Silencing onion lachrymatory factor synthase causes a significant change in the sulfur secondary metabolite profile

Corresponding Author:

Colin C. Eady, New Zealand Institute for Crop & Food Research Limited, Private Bag 4704, Christchurch, New Zealand. Tel: 00 64 33 25 64 00; Fax: 00 64 33 25 20 74.

eadyc@crop.cri.nz

Research Category: System Biology, Molecular Biology and Gene Regulation
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Footnotes

Financial sources: New Zealand Foundation for Research, Science and Technology (FRST); House Foods Corporation, Japan.

Crop & Food Research manuscript no. 858

Author affiliations:

Colin C. Eady¹², Takahiro Kamoi¹³, Masahiro Kato³, Noel G. Porter², Sheree Davis², Martin Shaw², Akiko Kamoi³, Shinsuke Imai³

¹National Centre for Advanced Bio-Protection Technologies, Lincoln University, Christchurch, New Zealand.

²New Zealand Institute for Crop & Food Research Limited, Private Bag 4704, Christchurch, New Zealand.

³House Foods Corporation, 1-4 Takanodai, Yotsukaido, Chiba, Japan 284-0033.

Corresponding Author: Colin C. Eady: eadyc@crop.cri.nz
Abstract

Through a single genetic transformation in onion, a crop recalcitrant to genetic transformation, we suppressed the lachrymatory factor synthase gene (lfs) using RNAi silencing in six plants. This reduced lachrymatory synthase activity by up to 1544-fold, so that when wounded the onions produced significantly reduced levels of tear-inducing lachrymatory factor. We then confirmed, through a novel colorimetric assay, that this silencing had shifted the trans-S-1-propenyl-L-cysteine sulfoxide (1-PRENCSO) breakdown pathway so that more 1-propenyl sulfenic acid was converted into di-1-propenyl thiosulfinate. A consequence of this raised thiosulfinate level was a marked increase in the downstream production of a non-enzymatically produced zwiebelane isomer and other volatile sulfur compounds, di-1-propenyl disulfide and 2-mercapto-3,4-dimethyl-2,3-dihydrothiophene, which had previously been reported either in trace amounts or had not been detected in onion. The consequences of this dramatic simultaneous down- and up-regulation of secondary sulfur products on the health and flavour attributes of the onion are discussed.
Introduction

*Allium* species synthesize a unique set of secondary sulfur metabolites derived from cysteine. Most notably the S-alk(en)yl-L-cysteine sulfoxides (ACSOs), including S-2-propenyl-L-cysteine sulfoxide (Alliin, 2-PRENSO), and *trans*-S-1-propenyl-L-cysteine sulfoxide (Isoalliin, 1-PRENSO) (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 sulphenic acids and volatile sulfur compounds are produced that give the characteristic flavor and bioactivity of the species. In garlic, 2-PRENSO is the major sulfoxide (Fritsch and Keusgen, 2006) 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., 1992a, 1992b; Rose et al., 2005). In onion (*Allium cepa* L.), 1-PRENSO is the major sulfoxide (Fritsch and Keusgen, 2006). 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 only tentatively been reported in trace amounts (Boelens et al., 1971). Instead, propanthial S-oxide (lachrymatory factor, LF), 1-propenyl methane thiosulfinate, and di-propyl disulfide are dominant (Block et al., 1992a, 1992b; Rose et al., 2005). LF is a critical practical point of difference between onion and garlic. It is the chemical responsible for inducing tearing in onion, a undesirable irritant, and it is hypothesised that LF production causes the absence of otherwise predicted sulfur volatiles (Randle and Lancaster, 2002), analogues of which in garlic are known for their health attributes (Griffiths et al., 2002).

Imai et al. (2002) discovered that the conversion of 1-propenyl sulfenic acid to LF is mediated by an enzyme they named lachrymatory factor synthase (LFS, Figure 1). LFS acts specifically upon 1-propenyl sulfenic acid, following the action of alliinase on 1-PRENSO to produce LF. The specificity of this end pathway reaction suggests that the production of LF could be reduced by genetic manipulation of the LFS transcript using RNAi silencing. In the absence of LFS, the unstable 1-propenyl sulfenic acid would undergo spontaneous self-condensation to di-1-propenyl thiosulfinate (Imai et al., 2002).
This raised thiosulfinate level would then be available for conversion, via the non-enzymatic part of the pathway (Figure 1), into a cascade of predicted secondary compounds that have not been detected previously in onion or have only been reported in trace amounts. These compounds are responsible for the unique sensory, odour and flavour notes (Block 1992c) as well as the health-promoting attributes of the edible Allium species (e.g. anti-inflammatory, anti-platelet aggregation, anti-cancer, lipid-lowering) (Griffiths et al., 2002; Lanzotti, 2006; Randle and Lancaster, 2002; Morimitsu et al., 1992; Block et al., 1996a).

