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Hydrogen sulfide and rhizobia synergistically regulate nitrogen (N) assimilation and remobilization during N deficiency-induced senescence in soybean

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Abstract

Hydrogen sulfide (H_2S) is emerging as an important signalling molecule that regulates plant growth and abiotic stress responses. However, the roles of H_2S in symbiotic nitrogen (N) assimilation and remobilization have not been characterized. Therefore, we examined how H_2S influences the soybean (*Glycine max*)/rhizobia interaction in terms of symbiotic N fixation and mobilization during N deficiency-induced senescence. H_2S enhanced biomass accumulation and delayed leaf senescence through effects on nodule numbers, leaf chlorophyll contents, leaf N resorption efficiency, and the N contents in different tissues. Moreover, grain numbers and yield were regulated by H_2S and rhizobia, together with N accumulation in the organs, and N use efficiency. The synergistic effects of H_2S and rhizobia were also demonstrated by effects on the enzyme activities, protein abundances, and gene expressions associated with N metabolism, and senescence-associated genes (*SAG*s) expression in soybeans grown under conditions of N deficiency. Taken together, these results show that H_2S and rhizobia accelerate N assimilation and remobilization by regulation of the expression of *SAG*s during N deficiency-induced senescence. Thus, H_2S enhances the vegetative and reproductive growth of soybean, presumably through interactions with rhizobia under conditions of N deficiency.

KEYWORDS

assimilation, hydrogen sulfide (H_2S), nitrogen, remobilization, rhizobia, soybean (*Glycine max*)

1 | INTRODUCTION

As one of the most abundant elements on the planet, nitrogen (N) is a nutrient necessary for plant growth and development, but its availability directly limits crop production (Robertson & Vitousek, 2009). Although N is widely present in the atmosphere, it primarily exists in the form of dinitrogen, which cannot be used to synthesize constituent elements such as the nucleic acids, enzymes, and proteins used directly by plants (Sinclair & Horie, 1989). Therefore, to convert N into a form that can be utilized by plants, chemical fixation via industrial production and biological N fixation (BNF) via microorganisms are often employed in agriculture. For example, legumes, including alfalfa (*Medicago sativa*) and soybean (*Glycine max*), are capable of BNF using nodules formed by symbiosis with rhizobia (Berman-Frank, Lundgren, & Falkowski, 2003). This symbiotic relationship between rhizobia and legumes is beneficial to both partners: The plants provide energy (in the form of carbohydrates) to bacteria that convert gaseous N_2 to ammonia via the action of nitrogenase; bacterially produced ammonia is subsequently utilized for plant growth (Chakrabarti &

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Mukherji, 2003). Nitrate absorbed by the plant itself is also converted to ammonium by the action of nitrate reductase (NR) and nitrite reductase (NiR). Subsequently, a series of N assimilation enzymes including glutamine synthetase (GS), glutamate synthetase (GOGAT), and glutamate dehydrogenase (GDH) transform ammonium into amino acids that can be utilized for biomacromolecular synthesis (Becker, Carrayol, & Hirel, 2000). Therefore, it is possible to promote N assimilation and transport by increasing the activities of nitrogenase and N metabolic enzymes in legumes (Tian et al., 2010).

Senescence is not only a highly regulated genetic control process that leads to the death of specific organs or whole organisms but is also thought to be a developmental process in plants (Munné-Bosch, 2008). Because plants are sessile organisms that cannot escape from nonoptimal environmental conditions to obtain their desired mineral nutrients, their evolutionary history has led to programmed cell death and aging to manage the occasional nutritional shortages that they encounter (Lim, Kim, & Nam, 2007). During the process of aging, the plant cells undergo highly ordered decomposition through cellular metabolic processes and the degradation of cell structures (Gan & Amasino, 1997), and the phenomenon of leaf senescence is a clear example. Leaf senescence helps to eliminate inefficient and aging photosynthetic organs, and it is equally important for nutrient remobilization and recycling from leaves to other plant parts that are growing (Avilaospina, Moison, Yoshimoto, & Masclauxdaubresse, 2014; Have, Marmagne, Chardon, & Masclaux-Daubresse, 2017).

