Silencing Onion Lachrymatory Factor Synthase Causes a Significant Change in the Sulfur Secondary Metabolite Profile

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Through a single genetic transformation in onion (Allium cepa), a crop recalcitrant to genetic transformation, we suppressed the lachrymatory factor synthase gene using RNA interference silencing in six plants. This reduced lachrymatory synthase activity by up to 1,544-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-5-1-propenyl-L-cysteine sulfoxide 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 nonenzymatically produced zwiebelane isomer and other volatile sulfur compounds, di-1-propenyl disulfide and 2-mercapto-3,4-dimethyl-2,3-dihydropiophene, which had previously been reported 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 flavor attributes of the onion are discussed.

Allium species synthesize a unique set of secondary sulfur metabolites derived from Cys. Most notable are the S-alk(en)yl-i-Cys sulfoxides, including S-2-propenyl-S-1-cysteine sulfoxide (alliin; 2-PRENCSO) and trans-S-1-propenyl-L-cysteine sulfoxide (isalliin; 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 volatile sulfur compounds are produced that give the characteristic flavor and bioactivity of the species. In garlic (Allium sativum), 2-PRENCSO 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), 1-PRENCSO 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, an undesirable irritant, and it is hypothesized 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; Fig. 1). LFS acts specifically upon 1-propenyl sulfenic acid, following the action of alliinase on 1-PRENCSO 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 RNA interference (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 nonenzymatic part of the pathway (Fig. 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, odor, and flavor notes (Block, 1992) as well as for the health-promoting attributes of the edible Allium species (e.g. antiinflammatory, antiplatelet aggregation, anticancer, lipid-lowering; Morimitsu et al., 1992; Block et al., 1996b; Griffiths et al., 2002; Randle and Lancaster, 2002; Lanzotti, 2006).

Onion is a very difficult biological system to work with; it has a large genome, is heterozygous, has a slow generation time, and has a poor capacity for manipulation (Eady, 2002; Eady and Hunger, 2008). However, Allium 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 overregulate or underregulate 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 the conversion of 1-propenyl sulfenic acid to the undesirable LF, we tested the hypothesis that this would allow 1-propenyl sulfenic 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 (‘Enterprise’), a pungent open-pollinated (O) fresh onion (‘Pukekohe LongKeeper’), and a pungent dehydration (D) mid-daylength onion (Sensient Dehydrated Flavors). Eleven plants were evaluated, three nontransgenic plants (HN, ON, and DN) and eight transgenic plants (H1, H2, H3, O1, O2, O3, D1, and D2), from the hybrid, open-pollinated, and dehydration cultivars as indicated. These were the transformants recovered from approximately 15,000 immature embryos used in 16 transformation experiments (approximately 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 medium containing geneticin, except for plant O3, which had to be rescued onto nonselective medium. All transgenic plants grew and formed morphologically similar plants and bulbs to their nontransgenic counterparts (Fig. 2). Seed set and F1 progeny had been obtained from two lines by selfing or crossing onto nontransgenic counterparts.

T-DNA Integration and Integrity

Southern-blot 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 that plant O1 contained a multiple insert at a single locus. The remaining five plants, H2, H3, O2, O3, and D2, contained single-copy inserts (Fig. 3, top) integrated at different locations from each other, confirming the nonclonal nature of the transgenic events. PCR data (Table I) 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 nonselective medium resulted in the maintenance of this plant. In plant H2, the 5’ region of the lfsRNAi cauliflower mosaic virus (CaMV) 35S promoter sequence was truncated. However, this did not compromise transcription or small interfering RNA (siRNA) production (Fig. 3, center; Table I).

siRNA Production

Detection of lfsRNAi transcript by reverse transcription (RT)-PCR was used to indicate functionality of the
transgene. All transgenic plants except O1 produced \textit{lfs}RNAi transcript (Table I). 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 \textit{lfs} siRNA using a \textit{lfs} probe (Fig. 3, center) showed that six plants (H1, H2, H3, O2, O3, and D2) produced siRNA fragments corresponding to the LFS gene sequence. Interestingly, plant D1, which produced \textit{lfs}RNAi transcript, failed to produce \textit{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.