Onion is a very difficult biological system to work with; it has a large genome, is heterozygous, has a slow generation time and a poor capacity for manipulation (Eady & Hunger 2008). However, it is a genus with a unique, complex and important secondary sulfur pathway that has no model counterpart. Current ‘tearless’ onion cultivars (e.g. Vidalia) are achieved through deficient uptake and partitioning of sulfur and/or growth in sulfur-deficient soils, but in so doing they accumulate fewer secondary sulfur compounds in the bulb (Randle and Lancaster, 2002), reducing their sensory and health qualities compared with more pungent high-sulfur cultivars. In this research we set out to genetically manipulate the sulfur secondary metabolite pathway of onion using RNAi. Unlike previous research aiming to over- or under-regulate a particular enzyme within a secondary pathway to either increase consumer-desirable compounds (Davuluri et al., 2005) or remove deleterious ones (Capell and Christou, 2004; Sunilkumar et al., 2006), we aimed to achieve both. By reducing LFS and stopping conversion of 1-propenyl sulphenic acid to the undesirable LF, we tested the hypothesis that this would allow 1-propenyl sulphenic acid to be available for spontaneous conversion into thiosulfinate and thiosulfinate derived sulfur compounds, analogues of which are renowned for their desirable sensory and health promoting attributes.

Results

Three onion cultivars were studied; a mild hybrid (H) mid-daylength fresh onion cv. Enterprise, a pungent open pollinated (O) fresh onion cv. Pupekohe LongKeeper and a pungent dehydration (D) mid-daylength onion (Sensient Dehydrated Flavors, Co. Ca. USA). Eleven plants were evaluated, three non-transgenic plants, HN, ON, and DN, and
eight transgenic events, H1, H2, H3, O1, O2, O3, D1 and D2, from the hybrid, open-pollinated and dehydration cultivars respectively. These were the transformants recovered from ~15,000 immature embryos used in 16 transformation experiments (~0.05% transformation frequency).

Plant selection and regeneration

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, except for plant O3 which had to be rescued onto non-selective media. All transgenic plants grew and formed morphologically similar plants and bulbs to their non-transgenic counterparts (Figure 2). Seed set and F1 progeny has been obtained from two lines so far by selfing or crossing onto non-transgenic counterparts.

T-DNA integration and integrity

Southern analysis of onion plants, using a gfp gene probe (Eady et al., 2000) revealed that plants H1 and D1 contained two copies of the T-DNA construct at different loci, and plant O1 contained a multiple insert at a single locus. The remaining five plants, H2, H3, O2, O3, and D2, contained single copy inserts (Figure 3, top) integrated at different locations from each other, confirming the non-clonal nature of the transgenic events. PCR data (Table 1) indicated that the T-DNA cassette was not complete in all plants evaluated. In plant O3 the nptII gene sequence could not be detected. Initial identification of this transgenic event by GFP expression and rescue to non-selective media resulted in the maintenance of this plant. In plant H2 the 5′ region of the lfsRNAi CaMV35s promoter sequence was truncated. However, this did not compromise transcription or siRNA production (Figure 3, centre, and Table 1).

siRNA production

Detection of lfsRNAi transcript by RT-PCR was used to indicate functionality of the transgene. All transgenic plants except O1 produced lfsRNAi transcript (Table 1). Such observation of transgene inactivation due to multiple copy inserts at a single locus is common (Muskens et al., 2000; Tang et al., 2007). Detection of lfs siRNA using a lfs
probe (Figure 3, centre) showed that six plants, H1, H2, H3, O2, O3, and D2, were producing siRNA fragments corresponding to the LFS gene sequence. Interestingly, plant D1, which produced lfsRNAi transcript, failed to produce lfs siRNA at detectable levels. We are unaware of other research that has used RT-PCR to detect hairpin transcripts and suggest that it is a valuable tool to differentiate reasons for hairpins failing to silence. In this case we can assert that the cause was not transcriptional inactivation.

Lachrymatory factor synthase levels

lfs transcript levels (Figure 3, bottom) were compared in cDNA samples from transgenic and non-transgenic plants by qRT-PCR. Low levels of transcript corresponded well with the presence of lfs siRNA fragments.

No LFS protein could be detected in plants that produced lfs siRNA. Plants O1 and D1 with no observable lfs siRNA had LFS protein levels that fell well within the range of their respective control non-transgenic plants.