Currently, many studies have confirmed that hydrogen sulfide (H_2S), like nitric oxide (NO) and carbon monoxide (CO), plays a pivotal role in plant growth and abiotic stress response processes. For example, H_2S had a conspicuous regulatory effect on the seed germination and stomatal movement of *Arabidopsis* (Baudouin et al., 2016; Jin et al., 2017) and the formation of lateral roots in tomato (Fang, Cao, Li, Shen, & Huang, 2014). Additionally, our previous research has determined that H_2S promotes photosynthesis in spinach (*Spinacia oleracea*) and maize (*Zea mays*) seedlings (Chen et al., 2011; Chen et al., 2015), is involved in drought tolerance in spinach (Chen et al., 2016), and alleviates high salt stress in barley (Chen et al., 2015). The fumigation of *Ipomoea aquatica* with NaHS improved the energy status and antioxidant capacity and inhibited the respiratory rate of the plant, ultimately alleviating leaf yellowing and senescence (Hu, Liu, Li, & Shen, 2015). Surprisingly, H_2S seemed to affect energy production regulated by mitochondria and prevent cellular senescence, ultimately delaying leaf senescence in *Arabidopsis* under drought stress (Jin, Sun, Yang, & Pei, 2018). Moreover, we recently demonstrated that H_2S was of the utmost importance in soybean seedlings for its ability to improve the plant biomass and cause the accumulation of nutrients such as Fe and N under conditions of iron deficiency (Chen et al., 2018). Moreover, the nodulation and N fixation capacity of the soybean symbiotic system were reinforced by the action of H_2S (Zou et al., 2019).

The growing interest in H_2S function led to our examination of the synergistic function of H_2S and rhizobia in N deficiency signalling in this study. It has been effectively demonstrated from plant vegetative and reproductive growth that H_2S could act synergistically with rhizobia to promote N assimilation and remobilization by regulating N metabolism and senescence under N deficiency. Consequently, H_2S contributed to the improvement of plant biomass, N content in different tissues, and grain yield during the soybean-rhizobia symbiosis.

2 | MATERIALS AND METHODS

2.1 | Plant growth and treatments

Soybean seeds (*Glycine max*, Zhonghuang 13) were surface sterilized with 75% ethanol for 30 s and 50% sodium hypochlorite solution for an additional 4 min and then placed on 1.0% sterile agar medium for approximately 72 hr in a 28°C constant temperature incubator. After the seeds germinated, they were sown in 700-ml growth substrate (vermiculite and perlite, V:V = 2:1) containing 300-ml N-free nutrient solution, which was sterilized in a polypropylene planting bag. The composition of the nutrient solution was as follows: 0.68-mM $CaCl_2{\cdot}2H_2O$, 0.73-mM KH_2PO_4, 0.02-mM $FeC_6H_5O_7$, 1.25-mM NaH_2PO_4, 0.49-mM $MgSO_4{\cdot}7H_2O$, 46.28-μM H_3BO_3, 9.1-μM $MnSO_4{\cdot}4H_2O$, 0.77-μM $ZnSO_4{\cdot}7H_2O$, 0.16-μM $Na_2MnO_4{\cdot}2H_2O$, and 0.32-μM $CuSO_4{\cdot}5H_2O$. The plants were grown in a constant temperature incubator with a light/dark regime of 12/12 hr, relative humidity of 80%, temperature of 23/25°C, and photosynthetically active radiation of 190 μmol m^{-2} s^{-1}.

NaHS was used as an exogenous H_2S donor (Hosoki, Matsuki, & Kimura, 1997). After the first true leaves of the plants were fully expanded, some plants were inoculated with rhizobia (*Sinorhizobium fredii* Q8 strain), by the addition of 10-ml of a rhizobia suspension (OD_{600} = 0.05) to each bag. Thereafter, some seedlings were watered with 10-ml of 100-μM NaHS solution every 3 days (Zou et al., 2019), and blank controls were watered with sterilized distilled water. Additionally, 50-ml of sterile N-free nutrient solution was added to each bag every week to maintain constant humidity and nutrition. Therefore, all the seedlings were divided into the following four groups after treatment: (a) Control, without rhizobia or NaHS; (b) NaHS, with 100-μM NaHS; (c) Q8, with rhizobia inoculation; and (d) Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS. The experiment was repeated four times with three biological replicates during each experiment and three plants per replicate. In addition, each experimental index was measured by at least four replicates. The data were obtained from a pool of four experiments.

