Hydrogen Sulfide Alleviates Dark-promoted Senescence in Postharvest Broccoli

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Abstract. Broccoli (Brassica oleracea var. italica) is an important vegetable crop rich in vitamins and sulfurophane. However, the floral heads of broccoli experience rapid postharvest senescence. Here we found that hydrogen sulfide (H2S) treatment alleviated dark-promoted senescence in broccoli florets. H2S delayed the symptoms of senescence and maintained higher levels of chlorophyll and Rubisco and lower protease activity compared with water control. Gene expression analysis showed that H2S down-regulated the expression of chlorophyll degradation-related genes BoSGR, BoNYC, BoCLH1, BoPPH, and BoRCCR. Expression of lipoygenase gene BoLOX1 and the genes involved in the ethylene synthesis pathway, BoACS2 and BoACS3, were also down-regulated by H2S. The reduced expression level in cysteine protease gene BoCP3 and aspartic protease gene BoLSC807 suggested the role of H2S in alleviating protein degradation during broccoli senescence. H2S up-regulated the expression of sulfur metabolism genes BoSR and BoOASTL, and the antioxidant gene BoCAT. These results show that H2S plays a vital role in alleviating broccoli senescence through a broad regulation on gene expression of reactive oxygen species (ROS) metabolism genes, ethylene synthesis genes, and protease genes.

Broccoli (Brassica oleracea var. italica) is an important vegetable of high nutritional value and a common component of the human diet. Floral heads of broccoli are harvested during the immature stage when florets are composed of male and female reproductive structures surrounded by immature petals and enclosed with chlorophyll-containing sepalas (Chen et al., 2008). Postharvest broccoli florets experience senescence similar to those seen in developmental leaf senescence at biochemical and transcriptional levels, involving decline in photosynthesis and degradation of macromolecules such as proteins, lipids, and chlorophyll (Buchanan-Wollaston, 1997; Smart, 1994). Production of ethylene plays an important role in regulating the yellowing of florets after harvest, because chlorophyll loss is associated with an increase in ethylene synthesis in florets (Tian et al., 1994). Dark treatment has been shown to accelerate leaf senescence (Quirino et al., 2000). In many plant species, dark treatment causes chlorophyll and protein degradation, which also occurs in developmental senescent leaves (Biswal and Biswal, 1988). When stored in darkness, broccoli florets experienced more rapid deterioration in quality compared with those under light (Li et al., 2014). To extend the shelf life of broccoli, various methods have been developed, including modified atmosphere packaging, ultraviolet-C and light irradiance, and appropriate refrigerated storage (Chen et al., 2008).

H2S has been shown to play various physiological roles in animal cells (Wang, 2002). Recently, more evidence has demonstrated that H2S plays multiple roles in plants, including seed germination, root organogenesis, abiotic stress response, and photosynthesis (Jin et al., 2011, 2013; Lisjak et al., 2013; Zhang et al., 2008, 2009). More recently, H2S was found to prolong postharvest shelf life of strawberry, kiwifruit, and broccoli by acting as an antioxidant (Gao et al., 2013; Hu et al., 2012; Li et al., 2014). Previously we reported that H2S fumigation effectively alleviates postharvest senescence of broccoli through modulation of the endogenous antioxidant system. In this article we investigate accelerated senescence of broccoli florets in darkness using biochemical and molecular analysis and the interplay of H2S with sulfur metabolism, the ethylene pathway, and protein degradation.

Materials and Methods

Plant materials and treatment. Fresh broccoli (Brassica oleracea var. italica) heads were kindly supplied by Anhui Academy of Agricultural Sciences, Hefei, Anhui province, China. Broccoli heads having similar size and maturity and florets without physical damage or microbial infection were used in this study. The H2S donor, sodium hydrosulfide (NaHS; Sigma, St. Louis, MO) was used in aqueous solutions (150 mL at concentrations of 0.24, 0.48, 0.72, 0.96, and 1.2 mmol·L-1) and applied to florets in sealed containers (volume 3 L). Six pieces of fresh-cut broccoli florets (16 ± 2 g per piece) from six different broccoli heads allocated on board with pores that separated florets and NaHS solution were fumigated with H2S in the sealed containers at 25°C with 85% to 90% relative humidity and stored in darkness. NaHS solutions were renewed every 24 h. Florets were sampled from different broccoli for RNA extraction and other experiments at the time points indicated in figure legends. Each experiment was repeated three times.