Assays of LFS activity in both leaf and bulb measured by in vitro generation of LF demonstrated that plants with no detectable LFS, as measured by Western analysis, also had significantly reduced LFS activity (Figure 4, middle). This activity in plants H1, H2, and H3 was reduced by between 21 and 103-fold in leaf tissue and by between 18 and 1168-fold in bulb tissue. Activity in plants O2 and O3 was reduced by between 38 and 70-fold in leaf tissue and between 1515 and 1544-fold in bulb tissue. Activity in plant D2 was reduced by 396-fold in leaf and 501-fold in bulb tissue. The more pronounced reduction observed in bulb tissue over leaf tissue suggests that LFS is probably a relatively major protein within aestivating storage bulb tissue compared with leaf material. Plants D1 and O1 failed to produce the LFS silencing signal or reduce LFS activity.

Precursor 1-PRENSO levels and alliinase activity
Biochemical analysis showed that the 1-PRENSO levels in the transgenic and control plants were between 4 and 13 mg/g DWt. Alliinase activity was between 15.8 and 42.4 nKats/mg protein. These substrate and enzyme levels are within the normal physiological range reported for onions (Kitamura et al., 1997; Kopsel and Randle, 1999). This suggests that in our transgenic onions, silencing lfs transcripts did not affect alliinase activity or 1-PRENSO levels.

Phenotype analysis of secondary sulfur chemistry

In order to identify all the possible changes to onion secondary sulfur metabolism three established techniques were used: GC with flame photometric detection (GC-FPD), solid-phase microextraction (SPME) GCMS, and solvent extraction GCMS. In addition, to detect the previously undetected in onion di-1-propenyl thiosulfinate a novel colorimetric (“pinking”) assay was developed and used.

Volatile sulfur compounds

GC-FPD analysis of LF levels from freshly crushed leaf material demonstrated that H1, H2 and H3 were reduced by 13.5, 35.5, and 30-fold respectively compared with HN, that O2 and O3 were reduced by 30 and 67-fold compared with ON, and that D2 was reduced by 36-fold compared with DN. In bulb material, LF was reduced by 10.2 and 28.2-fold for H1 and H3 compared with HN (H2 was not measured as the bulb was infected), by 6.4 and 28-fold for O2 and O3 compared with ON, and by 12.8-fold for D2 compared with DN (Figure 4, bottom). Analysis of F1 progeny from line D2 has demonstrated that the reduced LF level is inherited along with GFP expression (data not shown).

Other sulfur volatile components given off by damaged onion tissue were detected by SPME-GCMS analysis of headspace as proposed by Arnault et al. (2000). Healthy cut leaf blade material was analysed using a protocol similar to that described by Hori (2007). The identification of the volatile compounds was based on comparisons of mass spectral data (Supplemental Figure S1) with published work (Hiramitsu, 1989; Sinha et al, 1992; Block et al, 1996b). This analysis (Figure 5) revealed that the LF-reduced
plants produced a significantly decreased di-propyl disulfide peak and a much increased 1-propenyl propyl disulfide peak compared with the control. In addition, the LF-reduced plants produced five peaks not detected in the control onion, three peaks representing di-1-propenyl disulfide isomers and two peaks representing 2-mercapto-3,4-dimethyl-2,3-dihydrothiophene isomers. The disulfides have only previously been reported in trace amounts in onion (Boelens et al., 1971). The dihydrothiophenes have only previously been reported in trace amounts in Welsh onion (Kuo et al., 1992a; 1992b) but have never been detected in onion.

Subjective sensory evaluation of the reduced LF plants by the authors supported the GC analysis. 0.5 cm² of onion leaf and bulb samples crushed between fingernails and placed touching the eyelashes caused a stinging and tearing sensation with control tissue but no tearing or stinging when reduced LF material was used. The same material when placed next to the nose revealed an aroma in the reduced LF plants that was less pungent and sweeter than that given off by the non-transgenic counterparts.

