrane lipids and LNA oxygenation by LOX, even though LNA is likely to be liberated from chloroplast membrane lipids and to be oxydized by LOX in the chloroplast.

5. Oxylipin pathway: hydroperoxide lyase and allene oxide synthase

The hydroperoxy fatty acids are reactive molecules that could be toxic to cells, and they are immediately metabolized by HPL, AOS, divinyl ether synthase (DES), or peroxygenase (POX) (Rustérucci *et al*., 1999; Noordermeer *et al*., 2001; Alméras *et al*, 2003). As described above, HPL and AOS lead to the biosynthesis of C_6 aldehydes and jasmonates, respectively (Fig. 1.1). In addition to the two major pathways, DES and POX catalyze LOX products to form divinyl ether and peroxy fatty acids, respectively. However, the minor pathways have been less well characterized.

The HPLs, which have been found to be part of the cytochrome P450 monooxygenase family and categorized as a CYP74B, have been purified and cloned from plant species (Noordermeer *et al*., 2001; Feussner and Waternack, 2002). There are two types of HPLs

based on substrate preference. The 9-HPL cleaves 9-HPOT and generates two C_9 fragments (*cis*-3 *cis*-6-nonedianal and 9-oxo-nonanoic acid). The 13-HPL cleaves 13-HPOT and generates *c*-3-H and a C_{12} fragment. The AOS is also a member of the cytochrome P450 monooxygenase family and categorized as a CYP74A. AOS has been purified and cloned from several species and found to be a single gene (Schaller *et al*., 2000). Both HPL and AOS do not need molecular oxygen and NADPH for activity. Both HPL and AOS seem to share a common reaction through an intermediate epoxy allylic carbocation. Tijet *et al*. (2001) found that HPL with dual activity on both 13- and 9-HPOT from melon fruit has more amino acid residue homology to AOS than to 13-HPL, suggesting that AOS could bind and metabolize both 9- and 13-HPOT. Interestingly, Kandzia *et al*. (2003) suggested that C_6 and C_9 aldehydes can be produced by the same enzyme by using two different substrates. However, those possibilities remain to be elucidated.

Little information is available on intracellular localization of HPL because HPL is a membrane-bound enzyme and exists in small amounts in plant tissue (Fauconnier *et al*., 1997). Both HPL and AOS appear to be in the chloroplast (Feussner and Waternack, 2002) but could use different pools of 13-HPOT (Froehlich *et al*., 2001). Ziegler *et al*. (2000) revealed a single AOS gene in tomato and the protein was localized to the chloroplast. Froehlich *et al*. (2001) found that AOS, which contains a typical N-terminal transit peptide, was targeted to the inner envelope membrane and that AOS was peripherally associated with the inner envelope but the bulk of the protein faced the stroma. However, AOS, which does not have a transit peptide, has been reported from guayule and barley (Schaller *et al*., 2000), suggesting other sub-cellular localizations.

These findings indicate that the biosynthesis of JA may not be limited to the chloroplast and localization may be dependent on species.

Functional analyses of HPL and AOS have been studied using transgenic plants. Vancanneyt *et al*. (2001) found that HPL-depleted potato leaves had lowered HPL-derived compounds, leading to an increase in aphid feeding. The role of HPL was further supported by Kessler *et al*. (2004) who showed that silencing HPL significantly reduced the release of *c*-3-H while silencing AOS significantly increased the release of *c*-3-H, suggesting the change of LOX-derived regulation to the HPL cascade. Halitschke *et al*. (2004) also reported similar results by using antisense genotypes of HPL and AOS. Therefore, the studies indicate a role of HPL and AOS in regulating the flux of the pathway.

Wounding and fungal attack induced HPL and AOS within 30 minutes in Arabidopsis and lemon leaves (Bate *et al*., 1998; Kubigsteltig *et al*., 1999; Gomi *et al*., 2003). In addition, Howe *et al*. (2000) found that HPL was induced in response to insect attack and mechanical wounding. The study of Kubigsteltig *et al*. (1999) showed that transcription of the AOS gene in Arabidopsis was activated in the abscission zones of floral organs. Since the expressions of HPL and AOS have been tightly linked with elevated C_6 aldehyde and JA content, the results support that HPL and AOS play a decisive role in C_6 aldehyde and JA biosynthesis upon stress and developmental cues.

6. Physiological roles of oxylipins

It has been suggested that C_6 volatile compounds play a role in the defense mechanism by inducing prosystemin gene expression (Sivasankar *et al*., 2000), defense-related genes (Bate and Rothstein, 1998; Gomi *et al*., 2003), and stress and defense responses in plants (Vancanneyt *et al*., 2001; Farag and Paré, 2002) as well as in neighboring plants (Farmer, 2001). LOX and other defense-related genes were expressed following *t*-2-H exposure but not by *trans*-2-hexenol exposure (Bate and Rothstein, 1998). Gomi *et al*. (2003) showed that *t*-2-H induced LOX, HPL, and AOS, while *cis*-3-hexenol and *trans*-2-hexenol increased expression of AOS. Farag and Paré (2002) detected enhanced emission of monoterpenes and sesquiterpenes by treatment of *t*-2-H in tomato, and Farag *et al*. (2005) found that *cis*-3-hexenol and *t*-2-H were most effective in triggering emission of a group of volatile compounds. In contrast, terpenoid biosynthesis was only elicited by JA (Halitschke *et al*., 2004). Meanwhile, *t*-2-H alone did not activate a pathogenesis related gene (Alméras *et al*., 2003) but it activated protease inhibitor combined only with JA (Halitschke *et al*., 2004). Interestingly, some plants emit volatile compounds as an indirect defense mechanism to attract the natural enemies against herbivorous insects (Paré and Tumlinson, 1999; van Poecke and Dicke, 2002). Consequently, the evidence suggests that wound-induced C_6 volatile compounds, especially *t*-2-H, play a role in inducing defense-related genes that produce a signal molecule. The role of the compounds may overlap with JA for triggering defense responses. However, how gene expression is regulated by C_6 aldehydes is not known.

The role of oxygenated fatty acids in a plant-fungus interaction was demonstrated in tobacco plants depleted in a pathogen-induced LOX (Rancé *et al*., 1998). Similarly, Kessler *et al*. (2004) also reported the importance of JA in plant-insect interaction by using LOX silencing. JA has been known to act as a multifunctional growth regulator that modulates reproduction, metabolic regulation, and plant resistance to insects and pathogens (Ishiguro *et al*., 2001; Liechti and Farmer, 2002; Li *et al*., 2003). Stintzi and Browse (2000) showed that *opr3* sterile plants, which lack JA, were rendered fertile by exogenous JA, not by OPDA, indicating that JA and not OPDA is the signaling molecule in the production of viable pollen. Stintzi *et al*. (2001) further investigated the role of OPDA by using *opr3* plants. In their study, the *op*r3 plants treated with OPDA showed resistance to attack by an insect and a fungal pathogen and expressed wound-induced genes, suggesting that OPDA can fulfill a similar role to JA in the absence of JA. Li *et al*. (2003) used *spr*2, a mutant defective in ω3 desaturase, and clearly suggested a role of LNA and JA in defense against insects. Recently, Halitschke *et al*. (2004) demonstrated that larval development and feeding on leaves could be stimulated by volatiles including *c*-3-H.

The exact role and relative importance of each JA derivative for the cellular responses has not been clearly defined. However, MeJA has appeared to be an important cellular signal molecule mediating diverse developmental processes and defense responses. Choi *et al*. (1994) demonstrated the stimulatory effect of MeJA on 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a key enzyme to produce mevalonic acid in the pathway of isoprenoid biosynthesis, suggesting MeJA as a signaling molecule. MeJA application led to a significant decrease of thylakoid membrane LNA, increased LNA

accumulation, induced LOX mRNA, accumulated LOX protein, enhanced LOX and HPL activities, and increased 13-HPOT and C_6 aldehyde formation (Avdiushko *et al*., 1995; Bate and Rothstein, 1998; Bachmann *et al*., 2002). This work has clearly indicated that MeJA activates the HPL branch of the oxylipin pathway. Seo *et al*. (2001) hypothesized that MeJA may have its own role in developmental processes and defense responses, even though JA is the primary intracellular signal transducer. Additionally, unstable *epi*-MeJA, a precursor of MeJA, increased polyphenol oxidase, a putative defense enzyme, and reduced damage by insects (Pickett and Poppy, 2001). Jasmonates had a maximum concentration in immature strawberry fruit and then steadily decreased during ripening (Gansser *et al*., 1997). It was also reported that raspberries treated with MeJA had higher soluble solids content, total sugars, fructose, glucose, sucrose, and antioxidant capacity but had lower titratable acids, malic acid, and citric acid than untreated fruit (Wang and Zheng, 2005).

The release of jasmone can be induced by damage but little is known about its biological function. Birkett *et al*. (2000) examined the possible role of jasmone and speculated that jasmone could act as an aphid repellent and an attractant for insects that feed on ahpids, suggesting that the role of jasmone is different from that of MeJA. Bruce *et al*. (2003) confirmed the effectiveness of jasmone as a repellent to aphids. Pickett *et al*. (2003) also supported a different role of jasmone from MeJA by discussing a number of differences in gene regulation in bean plants between MeJA and jasmone. They also determined the effect of jasmone on inducing defense in intact plants. However, the mechanism by which genes are activated by jasmone has not been identified.

A diverse group of oxygenated metabolites, oxylipins in plant and eicosanoids in animals, are produced by the oxidative transformation of unsaturated fatty acids via a series of metabolic pathways (Fritzpatrick and Soberman, 2001; Blée, 2002; Weber, 2002). In plants, the biosynthesis of oxylipins is initiated mainly from LNA, while that of eicosanoids is initiated mainly from arachidonic acid in animals. Among oxylipins and eicosanoids, two major derivatives, jasmonates in plants and prostaglandins in animals, have been studied extensively. Increasing evidence indicates that the biological importance of jasmonates in plants is comparable to that of prostaglandins in animals.

7. Aldehydes as an effective natural product for control of *Botrytis cinerea*

Plants produce numerous volatile compounds and the compounds have been studied for their biological effects on fungal spores, seeds, bacteria, and plant tissues (French, 1985). Since gray mold, caused by *B. cinerea*, has been regarded as one of the important diseases in fresh produce, numerous volatile compounds have been evaluated for control of the disease. Hamilton-Kemp *et al*. (1992) developed a bioassay system, tested the effects of volatile compounds on fungal growth, and showed the effectiveness of C_6 and C_9 aldehydes, which have antifungal activity at low concentration. In addition, Hamilton-Kemp *et al*. (1992) and Andersen *et al*. (1994) demonstrated that *t*-2-H was more effective in inhibiting hyphal growth of *B. cinerea* than the saturated aldehyde, hexanal. The C_9 aldehydes and ketones were generally more effective on *Alternaria alternata* than their C_6 aldehydes. Archbold *et al*. (1997) demonstrated that *B. cinerea* could grow

slowly in the presence of many volatile compounds at low concentrations, suggesting an inhibitory effect. Among the volatile compounds, *t*-2-H has been found to both inhibit and promote germ tube elongation in a concentration-dependent manner (Fallik *et al*., 1998). Other compounds, which have shown an inhibitory or stimulatory effect on *B. cinerea*, include acetate esters such as butyl acetate and hexyl acetate (Filonow, 1999).

The biologically active volatile compounds have carbon chains with various reactive groups. Important properties for the compounds include carbon chain length, the degree of unsaturation, and functional group (French, 1985). Among the compounds, aldehydes have been extensively studied due to their effective antimicrobial activity. There have been several studies to elucidate the mechanisms involved in the antimicrobial activity (Trombetta *et al*., 2002; Kubo *et al*., 2003). The presence of an α,β-unsaturated bond adjacent to the carbonyl moiety has been shown to be significant in the antifungal and antibacterial activity (Andersen *et al*., 1994; Trombetta *et al*., 2002). As the carbon chain length increased until maximum activity was reached, the antimicrobial activity increased (Kubo *et al*., 2003). Trombetta *et al*. (2002) demonstrated that aldehydes including *t*-2-H caused significant changes in cellular membrane permeability, suggesting that they can penetrate through membranes and interact with intercellular components. Kubo *et al*. (2003) hypothesized that the aldehydes bind nonspecifically, disrupt the hydrogen bonds in the lipid bilayer, and affect fluidity of membrane lipids.

The compound *t*-2-H is reactive, featuring two functional groups, an aldehyde group at C1 and a double bond between C2 and C3. The functional groups can attack proteins (Baker *et al*., 1999; Ichihashi *et al*., 2001). It has been suggested that the aldehyde reacts with the sulfhydryl group of cysteine, the ε-amino group of lysine, and the imidazole

group of histidine in protein and forms adducts via Michael addition or Schiff base reactions (Fig. 1.3). Evidence of a covalent cross-link between *t*-2-H and proteins has been reported (Židek *et al*., 1997; Zhou and Decker, 1999; Meynier *et al*., 2004). Amarnath *et al*. (1998) showed that the major reaction of *t*-2-H in the presence of protein *in vitro* was Michael addition. The protein adduct formed is mostly free aldehyde which can further undergo several types of reactions. Baker *et al*. (1999) concluded that *t*-2-H modified lysine and histidine residues and the N-terminus of proteins. Ichihashi *et al*. (2001) characterized a covalent modification of a protein and showed that the protein adduct forms were specific to 2-alkenals. The result was in agreement with the previous findings in which the covalent modified adducts had been obtained between various 2-alkenals and protein (Amarnath *et al*., 1998; Zhou and Decker, 1999). Therefore, the chemical properties of aldehydes contribute to the reactivity with proteins in biological systems. However, since the studies have been mostly performed *in vitro*, it is not known whether aldehydes can modify proteins *in vivo* in a similar way.

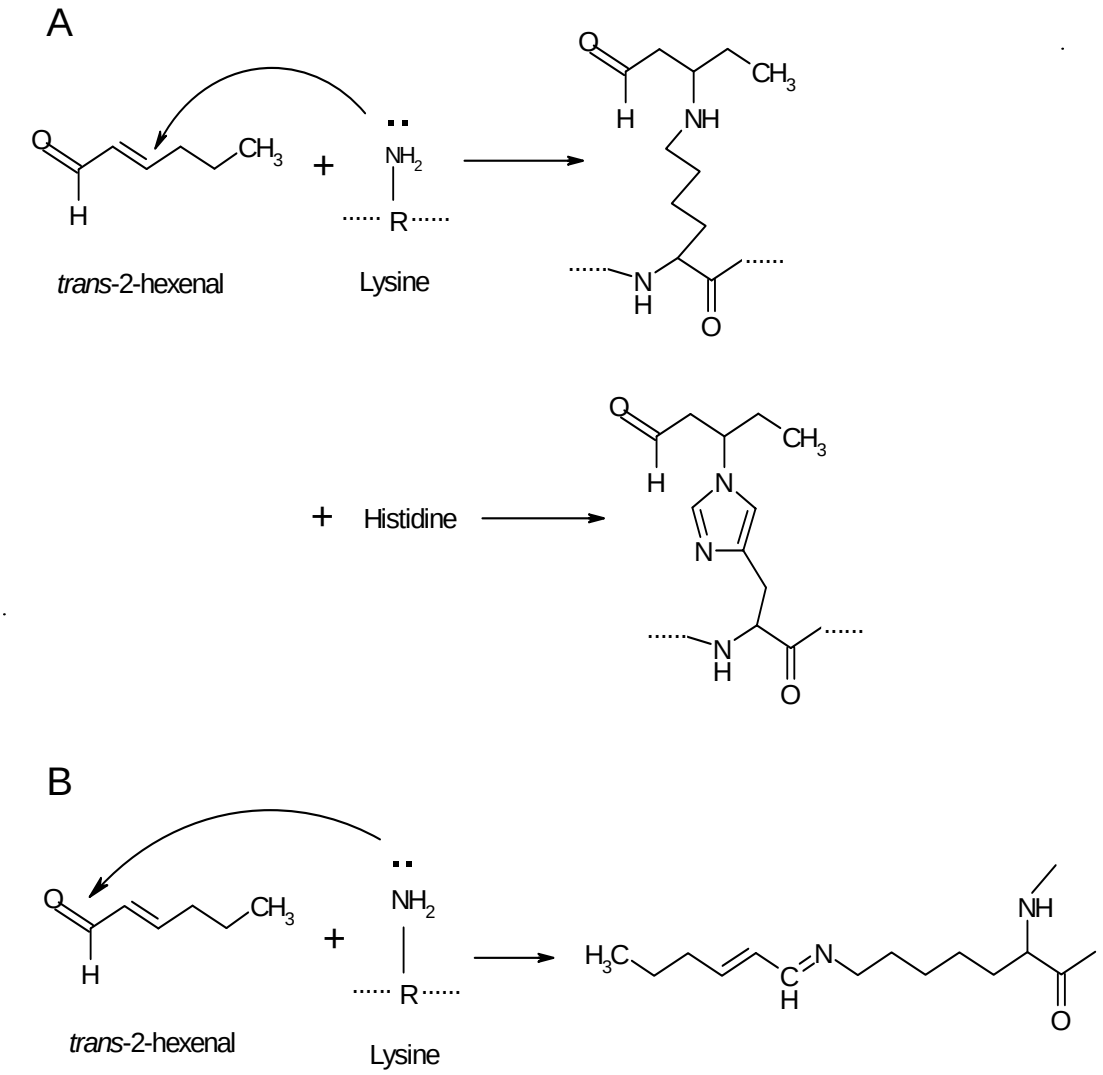


Figure 1.3 Reactions proposed for the formation of adducts of lysine or histidine-containing proteins and *trans*-2-hexenal (*t*-2-H). The double bond between C2 and C3 of *t*-2-H reacts with primary amine of lysine or imidazole ring of histidine, forming Michael adducts (A. Michael addition reaction). The carbonyl group of *t*-2-H reacts with the primary amine of lysine, forming a Schiff base adduct (B. Schiff base reaction).

8. Research objectives

The overall aim of the research is to understand the regulation of *t*-2-H production in strawberry in response to wounding and the interaction of *t*-2-H with *B. cinerea*.

Objective 1: It is not known how *t*-2-H biosynthesis is regulated in strawberry fruit in response to wounding. Thus objective 1 was to determine if the pool of free LNA and the activities of LOX and HPL responsible for the production of *t*-2-H are changed in response to wounding.

Objective 2: It is not clear how C_6 aldehydes can interact with the protein and lipid fractions of *B. cinerea*. Thus objective 2 was to develop an efficient system of radiolabeling C_6 aldehyde and demonstrate if protein and/or lipid fractions exhibit radiolabel from C_6 aldehyde.

Objective 3: It is not clear if *t*-2-H affects *B. cinerea* metabolism. Thus objective 3 was to determine if *t*-2-H affects protein expression patterns of *B. cinerea.*

CHAPTER TWO

BIOSYNTHESIS OF *trans*-2-HEXENAL IN RESPONSE TO WOUNDING IN STRAWBERRY FRUIT

Introduction

Oxylipins are a group of biologically active compounds derived from polyunsaturated fatty acids in oxylipin metabolism, mainly from α-linolenic acid (LNA), an eighteen-carbon fatty acid (Howe and Schilmiller, 2002). Jasmonates, aldehydes, ketols, and epoxy-, hydroxy-, and divinyl-ether derivatives are among many fatty acid derivatives that plants synthesize (Blée, 2002; Howe and Schilmiller, 2002; Weber, 2002). Oxylipins are known to be synthesized *de novo* in response to stress; polyunsaturated fatty acids are liberated from membrane lipids and converted into various oxylipins via several enzymatic steps.

During harvest, handling, and postharvest transport of fruits and vegetables, especially soft fruit like strawberry fruit, they are subject to a variety of types of injury. The injuries disrupt the tissue surface and allow pathogens to penetrate into the interior. A number of volatile compounds are released upon wounding including terpenoids, phenylpropanoids, and lipoxygenase (LOX)-derived volatiles. Strawberry fruit produces a diverse group of wound volatile compounds, aldehydes, alcohols, and esters derived from the LOX and hydroperoxide lyase (HPL) pathway (Hamilton-Kemp *et al*., 2003).

One of the oxylipins, *trans*-2-hexenal (*t*-2-H), is released from macerated leaves and fruits (Hatanaka and Harada, 1973; Gray *et al*., 1999). The *t*-2-H, a member of a class of volatiles produced by all green plant tissue, is a six-carbon aldehyde derived from the LOX and HPL pathway (Fig. 1.1) (Hildebrand, 1989; Feussner and Waternack, 2002; Noordermeer *et al*., 2002). It may be produced from LNA released from galactolipids by a lipase or a lipase-like activity (Matsui *et al*., 2000). It has been demonstrated that *t*-2-H formation occurs on a membrane where LOX and HPL activities are exhibited (Blée and Joyard, 1996; Riley *et al*., 1996; Froehlich *et al*., 2001). In addition, 13-LOX involved in C_6 aldehyde synthesis was localized in chloroplasts (Chen *et al*., 2004). Through the reaction of LOX, a molecular oxygen is inserted between C and H at position 13 of LNA. The resulting fatty acid hydroperoxide [(13*S*)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid, 13-HPOT] is cleaved by HPL, resulting in the production of 12-oxo-(9*Z*)-dodecenoic acid and *cis*-3-hexenal (*c*-3-H). The *c*-3-H isomerizes spontaneously or enzymatically into more stable *t*-2-H (Hildebrand, 1989). The C_6 aldehydes are further reduced to alcohols by alcohol dehydrogenase. The C_{12} oxo-acid is the precursor of traumatin, previously known as wound signal (Zimmerman and Coudron, 1979).

It is well understood that *t*-2-H is synthesized from LNA through LOX and HPL in response to wounding (Hildebrand *et al*., 1989; Howe *et al*., 2000; Gomi *et al*., 2003). The *t*-2-H could be detected within as little as 20 seconds after tissue disruption (Matsui *et al*., 2000). Most of the *t*-2-H emitted from wounded strawberry fruit was within 15 minutes following injury (Hamilton-Kemp *et al*., 2003). However, it is not known how *t*-2-H biosynthesis is regulated in strawberry fruit in response to wounding. The production

of free 18:3 and activity of the enzymes responsible for *t*-2-H production following wounding have not been reported from strawberry fruit.

The goal of this study was to provide knowledge about the biosynthesis of *t*-2-H in strawberry fruit upon wounding. To that end, composition and quantity of total and free fatty acids of control and wounded strawberry fruit were analyzed. In addition, key enzyme activities of the LOX pathway and production of C_6 aldehydes were determined.

