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DEVELOPING AN OPTIMAL METHOD FOR PRODUCING A TEARLESS ONION

A thesis

submitted in partial fulfilment

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Abstract of thesis submitted in partial fulfillment of the requirements for the

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Developing an optimal method for producing a tearless onion

by Takahiro Kamoi

People experience the irritating tearing and burning sensation of lachrymatory factor (LF, propanthial *S*-oxide) when cutting or chopping onion bulbs. LF is produced by lachrymatory factor synthase (LFS) specifically from 1-propenyl sulfenic acid, a breakdown product of *trans*-1-propenyl-L-cysteine sulfoxide (1-PRENCSO) by alliinase. This thesis describes strategies to produce a tearless onion by using RNA interference (RNAi) silencing.

To determine whether a gene silencing cassette can silence *lfs* gene transcripts from onion (*Allium cepa* L.), a crop recalcitrant to genetic transformation, a gene silencing assessment system was developed by using a model plant as a host for the gene of interest. Tobacco (*Nicotiana tabacum*) plants transgenic for LFS enzyme activity from onion were first produced by introducing a CaMV 35S-onion-*lfs* gene construct. These plants were then subjected to a second transformation with an RNAi construct directed against the *lfs* gene sequence. LFS enzyme activity assay showed that the transgenic plants, containing both the *lfs* gene and the RNAi construct, had significantly reduced LFS activity. This observation was supported by Western analysis for the LFS protein and further validated by quantitative RT-PCR analysis that demonstrated a significant reduction in the *lfs* transcript level in the dual transformants. This work demonstrated that the RNAi construct is a suitable candidate for the development of a tearless onion. This model plant RNAi system has wide reaching applications for assessment and targeting of plant secondary pathway genes, from poorly studied or recalcitrant plant species, that are important in pharmacological, food and process industries.

The functional RNAi vector identified in the model system was transformed into onion. Endogenous *lfs* transcript levels were successfully reduced by up to 43-fold in six transgenic lines. In consequence, LFS enzyme activity was decreased by up to 1573-fold

and this observation was supported by Western analysis for the LFS protein. Furthermore, the production of the deterrent LF upon tissue disruption was reduced up to 67-fold. Subjective olfactory assessment of silenced lines indicated that the pungent odour given off by the leaf and bulb material was much reduced compared with that of non-transgenic counterparts, and that this was replaced by a sweeter milder onion odour. A novel colorimetric assay demonstrated that this silencing had shifted the 1-PRENCSO breakdown pathway so that by reducing LFS protein, more 1-propenyl sulfenic acid was converted into di-1-propenyl thiosulfinate. A consequence of the raised thiosulfinates levels was a marked increase in the downstream production of a non-enzymatically produced zwiebelane isomer that has never previously been identified, and other volatile compounds, di-1-propenyl disulfides and 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes, which had previously been reported either in small amounts or had not been detected in onions. These raised volatile sulfur compounds provide an explanation for the unique flavour notes of the LF reduced onion and are predicted to have health benefits akin to those found in garlic. These results demonstrated that silencing of LFS enzyme activity by introducing an RNAi construct directed against the *lfs* gene sequence simultaneously reduced levels of the deterrent LF and increased the desirable thiosulfinates in onions.

Keywords: Onion, lachrymatory factor synthase, propanthial *S*-oxide, RNA interference, gene silencing, model system, colorimetric assay, thiosulfinates, zwiebelane isomer, disulfides, and dihydrothiophenes.

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Abbreviations

bp	base pair
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
^{o}C	Degrees centigrade
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
g	gram
g	gravity
GC	gas chromatography
GFP	green fluorescent protein
HPLC	high pressure liquid chromatography
hr	hour
kb	kilobase
kDa	kiloDaltons
L	litre
mg	milligram
min	minute
ml	millilitre
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
MS	mass spectrometry
ng	nanogram
nm	nanometer
nM	nanomolar
ODS	octadecylsilica
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
psi	pound per square inch
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
qRT-PCR	quantitative RT-PCR
SDS	sodium dodecyl sulphate
sec	second
T-DNA	transfer DNA
Tris	tris(hyroxylmethyl)methylamine
µg	microgram
µl	microlitre
µm	micrometer

Chapter 1: Literature Review

1.1 Introduction

Onion is now the world's second most produced vegetable after tomato (FAOSTAT, 2006). In New Zealand it is one of the most important vegetable crops with 0.21 million tons produced in 2005 from 4,855 hectares, which represented about 9% of the total vegetable area under cultivation. The sales value of this was NZ$25 million in domestic and NZ$77.7 million in exports which accounted for approximately 40% of fresh vegetables exported (FreshFacts, 2006). Onions produced in New Zealand are world renowned for their storage and high-pungency characteristics. Pungency has been correlated with production of lachrymatory factor (LF) upon cutting or chopping (Randle, 1997). High pungency and LF induced tearing generally provide a detrimental experience for consumers who seem to prefer low-pungency types. 'Vidalia', one of the most popular low-pungency onions, commands approximately three times the value of medium to high pungency counterparts (Carter *et al*., 2006). The market for low-pungency onions is expanding in the United States and the United Kingdom. The consumption of the low-pungency onions now accounts for 15%-25% of total onion consumption in the United States. Current low pungency onions have poor storage characteristics thus there is an obvious niche for a long storage (i.e. Pukekohe Longkeeper, NZ type onion) low-pungency type onions.

1.2 Sulfur compounds in *Allium* species

Allium species have been used as foods, spices, and in therapeutic diets all around the world since ancient times. Approximately 750 species have been estimated in the genus *Allium* (Fritsch & Friesen, 2002). Of these species, onion and shallot (*A. cepa*), garlic (*A. sativum*), leek (*A. ampeloprasum*), and Welsh onion (*A. fistulosum*) have been commonly consumed in Europe, Asia, North and South America, or Africa. These edible species exhibit a range of sulfur related compounds responsible for specific flavour, odour and health attributes, including lachrymatory activity (universally associated with onion) but only garlic produces no lachrymatory factor (LF) upon tissue disruption (Imai *et al*., 2002; Block, 1992a). The sulfur chemistry behind these compounds is unique to *Allium* species, fascinating and intriguing. Many compounds and pathways have only recently been elucidated and even now several mechanisms and pathways are still predictions based on partial evidence.

1.2.1 Non-volatile sulfur compounds

Allium species synthesize unique sulfur compounds known as secondary metabolites. The main sulfur compounds exist in the form of non-volatile amino acid derivatives, *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs). To date, four major ACSOs, *S*-methyl-L-cysteine sulfoxide (Methiin, MCSO), *S*-2-propenyl-L-cysteine sulfoxide (*S*-allyl-L-cysteine sulfoxide, Alliin, 2-PRENCSO), *trans*-*S*-1-propenyl-L-cysteine sulfoxide (Isoalliin, 1-PRENCSO) and *S*-propyl-L-cysteine sulfoxide (Propiin, PCSO), and two minor ACSOs, *S*-ethyl-L-cysteine sulfoxide (Ethiin, ECSO) and *S*-n-butyl-L-cysteine sulfoxide (Butiin, BCSO) have been identified (Table 1.1) (Rose *et al*., 2005). The relative amount of these parental sulfur compounds varies among *Allium* species (Table 1.2). MCSO is the most ubiquitous cysteine sulfoxide in all *Allium* species, and in addition observed in Brassicaceae (Fritsch & Keusgen, 2006). 2-PRENCSO is the dominant sulfoxide in garlic-type *Allium* species. On the other hand, 1-PRENCSO is the major cysteine sulfoxide in onion-type although a previous study reported that its levels increase in garlic with cold storage (Lukes, 1986). To date, conflicting observations have been reported about the existence of PCSO in *Allium* species. Some studies reported PCSO is present in trace amounts, others reported absent. This can be attributed to cultivar variations and analysis methods (Randle *et al*., 1995). Some methods can lead to misidentification of peaks because of their short run times, or undetectable levels because of their detection limits. Further investigation is required for the PCSO distribution.

Table 1.1 *S*-alk(en)yl-L-cysteine sulfoxides in *Allium* species.

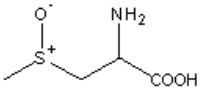
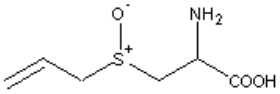
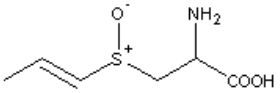
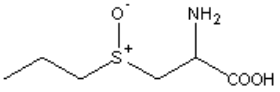
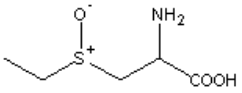
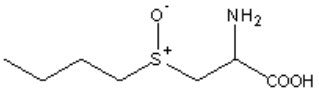
Chemical name	Common name	Abbreviation	Formula
S-methyl-L-cysteine sulfoxide	Methiin	MCSO	
S-2-propenyl-L-cysteine sulfoxide	Alliin	2-PRENCSO	
S-1-propenyl-L-cysteine sulfoxide	Isoalliin	1-PRENCSO	
S-propyl-L-cysteine sulfoxide	Propiin	PCSO	
S-ethyl-L-cysteine sulfoxide	Ethiin	ECSO	
S-butyl-L-cysteine sulfoxide	Butiin	BCSO	

Table 1.2 Content of *S*-alk(en)yl-L-cysteine sulfoxides in *Allium* species.

Taxonomic groups	Common name	Type	Tissues	Content (mg/100g fresh weight)				Reference	Method
				MSCO	**2-PRENCSO**	**1-PRENCSO**	**PCSO**		
A. cepa	Onion	Rijnsburger	Bulbs	70	T	357	28	Edwards et al., 1994	HPLC
		White	Bulbs	24	ND	131	ND	Thomas & Parkin, 1994	HPLC
		cv. TG1015Y (Mild)	Bulbs	14	ND	78	ND	Yoo & Pike, 1998	HPLC
		Dehydrator	Bulbs	15	ND	98	ND	Thomas & Parkin, 1994	HPLC
		Dehydrator	Bulbs	17	ND	232	ND	Yoo & Pike, 1998	HPLC
A. cepa	Shallot		Bulbs	10	ND	217	ND	Yoo & Pike, 1998	HPLC
			Bulbs	41	1	93	18	Kubec et al., 2000	GC
A. fistulosum	Welsh onion		Leaves	16	ND	156	ND	Yoo & Pike, 1998	HPLC
			Leaves	6	T	13	2	Kubec et al., 2000	GC
A. schoenoprasum	Chive		Leaves	80	ND	40	2	Edwards et al., 1994	HPLC
			Leaves	68	9	65	ND	Yoo & Pike, 1998	HPLC
			Leaves	32	2	31	7	Kubec et al., 2000	GC
A. ampeloprasum	Leek		Bulbs	28	ND	75	ND	Thomas & Parkin, 1994	HPLC
			Leaves	19	ND	228	ND	Yoo & Pike, 1998	HPLC
			Stem	4	T	18	T	Kubec et al., 2000	GC
A. ampeloprasum	Elephant garlic		Leaves	97	208	T	3	Kubec et al., 1999	GC
A. sativum	Garlic		Cloves	T	790	T	T	Edwards et al., 1994	HPLC
			Cloves	60	305	±	ND	Thomas & Parkin, 1994	HPLC
			Cloves	56	983	135	ND	Yoo & Pike, 1998	HPLC
		cv. Tantal	Cloves	65	632	T	ND	Kubec et al., 1999	GC
		cv. OL1450	Cloves	78	1140	T	ND	Kubec et al., 1999	GC
A. chinese	Rakkyo		Leaves	200	14	T	T	Kubec et al., 2000	GC

T, trace amounts; ND, not detected; ±, detected in some samples. MCSO, Methyl cysteine sulfoxide; 2-PRENCSO, 2-propeny cysteine sulfoxide; 1-PRENCSO, 1-propeny cysteine sulfoxide; PCSO, propyl cysteine sulfoxide.

1.2.2 Volatile sulfur compounds

When the tissues of any *Allium* species are disrupted, the non-volatile ACSOs are cleaved by the enzyme alliinase (EC 4.4.1.4) and volatile sulfur compounds are produced that give the characteristic flavours and bioactivities of the species. The highly reactive sulfenic acids, released from ACSOs by alliinase, are immediately converted into lachrymatory factor (LF, propanthial *S*-oxide) or thiosulfinates. These unstable, some even at room temperature, organosulfur compounds are non-enzymatically rearranged or converted into predominantly polysulfides, zwiebelanes, cepaenes, and ajoenes. (Block, 1993; Rose *et al*., 2005) (Figure 1.1). The particular sulfur volatile profile of *Allium* species directly correlates to the relative amounts of the ACSOs present (Table 1.3). In garlic, 2-PRENCSO is the major cysteine sulfoxide (Table 1.2) and this produces di-2-propenyl-thiosulfinate (allicin) upon tissue disruption. The decomposition product of allicin, di-2-propenyl-disulfide, is the dominant volatile component liberated (Block *et al*., 1992b & 1992c; Rose *et al*., 2005). In onion, 1-PRENCSO is the major ACSO (Table 1.2). This would be predicted to produce di-1-propenyl thiosulfinate and di-1-propenyl disulfide. However, di-1-propenyl thiosulfinate has never been reported in onion and di-1- propenyl disulfide has been reported in small amounts (Boelens *et al*., 1971; Ferary & Auger, 1996; Mondy *et al*., 2001; Arnault *et al*., 2004). Instead, LF, 1-propenyl methane thiosulfinate, 1-propenyl propane thiosulfinate and di-propyl disulfide are dominant (Block *et al*., 1992b & 1992c; Rose *et al*., 2005). Furthermore, the total thiosulfinates level in onion is much less than that in garlic although there is not such a big difference in the total ACSOs levels (Table 1.2 & 1.3). It has been speculated this antinomy is a consequence of LF production in onion (Randle & Lancaster, 2002).

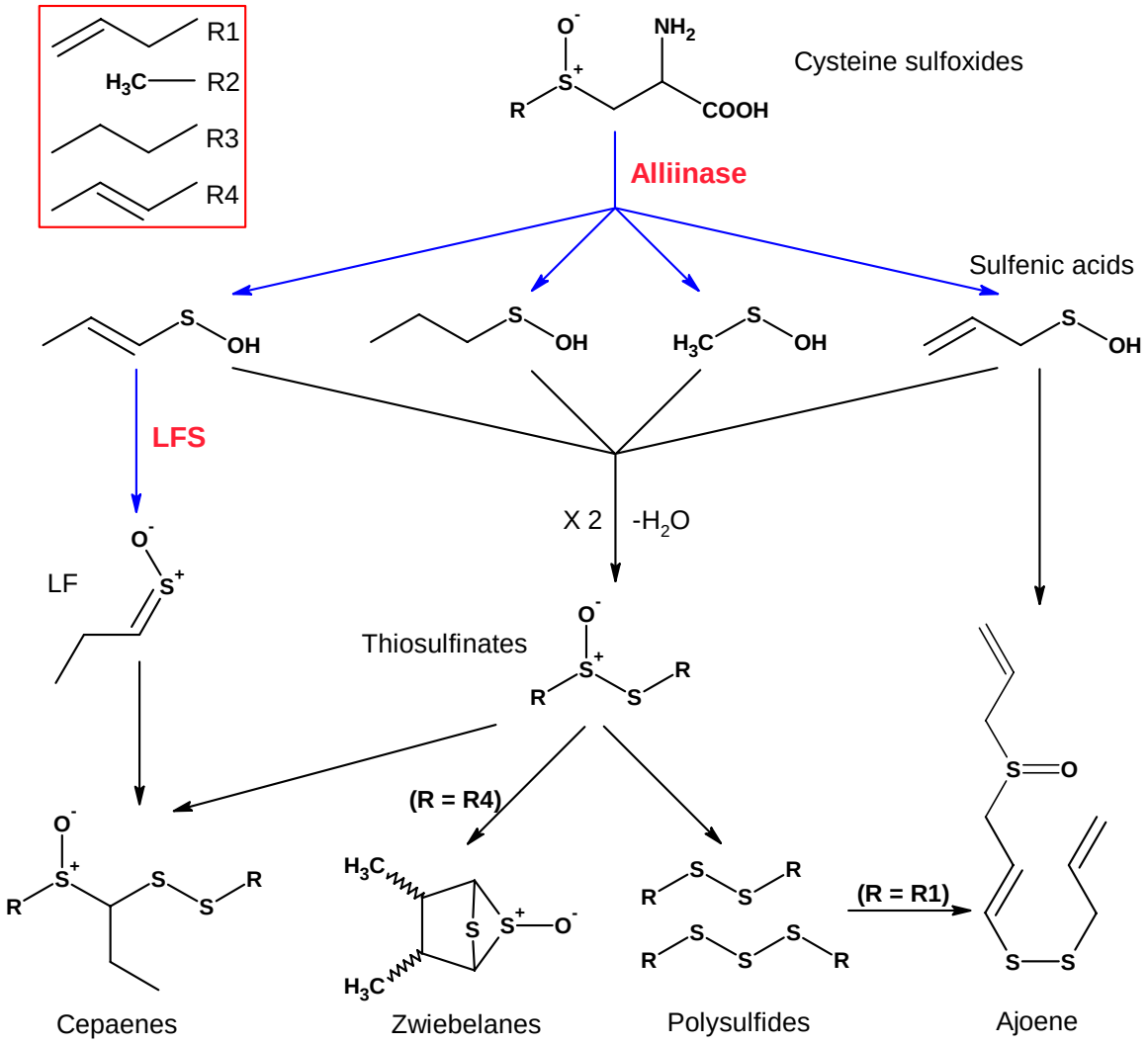


Figure 1.1 Major breakdown pathway of ACSOs in *Allium* species.

Blue arrows show enzymatic reactions.

Table 1.3 Relative content of thiosulfinates and zwiebelanes in *Allium* species (adapted from Block et al., 1992 b & c).

Thiosulfinate (TS) R-S(O)-R'	Onion (white)	Shallot	Welsh onion	Chive	Leek	Elephant garlic	Garlic
Me-S(O)S-Me	1	9	1	1.2	3	10	ND
Me-S(O)S-2Pe / 2Pe-S(O)S-Me	ND	ND	ND	ND	ND	42	4.3
Me-S(O)S-1Pe / 1Pe-S(O)S-Me	48	24	20.5	5.7	39	5.3	T
Me-S(O)S-Pr / Pr-S(O)S-Me	2	4	15	10.6	10	ND	ND
2Pe-S(O)S-2Pe	ND	ND	ND	ND	ND	38	89
2Pe-S(O)S-1Pe / 1Pe-S(O)S-2Pe	ND	ND	ND	ND	ND	5.1	6.9
Pr-S(O)S-Pr	9	27	35	57	25	ND	ND
Pr-S(O)S-1Pe / 1Pe-S(O)S-Pr	40	36	29.5	25.4	23	ND	ND
1Pe-S(O)S-1Pe	ND	ND	ND	ND	ND	ND	ND
Total TS (µmol/g fresh weight)	0.20	0.25	0.08	0.26	0.15	5.20	14.30
LF	+ +	+ +	+ +	ND	+	ND	ND
cis / *trans* zwiebelanes	29% TS	4% TS	8% TS	4% TS	11% TS	ND	ND
Analytical method	HPLCMS GCMS	HPLCMS GCMS	HPLCMS GCMS	HPLCMS GCMS	HPLCMS GCMS	HPLCMS	HPLCMS

Quantification was determined by solvent extraction-GCMS and HPLCMS analyses. Me, methyl; 2Pe, 2-propenyl (allyl); 1Pe, 1-propenyl; Pr, propyl; LF, lachrymatory factor; ND, not detected; T, trace amounts; ++, substantial amounts of LF detected; % TS, zwiebelanes indicated as % total thiosulfinates (TS) present.

1.2.3 Analytical methods for volatile sulfur compounds.

The sulfenic acids have been reported to be converted into various volatile sulfur compounds, for example, LF, thiosulfinates, polysulfides, zwiebelanes, cepaenes and ajoene which are responsible for characteristic flavours in *Allium* species upon tissue disruption. Detectable organosulfur constituents depend on the maceration time of samples and the thermal condition of the analyses. Since the volatiles are thermally unstable, they are subsequently decomposed into artifacts, which are absent in raw cut *Allium* species (Block, 1993). In the case of the highly volatile LF, a standard gas chromatography (GC) analysis of sample from quickly macerated tissue (several tens of seconds) is an appropriate method (Kopsell *et al*., 2002). Thiosulfinates analysis is more difficult because of their thermal instability although most of them are stable at room temperature in mildly acidic conditions (Shen *et al*., 2002). Especially, di-1-propenyl thiosulfinate is so unstable that it immediately rearranges into a zwiebelane even at -15^{o}C (Block, 1991). Block *et al*. (1992 b & c) and Calvey *et al*. (1997) developed analytical methods for all the organosulfur compounds except LF and polysulfides in a variety of *Allium* plants using long incubation time (several tens of minutes), and supercritical fluid extraction (SFE) and

solvent extraction (SE) high-performance liquid chromatography (HPLC)-mass spectrometry (MS) as well as SE-GCMS with on-column injection ramped from 0^{o}C. On the contrary, Auger's research team demonstrated that all the true onion volatile sulfur compounds were detected by analysis of the same sample using successive solid-phase microextraction (SPME)-GCMS and SE-GCMS with normal injection at 200^{o}C (Ferary & Auger, 1996; Arnault *et al*., 2000; Mondy *et al*., 2001). Aside from this, an indirect detection method to quantify thiosulfinates levels has been developed by Carson & Wong (1959) and Nakata *et al*. (1970). This method is colorimetric assay for detection of all thiosulfinates by using *N*-ethylmaleimide as a derivatizing agent of thiosulfinates into a pink to red color pigment.

1.2.4 Sensory notes of volatile sulfur compounds

Different volatile sulfur compounds contribute characteristic odour and taste of *Allium* species. Sensory notes of the wide-ranging volatile organosulfur volatiles given off by tissue disruption in *Allium* species have been examined (Table 1.4). LF has pungent, heat and mouth burn sensation. Thiosulfinates have raw flesh flavour notes (e.g. allicin provides the unique smell of fresh cut garlic) whilst polysulfides have the sweet aroma of cooked or fried *Allium* species. Zwiebelanes impart a sweet or brown sauté taste with liver and hydrogen sulfide notes. Cepaenes have a fresh onion and fruity-melon-like flavour (Shankaranarayana *et al*., 1982; Lancaster & Boland, 1990; Randle *et al*., 1994; Block *et al*., 1997; Randle, 1997).

Table 1.4 Sensory notes of volatile sulfur compounds in *Allium* species.

Compound	Sensory note	Reference
LF	onion, sulfur, gaseous, livery, heat, mouth burn, pungent	Randle *et al*., 1994
Me-S(O)S-Me	creamy, sulfury, cabbage, broccoli, califlower, green onion	Randle *et al*., 1994
Me-S(O)S-1Pe / 1Pe-S(O)S-Me	creamy, sulfury, cabbage, radish, meaty, shallot	Randle *et al*., 1994
Me-S(O)S-Pr / Pr-S(O)S-Me	creamy, sulfury, cabbage, green	Randle *et al*., 1994
2Pe-S(O)S-2Pe	fresh garlic	Shankaranarayana *et al*., 1982
Pr-S(O)S-Pr	fresh onion, chive, green onion	Randle *et al*., 1994
Pr-S(O)S-1Pe / 1Pe-S(O)S-Pr	green, raw fresh onion	Randle *et al*., 1994
2Pe-SS-2Pe	cooked garlic	Shankaranarayana *et al*., 1982
Pr-SS-Pr	cooked onion	Lancaster & Boland, 1990
1Pe-SS-1Pe	cooked onion	Lancaster & Boland, 1990
cis zwiebelanes	sweet brown saute taste, liver and hydrogen sulfide notes	Randle *et al*., 1994
trans zwiebelanes	sweet sulfur taste, green or raw onion	Randle *et al*., 1994
cepaenes	fresh onion, friuty-melon	Block *et al*., 1997

R-S(O)S-R', thiosulfinate; R-SS-R', disulfide; Me, methyl; 2Pe, 2-propenyl (allyl); 1Pe, 1-propenyl; Pr, propyl; LF, lachrymatory factor.

1.2.5 Physiological activity of volatile sulfur compounds

For centuries, the genus *Allium* plants, especially onion and garlic, have been used in the folklore in many countries as not only foods and spices but also therapeutic agents to treat cardiovascular, inflammatory and cancer diseases. In the last few decades, many *in vitro* and *in vivo* studies have demonstrated that the therapeutic properties are attributed to the volatile sulfur compounds.

1.2.5.1 Cardiovascular effects

Thrombosis causes serious coronary syndromes such as cerebral infarction, myocardial infarction and angina. Inhibitors of platelet aggregation such as aspirin (cyclooxygenase-1 (COX-1) inhibitor) or heparin (thrombin indirect inhibitor) have been used as medicine since the aggregation plays a key role in forming a thrombus. Aqueous extracts from raw onion and garlic have significant increased anti-platelet aggregation activity *in vitro* compared with those from boiled or cooked intact materials in which alliinase is inactivated before tissue disruption. Raw garlic is approximately 13 times more potent than raw onion (Ali *et al*., 1999) and the inhibiting activity of garlic correlates with the amounts of di-2-propenyl thiosulfinate (allicin) (Cavagnaro *et al*., 2007). This means that the breakdown volatile compounds are more potent than the cysteine sulfoxide precursors. A few mechanisms of the anti-platelet action have been suggested and several platelet

inhibitors have been identified from onion and garlic. Garlic inhibits COX-1 activity *in vitro* and *in vivo* (Ali *et al*., 1990; Ali, 1995). Allicin, dipropyl thiosulfinate and diethyl thiosulfinate have anti-platelet activity *in vitro* and the activity is stronger than aspirin (Briggs *et al*., 2000). Cepaenes and zweibelanes found in onion extracts have demonstrated anti-aggregation activity *in vitro* (Morimitsu *et al*., 1992; Block *et al*., 1996a; Block *et al*., 1997). Furthermore, ajoene and methyl allyl trisulfide, constituents of garlic extracts, have been shown to inhibit *in vitro* the platelet aggregation by preventing COX-1 activity (Srivastava & Tyagi, 1993; Ariga & Seki, 2006).

Vascular endothelium dysfunction results in Arteriosclerosis and Angina which are also serious cardiovascular diseases. Nitroglycerin, donor of nitric oxide (NO), has been used as a medicine since NO plays a key role in vasorelaxation. Recently, hydrogen sulfide (H_2S) has been shown to be a novel gaseous signaling molecule such as NO in the cardiovascular system (Pryor *et al*., 2006). Benavides *et al*. (2007) have demonstrated that di-2-propenyl (allyl) polysulfides, constituents in garlic extracts, are converted into H_2S by the red blood cells and results in vasorelaxation *in vitro*, and that unsaturated polysulfuides are more potent than saturated ones.

1.2.5.2 Anti-inflammatory properties

Onion and garlic are also used as traditional medicine for the treatment of bronchial asthma, one of inflammatory diseases. A few mechanisms of the anti-inflammatory action have been suggested and several inhibitors have been identified from onion and garlic. Thiosulfinates and cepaenes found in onion extracts have demonstrated their anti-inflammatory activity *in vitro* by inhibiting COX-2 and lipoxygenase (LOX). Uninhibited they trigger an arachidonic acid cascade which leads to bronchial restriction. The effect of unsaturated thiosulfinates is more potent than that of saturated ones (Wagner *et al*., 1990). Furthermore, ajoene, a constituent of garlic extracts, have been shown to inhibit the COX-2 activity *in vitro* (Dirsch *et al*., 2001).

1.2.5.3 Anti-carcinogenic properties

A protective effect toward cancer has been epidemiologically observed in *Allium* vegetable consumption, especially in onion and garlic (Rose *et al*., 2005). Onion and garlic have been reported to exert their anti-carcinogenic properties by different mechanisms: induction of

phase II detoxification enzymes such as glutathione *S*-transferase (GST) and quinone reductase (QR); inhibition of cytochrome P450 that activates pro-carcinogens; and inhibition of cellular proliferation by induction of apoptosis (Corzo-Martínez *et al*., 2007). Several key volatile sulfur compounds associated with this property have been identified although the mechanisms have not been fully understood. Di-2-propenyl (allyl) polysulfides increase activity of the phase II detoxification enzymes in a variety of tissues in rats more than diallyl monosulfide and dipropyl mono or polysulfides (Guyonnet *et al*., 1999; Munday *et al*., 2003). Di-1-propenyl monosulfide has more potential for the induction of the detoxification enzymes than diallyl disulfide in rat tissues (Munday *et al*., 2005). Diallyl polysulfides and ajoene, constituents in garlic, inhibit the cellular proliferation by inducing apoptosis in human cells (Sakamoto *et al*., 1997; Dirsch *et al*., 1998). Recently, Hosono *et al* (2005) demonstrated that diallyl trisulfide suppressed the proliferation and induced apoptosis of human colon cancer cells by oxidative modification of cysteine residues in β-tubulin, and also inhibited significantly the growth of human cancer cells in nude mice *in vivo*.

1.2.6 Roles of volatile sulfur compounds

The sulfur volatile metabolites in *Allium* species have been extensively studied for their defensive roles (Auger *et al*., 2004). Several *in vitro* studies have reported that *Allium*-derived volatile sulfur compounds, such as thiosulfinates and polysulfides, have nematocide activity (Tada *et al*., 1988), insecticidal and fungicidal activity (Auger *et al*., 2004), and anti-microbial activity (Ankri & Mirelman, 1999). However, only few *in vivo* studies on their defensive effects within the plants have been undertaken (Dugravot *et al*., 2005; Hori & Harada, 1995).

Conversely, several pests and diseases actively seek out the sulfur volatile compounds, such as polysulfides, from *Allium* species and use them to target the genus (Jones *et al*., 2004). Onion fly (*Delia antique*) is specifically attracted to the sulfur volatile compounds given off by *Allium* tissue (Romeis *et al*., 2003). Onion aphids (*Neotoxoptera formosana*) have also been shown to be attracted to specific sulfide volatiles from spring onion (*Allium fistulosum*) and chives (*Allium tuberosum*) (Hori, 2007; Hori & Komatsu, 1997). The disease-causing fungus *Sclerotium cepivorum* is known to be stimulated to germinate by the sulfur volatiles derived from alkyl cysteine sulfoxide (ACSO) (Jones *et al*., 2004).

Roles of LF in *Allium* species, by contrast, have been poorly studied.

1.3 Lachrymatory factor synthase (LFS)

1.3.1 Discovery of LFS

People experience the irritating tearing and burning sensation of LF when cutting or chopping onion bulbs. It is the LF that critically distinguishes onion from garlic. Very recently, the mechanism of producing LF has been revised. Previous studies suggested (Brodnitz & Pascale, 1971; Block *et al*., 1979) that in chopped onion 1-PRENCSO, the major cysteine sulfoxide in onion, is cleaved by alliinase to produce 1-propenyl sulfenic acid, and that the sulfenic acid is subsequently converted into LF and thiosulfinates spontaneously via different pathways because the sulfenic acid is very unstable and has never been isolated. However, Imai *et al*. (2002), when investigating discoloration in a mixture of onion and garlic, discovered that the reaction converting 1-propenyl sulfenic acid into LF is catalyzed by a novel enzyme, lachrymatory factor synthase (LFS) encoded by *lfs* gene (GenBank accession number AB089203) (Figure 1.1). The *lfs* gene has no intron region and a predicted gene product of 169 amino acids. The LFS protein acts specifically upon 1-propeny sulfenic acid and contains no defined structural domain. Interestingly, there is no LFS activity in garlic (Imai *et al*., 2002).

1.3.2 LFS in *Allium* species

The *lfs* genes are highly conserved among certain members of the *Allium* species. The *lfs* cDNAs cloned from shallot (GenBank accession number AB094593), Welsh onion (GenBank accession number AB094590, AB094591), leek (GenBank accession number AB094594, AB094595), elephant garlic (GenBank accession number AB094596) and rakkyo (GenBank accession number AB094592) have very similar nucleotide and deduced amino acid sequences compared with onion cDNA. All the recombinant LFS proteins from these *Allium* vegetables expressed in *Escherichia coli* (*E. coli*) demonstrate LFS activity. Very interestingly, garlic only has a truncated *lfs* cDNA sequence and subsequently results in the absence of LFS activity in garlic (N. Masamura, personal communication, 31 July 2008).

1.3.3 LFS mutant

Two putative active center residues of LFS were identified by analysing the amino-acid homology sequence and using GeneTailor™ Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA) (Masamura *et al*., 2008). The first is arginine (Arg) at the 71st position and the second is glutamic acid (Glu) at the 88th position. Recombinant LFS mutant proteins expressed in *E. coli*, containing either substituted leucine (Leu) for Arg (R71L) or Glutamine (Gln) for Glu (E88Q), showed no LFS activity. The three-dimensional structure analysis of LFS is currently being performed (Masamura *et al*., 2008) so that the active center residues will be clarified.