2.2 | Sample harvesting

Samples were harvested on the 12th, 17th, 22nd, 26th, 33rd, and 40th days after the plants were inoculated. We conducted a preliminary experiment that identified the following: Day 12 (the soybean basically has produced leaves in the different leaf blades); Day 17 (pre-flowering); Days 20-22 (blossoming); Days 26-27 (the soybean has finished flowering and begun to form bean pods); Day 33 (The pod is forming); and Day 40 (the pod has formed, but it is not full). Each soybean plant was removed from the substrate when harvested. The shoot of the plant was then separated from the roots, and the roots were washed with distilled water. The shoots included

stems and leaves from different blades, whereas the roots and nodules were underground (Figure 1). As in Figure 1, the leaves were divided into five different blades to better explore N transport and remobilization in the soybean leaves. Dry samples were harvested at each time point to measure the biomass and N content. The only difference was the situation that on the 26th day, in addition to the collection of dry samples, some fresh samples were also harvested in liquid N and stored in −80°C refrigerator for RNA extraction and additional experiments. L-3 leaves growing vigorously at that time were selected. To explore the response mechanism of soybean to vigorous N metabolism, we screened separate periods during a preliminary experiment. The records of soybean development showed that the plants began to bloom around the 20th day post inoculation, and by the 26th day, signs of the pods began to appear. Simultaneously, initial experiments indicated that on the 26th day, the N content in roots and leaves increased prominently, and the N resorption efficiency (NRE) of the leaves was also significantly enhanced (Figure 4). These data indicate our reasons for selecting the 26th day as an optimal checkpoint to collect fresh samples for additional measurements. On the 26th day, the L-1 and L-2 leaves began to yellow and fall; the L-4 and L-5 leaves had grown and were tender, whereas the L-3 leaves were growing vigorously and robustly. Therefore, the active phase of N metabolism and transport was most likely to occur in L-3 leaves. Thus, that is why we chose the L-3 leaves for this experiment.

In addition, samples from the 47th day at maturity were harvested to evaluate the long-term changes on the N content in different tissues, grain yield, and N use efficiency (NUE). The soybeans were separated into dry remains during harvest (DR; roots + stems + leaves + pods) and total seeds (SEEDS).

2.3 | Statistics and analysis of the plant biomass, nodule number, and number of fallen leaves

The shoots, roots, and nodules of each of the three plants were placed separately in envelopes and then in an oven at 65°C for 48 hr and weighed to obtain the biomass of the shoots and roots. Moreover, the dry weight (DW) of the DR and SEEDS and the grain yield (pods + seeds) on the 47th day were determined in the same manner. The root nodules from every three plants of both Q8 and Q8 + NaHS treatments were removed and counted after the plants were removed from the substrate. When most of the plants had defoliated, the number of fallen leaves (actual leaves) was recorded on the 15th, 19th, 24th, 29th, 33rd, 38th, and 40th days based on the traces they left behind. When harvesting at maturity, the amount of grain per three plants was also recorded.

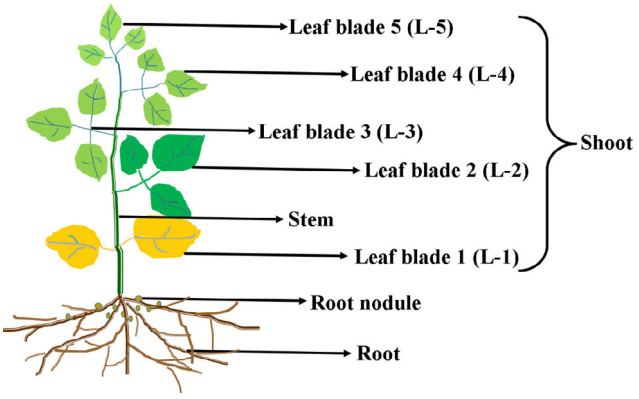

FIGURE 1 An illustration of the different soybean tissues. Soybean is made up of shoot, root, and root nodule. And the shoot contains stem and leaf, which includes Leaf Blade 1 (L-1), Leaf Blade 2 (L-2), Leaf Blade 3 (L-3), Leaf Blade 4 (L-4), and Leaf Blade 5 (L-5)

2.4 | Determination of the chlorophyll and N contents, and evaluation of the NRE and NUE

The chlorophyll contents of the leaves were identified using a chlorophyll meter (SPAD-502Plus, Konica Minolta, Japan). The N contents were determined by the Kjeldahl method with slight modifications (Zdravko, Trajče, Ivana, & Marin, 2014). First, 0.2 g of dry sample ground into powder was placed into a digestive tube. Then, 5-ml of concentrated H_2SO_4 was added, followed by shaking and mixing. Next, the mixture was digested in a microwave digestion system (Labtec™ Line, FOSS, Denmark) at 365°C, and seven to eight drops of 30% H_2O_2 were added every 30 min. This was repeated three to four times until the digestion solution changed from black to clear. Finally, the digestion solution was diluted to a constant volume with distilled water, followed by the determination using an automatic Kjeldahl apparatus (Kjeltec™ 8400, FOSS). Based on the results, the N content was calculated as the dry weight (DW).