LFS Levels

\textit{lfs} transcript levels (Fig. 3, bottom) were compared in cDNA samples from transgenic and nontransgenic plants by quantitative RT-PCR. Low levels of transcript corresponded well with the presence of \textit{lfs} siRNA fragments.

No LFS protein could be detected in plants that produced \textit{lfs} siRNA. Plants O1 and D1, with no observable \textit{lfs} siRNA, had LFS protein levels that fell well within the range of their respective control nontransgenic plants (Fig. 4, top).

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-blot analysis, also had significantly reduced LFS activity (Fig. 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 1,168-fold in bulb tissue. Activity in plants O2 and O3 was reduced by between 38- and 70-fold in leaf tissue and by between 1,515- and 1,544-fold in bulb tissue. Activity in plant D2 was reduced by 396-fold in leaf and by 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.

Table I. Summary of molecular and biochemical data for transgenic onion plants

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bulb tissue compared with leaf material. Plants D1 and O1 failed to produce the LFS silencing signal or to reduce LFS activity.

Precursor 1-PRENCSO Levels and Alliinase Activity

Biochemical analysis showed that the 1-PRENCSO levels in the transgenic and control plants were between 4 and 13 mg g\(^{-1}\) dry weight. Alliinase activity was between 15.8 and 42.4 nkat mg\(^{-1}\) protein. These substrate and enzyme levels are within the normal physiological range reported for onion (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-PRENCSO levels.

Phenotype Analysis of Secondary Sulfur Chemistry

In order to identify all of the possible changes to onion secondary sulfur metabolism, three established techniques were used: gas chromatography (GC) with flame photometric detection, solid-phase microextraction (SPME) GC-mass spectrometry (MS), and solvent extraction GC-MS. 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-flame photometric detection 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

Figure 3. Molecular analysis. Top, Southern analysis of lfsRNAi transgenic onion plants probed with gfp probe. Center, RNAi analysis of small RNA probed with lfs probe. Bottom, Quantitative RT-PCR measurement of lfs transcript compared with nontransgenic (NT). HN, ON, and DN indicate nontransgenic hybrid, open-pollinated, and dehydration control, respectively (stars); H1, H2, H3, O1, O2, O3, D1, and D2 represent specific transgenic hybrid, open-pollinated, and dehydration onion plants (circles). Far right lanes show one-, five-, and 10-copy control, respectively (on Southern-blot gel) and LFS sequence control (on RNAi gel). The 95% confidence limit is calculated from an ANOVA 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-blot analysis of LFS. Middle, In vitro LF peak area (LPA). Bottom, In vivo LF measurements. HN, ON, and DN indicate nontransgenic hybrid, open-pollinated, and dehydration control, respectively; H1, H2, H3, O1, O2, O3, D1, and D2 represent specific transgenic hybrid, open-pollinated, and dehydration onion plants. The 95% confidence limit is calculated by analysis of log-transformed data of within-bulb and within-leaf variation to account for the variance difference between the low and high LF values. ND, Not detected.
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 (Fig. 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-GC-MS analysis of head space, as proposed by Arnault et al. (2000). Healthy cut leaf blade material was analyzed 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 Fig. S1) with published work (Hiramitsu, 1989; Sinha et al., 1992; Block et al., 1996a). This analysis (Fig. 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 control plants. 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 and Ho, 1992a, 1992b) but have never been detected in onion.

Subjective sensory evaluation of the reduced-LF plants by the authors supported the GC analysis. Onion leaf and bulb samples (0.5 cm²) 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 nontransgenic 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

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**Figure 5.** GC analysis of solid-phase microextraction sulfur components from the head space 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.
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, 2006b) demonstrated that 1-propenyl-containing thiosulfinates produce a pink pigment (of unknown structure) when mixed with Gly and formaldehyde. We exploited this reaction to develop a simple colorimetric assay (pinking assay) to detect 1-PRENCSO-derived thiosulfinate (Supplemental Fig. S2). The 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 (Fig. 6). The 1-PRENCSO-derived product correlated with a pink color, while the alliin-derived product produced no pink color. Pinking analysis of the reduced-LF plants confirmed increased levels of 1-propenyl-containing thiosulfinates. Furthermore, addition of recombinant LFS to the extract lowered the pinking level and confirmed that the absence of LFS was the cause of pinking (Fig. 7). In vitro analysis indicated that not all 1-PRENCSO-derived thiosulfinates are captured in the color reaction and that LFS is a strong competitor for 1-propenyl sulfenic acid. In in vitro assays, one to two times normal physiological levels of LFS (using recombinant LFS) converted more than 85% of 1-PRENCSO to LF (data not shown).