1.3.4 Tearless onion

Currently almost tearless (low-pungency) onions are produced by growing onion varieties that contain low levels of 1-PRENCSO under low sulfur fertilizer (Randle *et al*., 1994 & 1995), for example the famous Vidalia® or Supasweet™ onion. Attempts have also been made to silence the *alliinase* gene and so prevent initial ACSOs breakdown upon tissue disruption (Eady *et al*., 2005d). However, onions produced by either of these ways would reduce not only LF but also thiosulfinates and their converted volatile sulfur compounds (Figure 1.1), thus potentially losing many of the characteristic flavour notes and health benefits related to these volatiles. Furthermore, the shelf life of Vidalia® onions is limited to around four months so that there is no way to store excess supply (Clemens, 2002), and the cultivated area is also limited to sulfur-deficient soils. However, such spatial and temporal limitations on supply allow it to maintain a market premium.

The discovery of LFS has led to the proposal that tearless onions unable to produce LF might be possible by silencing LFS enzyme activity. In the absence of LFS, by stopping conversion of 1-propenyl sulfenic acid to the undesirable LF, the unstable sulfenic acid would be predicted to undergo spontaneous self-condensation to thiosulfinate (Imai *et al*., 2002). This raised thiosulfinate level would then be available for conversion into a cascade of secondary volatile sulfur compounds as described earlier. Unlike the current almost tearless onion, which relies on reduced levels of sulfur compounds, a silenced LFS tearless onion would be predicted to maintain normal levels of nutritional sulfur precursor compounds and, due to increased conversion down the thiosulfinate pathway, have improved flavour, odour and bioactive properties compared to a normal onion.

1.3.5 *In vitro* assay

To examine the possibility of producing a tearless onion, *in vitro* assays were performed by Imai *et al* (House Foods Corporation, Chiba, Japan, unpublished data, 2002). LF and thiosulfinates levels were analysed in a reaction mixture comprising of; *trans*-1-PRENCSO purified from onion, alliinase purified from garlic, and various amounts of recombinant LFS protein purified from *E. coli*. LF levels were measured by HPLC and thiosulfinates levels were determined by a colorimetric assay using *N*-ethylmaleimide and absorbance at 515 nm. This analysis showed that the yield of thiosulfinates increased while that of LF decreased as the amount of recombinant LFS protein decreases (Figure 1.2). These results indicated that a non-lachrymatory onion can be created by suppressing LFS enzyme activity.

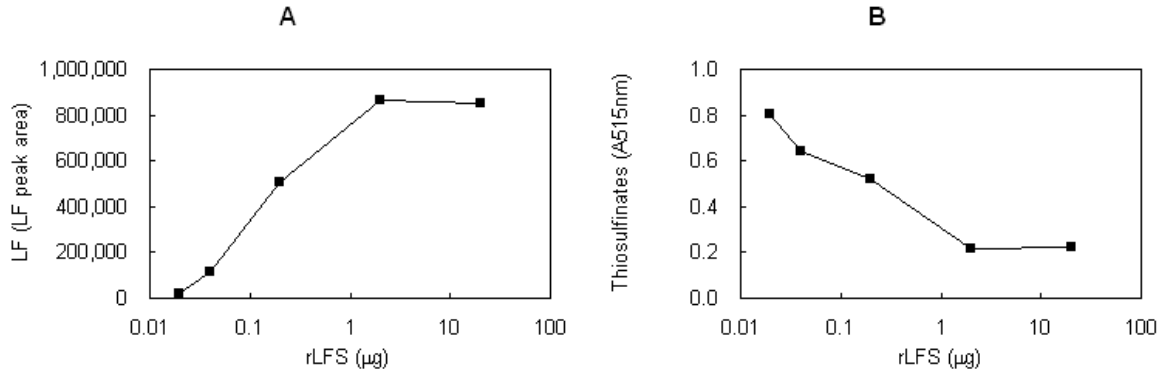


Figure 1.2 Effects on LF and thiosulfinates levels in *in vitro* assay performed by Imai *et al* (2002).

(A) LF levels in the reaction mixture; *trans*-1-PRENCSO purified from onion, purified garlic alliinase, and various amount of recombinant LFS (rLFS) protein expressed in *E. coli*. LF levels were measured by HPLC as LF peak area. These levels are mean of values obtained from two separate sets. (B) Thiosulfinates levels in the reaction mixture. Thiosulfinates levels were determined by colorimetric assay using *N*-ethylmaleimide as absorbance at 515 nm. These levels are mean of values obtained from two separate sets.

1.4 Strategies for silencing LFS enzyme activity

There are three approaches to silence LFS enzyme activity:

- *lfs* gene knockout by homologous recombination-dependent gene targeting or mutagenesis.

- *lfs* gene transcript degradation by RNA interference.
- LFS enzyme inhibition by competition with an inactive LFS mutant protein

1.4.1 Homologous recombination-dependent gene targeting

Homologous recombination-dependent gene targeting can induce targeted gene replacements and targeted point mutations. In general, targeted gene replacement is considered to occur via double crossover events at the flanking homologous regions. Similarly, targeted point mutation is regarded to occur by double crossover events (Figure 1.3). A few successful experiments for endogenous gene targeting by homologous recombination have been reported (Iida & Terada, 2005). In monocotyledonous plants, only two groups have succeeded in rice (Terada *et al*., 2002; Endo *et al*., 2007). The efficiency of gene targeting is very low because random integration of transgenes by non-homologous end-joining occurs much more frequently than targeted homologous recombination. It is still necessary to accumulate more knowledge about the mechanism of homologous recombination and to improve the procedures.

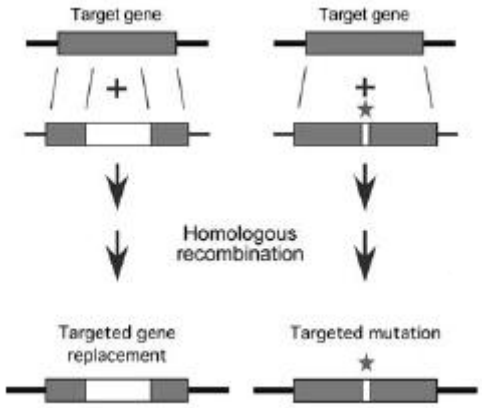


Figure 1.3 Homologous recombination-dependent gene targeting (adapted from Iida *et al*., 2005).

1.4.2 Mutagenesis

Mutagenesis might be also able to knock out targeted gene. Specific mutation in *lfs* gene or *lfs* transcriptional factor gene could create “tearless onion”. Current techniques include chemical mutagenesis using ethylmethane sulfonate (EMS), insertional mutagenesis using transposons or T-DNAs, and radiation mutagenesis using gamma ray, neutron ray, or

heavy-ion beams. These approaches have been applied to not only functional analysis of plant genes but also cultivar improvement. In these days, several successful studies using heavy-ion beams have been reported (Abe *et al*., 2000). However, the frequency of targeted mutagenesis is extremely poor because random mutagenesis occurs quite frequently. Simple and easy screening methods are necessary to select the targeted phenotype. Although it is easy to screen novel color flowers or salt tolerant plants, in the case of non-lachrymatory onions, we would have to damage onion tissue and screen for mutagenized onion plants that did not produce LF. Recently, a breakthrough method called TILLING (Targeting Induced Local Lesions in Genomes) has been developed. In this method, specific mutants are detected by use of a mass sequencing approach that can identify point mutations within mutagenized plants (McCallum *et al*., 2000). Moreover, a less-expensive and faster modification of this method enabled to screen in high-throughput manner (Colbert *et al*., 2001). However, several *lfs* loci can be detected in onion using Southern analysis indicating that it is either multi-copy and/or that similar pseudogenes exist (unpublished data), such sequences could compromise the case of undertaking a TILLING approach. A mutated transcription factor controlling the *lfs* gene could be used to knockdown *lfs* transcription but the targeted mutants cannot be screened by TILLING because the transcription factor gene is still unknown. These limitations imply that the application of mutagenesis on producing “tearless onion” would be a very inefficient and labour-intensive endeavour.

1.4.3 RNA interference (RNAi)

RNAi works by specifically degrading targeted transcript sequences. The gene silencing system is triggered in the cell by the detection of double-stranded RNA (dsRNA) that is complementary to the target sequence. Fire *et al*. (1998) discovered that the dsRNA could induce gene silencing in *Caenorhabditis elegans*. Since then, other components of the RNAi system have been clarified. The dsRNA is cleaved by the RNaseIII-type multidomain enzyme Dicer to give small dsRNA of 21-26-nucleotide named small interfering RNAs (siRNAs) which have 2-nt overhangs at their 3’ ends and phosphate groups at their 5’ ends. siRNAs are subsequently incorporated into a multi-subunit RNA-induced silencing complex (RISC) to form a RNA-protein complex. The antisense-strand-containing RISC binds to the target mRNA and suppresses gene expression by degrading the target sequence (Figure 1.4) (Waterhouse & Helliwell, 2003;

Baulcombe, 2004; Meister & Tuschl, 2004).

RNAi is a common phenomenon in animals, fungi and plants (Waterhouse *et al*., 1998; Hamilton & Baulcombe, 1999). In plants, RNAi is thought to be not only a regulation system for endogenous genes but also a defensive system against viruses (Waterhouse *et al*., 2001; Baulcombe, 2004) Over the past few years, RNAi has become a powerful tool to study gene function in major crop plants such as rice (Komiya *et al*., 2008), wheat (Travella *et al*., 2006) and corn (McGinnis *et al*., 2007). Furthermore, the RNAi approach has been successfully used for metabolic engineering to reduce compounds such as caffeine in coffee (Ogita *et al*., 2003), toxic gossypol in cottonseed (Sunilkumar *et al*., 2006) and allergen in peanut (Dodo *et al*., 2008), and also to elevate desirable compounds such as non-narcotic alkaloid reticuline in opium poppy (Allen *et al*., 2004), and carotenoid and flavonoid in tomato (Davuluri *et al*., 2005). This useful method has been applied to a variety of plants but never to *Allium* species. Because of these previous applications RNAi mediated silencing of *lfs* gene transcripts in onion would probably have a high likelihood of success.

However, some reports have observed that in some transgenic lines RNAi failed or silenced inefficiently (Kerschen *et al*., 2004; Xiong *et al*., 2005; McGinnis *et al*., 2007). Several studies have reported that position effect and copy number of a transgene affect its expression level (Gallie, 1998; Matzke & Matzke, 1998) and this may reduce RNAi transcription to ineffective levels. Spiker & Thompson (1996) demonstrated that transgene expression is silenced by methylation when multiple inserts are integrated at a single locus. Also, target sequences have their own inherent sensitivity to RNAi (Kerschen *et al*., 2004) possibly due to sequence composition, gene expression patterns, and RNA turnover rate. RNAi can also cause ‘off-target’ effects which silence non-target genes. These arise when full or partial complementarity between the siRNA and random mRNA transcripts occurs. It has been reported that ‘off-target’ effects can generate abnormal phenotypes although most reports concerning the effects are due to partial homology of siRNA to the 3’ untranslated region in the genes of animals (Lin *et al*., 2005; Birmingham *et al*., 2006). Thus it is important to have a detailed knowledge of the target gene, the silencing sequences, and several transgenic events for analysis in order to effectively assess the potential of a silencing strategy.

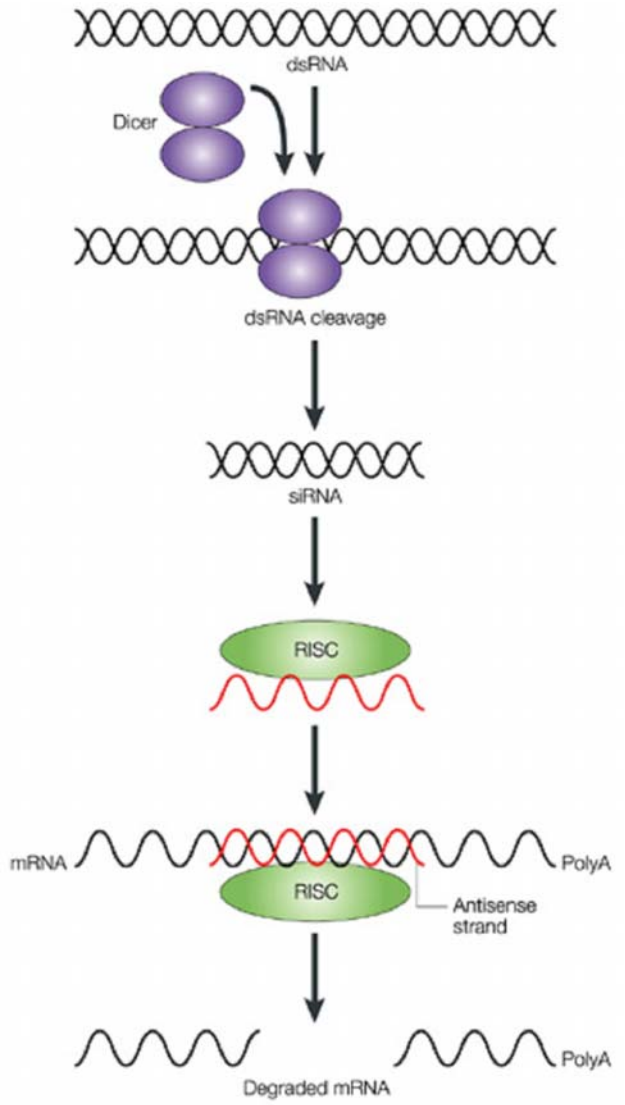


Figure 1.4 The current model of RNAi in plants (adapted from Waterhouse *et al*., 2003).

1.4.4 Competitive inhibition with LFS mutant

LFS mutant enzymes might be able to competitively inhibit the action of native LFS enzyme. However, in an *in vitro* assay, a mixture of approximately 4 ng of rLFS and 970 ng of rLFS mutant (R71L or E88Q) gave only a half fold reduced LFS enzyme activity compared with only rLFS (Imai *et al*., unpublished data). Although this concluded that competitive inhibition is unlikely to work *in planta*, transgenic tobacco plants containing both LFS and E88Q mutant were produced in order to confirm this possibility (Kamoi *et al*., unpublished data).

Considering possibilities and limitations of the approaches above, it is concluded that the RNAi approach is the most suitable to investigate in order to develop an onion line with significantly reduced LFS enzyme activity.

1.5 Plant transformation

Agrobacterium tumefaciens-mediated transformation protocols of such model plants as tobacco have been used for over 20 years (Horsch *et al*., 1985). However, these methods initially did not work on monocotyledonous plants because the plants were not natural hosts of *Agrobacterium*. Some alternative methods, such as electroporation, polyethyleneglycol, and particle bombardment, have been developed to deliver foreign genes into monocotyledonous plants directly. However, it has been demonstrated that direct DNA delivery techniques frequently produce transformants that contain multiple copies of the transgenes and truncated transgenes (Spencer *et al*., 1992).

Eventually, co-cultivation techniques of particular monocotyledonous cell types were developed and *Agrobacterium tumefaciens*-mediated transformation protocols were established for rice (Hiei *et al*., 1994). As a result, many successful transformation protocols in other monocotyledonous plants using *Agrobacterium* have been reported. Eady *et al*. developed the first routine transformation system for onion (2000), leek and garlic (2005c), and also demonstrated the inheritance and expression of the transgenes in transgenic onion plants (2005a & b). However, the system has not been without its problems. Transgenic onions are produced quite inefficiently and the method takes 1 year from co-cultivation to transgenic bulb production. Moreover, the season for transformation is limited because this method uses onion immature embryos as the explant source. It is still necessary to accumulate more know-how about onion transformation and to improve the procedures.

On the contrary, *Agrobacterium*-mediated transformation of tobacco is easy and efficient. It takes approximately 2 months from transformation to plant regeneration. Therefore, it was proposed that an optimal method for producing a non-lachrymatory onion would be to assess RNAi constructs in tobacco as a model prior to attempting transformation in recalcitrant onion.

1.6 Hypothesis and key objectives

The hypothesis for this study is that an RNAi construct directed against the *lfs* gene sequence will, when introduced into a plant expressing the *lfs* gene, induce silencing of the *lfs* gene and reduce LFS enzyme activity. Furthermore, in the case of onion, the gene silencing will produce a tearless onion with significantly reduced levels of LF which would be matched by a corresponding increase thiosulfinates levels. Hence, 1-PRENCSO breakdown pathway will shift from production of LF to thiosulfinates.

The key objectives are:

- To develop a model system by transforming tobacco with a constitutively expressed *lfs* gene followed by a second transformation with a *lfs*RNAi construct and then evaluating the silencing efficiency of the RNAi by assessment of *in vitro* LFS enzyme activity.
- To demonstrate silencing of native LFS enzyme activity by transforming onion with a functional *lfs*RNAi construct (from above).
- To demonstrate that an onion with silenced LFS enzyme activity has decreased LF levels and increased thiosulfinates levels.
- To determine the changes to the volatile sulfur profile and predict the 1-PRENCSO breakdown pathway in tearless onion.

Chapter 2: Materials and methods

2.1 Construction of vectors

2.1.1 Standard techniques

High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) was used to purify DNA fragments from reaction mixtures. QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) was used to purify DNA fragments from gels. T4 DNA Ligase (Invitrogen, Carlsbad, CA, USA) was used to join DNA fragments with staggered or blunt ends. QIAprep Spin Miniprep Kit (QIAGEN) was used to purify plasmids from *Escherichia coli* cultures. *E. coli* transformation used Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). *Agrobacterium* transformation used *Agrobacterium* LBA4404 (Invitrogen). All procedures followed the manufacturer's instructions. Other standard molecular biology techniques followed those outlined in Sambrook & Russell (2001) unless otherwise stated.

2.1.2 *lfs* gene expressing vector

An *lfs* gene expressing vector was constructed using the pCAMBIA1302 binary vector (CSIRO Plant Industry, Canberra, Australia). A 510 bp DNA fragment derived from *lfs* cDNA (GenBank accession number AB089203) (Figure 2.1) at positions 53-562 was amplified by polymerase chain reaction (PCR) using 20 ng of onion genomic DNA as a template and primers (*Nco*I-LFS-f1/*Pml*I-LFSanalog-r1, Table 2.1). The PCR reaction was performed using *Taq* DNA polymerase (ABgene, Epsom, UK) with all reagents at concentrations according to the manufacturer's recommendations and subjected to the following cycling conditions: 1 cycle of 94 ^{o}C for 5 min; 35 cycles of 94 ^{o}C for 1 min; 55 ^{o}C for 1 min; 72 ^{o}C for 1 min; and a final cycle of 7 min at 72 ^{o}C using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). These primers were designed to contain the restriction sites *Nco*I and *Pml*I respectively. The PCR fragment was cleaved with *Nco*I and *Pml*I and purified from the reaction mixture using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's instructions. pCAMBIA1302 was cleaved with *Nco*I and *Pml*I to liberate the original *mgfp5* sequence at the *Nco*I and *Pml*I sites between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) terminator (Figure 2.2)**,** and the resulting fragments were separated by gel electrophoresis and the vector backbone was purified from the gel using

the QIAquick Gel Extraction Kit (QIAGEN) according to the manufactures instruction. The 510 bp *lfs* fragment was ligated into the vector backbone using T4 DNA Ligase (Invitrogen) according to the manufactures instruction. The ligated vector was then transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) according to the manufactures instruction. Transformed colonies were identified by growth on LB medium containing 50 mg/L kanamycin. Plasmid was isolated from these colonies using a QIAprep Spin Miniprep Kit (QIAGEN, Germany) according to the manufactures instruction, and analysed by restriction digest, PCR, and sequencing to confirm the integrity of the newly constructed plasmid. Confirmed transformed colonies were grown to log phase and stored in 30% glycerol stocks at -80 ^{o}C. Plasmid isolated from this was used to transform electrocompetent *Agrobacterium* LBA4404 (Invitrogen) according to the manufactures instruction. Transformed colonies were selected by growth on YM medium containing 50 mg/L kanamycin and 100 mg/L streptomycin. Plasmid presence was confirmed by colony PCR. Confirmed transformed colonies were grown to log phase and stored in 30% glycerol stocks at -80 ^{o}C.

Figure 2.1 cDNA sequence of *lfs* (GenBank accession number AB089203)

The start and stop codons are highlighted in red.

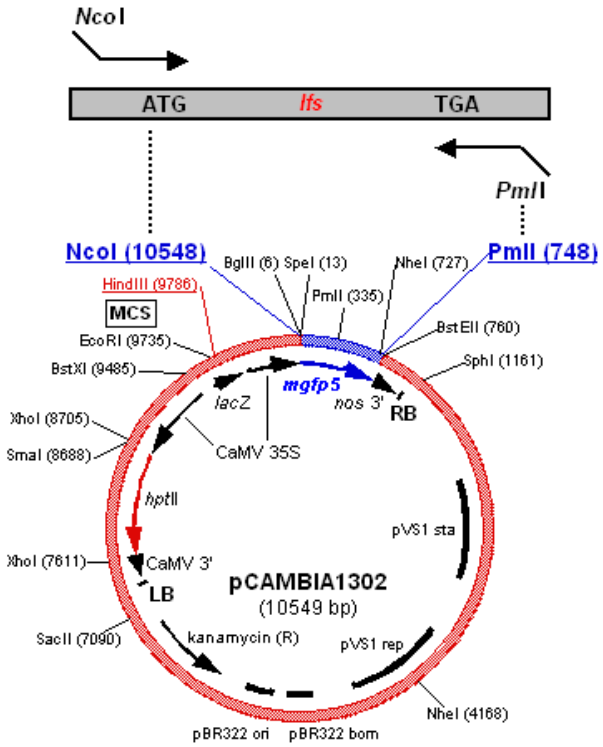


Figure 2.2 Map of pCAMBIA1302 binary vector and scheme for constructing *lfs* expressing vector.

A 510 bp DNA fragment derived from *lfs* cDNA (AB089203) at positions 53 to 562 was selected for *lfs* region and inserted into the *Nco*I and *Pml*I sites by replacing the *mgfp5* original sequence in pCAMBIA1302. LB, left T-DNA border; CaMV 3', cauliflower mosaic virus 35S terminator; *hpt*II, hygromycin phosphotransferase II; CaMV 35S, cauliflower mosaic virus 35S promoter; *lacZ*, a fragment of LacZ alpha-coding sequence; MCS, multi cloning sites; *mgfp5*, a fragment of mGFP5-coding sequence; *nos* 3', nopaline synthase terminator; RB, right T-DNA border.

2.1.3 *lfs*RNAi expressing vector

An *lfs*RNAi vector was constructed using the pART27 binary vector (Gleave, 1992) containing the pHANNIBAL-based RNAi cassette (Wesley *et al*., 2001). A 521-bp DNA fragment derived from *lfs* cDNA (GenBank accession number AB089203) (Figure 2.1) at positions 56-576 was selected for a sense *lfs* region of an RNAi construct and inserted into the *Xho*I and *Kpn*I sites in the vector pHANNIBAL (CSIRO Plant Industry) (Figure 2.3). Similarly, a 512-bp DNA fragment at positions 63-574 was selected for an antisense *lfs* region and inserted into the *Cla*I and *BamH*I sites of the pHANNIBAL (Figure 2.3). The sense and antisense fragments were amplified by PCR from the full length *lfs* sequence using primers containing the restriction sites *Xho*I and *Kpn*I (*Xho*I-LFS/*Kpn*I-LFS, Table 2.1), and *Cla*I and *BamH*I (*Cla*I-LFS/*BamH*I-LFS, Table 2.1) respectively. Amplified

fragments were cleaved with the appropriate restriction enzymes and purified from the reaction mixture using a High Pure PCR Product Purification Kit (Roche). pHANNIBAL was initially cleaved with the *Xho*I and *Kpn*I restriction enzymes and the vector backbone separated by gel electrophoresis and then purified from the gel using a QIAquick Gel Extraction Kit (QIAGEN). This cleaved vector and the sense *lfs* region were ligated using T4 DNA Ligase (Invitrogen) to create the pHANNIBAL-sense *lfs*. The ligated vector was then transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). Transformed colonies were identified by growth on LB medium containing 50 mg/L ampicillin. Plasmid was isolated from these colonies using a QIAprep Spin Miniprep Kit (QIAGEN) and analysed by restriction digest. This pHANNIBAL-sense *lfs* was cleaved with *Cla*I and *BamH*I restriction enzymes and the vector backbone, separated by gel electrophoresis, purified from the gel using a QIAquick Gel Extraction Kit (QIAGEN). This cleaved vector and the antisense *lfs* region were ligated using T4 DNA Ligase (Invitrogen) to create the pHANNIBAL-*lfs*RNAi. The ligated vector was then transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). Transformed colonies were identified by growth on LB medium containing 50 mg/L ampicillin. Plasmid was isolated from these colonies using a QIAprep Spin Miniprep Kit (QIAGEN) and analysed by restriction digest.

pART27-*lfs*RNAi-*mgfp5*ER (Figure 2.4) was created by ligating the *Not*I fragment of the pHANNIBAL-*lfs*RNAi into the *Not*I site in the pART27 binary vector containing the *mgfp5*ER cassette (Haseloff *et al*., 1997) using T4 DNA Ligase (Invitrogen). The ligated vector was then transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). Transformed colonies were identified by growth on LB medium containing 100 mg/L spectinomycin. Plasmid was isolated from these colonies using a QIAprep Spin Miniprep Kit (QIAGEN) and analysed by restriction digest, PCR, and sequencing to confirm the integrity of the newly constructed plasmid. Confirmed transformed colonies were grown to log phase and stored in 30% glycerol stocks at –80 ^{o}C. Plasmid isolated from these bacteria was used to transform electrocompetent *Agrobacterium* LBA4404 (Invitrogen). Transformed colonies were selected by growth on YM medium containing 100 mg/L spectinomycin and 100 mg/L streptomycin and plasmid presence confirmed by colony PCR. Confirmed transformed colonies were grown to log phase and stored in 30% glycerol stocks at –80 ^{o}C.

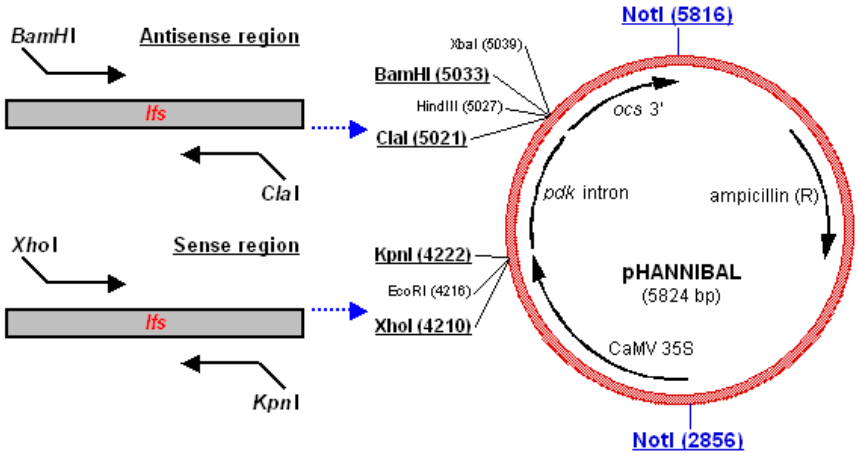


Figure 2.3 Map of pHANNIBAL vector and scheme for constructing *lfs*RNAi cassette.

A 521 bp DNA fragment derived from *lfs* cDNA (AB089203) at positions 56 to 576 was selected for a sense *lfs* region of an RNAi cassette and integrated into *Xho*I and *Kpn*I sites in pHANNIBAL. Similarly, a 512 bp fragment at positions 63 to 574 was selected for an antisense *lfs* region and inserted into *Cla*I and *Bam*HI sites. The RNAi cassette was excised from the pHANNIBAL using the restriction enzyme *Not*I. CaMV 35S, cauliflower mosaic virus 35S promoter; *pdk* intron, intron region of pyruvate orthophosphate dikinase; *ocs* 3', octopine synthase terminator.

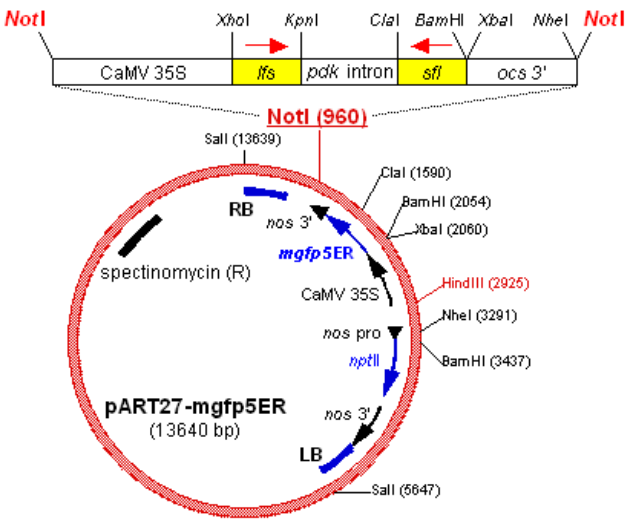


Figure 2.4 Map of pART27-*mgfp5*ER binary vector and scheme for constructing *lfs*RNAi vector.

The RNAi cassette, excised from the pHANNIBAL using the restriction enzyme *Not*I, was inserted into the *Not*I site in the pART27-*mgfp5*ER. RB, right T-DNA border; CaMV 35S, cauliflower mosaic virus 35S promoter; *lfs*, a sense fragment of LFS-coding sequence; *pdk* intron, intron region of pyruvate orthophosphate dikinase; *sfl*, an antisense fragment of LFS-coding sequence; *ocs* 3', octopine synthase terminator; *nos* 3', nopaline synthase terminator; *mgfp5*ER; a fragment of mGFP5ER-coding sequence; *nos* pro, nopaline synthase promoter; *npt*II, neomycin phosphotransferase II; LB, left T-DNA border.

Table 2.1 Primers sequences used for constructing vectors.

Primer name	Primer sequence (5' - 3')
*Nco*I-LFS-f1	CATGCCATGGAGCTAAATCCTGGTGC
*Pml*I-LFSanalog-r1	TCACACGTGTCAAGCACTGCAAACCTCTT
*Xho*I-LFS	CCGCTCGAGCTAAATCCTGGTGC
*Kpn*I-LFS	CGGGGTACCGATATTCAAGATCAAG
*Cla*I-LFS	CCATCGATATTCAAGATCAAGC
*Bam*HI-LFS	CGCGGATCCTGGTGCACCTGC

The bases underlined represent the restriction enzyme sites.

2.2 Production of transgenic plants

2.2.1 Tobacco leaf disc transformation

i) Plant material

Virus-free tobacco (*Nicotiana tabacum*, Petit Havana SR1) plants were multiplied on MS30 medium consisting of MS salts and vitamins (Murashige & Skoog, 1962) plus 30 g/L sucrose and 3.5 g/L Phytagel™ (SIGMA, St. Louis, MO, USA). The gelling agent was added after the pH was adjusted to 5.8 with 0.1 M KOH, and then the medium was autoclaved at 121^{o}C for 15 min. Fifty ml aliquots of the medium were dispensed into sterilized plastic pottles (Vertex Pacific, Hamilton, New Zealand). Plants were subcultured every 3-4 weeks and incubated at 25^{o}C under cool white fluorescent lamps (80-100 μmol/m^{2}/s; 16 h photoperiod).

ii) Bacteria

Agrobacterium tumefaciens strain LBA4404 containing the binary vector pART27-*lfs*RNAi-*mgfp5*ER or pCAMBIA1302-*lfs* was used for transformation. A 0.5 ml aliquot of frozen glycerol stock was inoculated into 50 ml of LB media containing 100 mg/L streptomycin and 100 mg/L spectinomycin for the pART27 based vector, or 100 mg/L streptomycin and 50 mg/L kanamycin for the pCAMBIA1302 vector. These were cultured overnight on a rotary shaker at approximately 200 rpm at 28^{o}C. The absorbance at 660nm of the overnight *Agrobacterium* cultures was adjusted to approximately 0.5 by dilution with LB media.

iii) Transformation

Tobacco leaf discs from *in vitro* grown cultures were used for the transformation (Horsch *et al*., 1985). Cut leaf discs (1cm in diameter) were infected with *Agrobacterium* cultures, by dipping the leaf discs into the *Agrobacterium* for approximately 2 minutes. Following these discs were blotted dry with filter paper and placed on MS30 medium containing 1 mg/L benzylaminopurine (BAP) for 3 days. After co-cultivation, the discs were transferred to MS30 plus 1 mg/L BAP, 200 mg/L timentin (SmithKline Beecham, Pittsburgh, PA, USA), and 10 mg/L geneticin or hygromycin, depending on which T-DNA insert was being selected for. Approximately 3-4 weeks later, the shoots regenerating from the callus were isolated and subcultured to MS30 media containing 200 mg/L timentin and 10 mg/L geneticin or hygromycin respectively. Thereafter, healthy and well-formed shoots were transferred to MS30 media plus 100 mg/L timentin and 10 mg/L geneticin or hygromycin to initiate root growth.