Thiosulfinate detection and abundance

A key prediction was that the reduction of LFS levels would leave more 1-PRENCSO-derived 1-propenyl sulfenic acid available for thiosulfinate production. However, no simple method of specifically detecting di-1-propenyl thiosulfinate was available. Block (1991) synthesized the thiosulfinate by oxidation of di-1-propenyl disulfide and demonstrated by low temperature NMR analysis at -15°C that it rapidly changed form to become a zwiebelane, thus its thermal instability ruled out the use of standard GC techniques for analysis. Recently, observations by Imai et al. (2006a; b) demonstrated that 1-propenyl-containing thiosulfinates produce a pink pigment (of unknown structure) when mixed with glycine and formaldehyde. We exploited this reaction to develop a simple colorimetric assay (pinking assay) to detect 1-PRENCSO-derived thiosulfinate (Supplemental Figure S2). Specificity of detection was confirmed by in vitro analysis of 1-PRENCSO or alliin (2-PRENCSO) derived products incubated in the presence of garlic alliinase (Figure 6). 1-PRENCSO derived product correlated with a pink colour whilst alliin-derived product produced no pink colour. ‘Pinking” analysis of
the reduced LF plants confirmed increased levels of 1-propenyl-containing thiosulfinates. Further, addition of recombinant LFS to the extract lowered the ‘pinking’ level and confirmed that the absence of LFS was the cause of pinking (Figure 7). *In vitro* analysis indicated that not all 1-PRENCSO-derived thiosulfinates are captured in the colour reaction, and that LFS is a strong competitor for 1-propenyl sulfenic acid. In *in vitro* assays, 1-2 times normal physiological levels of LFS (using recombinant LFS) converted more than 85% of 1-PRENCSO to LF (data not shown).

Non-enzymatic thiosulfinate-derived reaction products

The thiosulfinates are known to convert spontaneously to a number of other sulfur compounds (Griffiths et al., 2002) and solvent extraction-GCMS analysis of crushed reduced LF plants was undertaken in an effort to identify differences from non-transgenic onions. The total ion-chromatogram produced by GCMS analysis of incubated reduced LF extract from plants H3, O3 and D2 compared with their non-transgenic counterparts detected a large peak difference in the transgenic extracts (Figure 8), which was confirmed by the mass spectra fingerprint (Supplemental Figure S1) to be a zwiebelane isomer. Zwiebelane isomers were first reported by Arnault (2000), although he did not identify the chemical structure. Our analysis demonstrated that at least one of the proposed downstream sulfur compounds was elevated in the reduced LF onions.

**Discussion**

This report is the first demonstration of gene silencing in an *Allium* species. It also shows that the manipulation of plant secondary metabolite pathways can result in dramatic simultaneous down- and up-regulation of products within that pathway and the production of novel products upon tissue disruption.

Earlier work using antisense technology to silence the alliinase gene in onion produced some transgenic plants but conclusive proof of silencing was not obtained (Eady et al. 2004). In this work we successfully decreased endogenous LFS production through a single simple transformation event. This unambiguously lowered LFS activity in the
plant, and the consequence of this was a significant reduction in the production of the irritant LF. This caused a large shift in the 1-PRENCSO breakdown pathway such that much more 1-propenyl sulfenic acid was available for conversion into thiosulfimates. Raised levels of 1-propenyl-containing thiosulfinate (detected using the novel pinking assay), predictably increased levels of the non-enzymatically produced downstream product, a zwiebelane isomer and di-1-propenyl disulfides. However, much more surprising and unpredicted was the production of 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes.

Silencing the lfs transcript has confirmed the hypothesis that LF production prevents the spontaneous production, from 1-propenyl sulfenic acid, of 1-propenyl-containing thiosulfimates in crushed onion. This demonstrates the specificity and affinity of LFS for 1-propenyl sulfenic acid and would indicate that alliinase and LFS must work in close proximity, as negligible amounts escape conversion into LF.

The development of the ‘pinking’ assay reported here originated from in-depth studies of the cause of ‘off-colours’ in processed onion and garlic products (Imai et al., 2006a,b). It provides a unique and simple method for detecting the 1-PRENCSO-derived thiosulfinate. Unlike the N-ethylmaleimide method of Lee and Parkin (1998), which detects all thiosulfimates, this ‘pinking’ method is specific to 1-propenyl thiosulfinate. The development of a high-throughput protocol for this assay will provide allium breeders and processors with a simple tool to quantify this important precursor.

Previous research in Allium species (Boelens et al., 1971; Block and Zhao, 1990; Arnault et al., 2000) made it possible to predict the non-enzymatic breakdown of 1-propenyl-containing thiosulfinate (Figure 9). Much of what was predicted was indeed confirmed by our research, with a zwiebelane isomer and 1-propenyl propyl disulfide and the di-1-propenyl disulfides being detected in much greater abundance in volatiles from the reduced LF plants. Arnault et al. (2000; 2004) reported that thiosulfimates and zwiebelanes are converted into disulfides in SPME-GCMS analysis because of its thermally severe condition compared with solvent extraction-GCMS. The increase of 1-propenyl containing disulfides in the SPME-GCMS analysis corresponded to increased absorbance in the pinking assay of the reduced LF plant extracts indicating that the
disulfides are breakdown products from the 1-propenyl thiosulfinate. The decrease in dipropyl disulfide level is most likely a result of the greater ability of the propyl sulfenic acid to react with the propenyl form, or a result of the decrease of LF (Block, 1991).