Visual evaluation of postharvest broccoli. The appearance of fresh-cut broccoli florets fumigated with H2S gas was evaluated subjectively by 20 people using five indices, including freshness, yellowness, texture, flavor, and rotting rate (Takamiya et al., 2000). Each index was divided into four ranks and the score was 0, 1.0, 1.5, and 2.0 with lower numbers indicating lower quality. A sum of the five indices ranging from 0 to 10 was used for visual evaluation.

Determination of chlorophyll content. Chlorophyll content of broccoli florets was determined according to the method of Lichtenthaler and Wellburn (1983) with minor modifications. Broccoli florets (0.5 ± 0.01 g) were homogenized using a pestle and mortar in the presence of ice and then incubated in...
10 mL of 80% acetone for 24 h in darkness at 4 °C. The supernatant was measured at 665 and 645 nm, respectively. Chlorophyll content was calculated using the following formulas: total chlorophyll (Chl) (mg g⁻¹) = (20.2A645 + 8.02A633)/V W; Chl a (mg g⁻¹) = (12.7A645 - 2.69A645)/V W; Chl b (mg g⁻¹) = (22.9A645 - 4.68A643)/V W. Each analysis was repeated three times and the results were expressed as mg g⁻¹ fresh weight.

**Results**

H₂S alleviates dark-induced senescence in broccoli. Broccoli florets stored in darkness were fumigated with different concentrations of NaHS. As shown in Figure 1A, H₂S effectively alleviated dark-induced senescence in broccoli florets in a dose-dependent manner. Control broccoli yellowed quickly after 3 d of storage, whereas NaHS at an optimal concentration of 0.96 mmol L⁻¹ postponed yellowing process until Day 5. Florets exposed to 0.96 mmol L⁻¹ NaHS showed the highest visual value according to the five indices of freshness, yellowness, texture, flavor, and rotting rate (Fig. 1B).

**Effects of H₂S fumigation on content of chlorophyll, activity of protease, and degradation of Rubisco.** We determined the effects of H₂S fumigation on Chl degradation, which is the principle symptom of broccoli senescence. As shown in Figure 2A–C, total Chl, Chl a, and Chl b in both control and H₂S-fumigated broccoli decreased gradually during the whole storage time. However, H₂S fumigation maintained significantly higher Chl level than a water control (Fig. 2A–C).

Senescence-associated proteases, which are accumulated in the vacuole, play a vital role in protein degradation during plant senescence (Li et al., 2014). As expected, protease activity in control samples increased steadily during the first d of storage followed by a decrease on Day 5 (Fig. 2D). In contrast, H₂S sustained stable and lower levels of protease activity in broccoli (Fig. 2D). During leaf senescence, Rubisco is gradually degraded for recycling within the plant. As shown in Figure 2E, Rubisco was degraded gradually during dark senescence in control broccoli florets, whereas H₂S fumigation alleviated the degradation of Rubisco, especially on Days 4, 5, and 6.

**Discussion**

The results presented in this study show that H₂S alleviates dark-induced senescence in broccoli. We demonstrated that H₂S alleviates chlorophyll degradation and protease activity, thereby delaying senescence in broccoli florets. These findings suggest that H₂S may serve as a potential agent for delaying senescence and maintaining chlorophyll content in broccoli. Further research is needed to elucidate the underlying mechanisms of H₂S in delaying senescence and maintaining chlorophyll content in broccoli.
As shown in Figure 4, ethylene biosynthesis and signal transduction were inhibited by H2S. As shown in Figure 5A, H2S fumigation induced higher expression of BoOASTL and BoSR on Day 3 of storage compared with water control. In contrast, the expression levels of BoAPX and BoGR were not significantly changed in H2S-fumigated broccoli.

Effects of H2S on the transcription of protein degrading genes. Genes involved in protein degradation and in amino acids recycling from degraded protein are thought to be up-regulated in senescing tissues (Chen et al., 2008). Expression of senescence-related protease genes BoCP1, BoCP3, and BoLSC807 were assayed in broccoli (Fig. 5C). BoCP3 was activated on Days 1 and 3 of storage, whereas H2S fumigation significantly decreased BoCP3 expression (Fig. 5C). Meanwhile, expression of BoLSC807 (aspartic protease encoding gene) was also repressed by H2S fumigation on Day 3 of storage, suggesting the role of H2S in inhibiting protein degradation (Fig. 5C).