2.2.2 Immature onion embryo transformation

Immature onion embryos were transformed according to Eady *et al*. (2000).

i) Plant material

Field-grown umbels of onion (*Allium cepa* L.) were used as a source of immature embryos. A mild hybrid mid-daylength onion (cv. 'Enterprise') and a pungent open-pollinated onion (cv. 'Pukekohe Longkeeper') were field grown in Christchurch, New Zealand. A pungent dehydration mid-daylength onion (breeding line from Sensient Dehydrated Flavors, CA, USA) was field grown in California, USA.

ii) Bacteria

Agrobacterium tumefaciens strain LBA4404 containing the binary vector pART27-*lfs*RNAi-*mgfp5*ER was used for transformation. The pART27 vector was inoculated into 50 ml of LB media as outlined in 2.2.1 above. The overnight *Agrobacterium* cultures were replenished with an equal volume of LB media containing the same amount of antibiotic reagents and 100 mM acetosyringone and grown for at least 3 hours. Agrobacteria were precipitated by 5-min centrifugation at 4,000 rpm and resuspended in P5 media (Eady *et*

al., 1998a & b) containing 200mM acetosyringone. The absorbance at 550nm of the resuspended solutions was adjusted to approximately 0.3.

iii) Isolation of embryos

Approximately 10 g of immature seed, with dark seed coat but soft endosperm, was isolated from onion umbels, placed in about 10 ml of distilled water, and then stored at 4^{o}C overnight. After incubation, the seeds were sterilized by a 30 second wash ethanol followed by 10 min. in 1.44% sodium hypochlorite and four rinses in sterile distilled water. Immature embryos were isolated from the seeds and cut into small pieces. Batches of fifty embryos were then placed in 1.5 ml tubes containing 50 µl of P5 media.

iii) Transformation

A 400 µl aliquot of the *Agrobacterium* culture was added to the embryos and the mixture was vortexed for 30 seconds. Following this treatment, the embryos were placed in a vacuum chamber at 2500 psi for 30 minutes. After this they were carefully removed from the tube and air dried for approximately 2 minutes on filter papers before transfer and careful plating out onto P5 medium. After 5 days co-cultivation on P5 media, the embryos were transferred to P5 plus 100 mg/L timentin and cultured for 1 week. Embryo pieces expressing GFP were then transferred to P5 plus 100 mg/L timentin and 10 mg/L geneticin. The embryos were subcultured to fresh medium every 2 weeks until transgenic secondary embryogenic cultures were obvious. These embryogenic cultures were transferred to SM4 shooting medium (Eady *et al.*, 1998a & b) containing 100 mg/L timentin and 10 mg/L geneticin and subcultured on to fresh SM4 media every 3 weeks for 12 weeks. Plant tissues producing well-formed and healthy shoots were transferred to 1/2MS30 rooting media (Eady *et al.*, 1998a & b) containing 10 mg/L geneticin.

Plants material exhibiting a healthy shoot and well-formed roots were transferred onto soil in the glasshouse (Crop & Food Research). The plants was grown in the onion soil mix (Eady *et al*., 2002), in pots on a plastic tray with water and covered with plastic film to prevent water evaporating. After one to two weeks of acclimatization pots were transferred to the bench and the plastic film removed. Following that plants were repotted as required.

2.3 Analyses

2.3.1 Sample preparation for analysis

i) Tobacco plants

Young and healthy *in vitro* plant leaves were harvested into dry ice and used for each analysis after crushing.

ii) Onion plants

Preliminary analysis of shoot culture, glasshouse leaves, and bulbs (data not shown) demonstrated that *lfs* transcripts and LFS activity levels altered within (top to bottom) leaf sample or within (inner to outer) bulb sample. To counter this both leaf and bulb samples were grinded uniformly into powder for analysis. Approximately 2 g of young healthy leaf (10-20 cm in length) from the second or third innermost leaf of actively growing glasshouse plants and top half mature bulb samples bisected longitudinally were harvested into liquid N_2. Samples were disrupted by grinding in liquid nitrogen and stored at -80ºC until ready for analysis. Half of each leaf and bulb sample was freeze-dried, disrupted by grinding, and stored at room temperature in a desiccator. Frozen leaf samples were used for PCR analysis, Southern analysis, siRNAs analysis, and LF assay. Freeze-dried leaf samples were used for RT-PCR analysis, quantitative real-time RT-PCR (qRT-PCR) analysis, western analysis, and LFS enzyme activity assay. Freeze-dried bulb samples were used for LFS enzyme activity assay, LF assay, pinking assay, and thiosulfinates profile analysis. Fresh leaf samples were used for headspace GCMS analysis. To avoid any confusion only a single plant from each line was used for analysis, or if a clone was used then Southern analysis was performed to demonstrate the clonal nature of the plants.

2.3.2 PCR analysis

PCR analysis was used for testing the presence of transgenes. Genomic DNA was extracted from approximately 50~100 mg of plant leaf material using a DNeasy Plant Mini Kit (QIAGEN) according to the manufacture's instruction. Polymerase chain reaction (PCR) was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using primers specific for the transgenes (Table 2.2, Figure 2.5), 18S rRNA

primers (QuantumRNA™ 18S Internal Standards, Ambion), and *Taq* DNA polymerase (QIAGEN or ABgene) with all reagents at concentrations according to the manufacturer's recommendations. The PCR reaction was subjected to the following cycling conditions: 1 cycle of 95°C for 15 min; 35 cycles of 94°C for 1 min; 60°C for 1 min; 72°C for 1 min; and a final cycle of 10 min at 72°C for QIAGEN polymerase, and similarly, 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 1 min; 60°C for 1 min; 72°C for 1 min; and a final cycle of 7 min at 72°C for ABgene polymerase. Amplified DNA fragments were separated by electrophoresis in a 1% or 2% agarose gel containing ethidium bromide and visualized under UV light.

2.3.3 Southern analysis

Genomic DNA was extracted based on the method of Doyle & Doyle (1990) from tobacco leaf materials and using a Nucleon PhytoPure (GE Healthcare Bioscience, Little Chalfont, UK) from onion leaf materials according to manufacturer's instructions.

Southern blot analysis was performed to identify individual transformation events essentially according to the method described by Eady *et al*. (2000). The genomic DNA was digested using *Hin*dIII. Ten μg of the of the digested DNA from tobacco or 20 μg of the DNA from onion was electrophoresed on a 0.8% TBE-buffered agarose gel. The separated DNA was transferred onto a Zeta-Probe GT Blotting Membrane (Bio-Rad, Hercules, CA, USA) and hybridized with PCR-amplified DNA probes. A 525 bp *lfs* probe was amplified using primers (NcoI-LFS-f1/Pml-LFSana-r1, Table 2.1, Figure 2.5), and similarly, a 834 bp *gfp* probe was amplified using primers (GFP-a/GFP-b, Table 2.2, Figure 2.5). Both probes were labeled radioactively with [α-^{32}P] dCTP using a Megaprime DNA Labeling System (GE Healthcare Bioscience). Membranes were pre-hybridized with 30 ml of pre-hybridization buffer consisting of 5 X SSPE, 5 X Denhardts, 0.5% SDS, 1.5 g of Dextran sulphate, and 9 mg of denatured herring sperm DNA at 65°C overnight. Then, the labelled probe was added into the pre-hybridization buffer and hybridised at 65°C overnight. The following day membranes were washed at 65°C in 50 ml of 2 X SSC plus 0.1% SDS for 30 min and 50 ml of 1 X SSC plus 0.1% SDS for 15 min. Washed membranes were placed in cassettes containing Kodak BioMax MR films (Eastman Kodak Company, Rochester, NY, USA) and placed at -80°C. Films were developed after 1-2 weeks exposure to the membrane.

2.3.4 RT-PCR analysis

RT-PCR analysis was performed to test the presence of transcripts of transgenes in plants. Total RNA was isolated from approximately ~100 mg of plant leaf material using an RNeasy Plant Mini Kit (QIAGEN) and treated with a TURBO DNA-*free* (Ambion, Austin, TX, USA) to remove contaminated DNA following the manufacturers instructions. After DNase treatment, the RNA samples were tested for the presence of contaminating DNA by PCR. RNA was quantified using a ND-1000 Spectrophotometer (Nano Drop Technologies, Rockland, DE, USA). Approximately 1 µg or 200 ng of the total tobacco RNA, or 200 ng of the total onion RNA was reverse-transcribed to first strand cDNA using an Omniscript RT Kit (QIAGEN) and oligo-dT(21) primer or oligo-dT plus adaptor primer (5'-AACTGGAAGAATTCGCGGCCGCAGGAA$(T)_{18}$-3') at 37ºC for 60 min, and then the reverse transcriptase was inactivated at 93ºC for 3 min. PCR was performed using the first strand cDNA, equivalent to approximately 100 ng or 10 ng of the total tobacco RNA, or 10 ng of the total onion RNA, as a template. For *lfs* expressing tobacco, *lfs* transcripts were amplified using primers (LFS-f2/LFS-r2, Table 2.2, Figure 2.5). For dual transgenic tobacco plants (*lfs* + *lfs*RNAi) and transgenic tearless onion plants, *lfs*RNAi transcripts were amplified using primers (LFS-r2/OCS-r1, Table 2.2, Figure 2.5). These primers were designed at the border region between antisense *lfs* and *ocs* terminator so that they did not amplify the endogenous *lfs* transcripts. The PCR reaction was carried out using *Taq* DNA polymerase (QIAGEN) as described in section 2.3.2 above.

2.3.5 Small interfering RNAs analysis (I would like to thank my external supervisor, Dr. Colin Eady, for this analysis)

Small interfering RNAs (siRNAs) analysis was performed to examine the presence of *lfs* siRNA molecules essentially according to the protocol described by Hamilton & Baulcombe (1999). Total small RNA was isolated from approximately ~450 mg of plant leaf material using a *mir*Vana miRNA Isolation Kit (Ambion) and quantified using a GeneQuant®II (GE Healthcare Bioscience) according to the manufacturers instructions. Approximately 5 µg of the small RNA was loaded onto a 15% polyacrylamide gel and electrophoresed following *mir*Vana (Ambion) instructions. The separated small RNA was capillary transferred onto a Zeta-Probe GT Blotting Membrane (Bio-Rad) and UV

crosslinked to the membrane using Bio-Link BLX-E254 (Vilber Lourmat, Marne-la-Vallée Cedex, France) according to the manufacturers instructions. The membrane was pre-hybridized according to the method described by Hamilton & Baulcombe (1999) and then, hybridized with PCR-amplified 525 bp DNA probes of *lfs* (see 2.3.3 above) at 40ºC overnight. Excess probe was washed by 6 x SSC plus 0.2% SDS for 30 min twice and 2 x SSC plus 0.1% SDS for 30 min. The membrane was placed in a cassette containing a Kodak BioMax MR film (Eastman Kodak Company, Rochester, NY, USA) and placed at -80^{o}C. Films were developed after 1-2 weeks exposure to the membrane.

Table 2.2 Primers list for PCR, Southern, RT-PCR, and siRNAs analysis and expected product size.

Primer name	Primer sequence (5' - 3')	Product
CaMV35S-f2	AGCAGCTGACGCGTACACA	251 bp
CaMV35S-r1	CACCTTCGAACTTCCTTCCTAGA	
CaMV35S-f1	TCTGTCACTTCATCGAAAGGAC	784 bp
LFS-r2	CTCTTCGATTTTCTGACCTATCTCAGTAGC	
LFS-f5	TTCTGCAGCATTGGGCTACT	265 bp
PDK-r2	CTTCTTCGTCTTACACATCACTTGT	
PDK-f2	GCTAATATAACAAAGCGCAAGA	350 bp
antiLFS-r1	TTCAGTGCAAGTATATCGAGGGTAT	
LFS-r2	CTCTTCGATTTTCTGACCTATCTCAGTAGC	561 bp
OCS-r1	TGCACAACAGAATTGAAAGC	
pBINmgfp5ER-f1	GAGAGGGTGAAGGTGATGCAA	423 bp
pBINmgfp5ER-r1	CGATGTTGTGGCGGGTCTT	
NPT-f2	CCGTGATATTGCTGAAGAGCTT	800 bp
NOST-r2	ACCGGCAACAGGATTCAAT	
hptII-f1	CCGCAAGGAATCGGTCAATA	454 bp
hptII-r1	CCCAAGCTGCATCATCGAAA	
LFS-f2	GAATTTTGGGCCAAGGAGAAGCTGG	350 bp
NOST-r2	ACCGGCAACAGGATTCAAT	
GFP-a	ACGTCTCGAGGATCCAAGGAGATATAACA	834 bp
GFP-b	ACGTCTCGAGCTCTTAAAGCTCATCAT	
LFS-f2	GAATTTTGGGCCAAGGAGAAGCTGG	240 bp
LFS-r2	CTCTTCGATTTTCTGACCTATCTCAGTAGC	

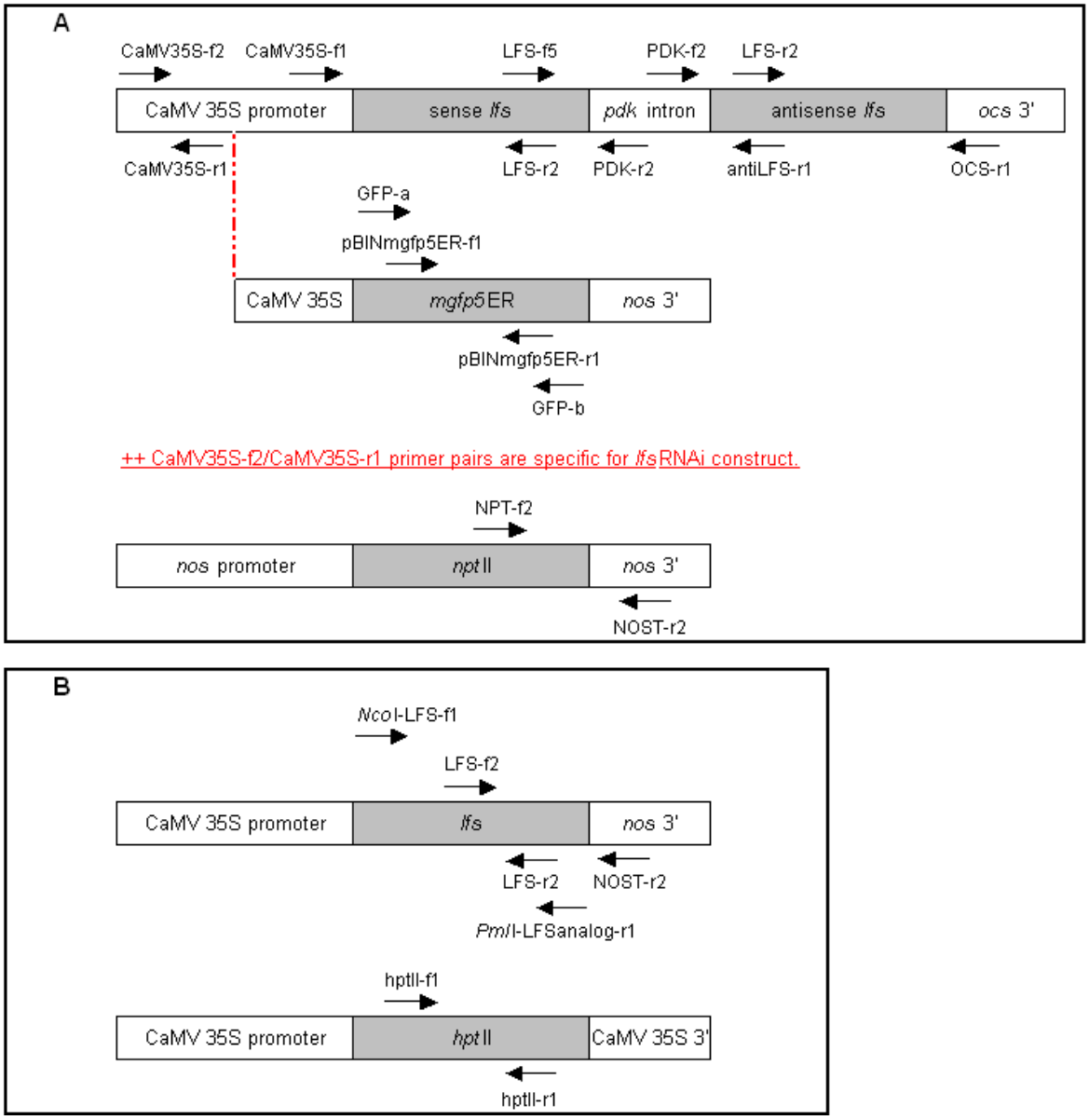


Figure 2.5 Primer sites for pART27-*lfs*RNAi-*mgfp5*ER (A) and pCAMBIA1302-*lfs* (B) vector.

lfs, a sense fragment of LFS-coding sequence; *pdk* intron, intron region of pyruvate orthophosphate dikinase; *ocs* 3', octopine synthase terminator; *mgfp5*ER; a fragment of mGFP5ER-coding sequence; *nos* 3', nopaline synthase terminator; *npt*II, neomycin phosphotransferase II; *hpt*II, hygromycin phosphotransferase II; CaMV 35S 3', cauliflower mosaic virus 35S terminator.

2.3.6 Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR (qRT-PCR) analysis was performed to examine *lfs* transcript levels in transgenic plants. Total RNA was prepared in triplicate from each plant as described above 2.3.4. Similarly, 200 ng of the each total RNA was reverse-transcribed

to first strand cDNA using oligo-dT(21) primer.

i) Dual transgenic tobacco plants (*lfs* + *lfs*RNAi)

The region between +24 and +110 in the *lfs* gene expressing construct was selected as a target to be amplified using specific primers (LFS525Quant-f1/LFS525Quant-r1, Table 2.3, Figure 2.6). The forward primer was designed at the junction of the *lfs* sequence and the plasmid DNA as this region has low homology with the *lfs*RNAi construct (Figure 2.6) so that the *lfs*RNAi transcripts would not be amplified. Tobacco *ubiquitin* sequence (GenBank accession number U66264) was selected as an internal control gene to be amplified using specific primers (tUbiQuant-f2/tUbiQuant-r2, Table 2.3). Each sequence was amplified in triplicate on the same plate in 25 µl reaction volume containing the each first strand cDNA, equivalent to 10 ng of the total RNA, using a QuantiTect SYBR Green PCR Kit (QIAGEN) according to the manufacturer's instruction. PCR, performed in an ABI PRISM 7700 (Applied Biosystems), was set with the following PCR cycle program: 15 min at 95ºC and 40 cycles, 15 sec at 94ºC, 30 sec at 57ºC, and 30 sec at 72ºC.

Each standard curve was calibrated by using the five concentrations of RT-PCR amplicon containing the targeted *lfs* or *ubiquitin* sequence, such as 100, 1000, 10000, 100000, and 1000000 copies per reaction. The *lfs* standard DNA fragment was amplified using primers (LFS525STD-f1/LFS525STD-r1, Table 2.3, Figure 2.6) and *Taq* DNA polymerase (QIAGEN), and purified from the PCR reaction mixture using a QIAquick PCR Purification Kit (QIAGEN) according to manufacture's instructions. Similarly, the *ubiquitin* standard DNA fragment was amplified using primers (tUbiSTD-f1/tUbiSTD-r1, Table 2.3). The process for drawing threshold lines was followed by Kuribara *et al*. (2002). The relative *lfs* mRNA level was determined by dividing the *lfs* copy number by the internal standard gene copy number.

Table 2.3 Primers list for tobacco qRT-PCR analysis and expected product size.

Primer name	Primer sequence (5’ - 3’)	Product
LFS525Quant-f1	GGACTCTTGACCATGGAGCTAAA	87 bp
LFS525Quant-r1	GACTTTGCCGCTCCATTTTC	
tUbiQuant-f2	CCAGAAAGAGTCAACCCGTCA	101 bp
tUbiQuant-r2	AACAGACACAGTCAATCAACACGAC	
LFS525STD-f1	CATTTCATTTGGAGAGAACACGG	651 bp
LFS525STD-r1	ATGATAATCATCGCAAGACCGG	
tUbiSTD-f1	AGCTCTGACACCATCGACAATG	255 bp
tUbiSTD-r1	CAAAGCACATCACGACCACAA	

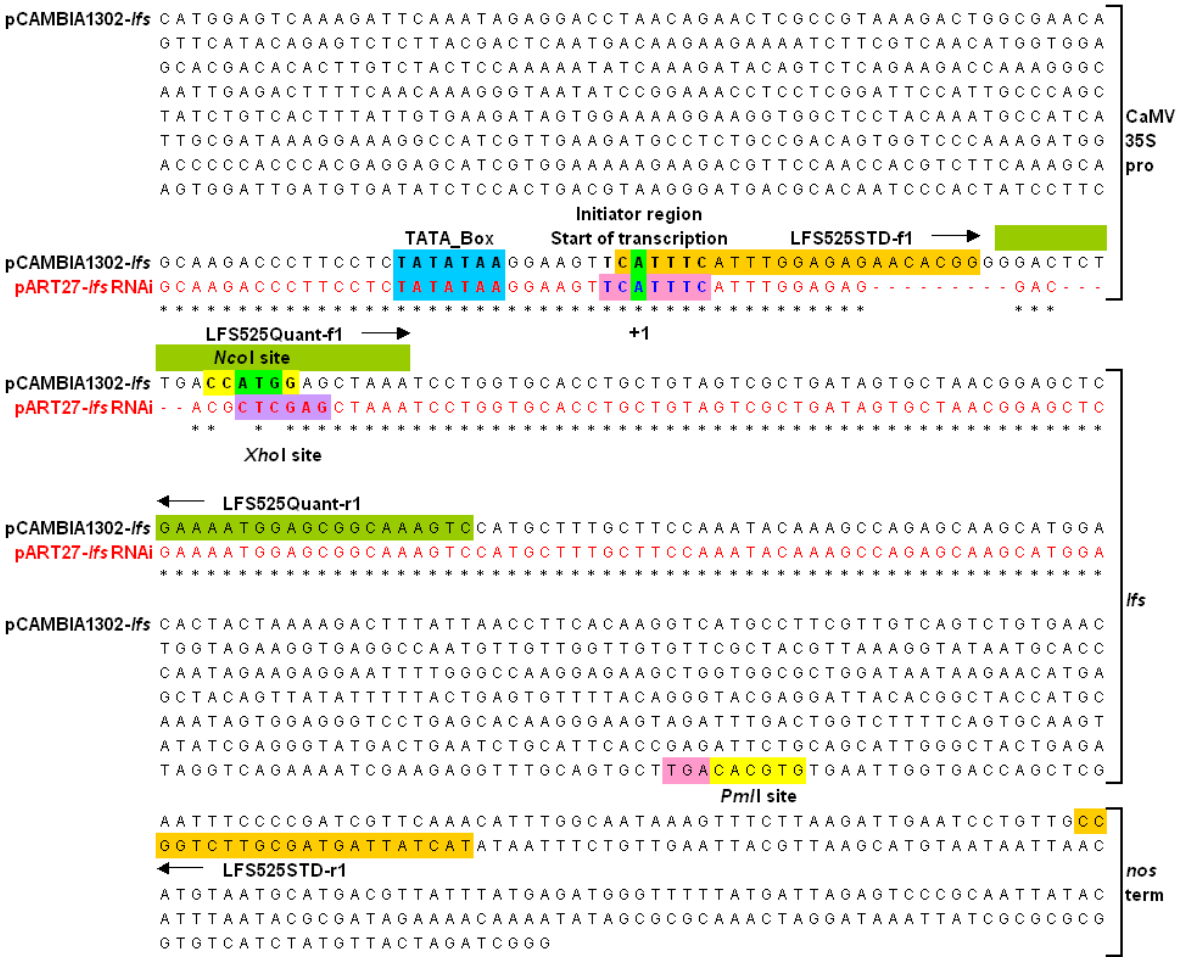


Figure 2.6 Full DNA sequence of *lfs* expressing construct, partial sequence of *lfs*RNAi expressing construct (red), and primer sites for tobacco qRT-PCR analysis.

LFS525Quant-f1 primer was designed at the junction of the *lfs* and CaMV 35S promoter sequence in pCAMBIA1302-*lfs* vector as this region has low homology with the *lfs*RNAi construct so that the *lfs*RNAi transcripts would not be amplified. *, homologous DNA sequences. CaMV 35S pro, cauliflower mosaic virus 35S promoter; *lfs*, a fragment of LFS-coding sequence; *nos* term, nopaline synthase terminator.

ii) Transgenic tearless onions

The 5' untranslated region (UTR) of the *lfs* sequence (GenBank accession number AB089203) was selected as a target to be amplified using specific primers (LFSQuant-f1/LFSQuant-r1, Table 2.4, Figure 2.7). The forward primer was designed at the 5' UTR so that the *lfs*RNAi transcripts would not be amplified. An onion putative *ubiquitin* sequence (GenBank accession number AA451588) was selected as an internal control gene to be amplified using specific primers (UbiQuant-f1/UbiQuant-r1). Each sequence was amplified as described above.

Similarly, each standard curve was calibrated by using the five concentrations of RT-PCR amplicon containing the targeted *lfs* or putative *ubiquitin* sequence. The *lfs* standard DNA fragment was amplified using primers (LFS-f3/LFS-r5, Table 2.4, Figure 2.7) The internal standard DNA fragment was amplified using primers (Ubi-f1/Ubi-r1). The process for drawing threshold lines was followed by Kuribara *et al*. (2002). The relative *lfs* mRNA level was determined by dividing the *lfs* copy number by the internal standard gene copy number.

Table 2.4 Primers list for onion qRT-PCR analysis and expected product size.

Primer name	Primer sequence (5' - 3')	Product
LFSQuant-f1	ACGTTATATCAAGAAGATTGTCCAA	85 bp
LFSQuant-r1	TCCGTTAGCACTATCAGCGAC	
UbiQuant-f1	ACGATTACACTAGAGGTGGAGAGCTC	107 bp
UbiQuant-r1	CCTGCAAATATCAGCCTCTGCT	
LFS-f3	CAATTCAGACTCACATTACG	678 bp
LFS-r5	AACACACAACACTCAGTCTTAC	
Ubi-f1	CAACATCCAAAAGGAGTCGACC	275 bp
Ubi-r1	CACGAAGACGGAGCACAAG	

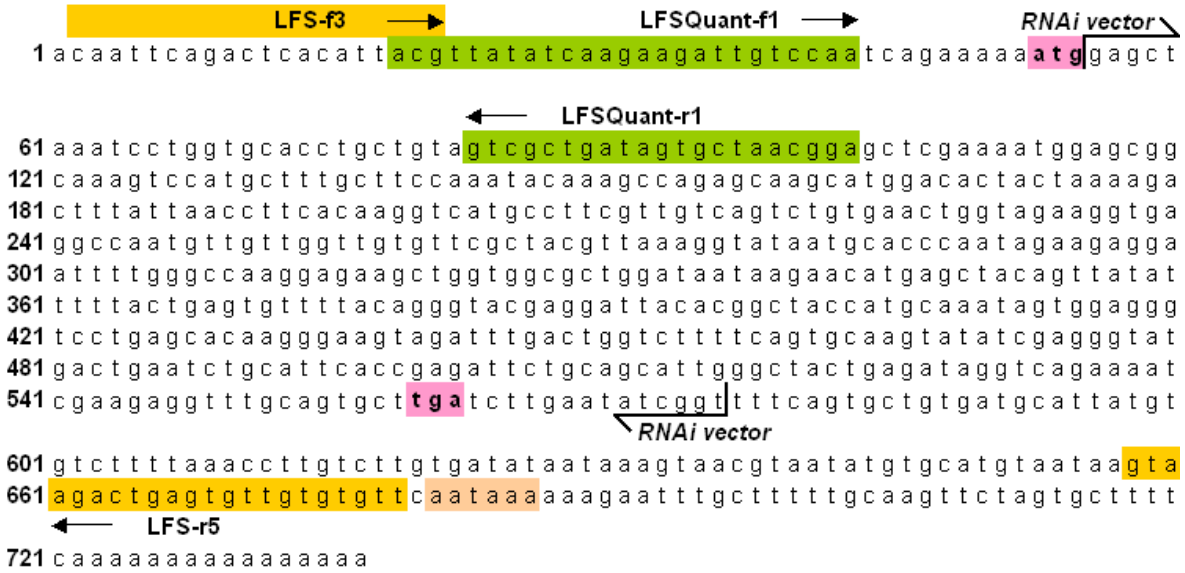


Figure 2.7 *lfs* cDNA sequence and primer sites for onion qRT-PCR analysis.

LFSQuant-f1 primer was designed at 5' UTR region of *lfs* sequence so that the *lfs*RNAi transcripts would not be amplified.

2.3.7 Western analysis

Western analysis was performed to examine the presence of LFS protein in transgenic plants. For tobacco plants, total soluble protein was extracted from approximately 100 mg of frozen leaf material by PBS buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) at a ratio of 1 g tissue:2 ml buffer and centrifuged at 16,000 x g at 4ºC

for 5 min. For onion plants, similarly, the protein was extracted from approximately 20 mg of freeze-dried leaf powder material in PBS buffer at a ratio of 1 g tissue:17.5 ml buffer. The supernatant fraction was used as a protein sample and quantified using a Protein Assay Kit (Bio-Rad) with standard curves generated using bovine serum albumin (BSA) (SIGMA, St. Louis, MO, USA). Fifty µg of the tobacco soluble protein or 100 µg of the onion soluble protein containing Laemmli Sample Buffer (Bio-Rad) was loaded onto a 15% polyacrylamide gel and electrophoresed. The separated protein was electrically blotted using a Trans-Blot SD Cell (BIO-RAD) onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) following the manufacturer's instructions. The blot was blocked by BSA solution consisting of 0.1 M maleic acid, 0.15 M NaCl, and 1% BSA for 1 h and then, incubated for 1 h with an LFS polyclonal antibody raised in rabbits (Imai *et al*., 2003). Excess antibody was removed by two 20 min washes in PBS buffer and the membrane was incubated for 1 h with an anti-rabbit IgG solution. Again, excess antibody was removed by three 20 min washes in PBS buffer and the LFS protein-antibody conjugates were detected using a HRP Conjugate Substrate Kit (Bio-Rad) according to the manufacturer's instructions. The detected membranes were incorporated by ChemiDoc XRS (Bio-Rad) according to the manufacturer's instructions. Both LFS and degraded LFS bands were selected and quantified using Quantity One Software ver. 4.6 (Bio-Rad) according to the manufacturer's instructions.

2.3.8 LFS enzyme activity assay

LFS enzyme activity was evaluated by lachrymatory factor (LF) forming capability. For tobacco plants, total soluble protein was extracted from approximately 100 mg of frozen leaf material by PBS buffer at a ratio of 1 g tissue:10 ml buffer and quantified according to the method as described above (2.3.7). For onion plants, similarly, the protein was extracted from approximately 15 mg of freeze-dried leaf or bulb material by PBS buffer at a ratio of 1 g tissue:100 ml buffer. For the LF-forming capability assay, 10 µl of the extracted protein solution, 40 µl of purified garlic alliinase (555 nKats/mg protein), and 20 µl of 20 mg/ml *trans*-1-PRENCSO purified from onion (Imai *et al*., 2006b) were mixed and left for 3 min at room temperature. Two µl of the resulting mixture was analysed by HPLC (SHIMADZU, Kyoto, Japan) using an ODS Hypersil column (250 mm x 4.6 mm, particle size 5 µm) (Thermo Fisher Scientific, Waltham, MA, USA) at 35ºC. The column was eluted with 30% (v/v) acidic methanol (pH 3.3) at a flow rate of 0.6 ml/min and LF

was monitored by SPD-10AV UV-VIS Detector (SHIMADZU) at 254 nm. The LFS enzyme activity was assessed by calculating LF peak area (LPA) per ng of protein in 2 µl of the reaction mixture.