The NRE of leaves was expressed on a mass basis using the following equation with slight modifications (Wang et al., 2014):

$$\mathrm{NRE} = \frac{Ngreen - Nsenescent}{Ngreen} \times 100\%,$$

where *Ngreen* is the N content in growing leaves and *Nsenescent* is the N content in fallen leaves.

The harvest index (HI) during the harvest was estimated as the $(DW_{SEEDS})/(DW_{DR} + DW_{SEEDS})$ ratio, which indicates the yield. The DW and N content (N%) were combined to determine the N harvest index (NHI), a key indicator of grain filling with N, as $(N\%_{SEEDS}\ DW_{SEEDS})/(N\%_{DR}\ DW_{DR} + N\%_{SEEDS}\ DW_{SEEDS})$. In addition, $(N\%_{DR}\ DW_{DR} + N\%_{SEEDS}\ DW_{SEEDS})$ was considered to be the N accumulation in plants. Therefore, the NUE was evaluated as the ratio of NHI/HI (Guiboileau, Yoshimoto, Soulay, & Avice, 2012).

2.5 | Determination of N metabolic enzyme activities and the leghaemoglobin content

NR, NiR, GS, GOGAT, and GDH activities in L-3 leaves and entire roots from the 26th day were measured using a micromethod test kit (Suzhou Comin, China). Different crude enzyme solutions were

extracted from the fresh plant samples (0.1 g) using the respective kits. Simultaneously, the protein concentration of different samples was determined as described by Chen et al. (2011). Based on the catalytic principle of each enzyme, extracted crude enzyme solutions were used to conduct a series of reactions with the substrate. Finally, the absorbance was measured at the corresponding wavelength using a micro ultraviolet spectrophotometer (Epoch, Bio Tek, USA), which was used to calculate the enzyme activity in the protein concentration of the sample based on the corresponding formula in the protocols.

Nitrogenase activity was measured by acetylene reduction assay (ARA) in nodules on the 26th day (Wych & Rains, 1978; Zou et al., 2019). Fresh root nodules (0.2 g) were weighed into a 25-ml vial, and 200-μl of acetylene was added. The vial was then sealed and incubated for 3 hr at 28°C. Finally, the ethylene content produced by the reduction of acetylene was determined using a gas chromatograph (GC-14C, Shimadzu, Japan) and calculated in fresh weight. Standard curves prepared with a pure ethylene standard were utilized to calibrate the gas chromatography results.

Leghaemoglobin (Lb) was extracted from the nodules on the 26th day and measured as previously described (Riley & Dilworth, 1985). The fresh nodules were ground to a powder in liquid N, which was mixed in a phosphate buffer (0.1-M, pH 6.8) at 5°C. The amount of phosphate buffer was approximately four times the volume of the nodule. After centrifugation at 100 *g* at 5°C for 15 min, the precipitate was discarded, and the supernatant was centrifuged at 21,460 *g* at 5°C for 20 min. The resulting supernatant was measured at 540 nm using a spectrophotometer. The leghaemoglobin content was calculated in fresh weight based on a standard curve, which was prepared using bovine haemoglobin as a standard protein. All the experiments were established with three biological replicates, each with three technical replicates.

2.6 | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis

The total protein of soybean L-3 leaves and entire roots from the 26th day was extracted as described by Chen et al. (2011). The protein concentrations were established using a Bradford Protein Assay Kit (TIANGEN, Beijing, China).

For the western blot analysis, proteins (45 μg per sample) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a 12% or 10% (w/v) acrylamide gel and transferred to a polyvinylidene difluoride membrane for 20 min using a semidry transfer film (Trans-Blot SD, Bio-Rad, USA). The membrane was blocked overnight in 5% skim milk powder dissolved in phosphate-buffered