Discussion

Dark exposure is used as a conventional method to induce plant senescence (Smart, 1994). Our results suggest that dark treatment induces broccoli senescence by Chl and protein degradation, and H2S effectively alleviates the senescence symptom (Fig. 1). Chlorophyll content decreases dramatically in control broccoli during storage, whereas H2S application sustains higher Chl content (Fig. 2A–C). Consistently, H2S application was found to delay Chl degradation under normal light storage conditions (Li et al., 2014). However, we find that broccoli stored in darkness becomes yellow quickly compared with broccoli under normal light conditions, suggesting that dark treatment is effective in triggering broccoli senescence (data not shown).

Chlorophyll degradation is the first visible symptom of senescence exhibited by a loss of green color and shortening of shelf life (Chen et al., 2008). SGR, a chloroplast-located protein, destabilizes light harvesting complex subunits to release Chls. During Chl degradation, transformation from Chl b to Chl a is catalyzed by a Chl(ide) b reductase [non-yellow coloring 1 (NYC1)] (Hörtensteiner and Kräutler, 2011). The enzyme, PPH, is involved in dephytlation of phophytine to generate phosphorhbid (Hörtensteiner and Kräutler, 2011). PaO catalyzes the opening of the porphyrin ring of phophorhbid and generates red chlorophyll catabolites (RCC) followed by the reduction of RCC by RCC reductase (RCCR) (Hasperue et al., 2013). In this work, we analyze the expression of Chl degradation-related genes (BoSGR, BoCLH1, BoCLH2, BoPPH, BoRCCR, BoPaO, and BoNYC), and H2S is able to sustain a lower expression level of BoSGR, BoCLH1, BoPPH, and BoRCCR in broccoli. BoCLH1 and BoCLH2 are involved in ethylene-induced Chl degradation (Chen et al., 2008). The activation of BoCLH1 and BoCLH2 expression in control broccoli suggests that their expression levels were not significantly changed by H2S fumigation.
expression might be induced by endogenous ethylene generated during senescence (Fig. 3). Ethylene plays an important role in floret yellowing of broccoli (Tian et al., 1994). 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) are key enzymes for ethylene production, which catalyze the conversion of S-adenosylmethionine to ACC and ACC to ethylene, respectively (Hamilton et al., 1991; Lin et al., 2009). In our experiment, increased expression of ethylene synthesis and signaling related genes BoACS2, BoACS3, BoERS, and BoETR1 during storage suggests the key role of the ethylene pathway in inducing broccoli senescence (Fig. 4). However, we first present evidence that H2S application effectively blocks the ethylene synthesis pathway through reducing the transcripts of BoACS2 and BoACS3 (Fig. 4, suggesting the role of H2S in counteracting ethylene synthesis. The lowered ethylene synthesis in H2S treatment will also help to reduce Chl and protein degradation (Chen et al., 2008). Similarly, nitric oxide has been shown to transcriptionally repress the expression of genes of ethylene biosynthesis enzymes, suggesting a similar mechanism in decreasing ethylene synthesis (Manjunatha et al., 2012). Previous study shows that exogenous H2S affects sulfur metabolism on enzymatic activity and gene expression levels (Lai et al., 2014). In the present study, the expression of BoOASTL and BoSR is up-regulated by H2S fumigation, implicating the accelerated sulfur metabolism induced by exogenous H2S. Reactive oxygen species overproduction and oxidative damage are essential events in the senescence of postharvest vegetables (Li et al., 2014). LOX catalyze the hydroperoxidation of polyunsaturated fatty acids and contribute to membrane deterioration (Gomez-Lobato et al., 2012). However, plants have developed the ability to eliminate ROS with an efficient ROS-scavenging system (Mittler et al., 2004).
In conclusion, we provide evidence that H$_2$S delays dark-promoted senescence in broccoli by inhibiting Chl and protein degradation, and this observation offers the prospect of H$_2$S application in managing post-harvest storage of horticultural products. The gene expression assays confirms the multiple roles of H$_2$S in regulating Chl and protein degrading genes and ethylene synthesis pathway. Senescence is a complex, highly regulated process in plants (Buchanan-Wollaston, 1997; Smart, 1994), which involved ethylene, H$_2$O$_2$, cytokinins, jasmonic acid, etc. Thus, more works regarding the interplay between H$_2$S and other signals are required to elucidate the senescence regulator role of H$_2$S.