2.3.9 Lachrymatory Factor (LF) assay (I would like to thank Dr. Noel Porter for this assay)

The lachrymatory factor (LF) assay was performed to examine the LF production when onion tissue is crushed. LF was measured using gas chromatography (GC) basically based on the method described by McCallum *et a*l. (2005). Approximately 100 mg of frozen leaf powder was suspended with 500 µl of deionised water and held at room temperature with stirring every minute. Three min after the initial mixing, 500 µl of dichloromethane containing 0.06 µl/ml of heptane thiol as an internal standard was added and rolled gently for 2 min to extract the sulfur compounds. The dichloromethane phase was transferred to a GC auto-injector vial for immediate analysis. Similarly, approximately 30 mg of freeze-dried bulb powder was suspended with 500 µl plus seven times the sample weight of deionised water to replace the water removed by freeze-drying and the sulfur compounds were extracted as described above. One µl of the dichloromethane phase was injected with splitless mode at 160ºC into a 6890 GC (Agilent Technologies, Santa Clara, CA, USA). GC separation was carried out using a HP-Innowax column (30 m, 0.32 mm id, 0.25 µm film thickness, Agilent Technologies). The carrier gas was hydrogen at a constant 60 cm/sec and the column temperature was isothermal at 55ºC. LF was detected using a flame photometric detector (FPD) at 180ºC. The detector was calibrated against the internal standard. The sulfur samples were prepared in triplicate from each leaf and bulb powder sample, and GC analysis was performed in duplicate for each extracted sulfur sample. Mean (n = 6) LF levels were expressed as ng LF/g fresh weight.

2.3.10 Pinking assay (I would like to thank Masahiro Kato for this assay)

trans-1-PRENCSO-derived thiosulfinate reacts with glycine and formaldehyde to develop pink colour pigments (Imai *et al*., 2006a & b). The thiosulfinate levels were evaluated by measuring the pigments.

The range and reliability of the 'pinking assay' were examined. Several volumes of 20 mg/ml *trans*-1-PRENCSO or 2-PRENCSO (alliin) (0-850 n mol) were mixed with 40 µl of 5% glycine and 145 µl of purified garlic alliinase (555 nKats/mg protein) (Imai *et al*., 2006b). After 30 min at 25ºC, insoluble matter was removed by centrifugation at 16,000 x *g* at 25ºC for 3 min, and 10 µl of 100 ppm of formaldehyde solution was added to the 100 µl of the supernatant. This was then incubated at 40ºC for 6 h and then the absorbance at 520 nm was measured using a UV-3100PC spectrophotometer (SHIMADZU). Each assay was performed in triplicate.

The 'pinking assay' was developed as follows: three sets of 30 mg of freeze-dried onion bulb powder was resuspended in 200 µl of 1% glycine solution with and without 15 µg of recombinant LFS (rLFS) protein equivalent to 1 to 0.5 times normal physiological levels of LFS. After 30 min at 25ºC, insoluble matter was removed by centrifugation and 10 µl of 100 ppm formaldehyde solution was added to 100 µl of the supernatant. This was then incubated at 40ºC for 6 h and then absorbance at 520 nm was measured by spectrophotometer. Increasing absorbance (pinking) demonstrated the relative abundance of 1-propenyl-containing thiosulfinates.

2.3.11 GC-MS analysis

2.3.11.1 Solvent extraction (SE) analysis

i) Sample analysis

Thiosulfinates profile analysis was performed according to Arnault's (2000) method with slight modification. Forty mg of freeze-dried onion bulb powder from each line was incubated in 400 µl deionised water at room temperature and vortexed gently. After 80 min, 200 µl of diethyl ether (containing 0.01 µl benzyl alcohol as an internal standard) was added and vortexed briefly. After 1 min centrifugation at 16,000 x *g*, the diethyl ether phase was transferred to a GC auto-injector vial for immediate analysis. One µl was injected into a 5975C inert XL MSD MS (Agilent Technologies) equipped with a 7890A GC system (Agilent Technologies). Data processing used MSD ChemStation Data Analysis (Agilent Technologies). GC separation was achieved using a 15 m x 0.32 mm i.d. with a thick coating (5 µm) DB-1 column (Agilent Technologies). The carrier was 99.999% helium at 3.5 ml/min and the column temperature program was 5^{o}C/min from 70

to 250°C. The injector was set for splitless mode and at 200°C. The transfer line and detector temperature was held at 150°C and total ion chromatograms and mass spectra were analysed with the electron impact mode.

ii) Model study

Ten µl of 20 mg/ml purified onion *trans*-1-PRENCSO equivalent to 40 mg of freeze-dried onion bulb powder from transgenic plant line O3 and 20 µl of purified garlic alliinase (555 nKats/mg protein) (Imai *et al*., 2006b) mixed with or without 2.5 µg of rLFS were incubated at room temperature for 3 or 80 min. Then, the volatile compounds were extracted with 200 µl diethyl ether (containing 0.01 µl benzyl alcohol as an internal standard) and injected into GC-MS as described above.

2.3.11.2 Headspace solid-phase microextraction (SPME) analysis

i) Sample analysis

Headspace solid-phase microextraction (SPME) analysis was performed to examine the odour difference between transgenic and non-transgenic onion plants according to Hori's (2007) method with slight modification. Five gram of fresh leaf materials from each line was put into a 20 ml SPME vial and covered with lid immediately then left for 30 min at room temperature. The head space of the vial was extracted with SPME (100 µm polydimethylsiloxane; Supelco) at room temperature for 10 min. The fiber was inserted into a 5975C inert XL MSD MS (Agilent Technologies) equipped with a 7890A GC system (Agilent Technologies). Data processing used MSD ChemStation Data Analysis (Agilent Technologies). GC separation was achieved using a 30 m x 0.25 mm i.d. with 0.25 µm film DB-5ms column (Agilent Technologies). The carrier was 99.999% helium at a column pressure of 100 kPa and the column temperature program was 5 min at 35°C, 4°C/min to 200°C, 16 min at 200°C, 10°C/min to 250°C. The injector was set for splitless mode and at 220°C, and the desorption time was 3 min. Transfer line and detector temperature was held at 150°C and total ion chromatograms and mass spectra were analysed with the electron impact mode.

ii) Model study

Two hundred µl of 20 mg/ml purified onion *trans*-1-PRENCSO (equivalent to 800 mg of freeze-dried onion bulb powder from the O3 line) and 200 µl of purified garlic alliinase (555 nKats/mg protein) (Imai *et al*., 2006b) mixed with or without 12.5 µg of rLFS were incubated at room temperature for 30 min in the SPME vial. The head space of the vial was extracted with SPME (100 µm polydimethylsiloxane; Supelco) at room temperature for 10 min and then the fiber was inserted into GC-MS as described above.

2.3.12 Statistical analysis

In order to evaluate silencing efficacy in transgenic plants compared with each counterpart, the data from qRT-PCR, LFS enzyme activity assay, and LF assay was analysed by using Dunnett's multiple comparison test (Dunnett, 1955 & 1964) and the significance level used was $P<0.05$.

Chapter 3: Gene silencing assessment for *lfs* gene using tobacco as a model plant

3.1 Introduction

RNA interference (RNAi) is a common phenomenon in animals, fungi and plants (Waterhouse *et al*., 1998; Hamilton & Baulcombe 1999). In plants, RNAi is thought to be not only a regulation system for endogenous genes (Baulcombe, 2004) but also a defensive system against viruses (Waterhouse *et al*., 2001). Over the past few years RNAi has become a powerful tool to study the role of target genes, or change metabolite profiles, in various plants such as Arabidopsis (Kerschen *et al*., 2004), tobacco (Wesley *et al*., 2001), rice (Miki *et al*., 2005), wheat (Travella *et al*., 2006), corn (McGinnis *et al*., 2007), tomato (Davuluri *et al*., 2005), opium poppy (Allen *et al*., 2004), coffee (Ogita *et al*., 2003), and cotton (Sunilkumar *et al*., 2006). However, some reports have observed that in some transgenic lines RNAi failed or silenced inefficiently (Kerschen *et al*., 2004; Xiong et al., 2005; McGinnis *et al*., 2007). One reason for this is failure to express the integrated dsRNA coding sequence. Several studies have reported that position effect and copy number of a transgene affect its expression level (Gallie, 1998; Matzke & Matzke, 1998). Spiker and Thompson (1996) demonstrated that transgene expression is silenced by methylation when multiple inserts are integrated at a single locus, but that expression is strong and unaffected by gene silencing when transgenes are integrated with flanking matrix attachment regions (MARs). In addition, certain target RNA may not be degraded efficiently because of the sequence composition, gene expression patterns, and RNA turnover rate. Target sequences have their own inherent sensitivity to RNAi (Kerschen *et al*., 2004). Undesirable effects of RNAi include 'off-target' effects which silence non-target genes. These arise when full or partial complementarity between the siRNA and random mRNA transcripts occurs. It has been reported that 'off-target' effects can generate abnormal phenotypes although most reports concerning the effects are due to partial homology of siRNA to the 3' untranslated region in the genes of animals (Lin *et al*., 2005; Birmingham *et al*., 2006). Thus it is important to have a detailed knowledge of the target gene, the silencing sequences, and several transgenic events for analysis in order to evaluate the silencing potential.

The wider application of RNAi to the study of genes in unusual secondary pathways within recalcitrant or poorly studied plant species is enormous. However, transformation and analysis of such species can be time-consuming and problematic, especially if only a few

transformants are produced and RNAi silences inefficiently. For example, onion transformation is problematic, inefficient and takes about one year from co-cultivation to transgenic bulb production (Eady & Hunger, 2008). Yet, onion contains an fascinating secondary sulfur pathway of interest to biochemists and which could be better understood through RNAi investigations.

We have used components of this sulfur pathway to demonstrate the application of a model plant system for RNAi silencing of unusual secondary pathway genes. Lachrymatory factor synthase (LFS) is the last enzyme in the conversion of *trans*-*S*-1-propenyl-L-cysteine sulfoxide (1-PRENCSO) to lachrymatory factor (LF, propanthial *S*-oxide) in onion. Without LFS, the unstable 1-propenyl sulfenic acid will spontaneously convert into thiosulfinates (Imai *et al*., 2002) responsible for onion's characteristic flavours and bioactivity (Griffiths *et al*., 2002; Rose *et al*., 2005). Silencing *lfs* expression by RNAi could produce a non-lachrymatory (tearless) onion which accumulates thiosulfinates thus enhancing the onions health giving characteristics.

Currently, to test this theory the development of a number of RNAi vectors followed by their transformation into onion would be required. To test several RNAi cassettes directly in onion and to obtain several events for analysis would be a considerable undertaking. By contrast, in this chapter we demonstrated the quick and simple assessment of an *lfs*RNAi silencing cassette against an *lfs* gene through the use of tobacco as a model host for this intriguing secondary pathway gene.

3.2 Results

3.2.1 Production of *lfs* expressing transgenic tobacco plants

3.2.1.1 Plant selection and regeneration

pCAMBIA1302-*lfs* binary vector (Figure 3.1) was introduced, via *Agrobacterium*-mediated transformation, into tobacco plants (Petit Havana SR1) to produce *lfs* expressing transgenic tobacco plants. The transformed tissue regenerated and rooted well in media containing hygromycin. All events grew and formed morphologically equivalent plants to non-transgenic tobacco plant (data not shown).

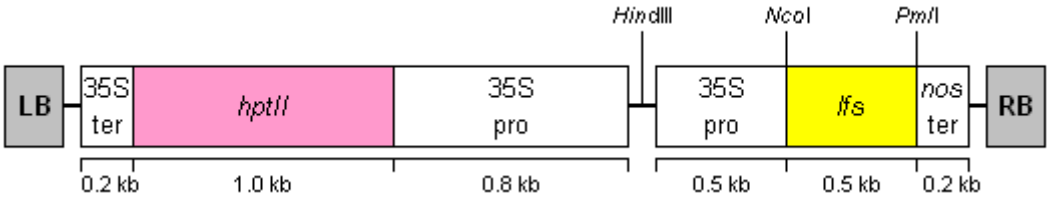


Figure 3.1 T-DNA region of pCAMBIA1302-*lfs* binary vector.

A 510 bp DNA fragment derived from *lfs* cDNA (AB089203) at positions 53 to 562 was selected for *lfs* region and inserted into the *Nco*I and *Pml*I sites by replacing *mgfp5* original sequence in pCAMBIA1302. LB, left T-DNA border; 35S ter, cauliflower mosaic virus 35S terminator; *hptII*, hygromycin phosphotransferase II; 35S pro, cauliflower mosaic virus 35S promoter; *lfs*, a fragment of LFS-coding sequence; *nos* ter, nopaline synthase terminator; RB, right T-DNA border.

3.2.1.2 PCR analysis

Eight transgenic plants were chosen for analysis. PCR analysis was used to test whether the transgenes were present in the transgenic plants. In order to determine whether the extracted DNA was suitable for PCR amplification, PCR using *18SrRNA* specific primers was performed. PCR amplification of *18SrRNA* gene showed that all plants produced band of the expected size, but pCAMBIA1302-*lfs* did not because there is no *18SrRNA* gene in the plasmid (Figure 3.2.C). Hence, these DNA samples were of sufficient quality for PCR analyses. PCR using *lfs* and *hptII* primers (NcoI-LFS-f1/PmlI-LFSanalog-r1, hptII-f1/hptII-r1, Table 2.2, Figure 2.5.B) showed that the observed amplicons from all transformants were of the same size as predicted (Figure 3.2.A & B). These results indicated the presence of both *lfs* and *hptII* genes in the transgenic tobacco plants.

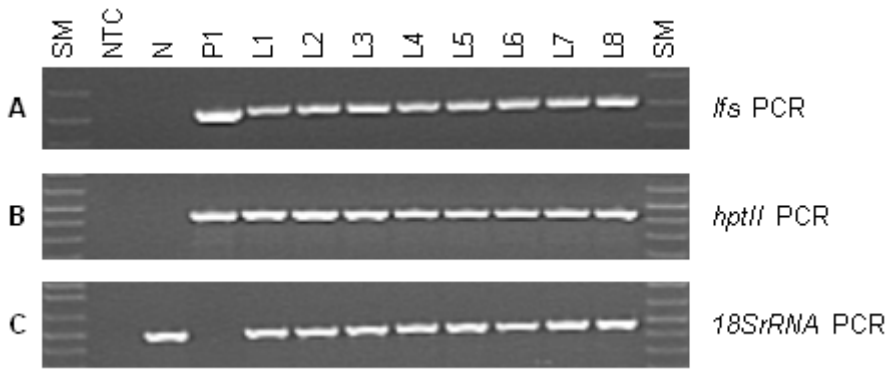


Figure 3.2 PCR analyses of *lfs* transgenic tobacco plants.

The analyses were performed using three primer sets, (A) producing an expected 525 bp product for *lfs* sequence, (B) producing an expected 454 bp product for *hptII* sequence, and (C) producing an expected 489 bp product for *18SrRNA* sequence, respectively. Amplified regions for each primer were described in Figure 2.5.B. SM, 1 kb plus DNA ladder 10787-018 (Invitrogen, Carlsbad, CA, USA) size marker; NTC, no DNA template control; N, non-transgenic tobacco plant; P1, pCAMBIA1302-*lfs* vector; L1-L8, eight *lfs*-expressing transgenic tobacco plants.

3.2.1.3 Southern blot analysis

The same transformants as used in PCR analysis (Figure 3.2) were tested by Southern hybridization to identify individual transformation events and deduce copy number of the integrated T-DNA region. Because there is only one *Hin*dIII site in the T-DNA region of pCAMBIA1302-*lfs*, located between the *lfs* and *hptII* genes (Figure 3.1), the second *Hin*dIII site must be located in the plant genome. Therefore, it is possible to estimate the copy number by probing with the *lfs* gene. pCAMBIA1302-*lfs* digested with *Hin*dIII was used as a positive control and to demonstrate equivalent copy number controls of the *lfs* gene. Five transformants, L1, L2, L4, L6, and L7 contained a single copy insert, and the other three plants, L3, L5, and L8, had multiple inserts at different loci. Moreover, all of these transgenic plants, except L4 and L6, were independent transgenic lines because the detected bands showed different patterns. L4 and L6 appeared clonal because of the same pattern of the detected bands (Figure 3.3).

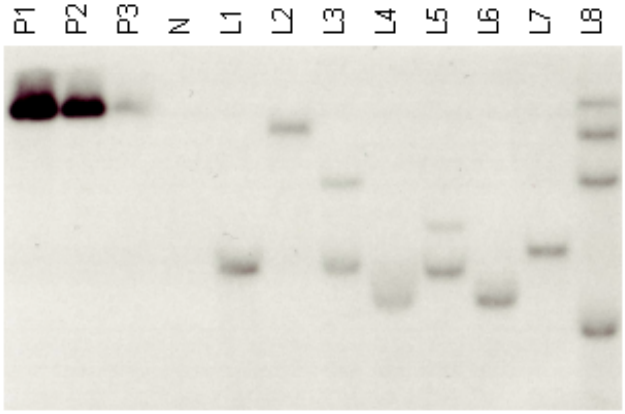


Figure 3.3 Southern analysis of *lfs* expressing tobacco plants probed with *lfs* probe.

Ten micrograms of DNA sample were digested with *Hin*dIII and hybridised with *lfs* probe. P1, two hundred and thirty picograms of pCAMBIA1302-*lfs* plasmid DNA digested with *Hin*dIII that represents 10 copies; P2, one hundred and fifteen picograms of pCAMBIA1302-*lfs* plasmid DNA digested with *Hin*dIII that represents 5 copies; P3, twenty-three picograms of pCAMBIA1302-*lfs* plasmid DNA digested with *Hin*dIII that represents 1 copy; N, non-transgenic tobacco plant; L1-L8, eight *lfs*-expressing transgenic tobacco plants.

3.2.1.4 RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed with RNA prepared from leaves of seven independent events in order to confirm the expression of the inserted *lfs* gene. After DNase treatment of the prepared RNA, DNA contamination was assessed by attempting PCR amplification of the RNA samples with *lfs* gene specific primers. No DNA was amplified indicating that no *lfs* gene DNA sequence was present (data not shown). RT-PCR analysis using *lfs* primers (LFS-f2/LFS-r2, Table 2.2, Figure 2.5.B) showed that the observed amplicons from all transformants were of the same size as predicted (Figure 3.4.A). This result indicated the presence of *lfs* gene transcripts in the transgenic tobacco plants.

3.2.1.5 Western blot analysis

The same transformants as in RT-PCR analysis were tested by western hybridization to visualize the translation of the expressed *lfs* transcript. Protein extracted from leaf material

of each event was separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane, and detected by an LFS polyclonal antibody. Western blot revealed that high levels of LFS protein were detected in four transgenic plants, L1, L2, L3, and L4, whereas levels were low in L7 and undetectable in L5 and L8 (Figure 3.4.B).

3.2.1.6 LFS enzyme activity assay

LFS enzyme activity was examined in the seven transgenic lines. The activity was evaluated by measuring the amount of LF liberated when transgenic tobacco leaf protein extract was mixed with alliinase and *trans*-1-PRENCSO. Identification of LF was performed by HPLC and a calculated value of LF peak area per ng of protein was used as a measure of LFS activity. Figure 3.4.C summarizes the results, showing that the LF-forming capability was verified in the transgenic tobacco events. Four events, L1, L2, L3, and L4, contained high enzyme activity equivalent to that observed in onion leaf (Figure 4.5.B). The three events, L5, L7, and L8, had low activity. These results corresponded to the western analysis and verified that the *lfs*-transgene was expressed and translated at a high level in four of the seven events tested. Ultimately, the L1 plant containing a single highly expressing copy of the *lfs* gene was selected as a model plant to determine the silencing assessment.

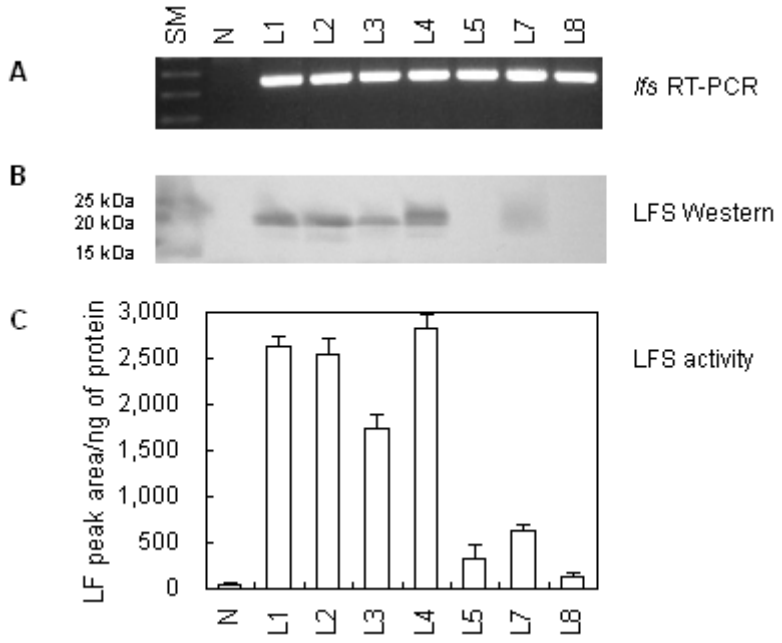


Figure 3.4 Expression analyses of integrated *lfs* gene in *lfs* expressing tobacco plants.

(A) RT-PCR analysis of *lfs* transcripts. The RT-PCR produced an expected 240 bp product for *lfs* sequence. (B) Western blot analysis of LFS protein. The western analysis produced an expected 20 kDa band for LFS protein. (C) LFS enzyme activity assay. The enzyme activity is presented as LF peak area per ng of protein in the assay reaction mixture. Enzyme activity results are mean (plus minus SEM) of values obtained from three separate sets of extracted protein samples from each plant. SM, 100 bp DNA ladder 3407A (TaKaRa, Shiga, Japan) and precision plus blue standard for protein 161-0373 (Bio-Rad, Hercules, CA, USA) size markers respectively; N, non-transgenic tobacco plant; L1-L8, seven transgenic tobacco lines.

3.2.2 Evaluation of *lfs* gene silencing

3.2.2.1 Plant selection and regeneration

The pART27-*lfs*RNAi-*mgfp5*ER binary vector (Figure 3.5) was introduced, via *Agrobacterium*-mediated transformation, into *lfs* expressing model transgenic tobacco plant (L1) to evaluate the *lfs* gene silencing. The dual transformed tissue regenerated and rooted well in media containing geneticin, and plants showed strong constitutive GFP expression. All transgenic lines grew and formed morphologically equivalent plants to the L1 model transgenic tobacco plant (Figure 3.6).

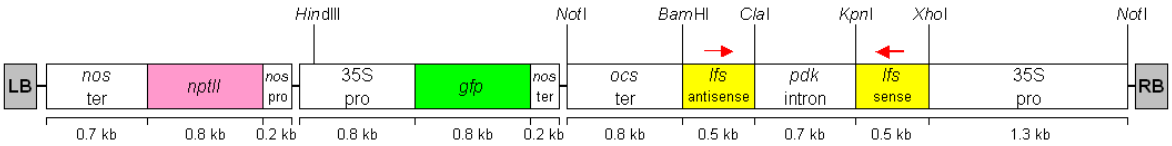


Figure 3.5 T-DNA region of pART27-*lfs*RNAi-*mgfp5*ER binary vector.

A 521 bp DNA fragment derived from *lfs* cDNA (AB089203) at positions 56 to 576 was selected for a sense *lfs* region of an RNAi cassette and integrated into *Xho*I and *Kpn*I sites in pHANNIBAL. Similarly, a 512 bp fragment at positions 63 to 574 was selected for an antisense *lfs* region and inserted into *Cla*I and *Bam*HI sites. The RNAi cassette was excised from the pHANNIBAL using the restriction enzyme *Not*I and inserted into the *Not*I site in the pART27-*mgfp5*ER. LB, left T-DNA border; *nos* ter, nopaline synthase terminator; *npt*II, neomycin phosphotransferase II; *nos* pro, nopaline synthase promoter; 35S pro, cauliflower mosaic virus 35S promoter; *gfp*; a fragment of mGFP5ER-coding sequence; *ocs* ter, octopine synthase terminator; *lfs*, a fragment of LFS-coding sequence; *pdk* intron, intron region of pyruvate orthophosphate dikinase; RB, right T-DNA border.

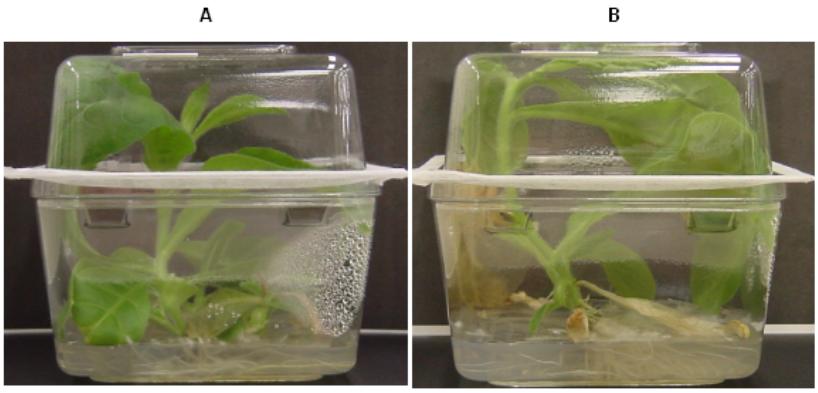


Figure 3.6 Tissue culture transgenic tobacco plants.

(A) *lfs* expressing tobacco plant [L1 event]. (B) Dual transformed tobacco plant [LR2 event].

3.2.2.2 PCR analysis

Twelve of a large number of transgenic plants regenerated were selected for analysis. PCR analysis was performed as described in 3.2.1.1. *lfs* expressing vector specific primers were designed to the *hptII* gene and boundary region between the *lfs* gene and *nos* terminator (hptII-f1/hptII-r1, LFS-f2/NOST-r2, Table 2.2, Figure 2.5.B). Similarly, *lfs*RNAi expressing vector specific primers were designed to the *gfp* gene and boundary region

between the antisense *lfs* and the *ocs* terminator (pBINmgfp5ER-f1/pBINmgfp5ER-r1, LFS-r2/OCS-r1, Table 2.2, Figure 2.5.A). For the *lfs* vector specific primers, an expected size band was detected from both the L1 host tobacco plant and all LR dual transformants as well as the *lfs* vector, but not from RNAi vector and a non-transgenic tobacco plant. For the *lfs*RNAi vector specific primers, an expected size band was detected from only the LR dual transformants as well as the RNAi vector, but not from the *lfs* vector, a non-transgenic tobacco plant, and the L1 host tobacco plant (Figure 3.7). This result indicated that the designed primers worked specifically, and all the dual transformed tobacco plants (the LR events) contained both the *lfs* and the *lfs*RNAi inserts.

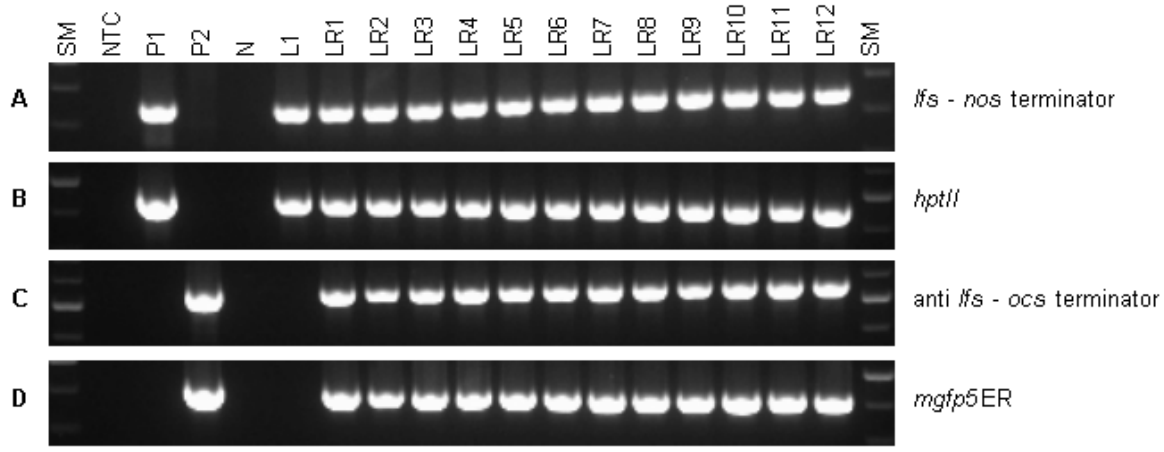


Figure 3.7 PCR analyses of secondary transgenic tobacco plants.

The analyses were performed using four primers, (A) producing an expected 350 bp product for border region between *lfs* and *nos* terminator, (B) producing an expected 454 bp product for *hptII* sequence, (C) producing an expected 561 bp product for border region between antisense *lfs* and *ocs* terminator, and (D) producing an expected 423 bp product for *mgfp5*ER sequence, respectively. Amplified regions for each primer were described in Figure 2.5.A. SM, 100 bp DNA ladder 3407A (TaKaRa, Shiga, Japan) size marker; NTC, no DNA template control; P1, pCAMBIA1302-*lfs* vector; P2, pART27-*lfs*RNAi-*mgfp5*ER vector; N, non-transgenic tobacco plant; L1, *lfs* expressing model host tobacco plant; LR1-LR12, twelve secondary transformants integrated *lfs*RNAi construct into *lfs*-expressing tobacco plant [L1].

3.2.2.3 Southern blot analysis

The same transformants as used in PCR analysis were tested by Southern hybridisation as described in 3.2.1.2. Because there is only one *Hin*dIII site in the T-DNA region of

pART27-*lfs*RNAi-*mgfp5*ER, located in 5' region of 35S promoter (Figure 2.4), the second *Hin*dIII site must be located in the plant genome. Therefore, it is possible to estimate the copy number by probing with the *gfp* gene. pART27-*lfs*RNAi-*mgfp5*ER digested with *Hin*dIII was used as a positive control to demonstrate equivalent copy numbers of the *gfp* gene. LR2 and LR9 transgenic plants contained a single copy of the RNAi construct and the others had multiple copies. LR3 was probably clonal with LR12, and LR10 was probably clonal with LR11, because they showed the same Southern profile (Figure 3.8).

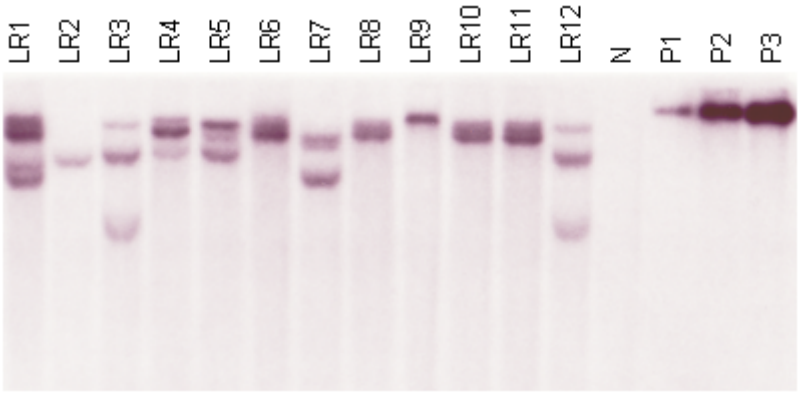


Figure 3.8 Southern analysis of secondary transgenic tobacco plants probed with *gfp* probe.

Ten micrograms of DNA sample were digested with *Hin*dIII and hybridised with the *gfp* probe. LR1-LR12, twelve secondary transgenic tobacco plants; N, non-transgenic tobacco plant; far right lanes, 1, 5, 10 copy equivalent plasmid controls respectively; P1, forty picograms of pART27-*lfs*RNAi-*mgfp5*ER plasmid DNA digested with *Hin*dIII that represents 1 copy; P2, two hundred picograms of pART27-*lfs*RNAi-*mgfp5*ER plasmid DNA digested with *Hin*dIII that represents 5 copies; P3, four hundred picograms of pART27-*lfs*RNAi-*mgfp5*ER plasmid DNA digested with *Hin*dIII that represents 10 copies.

3.2.2.4 RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed with RNA prepared from leaves of ten independent events in order to confirm the expression of the inserted *lfs*RNAi gene. After DNase treatment of the prepared RNA, DNA contamination was shown to be absent by using *ubiquitin* gene specific primers (tUbiQuant-f2/tUbiQuant-r2, Table 2.3) and the RNA samples as a template for PCR. No product was obtained (data not shown). *lfs*RNAi transcript specific primers were designed at the border region between the antisense *lfs* and the *ocs* terminator (LFS-r2/OCS-r1,

Table 2.2, Figure 2.5.A) so that they did not amplify the endogenous *lfs* transcripts. RT-PCR analysis using the primers showed that the observed amplicons from all transformants were of the same size as predicted (Figure 3.9.A) and that no amplicon was observed in non-transgenic and L1 model host tobacco plant. This result indicated the specificity of the designed primers and the presence of *lfs*RNAi gene transcripts in the transgenic tobacco plants. The next step was to evaluate if the presence of the *lfs*RNAi gene transcripts was producing a silencing signal and affecting the *lfs* gene transcripts.