Materials and Methods

Plant material

The strawberry (*Fragaria* x *ananassa* Duch.) cultivar 'Tribute' was used. Plants were grown in the greenhouse or outdoors in containers. Mature red fruit was selected for uniformity of color, size, and shape on the day of use. All fruit was harvested and placed at ambient laboratory temperature for 30 min before wounding treatment.

Wounding procedure

Wounding was performed by the method of Kemp *et al*. (2003). Four ripe fruits (30-35 g) were shaken in a 475 mL glass jar, closed with a Teflon lined screw cap lid, at 300 rpm for 30 sec in a shaker (Model 3540, Lab-Line Instruments Inc., Melrose Park, IL). The shaking did not cause visible disruption or exudate formation on the fruit. Control (unwounded) fruit was sampled without shaking and wounded fruit was sampled at 5, 10, and 15 min after shaking. There were four replicate experiments and four fruits were analyzed per treatment and sampling time, unless stated otherwise. Following wounding

treatments, the fruits were immediately used for analyses or immersed in liquid N_2 and stored at -80 ˚C for future analyses.

Lipid extraction and fatty acid analysis

For total lipid extraction, fruits (8 g) frozen in liquid N_2 were homogenized using a homogenizer (Model 5000, Omni International, Gainesville, VA) for 1 min in 40 mL methanol, chloroform, and water (2:1:0.8), vortexed for 1 min, and centrifuged at 6,000 g for 20 min (Couture *et al*., 1988). This was repeated four times. A suitable amount (630 μg/g fruit) of triheptadecanoin was added to the homogenate as an internal standard. Combined supernatants were dried using a rotary evaporator, and the residue was redissolved in 10 mL chloroform. One mL of extract was dried under N_2.

For total fatty acid analysis, the dried lipid sample was transmethylated with 0.5 mL 1% sodium methoxide. The sample was shaken for 45 min and the resulting fatty acid methyl esters were extracted with hexane. The sample was partitioned against 0.9% KCl, and aliquots of the upper hexane layer were analyzed using a Hewlett Packard 5890 (Agilent, Wilmington, DE) gas chromatograph (GC) equipped with a flame ionization detector (FID) and a FFAP column (14 m X 0.25 mm, 0.33 μm film thickness, Agilent). Samples were analyzed using a temperature gradient of 140 °C for 1 min, increased to 235 °C at a rate of 10 °C per min, and then held at this temperature for 20 min. Helium was used as the carrier gas at a flow rate of 1 mL per min.

For free fatty acid analysis, total lipid samples were prepared in 10 mL chloroform as described above, except that a suitable amount (5 μg/g fruit) of free heptadecanoic acid (17:0) was added to the homogenate as an internal standard. Samples were separated on

250 μm silica TLC plates (Whatman, Clifton, NJ). Sample-loaded plates were developed in hexane/methyl tertiary butyl ether (MTBE)/acetic acid (HOAc) (80:20:1). Authentic free linoleic acid (18:2) was used as a loading standard. The plates were sprayed with 0.01% primulin in 80% acetone, and fatty acids were visualized under UV light (300 nm). A suitable amount (1 μg/g fruit) of free nonadecanoic acid (19:0) was added to the fatty acid spot on the plate. The spot was scraped from the plate and eluted through a fiber glass column (Corning Glass Works, Corning, NY) with MTBE. The sample was evaporated under N_2 and methylated with a few drops of diazomethane. The ether was dried with N_2 and the sample was dissolved in hexane. The sample was partitioned against 0.9% KCl, and the hexane layer was analyzed by GC as described above.

Preparation of LOX and HPL crude extracts

LOX and HPL crude extracts were prepared from fruits with or without freezing in liquid nitrogen over a period of 15 min after wounding as freezing may affect the enzyme activities (Fall *et al*., 2001). The activities of frozen fruits did not differ from those of non-frozen fruits (data not shown). This result eliminates the possibility that LOX and HPL activities were affected by a freezing injury. Therefore, the activities of LOX and HPL were determined using fruit which was immediately immersed in liquid nitrogen after wounding treatment and sampling time periods. Fresh or frozen fruits (30 g) were homogenized with 4.8 g of polyvinylpolypyrrolidone (PVPP), 40 mL of 0.1 M Tris-HCl (pH 8.0), and 1 M KCl for LOX extraction (Pérez *et al*., 1999). For HPL extraction, 0.1% Triton X-100 was added to the buffer used for LOX extraction. The resulting homogenates were centrifuged at 20,000 g for 20 min. The supernatants were used for

enzyme assays of LOX and HPL. Protein concentration was measured with Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as standard protein at 595 nm using a spectrophotometer (Cary 50 Bio, Varian, Walnut Creek, CA) (Bradford, 1976).

LOX assay

LOX activity was determined by monitoring the conversion of LNA to diene product at 234 nm (Axelrod *et al*., 1981). The crude extract prepared as described above was added to a standard assay mixture. The mixture contained 1 mL of 0.1 M sodium phosphate buffer, pH 6.0, 10 mM LNA, and an appropriate amount (5-20 μL) of LOX extract. The increase in absorbance at 234 nm was recorded for 5 min. LOX activity was determined as the formation of 13-HPOT in nmol per min per mg protein, using an extinction coefficient of 25,000 M^{-1} cm^{-1} for 13-HPOT.

Preparation of LOX from soybean for synthesis of 13-HPOT

For the enzymatic reaction to synthesize 13-HPOT from LNA, LOX was extracted from a soybean genotype (-LOX3), which is designated as a null for LOX3 with normal levels of LOX1 and LOX2 (Hildebrand *et al*., 1990). Soybeans (3 g) were ground using a coffee grinder and macerated in a mortar with 5 mL of acetone stored at -20 ˚C. The residue in the mortar was washed twice with 5 mL of acetone and 10 mL of ethyl ether and filtered on Whatman paper with vacuum. The defatted soybean powder on the filter paper was dried under N_2 and stored at 4 ˚C. The soybean powder (100 mg) was extracted with 1 mL 0.2 M of sodium acetate, pH 4.5, for 1 hr on a shaker and filtered

through Miracloth. Ammonium sulfate (40%) was added to the filtrate, which was centrifuged at 8,000 g for 10 min. The supernatant was precipitated with ammonium sulfate (60%) and centrifuged at 8,000 g for 10 min. The pellet was dissolved in 100 μL of sodium borate buffer, pH 9.0, which was used for LOX activity. LOX activity was determined by the method described above, except that the LOX assay mixture for soybean contained 0.2 M sodium borate buffer, pH 9.0. LOX activity was defined as a 0.001 increase of absorbance at 234 nm per min.

Preparation of 13-HPOT and HPL assay

The 13-HPOT was prepared from LNA using crude soybean LOX. One hundred μL of 2.4 mM LNA and 3750 units of crude soybean LOX were added to 15 mL of oxygenated 0.2 M sodium borate buffer, pH 9.0. The reaction was allowed to proceed on ice for 1 hr under a constant flow of oxygen. The pH of the reaction mixture was adjusted to 4. The 13-HPOT produced was extracted with 20 mL of diethyl ether and the extract was dried under N_2. The residue was dissolved in 500 μL of hexane and ethyl ether and applied to a 40 μm silica column with a 20 cm length, equilibrated with 40 mL of hexane (Fauconnier and Marlier, 1996). The column was eluted first with 50 mL of hexane/ HOAc (100:1) and then 50 mL of hexane/MTBE/ HOAc (90:10:1). The 13-HPOT was collected in the next fraction eluted with 50 mL of hexane/MTBE/ HOAc (75:25:1). The product was dried under N_2, dissolved in ethanol, and stored at -80 ˚C. The final concentration of 13-HPOT, estimated from the absorbance, using the extinction coefficient of 25,000 M^{-1} cm^{-1} at 234 nm, was approximately 0.36 mM.

The crude extract prepared as described above was added to a standard assay mixture which contained 1 mL of 0.1 M potassium phosphate buffer, pH 6.0, 0.1 mM 13-HPOT, 0.1 mM NADH, 50 units of alcohol dehydrogenase (one unit converts 1.0 μmol of ethanol to acetaldehyde per min at pH 8.8 at 25 ˚C), and an appropriate amount (5-50 μL) of HPL extract. HPL activity was determined spectrophotometrically by monitoring the oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm in a coupled enzyme assay (Vick, 1991). The decrease in absorbance at 340 nm was measured for 5 min. HPL activity was expressed as the oxidation of NADH in nmol per min per mg protein, using an extinction coefficient of 6,220 M^{-1} cm^{-1} for NADH.

Headspace sampling and analysis of C_6 aldehydes

For C_6 aldehyde measurement, a 495 mL glass jar containing the fruit was sealed with a Teflon-lined plastic screw cap prior to shaking. At 5, 10, and 15 min following the wounding treatments, vapor phase volatile compounds were directly sampled from headspace of the jar using a gas tight syringe. The volatiles collected (400 μL) were injected into a GC (Hewlett Packard 5890 II, Agilent), equipped with a 60 m x 0.32 mm DB-5 column with a 1 μm film thickness (J & W Scientific, Folsom, CA) and a FID. The operating conditions were 50 ˚C for 5 min, and then a temperature increase of 2 ˚C per min to 200 ˚C. Helium was used as the carrier gas at a flow rate of 30 cm per sec. The volatile compounds were identified by GC-MS (Hewlett Packard GCD 1800B, Agilent) fitted with a 25 m x 0.25 mm DB-5 column (Agilent). The operating conditions were 40 ˚C for 5 min, and the temperature was increased by 2 ˚C per min to 200 ˚C. Spectra were matched to those in the National Institute of Standards and Technology library and

confirmed by comparing the retention times of strawberry fruit compounds with those of authentic compounds. Total headspace concentration was calculated using the response factor as in Hamilton-Kemp *et al*. (1992).

Results

Fatty acid content and composition of strawberry fruit

The total and free fatty acid content and composition of strawberry fruits are shown in Table 2.1. Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (LNA, 18:3) acids were detected as components of the total and free fatty acids. Total fatty acids comprised more than 0.2% of fruit tissue while free fatty acids comprised less than 0.3% of the total fatty acids. The major components of the total fatty acids in strawberry fruit were 18:2 and 18:3. The total fatty acid contents were slightly higher (approximately 38%) than those measured by Couture *et al*. (1988). The major components of the free fatty acids in strawberry fruit were 18:2 and 16:0, followed by 18:3. Trace amounts of palmitoleic acid ($16:1\Delta^9$ and $16:1\Delta^7$) from total and free fatty acids were detected (data not shown).

Changes in total and free fatty acid content in strawberry fruit after wounding

To investigate the effect of wounding on changes in total and free fatty acid content in strawberry fruit, the total and free fatty acids were quantified from unwounded fruit, and 5, 10, and 15 min after wounding. There was a trend in which wounding of strawberry fruit increased total fatty acid amount about two fold up to 10 min with a

Table 2.1 Fatty acid content and composition of strawberry fruit

Fatty acids	Total		Free	
	μg/g fruit	%	ng/g fruit	%
16:0	307 ± 171	11.6 ± 1.8	1630 ± 265	27.9 ± 2.1
18:0	36 ± 16	1.5 ± 0.1	510 ± 92	8.6 ± 0.2
18:1	308 ± 108	14.0 ± 1.2	592 ± 114	10.1 ± 0.7
18:2	1020 ± 395	42.5 ± 0.4	2400 ± 559	39.7 ± 3.2
18:3	776 ± 273	30.4 ± 0.6	755 ± 85	13.7 ± 1.9
Total	2250± 963	100	5890 ± 1125	100

Data are means ± standard errors of 4 independent extractions of ripe fruit.

Fruit weight represents fresh weight of fruit.

slight decrease at 15 min (Fig. 2.1). The relative contribution of each fatty acid to total fatty acids remained unchanged over 15 min upon wounding (data not shown). The increased pool of total fatty acids with no change in the composition suggests that the complete fatty acid biosynthetic pathway may be regulated in the wounded tissue, i.e., immediate activation of all enzymes in the pathway, resulting in an increased pool of total fatty acids.

The amounts of free 16:0 and 18:0 were not greatly changed over time after wounding (Fig. 2.2). Free 18:1 and 18:2 showed increased concentrations up to 10 min after wounding but decreased by 15 min. It is also interesting to note that free 16:1 (63 ng/g fruit) in unwounded fruit was increased 3-fold to 183 ng/g fruit at 10 min after wounding (data not shown). A decrease in free 18:3 content was detected within 5 min in response to wounding but remained unchanged through 15 min. The accumulation of free 18:1 and 18:2 in wounded fruit along with the accumulation of total 18:1 and 18:2 indicates that 18:1 and 18:2 are hydrolyzed from membranes upon wounding, implying the presence of lipase activities. However, changes in the pool of free 18:3 upon wounding did not show a pattern similar to those of free 18:1 and 18:2. Free 18:3 comprised 12.5% of total free fatty acids in unwounded fruit and decreased rapidly to 7% at 5 min after wounding, but increased to the original level of unwounded fruit by 15 min after wounding. Since lipase activity is required for the production of free 18:3 for C_6 aldehyde biosynthesis in response to wounding (Matsui *et al*., 2000), the decrease of free 18:3 content at 5 min after wounding, despite increased total 18:3 content (Fig. 2.1), implies that free 18:3 is metabolized into oxylipins.

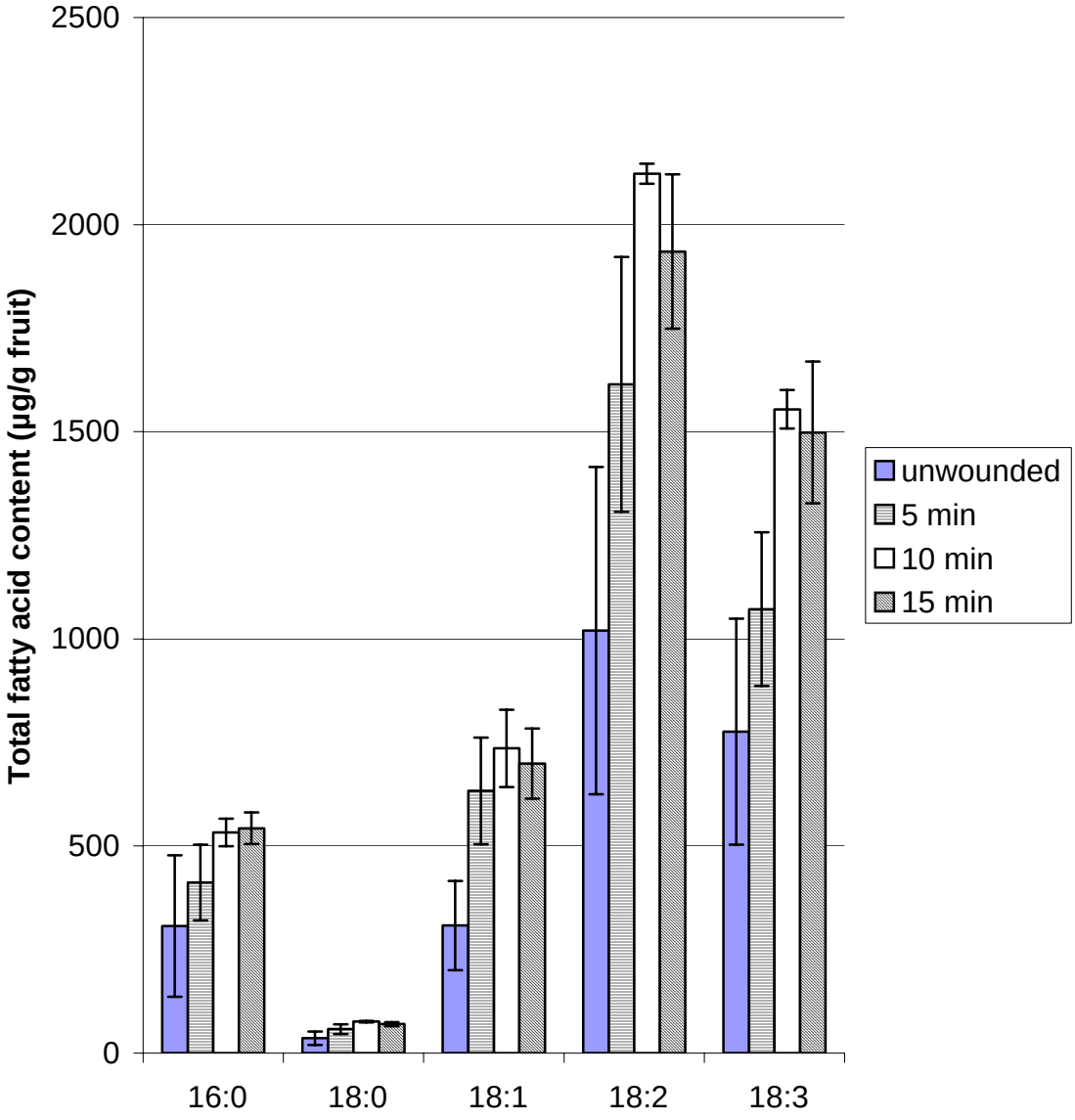


Figure 2.1 Change in total fatty acid content in strawberry fruit in response to wounding.

Bars indicate standard errors of 4 independent extractions of ripe fruit.

Fruit weight represents fresh weight of fruit.

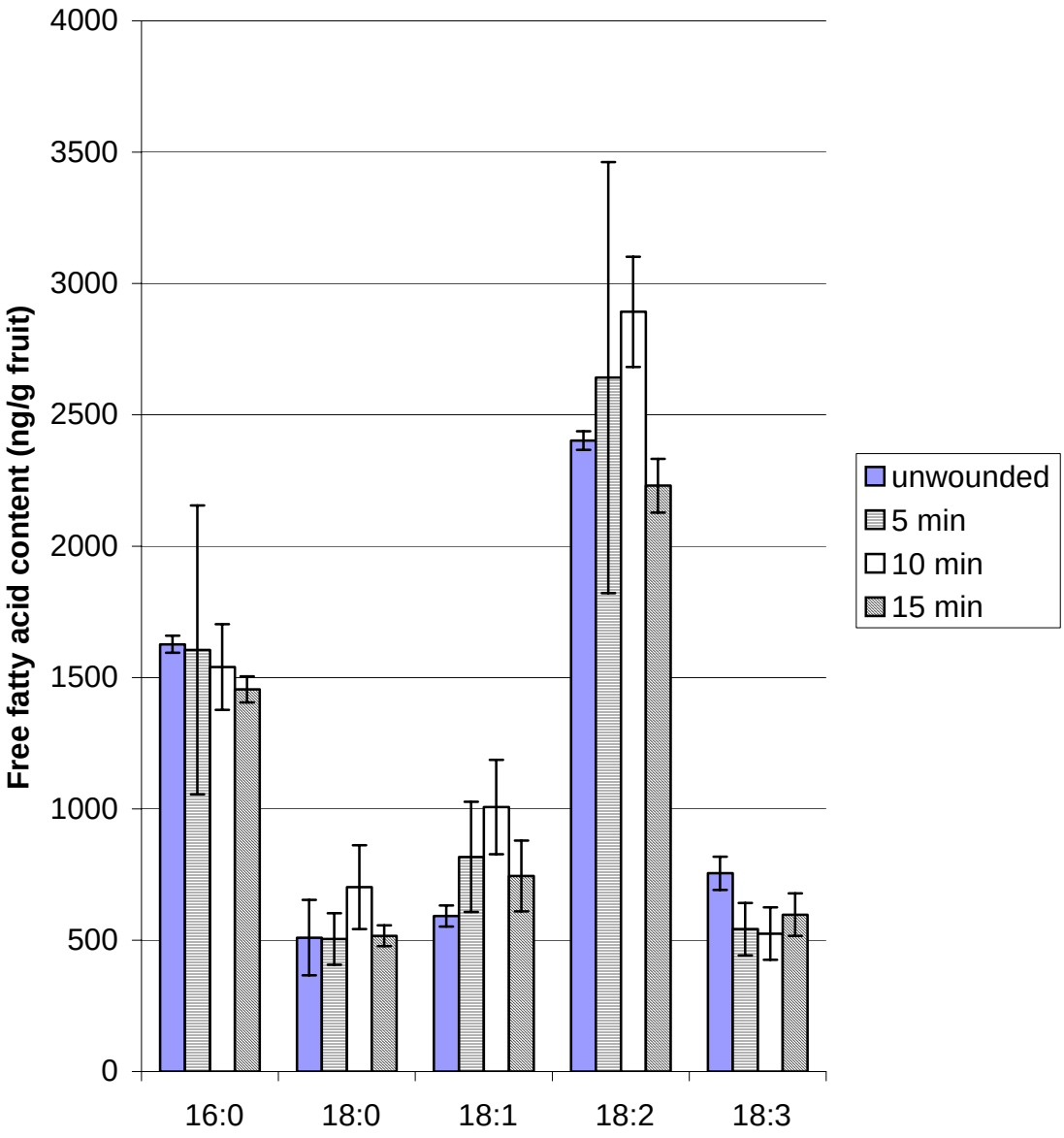


Figure 2.2 Change in free fatty acid content in strawberry fruit in response to wounding.

Bars indicate standard errors of 4 independent extractions of ripe fruit.

Fruit weight represents fresh weight of fruit.

Changes in LOX and HPL activities in strawberry fruit after wounding

Wounding resulted in a change in LOX and HPL activity over 15 min (Fig. 2.3). Prior to wounding, the activities of LOX and HPL were comparable to those measured by Pérez *et al*. (1999). An activation of LOX was observed within 10 min after wounding, but this decreased to the initial activity of unwounded fruit by 15 min. In response to wounding, HPL showed a similar pattern as that of LOX. The decreased free 18:3 shown in Fig 2.2 and stimulated LOX activity suggests that wounding enhances synthesis of 13-HPOT, a product of LOX and substrate of HPL, and that 13-HPOT is metabolized to *c*-3-H through the activation of HPL.

Changes in C_6 aldehyde production in strawberry fruit after wounding

In response to wounding, aldehydes, alcohols, and esters were detected from strawberry fruit over 15 min (data not shown). The vapor-phase concentrations of C_6 aldehydes measured at 5, 10, and 15 min represent total accumulation from 0-5, 0-10, and 0-15 min, respectively. The *c*-3-H was produced by unwounded fruit, while *t*-2-H was not detected (Table 2.2). Wounding induced significant changes in C_6 aldehyde production in fruit within 5 min. The vapor-phase concentration of *c*-3-H increased gradually over 15 min after wounding. In contrast, *t*-2-H was detected in highest quantity at 5 min after wounding, maintained that level through 10 min, but declined by 15 min. The *t*-2-H production coincided with activities of LOX and HPL shown in Fig. 2.3. The gradual emission of *c*-3-H and rapid emission of *t*-2-H within 5 min after wounding indicate a wounding-induced synthesis of C_6 aldehydes.