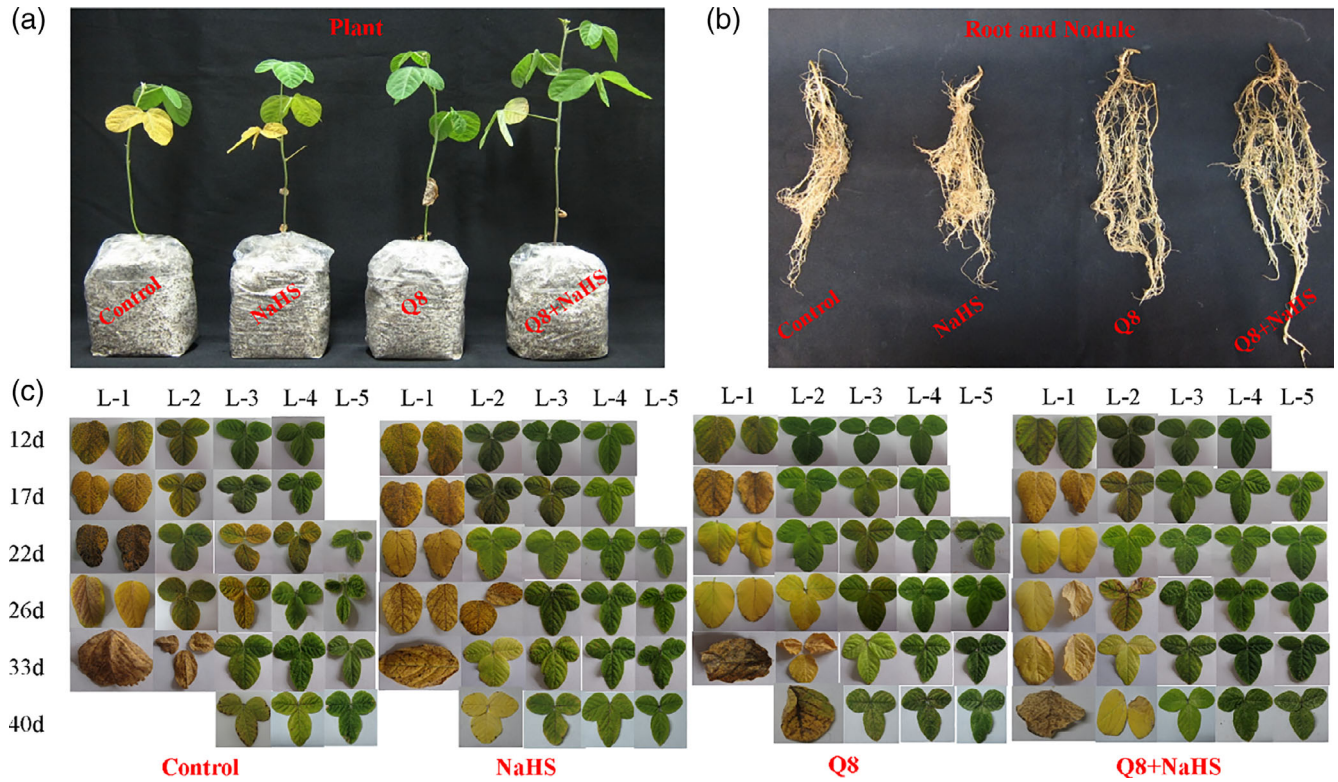

FIGURE 2 H_2S and rhizobia exerted an effect on plant phenotype (a), root shape, and nodule amount (b). In addition, the leaf phenotypes of different leaf blades under the four different treatments also changed as soybean grew (c). Soybean seedlings were first grown in N-free nutrient media. The rhizobia inoculation treatment was carried out after the first true leaves of soybean were fully expanded, and then 100-μM NaHS was added every 3 days. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

saline/Tween (137-mM NaCl, 2.7-mM KCl, 10-mM Na_2HPO_4, 1.76-mM KH_2PO_4, and 0.05% Tween-20, pH 7.2-7.4). The protein blot was probed with primary antibodies for the Rubisco large subunit (RbcL; 1:10,000, AS03 037, Agrisera), NR (1:1,000, AS08 310, Agrisera), and GS1 GS2 glutamine synthetase (GLN1 GLN2; 1:10,000, AS08 310, Agrisera) and then incubated for 12 hr at 4°C. The blot was washed three times with phosphate-buffered saline/Tween, followed by incubation with goat anti-rabbit IgG (H + L)-HRP (1:5,000, Sungene, China) secondary antibody for 12 hr at 4°C. Actin (1:5,000, AS13 2,640, Agrisera) was used as an internal control. The blots were finally washed as described above and coloured with ECL luminescence (Vazyme, China). Images of the blots were generated using a chemiluminescence imaging system (Chemidoc XRS$^+$, Bio-Rad, USA). The optical density value was determined using ImageJ software (NIH, USA) and used to estimate the protein expression. Three biological replicates were performed for this experiment, each with three technical replicates.