Nonenzymatic 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 GC-MS analysis of crushed reduced-LF plants was undertaken in an effort to identify differences from nontransgenic onions. The total ion chromatogram produced by GC-MS analysis of incubated reduced-LF extract from plants H3, O3, and D2 compared with their nontransgenic counterparts detected a large peak difference in the transgenic extracts (Fig. 8), which was confirmed by the mass spectra fingerprint (Supplemental Fig. S1) to be a zwiebelane isomer. Zwiebelane isomers were first reported by Arnault et al. (2000), although they 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, to our knowledge, 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 thiosulfinates. Raised levels of 1-propenyl-containing thiosulfinate (detected using the novel pinking assay) predictably increased levels of the nonenzymatically 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 thiosulfinates 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 colors” in processed onion and garlic products (Imai et al., 2006a, 2006b). 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 thiosulfinates, 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 nonenzymatic breakdown of 1-propenyl-containing thiosulfinate (Fig. 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 thiosulfinates and zwiebelanes are converted into disulfides in SPME-GC-MS analysis because of its thermally severe condition compared with solvent extraction GC-MS. The increase of 1-propenyl-containing disulfides in the SPME-GC-MS 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 di-

Figure 8. Total ion chromatograms of incubated onion extract. Chromatograms for H3 and HN, O3 and ON, and D2 and DN show the control benzyl alcohol and the zwiebelane isomer.
sulfide 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-GC-MS analysis. The results presented here 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 GC-MS protocols (Arnault et al., 2000, 2004; Hori, 2007). Thus, while 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 flavor of the lower LF levels combined with the 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 flavor notes (Randle, 1997). Disulfide and dihydrothiophene compounds are associated with the sweeter aroma of cooked or fried onions (Lancaster and Boland, 1990; Albanese and Fontijne, 2002). Trans- and cis-zwiebelanes are associated with sweet raw onion sulfur tastes and sweet brown sauce flavor notes, respectively (Block et al., 1996b). 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 sauce flavor 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 flavor roles 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; as such, they are being further investigated. For example, thiosulfinates have antiasthmatic 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, 1992; Block et al., 1996b; 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 signaling molecule that has an ideal physiological profile for cardiovascular protection (Lefer, 2007). The nature and similarity of

Figure 9. The proposed sulfur pathway following tissue disruption in transgenic onion. * Detected by pinking assay; ** detected by solvent extraction GC-MS; *** detected by SPME-GC-MS.
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 mid-daylength fresh onion (Allium cepa (’Enterprise’)), a pungent open-pollinated fresh onion (’Pukekeo LongKeeper’), and a pungent dehydration mid-daylength onion (Sensient Dehydrated Flavours) were selected as control onion (Eady et al., 2000). The T-DNA cassette designed for silencing in onion (Fig. 10) was contained within a pArt binary vector (Gleave, 1992) with a nos promoter under the control of a constitutive promoter. The T-DNA cassette designed for silencing in onion (Fig. 10) was contained within a pArt binary vector (Gleave, 1992) with a nos promoter under the control of a constitutive promoter.

Biochemical Analysis of Plants

Western Analysis of LFS Protein

Total protein was extracted from approximately 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. One hundred micrograms of protein in Laemmli Sample Buffer (Bio-Rad) was loaded onto a 15% polyacrylamide gel and electrophoresed. The gel was then stained with a Trans-Blot SD Cell (Bio-Rad) onto a polyvinylidene difluoride membrane (Bio-Rad) following the manufacturer’s instructions. The blots were 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 the 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 version 4.6 (Bio-Rad) according to the manufacturer’s instructions.