3.2.2.5 Quantitative RT-PCR analysis

A quantitative real-time RT-PCR (qRT-PCR) assay was used to evaluate the extent of the *lfs* gene silencing in the dual transgenic tobacco plants. The *lfs* mRNA level was determined relative to an *ubiquitin* mRNA sequence used as an internal standard gene. RNA extracted, as described in 3.2.2.4, in triplicate from leaf material of ten plants, LR1-LR10, was reverse-transcribed using an oligo-dT primer and amplified with *lfs* or *ubiquitin* transcript specific primers (LFS525Quant-f1/LFS525Quant-r1, tUbiQuant-f2/tUbiQuant-r2, Table 2.3) respectively in separate reaction mixtures on the same PCR plate. The *lfs* forward primer specific to the *lfs* transcripts from the *lfs* expressing cassette was designed at the junction of the *lfs* sequence and the plasmid DNA to specifically amplify the *lfs* transcripts but not the *lfs*RNAi transcript (Figure 2.6). qRT-PCR analysis showed that the *lfs* transcript level in all LR plants was reduced at least 5-fold compared with the L1 model tobacco plant. No *lfs* transcript was detected in the non-transgenic control tobacco plant (Figure 3.9.B).

3.2.2.6 Western blot analysis

Western analysis was performed, as described in 3.2.1.4, using protein prepared from leaves of the same LR1-LR10 plants used in the qRT-PCR assay. Western blot indicated that in the ten LR events, LFS protein was either not detectable or detectable at low levels (LR4 and LR7), compared with the high levels observed in L1 model host plant (Figure 3.9.C). This result corresponded to the qRT-PCR results.

3.2.2.7 LFS enzyme activity assay

LFS enzyme activity assay was performed, as described in 3.2.1.5, using protein prepared in triplicate from leaves of the same LR1-LR10 plants used in the qRT-PCR assay. The enzyme assay showed that all LR plants had significantly reduced LFS activity compared with the L1 model *lfs* expressing tobacco plant (Figure 3.9.D). These biochemical results supported the qRT-PCR and the western results, and confirmed that the *lfs*RNAi cassette could efficiently silence *lfs* transcripts.

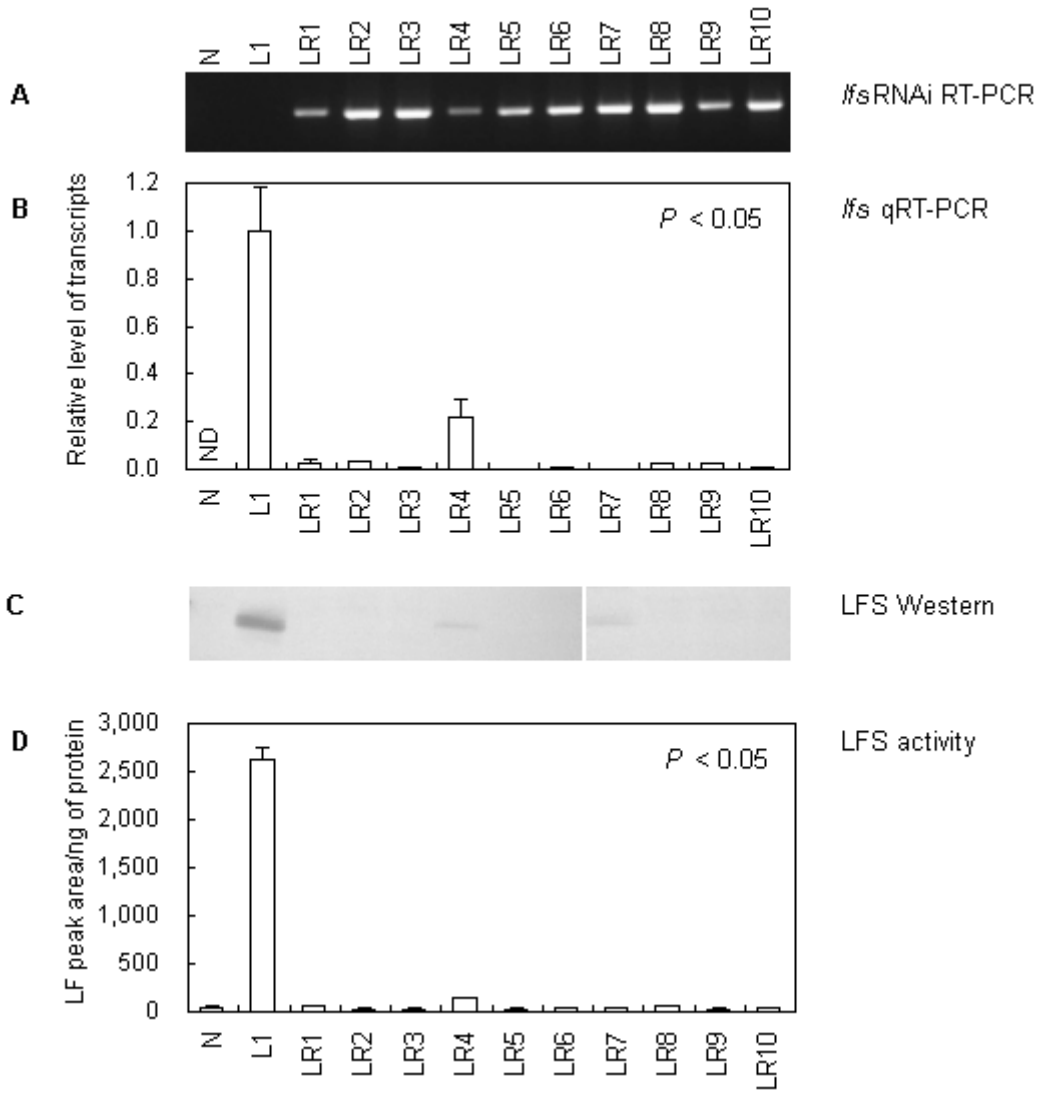


Figure 3.9 Expression analysis of integrated RNAi gene and evaluation of *lfs* gene silencing.

(A) RT-PCR analysis of *lfs*RNAi transcripts. The RT-PCR produced an expected 561 bp product for border region between antisense *lfs* and *ocs* terminator. (B) Quantitative real-time RT-PCR analysis of secondary transgenic tobacco plants. *lfs* mRNA level is shown relative to *ubiquitin* mRNA used as an internal standard gene. The transcripts results are mean (plus minus SEM) of values obtained from three separate sets of extracted RNA samples from each plant. The values for the dual transgenic events are significantly smaller than that for the control (L1) value at P<0.05 performed using Dunnett's multiple comparison test. (C) Western blot analysis of LFS protein. The western analysis produced an expected 20 kDa band for LFS protein. (D) LFS enzyme activity assay. The enzyme activity is presented as LF peak area per ng of protein in the assay reaction mixture. Enzyme activity results are mean (plus minus SEM) of values obtained from three separate sets of extracted protein samples from each plant. The values for the dual transgenic events are significantly smaller than that for the control (L1) value at P<0.05 performed using Dunnett's multiple comparison test. N, non-transgenic tobacco plant; L1, *lfs*-expressing model tobacco plant; LR1-LR10, ten secondary transformants.

3.3 Discussion

The focus of this chapter was to develop a rapid test system to evaluate RNAi based silencing of the onion *lfs* gene. To achieve this objective, firstly, a transgenic model host tobacco plant, containing and expressing the *lfs* gene, was produced. Then, this plant was re-transformed with an *lfs*RNAi construct and the dual transformants were assessed for silencing. This whole process took around 5 months which was a very rapid procedure compared to the transformation and regeneration of onion plants which could easily takes 5 months to get just a few transgenic events back to initial shoot culture stage.

Selection of a primary transgenic event was necessary as LFS protein level varied dramatically among different transgenic events. A big variation was observed among both single and multiple copy events. This was as expected as position effect and copy number of an integrated gene affect its expression level (Gallie 1998; Matzke & Matzke 1998). Quantitative RT-PCR analysis to further examine *lfs* transcript levels will provide more corresponding data to the LFS activity and western analyses results. One event (L1), which contained a single copy of the *lfs* gene and exhibited the same high level of LFS enzyme activity as onion leaf, was selected as a model plant for silencing tests.

The second transformation of L1 with the *lfs*RNAi construct produced a similar range of additional integration events. Molecular and biochemical analyses of these events demonstrated that the RNAi construct efficiently silenced the *lfs* gene expression. However, it might be possible that this was caused by the RNAi cassette integrating into the *lfs* cassette and disrupting the gene function. LFS activity assay and siRNA detection in segregating offspring from a selfed LR plant, containing a single copy of the *lfs* and the *lfs*RNAi construct, will confirm that RNAi construct was responsible for the silencing.

RT-PCR analysis to detect transcripts of the transformed RNAi gene allowed reasons for failure of silencing to be determined. In order for RNAi system to succeed, at least three steps must work. First, transformed RNAi gene is transcribed and forms the double-stranded RNA (dsRNA). Second, the dsRNA is degraded into small interfering RNAs

(siRNAs). Third, the target RNA is cleaved by the siRNAs' guide. These three steps can be verified by molecular analyses: RNAi transcripts detection by RT-PCR (as described in this thesis), siRNAs detection by Northern (Hamilton & Baulcombe, 1999), and quantification of the target RNA by quantitative real-time RT-PCR (qRT-PCR), respectively. Use of these different analyses allow us to determine whether the transgenic events that do not silence the target gene fail to produce dsRNA, or fail to produce siRNAs or fail to cleave the target RNA. In this chapter, we examined the first and third steps to show the successful gene silencing by RNAi in our dual transgenic tobacco plants. Subsequent research (Chapter 4) has demonstrated that a combination of these analyses is a valuable tool to differentiate the reasons for failure of silencing.

The use of RT-PCR to detect the RNAi gene transcripts demonstrated that we had to be careful in designing primers specific to the target RNA for qRT-PCR. The positive RT-PCR result indicated that transcripts of the transformed RNAi gene are present in the cell, prior to them being degraded into siRNAs by Dicer. These could interfere with attempts to amplify target gene RNA transcripts. Therefore, the target gene primers must be designed so that they specifically amplify target RNA sequence and do not amplify RNAi transcripts. As part of the target sequence is incorporated into the RNAi construct, specific primers can only be designed at the 5' and 3' regions which do not overlap with the RNAi construct. However, amplicons spanning almost the entire *lfs* sequence are not suitable for qRT-PCR because such a amplicon is too long to be analysed by qRT-PCR (according to manufacturer's instructions no longer than 150 bp is recommended). Also, primers at the 3' region would detect residual target RNA after being cleaved by RISC. On the contrary, primers at 5' region would not amplify the residual target RNA (Figure 3.10). Hence, we designed specific primers at the 5' region of the target RNA sequence that only partially overlaps with the fragment incorporated into the RNAi construct.

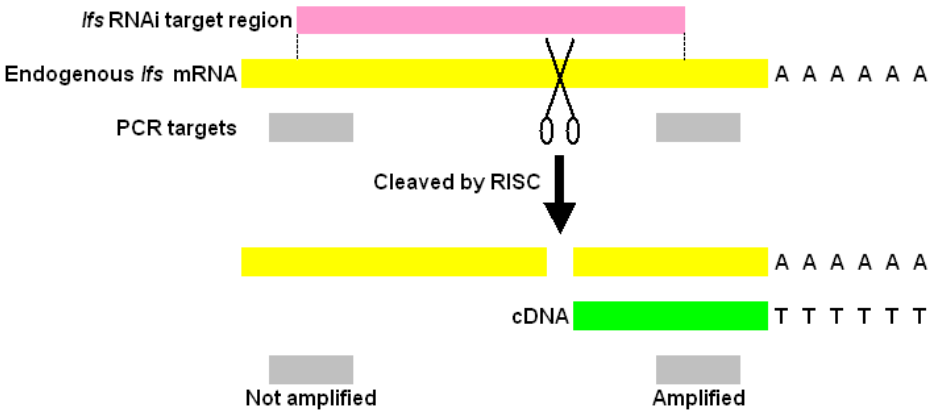


Figure 3.10 Target region for qRT-PCR analysis.

It has been reported that silencing efficiency is not related to the RNAi cassette copy number (Travella *et al*. 2006; McGinnis *et al*. 2007), but others have reported that single copy RNAi cassette insertions silenced genes more efficiently and were inherited more stably than multiple copies of insertions (Kerschen *et al*. 2004). In general it appears that silencing efficiency depends upon the relationship between target gene expression level and the silencing signal expression level. Our transgenic events contained both single and multiple copy RNAi cassettes, and all silenced almost completely. Thus, we can presume that the RNAi construct integrated was transcriptionally active. However, such efficient silencing is not always observed and multiple insertions can cause transcriptional inactivation (Fagard & Vaucheret, 2000). Thus it is necessary to produce a number of transgenic events for assessment. However, in onion, to produce several events for analysis would require considerable time and effort. Our previous assessment of a poorly functioning *lfs*RNAi cassette directly into onion produced only a few transformants and it was time-consuming to ascertain why it did not function correctly (data not shown). This model assessment system would have saved considerable time in this respect.

One drawback of the model system is its inability to detect the consequences of silencing on the whole metabolic pathway under investigation. For example, in the work of Allen *et al*. (2004) it could not have shown that silencing the codeinone reductase gene in opium poppy affects a feedback mechanism resulting in the accumulation of (S)-reticuline seven

steps upstream of codeinone. Our model system can not build up the whole metabolic pathway surrounding the target gene but it is a valuable tool to estimate silencing efficiency of the target gene. Therefore, the system is unable to examine 'feedback' effects caused by target gene silencing on other reactions within the metabolic pathway involved.

All of the silencing events (LR) grew and formed morphologically equivalent plants to non-transgenic tobacco plants. This indicated that 'off-target' silencing leading to phenotypic abnormalities did not arise in the transgenic plants. Due to the broad conservation of 'housekeeping' genes between species this would indicate that the RNAi cassette evaluated here would function in onion. However, secondary structure, expression pattern, and RNA turn over of onion native *lfs* RNA sequence may affect silencing efficiency in a different way from those of *lfs* RNA in a tobacco host plant. Subsequent research (Chapter 4) has demonstrated that this model assessment does relate well to the natural host of the *lfs* gene. The model system is now being used to assess new shorter *lfs*RNAi constructs to determine the minimal efficient sequence size required for silencing so that efficient multiple target chimeric silencing constructs can be created.

In conclusion, we have developed a practical rapid system to first assess silencing of secondary metabolite pathway genes originating from recalcitrant or poorly studied plant species. This approach is novel and of great advantage in obtaining a reliable RNAi cassette before use in recalcitrant target plant species, and in consequence, saves considerable time and effort. As it is important to have a detailed knowledge of the target gene and the silencing sequences and several transgenic events for analysis in order to assess silencing efficiency, this model could prove invaluable for the study of many plant secondary metabolite products used in pharmacological, food and process industries.

Chapter 4: Silencing lachrymatory factor synthase produces a tearless onion

4.1 Introduction

Allium species synthesize a unique set of sulfur secondary metabolites, the majority of which exist in the form of non-volatile amino acid derivatives, *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs), including *S*-2-propenyl-L-cysteine sulfoxide (alliin, 2-PRENCSO) and *trans*-*S*-1-propenyl-L-cysteine sulfoxide (isoalliin, 1-PRENCSO) (Rose *et al*., 2005). When the tissues of any *Allium* species are disrupted, these amino acid derivatives are cleaved by the enzyme alliinase (EC 4.4.1.4) into their corresponding sulfenic acids and then the acids are converted into volatile sulfur compounds which give the characteristic flavour and bioactivity of the species.

The particular profile of sulfur volatile compounds in *Allium* species directly correlates with the relative amount of the cysteine sulfoxides. In garlic, 2-PRENCSO is the dominant precursor (Fritsch & Keusgen, 2006) and this produces di-2-propenyl thiosulfinate (allicin) upon tissue disruption. The decomposition product of allicin, di-2-propenyl disulfide, is the major volatile component liberated (Block *et al*., 1992b & c; Rose *et al*., 2005). In onion, 1-PRENCSO is the major sulfoxide (Fritsch & Keusgen, 2006). This would be predicted to liberate di-1-propenyl thiosulfinate and di-1-propenyl disulfide. However, di-1-propenyl thiosulfinate has never been reported in onion and di-1-propenyl disulfide has only been reported in small amounts (Boelens *et al*., 1971; Ferary & Auger, 1996; Mondy *et al*., 2001; Arnault *et al*., 2004). Instead, propanthial *S*-oxide (lachrymatory factor, LF), 1-propenyl methane thiosulfinate, and dipropyl disulfide are present dominantly (Block *et al*., 1992b & c; Rose *et al*., 2005). It has been speculated that the absence of the predicted sulfur volatiles in onion is a consequence of LF production (Randle & Lancaster, 2002).

The disruption of raw onion by cutting and chopping releases distinctive volatile sulfur flavours and an attendant lachrymator that is an experience familiar to us all. The mechanism of this reaction was explained as cleavage of 1-PRENCSO by enzyme alliinase into sulfenic acids, followed by spontaneous rearrangement to form LF and a cascade of other sulfur volatiles. However, Imai *et al*. (2002) discovered that the conversion of 1-propenyl sulfenic acid to LF is catalysed by a novel enzyme, lachrymatory factor synthase (LFS) (Figure 4.1). This discovery suggests that the production of LF could be reduced by silencing *lfs* gene transcripts by the use of RNAi. In the absence of LFS, the unstable 1-propenyl sulfenic acid would be predicted to undergo spontaneous self-condensation to di-

1-propenyl thiosulfinate (Imai *et al*., 2002). This increased thiosulfinate would then be available for non-enzymatic conversion into a cascade of predicted volatile sulfur metabolites (Figure 4.1). These include polysulfides and zwiebelanes that are responsible for characteristic allium flavours (Block 1992a) as well as the health-promoting properties of alliums such as anti-inflammatory, anti-platelet aggregation, anti-cancer, and lipid-lowering effects (Morimitsu *et al*., 1992; Block *et al*., 1996a; Griffiths *et al*., 2002; Randle & Lancaster, 2002; Lanzotti, 2006). In this chapter, we aimed to genetically manipulate the sulfur secondary metabolite pathway of onion, by using the RNAi vector that was tested in the tobacco model system described in chapter 3, to simultaneously increase levels of desirable compounds and reduce the irritant LF.

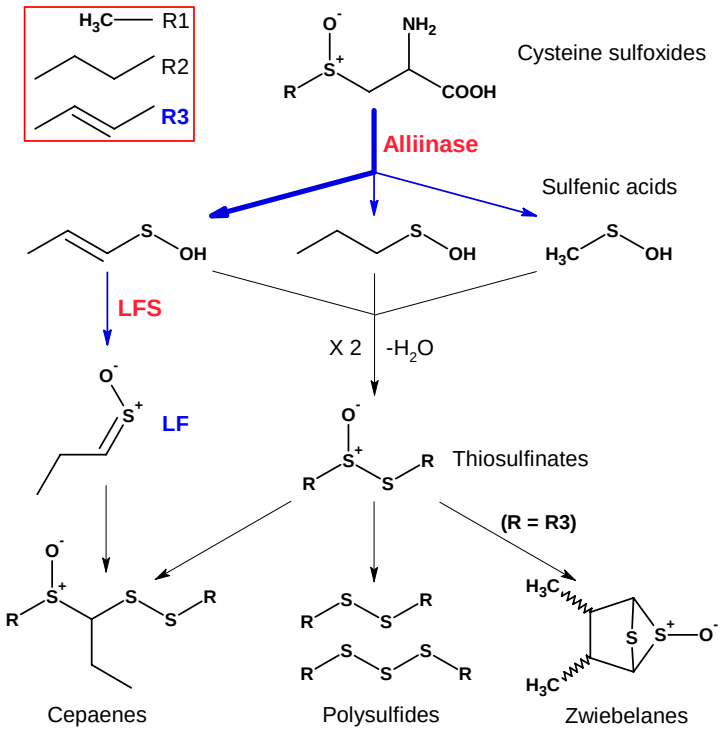


Figure 4.1 Major breakdown pathway of cysteine sulfoxides in onion.

Blue arrows show enzymatic reactions.

4.2 Results

4.2.1 Production of transgenic tearless onions

4.2.1.1 Plant selection and regeneration

The pART27-*lfs*RNAi-*mgfp5*ER binary vector (Figure 3.5), verified to silence *lfs* gene in the tobacco model system described in chapter 3, was transformed into hybrid (H) (mild, cv. 'Enterprise'), open-pollinated (O) (pungent, cv. 'Pukekohe Longkeeper'), and dehydration (D) (very pungent, breeding line from Sensient Dehydrated Flavors, CA, UAS) onions via *Agrobacterium*-mediated transformation (Eady *et al*., 2000). Under selection and regeneration, the transformed tissue behaved in a similar manner to that observed in previous onion transformations (Eady *et al*., 2000 & 2002). Transgenic shoot cultures rooted well in media containing geneticin and showed strong constitutive GFP expression except for line O3 which had to be rescued on to non-selective media. All lines grew and formed morphologically equivalent plants and bulbs to their non-transgenic counterparts (Figure 4.2). No morphological differences were observed in the glasshouse between transgenic and non-transgenic lines apart from GFP expression, which was apparent in all transgenic lines viewed under blue light excitation. Normal physiological growth, bulb development, and viable seed set (data not shown) all confirmed that transgenic and non-transgenic plants behave physiologically in a very similar manner, and indicated that the performance of the transgenic onions under glasshouse conditions is not compromised in any way. Plants from eight different transgenic events, H1, H2, H3, O1, O2, O3, D1, and D2, were analysed along with three non-transgenic plants, HN, ON, and DN from hybrid, open-pollinated, and dehydration lines respectively.

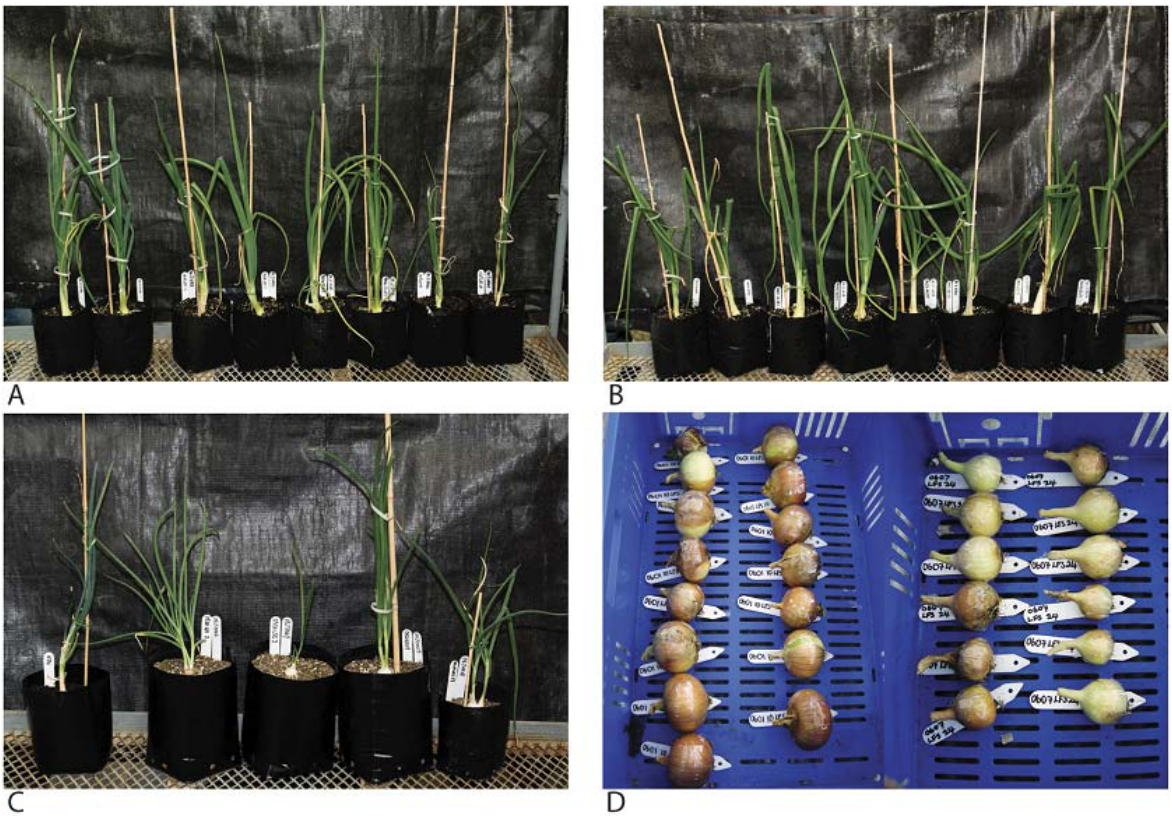


Figure 4.2 Ex-flasked intermediate daylength onions transformed with pART27-*lfs*RNAi-*mgfp5*ER.

(A) Open-pollinated onions [O lines]; far left two plants, non-transgenic onions. (B) Hybrid onions [H lines]; far left two plants, non-transgenic onions. (C) Dehydration onions [D lines]; far left plant, non-transgenic onion. (D) LFS-silenced bulbs.

4.2.1.2 PCR analysis

PCR analysis was used to test whether the transgenes were present in the transgenic plants. In order to determine whether extracted DNA was suitable for PCR amplification, PCR using *18SrRNA* specific primers was performed. PCR amplification of *18SrRNA* gene showed that all plants produced a band of an expected size, but pART27-*lfs*RNAi-*mgfp5*ER did not because there is no *18SrRNA* gene in the plasmid (Figure 4.3.H). Hence, the extracted DNA had enough quality to be amplified. PCR analysis using *lfs*RNAi, *mgfp5*ER, and *nptII* primers (CaMV35S-f2/CaMV35S-r1, CaMV35S-f1/LFS-r2, LFS-f5/PDK-r2, PDK-f2/antiLFS-r1, LFS-r2/OCS-r1, pBINmgfp5ER-f1/pBINmgfp5ER-r1, NPT-f2/NOST-r2, Table 2.2, Figure 2.5.A) showed that the observed amplicons from all transformants except H2 and O3 were of the predicted size. In H2, the 5' region of *lfs*RNAi

CaMV35S promoter was not detected. In O3, the *nptII* gene was not detected (Figure 4.3.A-G). These results indicated the absence of part of the *lfs*RNAi CaMV35S promoter and *nptII* gene sequences in H2 and O3 respectively, and the presence of intact T-DNA region in the other transgenic onion plants. This result in O3 line corresponded to the observation that O3 did not grow well in media containing geneticin.

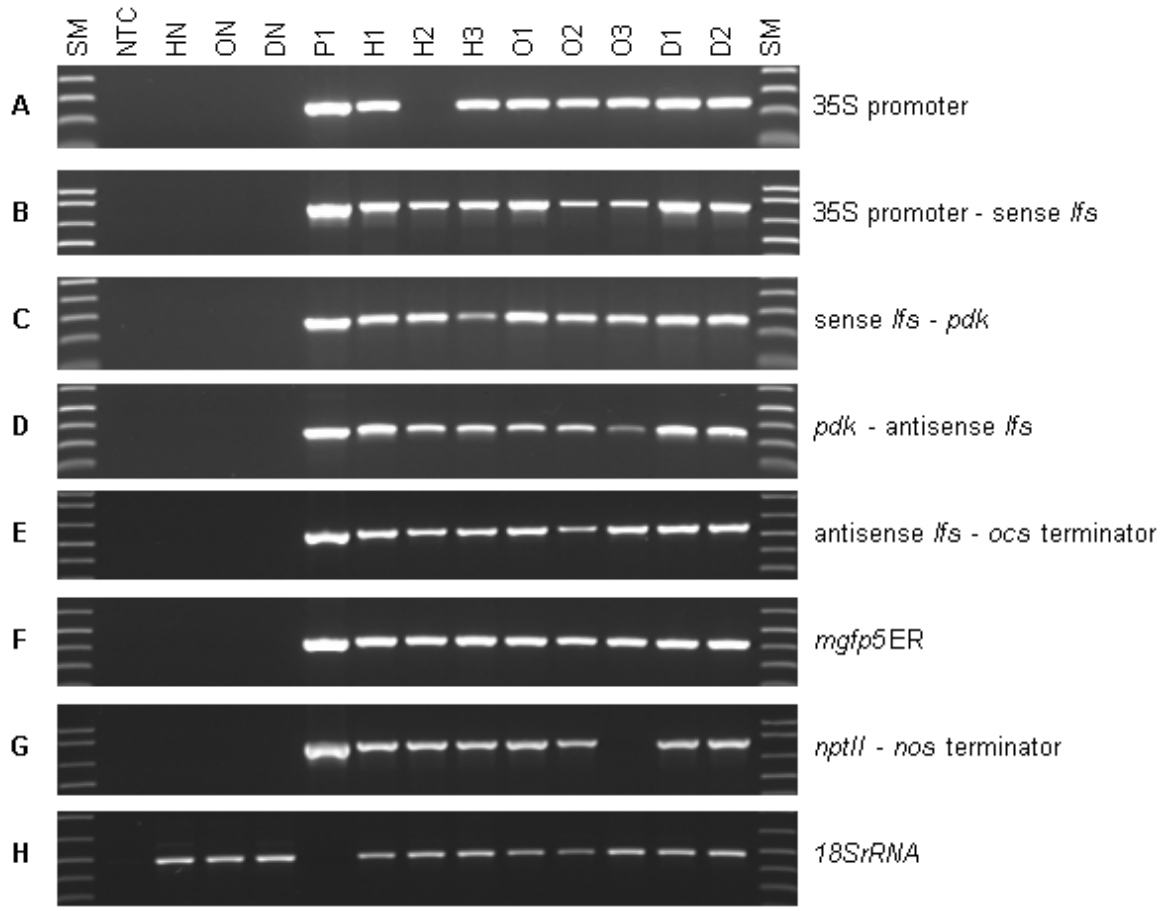


Figure 4.3 PCR analyses of *lfs*RNAi transgenic onion plants.

The analyses were performed using eight primer pairs, (A) producing an expected 251 bp product from the 5' region of *lfs*RNAi CaMV35S promoter sequence, (B) producing an expected 784 bp product from the border region between CaMV35S promoter and sense *lfs*, (C) producing an expected 265 bp product from the border region between sense *lfs* and *pdk* intron, (D) producing an expected 350 bp product from the border region between *pdk* intron and antisense *lfs*, (E) producing an expected 561 bp product from the border region between antisense *lfs* and *ocs* terminator, (F) producing an expected 423 bp product from the *mgfp5*ER sequence, (G) producing an expected 800 bp product from the border region between *nptII* and *nos* terminator, and (H) producing an expected 489 bp product from the *18SrRNA* sequence, respectively. Amplified regions for each primer are described in Figure 2.5.A. SM, 1 kb plus DNA ladder 10787-018 (Invitrogen, Carlsbad, CA, USA) size marker; NTC, no DNA template control; HN, ON, and DN, non-transgenic hybrid, open-pollinated, and dehydration onion controls, respectively; P1, pART27-*lfs*RNAi-*mgfp5*ER vector; H1, H2, H3, O1, O2, O3, D1, and D2 are transgenic hybrid, open-pollinated, and dehydration onion lines, respectively.

4.2.1.3 Southern blot analysis

The same transformants used in the PCR analysis were tested by Southern hybridization to identify individual transformation events and deduce the copy number of integrated T-DNA region. Because there is only one *Hin*dIII site in the T-DNA region of pART27-*lfs*RNAi-*mgfp5*ER, located in the 35S promoter (Figure 2.4), the second *Hin*dIII site must be located in the plant genome. Therefore, it is possible to estimate the copy number by probing with the *gfp* gene. The pART27-*lfs*RNAi-*mgfp5*ER plasmid, digested with *Hin*dIII, was used as a positive control and to demonstrate equivalent copy number controls of *gfp* gene. The H1 and D1 lines contained two copies inserted at different loci, and the O1 line had multiple inserts at a single locus. The remaining five lines, H2, H3, O2, O3, and D2, contained a single copy of the T-DNA insert integrated at obviously different locations from each other (Figure 4.4). This confirmed the non-clonal nature of the transgenic events.