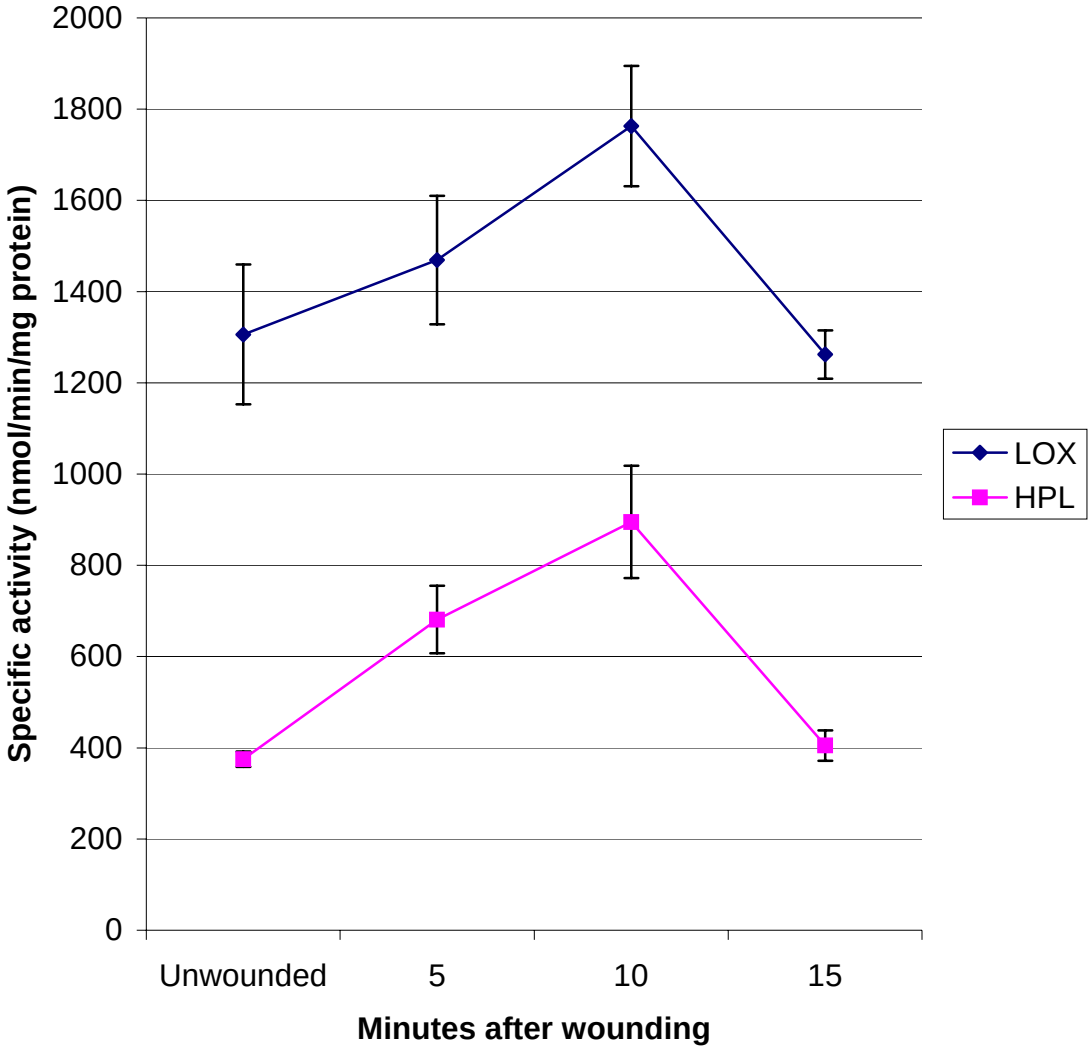


Figure 2.3 Change in LOX and HPL activities in strawberry fruit in response to wounding. LOX activity was determined as the formation of 13-HPOT in nmol per min per mg protein. HPL activity was determined as the oxidation of NADH in nmol per min per mg protein. Bars indicate standard errors of 4 independent measurements of ripe fruit.

Table 2.2 Change in vapor-phase concentrations of *cis*-3-hexenal (*c*-3-H) and *trans*-2-hexenal (*t*-2-H) in strawberry fruit in response to wounding

	Minutes after wounding			
	Unwounded	5	10	15
	Vapor-phase concentration(ng/g fruit)			
c-3-H	2.3 ± 2.3[a]	5.2 ± 3.0	10.8 ± 0.9	12.4 ± 1.1
t-2-H	0.0 ± 0.0	13.0 ± 2.5	11.9 ± 0.2	5.9 ± 0.5

Data are means ± standard errors of 4 independent measurements. [a]The *c*-3-H concentration in unwounded fruit was determined from 2 jars. Each jar contained 4 individual fruit. Fruit weight represents fresh weight of fruit. The quantity of *c*-3-H and *t*-2-H (ng/L in air) calculated by response factor was converted to the equivalent quantity (ng) by conversion factor (0.45) between the units.

Discussion

It is well known that the susceptibility of fatty acids to oxidative damage increases as fatty acid unsaturation in membranes increases. It is interesting to note that total 18:2 and 18:3 in ripe strawberry fruit comprised more than 70% of total fatty acids (Table 2.1). The high degree of fatty acid polyunsaturation of strawberry fruit may reflect sensitivity to lipid peroxidation. In contrast, 18:1, 18:2 and saturated fatty acids such as myristic acid (14:0) and 18:0 are the most abundant fatty acids in apple, apricot, mango, and tomato fruits (Sharaf *et al*., 1989; Wang and Faust, 1992; Gray *et al*., 1999). Lipid peroxidation is an oxidative deterioration of polyunsaturated fatty acids with formation of peroxides and aldehydes. Total fatty acid content of strawberry fruit was less than two-fold higher or lower than that of fruit of other species.

The two-fold increase in content of total unsaturated fatty acids up to 10 min after wounding in strawberry fruit (Fig. 2.1) was unexpected as previous studies showed that there was no significant change or decrease in total amount of lipids and fatty acids in leaves up to 30 min after wounding (Conconi *et al*., 1996a; Ryu and Wang, 1998). Wounding effects on strawberry fruits may differ from those of leaves since the external skin of strawberry fruits can be more easily damaged than that of leaves. It should be noted that the shaking procedure used in this experiment cannot be applied to leaves. Saturated fatty acids (16:0 and 18:0) are synthesized before being incorporated into membrane lipids, while 18:3 can be synthesized from 18:1 via 18:2 by a class of desaturases on membrane lipids (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). The increased total fatty acids may be a consequence of cellular events in which

enzymes involved in biosynthesis of fatty acids, fatty acid binding-proteins involved in transfer of fatty acids to membranes, or desaturases are activated to fill disrupted membrane bilayers, as a wound healing process. Rapid intracellular removal of fatty acids from the cytofacial side of the plasma membrane and their subsequent esterification occurs with half-times typically less than 1 min in the cell (McArthur *et al*., 1999). Even though no evidence of the wound-induced increase of unsaturated fatty acids has been available, Spiteller (2003) suggested that any alteration of membrane fatty acids is related to lipid peroxidation. However, the levels for total fatty acids in this study may be due to artifact generation, perhaps increasing them above *in vivo* amount. Biological samples need to be frozen and maintained at - 80 ºC prior to solvent extraction to minimize such artifact generation (Schmelz *et al*., 2004). However, differences from wound treatment were consistent and likely reflect real wound responses.

The fatty acids are known to be released from the plasmalemma and plastid membranes by lipases after wounding (Narváez-Vásquez *et al*., 1999; Wang *et al*., 2000). Polyunsaturated free fatty acids liberated from membranes are oxidized by LOX or chemically metabolized to other products (Blée, 2002; Howe and Schilmiller, 2002; Weber, 2002). In this study, the changes in free fatty acids reflect biochemical events in strawberry fruits after wounding. The continued increase in free 18:1 and 18:2 content through 10 min after wounding, shown in Fig. 2.2, is likely caused by build-up of fatty acids liberated from membrane lipids. The free fatty acid contents may not represent accurate levels, as noted above, because of lipase activity in extraction solution. The activation of lipid hydrolysis indicates the presence of a lipase and its immediate activation within 5 min in response to wounding, even though it has not been determined

whether a specific lipase is related to this early wounding response. It seems that the predominant substrate of LOX is free 18:3 because free 18:2 was increased through 10 min after wounding while free 18:3 was reduced. If LOX preferred free 18:2 to free 18:3 as a substrate, free 18:3 would have presumably increased. The substrate specificity to free 18:3 of LOX was previously reported (Pérez *et al*., 1999). In Fig. 2.2, it was shown that the accumulated free 18:1 and 18:2 levels, having increased through 10 min after wounding, were decreased to the levels observed in unwounded fruit by 15 min. Free unsaturated fatty acids are toxic to cells. It is possible that accumulation of excessive free fatty acids as part of normal cellular metabolism may trigger a protective mechanism, detoxifying harmful unsaturated free fatty acids. A fatty acid hydroxylase can mediate the detoxification of unsaturated fatty acids as a protective response to toxic fatty acids (Palmer *et al*., 1998). The free 18:1 and 18:2 levels at 10 min after wounding may represent a maximal concentration in strawberry fruit that do not cause toxicity.

Changes in free 16:0 levels did not show a similar pattern to those observed in free 18:1 and 18:2 (Fig. 2.2). The lack of apparent wound-induced effect on free 16:0 level may be correlated to the free 16:1 level. Even though a small amount of free 16:1 existed in fruit (data not shown), the increase (63 to 183 ng per g fruit at 10 min after wounding) in free 16:1 argues for the existence and activation upon wounding of a $16{:}0\Delta^7$ desaturase (FAD5). The $16{:}0\Delta^7$ desaturase functions by a *cis* double bond insertion at the Δ^7 position of 16:0, mainly at the *sn*-2 position of plastidal MGDG (Heilmann *et al*., 2004). The $16{:}0\Delta^7$ desaturase can produce both $16{:}1\Delta^9$ on plasma membranes and $16{:}1\Delta^7$ on plastid membranes, suggesting changes in regiospecificity by subcellular targeting. Interestingly, we observed that free 16:1 detected in strawberry fruit had a *cis* double bond at the Δ^7

position while total 16:1 detected in strawberry fruit had a *cis* double bond at the Δ^9 position (data not shown). It seems that membrane 16:1Δ^7 and free 16:1Δ^9 quantities were below the detection limits. Therefore, it is likely that a 16:0Δ^7 desaturase is expressed in the cytoplasm of fruit but a lipase is exclusively activated on galactolipids in plastids in response to wounding. If phospholipases were activated, free 16:1Δ^9 should have been detected.

Since free 18:3 is a precursor to C_6 aldehydes, it is crucial to take a look into the changes of free 18:3 levels upon wounding. If a lipase is activated upon wounding in strawberry fruit, the increase in free 18:3 should be accompanied by lipid turnover. Indeed, the decrease in free 18:3 levels at 5 min after wounding, along with the increase in free 18:1 and 18:2 levels (Fig. 2.2), indicates that free 18:3 released from the plastid membrane is metabolized. The correlation between the decrease in free 18:3 level and increase in LOX and HPL activities further supports the possibility that C_6 aldehydes are produced from *de novo* synthesis of free 18:3. Further analyses will be necessary to understand the metabolic flux in strawberry fruit in response to wounding. Nevertheless, the data presented in this study suggest that free 18:3 generated from membrane 18:3 upon wounding is used as a source for the C_6 aldehyde biosynthesis.

It is likely that levels of both *c*-3-H and *t*-2-H are closely related to the activities of LOX and HPL over 15 min after wounding (Fig. 2.3, Table 2.2). The results are in good agreement with the work by Hong *et al*. (2004) who showed that an elevated level of *c*-3-H was a consequence of an increase in endogenous 13-LOX activity. In addition, *c*-3-H production required the activity of HPL (Kessler *et al*., 2004). LOX gene expression, enzymatic activity, and C_6 aldehyde synthesis have shown similar trends in other species,

suggesting that the synthesis of C_6 aldehydes is a consequence of increased LOX and HPL activities (Santino *et al*., 2003; Ongena *et al*., 2004). Since the increase occurred within 10 min in response to wounding, the enhanced LOX and HPL activities may be related to posttranslational mechanisms, such as enzyme stability, presence of inhibitors, and enzyme modification. However, the possibility cannot be excluded that increased gene expression, as mRNAs or inactive proenzyme forms of LOX and HPL are present in sufficient amounts in cells and are rapidly translated to the enzymes upon wounding, resulting in accumulation of enzymes responsible for the rapid synthesis of *t*-2-H. Even though LOX and HPL mRNA and proenzymes are known to preexist in the intact tissues of other species (Wang *et al*., 2000; Noordermeer *et al*., 2001; Vancanneyt *et al*., 2001), it remains to be elucidated whether the quantity of LOX is actually enhanced within 10 min upon wounding in strawberry fruit.

It is important to investigate the metabolic flow from free 18:3 to C_6 aldehydes. Assuming that the proportion of free fatty acid increases were consistent for free 18:1, 18:2, and 18:3, free 18:3 should have been accumulated like free 18:1 and 18:2, if free 18:3 was not metabolized into products. Since free 18:2 may also be catalyzed by strawberry LOX (Pérez *et al*., 1999), free 18:1 can be used as a standard to estimate free 18:3. Free 18:1 increased 23%, 54%, and 15% at 5, 10, and 15 min after wounding, respectively (Fig. 2.2). Therefore, the amounts of free 18:3 produced can be estimated as 920, 1150, and 860 ng/g fruit at 5, 10, and 15 min after wounding, respectively. Free 18:3 available for C_6 aldehydes, i.e., the difference between free 18:3 levels measured (Fig. 2.2) and estimated above, would be 370, 600, and 260 ng/g fruit. If 100 % of 18:3 was converted into C_6 aldehydes, 120, 200, 90 ng of C_6 aldehydes per g fruit would have been

produced because the 13-HPOT is cleaved into a C_{12} compound and *c*-3-H. The vapor-phase quantity of total C_6 aldehydes measured within 15 min (Table 2.2) seems to be a 10-25% conversion of free 18:3 to C_6 aldehydes. However, given that strawberry fruit has the capacity to absorb C_6 aldehydes from atmosphere and metabolize them (Hamilton-Kemp *et al*., 1996) and free 18:3 may be metabolized into a number of compounds in the oxylipin pathway, reducing the vapor-phase concentration of C_6 aldehydes, it is not surprising that the vapor-phase concentration of C_6 aldehydes represents a fraction of free 18:3 content. It is noted that free 18:3 was sufficient for total C_6 aldehyde production.

Our data contradict the results reported by Yilmaz *et al*. (2001) who indicated that enzyme activities of LOX and HPL were not a good indicator of the amount of C_6 volatiles produced by tomato fruit. Howe *et al*. (2000) also suggested that HPL plays a minor role in the production of C_6 volatile compounds during tomato fruit ripening since a paucity of HPL mRNA accumulation in mature green and red fruit was determined. Matsui *et al*. (2001) indicated that 9-LOX, the major LOX form in tomato fruit, might not contribute to the formation of flavor volatiles but that a very low level of 13-LOX could be sufficient for C_6 production. Similarly, tomato had low C_6 aldehyde-forming activity (Sekiya *et al*., 1983). Strawberry and tomato fruit may exhibit different metabolic flows through the pathway of LOX and HPL for the biosynthesis of *c*-3-H and *t*-2-H. The *t*-2-H was detected in unwounded tomato fruit (Yilmaz *et al*., 2001), while *t*-2-H was detected in strawberry fruit only when the fruit was subjected to wounding as shown in this study. The differing profiles of *t*-2-H emission between tomato and strawberry fruit may indicate a difference in regulation of LOX and HPL activities. It is possible that synthesis of C_6 aldehydes may not be affected upon wounding in plant species that favor 9-HPOT

as a product. Therefore, the diverse regulatory processes involved in C_6 aldehyde production may provide an understanding of biochemical mechanisms of wound responses.

From the results obtained in this study, a model for regulation of wound-induced biosynthesis of C_6 aldehydes by strawberry fruit can be proposed. At 5 min after wounding, free 18:3, released from galactolipids, is converted to *c*-3-H by LOX and HPL, and the *c*-3-H is rapidly isomerized to *t*-2-H. At 10 min after wounding, a continuing supply of free 18:3 from plastid 18:3 continues to increase activity of LOX and HPL and increase *c*-3-H production, isomerizing to maintain production of *t*-2-H. At 15 min after wounding, the substrate (free 18:3) availability is lower, leading to lower activaties of LOX and HPL and a decreased overall production of the C_6 aldehydes. Such stimulation by free 18:3 has been reported (Vancanneyt *et al*., 2001). However, it is difficult to associate the apparent continuing emission of *c*-3-H with decrease in free 18:3 availability and decrease in LOX and HPL activities at 15 min after wounding, though levels of free 18:3 may be sufficient for this. It may be possible that an isomerization factor is activated upon wounding and *c*-3-H is rapidly converted to *t*-2-H, but the factor returns to a deactivated state by 15 min after wounding resulting in the accumulation of *c*-3-H. However, it is still unclear whether the conversion of *c*-3-H to *t*-2-H *in vivo* can be accelerated by an enzyme in strawberry fruit, as *c*-3-H may isomerize spontaneously into more stable *t*-2-H (Hildebrand, 1989). Nevertheless, the proposed model emphasizes the involvement of substrate availability and increased LOX and HPL activities with the initial burst of *t*-2-H synthesis. With regard to this, Vancanneyt *et al*. (2001) suggested

that *c*-3-H production is determined by substrate availability to HPL in potato leaves rather than by the abundance of HPL activity.

Cuticle and cell wall of fruit tissue constitutes a physical barrier against fungal pathogens. *B. cinerea* is able to penetrate through undamaged or damaged structures by secreting cuticle and cell wall degrading enzymes (Staples and Mayer, 1995). Wounding treatments in this study could provide a site of entry through damaged tissue without enzymatically degrading the physical networks of intact fruit. The headspace quantity of *t*-2-H produced from fruit (28 ng/L in air) in response to wounding at 5 min in this study (Table 2.2) was far below the level of *t*-2-H (480 ng/L in air) which inhibited the growth of *B. cinerea* (Fallik *et al*., 1998), suggesting the possibility that the quantity might be at a level which stimulates fungal growth. The rapid emission of *t*-2-H from wounded strawberry fruit might be an activator of early fungal growth or spore germination, along with damage to physical structures of strawberry fruit. This is supported by work of Archbold *et al*. (2002) that volatile compounds emitted from strawberry fruit following wounding promoted *Botrytis* development. Since a jar containing strawberry fruit was sealed after wounding, the vapor-phase concentrations of C_6 aldehydes measured from the damaged fruit in this study likely reflect continuing release, reabsorption, or re-release of C_6 aldehydes by strawberry fruit (Hamilton-Kemp *et al*., 1996).

In contrast to the headspace quantity of *t*-2-H produced from wounded fruit at 5 min in this study (13 ng/g), a thousand-fold more *t*-2-H (12 μg/g) was collected from macerated tea leaves at 3 min (Hatanaka and Harada, 1973). Also, wounding led to the release of more C_6 aldehydes (60 μg/g dry weight) after 1 hr from alfalfa and clover leaves (Gouw *et al*., 2000). The larger amount of *t*-2-H produced from vegetative tissues,

compared to that produced from strawberry fruit within 15 min, would be higher than the level which inhibited the growth of *B. cinerea* (Fallik *et al*., 1998), suggesting that *t*-2-H may play a antimicrobial compound in pathogenesis in green leaf tissues. It is conceivable that changes in membrane structure and the resulting emission of *t*-2-H due to injury during postharvest handling, transport, and marketing of strawberry fruit might be responsible for triggering the attack of microorganisms, increasing the susceptibility of strawberry fruit to *B. cinerea*. It is also possible that *t*-2-H could be repeatedly emitted at intervals at any time wounding occurs to strawberry fruit during postharvest handling.

Besides its potential stimulatory effect on fungal growth, *t*-2-H can induce defense-related genes (Bate and Rothstein, 1998; Gomi *et al*., 2003). Recently, it was reported that *c*-3-H also induced defense-related genes (Farag *et al*., 2005). Although there have been intensive surveys of volatile compounds from strawberry fruit (Ménager *et al*., 2004), knowledge of endogenous concentrations of C_6 aldehydes in fruit is limited because i) destructive sampling of fruit, which may mimic wounding effects, can increase the level of the compounds, ii) evaporation of volatile compounds during volatile preparation steps after extraction can give an inaccurate concentration of volatile compounds, and iii) particularly, *t*-2-H is a highly reactive compound, featuring two functional groups, an aldehyde group at C1 and a double bond between C2 and C3. The two functional groups can readily react with proteins (Baker *et al*., 1999; Ichihashi *et al*., 2001), and some *t*-2-H may form adducts with proteins in strawberry fruit, resulting in a reduced endogenous level of *t*-2-H. The endogenous levels of *t*-2-H were measurable (379-982 ng/g fruit) with a trace amount of *c*-3-H from unwounded ripe strawberry fruit (Pérez *et al*., 1999; Ménager *et al*., 2004). It may be that this *t*-2-H is compartmentalized

or resides in the lipid fraction or as adducts. It is unclear why such high levels exist in unwounded fruit as little as found in control fruit in our studies. It is possible that increased C_6 aldehyde in response to wounding acts as an elicitor of the defense mechanism in wounded strawberry fruit. However, it is unknown whether *t*-2-H and/or *c*-3-H play a role in inducing defense mechanisms in strawberry fruit, even though *c*-3-H is quickly isomerized to *t*-2-H by the fruit (Pérez *et al*., 1999). Further studies will be needed to understand the potential roles of *t*-2-H in influencing fungal development and defense mechanisms of strawberry fruit.

In conclusion, this is the first study to demonstrate the regulation of *t*-2-H biosynthesis in strawberry fruit in response to wounding. Our data demonstrate that LOX and HPL activities rapidly increased upon wounding. The patterns were consistent with increased C_6 aldehyde biosynthesis. With the presence of lipase activity indicated by the increased pool of free 18:1 and 18:2, the decreased free 18:3 level and the increased *t*-2-H production from wounded fruit implies that *de novo* synthesis of free 18:3 is required for *t*-2-H biosynthesis in response to wounding.