LF 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 −80°C until ready for analysis. Half of each leaf and bulb sample was frozen, stored at room temperature in a desicator, and used for LF assays and precursor evaluation (in vivo LF leaf assays used fresh frozen material).

In Vivo LF Assays

Sample preparation and GC 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 deionized water in 1.5-mL microcentrifuge tubes. The tubes were held at room temperature (approximately 20°C) with stirring every minute. Three minutes after the initial mixing, 500 μL of dichloromethane (containing 0.06 μL/mL −1 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 autoinjector vial for immediate analysis.

Samples of bulb powder (10–30 mg) were prepared as above, except that in addition to 500 μL of deionized 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 160°C; hydrogen carrier gas at constant 60 cm s⁻¹ flow; isothermal 55°C oven temperature; flame photometric detector at 180°C. 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 picograms of LF per gram fresh weight using the dry weights of the bulb samples to adjust to a fresh weight basis.

In Vitro LF Assays

LF 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

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mt NaHPO₄, 2.68 mt KCl, and 1.47 mt KH₂PO₄) at a concentration of 1 g per 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 described. For the LF-forming capability assay, 10 μL of the extracted protein solution, 40 g mL⁻¹ purified garlic (Allium sativum) alliinase (555 nkat mg⁻¹ protein), and 20 g mL⁻¹ of 20 mg mL⁻¹ trans-PRENCSO purified from onion were mixed and left for 3 min at room temperature. Two microliters of the resulting mixture were analyzed by HPLC using an ODS column at 35°C. The column was eluted with 30% (v/v) acetic methanol (pH 3.3) at a flow rate of 0.6 mL min⁻¹, and LF was monitored by the A₂₅₀. LF enzyme activity was assessed by calculating LF peak area per nanogram of protein in 2 μL of the reaction mixture.

**PRENCSO Substrate and Alliinase Activity**

PRENCSO precursor levels were assayed as thiocarbamate derivatives by reverse-phase 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 the use of propyl-Cys sulfoxide 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 nmol) were mixed with 40 μL of 5% Gly solution and 145 μL of purified garlic alliinase (555 nkat mg⁻¹ protein). After 30 min at 25°C, insoluble matter was removed by centrifugation and 10 μL of 100 μL L⁻¹ formaldehyde solution was added to 100 μL of the supernatant. This was then incubated at 40°C for 6 h, and then A₂₅₀ was measured by spectrophotometry. Each assay was performed in triplicate.

The pinking assay was developed as follows: 30 mg of freeze-dried bulb powder was reconstituted in 200 μL of 1% Gly solution and with 15 μg of recombinant LFS. After 30 min at 25°C, insoluble matter was removed by centrifugation and 10 μL of 100 μL L⁻¹ formaldehyde solution was added to 100 μL of the supernatant. This was incubated at 40°C for 6 h, and then A₂₅₀ was measured by spectrophotometry. Increasing absorbance (pinking) demonstrated the relative abundance of 1-propenyl-containing thiocarbamates.

**Head Space SPME-GC-MS Analysis**

Head space analysis was performed according to Horii’s method (2007) with slight modifications. Five grams of sliced fresh leaf material from each plant was put into 20-mL SPME vials and covered with a lid immediately, then 1 min of centrifugation at 15,000 rpm, the diethyl ether phase was transferred to a GC autoinjector vial for immediate analysis. One microliter was injected into a 5975C inert XL MSD MS system (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. DB-5ms column with a column pressure of 100 kPa, and the column temperature program was 5 min at 35°C, 16 min at 200°C, and 10°C min⁻¹ to 250°C. Transfer line and detector temperature was held at 150°C, and total ion chromatograms and mass spectra were analyzed with the electron-impact mode. The compounds were identified by matching their mass spectra to the U.S. National Institute of Standards and Technology and Wiley mass spectral libraries and previous studies (Block et al., 1992b; Arnault et al., 2000).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB089203.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Mass spectral data.

**Supplemental Figure S2.** Proposed reaction mechanism for producing a pink pigment.

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