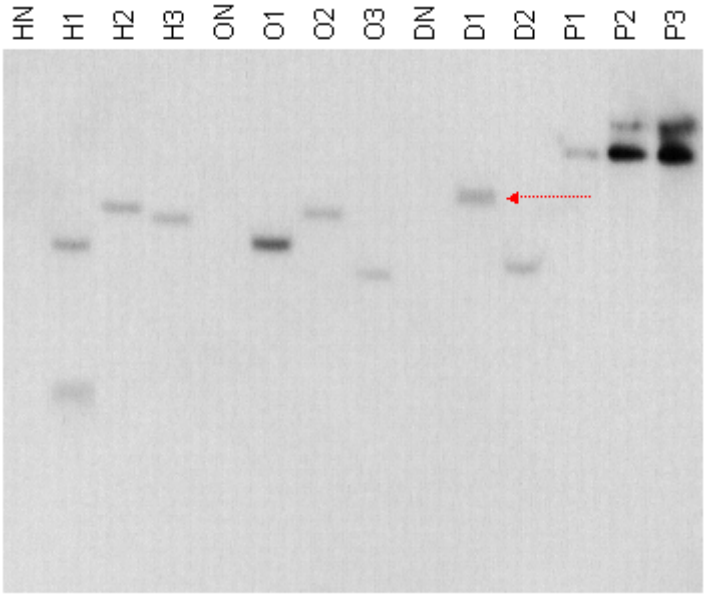


Figure 4.4 Southern analysis of *lfs*RNAi transgenic onion plants probed with *gfp* probe.

DNA was digested with *Hin*dIII and hybridised with *gfp* probe. HN, non-transgenic hybrid onion control; H1-H3, three transgenic hybrid onion lines; ON, non-transgenic open-pollinated onion control; O1-O3, three transgenic open-pollinated onion lines; DN, non-transgenic dehydration onion control; D1-D2, two transgenic dehydration onion lines; P1, twenty-three picograms of pART27-*lfs*RNAi-*mgfp5*ER plasmid DNA digested with *Hin*dIII that represents 1 copy; P2, one hundred fifteen picograms of pART27-*lfs*RNAi-*mgfp5*ER plasmid DNA digested with *Hin*dIII that represents 5 copies; P3, two hundred and thirty picograms of pART27-*lfs*RNAi-*mgfp5*ER plasmid DNA digested with *Hin*dIII that represents 10 copies; arrow, two close together bands in D1.

4.2.1.4 RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed with RNA prepared from leaves of each line in order to confirm the expression of the inserted *lfs*RNAi gene as described in 3.2.2.4. After DNase treatment of the prepared RNA, DNA contamination was shown to be absent using *lfs* gene specific primers (LFSQuant-f1/LFSQuant-r1, Table 2.4) and the RNA samples as a template for PCR. No product was obtained (data not shown). *lfs*RNAi transcript specific primers were designed at the border region between antisense *lfs* and *ocs* terminator (LFS-r2/OCS-r1, Table 2.2, Figure 2.5.A) so that the primers could not amplify the endogenous *lfs* transcripts. RT-PCR analysis using the primers showed that the observed amplicons from all lines except O1 were of the same size as predicted (Figure 4.5.A) and that no amplicon was observed in each non-transgenic counterpart. This result indicated the specificity of the designed primers and the presence of *lfs*RNAi gene transcripts in all transgenic onion lines except for O1. The next step was to see if the *lfs*RNAi gene transcript was producing a silencing signal that could reduce the *lfs* gene transcript level.

4.2.1.5 Small interfering RNAs (siRNAs) detection

siRNAs detection analysis was performed, according to Hamilton's (1999) method with slight modification (2.3.5, Chapter 2). Short-length RNA smaller than 200 bases was isolated from leaves of each line in order to confirm the presence of *lfs* siRNA fragments. The extracted small-length RNA was separated by polyacrylamide gel electrophoresis (PAGE), transferred onto a nylon membrane, and detected by randomly primed *lfs* probe. The result clearly revealed that H1, H2, H3, O2, O3, and D2 line produced *lfs* siRNA fragments but O1 line failed. This corresponded well to the result of *lfs*RNAi gene expression analysis. Interestingly, line D1, in which the integrated *lfs*RNAi gene was transcribed, failed to produce *lfs* siRNAs at detectable levels (Figure 4.5.B).

4.2.1.6 Quantitative RT-PCR analysis

Quantitative real-time RT-PCR (qRT-PCR) assay (2.3.6, Chapter 2) was used to evaluate the extent of the endogenous *lfs* gene silencing in the transgenic onion plants. The *lfs* mRNA level was determined relative to a putative *ubiquitin* mRNA sequence used as an internal standard gene. RNA extracted, as described in 4.2.1.4, in triplicate from leaf

material of each line, was reverse-transcribed using oligo-dT primer and amplified with *lfs* or *ubiquitin* transcripts specific primers (LFSQuant-f1/LFSQuant-r1, UbiQuant-f1/UbiQuant-r1, Table 2.4, Chapter 2) respectively in separate reaction mixtures on the same PCR plate. The *lfs* forward primer specific to the endogenous *lfs* transcripts was designed in the 5' untranscribed region (UTR) of the *lfs* sequence so that it did not overlap with the RNAi construct and amplify the *lfs*RNAi transcripts (Figure 2.7, Chapter 2). qRT-PCR analysis showed that all transgenic onion plants except for line O1 and D1 had significantly reduced *lfs* transcripts level compared to each non-transgenic counterpart. H1, H2, and H3 lines were decreased by between 10 and 17-fold. O2 and O3 lines were reduced by 8 and 5-fold, respectively. Line D2 was decreased by 43-fold (Figure 4.5.C). O1 and D1 lines failed to transcribe the integrated *lfs*RNAi gene or produce detectable *lfs* siRNAs were unsuccessful in silencing *lfs* gene. These results corresponded well to *lfs*RNAi gene expression and *lfs* siRNAs analyses.

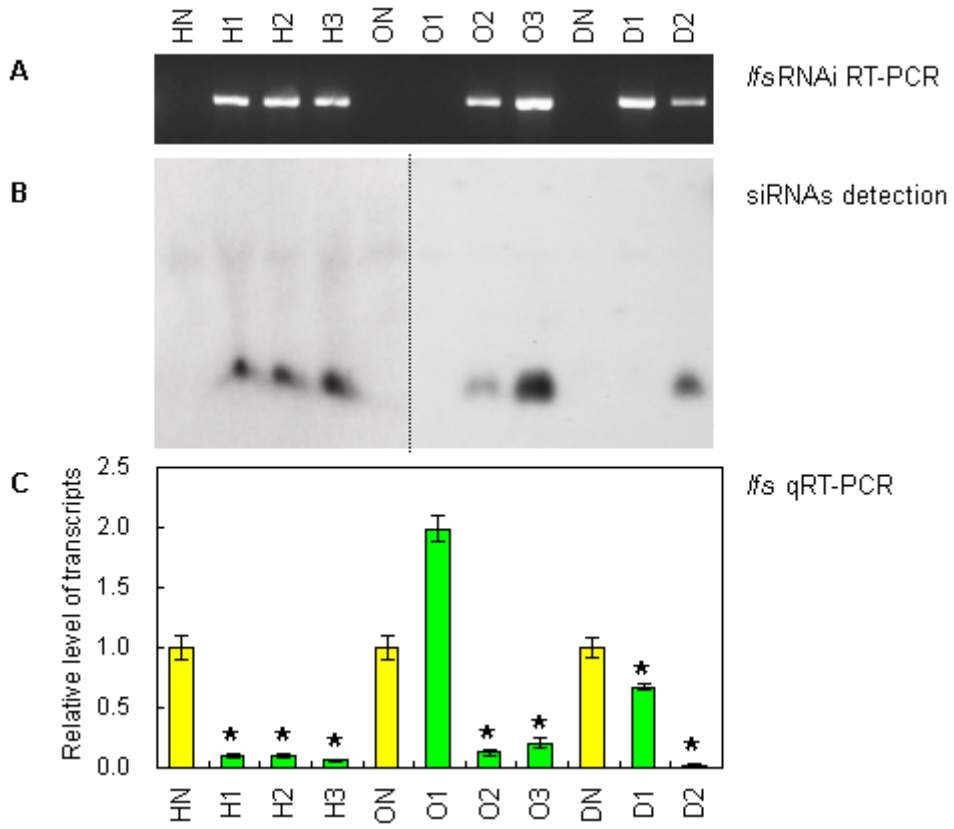


Figure 4.5 Expression analyses of integrated *lfs*RNAi gene and evaluation of *lfs* gene silencing.

(A) RT-PCR analysis of *lfs*RNAi transcripts in leaf tissue. The RT-PCR produced an expected 561 bp product for border region between antisense *lfs* and *ocs* terminator. (B) siRNAs detection analysis probed with randomly primed *lfs* probe in leaf tissue. (C) Quantitative real-time RT-PCR (qRT-PCR) analysis in leaf tissue of *lfs*RNAi transformed onion plants. The *lfs* mRNA level was determined by dividing the *lfs* copy number by the internal standard gene copy number as relative mRNA level. The transgenic samples were shown relative to the respective HN, ON and DN *lfs* transcript levels The transcripts results are mean (plus minus SEM) of values obtained from three separate sets of extracted RNA samples from each plant. *, The values for the transgenic onion lines are significantly smaller than those for each respective non-transgenic control (HN, ON, and DN) at $P<0.05$ performed using Dunnett's multiple comparison test; HN, non-transgenic hybrid onion control; H1-H3, three transgenic hybrid onion lines; ON, non-transgenic open-pollinated onion control; O1-O3, three transgenic open-pollinated onion lines; DN, non-transgenic dehydration onion control; D1-D2, two transgenic dehydration onion lines.

4.2.1.7 Western blot analysis

Western blot analysis was performed to visualize LFS protein levels and examine the consequence of *lfs* gene silencing. Protein extracted from leaf material of each line was

separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane, and detected with an LFS polyclonal antibody (2.3.7, Chapter 2). The densitometry of detected bands was quantified by using Quantity One Software (Bio-Rad). Western blot (Figure 4.6.A) revealed that no LFS protein was detected in six lines, H1, H2, H3, O2, O3, and D2, which contained significantly reduced *lfs* transcripts level. On the contrary, Lines O1 and D1 that failed to silence or silenced slightly *lfs* gene had clearly discernible LFS protein levels that fell well within the range of the control non-transgenic lines HN, ON, and DN.

4.2.1.8 LFS enzyme activity assay

LFS enzyme activity was examined in both leaf and bulb samples. The activity was evaluated by measuring the amount of LF liberated when onion protein extract was mixed with alliinase and *trans*-1-PRENCSO. Identification of LF was performed by HPLC and a calculated value of LF peak area per ng of protein was used as a measure of LFS activity (2.3.8, Chapter 2). Figure 4.6.B & C summarizes the results, showing that lines with no detectable LFS, as measured by western analysis, also had significantly reduced LFS activity. H1, H2, and H3 lines were reduced by between 21 and 102-fold in leaf tissue and between 732 and 1155-fold in bulb tissue compared with HN. O2 and O3 lines were decreased by between 39 and 71-fold in leaf tissue and between 1509 and 1573-fold in bulb tissue compared with ON. Line D2 was reduced by 393-fold in leaf tissue and 500-fold in bulb tissue compared with DN. Line O1 and D1 failed to produce *lfs* siRNA fragments were unsuccessful in reducing completely LFS activity. Leaf results corresponded well to bulb results and western analysis.

4.2.1.9 Lachrymatory factor (LF) measurement

LF was measured in both leaf and bulb samples to confirm whether *lfs* gene silencing produces a tearless onion. The quantification was performed by measuring the amount of LF produced when onion tissue was crushed. Identification of LF was performed by GC-FPD (flame photometric detector) (2.3.9, Chapter 2). LF levels in leaf tissue were reduced by 14, 35, and 30-fold for H1, H2, and H3 respectively compared with HN, were reduced by 30 and 67-fold for O2 and O3 compared with ON, and were reduced by 25-fold for D2 compared with DN (Figure 4.6.D). In bulb tissue LF levels were decreased by 3 and 7-fold

for H1 and H3 compared with HN, were decreased by 5 and 22-fold for O2 and O3 compared with ON, and were decreased by 14-fold for D2 compared with DN (Figure 4.6.E). H2 line was not measured as the bulb was infected. Leaf results corresponded well to bulb results as well as LFS enzyme activity assay and western analysis.

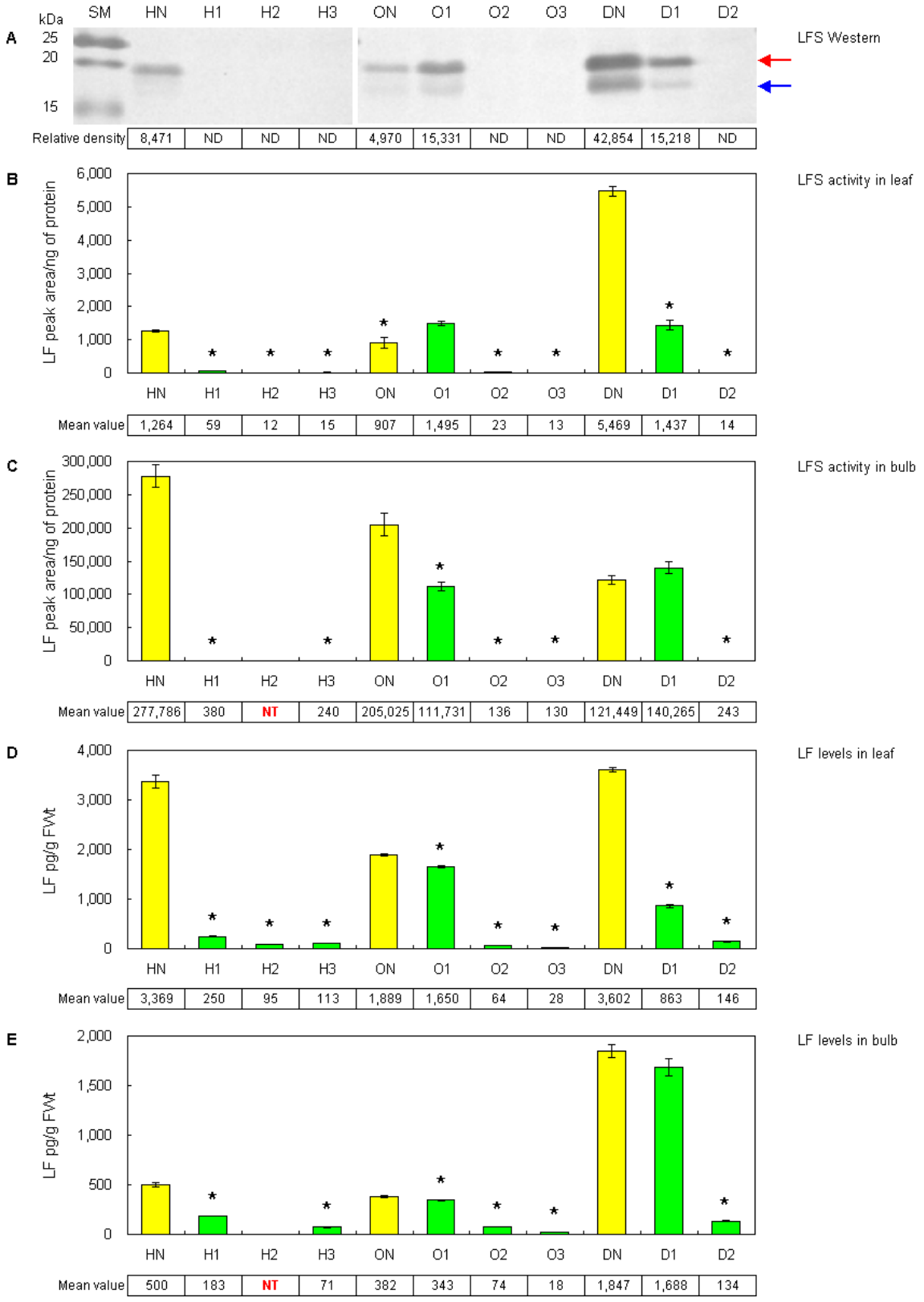

A
kDa
25
20
15
SM HN H1 H2 H3 ON O1 O2 O3 DN D1 D2
LFS Western
Relative density 8,471 ND ND ND 4,970 15,331 ND ND 42,854 15,218 ND
B
LF peak area/ng of protein
6,000
5,000
4,000
3,000
2,000
1,000
0
HN H1 H2 H3 ON O1 O2 O3 DN D1 D2
LFS activity in leaf
Mean value 1,264 59 12 15 907 1,495 23 13 5,469 1,437 14
C
LF peak area/ng of protein
300,000
250,000
200,000
150,000
100,000
50,000
0
HN H1 H2 H3 ON O1 O2 O3 DN D1 D2
LFS activity in bulb
Mean value 277,786 380 NT 240 205,025 111,731 136 130 121,449 140,265 243
D
LF pg/g FWt
4,000
3,000
2,000
1,000
0
HN H1 H2 H3 ON O1 O2 O3 DN D1 D2
LF levels in leaf
Mean value 3,369 250 95 113 1,889 1,650 64 28 3,602 863 146
E
LF pg/g FWt
2,000
1,500
1,000
500
0
HN H1 H2 H3 ON O1 O2 O3 DN D1 D2
LF levels in bulb
Mean value 500 183 NT 71 382 343 74 18 1,847 1,688 134

Figure 4.6 Analyses of LFS protein and LF in *lfs*RNAi transgenic onion plants.

(A) Western blot analysis of LFS protein in leaf tissue. (B) and (C) LFS enzyme activity assay in leaf and bulb tissue, respectively. The enzyme activity is presented as LF peak area per ng of protein in the assay reaction mixture. Enzyme activity results are mean (plus minus SEM) of values obtained from at least three separate sets of extracted protein samples from each plant. (D) and (E) LF measurements in leaf and bulb tissue, respectively. The results are mean (plus minus SEM) of values obtained from at least three separate sets of extracted protein samples from each plant. *, The values for the transgenic onion lines are significantly smaller than those for each non-transgenic control (HN, ON, and DN) at $P<0.05$ performed using Dunnett's multiple comparison test; SM, precision plus blue standard for protein 161-0373 (Bio-Rad, Hercules, CA, USA) size marker; HN, non-transgenic hybrid onion control; H1-H3, three transgenic hybrid onion lines; ON, non-transgenic open-pollinated onion control; O1-O3, three transgenic open-pollinated onion lines; DN, non-transgenic dehydration onion control; D1-D2, two transgenic dehydration onion lines; ND, not detected; NT, not tested; red arrow, LFS protein; blue arrow, degraded LFS protein.

Table 4.1 Summary of molecular and biochemical analyses.

Summary of molecular and biochemical analyses data of *lfs*RNAi transformed onion plants.

Analyses	Transgenic lines							
	H1	H2	H3	O1	O2	O3	D1	D2
PCR	Intact T-DNA	*lfs*RNAi promoter truncated	Intact T-DNA	Intact T-DNA	Intact T-DNA	*npt*II deleted	Intact T-DNA	Intact T-DNA
Southern	Two at different loci	Single	Single	Multi at one locus	Single	Single	Two at different loci	Single
*lfs*RNAi transcripts	Present	Present	Present	**Absent**	Present	Present	Present	Present
siRNAs	Present	Present	Present	**Absent**	Present	Present	**Absent**	Present
lfs transcripts	Reduced	Reduced	Reduced	**Normal**	Reduced	Reduced	**Normal**	Reduced
LFS protein	Reduced	Reduced	Reduced	**Normal**	Reduced	Reduced	**Normal**	Reduced
LFS activity	Reduced	Reduced	Reduced	**Normal**	Reduced	Reduced	**Normal**	Reduced
LF	Reduced	Reduced	Reduced	**Normal**	Reduced	Reduced	**Normal**	Reduced

4.2.2 Analysis of the manipulated sulfur secondary metabolite pathway

We predicted that by reducing LFS protein, more 1-PRENCSO-derived sulfenic acid would be available for thiosufinates production and, in consequence, the raised thiosulfinates would be converted non-enzymatically into the downstream volatile compounds. Subjective olfactory assessment of silenced lines indicated that the pungent odour given off by the leaf and bulb material was much reduced compared with that of non-transgenic counterparts, and that this was replaced by a sweeter milder onion odour. Here we investigated the changes in the sulfur secondary metabolites profile derived from 1-PRENCSO substrate in a representative tearless onion and non-transgenic onion plant from each cultivar.

4.2.2.1 Pinking assay

The identity of *trans*-1-PRENCSO-derived thiosulfinate is difficult to determine due to its instability. Chemical synthesis of the thiosulfinate by oxidation of di-1-propenyl disulfide demonstrated by low temperature NMR analysis at –15 ^{o}C that it rapidly changed form to become a zwiebelane (Block, 1991). However, recent observations by Imai *et al*. (2006a & b) demonstrated that 1-propenyl-containing thiosulfinates produce a pink pigment when mixed with glycine and formaldehyde (Figure 4.7). This reaction was developed into a simple ‘pinking assay’ to detect 1-PRENCSO-derived thiosulfinate (2.3.10, Chapter 2).

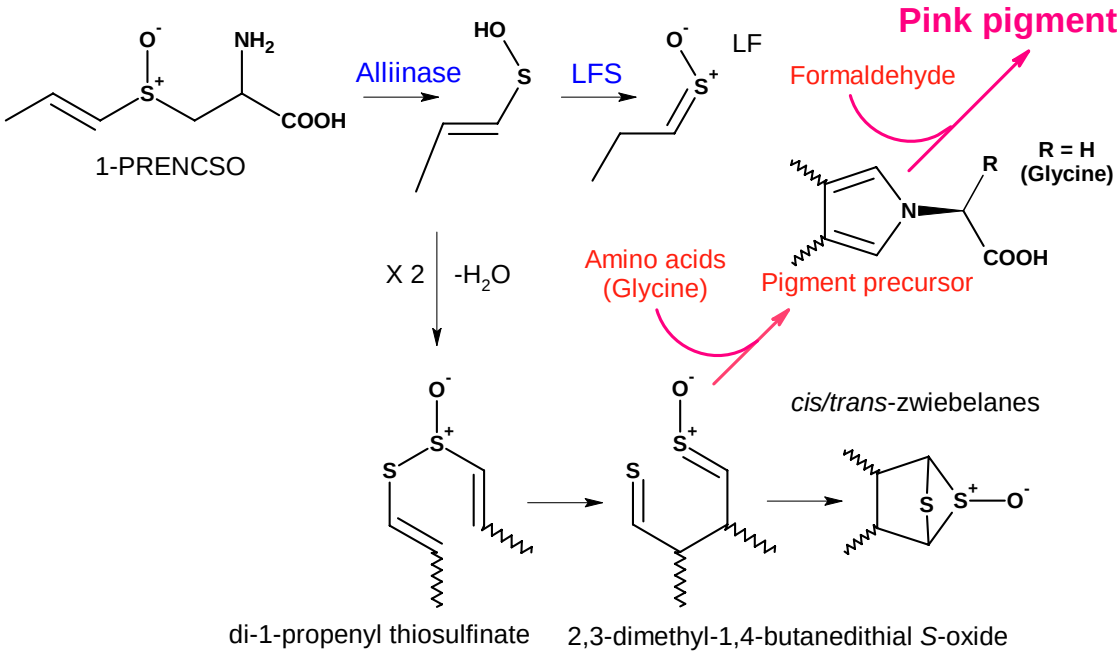


Figure 4.7 Proposed reaction mechanisms for producing a pink pigment.

i) Model study

Reliability and specificity of the assay was confirmed by model reaction analysis. Several amounts (0, 57, 113, 226, 339, 424, and 849 nmol) of 1-PRENCSO or 2-PRENCSO (alliin) mixed with garlic alliinase, glycine, and formaldehyde were incubated, and then absorbance in the reaction mixture was measured by spectrophotometer at 520 nm. 1-PRENCSO-derived thiosulfinate correlated with a pink colour, whilst 2-PRENCSO-derived thiosulfinate produced no pink colour (Figure 4.8.A). This result demonstrated that this 'pinking assay' is specific to the 1-PRENCSO-derived thiosulfinate. Another *in vitro* model study indicated that not all 1-PRENCSO-derived thiosulfinate is captured in the colour formation (data not shown). In further *in vitro* assays, rLFS, equivalent to 1 to 0.5 times normal physiological bulb levels of LFS, converted more than 85% of 1-PRENCSO to LF (data not shown). This demonstrated that LFS protein is a strong competitor for 1-propenyl sulfenic acid.

ii) Sample analysis

The 'pinking assay' was performed in bulb tissue of the representative lines (H3, O3, and D2) from each cultivar to confirm the levels of 1-propenyl-containing thiosulfinates. Onion extract was incubated in the presence of glycine and formaldehyde and then absorbance was measured by spectrophotometer at 520 nm. Levels of 1-propenyl-containing thiosulfinates were significantly increased in all transgenic lines compared with each non-transgenic counterpart (Figure 4.8.B). Furthermore, to confirm the absence of LFS protein as the cause of the pinking, onion extracts with added recombinant LFS (rLFS) protein, equivalent to 1 to $^{1}/_{2}$ times normal physiological levels of LFS, were examined similarly. The thiosulfinates level in all transformants significantly decreased compared to those without rLFS, whilst there was no change in non-transgenic onion plants with or without rLFS (Figure 4.8.B). These results indicated that 1-PRENCSO breakdown pathway shifts to thiosulfinate conversion in tearless onions, but is predominantly directed to LF conversion in normal onions, and that LFS protein is a strong competitor for 1-propenyl sulfenic acid.

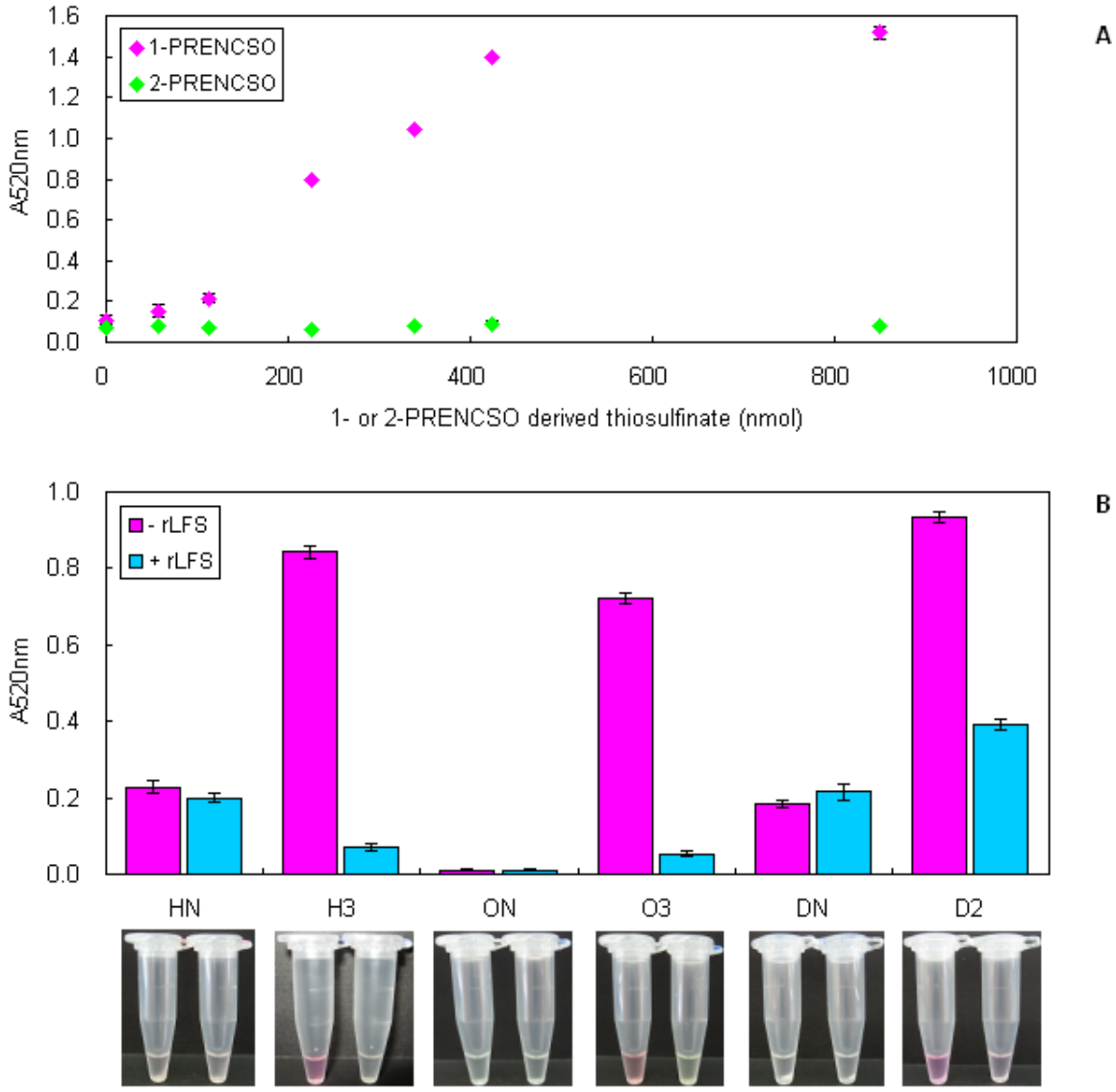


Figure 4.8 Results of pinking assay in model studies and in *lfs*RNAi transgenic onion plants.

(A) Specificity tests for pinking assay. The assay results are mean (plus minus SEM) of absorbances obtained from three separate sets of each amount of the substrates. Pink and green dots indicate 1-PRENCSO and 2-PRENCSO derived thiosulfinate levels, respectively. (B) Pinking assay results in transgenic and non-transgenic-lines. The values are mean (plus minus SEM) of absorbances obtained from three separate sets of extracted samples from each plant. Pink and blue bars indicate the pinking assay with and without rLFS, respectively. Images below show pinking colour for the respective lines (the left tube is without rLFS, the right is with rLFS). HN, ON, and DN, non-transgenic hybrid, open-pollinated, and dehydration onion controls, respectively; H3, O3, and D2, respective transgenic hybrid, open-pollinated, and dehydration onion lines.

4.2.2.2 Analysis of onion volatiles

The next step was to analyse onion sulfur volatiles produced by the increased thiosulfinates in the tearless transformants. Thiosulfinates have been reported to be converted non-enzymatically into volatile odours, for example, cepaenes, zwiebelanes, and polysulfides (Rose *et al*., 2005). To characterise all the true onion volatiles, Arnault *et al*. (2000) proposed that analysis by both solvent extraction (SE) GCMS and solid-phase microextraction (SPME) GCMS would be required due to the thermal instability of some of the volatile compounds. In general, SE injection is thermally milder than SPME injection because vaporization heat is deprived from sample solution in SE injection. Previous studies demonstrated that thiosulfinates, cepaenes, and zwiebelanes were detected in SE-GCMS, whilst disulfides required SPME-GCMS for detection (Ferary & Auger, 1996; Arnault *et al*., 2000). Disulfide compounds have been reported to be converted from thiosulfinates and zwiebelanes because of their thermally unstable property, in some cases even at room temperature (Arnault *et al*., 2000 & 2004; Block, 1993). In this study, we analysed onion volatiles by using both SE and SPME-GCMS.