CHAPTER THREE

SITE AND MODE OF INTERACTION OF C_6 ALDEHYDES WITH *BOTRYTIS CINEREA*

Introduction

Plants produce numerous volatile compounds, and the compounds have been studied for their biological effects on fungal growth (French, 1985). Since gray mold, caused by *Botrytis cinerea*, has been regarded as a major disease of fresh produce, numerous volatile compounds have been evaluated for their effects on disease development. Among theses compounds, aldehydes have been extensively studied due to their effective antimicrobial activity and potential as biologically based fumigants. Hamilton-Kemp *et al*. (1992) developed a bioassay system testing the effects of volatile compounds on fungal growth, and showed the effectiveness of six carbon (C_6) and nine carbon (C_9) aldehydes, which have antifungal activity at low concentrations. In addition, Hamilton-Kemp *et al*. (1992) and Andersen *et al*. (1994) demonstrated that *trans*-2-hexenal (*t*-2-H) was more effective in inhibiting hyphal growth of *B. cinerea* than the saturated aldehyde, hexanal. Archbold *et al*. (1997) demonstrated that *B. cinerea* grew slowly on strawberry fruit in the presence of many volatile compounds including aldehydes and alcohols at low concentrations, suggesting an inhibitory effect. Vapor produced in 250 mL bottles from

5.4 and 85.6 µmol of liquid *t*-2-H (low vs high headspace concentrations) were found to promote and inhibit growth of *B. cinerea*, respectively (Fallik *et al*., 1998).

The C_6 aldehydes, *cis*-3-hexenal (*c*-3-H) and *t*-2-H, are released from damaged leaves and fruits via the lipoxygenase (LOX) pathway (Hatanaka and Harada, 1973; Hatanaka *et al*., 1987; Gray *et al*., 1999). Besides their direct effect on pathogens, they are thought to be involved in the defense mechanism of plants by inducing prosystemin gene expression (Sivasankar *et al*., 2000), defense-related genes (Bate and Rothstein, 1998; Gomi *et al*., 2003), and stress and defense responses (Vancanneyt *et al*., 2001; Farag and Paré, 2002) in plants that produce them as well as in neighboring plants (Farmer, 2001). Linolenic acid (LNA, 18:3) released from membrane lipids by lipases is converted to 13-HPOT [(13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid] by LOX, non-heme iron-containing dioxygenases (Feussner and Waternack, 2002). 13-HPOT formed by LOX is immediately metabolized by hydroperoxide lyase (HPL) (Noordermeer *et al*., 2002). HPL cleaves 13-HPOT into 12-oxo-(9*Z*)-dodecenoic acid and *c*-3-H (Grechkin and Hamberg, 2004). The *c*-3-H isomerizes spontaneously or enzymatically into more stable *t*-2-H (Hildebrand, 1989). The C_6 aldehydes are further reduced to alcohols by alcohol dehydrogenase.

There have been several studies to elucidate the mechanisms involved in antimicrobial activity (Trombetta *et al*., 2002; Kubo *et al*., 2003). The presence of an α,β-unsaturated bond adjacent to the carbonyl moiety has been shown to be significant in antifungal and antibacterial activity (Andersen *et al*., 1994; Trombetta *et al*., 2002). The inhibitory effects of *t*-2-H against *B. cinerea* may be due to severe damage to fungal membranes and cell walls (Fallik *et al*., 1998). Trombetta *et al*. (2002) demonstrated that

aldehydes including *t*-2-H caused significant changes in cellular membrane permeability, suggesting that they can penetrate through membranes and interact with intercellular components. Kubo *et al*. (2003) hypothesized that the aldehydes bind nonspecifically, disrupt the hydrogen bond in the lipid bilayer, and affect fluidity of membrane lipids.

It is well established that *t*-2-H can react with and modify proteins (Refsgaard *et al*., 2000; Ichihashi *et al*., 2001; Meynier *et al*., 2004). Several lines of evidence have indicated that *t*-2-H is a reactive compound featuring two functional groups, an aldehyde group at C1 and a double bond between C2 and C3. The two functional groups can readily attack a nucleophile such as protein (Baker *et al*., 1999; Ichihashi *et al*., 2001). It has been suggested that the α,β-unsaturated aldehyde reacts with the sulfhydryl group of cysteine, the ε-amino group of lysine, and the imidazole group of histidine in protein and forms adducts via Michael addition or Schiff base reactions. Evidence of covalent cross-linking between *t*-2-H and proteins via Michael addition or Schiff base reactions has been reported (Židek *et al*., 1997; Zhou and Decker, 1999; Meynier *et al*., 2004). Amarnath *et al*. (1998) showed that the major reaction of *t*-2-H in the presence of protein *in vitro* was Michael addition. The protein adduct formed can further undergo several types of reactions. Baker *et al*. (1999) concluded that *t*-2-H modified lysine and histidine residues and the N-terminus of proteins. Ichihashi *et al*. (2001) characterized a covalent modification of a protein and showed that the protein adduct forms were specific to 2-alkenals. The result was in agreement with the previous findings in which the covalent modified adducts had been obtained between various 2-alkenals and protein (Amarnath *et al*., 1998; Zhou and Decker, 1999). Therefore, the chemical properties of C_6 aldehydes contribute to their reactivity with proteins in biological systems. However, since the

studies have been mostly performed *in vitro*, it is not known whether aldehydes can modify proteins *in vivo* in a similar way. The capability of *c*-3-H in forming adducts with proteins has not been studied due to instability of *c*-3-H.

Since radiolabeled C_6 aldehydes are commercially unavailable, synthesis of radiolabeled *t*-2-H is needed to study the interaction of C_6 aldehydes with *B. cinerea*. Vick and Zimmerman (1983) attempted to generate [U-^{14}C] LNA from [1,2-^{14}C] sodium acetate, but the specific activity was low due to a large pool of pre-existing non-radiolabeled LNA. Therefore, alternative synthesis procedures are needed. There have been several attempts to develop a process for C_6 aldehyde production from LNA (Berger *et al*., 1986; Whitehead *et al*., 1995; Noordermeer *et al*., 2002). Soybean LOX and watermelon HPL are known to yield large amounts of C_6 aldehyde (Sekiya *et al*., 1983; Hildebrand *et al*., 1990; Hatanaka, 1993; Hildebrand *et al*., 2000) when used together. Plant enzyme extracts are currently regarded as the most common sources for production of C_6 aldehydes.

The goal of this study was to develop an efficient system of labeling C_6 aldehydes and to determine if radiolabeled C_6 aldehydes can be extracted from sub-cellular fractions of *B. cinerea*. This chapter presents data on production of labeled [^{14}C] LNA and [^{3}H] C_6 aldehyde, use of radiolabeled C_6 aldehyde to determine its interaction with *B. cinerea*, and the uptake and subcellular targets of C_6 aldehyde.

Materials and Methods

Lemna gibba growth and *in vivo* labeling with [U-^{14}C] sucrose

A frond of sterile *Lemna gibba* cultures, which was obtained from a local pond, was placed in each of nine flasks containing 25 mL of 1% sucrose E medium as previously described (Cleland and Tanaka, 1986). Nine flasks were fed in triplicate per control and two concentrations with 12.5 μCi or 25.0 μCi of [U-^{14}C] sucrose (552 mCi/mmole at 0.2 mCi/mL, Amersham Biosciences, Piscataway, NJ) or non-radiolabeled sucrose, respectively. Upon the sucrose addition, the 12.5 μCi and 25.0 μCi of [U-^{14}C] sucrose were diluted to a final specific radiolabel of 50 μCi/mmol and 100 μCi/mmol, respectively. The flasks were covered with aluminum foil to prevent contamination and placed under continuous light at 25 °C for optimal growth.

Lipid and fatty acid extractions and analysis from *Lemna. gibba*

After 20 days of culture, fronds of *L. gibba* were harvested from each flask and freeze-dried. Approximately 30 mg of freeze-dried fronds were obtained per flask. A 10 mg dried sample was ground with a glass rod in a test tube and extracted with 1 mL of methanol, chloroform, and water (2:1:0.8) containing 0.1% butylated hydroxytoluene (BHT) and a few drops of 1 M oxalic acid. The sample, to which a suitable amount of the internal standard triheptadecanoin (0.3 mg/10 mg dried fronds) was added, was further extracted with 1 mL of chloroform and methanol (2:1). Following centrifugation, the chloroform layer was collected and evaporated under a steam of nitrogen.

After lipid extraction, a few drops of diazomethane and 0.5 mL of 1% sodium methoxide were added to the remaining residue and samples were shaken for 45 min. The resulting fatty acid methyl esters were extracted with hexane. Samples were washed with 0.9% KCl, the hexane layer was collected, and fatty acid methyl esters were analyzed by gas chromatography (GC) as described previously (Chapter 2).

After lipid extraction, separation of LNA from other fatty acids was performed according to the methods of Liu *et al*. (1995). The remaining residue was saponified for 45 min at 80 °C in 2 mL of 1 N KOH in 95% ethanol. Then, 5 mL of water was added and followed by a few drops of concentrated HCl to acidify the solution to pH 3. The fatty acids were extracted with hexane and applied to a reverse phase TLC plate (Sigma, St. Louis, MO), which was developed in acetonitrile/water/acetic acid (95:5:1, v/v). Authentic free LNA was used as a loading standard to identify free LNA. The plate was cut into two pieces; one had a band of loading standard and the other had a band of samples. The piece of plate with loading standard was placed in a tank containing iodine to be visualized under UV (300 nm). A suitable internal standard (nonadecanoin) was added (0.3 mg/10 mg dried fronds) to the free LNA spot of the sample before scraping the zone with free LNA from the plate. The LNA samples were eluted with diethyl ether and methylated with diazomethane. The ether was evaporated with N_2 and the residue was dissolved in hexane.

Total recovery of radiolabel from lipid extracts were estimated in aliquots sampled after three different steps; 1) total lipid extraction, 2) total fatty acid extraction after saponification, and 3) total fatty acid extraction before TLC loading. Radiolabel was

determined by liquid scintillation counting (TRI-CARB 1900CA, PerkinElmer Inc., Boston, MA). Specific radiolabel of [U-^{14}C] LNA collected was also measured.

In vitro C_6 aldehyde production

For the production of C_6 aldehyde, the enzymatic reaction was performed *in vitro* by incubation of LNA with LOX and HPL extracted from soybeans and watermelon leaves, respectively. LOX crude extract was prepared as described above (Chapter 2). For preparation of HPL crude extract from watermelon leaves, leaves (0.5 g) were frozen in liquid nitrogen, ground to a powder, and extracted with 1 mL of 50 mM potassium phosphate buffer, pH 6.8, 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM dithiothreitol (DTT), 1% protease inhibitor cocktail (Sigma), 0.5% Triton X-100R, and 50 mg polyvinylpolypyrrolidone (PVPP). After the sample was centrifuged at 14,000 rpm for 15 min, the supernatant was collected and used as a crude extract. LOX and HPL activities were measured by the methods described above (Chapter 2). The activities of LOX and HPL extracts were approximately 30.0 μmol/min/mg protein and 2.0 μmol/min /mg protein, respectively.

The reaction was initiated in a 10 mL serum bottle, which contained 4 mL of 0.012 μM LNA and 0.2 M borate buffer, pH 9.0. LOX was added to the mixture saturated with oxygen. After 2 hr incubation, the pH was lowered to 6-7 and HPL was added to the mixture. In preliminary experiments, a range of ratios among LOX, HPL, and LNA were tested, and highest *t*-2-H production was obtained from the ratio of 5 μg LOX protein extract, 15 μg HPL protein extract, 3.5 μg LNA, and 2 mL reaction volume. Following HPL addition, the bottle was immediately sealed with a 20 mm polytetrafluoroethylene

(TFE)/silicon liner and aluminum cap and placed at room temperature (23 ˚C) for 18 hr. The content of vapor-phase volatile compounds was determined at 2 and 18 hrs after incubation by using solid phase microextraction (SPME, Supelco, Bellefonte, PA). The SPME was carried out by inserting the SPME apparatus needle through the septum of the bottle and deploying the fiber (100 μm polydimethylsiloxane, Supelco). The air in the bottle was sampled for 15 min prior to analysis by GC described above (Chapter 2).

Incubation of radiolabeled C_6 aldehydes with *Botrytis cinerea* cultures

The process of producing C_6 aldehydes from radiolabeled [9,10,12,13,15,16-^{3}H] LNA (120 Ci/mmole at 1 mCi/mL; American Radiolabeled Chemicals Inc., St. Louis, MO) was performed according to the method described above with some modifications (Fig. 3.1). After 2 hr incubation of the radiolabeled LNA with LOX and pH adjustment, the mixture was placed in 1 or 2 cm-diameter glass vials, depending on the volume of the mixture, to which HPL was added. Spores of *B. cinerea* isolated from strawberry fruit used in the previous study (Chapter 2) were used to inoculate a culture on potato dextrose agar (PDA) at 22 ˚C. Subsequently, a single 14 day-old PDA culture and glass vials containing the reaction mixture were placed in a 14 cm-diameter glass petri dish, which was covered and immediately wrapped in Parafilm. This bioassay system (Fig. 3.1) was modified from that developed by Hamilton-Kemp *et al*. (1992). Three different amounts (0.75, 7.5, and 15 μCi) of [^{3}H] LNA, were used in this experiment. Volumes of reaction mixture used were in proportion to LNA added (2 mL per 1.5 μCi LNA). The system was sealed for 18 h. Each experiment was repeated three times.

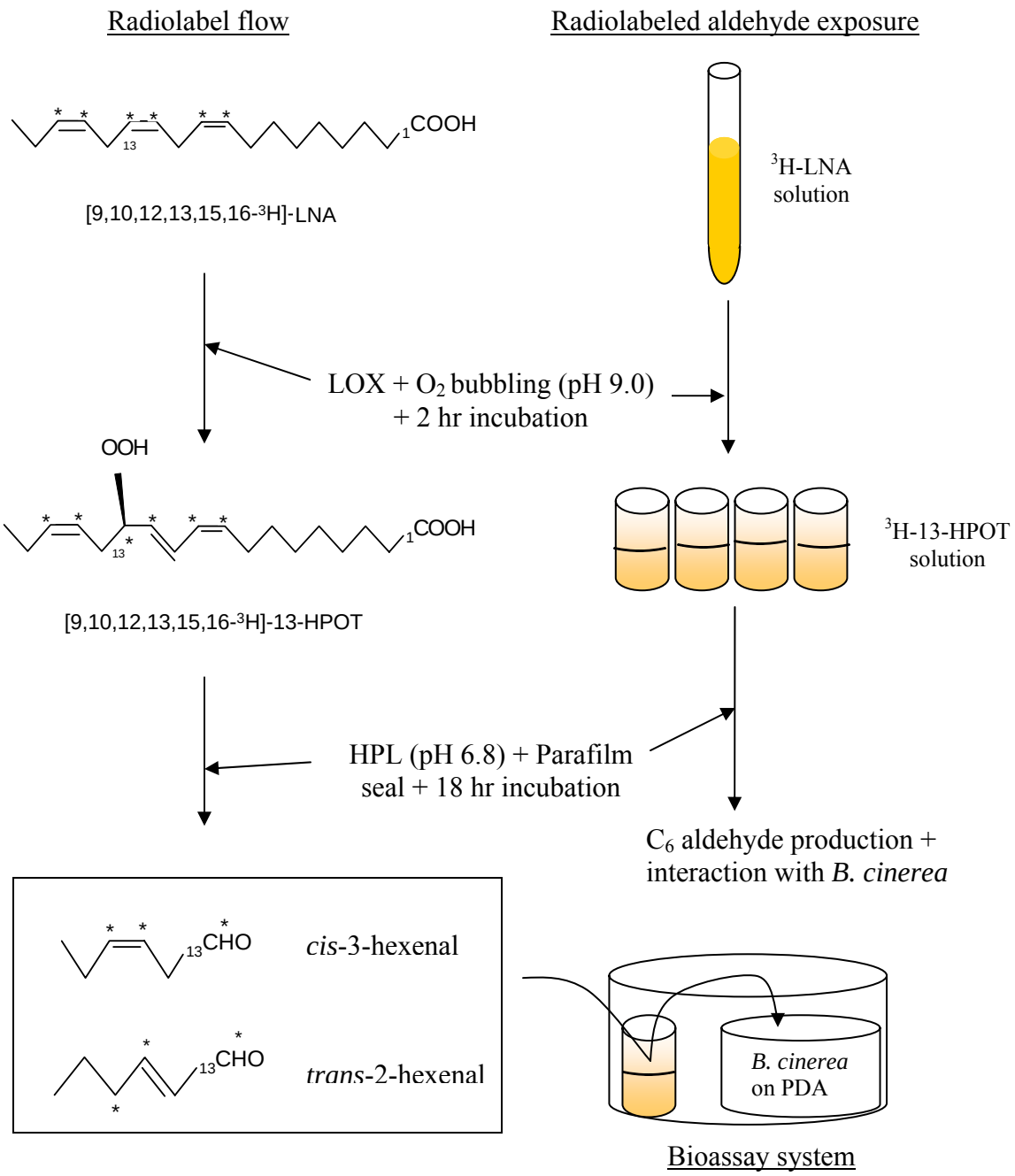


Figure 3.1 System for exposing *B. cinerea* to radiolabeled aldehydes. Flow of radiolabel and reaction procedures for C_6 aldehyde production are outlined.

* indicates ^3H label.

Extraction of radiolabel from *Botrytis cinerea* conidia and mycelia

After 18 hr incubation, the petri dish was opened and allowed to remain in place for 30 min prior to use. The fungal tissue was harvested from PDA medium by gentle pouring of ice-chilled water (10 mL) across on the PDA medium (Fig. 3.2) and suspending the culture. The suspension was further passed through Miracloth to separate mycelia from conidia. The residue on the Miracloth was washed with water (3 x 10 mL), and the filtrates were combined and centrifuged at 7,000 g for 30 min. It was previously shown that vigorous washing and physical force readily removed fungal mycelia (Doss *et al*., 1995; Doss, 1999). The pellet contained mostly conidia with some pieces of mycelia, confirmed by microscopy (Stemi SV11, Zeiss). Therefore, the residue on the Miracloth was enriched in labeled mycelia while the resulting pellet was enriched in labeled conidia. Total lipid was extracted from the conidia three times with methanol, chloroform, and water (2:1:0.8). For total protein extraction, conidia were boiled with sodium dodecyl sulfate (SDS) extraction buffer (950 μL Laemmli sample buffer (Bio-Rad, Hercules, CA) mixed with 50 μL β-mercaptoethanol) for 10 min and centrifuged at 14,000 g for 5 min. Total protein was retained in the supernatant. A portion of mycelia was also divided into lipid and protein fractions, prepared the same as for conidia. Portions of all samples were subjected to scintillation counting for measuring radiolabel.

Subcellular fractionation and SDS-PAGE

A. Wash protein

The resulting supernatant of the filtrates, which passed through Miracloth as described above, was used as the wash protein fraction. The volume was concentrated by

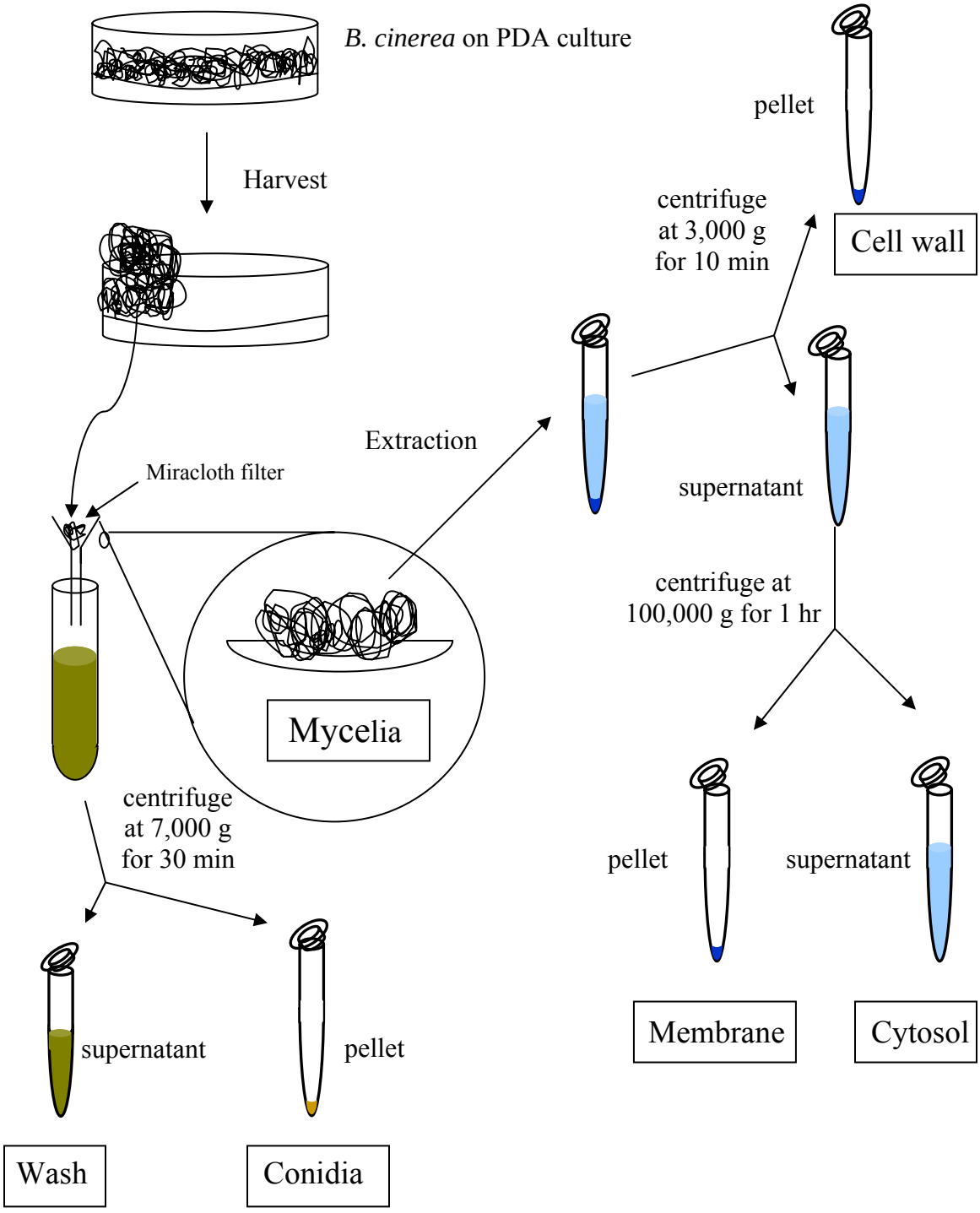


Figure 3.2 Scheme of protein sampling and subcelullar fractionation

passing through several rounds of a 3000-kDa-cutoff Centricon YM-3 ultrafiltration membrane (Millipore, Bedford, MA) according to the manufacturer's protocol. Final protein concentration was determined by the method of Bradford (1976). All samples were stored at -80 ˚C until further use.