2.7 | Total RNA extraction, reverse transcription, and quantitative real-time PCR

To analyse the level of gene expression, the total RNA in soybean tissues, including L-3 leaves and entire roots on the 26th day, was extracted using a plant RNA extraction kit (TaKaRa, China). The RNA concentration was determined using a micro ultraviolet spectrophotometer (Epoch, Bio Tek), whereas the RNA integrity was tested by 1.0% agarose gel electrophoresis. Reverse transcription of the total RNAs was performed using a reverse transcriptase kit (Vazyme, China), and the resulting products were utilized as templates for quantitative real-time PCR analysis. A 10-μl of real-time PCR contained 1-μl of forward and reverse primers, including those for N metabolism-related genes and senescence-associated genes (*SAGs*; Table S1), 600 ng of cDNA, 10-μl of EvaGreen 2× qPCR MasterMix (Abm, China), and 6-μl of sterilized water. The genes involved in N metabolism included *GmNR*, *GmNiR*, *GmGS1*, *GmGOGAT*, *GmGDH*, and *GmRubisco LSU*. The *SAGs* included cysteine proteinase (*GmCysP1*), ubiquitin-activating enzyme E1 (*GmUBE1*), ubiquitin-conjugating enzyme E2 (*GmUBE2*), ubiquitin carrier protein 4 (*GmUBC4*), endonuclease (*GmEN2*), NAC domain protein (*GmNAC1*, *GmNAC2*, *GmNAC3*, *GmNAC4*, *GmNAC5*, and *GmNAC6*), and receptor-like protein kinase (*GmRLK*). The dsDNA synthesis of these genes was amplified and detected using a real-time PCR system (Quantstudio 6 Flex, Thermo Fisher Scientific, USA) with actin as an internal control and the PCR conditions described in Table S2. Three independent replicates were performed for each sample. The relative transcriptional abundance of these genes was expressed as $2^{-\Delta\Delta Ct}$ using the comparative threshold cycle (C_t) method (Livak & Schmittgen, 2001). The experiment was performed with three biological replicates, each with three technical replicates.

2.8 | Statistical analysis

At least three replicates were measured for the physiological and biochemical analyses. Statistical analyses of the time-course experiments were performed with the repeated measurement of a general linear model procedure in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). A one-way

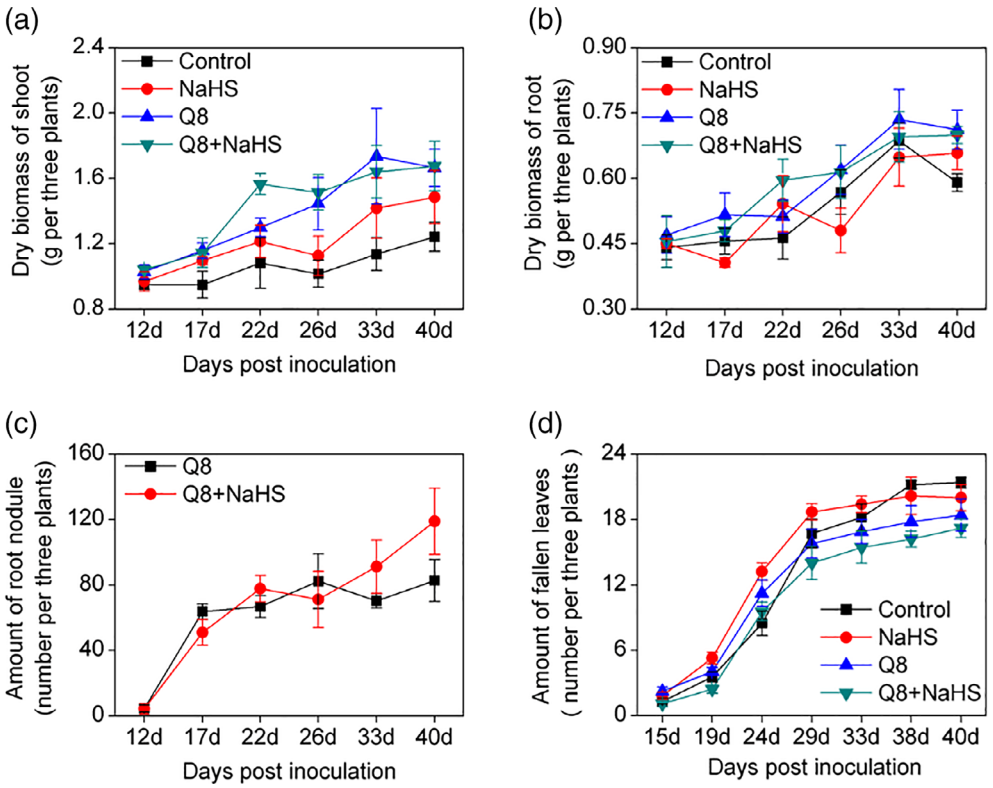

FIGURE 3 With time exposed to N deficiency, H_2S and rhizobia regulated the biomass of soybean shoot (a) and root (b). Moreover, H_2S enhanced the nodulation in the plants inoculated rhizobia (c), whereas Q8 and Q8 + NaHS treatments alleviated the senescence and wilting of leaves (d). Data were expressed as the mean ± *SE*. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

analysis of variance was adopted for significant differences in the histogram, and the results were expressed as the mean ± *SE*. Post hoc comparisons were tested using a Tukey test at a significance level of $P < .05$.