The presence of 2-mercapto-3,4-dimethyl-2,3-dihydrothiophenes was not predicted. The dihydrothiophenes were first reported to be formed by heating di-1-propenyl disulfide (Block and Zhao, 1990). Thus, the dihydrothiophenes detected in reduced LF plants are likely to be formed from di-1-propenyl disulfide in thermally severe SPME-GCMS analysis. The results presented here would indicate that the di-1-propenyl thiosulfinate and its corresponding di-1-propenyl disulfide are thermally very unstable and as such difficult to assess quantitatively despite the use of standard GCMS protocols (Arnault et al., 2000; 2004; Hori 2007). Thus, whilst the disulfides and dihydrothiophenes may not be present in raw reduced-LF onion, they are likely to be produced in a cooked reduced LF onion. Further research will clarify the levels of these reaction products in onion.

The impact on flavour of the lower LF levels combined with raised levels of thiosulfinate derived sulfur compounds in the reduced LF onion can be predicted from the existing literature. LF is the cause of the unpleasant pungent aroma associated with cut onions and the cause of heat and pungency flavour notes (Randle, 1997). Disulfide and dihydrothiophene compounds are associated with the sweeter aroma of cooked or fried onions (Lancaster and Boland, 1990; Albanese and Fontijine, 2002). Trans and cis zwiebelanes are associated with sweet raw onion sulfur tastes and sweet brown sautee flavour notes, respectively (Block, 1996a). Initial olfactory assessment by the authors (smelling the crushed onion samples) indicated that the pungent aroma was absent in the reduced LF plants and that the sweeter aroma of cooked onions was present. The literature suggests that the reduced LF onions will have low heat and pungency combined with enhanced sweet raw onion and sautee flavour notes. Formalized taste evaluation of the transgenic plants requires regulatory approval and this has not yet been granted. Sensory evaluation panels are planned to test these predictions. We also hope to determine the precise olfactory or flavour role of the other polysulfur volatiles that have been produced
Allium sulfur compounds are renowned for their human health giving attributes. We predict that the altered profiles present in the reduced LF plants are likely to have significant consequences for these attributes and as such they are being further investigated. For example, thiosulfinates have anti-asthmatic activity (Griffiths et al., 2002). There is a clear rank order of pharmacological activity, with saturated thiosulfinates being less active than unsaturated ones. The unsaturated 1-propenyl-containing thiosulfinate in the reduced-LF onions may confer health properties to onion that have previously been associated with the unsaturated allicin thiosulfinate in garlic. In addition, zwiebelanes have been associated with anti-platelet aggregation activity (Block et al., 1992c, 1996a; Keusgen, 2002). We are currently investigating the role of the zwiebelane isomer from our reduced LF plants on platelet aggregation. Benavides et al. (2007) demonstrated that di-2-propenyl disulfide from garlic is converted after ingestion, by red blood cells, into hydrogen sulfide, a powerful signalling molecule which has an ideal physiological profile for cardiovascular protection (Lefer, 2007). The nature and similarity of garlic disulfides to the disulfide compounds produced in the reduced LF plants warrants investigation to determine if they are also converted to hydrogen sulfide in the blood.

In summary, these reduced LF onions are a unique resource for understanding the role of specific sulfur secondary metabolites in plant biology, in human health and in terms of their potential value to the agrifood industry.