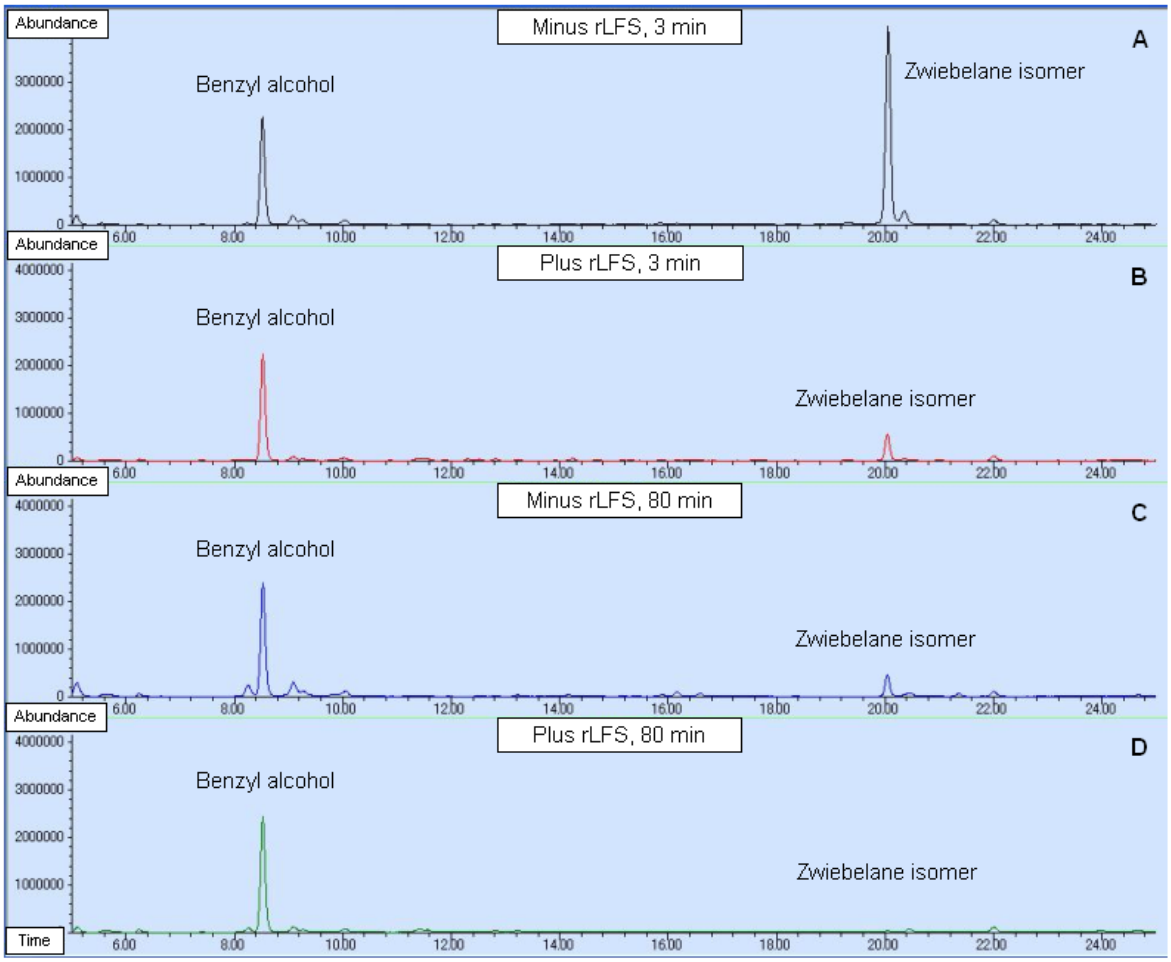
4.2.2.2.1 Solvent extraction-GCMS analysis

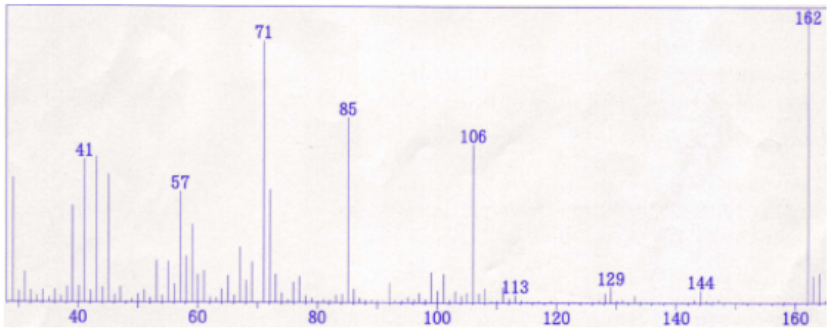
GCMS analysis of onion volatiles extracted with solvent was undertaken, according to Arnault's method (2000) with slight modification (7-fold diluted scale) (2.3.11, Chapter 2), to compare profile of thiosulfinates and their breakdown products between tearless onion and non-transgenic onion.

i) Model study

The different profile was examined by a model reaction analysis at first. 1-PRENCSO and garlic alliinase mixed with or without rLFS were incubated at room temperature for 3 or 80 min, and then, a white cloudy precipitate increased dramatically with incubation time in minus-rLFS reaction mixture (data not shown). The volatile compounds were extracted with diethyl ether containing benzyl alcohol as an internal standard and injected into GCMS. The total ion-chromatogram (Figure 4.9.A-D) produced by GCMS assay of the model reactions detected a large peak difference in the reaction without rLFS, which was confirmed by the mass spectral analysis, contrary to our expectations, to be a 'zwiebelane isomer' first reported in onion by Arnault (2000), although he has never identified the

chemical structure. The mass spectra of the ‘zwiebelane isomer’ was quite different from that of *cis* or *trans* zwiebelanes which were not detected, although the putative parent ion (m/z: 162) was the same (Figure 4.9.E & F). Furthermore, the ‘zwiebelane isomer’ dramatically decreased (90%) with incubation time. No compounds could be detected that increased with incubation time (Figure 4.9.A & C). This indicated that the ‘zwiebelane isomer’ is one of reaction intermediates arising from 1-PRENCSO breakdown.


Abundance
Minus rLFS, 3 min
A
Benzyl alcohol
Zwiebelane isomer
Abundance
Plus rLFS, 3 min
B
Benzyl alcohol
Zwiebelane isomer
Abundance
Minus rLFS, 80 min
C
Benzyl alcohol
Zwiebelane isomer
Abundance
Plus rLFS, 80 min
D
Benzyl alcohol
Zwiebelane isomer
Time


71
162
85
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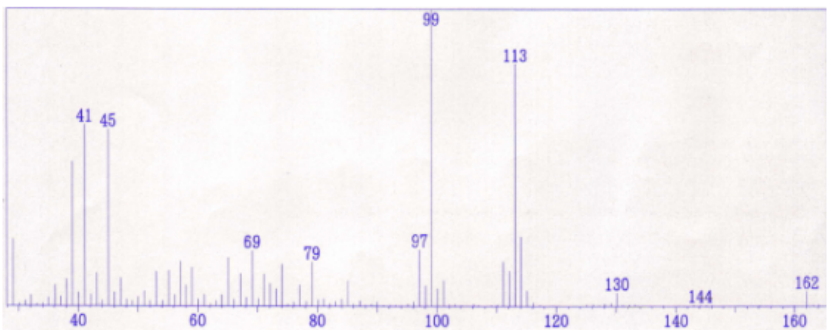
E


99
113
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162
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140
160

F

Figure 4.9 Results of solvent extraction-GCMS analysis in model studies.

(A) Total ion chromatogram of a model reaction without rLFS after an incubation period of 3 min. (B) Total ion chromatogram of a model reaction with rLFS after an incubation period of 3 min. (C) Total ion chromatogram of a model reaction without rLFS after an incubation period of 80 min. (D) Total ion chromatogram of a model reaction with rLFS after an incubation period of 80 min. (E) Mass spectrum of 'zwiebelane isomer'. (F) Mass spectrum of *cis* or *trans* zwiebelane.

ii) Sample analysis

The changes in the sulfur metabolites profile were examined in onion bulb materials similarly as described in a model reaction. Crushed onion was incubated at room temperature for 80 min to produce non-enzymatic reaction products, and the volatile compounds were extracted and analysed by GCMS. The total ion-chromatograms of transgenic onion volatiles extracted from lines H3, O3, and D2 compared with their non-transgenic counterparts detected a large peak difference in the transgenic extract, which corresponded to the 'zwiebelane isomer' in the model study (Figure 4.10). The 'zwiebelane isomer' in transgenic onion samples decreased more slowly (30%-70%) with incubation time than that in the model reaction (data not shown). This demonstrated that at least one of the proposed downstream sulfur compounds was elevated in the transgenic onions.

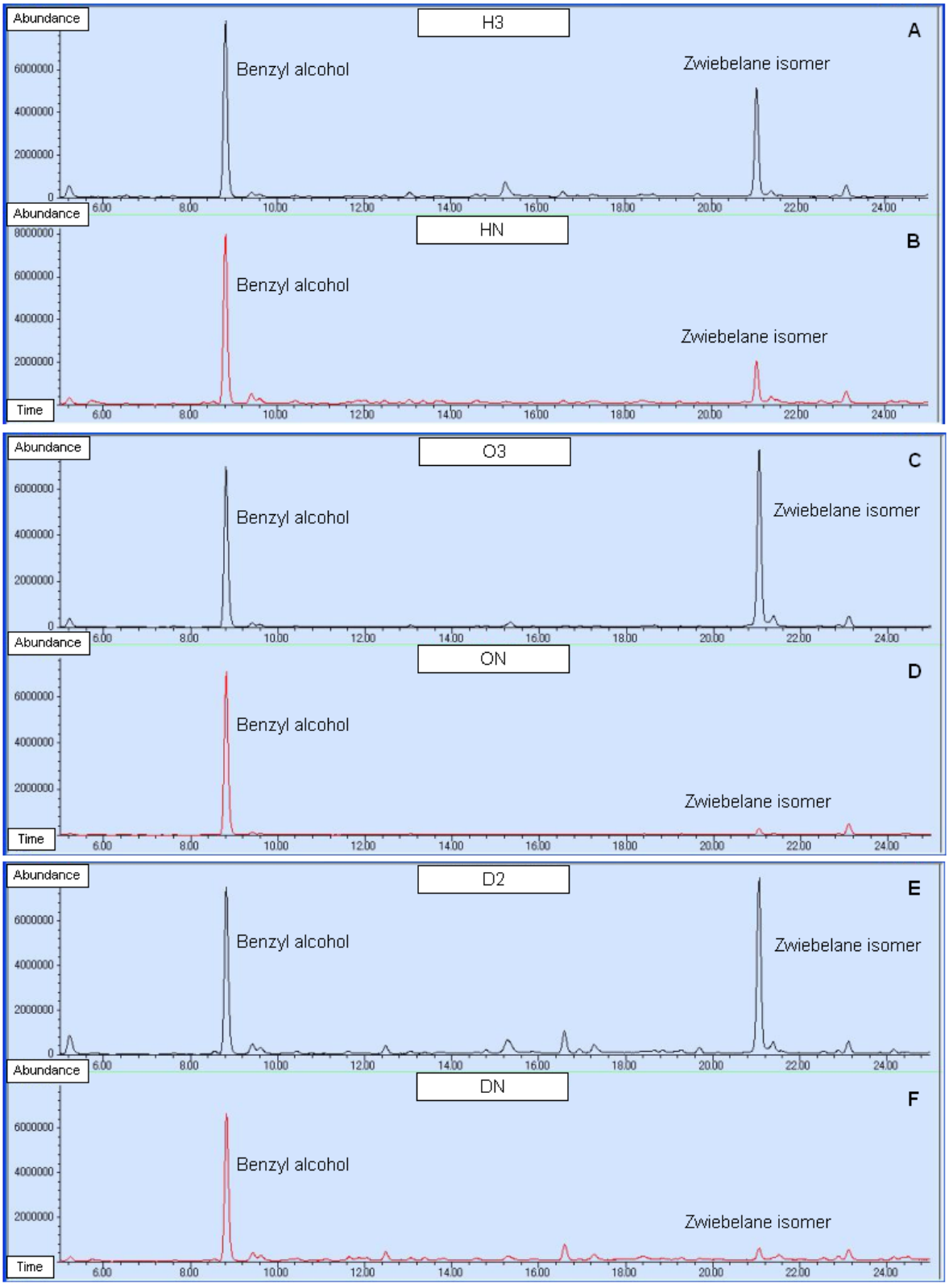

Figure 4.10 Results of solvent extraction-GCMS analysis in *lfs*RNAi transgenic onion plants.

HN, ON, and DN, non-transgenic hybrid, open-pollinated, and dehydration onion controls, respectively; H3, O3, and D2, respective transgenic hybrid, open-pollinated, and dehydration onion lines.

4.2.2.2.2 Headspace solid-phase microextraction-GCMS analysis

Headspace components of onion leaves were extracted with solid-phase microextraction (SPME) and analysed by GCMS, according to Hori's (2007) method with slight modification (2.3.11, Chapter 2), to examine difference in sulfur volatiles between tearless onion and non-transgenic onion. The identification of the volatile compounds was based on comparisons of mass spectral data with published work (Block *et al*., 1996b; Sinha *et al*., 1992).

i) Model study

The different volatiles were investigated by model reaction analysis at first. 1-PRENCSO and garlic alliinase mixed with or without rLFS in a vial were incubated at room temperature for 30 min. Then, the headspace components were extracted with SPME and analysed by GCMS. In consequence, the reaction without rLFS produced much increased di-1-propenyl disulfide isomers (Peak 3, 4 and 5) and 2-mercapto-3,4-dimethyl-2,3-dihydrothiophene isomers (Peak 6 and 7) compared to that with rLFS (Figure 4.11). These compounds have only tentatively been reported in trace or small amounts in onion or other *Allium* species (Boelens *et al*., 1971; Kuo & Ho, 1992a & b; Ferary & Auger, 1996; Mondy *et al*., 2001; Arnault *et al*., 2004). The dihydrothiophenes were first reported to be formed from pyrolysis of di-1-propenyl disulfide with heat (Block & Zhao, 1990) and have never been detected in onion. The detection of the dihydrothiophenes is attributed to the thermally severe condition of SPME-GCMS.

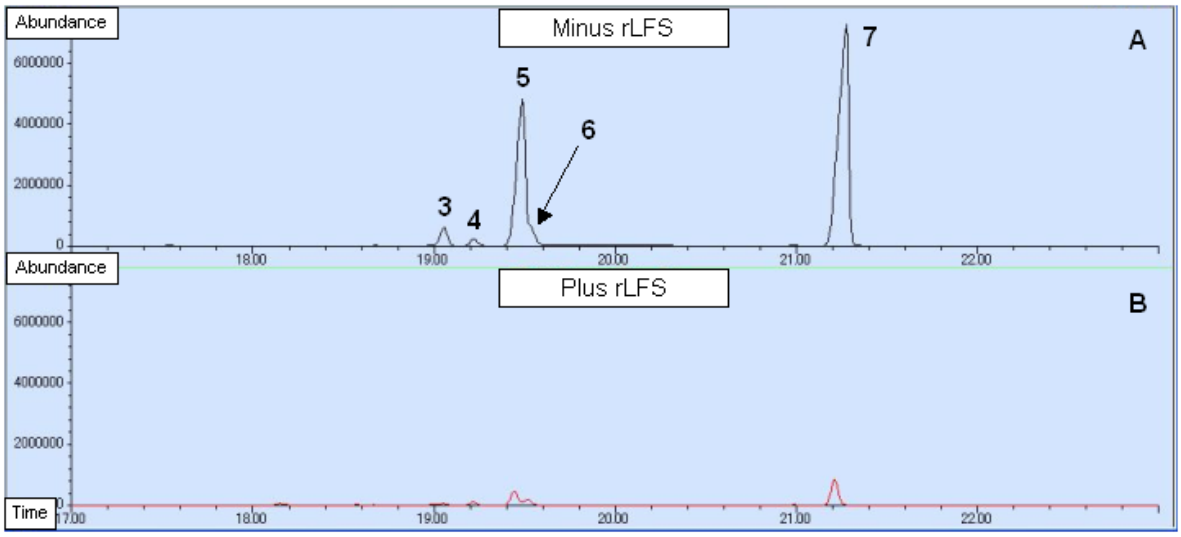

Figure 4.11 Results of SPME-GCMS analysis in model studies.

(A) Total ion chromatogram of a model reaction without rLFS. (B) Total ion chromatogram of a model reaction with rLFS. Peak 3, di-1-propenyl disulfide isomer 1; Peak 4, di-1-propenyl disulfide isomer 2; Peak 5, di-1-propenyl disulfide isomer 3; Peak 6, *syn*-2-mercapto-3,4-dimethyl-2,3-dihydrothiophene; Peak 7, *anti*-2-mercapto-3,4-dimethyl-2,3-dihydrothiophene.

ii) Sample analysis

The changes in the sulfur volatiles profile were examined in onion leaf materials. Similarly, test plant cut leaf materials were incubated in vials at room temperature for 30 min, and the headspace components were extracted by SPME and examined by GCMS. This analysis revealed that the tearless lines produced a decreased dipropyl disulfide peak and a much increased 1-propenyl propyl disulfide peak compared with the non-transgenic onion plant. In addition, as observed in the model study, the tearless lines produced five peaks not detected in the control onion, di-1-propenyl disulfide isomers and 2-mercapto-3,4-dimethyl-2,3-dihydrothiophene isomers (Figure 4.12). This demonstrated that the raised thiosulfinates was converted non-enzymatically into the downstream volatile sulfur compounds in the transgenic tearless onions.

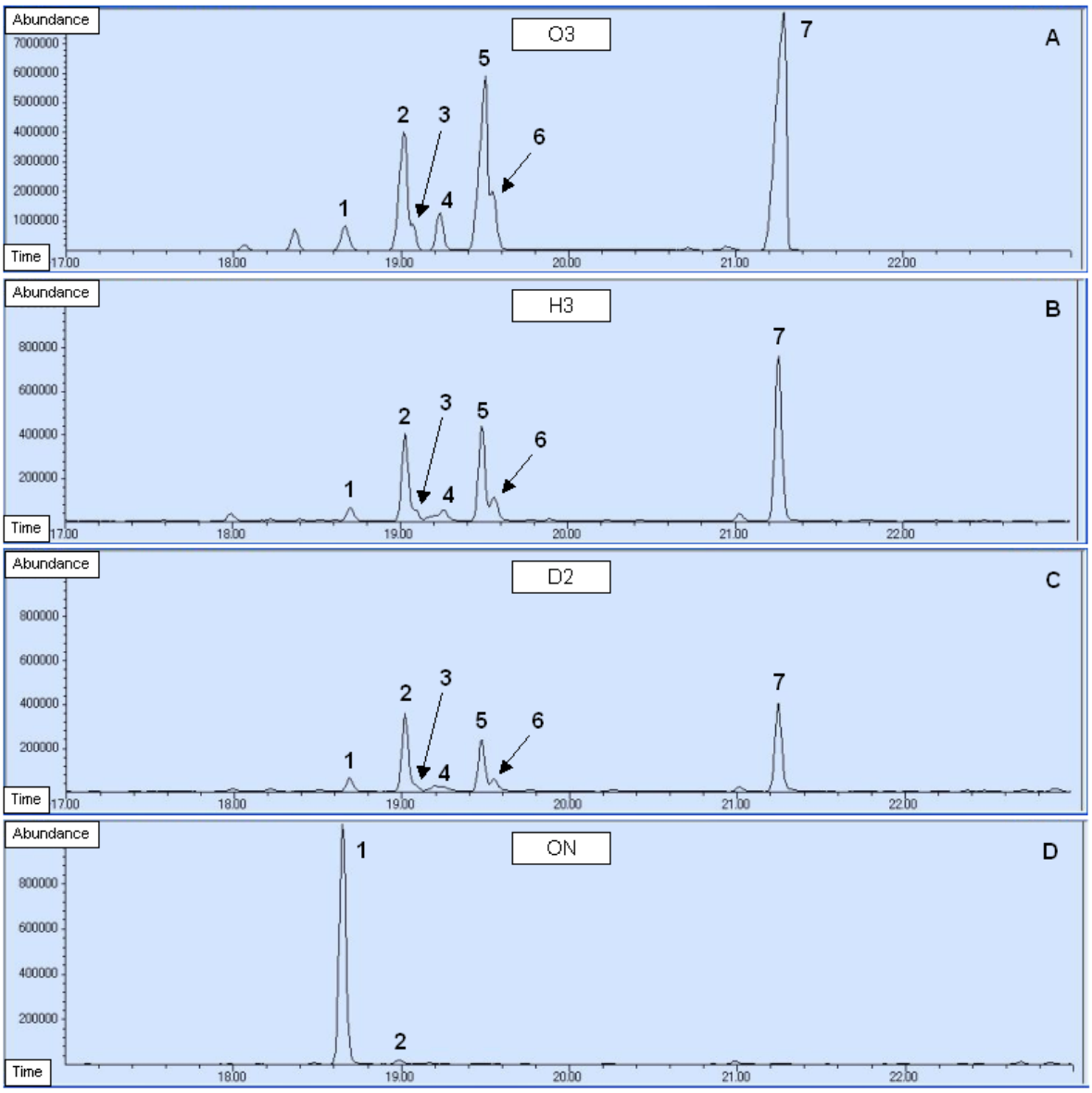

Figure 4.12 Results of SPME-GCMS analysis in *lfs*RNAi transgenic onion plants.

Peak 1, dipropyl disulfide; Peak 2, 1-propenyl propyl disulfide; Peak 3, di-1-propenyl disulfide isomer 1; Peak 4, di-1-propenyl disulfide isomer 2; Peak 5, di-1-propenyl disulfide isomer 3; Peak 6, *syn*-2-mercapto-3,4-dimethyl-2,3-dihydrothiophene; Peak 7, *anti*-2-mercapto-3,4-dimethyl-2,3-dihydrothiophene; ON, non-transgenic open-pollinated onion control; H3, O3, and D2, respective transgenic hybrid, open-pollinated, and dehydration onion lines.

4.3 Discussion

4.3.1 Summary of results

People, especially in the kitchen and in factories for processing onions, have suffered from a tear-inducing sensation upon the cutting or chopping of onion bulbs. Currently, 'tearless' onion cultivars (e.g. Vidalia), produced by growing under low sulfur fertilizer or sulfur-deficient soils, accumulate low amounts of cysteine sulfoxide. As a consequence, not only the pungent odour but also their characteristic flavours and health benefits related compounds reduce compared with more pungent high sulfur cultivars. In this study, we have for the first time successfully demonstrated the use of RNAi in *Allium* species, and showed that the single manipulation dramatically and simultaneously reduced the irritant LF and increased sulfur compounds related to flavour and health characteristics of alliums. Endogenous *lfs* transcripts levels were successfully reduced by up to 43-fold through a simple genetic transformation. In consequence, LFS enzyme activity was decreased by up to 1573-fold and the production of the deterrent LF was reduced by up to 67-fold in the transformants. This caused a large shift in the 1-PRENCSO breakdown pathway such that much more 1-propenyl sulfenic acid was available for conversion into thiosulfinates. 1-propenyl containing thiosulfinates levels were raised significantly and their non-enzymatically breakdown products, zwiebelane isomer, di-1-propenyl disulfide, and 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes were increased dramatically in the reduced LF onion plants.

4.3.2 Silencing in onion

This study is the first report of proven gene silencing in an *Allium* species. Although earlier work using antisense method to silence the *alliinase* gene produced some transgenic lines, conclusive proof of silencing was not obtained (Eady *et al*., 2005d). We have also demonstrated for the first time a dramatic simultaneous down- and up-regulation of products within plant secondary metabolic pathway through a single manipulation of that pathway. The RNAi approach has been successfully used to reduce compounds such as toxic gossypol in cottonseed (Sunilkumar *et al*., 2006) and allergen in peanut (Dodo *et al*., 2008), and also to elevate desirable compounds such as carotenoids and flavonoids in tomato (Davuluri *et al*., 2005), and the non-narcotic alkaloid reticuline in opium poppy (Allen *et al*., 2004). To our knowledge, there have been no previous examples of metabolic

engineering resulting in the simultaneous decrease of undesirable and increase of desirable biosynthetic products.

Analyses of 8 transgenic lines by molecular and biochemical assay revealed that 6 lines, H1, H2, H3, O2, O3, and D2, were dramatically silenced at a range of levels as expected but 2 lines, O1 and D1, were not. Line H2 succeeded in producing transcripts of *lfs*RNAi gene and silencing *lfs* gene although the line contained a truncated CaMV35S promoter deleted in 5' region of *lfs*RNAi cassette. This suggests that the truncated promoter still worked because it still contained, in the 3' region, the same functional sequence as the CaMV35S promoter of *gfp* expression cassette (Figure 2.5.A). This deletion, in the right border region, was unusual. A left border deletion, as observed in line O3 with a deleted in *nptII* gene, is more common and the deletion is not predicted to affect sequence 5' to the deletion (Tzfira *et al*., 2004). The deletion in O3 corresponded to the observation that the line grew poorly on media containing geneticin. The extensive PCR analysis of the full-length T-DNA region was vital for the elucidation of such T-DNA integration errors and provided data that supported our observations. Line O1 failed to produce detectable *lfs*RNAi transcripts and this contained multiple copies at a single locus, a phenomenon that is known for inducing transcriptional inactivation (Muskens *et al*., 2000; Tang *et al*., 2007). Interestingly, line D1 contained two copies at different loci and produced transcripts of *lfs*RNAi gene but failed to show detectable siRNA levels and silence the *lfs* gene. The analysis of hairpin transcript detection indicates that the inability to silence was not due to transcriptional inactivation but some other cause. Further research would be required to deduce the reason behind the lack of siRNA in this line.

The more significant production or reduction of LFS activity observed in bulb tissue over leaf tissue suggests that LFS is probably a relatively major protein within aestivating storage bulb tissue, whilst within leaf tissue it is probably less abundant due to the requirement for much more 'housekeeping' functions (e.g. photosynthesis). In addition, especially in bulb materials, the extent of the relative reduction of LF compared to control material was much less than the reduction observed in LFS activity. Analysing the number of moles of 1-PRENCSO and LF produced in the control onion lines (data not shown) suggests that the inconsistency might be due to the analytical method of LF detection. Kopsell *et al*. (2002) and our *in vitro* assay using model reaction system (data not shown) demonstrated that several tens of seconds, much shorter than the incubation time used in this study, give a more accurate quantification of LF in onion bulb because of the unstable,

reactive, and highly-volatile property of LF. This indicates that our control levels may have been significantly lower than actual LF liberated and that further investigation is required to develop a method for accurate LF quantification.

A previous study reported that RNAi silencing of codeinone reductase in opium poppy, at the end of the biosynthetic pathway leading to morphine, resulted in an accumulation of the precursor alkaloid (*S*)-reticuline that is seven enzymatic steps upstream of the secondary pathway (Allen *et al*., 2004). Both alliinase activity and 1-PRENCSO levels in the transgenic and control lines (data not shown) were within the normal physiological range reported for onions (Kitamura *et al*., 1997; Kopsell *et al*., 1999). This suggests that silencing *lfs* transcripts affected neither alliinase activity nor 1-PRENCSO levels in our transgenic onion plants.

4.3.3 Sulfur secondary metabolite profile changes

Analyses of the 3 representative tearless onion lines by pinking and GCMS assays revealed a dramatic profile alteration of sulfur secondary metabolites. The inability of the tearless onion to convert the 1-propenyl sulfenic acid to LF provided an opportunity for this reactive compound to non-enzymatically be converted into a series of sulfur compounds (Figure 4.13.A). The alk(en)yl cysteine sulfoxides (ACSOs) breakdown pathway in the tearless onion becomes more similar to that in garlic (Figure 4.13.B). We observed using the pinking assay that in the transgenic onions 1-propenyl sulfenic acid is predominantly condensed to 1-propenyl containing thiosulfinate, and from the GCMS analyses we could deduce that the thiosulfinate is then converted to ‘zwiebelane isomer’, di-1-propenyl disulfides and 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes. Surprisingly, many of these compounds, which were present in much greater abundance than expected in the tearless onion, have never been detected or have been detected only in small amounts in onion (Boelens *et al*., 1971; Ferary & Auger, 1996; Mondy *et al*., 2001; Arnault *et al*., 2004). On the contrary, sulfur volatile compounds, such as 1-propenyl methane thiosulfinate, *cis* or *trans*-zwiebelanes and dipropyl disulfide, which have been reported to be present dominantly in onion (Block *et al*., 1992b & c; Keusgen *et al*., 2002), were not detected in the tearless onion. The data presented here suggests that 1-PRENCSO breakdown pathway predominantly produced LF in onion. This is supported by the *in vitro* model study, whilst a previous study reported that approximately 70% of 1-sulfenic acid is converted to LF (Block *et al*., 1996a).

The non-LFS mediated 1-PRENCSO breakdown pathway has so far remained largely unknown in onion. Bayer *et al*. (1989) isolated and identified unique cyclic organosulfur compounds, *cis* or *trans* zwiebelanes, for the first time in onion juice, and proposed a model production pathway. The 1-propenyl sulfenic acid self-condense to di-1-propenyl thiosulfinate, via a different pathway from LF, and then the thiosulfinate rearranges into the zwiebelanes through 2,3-dimethyl-1,4-butanedithial *S*-oxide (Figure 4.14). However, nobody has detected the di-1-propenyl thiosulfinate or the 2,3-dimethyl-1,4-butanedithial *S*-oxide in onion, and the absence of these compounds has been suggested to be due to their instability (Block *et al*., 1996a & b). Furthermore, Block *et al*. (1996b) succeeded in synthesizing *cis* or *trans* zwiebelanes from di-1-propenyl disulfide by oxidation and proposed the model reaction pathway (Figure 4.14). However, an enigma arose from the synthesis. The zwiebelanes were produced from *cis*, *cis*-di-1-propenyl disulfide and not from *trans*, *trans*-1-propenyl disulfide. This indicated that the cyclic organosulfur compounds are produced from *cis*-1-PRENCSO but not from *trans*-1-PRENCSO although the *trans* form was predicted to be dominant as the *cis* form has not been reported in onion. Instead, 5,6-dimethyl-2-oxa-3,7-dithiabicycloheptane which also has never been detected in onion was produced from the *trans*, *trans*-disulfide. These studies have never analysed LFS silenced onion or the *in vitro* model reaction comprising 1-PRENCSO and garlic alliinase. Although a previous study did analyse a similar model reaction, it used crude alliinase purified from onion so the model reaction probably would be contaminated with LFS (Shen & Parkin, 2000). Therefore, analyses of our tearless onion and model reaction has for the first time enabled detection several organosulfur volatiles in much greater abundance than previous reports, and might help resolve this enigma.

We observed in solvent extraction GCMS analysis that, contrary to our expectation, a ‘zwiebelane isomer’ was present in great abundance in both tearless onion and model reaction without rLFS, and that it decreased dramatically with incubation time (Figure 4.9 & 4.10). This suggests that ‘zwiebelane isomer’ is extremely volatile like LF, or one of reaction intermediates arising from 1-PRENCSO breakdown. Surprisingly, the mass spectrum of ‘zwiebelane isomer’ was quite different from that of *cis* or *trans*-zwiebelanes (Figure 4.9.E & F), although the putative parent ion (m/z: 162) was same. Furthermore, ‘zwiebelane isomer’ purified by thin layer chromatography (TLC) formed pink colour by the pinking assay (data not shown). Although the full chemical structure has not yet been elucidated, the data presented here suggest that the ‘zwiebelane isomer’ is not an isomer of

zwiebelane but 1-propenyl containing sulfur compound. The ‘zwiebelane isomer’ is now being purified and analysed using nuclear magnetic resonance (NMR).

On the other hand, although we expected that *cis* or *trans*-zwiebelanes, or the dithiabicycloheptane would be present at dramatically increased levels in the tearless onion and model reaction without rLFS, solvent extraction GCMS analysis indicated that neither of these compounds could be detected over the incubation times analysed (Figure 4.9 & 4.10). This observation of the absent *cis* or *trans*-zwiebelanes corresponds to the Block’s result that the zwiebelanes were not produced from *trans*, *trans*-1-propenyl disulfide. This suggests that *cis* or *trans*-zwiebelanes might be produced via a different pathway from Bayer’s proposal. Enzymatic reaction, redox reaction or LF reaction via *trans*-1-PRENCSO, or *cis*-1-PRENCSO breakdown reaction might be involved in the production of the zwiebelanes. Although further investigation is required, our tearless onions and *in vitro* model reactions will provide a valuable tool to elucidate them. Analyses of the *cis*-1-PRENCSO in onion, and an onion homogenate with 13C-labelled *cis* or *trans*-1-PRENCSO added might help resolve this enigma and help clarify how *cis* or *trans* zwiebelane are produced.

In SPME-GCMS analysis, di-1-propenyl disulfides and their derivatives, 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes, were present in great abundance in both tearless onion and model reaction without rLFS (Figure 4.11 & 4.12). The detection of di-1-propenyl disulfides indicates the presence of di-1-propenyl thiosulfinate because thiosulfinates have previously been reported to be converted into disulfides in SPME-GCMS analysis due to the thermal instability of thiosulfinates (Arnault *et al*., 2000 & 2004). This observation corresponded to the results of pinking assay. Furthermore, the analysis of both tearless onion and the *in vitro* model reaction also provided clues for the production of dipropyl disulfide. 1-propeny propyl disulfide increased and dipropyl disulfide decreased dramatically in tearless onion compared with non-transgenic onion, whilst these disulfides were not detected in both model reactions (Figure 4.11 & 4.12). This observation in the tearless onion is most likely a result of the greater ability of the propyl sulfenic acid to react with the propenyl form or the decrease of LF (Block, 1991). In general, dialk(en)yl disulfide is produced from the corresponding precursor compound, alk(en)yl- L-cysteine sulfoxide, via dialk(en)yl thiosulfinate. Therefore, dipropyl disulfide is proposed to have arisen from propyl-L-cysteine sulfoxide (PCSO). However, interestingly, the major component in the headspace analysis, dipropyl disulfide, is present to much greater extent

than is reflected in the amount of the PCSO, which has never been detected or only previously detected at low levels in onion (Table 1.2, Chapter 1). Furthermore, the dipropyl thiosulfinate has only been reported at low levels in onion (Table 1.3, Chapter 1, Block *et al*., 1992b & c). To explain the presence of dipropyl disulfide, Block (1991) suggested that LF is reduced to propanesulfenic acid and then to propanethiol. When propanesulfenic acid and propanethiol are combined, they produce dipropyl disulfide. However, the dipropyl disulfide was not detected in our model study with rLFS, seemingly contradicting the above, although the reducing agent might be absent in the model study (Figure 4.11.B). Further analysis of our tearless onions, the model reaction, and an onion homogenate with 13C-labelled 1-PRENCSO added might help resolve these enigmas and help elucidate the unknown pathway of producing dipropyl disulfide.