B. Cell wall protein

The mycelial fraction was subjected to differential centrifugation by the schemes previously reported (Pitarch *et al*., 2002; Augstein *et al*., 2003). The mycelial tissue was frozen in liquid nitrogen and ground to a powder. The tissue (500 mg) was suspended in 1.5 mL extraction buffer of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 μL chymostatin, 10 μL aprotinin, 10 μL leupeptin, 15 μg phenylmethanesulfonyl fluoride (PMSF), and 2% PVPP. The resulting suspension was centrifuged at 3,000 g for 10 min. The resulting pellet was washed five times with each of the following ice-chilled solutions; water, 5% NaCl, 2% NaCl, and 1 mM PMSF, and was extracted twice by boiling with 2 mL SDS extraction buffer of 50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 2% SDS, and 10 mM DTT for 10 min. Proteins in the cell wall fraction were precipitated with 50% acetone for protein determination as described above.

C. Membrane and cytosol protein

The supernatant after centrifugation at 3,000 g for 10 min as described above was further centrifuged at 10,000 g for 10 min to remove debris. The resulting supernatant was used as membrane plus cytosol protein fraction. The sample was further centrifuged at 100,000 g for 1 hr in a Ti-91 rotor using an ultracentrifuge (XL-80, Beckman, Palo

Alto, CA). The cytosolic supernatant was decanted and the membrane pellet was dissolved in 500 mM NaOH.

D. SDS-PAGE

Protein samples of wash, membrane plus cytosol, cytosol, membrane, and cell wall were dissolved in SDS sample buffer as described above and were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) according to the standard protocol (Bio-Rad).

Subcellular distribution of radiolabel in the presence of non-radiolabeled C_6 aldehydes

To determine if headspace levels of *t*-2-H or *c*-3-H affected their distribution on fungal tissue, non-radiolabeled *t*-2-H and *c*-3-H at three different concentrations, 5.4, 85.6, and 428.0 μmol, were added directly to separate 1 mL vials. These were placed in the bioassay system containing the radiolabeled C_6 aldehyde-forming reaction mixture as decribed above (Fig. 3.1). After 18 hr, mycelia were harvested, subcellular fractionations from wash, cell wall, and membrane and cytosol were prepared as above, and radiolabel of the protein fractions was measured.

Interaction of radiolabeled C_6 aldehydes with subcellular proteins

Crude extracts of proteins in the wash, cell wall, membrane, and cytosolic fraction were prepared according to the method described above. The cap of the eppendorf or test tube containing the extracts was opened and placed on ice for 24 hr to allow free C_6

aldehydes to evaporate. Proteins in the wash fraction or in the membrane plus cytosol fraction were further concentrated by ultrafiltration or were subjected to ultracentrifugation, as described above. The protein in the cytosolic fraction was precipitated with 50% acetone. The protein concentration and radiolabel were measured in the protein samples following each step described above.

Results

In vivo labeling of [U-^{14}C] sucrose to produce linolenic acid in *Lemna gibba*

[U-^{14}C] sucrose was incorporated into *Lemna gibba* to determine the efficiency of the production of radiolabeled LNA, a precursor of *t*-2-H. The logarithm of the number of fronds plotted against time (Fig. 3.3) shows that *L. gibba* grew rapidly and reached a high number of fronds within 2 weeks similar to the growth reported by Lehman *et al*. (1981). The growth of fronds in the presence of 12.5 and 25.0 μCi [U-^{14}C] sucrose did not differ from that of fronds in the absence of radiolabeled sucrose.

The fatty acid composition analysis of *L. gibba* showed that total LNA (18:3) comprised 67.7 ± 1.3% of total fatty acids followed by other fatty acids such as palmitic acid (16:0) (21.2 ± 0.8%), linoleic acid (18:2) (10.2 ± 0.3%), and oleic acid (18:1) (0.9 ± 0.1%) (data not shown). Since the vegetative cells of the Lemnaceae contain 1.8 to 9.2% lipid by dry weight (DW) (Landolt and Kandeler, 1987), 18 to 92 μg of lipid may be extracted per one mg of dried fronds. Fatty acids can account for 90% of the acyl chains of the structural glycerolipids of almost all plants (Ohlrogge and Browse, 1995). Therefore, the total amount of 18:3, 13.5 μg/mg DW, after separation by reverse phase

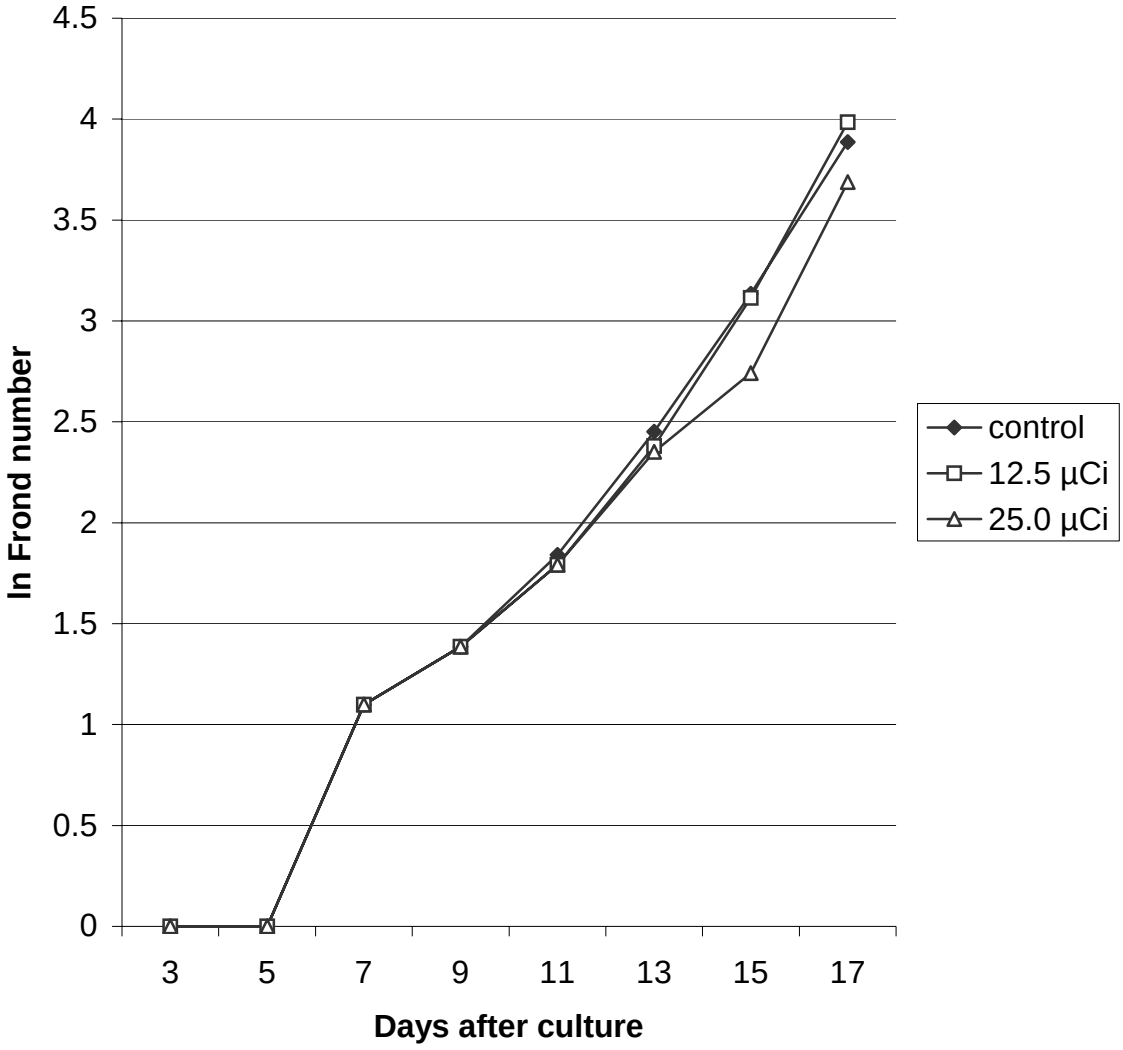


Figure 3.3 Growth curves of *Lemna gibba* fronds expressed as logarithm of the number of fronds against time in the presence of [U-^{14}C] sucrose. Fronds were grown in flasks containing non-radiolabel, 12.5 µCi, or 25.0 µCi [U-^{14}C] sucrose. Data shown are means of three replications.

TLC (data not shown), represents 16-85% recovery of the theoretical total amount of 18:3.

Radiolabel of [U-^{14}C] sucrose in the medium was negligible at harvest (data not shown), which is in agreement with results of Vaughan *et al*. (1994), indicating that [U-^{14}C] sucrose was taken up and either incorporated into daughter fronds or lost through respiration. After the first extraction step, 58 nCi/mg DW was recovered from fronds grown with 12.5 μCi sucrose (Table 3.1). After a second extraction step, 16 nCi/mg DW of radiolabel was recovered. The 42 nCi/mg DW of radiolabel lost from step 1 to 2 was because carbons in polar lipid head groups and other unsaponifiable compounds that remained in the extract were not extracted. Subsequent loss of 6 nCi/mg DW after a third extraction step occurred. The radiolabel of the final product, 18:3, was 130 nCi/mg 18:3. When 25.0 μCi sucrose was used, radiolabel of 120, 38, and 18 nCi/mg DW and 230 nCi/mg 18:3 was measured. The radiolabel measured was approximately two fold that recovered following culture on 12.5 μCi sucrose. This indicates that the radiolabel was incorporated into *L. gibba* and recovered in a concentration-dependent manner.

C_6 aldehyde production from linolenic acid

The final total amount of 18:3 recovered using *L. gibba* (Table 3.1) was not large enough to generate sufficient ^{14}C-*t*-2-H for subsequent interaction studies. However, incubation of commercially-available [9,10,12,13,15,16-^{3}H] LNA with LOX and HPL extracts produces [^{3}H] *t*-2-H. For a preliminary determination of the conversion efficiency of LNA to volatile compounds, non-radiolabeled LNA was used with LOX and HPL extracts. No volatile compounds other than C_6 aldehydes, *c*-3-H and *t*-2-H, were

Table 3.1 Incorporation of radiolabel into total lipids and linolenic acid (18:3) of *L. gibba* fronds grown for 20 days in the presence of 12.5 μCi or 25.0 μCi of [U-^{14}C] sucrose

Sucrose radiolabel	Radiolabel (μCi/g DW)			Specific radiolabel (μCi/g LNA)
	Extraction step			
	1	2	3	
12.5 μCi	58 ± 2	16 ± 1	10 ± 1	130 ± 10
25.0 μCi	120 ± 3	38 ± 2	18 ± 1	230 ± 10

Extraction steps: 1) total lipid extraction; 2) total fatty acid extraction after saponification; 3) total fatty acid extraction before TLC loading; 4) total 18:3.

Data shown are means ± standard errors of 3 replications.

produced using LNA as a substrate (data not shown). This agrees with the results of previous studies (Berger *et al*., 1986; Whitehead *et al*., 1995; Noordermeer *et al*., 2002), where C_6 aldehydes were the major volatiles derived from LNA. At 2 hr after the reaction started, *c*-3-H was the dominant volatile compound, then it decreased to the same extent as *t*-2-H increased by 18 hr (Fig. 3.4). The sum of the C_6 aldehydes was the same at the two times. The pattern of changes in C_6 aldehyde production was similar to that reported by Berger *et al*. (1986). The overall yield of C_6 aldehydes was approximately 1.22 μg, indicating that 25% of the LNA was converted to C_6 aldehydes. If 100% molar conversion occurred, 4.93 μg of C_6 aldehydes should have been produced from 14 μg of LNA, the initial amount of LNA used in the experiment. The 25% conversion shown in this study was similar to the result of Noordermeer *et al*. (2002) who reported 26% conversion of C_6 aldehydes from LNA by soybean LOX and recombinant HPL. The results demonstrate that *c*-3-H and *t*-2-H can be efficiently produced.

Incorporation of radiolabeled C_6 aldehydes into *Botrytis cinerea*

Using [9,10,12,13,15,16-^{3}H] LNA for the production and incorporation of radiolabeled C_6 aldehydes into *B. cinerea*, it was found that 70% of the total radiolabel remained in the reaction solution (Table 3.2), indicating that 30% of the radiolabel was incorporated into volatile compounds. This 30% of the radiolabel should be in C_6 aldehydes, even though a direct measurement of C_6 aldehyde production was not attempted. Upon extraction of radiolabel from the *B. cinerea* culture, only 0.6% of the radiolabel was recovered from the mycelia, suggesting that C_6 aldehydes were lost through evaporation when the closed incubation system was opened and/or were bound to

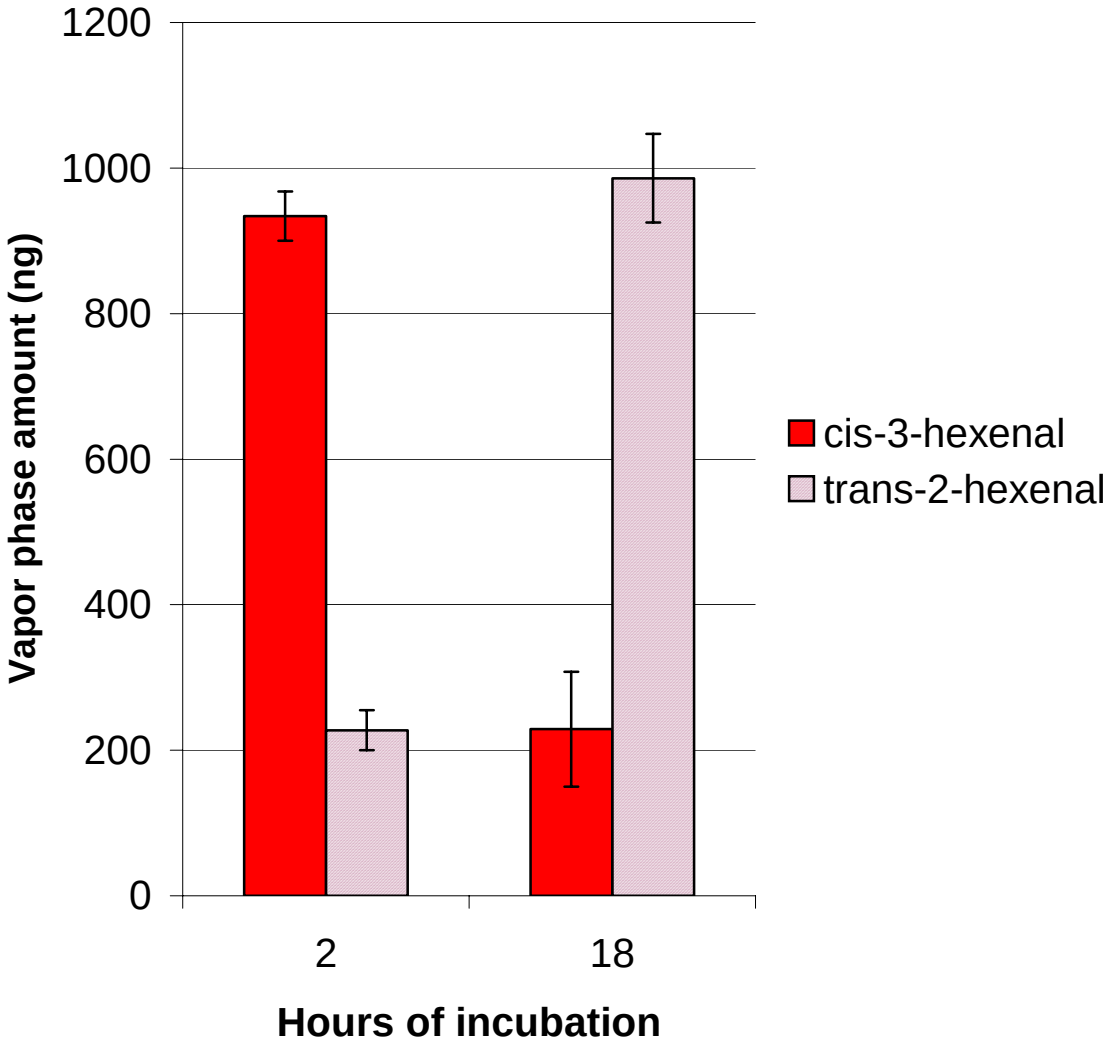


Figure 3.4 C_6 aldehyde production using linolenic acid as substrate with LOX and HPL extracts. The content of aldehydes was determined using solid phase microextraction (SPME). Bars indicate standard errors of 3 independent experiments.

Table 3.2 Distribution of radiolabeled volatile compounds produced from 0.75 μCi (6.25pmol) [^{3}H] linolenic acid by LOX and HPL extracts during an 18 hr exposure of *B. cinerea* to volatile compounds

	Total radiolabel ($\mu Ci \times 10^{-3}$)	Total recovery of radiolabel (%)
^{3}H-linolenic acid used in reaction solution	750.0	100
Non-volatile compounds in reaction solution after 18 hr incubation	528.0 ± 12.5	70.4
Volatile compounds emitted from reaction solution (by subtraction)	222.0 ± 5.2	29.6
Total incorporation into *B. cinerea* [a]	4.4 ± 0.1	0.6

[a] Total incorporation of radiolabel into *B. cinerea* was measured from all extracts obtained from a 14 day-old PDA culture. Data shown are means ± standard errors of 3 replications.

glass surfaces and media.

To investigate how the incorporation of radiolabeled C_6 aldehyde into *B. cinerea* is dependent on total radiolabel of LNA, the radiolabeled C_6 aldehydes generated from 0.75, 7.5, and 15.0 μCi [9,10,12,13,15,16-^{3}H] LNA (specific activity of 120 Ci/mmol) were allowed to interact with the fungus (Fig. 3.5). The incorporation of radiolabel was shown to be concentration-dependent. An increase of radiolabeled LNA quantity from 0.75 to 7.5 μCi resulted in a ten-fold increase of the incorporation of radiolabel into *B. cinerea*. In addition, when the concentration of radiolabeled LNA increased two-fold from 7.5 to 15 μCi, the radiolabel incorporated into *B. cinerea* also increased two-fold. This demonstrates that radiolabeled C_6 aldehydes can be consistently produced and incorporated into *B. cinerea*, and that incorporation is dependent on the initial concentration of radiolabeled LNA.

Interaction of radiolabeled C_6 aldehydes with conidia or mycelia of *Botrytis cinerea*

The location of incorporation of radiolabel into condia and mycelia was determined (Table 3.3). Radiolabel was not found in the total lipid of conidia or mycelia (data not shown), but it was recovered in total protein, indicating that C_6 aldehydes do not appreciably interact with lipids but do interact with proteins. Of the total radiolabel, approximately two-fold more C_6 aldehydes were incorporated into mycelia than into conidia. As total mass and protein content of mycelia on PDA medium was 12- and 8-fold greater, respectively, than those of conidia, radiolabel per unit FW and protein of conidia was greater than that of mycelia. Therefore, the results demonstrate that the relative amounts of C_6 aldehydes in conidia are higher than in mycelia.

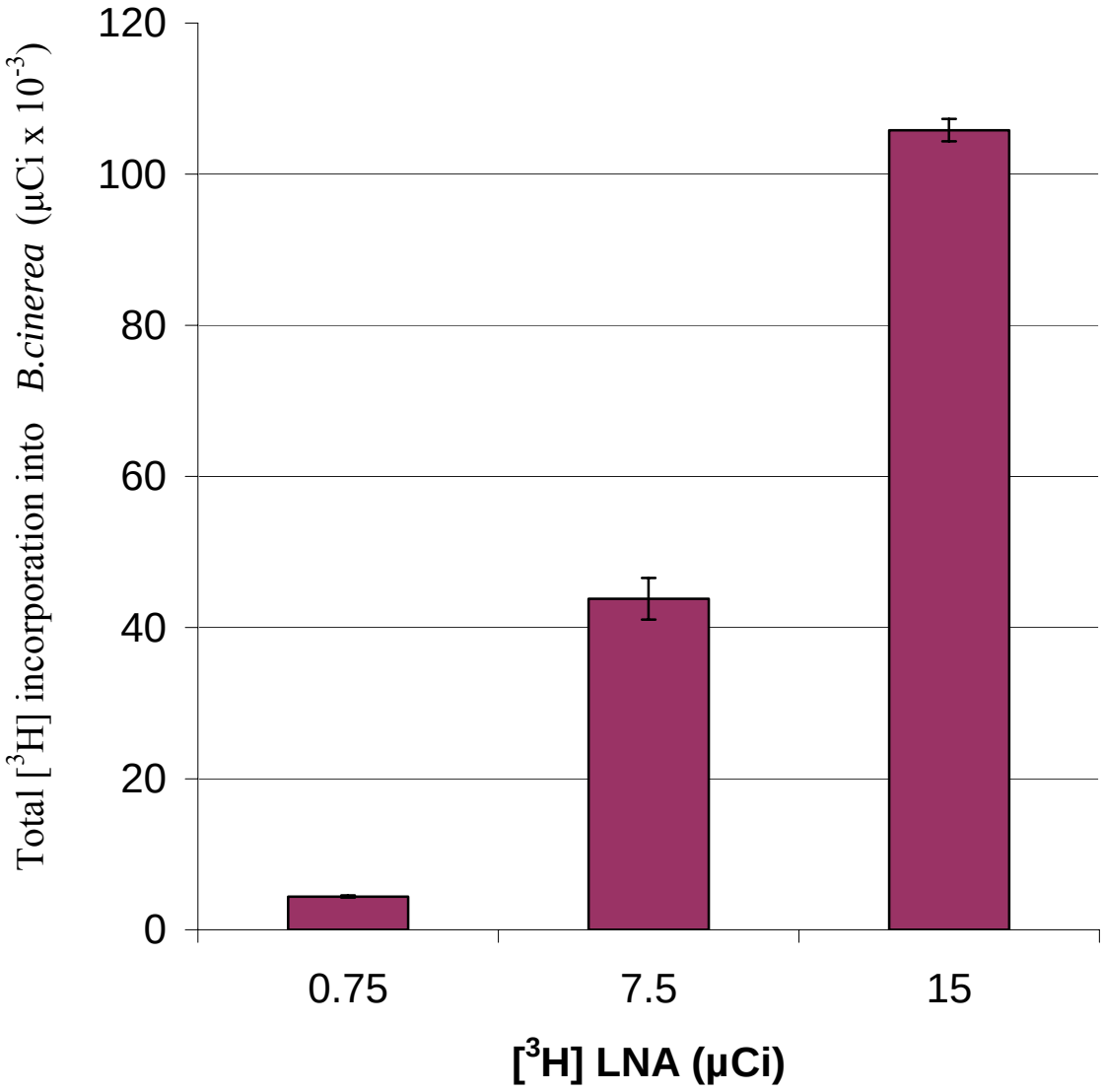


Figure 3.5 Total radiolabel from [^{3}H]-labeled volatiles incorporated into *B. cinerea*. Total incorporation of radiolabel into *B. cinerea* was measured from all fractions obtained from a 14 day-old PDA culture. Bars indicate standard errors of 3 independent experiments.