Materials and methods

Generation and molecular analysis of transgenic plants

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, Co. Ca. USA) were transformed regenerated and ex-flasked according to the method of Eady et al. (2000). The T-DNA cassette designed for silencing in onion (Figure 10) was contained within a pArt binary vector (Gleave, 1992) with a $m$-gfpER reporter gene under control of a CaMV 35s promoter (Haseloff, 1997) and a nptII gene under control of a nos promoter for ease of
detection and selection. It contained the pHannibal-based RNAi cassette (Wesley et al., 2001) containing a 512 bp hairpin of the lfs gene sequence under CaMV 35s promotional control. The CaMV 35s - mgfpER, and nos - nptII selection have all previously been shown to function well in onion (Eady et al., 2000). Southern analysis (Eady et al., 2000) gave integration details, loci and copy number. For subsequent leaf analysis only healthy inner leaves (10-20 cm in length) from actively growing plant material was used. To avoid any confusion only a single plant from each transgenic event, or a proven clone (by Southern analysis), was used for analysis. The presence of lfs siRNAi sequences was determined by the following simplified protocol of Hamilton and Baulcombe (1999). Small RNA was isolated from ~450 mg leaf tissue using a mirVana miRNA isolation kit (Ambion) following the manufacturer’s instructions. RNA was quantified using a GeneQuant®II (GE Healthcare Bioscience) and standardised to 250 ng/µl. Five µg in 40 µl (including loading buffer) was loaded on to a 15% polyacrylamide gel and electrophoresed following mirVana (Ambion) instructions. The separated small RNA was capillary blotted onto a Zeta-probe GT membrane (Bio-Rad) according to the manufacturer’s instructions, air-dried and UV crosslinked on to the membrane by a 120 mj burst (BLX254 Vilber Lourmat). The blot was prehybridised and incubated with an LFS probe, produced from a 450 bp LFS sequence using a P32 CTP random prime labelling kit (GE Healthcare Bioscience) according to the manufacturer’s instructions, at 40ºC overnight in a hybridiser HB-ID (Techne). Excess probe was removed by two 30 min washes in 6 x SSC, 0.2% SDS then 2 x SSC, 0.1% SDS. The washed membrane was placed in a Lanex cassette against a Bio-max film (Kodak) at -80ºC for 2 weeks before the film was developed.

For transcript analysis total RNA was isolated from ~10 mg of freeze-dried onion leaf tissue using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. RNA was quantified using a ND-1000 Spectrophotometer (Nano Drop Technologies) after treatment with TURBO DNA-free (Ambion). 200 ng of the total RNA was reverse-transcribed to first strand cDNA using an Omniscript RT Kit (Qiagen) and oligo-dT (21) primer.

For qualitative PCR to confirm the presence of lfsRNAi transcripts, the border region between antisense lfs of lfsRNAi cassette and ocs terminator was selected as a
target to be amplified using specific primers (forward, 5′ - CTCTTCGATTTTCTGACCTATCTCAGTAGC-3′ and reverse, 5′ - TGCACAACAGAATTGAAAGC-3′). The target sequence was amplified in 25 µl reaction volume containing the first strand cDNA, equivalent to 10 ng of total RNA, using a HotStarTaq Master Mix Kit (Qiagen) according to the manufacturer’s instructions. PCR, performed in a GeneAmp 9700 (Applied Biosystems), was set with the following PCR cycle program: 15 min at 95°C and 40 cycles, 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and 10 min at 72°C.

For quantitative real-time PCR, the 5′ region of lfs (accession No. AB089203) was selected as a target to be amplified using specific primers (forward, 5′-ACGTTATATCAAGAAGATTGTCCAA-3′ and reverse, 5′-TCCGTTAGCACTATCAGCGAC-3′) and putative ubiquitin sequence (Accession No. AA451588) was selected as an internal control gene to be amplified using specific primers (forward, 5′-ACGATTACACTAGAGGTGGAGAGCTC-3′ and reverse, 5′-CCTGCAAATATCAGCCTCTGCT-3′). Each sequence was amplified in 25 µl reaction volume containing the first strand cDNA, equivalent to 10 ng of total RNA, using a QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer’s instructions. PCR, performed in an ABI PRISM 7700 (Applied Biosystems), was set with the following PCR cycle program: 15 min at 95°C and 40 cycles, 15 sec at 94°C, 30 sec at 57°C, and 30 sec at 72°C. Each standard curve was calibrated by using the five concentrations of RT-PCR amplicon containing lfs or putative ubiquitin sequence, such as 100, 1000, 10000, 100000, and 1000000 copies per reaction. The lfs mRNA level was determined by dividing the lfs copy number by the internal standard gene copy number as a relative mRNA level.

Biochemical analysis of plants

Western analysis of LFS protein: Total protein was extracted from ~20 mg of freeze-dried onion leaf tissue by PBS buffer and quantified using a protein assay (Bio-Rad) with bovine serum albumin (Sigma) as a standard. 100 µg of protein in Laemmli Sample Buffer (Bio-Rad) was loaded onto a 15% polyacrylamide gel and electrophoresed. The protein was electrically blotted using Trans-Blot SD Cell (Bio-
Rad) onto a PVDF membrane (Bio-Rad) following the manufacturer’s instructions. The blot was blocked by BSA solution and incubated for 1 h with an LFS polyclonal antibody raised in a rabbit. 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 was detected using 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.