3 | RESULTS

3.1 | Effects of H_2S and rhizobia on the phenotypes of whole plants, roots, nodules, and different leaf blades

Soybean tissue includes leaves of different blades, stems, roots, and nodules (Figure 1). Treatment with H_2S and rhizobia not only promoted the growth of soybean but also contributed to the expansion of roots (Figure 2a,b). Nodule formation occurred in all the plants inoculated with rhizobia, which appeared to be promoted by H_2S (Figure 2b). As the plants grew, the leaves of different blades changed prominently. For example, as the lower (unifoliate and trifoliate) leaves (L-1 and L-2) continued to senescence and fall, the higher (trifoliate) leaves (L-3, L-4, and L-5) gradually generated and developed. Figure 2c showed that L-2, L-3, and L-4 leaves from the 12th day to the 17th day are more vigorous and green under the treatment of H_2S and rhizobia. Moreover, H_2S and rhizobia generated L-3, L-4, and L-5 leaves from the 22nd day to the 40th day that were more robust and emerald green. As expected, the synergistic effect of H_2S and rhizobia was the most obvious. Simultaneously, rhizobia inoculation rendered L-1 and L-2 leaves during the late growth more yellow than those of the control and NaHS (Figure 2c), which was consistent with the results of Figure S1. These results could be interpreted to show that the rhizobia contribute to the sufficient transport of nutrients from the lower leaves to the higher leaves.