Table 3.3 Radiolabel in protein from conidia or mycelia of *B. cinerea* after exposure to [^{3}H] C_6 aldehydes

	Total FW (mg)	Total protein (μg)	μCi x 10^{-6}	μCi x 10^{-6}/mg FW	μCi x 10^{-6}/μg protein
Conidia	42 ± 6	55 ± 9	2228 ± 300	53 ± 2	41 ± 7
Mycelia	501 ± 22	434 ± 31	4151 ± 242	8 ± 1	10 ± 1

The radiolabel was recovered from tissue after extensive gentle washing of conidia and mycelia for removal of external radiolabel. Total proteins were extracted by boiling fungal tissues with SDS sample buffer. Data shown are means ± standard errors of 3 replications. FW stands for fresh weight.

Subcellular distribution of C_6 aldehydes in *Botrytis cinerea*

Protein from different components of fungal tissue was fractionated, and radiolabel of each fraction was measured. Protein was obtained from wash, membrane, cytosol, and cell wall (Fig. 3.6). Although it was not tested whether some proteins in a fraction might carry over into other fractions, the protein profiles of the fractions on SDS-PAGE differed from each other, indicating that cross-contamination of each fractionation was minimal. The majority of the radiolabel was detected in the wash fraction (Table 3.4), followed by the membrane and the cytosolic and cell wall fractions.

To determine whether increasing amounts of vapor phase C_6 aldehydes to stimulatory and inhibitory levels can increase internal radiolabel, increasing amounts of non-radiolabeled C_6 aldehydes were used. When 5.4 μmol of non-radiolabeled *t*-2-H was used, radiolabel in wash and cell wall fractions decreased by approximately 50% but a subsequent decrease was not observed at the higher amounts, 85.6 and 428.0 μmol (Table 3.4). Radiolabel did not change in membrane and cytosolic fractions in the presence of 5.4 μmol of non-radiolabeled *t*-2-H, but it decreased at the higher concentrations, 85.6 and 428.0 μmol.

Changes in radiolabel were also observed when non-radiolabeled *c*-3-H was used (Table 3.4). Radiolabel in wash and cell wall protein extracts was not changed in the presence of 5.4 μmol of non-radiolabeled *c*-3-H, but levels decreased as the amount of non-radiolabeled *c*-3-H increased. Radiolabel in the membrane plus cytosolic protein extracts decreased by approximately 50% in the presence of 5.4 μmol of non-radiolabeled *c*-3-H and continued to decrease as amount of non-radiolabeled *c*-3-H increased. The results obtained in the presence of non-radiolabeled *c*-3-H were comparable to those in

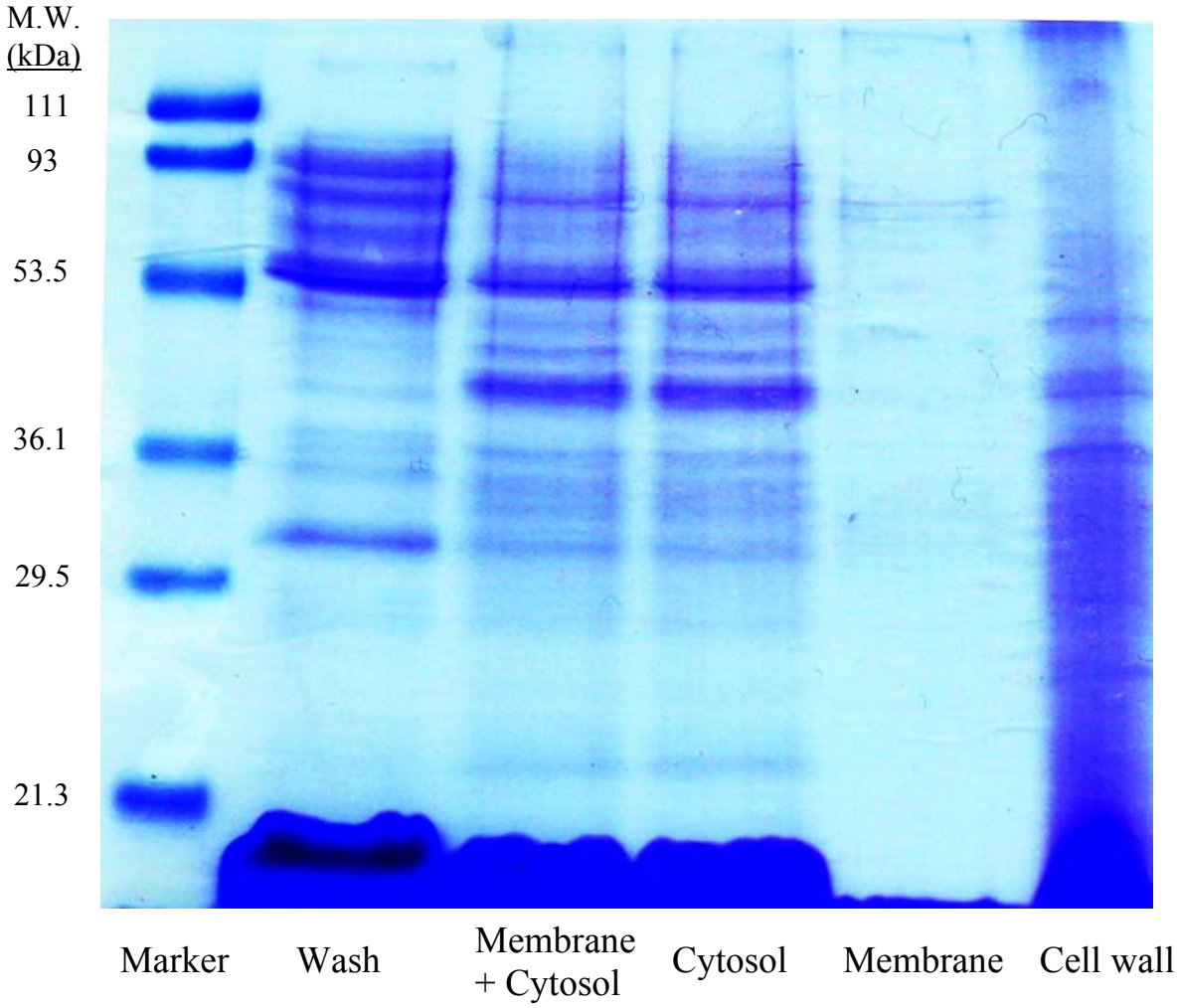


Figure 3.6 Profile of proteins on SDS-PAGE from different components of *B. cinerea*. Positions of prestained protein marker (Low Range, Bio-Rad) indicate molecular weight (M.W.) standards. Protein load from different fractions: Wash (25 µg), Membrane + Cytosol (25 µg), Cytosol (25 µg), Membrane (5 µg), and cell wall (25 µg).

Table 3.4 Subcellular distribution of label from [^{3}H] C_6 aldehydes in protein extracts of *B. cinerea* in the absence or presence of increasing amounts of non-radiolabeled *trans*-2-hexenal (*t*-2-H) and *cis*-3-hexenal (*c*-3-H)

	[^{3}H] C_6 aldehydes (μCi x 10^{-3}/fraction)						
		Non-radiolabeled *t*-2-H (μmol)			Non-radiolabeled *c*-3-H (μmol)		
	0	5.4	85.6	428.0	5.4	85.6	428.0
Wash	39.7 ± 3.8	19.1 ± 5.5	25.2 ± 10.5	15.8 ± 9.5	44.5 ± 4.8	20.8 ± 1.9	8.8 ± 4.6
Cell wall	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
Membrane + Cytosol	1.5 ± 0.4	1.4 ± 0.7	1.0 ± 0.3	0.4 ± 0.1	0.8 ± 0.2	0.1 ± 0.0	0.1 ± 0.0

[^{3}H] Linolenic acid at 7.5 μCi (62.5 pmol) was used to generate [^{3}H] C_6 aldehydes. Approximately 500 mg of mycelial tissue was collected from *B. cinerea* on a PDA medium. The radiolabel was measured from the same amount of protein per each fraction of wash (330 μg), cell wall (34 μg), and membrane plus cytosol (400 μg) across non-radiolabeled aldehyde concentrations. Data shown are means ± standard errors of 3 replications.

the presence of non-radiolabeled *t*-2-H. Overall, non-radiolabeled *t*-2-H at 5.4 μmol seemed to exhibit a lower competition for membrane plus cytosolic proteins but a higher competition for wash and cell wall proteins than non-radiolabeled *c*-3-H at the same amount.

Mode of interaction of C_6 aldehydes with *Botrytis cinerea*

Radiolabel in the wash, cell wall, and membrane plus cytosol fractions was not greatly reduced during a 24 hr air exposure following aldehyde exposure, indicating that the radiolabeled C_6 aldehydes did not easily evaporate (Table 3.5). Radiolabel in the wash protein fraction was greatly reduced after ultrafiltration, indicating that radiolabeled C_6 aldehydes passed through the size-exclusive membrane filter of Centricon YM-3. Radiolabel in the membrane and the cytosolic protein fractions was not greatly reduced after ultracentrifugation seperation of the membrane plus cytosolic protein fraction (data not shown). It appears that physical force does not affect the stability of adducts. However, most of the radiolabel in the cell wall and cytosolic protein fractions was lost during acetone precipitation, suggesting that the loss is due to a weak interaction of C_6 aldehyde to proteins.

Table 3.5 Free [^{3}H] C_6 aldehydes and binding of [^{3}H] C_6 aldehydes to wash, cell wall, membrane, and cytosolic proteins of *B. cinerea*

	Wash			Cell wall			Membrane + Cytosol		
Methods	Total radiolabel (μCi x 10^{-3})	Protein (μg)	μCi /g	Total radiolabel (μCi x 10^{-3})	Protein (μg)	μCi /g	Total radiolabel (μCi x 10^{-3})	Protein (μg)	μCi /g
Crude extract	39.7 ± 3.8	303.0 ± 28.1	131.0 ± 13.4	0.5 ± 0.1	NA	NA	1.5 ± 0.4	400.0 ± 25.4	3.8 ± 1.6
24h air exposure	35.3 ± 3.5	301.0 ± 28.0	117.3 ± 11.9	0.5 ± 0.1	NA	NA	1.5 ± 0.4	395.0 ± 25.5	3.8 ± 1.6
Ultrafiltration	0.4 ± 0.1	255.9 ± 23.5	1.7 ± 0.6						
Acetone precipitation				ND	34.0± 5.1	ND	0.2 ± 0.2[a]	240.9 ± 15.6[a]	0.7 ± 0.7[a]

Approximately 500 mg of mycelial tissue was collected from *B. cinerea* on a PDA medium. ND, none detected. NA, not available.

[a]The levels were determined from the cytosolic protein fraction after ultracentrifugation and acetone precipitation. 7.5 µCi (62.5 pmol) of [^{3}H] linolenic acid was used to generate [^{3}H] C_6 aldehydes.

Discussion

In vivo labeling with [U-^{14}C] sucrose to produce linolenic acid in *Lemna gibba*

One of objectives of this study was to generate radiolabeled *t*-2-H to characterize the interaction of *B. cinerea* and *t*-2-H. Free acetate has been found superior to pyruvate and other substrates as a precursor of fatty acid synthesis (Ohlrogge and Browse, 1995). Hurtubise *et al*. (1992) measured a maximum 3% incorporation of acetate into fatty acids, and Zimafuala *et al*. (1997) obtained less than 1% acetate incorporation into fatty acids. The low incorporation of free acetate into fatty acids was due to pre-existing non-radiolabeled acetate. In this study, the radiolabel incorporated into fatty acids from [U-^{14}C] sucrose was approximately 5% of total radiolabel (data not shown). The result indicates that use of [U-^{14}C] sucrose can generate an amount of radiolabeled fatty acids comparable to that from acetate. The specific radiolabel of 18:3 was estimated to be 130 nCi/mg when 12.5 μCi of [U-^{14}C] sucrose was used (Table 3.1). Vick and Zimmerman (1983), who used flax embryos, obtained a uniformly labeled 18:3 with a specific activity of 1.4 μCi/mg. The low specific radiolabel in our study may be due to low incorporation in relation to pool size of lipids (less than 10% of total dry weight). Incorporation of sucrose into fatty acids can be affected over time due to incorporation into other metabolites such as proteins, carbohydrates, etc, limiting its use for fatty acids.

The lower specific radiolabel may also be due to senescence of fronds. The whole population should be harvested before the onset of senescence, because fatty acids are not accumulated and galactolipids are degraded during senescence (Hurtubise *et al*., 1992). Yellowing of fronds was not detected in this experiment. Nonetheless, mother fronds may

senesce with flower induction in daughter fronds, and a decrease in the ratio of 18:3/18:2 may occur in connection with enhanced frond senescence (Landolt and Kandeler, 1987). It is also possible that C-18 unsaturated fatty acids were further metabolized to oxylipins including C_6-aldehydes before harvest (Hatanaka *et al*., 1978).

The specific radiolabel of labeled 18:3 proportionally increased with the [U-^{14}C] sucrose concentration (Table 3.1). This suggests that 18:3 with higher specific activity can be obtained by increasing the specific activity of the source [U-^{14}C] sucrose in the medium, up to a maximum level at which growth is normal. Vaughan *et al*. (1994) found that *L. gibba* with high specific activity was produced where growth was least. Taken together, it could be possible to obtain fatty acids and 18:3 with highest specific radiolabel immediately after most of the [U-^{14}C] sucrose is taken up by *L. gibba*. The results show the possibility of culturing *L. gibba* on [U-^{14}C] sucrose to elucidate biosynthetic or metabolic pathways of fatty acids.

In vitro C_6 aldehyde production from linolenic acid

In plants, volatile compounds such as C_6 aldehydes, alcohols, and acetates are produced from LNA by 13-LOX and 13-HPL (Hatanaka *et al*., 1987). In this study, the profile of volatile compounds could reflect the volatile characteristics of *c*-3-H. High *c*-3-H and low *t*-2-H production was detected with only trace amount of alcohols at 2 hr after incubation (Fig. 3.4), indicating that some of the *c*-3-H produced from LNA is emitted into air from the reaction solution due to its high volatility, but a part of the *c*-3-H produced is converted to *t*-2-H. It is possible that *c*-3-H can easily escape into the air. This may differ from a biological system, if trapping in a lipid-protein particle within

functional bilayer cell membranes increases the chances of isomerization to *t*-2-H and further metabolism to *t*-2-hexenol. Hudak and Thompson (1997) and Riley and Thompson (1997) suggested that *t*-2-H was accumulated to some extent in cytosolic lipid-protein particle blebbing from the cell membrane surface in carnation petal and tomato fruit, even though evidence that *c*-3-H is localized in a lipid-protein particle has not been available due to its rapid isomerization. It should be noted that total yield of C_6 aldehydes did not change during the period of measurement with slow isomerization of *c*-3-H to *t*-2-H (Fig. 3.4). It was reported that *t*-2-H could arise from *c*-3-H, a possibly spontaneous rearrangement of *c*-3-H (Hamilton-Kemp *et al*., 1996). Once *t*-2-H is emitted into air, *t*-2-H would not be isomerized to *c*-3-H because *t*-2-H is more stable than *c*-3-H. As C_6 aldehyde production from LNA by various sources of LOX and HPL in different incubation systems was completed within 1-4 hrs after incubation (Berger *et al*., 1986; Whitehead *et al*., 1995; Noordermeer *et al*., 2002), it is unlikely that the increased vapor phase concentration of *t*-2-H with decreased vapor phase concentration of *c*-3-H over the period is due to continuing production of *t*-2-H from *c*-3-H from the reaction mixture. The total vapor phase concentration of C_6 aldehydes at 2 and 18 hr after incubation (Fig. 3.4) likely reflects the maximum amount of C_6 aldehydes which could be obtained. Although it is unknown whether evaporation of *c*-3-H from the reaction mixture occurs more rapidly than metabolism to *t*-2-H in the reaction solution, the amount of *c*-3-H should have not decreased over the period if *t*-2-H was continuously produced by LOX and HPL in the reaction mixture and emitted into air and no spontaneous rearrangement of *c*-3-H to *t*-2-H in air occurred. In addition, it is also possible that *c*-3-H in the air may interact with an isomerization factor on the surface of the reaction mixture. Once *c*-3-H

has evaporated, it may not contact an isomerization factor with a high frequency, so rapid conversion of *c*-3-H may not occur. Taken together, a biochemical process is likely to occur in the C_6 aldehyde-producing system used in this study, i.e., the reaction is completed within 2 hr and *c*-3-H emitted slowly isomerizes to *t*-2-H by spontaneous or enzymatic reaction, resulting in altered ratio of *t*-2-H to *c*-3-H over the period.

Regarding the efficiency of the C_6 aldehyde-producing system, the total yield of C_6 aldehydes shown as a 25% conversion of LNA to C_6 aldehyde (Fig. 3.4) suggests two biochemical events occurred in the reaction *in vitro* during incubation. One is that the conversion of LNA to 13-HPOT is relatively low, leading to a lower yield of C_6 aldehydes. A direct measurement of 13-HPOT concentration produced in the system could be inaccurate because 13-HPOT is a very reactive compound (Noordermeer *et al*., 2001) and can be easily oxidized. Indeed, we were able to obtain only 15% yield of 13-HPOT from LNA using soybean LOX extracts (Chapter 2, Materials and Methods). Therefore, it is difficult to prove whether the synthesis of 13-HPOT from LNA was efficient in this system. The other is that LNA may be efficiently converted to 13-HPOT and then C_6 aldehydes, but C_6 aldehydes may not be volatilized. Since more than 90% of 13-HPOT may have been subject to turnover by HPL during incubation (Noordermeer *et al*., 2002), the low yield of C_6 aldehydes produced in this study suggests the potential for interacting with proteins, preventing evaporation. The C_6 aldehydes are regarded as highly reactive compounds because they have two functional groups, an aldehyde group and a double bond, which can readily react with an amine or sulfhydryl group on a protein (Baker *et al*., 1999; Ichihashi *et al*., 2001). Since LOX and HPL extracts were in the reaction mixture, the C_6 aldehydes produced could interact with those proteins,

preventing C_6 aldehyde emission into the air. Nevertheless, the results emphasize that C_6 aldehydes were consistently produced from LNA by LOX and HPL extracts, although C_6 aldehyde production was only 25% of the LNA utilized.

Incorporation of radiolabeled C_6 aldehydes into *Botrytis cinerea*

The concentration-dependent increase in incorporation of radiolabeled C_6 aldehydes into *B. cinerea* (Fig. 3.5) could be driven by diffusion, by an uptake mechanism, or both. Since diffusion is down a concentration gradient, saturation would not be seen until an equilibrium at higher concentrations of C_6 aldehydes. Alternatively, if there was an active uptake mechanism by *B. cinerea*, it is possible that a receptor for C_6 aldehydes may exist in *B. cinerea*. If true, the incorporation would be increased with an association rate constant until binding of C_6 aldehydes to a receptor approaches equilibrium. It should be noted that a concentration dependence of radiolabel recovered on PDA medium without *B. cinerea* was not detected (data not shown), indicating that presence of a mass of hyphae provides a site for C_6 aldehyde interaction.

As shown in Table 3.3, greater incorporation of C_6 aldehydes into conidia than mycelia per mg FW and μg protein was observed. Since mycelia comprised more than 90 % of the total mass of *B. cinerea* on PDA medium, a substantially greater incorporation of radiolabel should have been detected on mycelia. Previously, it was reported that spore germination was more sensitive to *t*-2-H concentration than was mycelial growth (Fallik *et al*., 1998). In addition, spores were more resistant toward toxic substances than mycelia of *Penicillium digitatum* except for acetaldehyde (Wolken *et al*., 2002). The susceptibility of spores to C_6 aldehydes, along with our results (Table 3.3) indicates that

conidia have more sites for uptake and binding of C_6 and other aldehydes. Conidia of *B. cinerea* had more protein per FW than mycelia, 1.3 µg/mg vs 0.9 µg/mg, respectively. The protein thiol content was 25% higher in conidia than in mycelia of *Neurospora crassa* (Brody *et al*., 1983) and thiols can react with aldehydes, suggesting the possibility of greater binding potential to greater protein. Therefore, higher conidial protein content and greater binding capacity of that protein may have led to the greater radiolabel in that fraction.