Lachrymatory factor analysis: Approximately 2 g of young leaf from the second or third innermost leaf of actively growing glasshouse plants and mature bulb samples (top half bisected longitudinally) was harvested into liquid N2. Samples were stored at -80ºC until ready for analysis. Half of each leaf and bulb sample was freeze-dried, stored at room temperature in a dessicator and used for LF assays, and precursor evaluation (in vivo LF leaf assays used fresh frozen material).

In vivo LF assays: Sample preparation and gas chromatographic analysis for LF were modified from a previously reported method (McCallum et al., 2005). Samples of leaf (20-100 mg) were crushed rapidly in 500 µl of deionised water in 1.5 ml microcentrifuge tubes. The tubes were held at room temperature (ca. 20ºC) with stirring every min. Three min after the initial mixing, 500 µl of dichloromethane (containing 0.06 µl/ml heptane thiol as an internal standard) was added. The tubes were gently rolled for 2 min to extract the sulfur compounds. A 300 µl sample of the dichloromethane phase was removed to a GC auto-injector vial for immediate analysis. Samples of bulb powder (10-30 mg) were prepared as above, except that in addition to 500 µl of deionised water, seven times the sample weight of water was added to the sample to replace the water removed by freeze-drying.

GC operating conditions were modified from the previous method as follows: 1 µl splitless injection with injector at 160ºC; hydrogen carrier gas at constant 60 cm/sec flow; isothermal 55ºC oven temperature; flame photometric detector at 180ºC. The
detector was calibrated against heptane thiol. Three samples were prepared for analysis from each leaf and bulb powder sample, and duplicate GC runs were done for each of the replicate samples. Mean (n = 6) LF levels are expressed as pg LF/g fresh weight using the dry weights of the bulb samples to adjust to a fresh weight basis.

In vitro LF assays: LFS enzyme activity was evaluated by LF-forming capability. Samples of freeze-dried powder (5-15 mg) were mixed with PBS buffer (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄) at a ratio of 1 g:100 ml. The mixture was centrifuged for 5 min at 15,000 rpm at 4°C and the resulting supernatant was used for the activity assay. Protein quantification was performed as above. 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-PRENCSO purified from onion were mixed and left for 3 min at room temperature. Two µl of the resulting mixture was analysed by HPLC using an ODS column at 35°C. 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 the absorbance 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.

PRENCSO substrate and alliinase activity: PRENCSO precursor levels were assayed as thiocarbamate derivatives by RP-HPLC following the protocol of Randle et al. (1995) Alliinase activity levels were determined following a modified Schwimmer and Mazelis method (Lancaster et al., 2000) further adapted by use of propylcysteine sulfoxide (PCSO) as the substrate and a higher, 30°C reaction temperature.

Pinking assay: The range and reliability of the ‘pinking assay’ were examined. Several volumes of 20 mg/ml 1-PRENCSO or alliin (0-850 n mol) were mixed with 40 µl of 5% of glycine solution and 145 µl (20 nKats) of purified garlic alliinase (555 nKats/mg protein). After 30 min at 25°C, insoluble matter was removed by centrifugation and 10 µl of 100 ppm of formaldehyde solution was added to the 100 µl of the supernatant. This was then incubated at 40°C for 6 h and then absorbance at 520 nm was measured by spectrophotometer. Each assay was performed in triplicate.

The ‘pinking assay’ was developed as follows: 30 mg of freeze-dried bulb powder was resuspended in 200 µl of 1% glycine solution with and without 15 µg of recombinant
LFS. After 30 min at 25°C, insoluble matter was removed by centrifugation and 10 µl of 100 ppm formaldehyde solution was added to 100 µl of the supernatant. This was incubated at 40°C for 6 h and then absorbance at 520 nm was measured by spectrophotometry. Increasing absorbance (pinking) demonstrated the relative abundance of 1-propenyl-containing thiosulfinates.

Headspace SPME-GCMS analysis: Headspace analysis was performed according to Hori’s method (2007) with slight modification. 5 g of sliced fresh leaf material from each plant was put into 20 ml of SPME vials and covered with a lid immediately, then left for 30 min at room temperature. The headspace of the vial was extracted with solid-phase microextraction (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. Transfer line and detector temperature was held at 150°C and total ion chromatograms and mass spectra were analysed with the electron impact mode. The compounds were identified by matching their mass spectra to the US National Institute of Standards and Technology (NIST), Wiley mass spectral libraries and previous studies (Hiramitsu, 1989; Sinha et al, 1992; Block et al., 1996b).