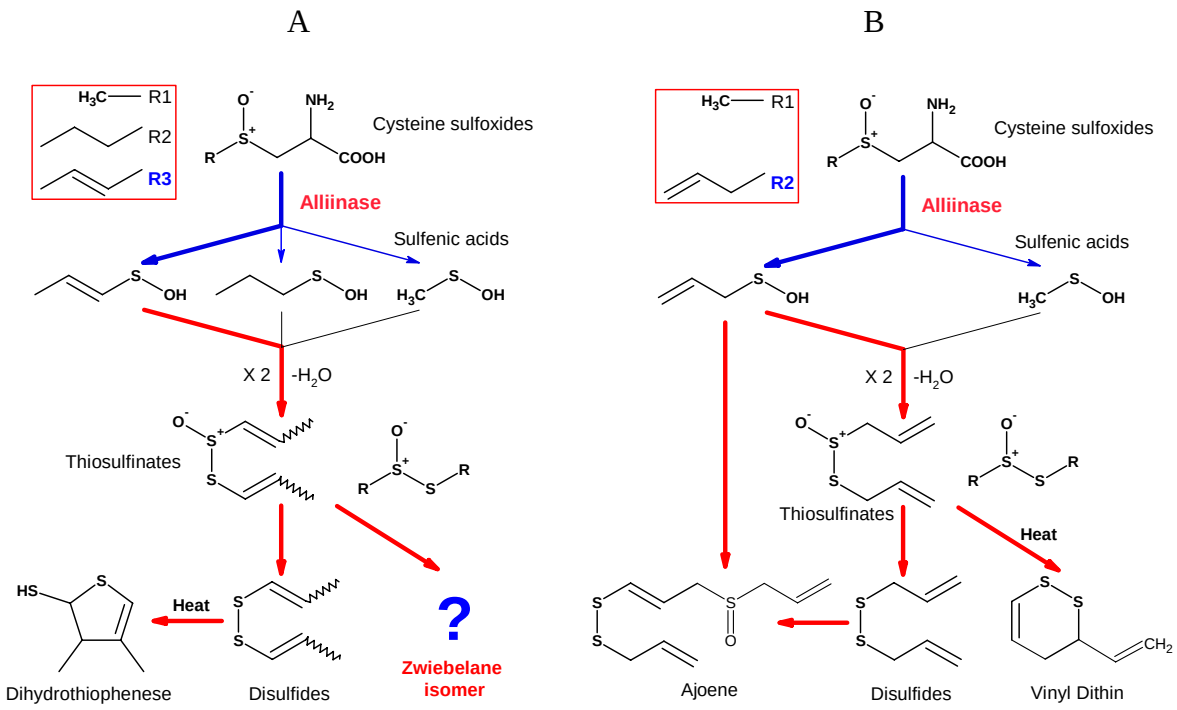

Figure 4.13 Major breakdown pathways of cysteine sulfoxides in tearless onion (A) and garlic (B).

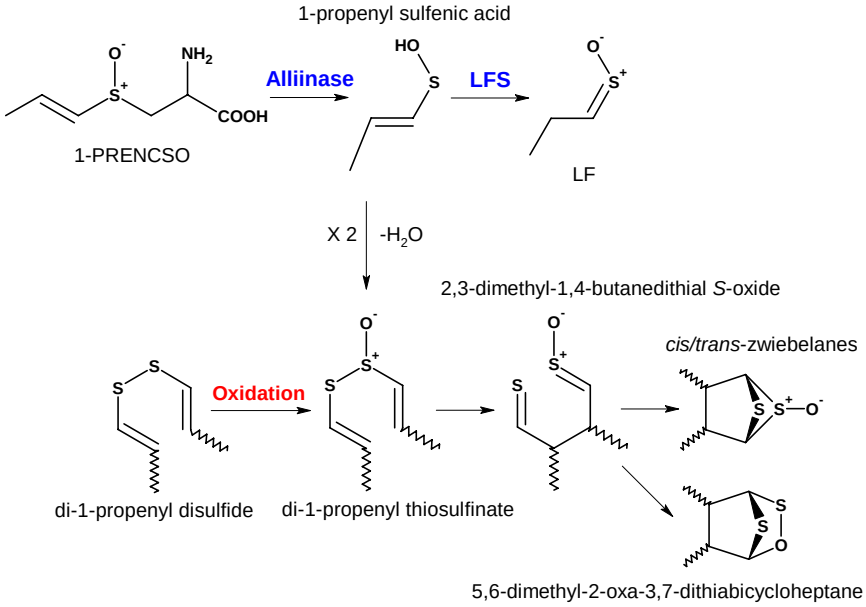

Figure 4.14 Proposed reaction mechanism for producing *cis* or *trans* zwiebelanes by Bayer (1989).

4.3.4 Sensory perception

From an olfactory viewpoint, the reduction of LF in our transgenic tearless onion lines would be predicted to reduce the pungent aroma associated with LF (Randle *et al*., 1994; Randle, 1997). The dramatically raised amount of 1-propenyl-containing thiosulfinates or disulfides and dihydrothiophenes would be predicted to increase the sweeter aroma of raw fresh and cooked or fried onions that are associated with these compounds (Lancaster & Boland, 1990; Randle *et al*., 1994; Randle, 1997; Albanese & Fontijine, 2002). The dihydrothiophenes were first reported by Block & Zhao (1990) to be formed from pyrolysis of di-1-propenyl disulfide with heat (Figure 4.11). Therefore, the detection of the dihydrothiophenes is attributed to the thermally severe condition of SPME-GCMS. Thus, whilst the disulfides and dihydrothiophenes may not be present in raw tearless onion, they are likely to be produced in cooked or fried tearless onion. These predictions indicate that the tearless onions may harbour enhanced desirable aromas and not lose the characteristic flavours that onions are renowned for. It is still necessary to determine the precise olfactory or flavour role of the other organosulfur volatiles that have been produced in our tearless onion lines. Subjective sensory evaluation (smell and tearing) by the authors concurred with the above predictions but sensory taste panels cannot be undertaken until regulatory approval is gained, this is currently being sought.

4.3.5 Health benefits

There are two major differences in the organosulfur compounds of garlic and onion – one is the loss of LF (Imai *et al*., 2002) and the other is the production of the substrate 2-PRENCSO in garlic compared with 1-PRENCSO in onion (Randle & Lancaster, 2002). The consequence of this is that onion produces mainly LF upon tissue disruption whilst garlic produces mainly allicin. Allicin has antimicrobial, antifungal, antiparasitic, and antiviral activity (Ankri & Mirelman, 1999) as well as beneficial health effects on cardiovascular, immune, and respiratory systems (Keusgen, 2002). There is a clear rank order of pharmacological activity with unsaturated thiosulfinates (like 1-propenyl-containing thiosulfinates) and polysulfides being more active than saturated ones (Breu & Dorsch, 1994; Benavides *et al*., 2007). Thiosulfinates, like allicin, have anti-asthmatic activity thought to be due to the suppression of cyclooxygenase and lipoxygenase-mediated reactions. These initiate eicosanoid metabolism and lead to bronchial restriction. This would indicate that our tearless onion, which now produce an analogous compound to

allicin with equivalent bioactivity could be predicted to have similar health benefits. In addition, thiosulfinates have anti-platelet aggregation activity (Briggs *et al*., 2000). However, garlic has 13 times more potent anti-aggregatory effect than onion (Ali *et al*., 1999). This might be because in normal onion 1-propenyl sulfenic acid is predominantly consumed to produce LF not thiosulfinates and, in consequence, total amount of thiosulfinates in onion is much less than that in garlic (Table 1.3, Chapter 1). The presence of thiosulfinates in high levels may confer more potent bioactivity to tearless onion than normal onion or considerable new properties that have previously been associated with garlic.

Furthermore, the raised levels in our tearless onion may further enhance the beneficial effects on cardiovascular function. Benavides *et al*. (2007) demonstrated that di-2-propenyl disulfide is converted into hydrogen sulfide (H_2S) by red blood cells and there is a clear rank order of H_2S producing ability with unsaturated disulfides being more active than saturated ones. This is currently known to be a powerful gaseous signalling molecule in humans which is responsible for vasodilation, the inhibition of leukocyte-endothelial cell interactions in the circulation. It is also a potent antioxidant and inhibits cellular apoptosis, and has been shown to transiently and reversibly inhibit mitochondrial respiration. Collectively this is an ideal physiological profile for cardiovascular protection (Lefer, 2007). Di-1-propenyl disulfide in high levels reported in our tearless onion could thus be predicted to have a more potent cardiovascular effect than normal onion. All this indicates that the raised organosulfur profiles observed in the tearless onions warrant much further and extensive investigation from a health perspective.

One caveat to this is the fact that disulfides have also been associated with haemolytic anaemia and that di-1-propenyl disulfide has been reported to be more toxic than the di-2-propenyl form (Munday & Manns, 1994). Cats and dogs are susceptible to such anaemia from onion and garlic, and so could be even more vulnerable to consumption of the tearless onions reported here. Nobody has reported about any role in human health for the increased levels of 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes.

4.3.6 Pinking assay

The development of the ‘pinking’ assay reported in this chapter was originated from in-depth studies of the cause of discoloration in processed onion and garlic products (Imai *et*

al., 2006a & b). It provides a unique and simple procedure for detecting the 1-PRENCSO-derived thiosulfinate which is difficult to determine due to its instability. Unlike a colorimetric procedure based on reaction with *N*-ethylmaleimide (Nakata *et al*., 1970), which detects all thiosulfinates, this 'pinking' method is specific to 1-PRENCSO-breakdown thiosulfinate. The development of a high-throughput protocol for this unique assay will provide onion researchers, breeders and processors with a simple tool to evaluate this valuable sulfur-derived bioactive and aroma compounds in onions, and perhaps help them identify a conventionally or mutagenically generated (non-genetically modified) tearless onions.

4.3.7 Conclusions

This study is the first report that a single manipulation of plant secondary metabolite pathways can provide dramatic simultaneous down and up regulation of products within that pathway. We demonstrated that a manipulation of onion sulfur metabolite pathway gene simultaneously increased levels of desirable compounds and decreased detrimental one. Our transgenic onions potentially offer a uniquely desirable commodity by combining tearlessness with higher levels of health-enhancing thiosulfinates and disulfides. The increased levels of these compounds and reduced levels of LF should enhance many of the desirable flavour and olfactory notes whilst reducing the pungency of onions. Our transgenic tearless onions will provide a valuable tool to dissect unknown metabolite pathway, flavour properties, and multifunctional health activities of onion.

Chapter 5: Concluding discussion

5.1 Overview of thesis

The overall goal of this thesis was to examine whether silencing of LFS enzyme activity would simultaneously reduce levels of the deterrent LF and increase the desirable thiosulfinates in onions.

RNAi technology was chosen for this study as a method to silence the LFS activity. Over the past few years, RNAi has become a powerful tool for metabolic engineering and virus resistance as well as functional gene analysis in a variety of plants (Mansoor *et al*., 2006; McGinnis *et al*., 2007). However, to test several RNAi cassettes directly in onion and to obtain several lines for analysis would be considerable undertaking because of inefficient onion transformation. Hence, an initial objective to develop a rapid and simple test system for the evaluation of *lfs*RNAi silencing through the use of tobacco as a model host was included.

The initial objective was divided into two parts. The first was to produce transgenic model host tobacco plants containing and expressing the *lfs* gene from onion by introducing a CaMV 35S-onion *lfs* gene construct. This was described in the first half of Chapter Three. Seven transgenic lines were characterized and demonstrated the presence of T-DNA region and *lfs* gene transcripts in all lines by PCR and RT-PCR analyses (Figure 3.2 & 3.4.A, Chapter 3). LFS protein levels, assessed by western blot analysis and LFS activity assay, varied dramatically among different lines (Figure 3.4.B & C, Chapter 3). A big variation was observed among both single and multiple copy lines. This was as expected as position effect and copy number of an integrated gene affect its expression level (Gallie, 1998; Matzke & Matzke, 1998). One line (L1), which contained a single copy of the *lfs* gene and exhibited the same high level of LFS enzyme activity as onion leaf, was selected as a model plant for the silencing test system. The second part of this objective was to subsequently transform the model plant with an RNAi construct directed against the *lfs* gene sequence and to evaluate the silencing efficiency of the RNAi by assessment of *in vitro* LFS enzyme activity. This was outlined in the second half of Chapter Three. The second transformation of the model plant with the *lfs*RNAi construct produced a similar range of additional integration lines. Ten transgenic lines were examined and demonstrated the presence of T-DNA region and *lfs*RNAi gene transcripts in all lines by PCR and RT-PCR analyses (Figure 3.7 & 3.9.A, Chapter 3). LFS enzyme activity assay showed that the dual transgenic tobacco plants, containing both the *lfs* gene and the RNAi construct, had at

least 18-fold reduction in the LFS activity (Figure 3.9.D, Chapter 3). This observation was supported by western analysis for the LFS protein and further validated by quantitative RT-PCR analysis that demonstrated significant reduction in the *lfs* transcript level in the dual transformants (Figure 3.9.B & C, Chapter 3). These results demonstrated that the RNAi construct is a suitable candidate for the development of a tearless onion.

The second and major objective was to produce a tearless onion by transformation using the functional RNAi vector identified in the model system. This was described in the first section of Chapter Four. The RNAi construct was transformed into three onion cultivars; a mild hybrid (H) mid-daylength fresh onion cv. Enterprise, a pungent open pollinated (O) fresh onion cv. Pukekohe LongKeeper and a pungent dehydration (D) mid-daylength onion (Sensient Dehydrated Flavors). Eight transgenic lines, H1, H2, H3, O1, O2, O3, D1 and D2, were characterized by molecular and biochemical analysis. Under selection and regeneration the transformed tissue behaved in a similar manner to that observed in previous onion transformations (Eady *et al*., 2000 & 2002). All lines grew and formed morphologically equivalent plants and bulbs to their non-transgenic counterparts (Figure 4.2, Chapter 4). Seed set and T1 progeny have been obtained from two lines by selfing or crossing onto their non-transgenic counterparts (data not shown). Six lines (H1, H2, H3, O2, O3 and D2) of eight transgenic onion lines were dramatically silenced at a range of levels as expected. *lfs* siRNA fragments were detectable and endogenous *lfs* transcripts levels were successfully reduced by up to 43-fold (Figure 4.5, Chapter 4). In consequence, LFS enzyme activity was decreased by up to 1573-fold and this observation was supported by western analysis for the LFS protein. Furthermore, the production of the deterrent LF upon tissue disruption was reduced up to 67-fold (Figure 4.6, Chapter 4). On the other hand, two lines, O1 and D1, failed to be silenced. Line O1 failed to produce detectable *lfs*RNAi transcripts and this contained multiple copies at a single locus, a phenomenon that is known for inducing transcriptional inactivation (Figure 4.4 & 4.5.A, Chapter 4) (Muskens *et al*., 2000; Tang *et al*., 2007). Interestingly, line D1 contained two copies of at different loci and produced transcripts of *lfs*RNAi gene but failed to show detectable siRNA levels and silence the *lfs* gene (Figure 4.4 & 4.5, Chapter 4). This inability to silence was not due to transcriptional inactivation but some other cause. Aside from these observations, both alliinase activity and 1-PRENCSO levels in the transgenic and control lines (data not shown) were within the normal physiological range reported for onions (Kitamura *et al*., 1997; Kopsel *et al*., 1999). This suggests that silencing *lfs* transcripts

affected neither alliinase activity nor 1-PRENCSO levels in the transgenic onion plants.

The third and final objective was to determine the change of volatile sulfur profiles containing thiosulfinates and predict the 1-PRENCSO breakdown pathway in the silenced transgenic onions. This was outlined in the second section of Chapter Four. We predicted that by reducing LFS protein, more 1-PRENCSO-derived sulfenic acid would be available for thiosulfinates production and, in consequence, the raised thiosulfinates would be converted non-enzymatically into the downstream volatile compounds. Subjective olfactory assessment of silenced lines indicated that the pungent odour given off by the leaf and bulb material was much reduced compared with that of non-transgenic counterparts, and that this was replaced by a sweeter milder onion odour. Three good silenced lines (H3, O3 and D2) were investigated by chemical analyses and these showed that there were dramatic changes in the volatile sulfur profiles compared with control counterparts. A novel colorimetric ('pinking') assay demonstrated that 1-PRENCSO derived thiosulfinates were significantly increased in all the transgenic lines. Addition of recombinant LFS protein to the onion extract decreased the 'pinking' level and confirmed the absence of LFS as the cause of producing pink pigment (Figure 4.8.B, Chapter 4). These results revealed that the 1-PRENCSO breakdown pathway shifts to thiosulfinates conversion in the silenced lines, but predominantly directs to LF production in control onions. Moreover, these observations demonstrated that LFS protein is a strong competitor for 1-propenyl sulfenic acid, and would indicate that alliinase and LFS should work in close proximity. Subsequent solvent extraction-GCMS and SPME-GCMS analysis (Figure 4.10 & 4.12, Chapter 4) demonstrated that a consequence of the raised thiosulfinates levels was a marked increase in the downstream production of a non-enzymatically produced 'zwiebelane isomer' that has never previously been identified, and other volatile sulfur compounds, di-1-propenyl disulfides and 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes, which had previously been reported either in small amounts or had not been detected in onions. These results and previous studies (Block & Zhao, 1990; Arnault *et al*., 2000 & 2004) made it possible to predict the 1-PRENCSO breakdown pathway (Figure 4.13.A, Chapter 4). 1-propenyl sulfenic acid produced from 1-PRENCSO by alliinase would be self-condensed into di-1-propenyl thiosulfinate, which would be subsequently converted into 'zwiebelane isomer' and di-1-propenyl disulfides. The disulfides would be decomposed into 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes with heat such as the thermally severe injection process of SPME-GCMS analysis.

These results demonstrated that the overall goal of this thesis was completed. This is the first report to demonstrate that silencing of LFS enzyme activity by introducing a RNAi construct directed against the *lfs* gene sequence simultaneously reduced levels of the deterrent LF and increased the desirable thiosulfinates in onions.

5.2 Contribution of this thesis to plant science and onion technology

5.2.1 Development of gene silencing assessment system in a model plant

A practical rapid system has been developed to first assess silencing of secondary metabolite pathway genes originating from recalcitrant or poorly studied plant species such as onions (Chapter 3; Kamoi *et al*., 2008). Previous studies (Kerschen *et al*., 2004; Xiong *et al*., 2005; McGinnis *et al*., 2007) have reported that in some transgenic lines RNAi failed or silenced inefficiently because of position effect or copy number of the transgene, or sequence composition, expression pattern or turnover rate of a target RNA. Therefore, it is important to have a detailed knowledge of the target gene, the silencing sequences, and several transgenic lines for analysis in order to evaluate the silencing potential. In onion, however, this would be a considerable undertaking because of its inefficient transformation. Our approach is novel and of great advantage in obtaining a reliable RNAi cassette before use in recalcitrant target plant species, and in consequence, saves considerable time and effort. This assessment system could prove invaluable for the study of many plant secondary metabolite products used in pharmacological, food and process industries.

5.2.2 Production of tearless onions

We have demonstrated for the first time dramatic simultaneous down- and up-regulation of products within plant secondary metabolite pathway through a single manipulation of that pathway (Chapter 4, Eady *et al*., 2008). The RNAi approach has been successfully used to reduce compounds such as toxic gossypol in cottonseed (Sunilkumar *et al*., 2006) and allergen in peanut (Dodo *et al*., 2008), and also to elevate desirable compounds such as carotenoid and flavonoid in tomato (Davuluri *et al*., 2005), and non-narcotic alkaloid reticuline in opium poppy (Allen *et al*., 2004). To our knowledge, there have been no previous examples of metabolic engineering resulting in the simultaneous decrease of deleterious and increase of desirable biosynthetic products.

Currently almost tearless (low-pungency) onions are produced by growing onion varieties that contain low levels of 1-PRENCSO under low sulfur fertilizer (Randle *et al*., 1994 & 1995), for example the famous Vidalia^{R} or Supasweet^{TM} onion. However, these onions would reduce not only LF but also thiosulfinates and their converted volatile sulfur compounds, thus potentially losing many of the characteristic flavour notes and health benefits related to these volatiles. Furthermore, the shelf life of Vidalia^{R} onions is limited to around four months so that there is no way to store excess supply (Clemens, 2002), and the cultivated area is also limited to sulfur-deficient soils. On the contrary, unlike the current almost tearless onions, our silenced transgenic onions reduced only LF but maintained normal levels of 1-PRENCSO and increased thiosulfinates and their converted volatile compounds responsible for the flavours and the bioactivities. Therefore, our transgenic tearless onions would be predicted to have less of the generally undesirable pungent flavour and aroma notes, and more of the desirable onion flavour and aroma notes, plus health benefits normally associated with the sulfur compounds in garlic. In addition, as tearless onions could be produced in any onion germplasm then shelf life and cultivation considerations, required for current almost tearless onions, would not be an issue. Thus our tearless onions could be considered as an example of 'next-generation biotech crops' which provide benefits to both farmers and consumers (Martino-Catt & Sachs, 2008).

Whilst breakdown pathways of ACSOs in garlic have been studied in details, those in onion have contained still unknown pathways. Onions represent a genus with an important, unique, complex, yet poorly understood secondary sulfur metabolite pathway because of the presence of LFS (Block, 1992a; Randle & Lancaster, 2002). The sulfur volatile metabolites in *Allium* species have been extensively studied for their pesticidal effects such as insecticides, acaricides, nematicides, herbicides, fungicides, bactericides and repellents against arthropods (Auger *et al*., 2004). Conversely, several pests and diseases actively seek out the sulfur volatile compounds from *Allium* species and use them to target the genus (Jones *et al*., 2004). The effects of thiosulfinates and their decomposed products, sulfides on the pesticidal and the attractant properties have been widely examined (Block, 1992a; Auger *et al*., 2004; Hori, 2007), whereas those of LF, by contrast, have been poorly studied. Hence, our transgenic tearless onions provide a valuable tool to dissect unknown sulfur metabolite pathways and the role of LF in *Allium* species.

5.2.3 Development of hairpin transcripts detection

In this research, we have developed RT-PCR analysis to detect transcripts of the transformed RNAi gene (Chapter 3 & 4). In order for RNAi to function, at least three steps must work. First, transformed RNAi gene is transcribed and forms the double-stranded RNA (dsRNA). Second, the dsRNA is degraded into small interfering RNAs (siRNAs). Third, the target RNA is cleaved by the siRNAs' guide. These three steps can be verified by molecular analyses: RNAi transcripts detection by RT-PCR (Chapter 3 & 4), siRNAs detection by Northern (Hamilton & Baulcombe, 1999), and quantification of the target RNA by quantitative real-time RT-PCR (qRT-PCR), respectively. Use of these different analyses allowed us to determine whether the transgenic lines that do not silence the target gene fail to produce dsRNA, fail to produce siRNAs, or fail to cleave the target RNA (Figure 4.5, Chapter 4). We believe that this is the first report of RNAi transcript detection by use of RT-PCR and suggest that it is a valuable tool to differentiate reasons for RNAi failing to silence.

Furthermore, the use of RT-PCR to detect the RNAi gene transcripts demonstrated that we had to be careful in designing primers specific to the target RNA for qRT-PCR. The positive RT-PCR result indicated that transcripts of the transformed RNAi gene are present in the cell, prior to them being degraded into siRNAs by Dicer. These could interfere with attempts to amplify target gene RNA transcripts. Therefore, the target gene primers for qRT-PCR must be designed so that they specifically amplify target RNA sequence and do not amplify RNAi transcripts.

5.2.4 Development of 'pinking' assay

We have developed a novel colorimetric 'pinking' assay to specifically detect di-1-propenyl thiosulfinate in onion (Figure 4.8, Chapter 4). Since this thiosulfinate is so unstable that it immediately rearranges into a zwiebelane even at -15^{o}C (Block, 1991), nobody has detected the thiosulfinate in onion by using GC and HPLC procedures. Unlike the other colorimetric assay that uses N-ethylmaleimide (Carson & Wong, 1959; Nakata *et al*., 1970), which detects all thiosulfinates, this 'pinking' method is specific to 1-PRENCSO breakdown thiosulfinate and provides a simple and unique tool to evaluate the specific thiosulfinates in onion. The development of a high-throughput protocol for this assay will provide onion breeders and processors with a simple tool to evaluate these important compounds and perhaps help screen tearless phenotype generated by conventional breeding or mutagenesis.

5.3 Directions for further research

5.3.1 Analysis of segregating progeny from tearless onions

We have characterized only T_0 transgenic tearless onion lines by molecular, biochemical and chemical analyses. So far, T_1 progeny has been obtained from two lines by selfing or crossing onto non-transgenic counterparts (data not shown). Preliminary GFP analysis and LF production upon leaf disruption in these lines indicate that the tearless phenotype is inherited as expected (data not shown). More thorough analyses of the progeny will be undertaken to confirm that stable integration and expression are inherited.

5.3.2 Elucidation of sulfur metabolites and their pathways

5.3.2.1 'zwiebelane isomer' and its derivative

We have observed in solvent extraction-GCMS analysis of both tearless onions and the model reaction without rLFS that, contrary to our expectation, a 'zwiebelane isomer' was present in great abundance, and that it decreased dramatically with incubation time, whilst conversely a white cloudy precipitate increased significantly. This precipitate was not detected as a signal in the analysis (Figure 4.9 & 4.10, Chapter 4). These observations suggest that 'zwiebelane isomer' is one of the reaction intermediates arising from 1-PRENCSO breakdown. The 'zwiebelane isomer' has only been reported in onions by Arnault *et al*. (2000) but it was never purified and identified. The mass spectra of the 'zwiebelane isomer' identified in this research was quite different from onion *cis* or *trans* zwiebelanes (Figure 4.9.E & F, Chapter 4) that have been proposed to be produced from 1-PRENCSO breakdown through di-1-propenyl thiosulfinate (Figure 4.14, Chapter 4) (Bayer *et al*., 1989). However, the actual breakdown pathway is still unknown in onion because the presence of LFS reduces the amount of 1-propenylsulfenic acid entering the pathway to such low levels that the compounds have not been readily identifiable. Thus the identification of the primary ('zwiebelane isomer') and the secondary (white cloudy precipitate) products identified in this research should allow the non-LFS mediated 1-PRENCSO breakdown pathway to be finally elucidated, and help study of health benefits associated with our tearless onions.

5.3.2.2 *cis* or *trans* zwiebelanes

Cis or *trans* zwiebelanes was first identified in onion and the production pathway has been proposed to be produced from *trans*-1-PRENCSO, via di-1-propenyl thiosulfinate (Figure 4.14, Chapter 4) (Bayer *et al*., 1989). A model reaction pathway that produces *cis* or *trans* zwiebelanes was proposed to demonstrate this. However, synthesis studies of the zwiebelanes from di-1-propenyl disulfide by oxidation indicated that the cyclic organosulfur compound is produced from *cis*-1-PRENCSO but not from *trans*-1-PRENCSO. This *cis*-1-PRENCSO has never been reported in onions (Block *et al*., 1996b). The latter observation corresponds to our results in solvent extraction-GCMS analysis of both model study without rLFS and tearless onions, in which *cis* or *trans* zwiebelanes were not detected (Figure 4.9 & 10, Chapter 4). This suggests that the zweibelanes might be produced via a different pathway from Bayer's proposal. Further analyses of the model study, *cis*-1-PRENCSO in onion, and an onion homogenate with 13C-labelled 1-PRENCSO added might help resolve this enigma and help clarify how *cis* or *trans* zwiebelane are produced.

5.3.2.3 Dipropyl disulfide

The disulfide analysis results described in Chapter 4 of this research highlighted an enigma in the currently proposed reaction mechanism for producing dipropyl disulfide. Dipropyl disulfide, the most predominant component in the headspace analysis of onions, is thought to be produced from propyl-L-cysteine sulfoxide (PCSO), via dipropyl thiosulfinate. However, PCSO has only previously been detected at low levels or has never been detected in onions (Table 1.2, Chapter 1). To explain the presence of dipropyl disulfide, Block (1991) suggested that the disulfide is formed from propanesulfenic acid and propanethiol which are reduced from LF. However, the dipropyl disulfide was not detected in our model study with recombinant LFS added, seemingly contradicting the above, although the reducing agent might be absent in the model reaction (Figure 4.11.B). Further analysis of the model study with a reducing agent added, and analysis of onion homogenate with 13C-labelled 1-PRENCSO added might help resolve this enigma.

5.3.2.4 Pink pigment

The 'pinking' assay developed in this thesis originated through studies into the cause of

discoloration in processed onion and garlic products. The reaction mechanism for producing pink pigment was proposed by model studies to be composed of three steps. First, di-1-propenyl thiosulfinate is formed from 1-PRENCSO after catalysis by alliinase. Second, a pigment precursor is formed from the reaction of glycine with 2,3-dimethyl-1,4-butanedithial *S*-oxide, the derivative of di-1-propenyl thiosulfinate. Third, a pink pigment is formed from the pigment precursor and formaldehyde (Figure 4.7, Chapter 4) (Imai *et al*., 2006a & b). However, the compounds produced within this pigment production pathway are still largely unknown. Hence, identification of the pink pigment and precursor, in both model studies and tearless onions, will resolve this and may assist in our understanding of how other sulfur metabolites are produced in tearless onions.

5.3.3 Taste and flavour tests of tearless onions

Previous studies that have identified the taste and flavour notes of particular sulfur volatile compounds made it possible to predict the taste and aroma of tearless onions. The tearless onions would be predicted to have reduced pungent aroma associated with LF, but increased sweeter aromas of raw fresh and cooked or fried onions that are associated with thiosulfinates, disulfides and dihydrothiophenes (Table 1.4, Chapter 1) (Albanese & Fontijine, 2002). We have subjectively performed sensory evaluation (smell & tearing) of the tearless onions. However, independent qualitative and quantitative smell and taste trials using panellists (Davidovich-Rikanati *et al*., 2007) or 'Taste Sensing System' (Intelligent Sensor Technology, *Inc*., Japan) (Miyanaga *et al*., 2003 & Hayashi *et al*., 2007) are required to confirm the taste and aroma predictions, and the possible benefits they may provide to consumers. Since the tearless onions are produced via genetic modification, regulatory approval will be required before panellists can be used.

5.3.4 Health benefits evaluation of tearless onions

Previous reports about physiological activities of sulfur volatile compounds made it possible to predict the health benefits in the tearless onions. The tearless onions would be predicted to have increased beneficial health effects on cardiovascular, anti-inflammatory and anti-carcinogenic effects that are associated with thiosulfinates and disulfides (Chapter 1.2.5). Garlic has much more potent physiological effects than onion. This might be because in normal onion 1-propenylsulfenic acid is predominantly converted into LF and

not thiosulfinates and, in consequence, the total amount of thiosulfinates in onion is much less than that in garlic (Table 1.3, Chapter 1). The presence of thiosulfinates in high levels may confer more potent bioactivity to tearless onions than normal onions or considerable new properties that have previously been associated with garlic. These predicted health benefits are currently being evaluated by *in vitro* and *in vivo* anti-platelet aggregation assays, anti-inflammatory assays measuring IL8 cytokine production in AGS cell lines, and in the future, in anti-carcinogenic trials using AGS cell proliferation assays.

5.3.5 Field tests of tearless onions

The tearless onions developed in this study were grown in the glasshouse not under field conditions. The roles of sulfur volatile compounds in *Allium* species have been studied widely and thought to be for defence against pests and predation. However, they also serve as attractants to specialized feeders and pathogens (Jones *et al*., 2004). Planned field tests of our tearless onions will be undertaken in order to study how a tearless phenotype with raised thiosulfinate levels will perform under normal field conditions. It will be of great interest to see how it withstands diseases and pest challenge and may help understand more precisely what the natural role is for LF and thiosulfinates in *Allium* species.

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