We could not detect any radiolabel from C_6 aldehydes in the lipid fraction of *B. cinerea* (Table 3.3); it was only in the protein fraction. This suggests that hydrophobicity may not be a critical factor affecting the reactive property of C_6 aldehydes. This is in agreement with Niknahad *et al*. (2003) who indicated that cytotoxicity of alkenals was related to their electrophilicity, not their hydrophobicity. Previously, Haynes *et al*. (2000) also suggested that hydrophobicity is not closely related to toxicity of aldehydes. In addition, the presence of an α,β-unsaturated bond adjacent to the carbonyl moiety has been shown to be involved in antifungal and antibacterial activity (Andersen *et al*., 1994; Trombetta *et al*., 2002). Therefore, the results support the concept that electrophilicity of C_6 aldehydes contributes to a reaction with proteins via Michael addition or Schiff base formation. It should be noted that *t*-2-H can react with proteins via irreversible or reversible Michael addition or reversible Schiff base reactions, while *c*-3-H can interact with proteins only via Schiff base reactions because of absence of the α,β-unsaturated bond in the latter compound. However, hydrophobic interaction is necessary for C_6 aldehydes to interact with or penetrate through hydrophobic surfaces and membranes of *B. cinerea*. The lipophilic nature of C_6 aldehydes may result in accumulation of

radiolabel in membrane without covalent linkage, causing loss of radiolabel in the lipid fraction during extraction procedures. This process can allow penetration of C_6 aldehyde into the phospholipid matrix of the plasma membrane, resulting in a disruption of membrane structure and a subsequent release of C_6 aldehyde into the cytosol. The free C_6 aldehyde could interact with proteins in the cytosol, and would not be extracted from the lipid fraction.

Site and mode of interaction of C_6 aldehydes with *Botrytis cinerea*

Once C_6 aldehydes are generated, they must interact with *B. cinerea* to induce biological responses, such as the previously observed stimulation or inhibition of growth (Fallik *et al*., 1998). Aldehydes including *t*-2-H can penetrate through membranes and interact with intracellular components by mediating significant changes in cellular membrane permeability (Trombetta *et al*., 2002). In this study, our data clearly demonstrate that C_6 aldehydes penetrated the fungal tissue (Table 3.4, Table 3.5). Surprisingly, the distribution of radiolabel recovered from *B. cinerea* indicates that approximately 95% of C_6 aldehydes did not penetrate through the fungal tissue while only 5% of C_6 aldehydes were internally located. Assuming that the concentration of C_6 aldehydes produced from LNA was too low to be detected in the fungal tissue, we speculated that use of non-radiolabeled C_6 aldehydes to increase haedspace concentration might increase overall membrane permeability into the fungal tissue. Thus, more radiolabeled C_6 aldehydes would be detected inside the tissue, as *t*-2-H was shown to damage the fungal cell membrane and wall (Fallik *et al*., 1998). However, no increase of radiolabel in cell wall or membrane and cytosolic fractions was detected when *B. cinerea*

was exposed to non-radiolabeled C_6 aldehydes at 5.4 and 85.6 μmol for 2 hrs prior to the exposure of fungus to radiolabeled C_6 aldehydes (data not shown). As described above, our results indicate that only 0.6% of radiolabeled C_6 aldehydes interacted with *B. cinerea* (Table 3.2) and 5% of the resulting 0.6% was internal tissue-associated (Table 3.4). If *t*-2-H and *c*-3-H generated in this system were equally interacting with proteins, 0.3% of C_6 aldehydes would be *t*-2-H. Given that the vapor-phase amount generated from 5.4 and 85.6 μmol of liquid *t*-2-H, which stimulated and inhibited the growth of *B. cinerea* (Fallik *et al*., 1998), would be estimated to yield 24.0 and 115.2 μmol, respectively, the amounts of *t*-2-H associated with *B. cinerea* would drop to 72.0 and 345.6 nmol, and only 3.6 and 17.2 nmol would be associated with internal tissue to stimulate and inhibit the growth of *B. cinerea*, respectively. It is interesting to note that C_6 aldehydes were detected from the cell wall protein fraction, suggesting that it is possible C_6 aldehydes interact with cell wall proteins. However, no evidence of interaction of C_6 aldehydes with cell wall protein has been reported elsewhere. The observation that C_6 aldehydes interact with cell wall protein emphasizes a potential role of wound-induced C_6 aldehydes in pathogenesis during penetration.

Proteins recovered in the wash fraction would be secreted from mycelia of *B. cinerea*. *B. cinerea* secretes cuticle and cell wall-degrading enzymes (Staples and Mayer, 1995). Since proteins which are secreted into the medium are thought to be involved in hydrolysis of cuticle, along with adhesion to the infection site, interaction of C_6 aldehydes with the secreted proteins, those found in the wash solution, could modify the proteins and affect the growth of *B. cinerea*. Protein modification at sufficient levels may cause loss of activity, resulting in a subsequent loss of infection progress. Alberti-Segui

et al. (2004) showed that absence of glycosidase activity, a secreted protein from yeast, reduced adherence to target cells and delayed colonization. Inactivation of $NADP^+$-isocitrate dehydrogenase occurred upon incubation with 4-hydroxynonenal via post-translational modifications, in which the aldehyde binds to a cysteine residue near the substrate's binding site (Benderdour *et al*., 2003). However, it is not known whether C_6 aldehydes can modify and affect the activity of secreted proteins.

Although the ratio of *t*-2-H to *c*-3-H of radiolabeled C_6 aldehydes produced from LNA likely varied over the incubation time, radiolabel was from either *t*-2-H or *c*-3-H as determined using non-radiolabeled LNA. Our data demonstrate that both non-radiolabeled *c*-3-H and *t*-2-H competed with radiolabeled C_6 aldehydes, reducing radiolabel in each fraction as non-radiolabeled amount increased (Table 3.4). The results were not surprising given that C_6 aldehydes are electrophiles, which can react with proteins. The results indicate that the binding of radiolabeled *t*-2-H to wash and cell wall proteins was reduced by non-radiolabeled *t*-2-H at higher amounts, but the low amount of non-radiolabeled *t*-2-H did not affect interaction of radiolabeled *t*-2-H to membrane plus cytosol fractions. It seems that *t*-2-H showed higher affinity for membrane plus cytosolic proteins while *c*-3-H showed higher affinity for wash and cell wall proteins. The existence of radiolabel in the presence of the highest amount (428 μmol) of non-radiolabeled C_6 aldehydes may be related to the abundance of interacting proteins where the concentration of the interacting proteins may be the determining factor affecting endogenous levels of C_6 aldehydes. It is noted that the existence of radiolabel in the presence of 428 μmol of *t*-2-H or *c*-3-H could reflect the existence of *c*-3-H-protein forms or *t*-2-H-protein forms, respectively, or their metabolites, such as alcohols and esters. If

free volatile C_6 aldehydes existed in the fractions, they should have evaporated (Table 3.5).

The radiolabel present after air exposure demonstrates that C_6 aldehydes retained in whole fractions corresponded mainly to either protein-bound forms or other non-volatile metabolites (Table 3.5). Myers *et al*. (1995) found a reduced content of ligand-binding forms after ultrafiltration, similar to this study, suggesting that proteolysis or chemical degradation of adducts can occur. Since only 15% of wash proteins were lost after ultracentrifugation, along with 99% loss of radiolabel (Table 3.5), the C_6 aldehydes-protein forms were likely unstable, suggesting that C_6 aldehydes interact with wash proteins via non-covalent or reversible covalent bonds. The loss of radiolabel from cell wall and cytosolic fractions supports the possibility that C_6 aldehydes do not interact with cell wall and cytosolic proteins via irreversible covalent bonds, as determined by acetone precipitation under conditions of denaturation (Table 3.5) where 100% and almost 90% loss of radiolabel was observed in cell wall and cytosolic protein fractions, respectively. Studies on protein modification by 2-alkenals have been done using mammalian cells *in vivo* or using known proteins *in vitro*. Even though the reactive aldehydes are considered to covalently modify proteins, Ichihashi *et al*. (2001) suggested that the aldehyde-protein adduct may be readily reversed in aqueous solution. With regard to the modification, Baker *et al*. (1999) suggested that the protein N-terminus might play a minor role in protein modification under native conditions because one N-terminus exists in a protein compared to many lysine and histidine residues. Therefore, our results indicate a mechanism in which C_6 aldehydes can form adducts by reversible binding to proteins. The reversible interaction of aldehydes with fungal proteins implies that aldehydes form

an intermediate, which may or may not proceed to completion of the Michael addition reaction or may proced to reversible Schiff base reaction. In this case, the aldehydes may bind in a pocket of a protein where a lysine or a cysteine residue is located and may exist as a reversible aldehyde-protein complex. However, how proteins may interact with aldehydes is currently unknown

It is possible that the aldehydes may be scavenged by proteins such as glutathione *S*-transferase (GST) and alcohol dehydrogenase and metabolized into forms, such as alcohols or esters, which are not capable of interacting with proteins. It is well known that α,β-unsaturated aldehydes are effective inducers of GST, which is involved in the detoxification of substrates including α,β-unsaturated aldehydes in plants (Vollenweider *et al*., 2000; Fujita and Hossain, 2003), even though a decisive role of GST has not been reported from the fungus. Alcohol dehydrogenase is known to be responsible for *t*-2-H metabolism to alcohol in cytosol (Niknahad *et al*., 2003) and *cis*-3-hexenyl acetate was biologically inactive; thus, acetylation of the alcohols causes inactivation, rapid turnover, and subsequent loss of activity (Farag *et al*., 2005). The possibility that aldehydes bind to proteins covalently cannot be ruled out. In that case, the modified protein could be degraded and aldehydes remain attached to the degraded peptide fragments, making them non-detectable in cell wall and cytosolic protein fractions after acetone precipitation (Table 3.5). Unfortunately, we were unable to find adduct forms using SDS and native PAGE because radiolabel produced in this study was below the detection limit of Phosphorimager. It may be possible to identify proteins interacting with C_6 aldehydes if a higher radiolabeled LNA is utilized.

It is of great interest to study how C_6 aldehydes, emitted from plant tissues in response to wounding, are distributed to induce a biological response of plants and fungi. Even though interaction of C_6 aldehydes with *B. cinerea* in this study was conducted in a closed system, localization of C_6 aldehydes in the fungus in an open system, such as in a greenhouse or field, would not likely be different. As mentioned above, our results indicated that only 0.6% of C_6 aldehydes interacted with *B. cinerea* (Table 3.2) and 5% of the resulting 0.6% became associated with internal fractions (Table 3.4). Even though little information on absorption or reabsorption of wound-induced C_6 aldehydes by a damaged plant itself or neighboring organisms is available from plant or fungus, the capacity of strawberry fruit (Hamilton Kemp *et al*., 1996) and *B. cinerea* in this study to metabolize aldehydes would argue that organisms may have developed their own strategies for absorbing and responding to C_6 aldehydes in the air to elicit an advantageous defense system for protecting themselves. Roles of volatile signaling between plant and plants, pathogens, and insects have been reviewed (Bruin and Dicke, 2001; Baldwin *et al*., 2002). Therefore, the significance of fungal protein interaction with C_6 aldehydes remains elusive but cannot be ignored.

In conclusion, using a procedure designed to produce *t*-2-H from [9,10,12,13,15,16-^{3}H] LNA with LOX and HPL extracts, we were able to synthesize the C_6 aldehydes *in vitro*. This is the first study to demonstrate the subcellular localization and possible mode of interaction of C_6 aldehydes with *B. cinerea*. Our results demonstrate that the majority of C_6 aldehydes remained on proteins on the surface of the fungal tissue but C_6 aldehydes could penetrate the tissue and be localized in the cytosol. C_6 aldehydes were more

effectively incorporated into conidia than mycelia and this may be related to the high sensitivity of the former to the volatile compounds.

CHAPTER FOUR

EFFECTS OF *trans*-2-HEXENAL ON SPORE GERMINATION AND PROTEINS OF *BOTRYTIS CINEREA*

Introduction

Botrytis cinerea causes a decay of strawberry, table grape, apple, and other fruits called gray mold. The fungal disease in strawberry is usually controlled by preharvest fungicide applications (Blacharski *et al*., 2001). However, since there has been increasing concern of fungicide residues, fungicide regulation for use on fresh produce, and fungicide resistance (Diánez *et al*., 2002), use of natural compounds has been regarded as a possible alternative. This is especially true for postharvest use where no chemicals are currently used. Several natural volatile compounds including aldehydes, acetate esters, alcohols, and terpenes have been tested for their antifungal effects (Archbold *et al*., 1999; Filonow, 2001; Harvey *et al*., 2002; Wolken *et al*., 2002; Chitarra *et al*., 2004).

It is well established that volatile compounds are ubiquitous in plants and contribute aroma and flavor characteristics. *trans*-2-Hexenal (*t*-2-H) is among the volatile compounds released from damaged tissue through the lipoxygenase (LOX) and hydroperoxide lyase pathway. It has been suggested that the wound volatile compounds may play a functional role in plant-pathogen interactions (Hamilton-Kemp *et al*., 2000). A vapor phase concentration from 5.4 μmol of *t*-2-H stimulated mycelial growth of *B.*

cinerea, while a vapor phase concentration from 85.4 μmol of *t*-2-H inhibited mycelial growth when the test was performed in a closed Petri dish (Fallik *et al*., 1998). However, the mechanism of *t*-2-H interaction with *B. cinerea* is not clear. Therefore, the goals of this study were to examine the effect of exposure of different concentrations of *t*-2-H on spore germination and mycelial protein expression of *B. cinerea* to further our understanding of the mode of action of *t*-2-H in the vapor phase.

Materials and Methods

Microphotograph of fungal spore germination

Spores were obtained from 14-day cultures isolated and managed by the methods described above (Chapter 3). A spore suspension and *t*-2-H solution were prepared according to Fallik *et al*. (1998). Aliquots (80 μL) of spore suspension (10^4 spore/mL) were streaked on a 5 cm petri dish containing 2% water agar and this dish was placed in 9 cm-diameter petri dish, described previously by Hamilton-Kemp *et al*. (1992). The spores were exposed to 10 μL of two different concentrations, 5.4 and 85.6 μmol of *t*-2-H dissolved in propylene glycol or a control (propylene glycol only) placed in a separate 1 cm-diameter glass vial within the 9 cm-diameter petri dish. The 9 cm-diameter petri dish was immediately wrapped in Parafilm and placed in an incubator at 22 ˚C for 24 h. The germinated spores were photographed after 24 hr using Stemi SV11 (Zeiss, Germany) equipped with a digital camera and computer-aided image analysis was performed, by using software (SigmaScan Pro, SPSS Inc., Chicago, IL).

Two-dimensional (2-D) gel electrophoresis and image analysis of proteins

Mycelial tissue (500 mg) was harvested from fungal cultures in the absence or presence of *t*-2-H (5.4 or 85.6 μmol) after 24 h and cytosolic protein samples of the tissue were prepared as described in Chapter 3. The samples precipitated with acetone were solublized in 100 μL of a lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris). After determination of protein concentration according to the method of Bradford (1976), 125 μL of rehydration buffer containing 8 M urea, 2% CHAPS, 18 mM dithiothreitol (DTT), 2% pH 3-10 ampholytes (Bio-Rad), and a trace of bromophenol blue was added to the protein sample (100 μg), which was centrifuged at 12,000 g for 2 min. The resulting soluble protein was added to a focusing tray (Amersham Bioscience, Piscataway, NJ) followed by loading on a 7 cm-long dry IPG strip (Bio-Rad, Hercules, CA). The strip was covered with mineral oil and left overnight for rehydration. The strip was removed from the tray and excessive oil was rinsed with water. Isoelectric focusing was performed on a Multiphor II (Amersham Bioscience) at 20 ˚C by running the program: 200 V for 1 min, 250 V for 2 min, 500 V for 10 min, increased by 250 V per 10 min up to 2750 V, and 3000 V for 1 hr 30 min. The immobilized strip was equilibrated in equilibration buffer (1.5 M Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 35% glycerol) with 1 % DTT for 15 min and then in the equilibration buffer with 2% iodoacetamide for 15 min. The equilibrated strip was laid on 12% SDS-PAGE, and 1% agarose solution (1% agarose, 0.1% SDS, and 0.125 M Tris, pH 6.8) was poured to join the strip to the top of SDS-PAGE. A second dimension run on SDS-PAGE was performed according to standard protocol (Bio-Rad). The 2-D gels were stained with SYPRO Ruby (Bio-Rad) and images of the gels were produced by fluorescence-based scanning using Storm 860 and ImageQuant software

(Molecular Dynamics, Sunnyvale, CA). The images produced were processed and analyzed using PDQuest 7.2 (Bio-Rad). Each control and treatment was carried out three times and the resulting triplicate gels were used for statistical analysis.

Trypsin digestion and MALDI Mass spectrometry (MS) analysis of proteins

Protein spots of interest were excised from the stained gels and digested with trypsin. The gel pieces were washed in 50 mM ammonium bicarbonate and 50% methyl cyanide, dried under vacuum for 20 min, then reduced with 10 mM DTT at 56 ˚C for 30 min, and alkylated with 50 mM iodoacetamide at room temperature in the dark for 30 min. After washing twice with 50 mM ammonium bicarbonate, the gel was dehydrated in 50% methyl cyanide and then dried under vacuum. A 10 μL trypsin solution (10 ng/ul Promega modified trypsin in 50 mM ammonium bicarbonate) was added to the gel on ice. After a 60 min rehydration, the remaining supernatant was removed. Then, 5 μL of 50 mM ammonium bicarbonate was added and digestion was allowed to proceed at 37 ˚C overnight. Peptides were extracted with two additions of 10-15 μL of 0.1% trifluoroacetic acid (TFA) and sonication for 10 min. Supernatants were combined and the volume of supernatant was reduced to about 10 μL under vacuum.

A 0.5 μL extracted peptide solution was mixed with 0.5 ul of matrix solution (5 mg of α-cyano-hydroxycinnamic acid in 1 mL of 0.1% TFA:methyl cyanide (1:1) and 10 mM monoammonium phosphate) and allowed to dry on the gold Matrix-Assisted Laser Desorption Ionization (MALDI) target plate. The spot was analyzed in a Ciphergen PBSIIc linear mass spectrometer (Ciphergen, Fremont, CA) at a mass range of 700-10000 Da. Spectra from 400 laser shots were averaged. Externally-calibrated masses were

submitted for database searches for similarity using the search engines ProFound and MASCOT. MALDI-Time of Flight (TOF) MS analysis was performed at Department of Biochemistry at University of Kentucky.

Results

Spore germination of *Botrytis cinerea* in the absence or presence of *trans*-2-hexenal

Spore germination was microscopically displayed to observe the germination characteristics of fungal spores in the absence or presence of *t*-2-H (Fig. 4.1). Compared to spore germination in the absence of *t*-2-H, it can be seen that spores normally germinate in the presence of 5.4 μmol *t*-2-H. However, no spore germination was observed when spores were exposed to 85.6 μmol *t*-2-H. The actual germ tube length in the presence of 5.4 μmol *t*-2-H did not differ from that in the absence of *t*-2-H even though it was observed that more than 50% of spores did not germinate in the presence of 5.4 μmol *t*-2-H (data not shown).

Comparison and analysis of proteins of *Botrytis cinerea* in the absence or presence of *trans*-2-hexenal

To characterize the effect of *t*-2-H on proteins of *B. cinerea*, the protein profiles in the absence or presence of 5.4 and 85.6 μmol *t*-2-H were analyzed using 2-D gel electrophoresis (Fig. 4.2). Similar protein patterns were reproducible in triplicate from three different preparations. Through quantitative analysis of 184 visible protein spots on

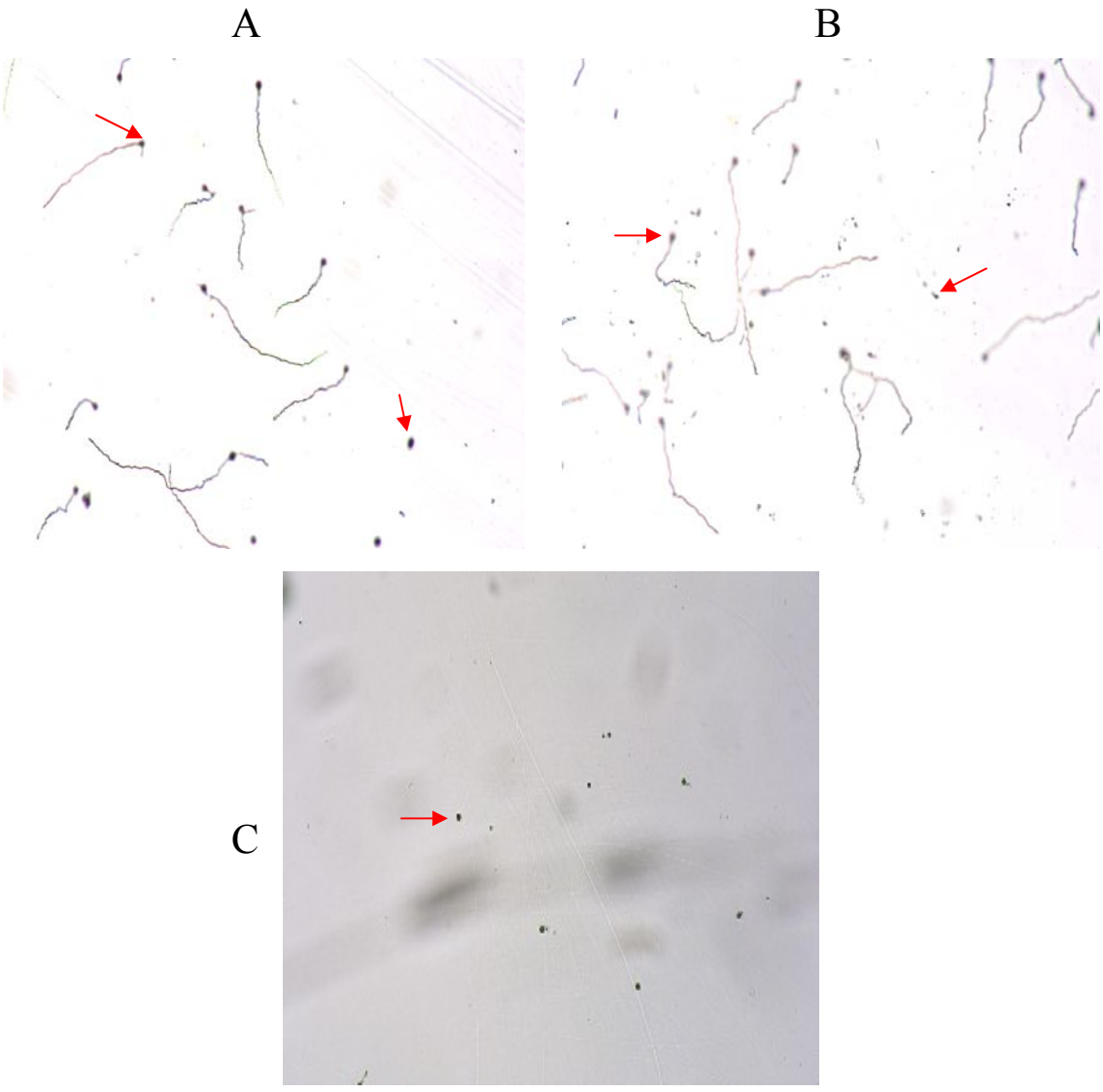


Figure 4.1 Spore germination of *B. cinerea* in the absence or presence of *trans*-2-hexenal (*t*-2-H). A. Control (No *t*-2-H), B. 5.4 µmol *t*-2-H, and C. 85.6 µmol *t*-2-H. Arrows indicate spores.