Solvent extraction-GCMS analysis: GCMS analysis was performed according to Arnault’s method (2000) with slight modification. 40 mg of freeze-dried bulb powder from each plant was incubated in 400 µl deionised water at room temperature and vortexed gently. After 80 min, 200 µl diethyl ether (containing 0.01 µl benzyl alcohol as an internal standard) was added and vortexed briefly. After 1 min centrifugation at 15000 rpm, the diethyl ether phase was transferred to a GC auto-injector vial for immediate analysis. 1 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°C/min from 70-250°C. Transfer line and detector temperature was held at 150°C and total ion chromatograms and mass spectra were analysed with the electron impact mode. The compounds were identified by matching their mass spectra to the US National Institute of Standards and Technology (NIST), Wiley mass spectral libraries and previous studies (Block et al., 1992b; Arnault et al., 2000).

Acknowledgements

We thank Peter Waterhouse of CSIRO for use of RNAi technology and critical review of the manuscript. Within Crop & Food Research, we thank Ruth Butler for statistical analysis, Doug Taylor for plant maintenance, and Daniel Park for editing.
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Figure legends

Figure 1. The main sulfur pathway following tissue disruption in onion.

Figure 2. Ex-flasked intermediate daylength A) open pollinated (O lines), B) hybrid (H lines) and C) dehydration (D lines) onions transformed with the lfs RNAi construct. (D) = LFS-silenced bulbs.

Figure 3. Molecular analysis. Top: Southern analysis of lfs RNAi transgenic onion plants probed with gfp probe. Centre: RNAi analysis of small RNA probed with lfs probe. Bottom: Quantitative RT-PCR measurement of lfs transcript, compared with non-transgenic (NT). HN, ON, and DN = non-transgenic hybrid (stars), open-pollinated and dehydration controls, respectively; H1, H2, H3, O1, O2, O3, D1, D2 = respective transgenic hybrid, open-pollinated and dehydration onion plants (squares). Far right-hand lanes, 1, 5, 10 copy control respectively (in Southern gel), LFS sequence control (RNAi gel). 95% confidence limit is calculated from an analysis of variance of logged data using the within-bulb variation. Data were log-transformed to adjust for variance heterogeneity between lines.

Figure 4. Biochemical analysis. Top: Western analysis of LFS, middle: in vitro LF peak area (LPA) and bottom: in vivo LF measurements. HN, ON and DN = non-transgenic hybrid, open-pollinated and dehydration controls, respectively; H1, H2, H3, O1, O2, O3, D1, D2 = respective transgenic hybrid, open-pollinated and dehydration onion plants. 95% confidence limit is calculated by analysis of log-transformed data of within-bulb and leaf variation to account for the variance difference between the low and high LF values. ND = not detected.

Figure 5. GC analysis of solid-phase microextraction sulfur components from the headspace of vials containing cut onion leaf material. 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.
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Figure 8. Total ion chromatograms of incubated onion extract. (a) H3 (top) and HN (bottom); b) O3 (top) and ON bottom; c) D2 (top) and DN (bottom) showing the control benzyl alcohol and the zwiebelane isomer.

Figure 9. The proposed sulfur pathway following tissue disruption in transgenic onion. *, Detected by pinking assay; **, Detected by solvent extraction-GCMS; ***, Detected by SPME-GCMS.

Figure 10. The T-DNA region of pART27-lfsRNAi-mgfp5ER silencing cassette used to silence LFS in onion. A 521-bp DNA fragment derived from lfs cDNA (AB089203) at positions 56-576 was selected for a sense lfs region of an RNAi construct and inserted into the XhoI and KpnI sites in pHANNIBAL. Similarly, a 512-bp DNA fragment at positions 63-574 was selected for an antisense lfs region and inserted into the Clal and BamHI sites. The RNAi construct was excised from pHANNIBAL using the restriction enzyme NotI and inserted into the NotI site in the pART27-mgfp5ER. The gfp marker gene was used to aid selection of transgenic events.

Figure S1. Mass spectral data.

Figure S2. Proposed reaction mechanism for producing a pink pigment.
Table 1: Summary of molecular and biochemical data for transgenic onion plants.

| Analysis          | H1              | H2                       | H3                      | O1     | O2     | O3     | D1    | D2    |
|-------------------|-----------------|--------------------------|-------------------------|--------|--------|--------|-------|-------|
| PCR               | Intact T-DNA    | CaMV35S deleted          | Intact T-DNA            | Intact T-DNA | Intact T-DNA | Intact T-DNA | Intact T-DNA |
| Southern analysis | Two at different loci | Single insert          | Single insert          | Multi inserts at one locus | Single insert | Two at different loci | Single insert |
| lfsRNA 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 |

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