**Literature Cited**

Biiswal, U.C. and B. Biiswal. 1988. Ultrastructural modifications and biochemical changes during senescence of chloroplasts. Intl. Rev. Cytol. 113:271–321.

Buchanan-Wollaston, V. 1997. The molecular biology of leaf senescence. J. Expt. Bot. 48:181–196.

Chen, Y.T., L.F.O. Chen, and J.F. Shaw. 2008. Senescence-associated genes in harvested broccoli florets. Plant Sci. 175:137–144.

Gao, S.P., K.D. Hu, L.Y. Hu, Y.H. Li, Y. Han, H.L. Wang, K. Lv, Y.S. Liu, and H. Zhang. 2013. Hydrogen sulfide delays postharvest senescence and plays an antioxidative role in fresh-cut kiwifruit. HortScience 48:1385–1392.

Gomez-Lobato, M.E., P.M. Civello, and G.A. Martinez. 2012. Expression of a lipoxygenase encoding gene (BoLOX) during postharvest senescence of broccoli. Postharvest Biol. Technol. 64:146–153.

Hamilton, A.J., M. Bouzayen, and D. Grierson. 1991. Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. Proc. Natl. Acad. Sci. USA 88:7434–7437.

Hasperue, J.H., M.E. Gomez-Lobato, A.R. Chaves, P.M. Civello, and G.A. Martinez. 2013. Time of day at harvest affects the expression of chlorophyll degrading genes during postharvest storage of broccoli. Postharvest Biol. Technol. 82:22–27.

Hörentsinner, S. and B. Kräutler. 2011. Chlorophyll breakdown in higher plants. Biochim. Biophys. Acta 1807:977–988.

Hu, L.Y., S.L. Hu, J. Wu, Y.H. Li, J.L. Zheng, Z.J. Wei, J. Liu, H.L. Wang, Y.S. Liu, and H. Zhang. 2012. Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. Plant Signal. Behav. 7:476–483.

Mittler, R., S. Vanderauwera, M. Gollery, and F. Van Breusegem. 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9:490–498.

Ono, Y., S. Wada, M. Izumi, A. Makino, and H. Ishida. 2013. Evidence for contribution of autophagy to Rubisco degradation during leaf senescence in Arabidopsis thaliana. Plant Cell Environ. 36:1147–1159.

Quirino, B.F., Y.S. Noh, E. Himelblau, and R.M. Amansino. 2000. Molecular aspects of leaf senescence. Trends Plant Sci. 5:278–282.

Reimerdes, E.H. and H. Klostermeyer. 1976. Determination of proteolytic activities on casein substrates. Methods Enzymol. 45:26–28.

Smart, C.M. 1994. Gene expression during leaf senescence. New Phyto. 126:419–448.

Takamiya, K.T., T. Tsuchiya, and H. Ohta. 2000. Degradation pathway(s) of chlorophyll: What has gene cloning revealed? Trends Plant Sci. 5:426–431.

Tian, M.S., C.G. Downs, R.E. Lill, and G.A. King. 1994. A role for ethylene in the yellowing of broccoli after harvest. J. Amer. Soc. Hort. Sci. 119:276–281.

Wang, R. 2002. Two’s company, three’s a crowd: Can H$_2$S be the third endogenous gaseous transmitter? FASEB J. 16:1792–1798.

Yang, G.D., K.X. Zhao, Y.J. Su, S. Mani, Q.H. Cao, S. Puukila, N. Khaper, L.Y. Wu, and R. Wang. 2013. Hydrogen sulfide protects against cellular senescence via S-sulfhydration of Keap1 and activation of Nrf2. Antioxid. Redox Signal. 18:1906–1919.

Zhang, H. L.Y. Hu, K.D. Hu, Y.D. He, S.H. Wang, and J.P. Luo. 2008. Hydrogen sulfide promotes wheat seed germination and alleviates the oxidative damage against copper stress. J. Integr. Plant Biol. 50:1518–1529.

Zhang, H., J. Tang, X.P. Liu, Y. Wang, W. Yu, W.Y. Peng, F. Fang, D.F. Ma, Z.J. Wei, and L.Y. Hu. 2009. Hydrogen sulfide promotes root organogenesis in Ipomoea beetata. Salixmadusana, and Glycine max. J. Integr. Plant Biol. 51:1084–1094.