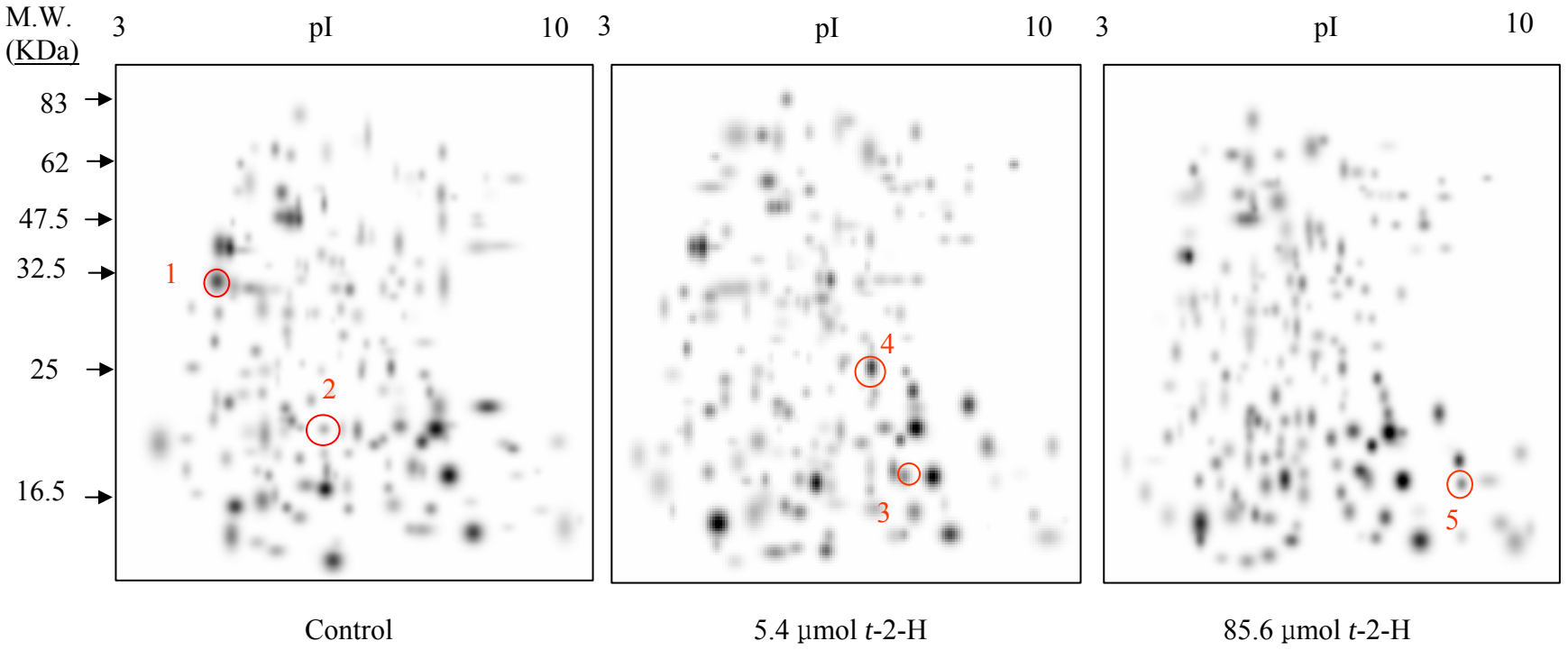


Figure 4.2 Comparisons of protein expression of *B. cinerea*, using two-dimensional gel electrophoresis, in the absence or presence of *trans*-2-hexenal (*t*-2-H). The three images were obtained from two-dimensional gel electrophoresis using image analysis software. Five protein spots in circles showed significant changes in intensity. Each number next to a circle matches the corresponding number of a spectrum shown in Fig. 4.3. Gels were reproducible three times and subjected to image analysis for statistics.

each gel, compared to controls, it was revealed that *t*-2-H clearly changed the intensity of protein expression of about one third to one half of the proteins (Table 4.1). Protein expression was up- or down-regulated in the presence of *t*-2-H. Of the 184 proteins, 15 and 18 proteins increased more than 4-fold, but 10 and 14 proteins decreased, in the presence of 5.4 and 85.6 μmol *t*-2-H, respectively. Significant intensity changes in proteins caused by 5.4 μmol *t*-2-H were not always observed in proteins affected by 85.6 μmol *t*-2-H, nor observed to occur in the same direction, increase or decrease (data not shown). The results indicate a concentration-dependent effect of *t*-2-H on differential protein expression. However, approximately 60% of the proteins were not affected (less than two-fold change) by *t*-2-H at either concentration.

Five proteins of interest were subjected to MALDI-TOF peptide analysis (Fig. 4.3). The protein spots 1 and 2 were reduced when exposed to both amounts of *t*-2-H, 3 and 4 were increased only when exposed to *t*-2-H 5.4 μmol, and 5 were increased only when exposed to 85.6μmol. A database search did not yield any significant matches of spectra to those spots, except spot 1, due to lack of proteins of *B. cinerea* on database. The spectrum of spot 1 matched that of a glucokinase from *Salmonella typhimurium* where 4 out of the 9 peptides matched. Activity of glucokinase from *B. cinerea* was measured to determine if activity was correlated to the decrease in the presence of *t*-2-H, but the activity did not differ between control and treatments (data not shown). Efforts using an in-gel activity assay to separate glucokinase from hexokinase and RT-PCR-based cloning of glucokinase mRNA with primers designed from known fungal glucokinases were attempted (data not shown). Unfortunately, we were not able to obtain significant results. The MALDI-TOF spectra of unidentified proteins are presented in Fig. 4.3.

Table 4.1 Changes in intensity of protein expression of *B. cinerea* in the absence or presence of *trans*-2-hexenal (*t*-2-H)

Degree of intensity changes of proteins	Regulation of protein expression	Number of proteins affected by concentration of *trans*-2-hexenal	
		5.4 μmole	85.6 μmole
High (> 4 fold)	Up	15	18
	Down	10	14
Moderate (2-4 fold)	Up	20	28
	Down	21	17
Little (< 2 fold)		118	107
Total		184	184

A total 184 protein spots were visible and analyzed by image anaysis software. The intensity levels of each protein spot obtained from triplicate samples of control and 5.4 μmol and 85.6 μmol *t*-2-H treatments were averaged and compared to that of control.

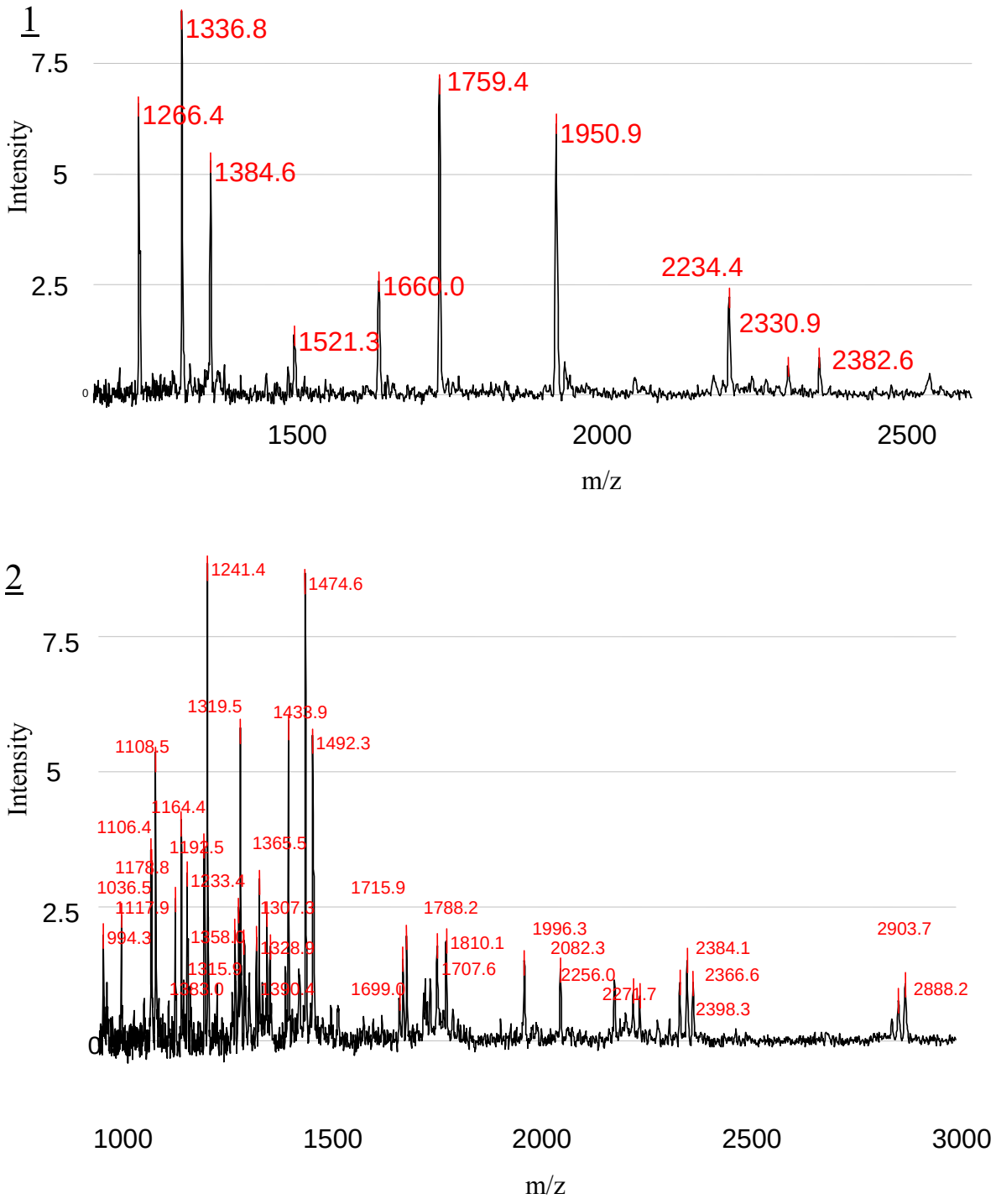
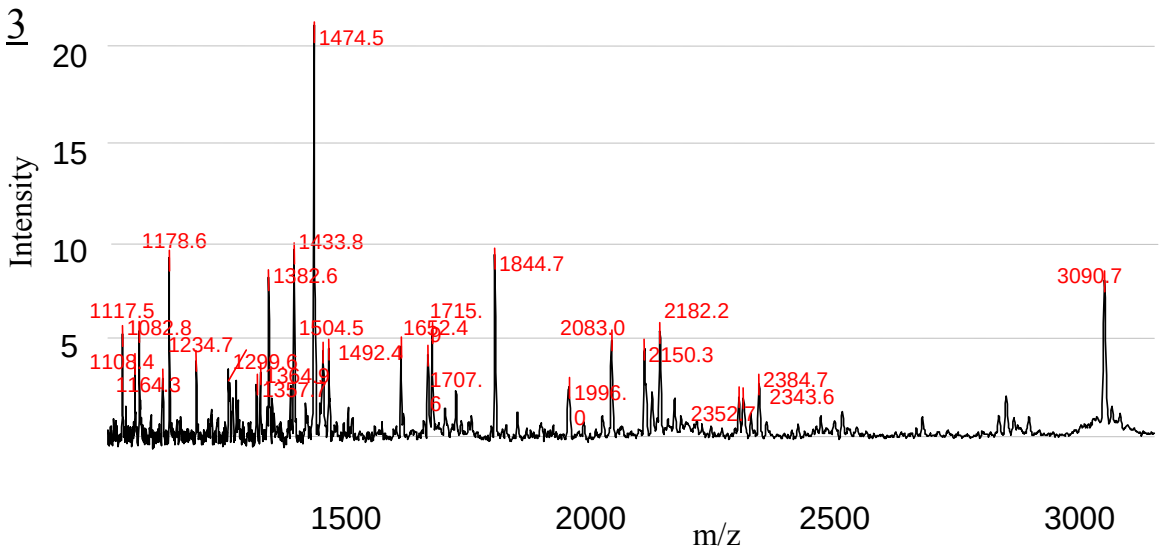
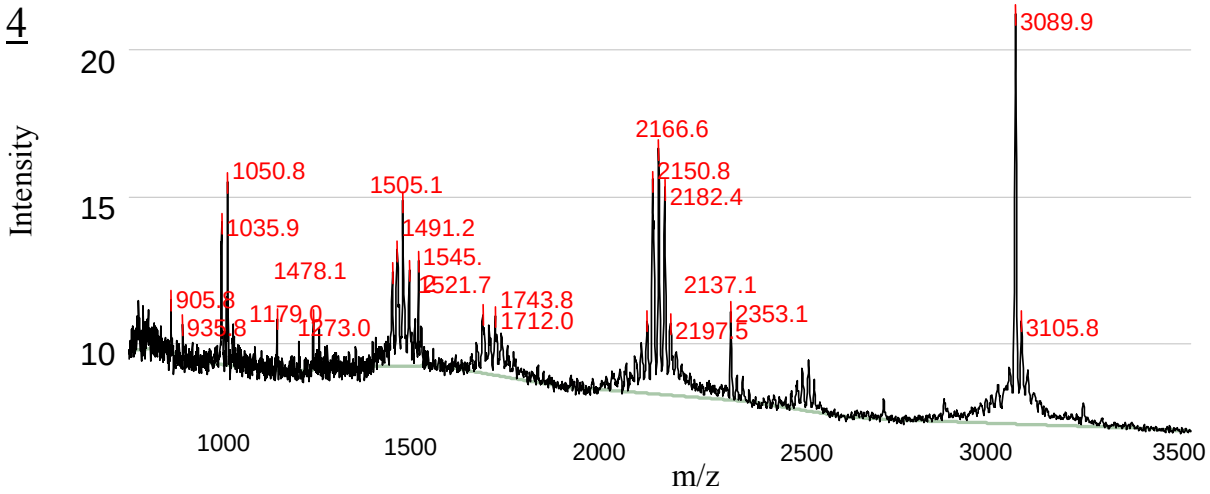
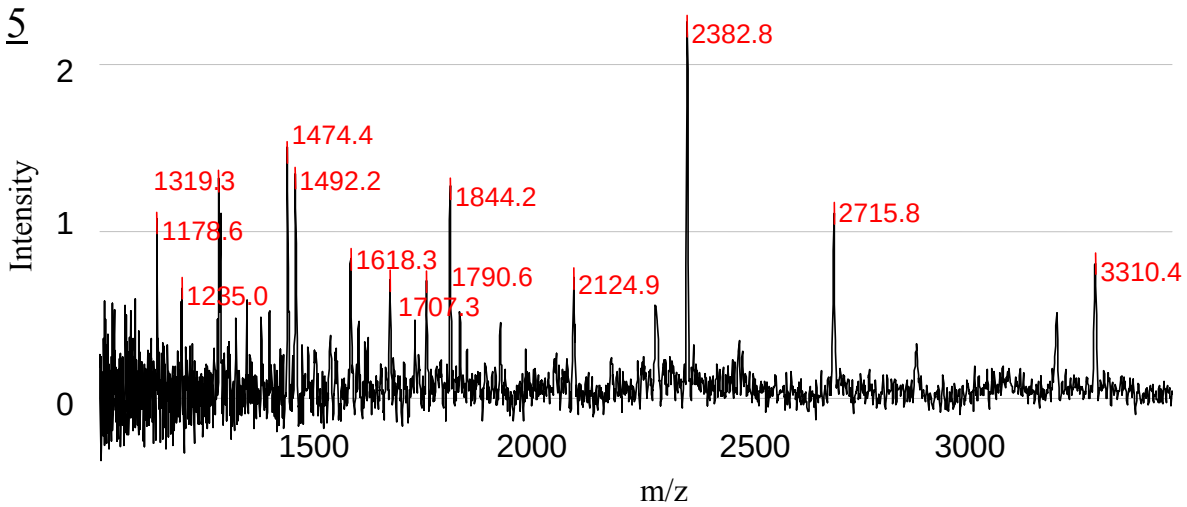


Figure 4.3 A MALDI-TOF spectra of peptides after trypsin digestion of five protein spots of interest. The corresponding number of a spectrum matches each number next to a circle shown in Fig. 4.2.


3
Intensity
20
15
10
5
1474.5
1178.6
1433.8
1382.6
1844.7
3090.7
1117.5
1082.8
1504.5
1715.
1652.4
2083.0
2182.2
1234.7
1492.4
2150.3
1108.4
1164.3
1707.
2384.7
2343.6
1996.
0
1500
2000
2500
3000
m/z


4
Intensity
20
15
10
3089.9
2166.6
1050.8
2150.8
1505.1
2182.4
1035.9
1491.2
1545.
1478.1
2521.7
2137.1
905.8
1743.8
1179.0
1712.0
2353.1
935.8
1473.0
2197.5
3105.8
1000
1500
2000
2500
3000
3500
m/z


5
Intensity
2
1
0
2382.8
1474.4
1319.3
1492.2
1844.2
2715.8
1178.6
1618.3
1790.6
2124.9
3310.4
1285.0
1707.3
1500
2000
2500
3000
m/z

Discussion

Germ tube growth of *B. cinerea* exposed to 5.4 μmol of *t*-2-H was not different from controls (Fig. 4.1). An inhibitory effect on percentage of germination as observed in this study (data not shown) was similar to results by Fallik *et al*. (1998) who showed that a vapor phase concentration from 5.4 μmol of *t*-2-H inhibited spore germination by 60%. The result suggests that once a germ tube from spores emerges, germ tube growth is normal. Fungicides that inhibit spore germination inhibit mycelial growth of *B. cinerea* at concentrations 5-45 fold higher than those for inhibition of spore germination, while fungicides that act after germination do not inhibit spore germination (Slawecki *et al*., 2002). The property of fungicides that inhibit spore germination is somewhat similar to that of *t*-2-H reported by Fallik *et al*. (1998). Therefore, the results imply that *t*-2-H may be active at a stage of germination but may not regulate germ tube growth. Consistent with a previous finding of more effective distribution of radiolabeled C_6 aldehydes into spores than mycelia (Chapter 3), we propose that *t*-2-H plays a major role in inhibiting spore germination in a concentration-dependent manner, but is much less active in inhibiting mycelial growth. Therefore, the stimulatory effect by *t*-2-H at a lower concentration observed by Fallik *et al*. (1998) may reflect a survival strategy of the fungus, increasing a population of mycelia when spore germination is inhibited. Since the fungus will be exposed directly to *t*-2-H during infection of a wounded plant, the toxic effects of the compound must be minimized by such a resistance strategy. Linoleic and linolenic acids and linoleic acid-derived compounds, which are produced by plant lipoxygenase during the defense response to *Aspergillus* species or are produced by the

fungus, can stimulate or inhibit conidium production or sporulation of several fungal species (Calvo *et al*., 2002). However, the mechanism of stimulation or inhibition of mycelial growth by *t*-2-H is not clear.

Treatment with *t*-2-H increased or decreased expression intensity of proteins of *B. cinerea* (Fig. 4.2 and Table 4.1). It is likely that the changes resulted from post-translational modification of proteins or a regulation of *de novo* biosynthesis of proteins because we incubated *t*-2-H with *B. cinerea* for 24 h. The period of incubation was chosen because it was previously shown that the headspace *t*-2-H concentration in the presence of *B. cinerea* markedly declined after 24 hr (Fallik *et al*., 1998), indicating that *B. cinerea* absorbed a significant amount of the volatile compound. As Alméras *et al*. (2003) suggested, *t*-2-H not only damages cells, but it can also selectively affect gene expression. There are three possibilities, which could explain the altered protein expression of *B. cinerea* in response to *t*-2-H. One is that existence of a receptor and signal transduction pathway specific to *t*-2-H leads to turning on or off the expression of genes, resulting in the increased or decreased intensity of proteins of *B. cinerea*. It is well known that several signaling pathways, such as the G-protein pathway, mitogen-activated protein kinase pathway, and cyclic AMP pathway, operate to regulate fungal growth and development (Tucker and Talbot, 2001; Calvo *et al*., 2002). To date, no signaling pathway associated with direct regulation of gene expression of *B. cinerea* by *t*-2-H has been reported. Another is that *t*-2-H diffuses into the cell wall and membrane and binds directly with cellular proteins, and the resulting protein modification can alter expression of one or more classes of genes. As shown in Chapter 3, *t*-2-H can interact with proteins inside the tissue. The third possibility is that *t*-2-H binds to proteins and the resulting

adducts destabilize proteins, leading to degradation. The *t*-2-H is highly reactive and can readily attack a nucleophile such as protein (Baker *et al*., 1999; Ichihashi *et al*., 2001). It is interesting to note that approximately 60% of proteins remain unaffected (less than two-fold change) by both amounts of *t*-2-H (Table 4.1), implying that adduct formation with proteins and a subsequent proteolysis may be a minor biochemical process. Indeed, we could not detect any degradation of proteins by SDS-PAGE when *B. cinerea* was exposed to 424 μmol of *t*-2-H (data not shown). As shown in Chapter 3, covalently-modified *t*-2-H-protein adducts were not obtained. Even though we were not able to identify a protein in this study, *t*-2-H at a lower concentration may influence growth-associated genes, stimulating mycelial growth when spore germination is hampered, but *t*-2-H at a higher concentration may induce defense-associated genes, minimizing damage to cell walls and membranes caused by *t*-2-H. As it has been known that *t*-2-H plays a role in defense-related gene expression in plants (Bate and Rothstein, 1998; Farag and Paré, 2002; Gomi *et al*., 2003), *t*-2-H may also induce the genes of *B. cinerea*.

In summary, our data show that germ tubes from germinated spores grow normally at a lower concentration of *t*-2-H, but spores did not germinate at a higher concentration. The effect of *t*-2-H on protein expression of *B. cinerea* was evaluated for the first time in this study. The results of this study showed that *t*-2-H treatment can alter the apparent intensity of fungal proteins. Further analysis will be needed to determine the significance of these changes and to further elucidate relationships between *t*-2-H and fungal growth.

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