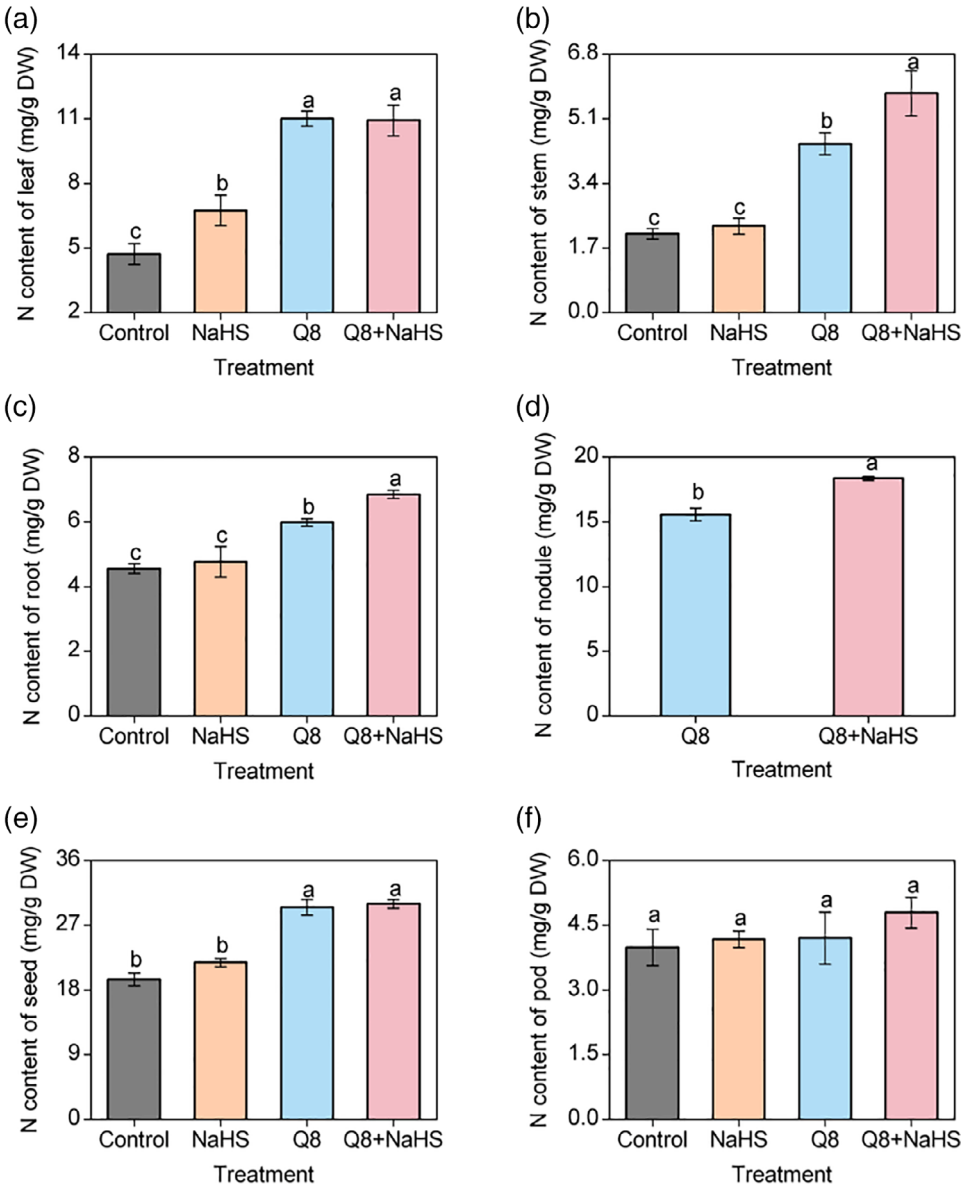

FIGURE 4 H_2S and rhizobia affected the N content in leaf (a), stem (b), root (c), nodule (d), seed (e), and pod (f) of soybean on the 47th day of reproductive growth, which was calculated in DW. Each value was the mean ± *SE*. Columns marked with different letters indicated significant differences, $P < .05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

3.2 | H_2S and rhizobia jointly affect the biomass, nodule numbers and fallen leaves in soybean, and the N content in different tissues during vegetative growth

Comparison of the shoot and root biomass in soybean clearly showed that H_2S and rhizobia markedly promoted the shoot biomass of the plant with an exposure time of N deficiency (RM GLM, F = 4.173, P = .028), whereas for the root biomass, this effect was not obvious (RM GLM, F = 2.940, P = .073, Figure 3a,b). The plants inoculated with rhizobia continued to produce and form nodules. Although the addition of H_2S tended to increase the number of nodules in the later growth stage, this effect was inconspicuous (RM GLM, F = 4.981, P = .067, Figure 3c). The number of fallen leaves under the different treatments all increased with time (Figure 3d), in which the deciduous rate was fast during the plant rapid growth period on the 19th-29th days, but it became slow during the steady growth period from the 29th to 40th days. In addition, only the Q8 and Q8 + NaHS treatments alleviated the senescence and withering of leaves compared with control during the later steady growth period (RM GLM, F = 4.185, P = .042). This was particularly true for the Q8 + NaHS treatment (RM GLM, F = 8.261, P = .021, Figure 3d).

Although the N content in leaves, stems, and roots of soybean tended to decrease overall, Q8 and Q8 + NaHS treatments significantly maintained the content of N compared with control during the late growth stage (RM GLM, F = 10.779, P = .010; F = 27.645, P = .000; F = 5.735, P = .025, Figure S2A–C). The N content in each leaf blade also declined to varying degrees with exposure time to N deficiency (Figure S2). In contrast, the chlorophyll content in L-1, L-2, and L-3 leaves decreased with time, whereas it increased in L-4 and L-5 leaves (Figure S1). Moreover, Q8 and Q8 + NaHS heightened the chlorophyll content in L-5 leaves (RM GLM, F = 7.394, P = .006) and the N contents in L-4 and L-5 leaves (RM GLM, F = 40.711, P = .000; F = 793.872, P = .000), particularly the Q8 + NaHS treatment (Figure S1D,E). In contrast, H_2S and rhizobia decreased the chlorophyll (RM GLM, F = 8.422, P = .000; F = 11.326, P = .000) and N contents (RM GLM, F = 4.047, P = .051; F = 22.27, P = .000) in L-1 and L-2 leaves (Figure S1A,B). The N content in fallen leaves decreased similarly to that of the growing tissue, but it was significantly reduced by Q8 and Q8 + NaHS (RM GLM, F = 15.951, P = .004, Figure S2D). A comparison of the two treatments inoculated with rhizobia indicated that the N content in nodules also decreased with time and was reduced by NaHS (RM GLM, F = 7.234, P = .055, Figure S2E). The NRE in leaves displayed a tendency to decrease initially and then increase, which was markedly enhanced by Q8 and Q8 + NaHS (RM GLM, F = 8.495, P = .018, Figure S2F).

3.3 | H_2S and rhizobia synergistically regulate the N content in different tissues and the yield parameters at maturity

To evaluate the longer term effects of H_2S and rhizobia on soybean under N deficiency, we performed additional experiments to determine when the reproductive harvest took place. We found that the N

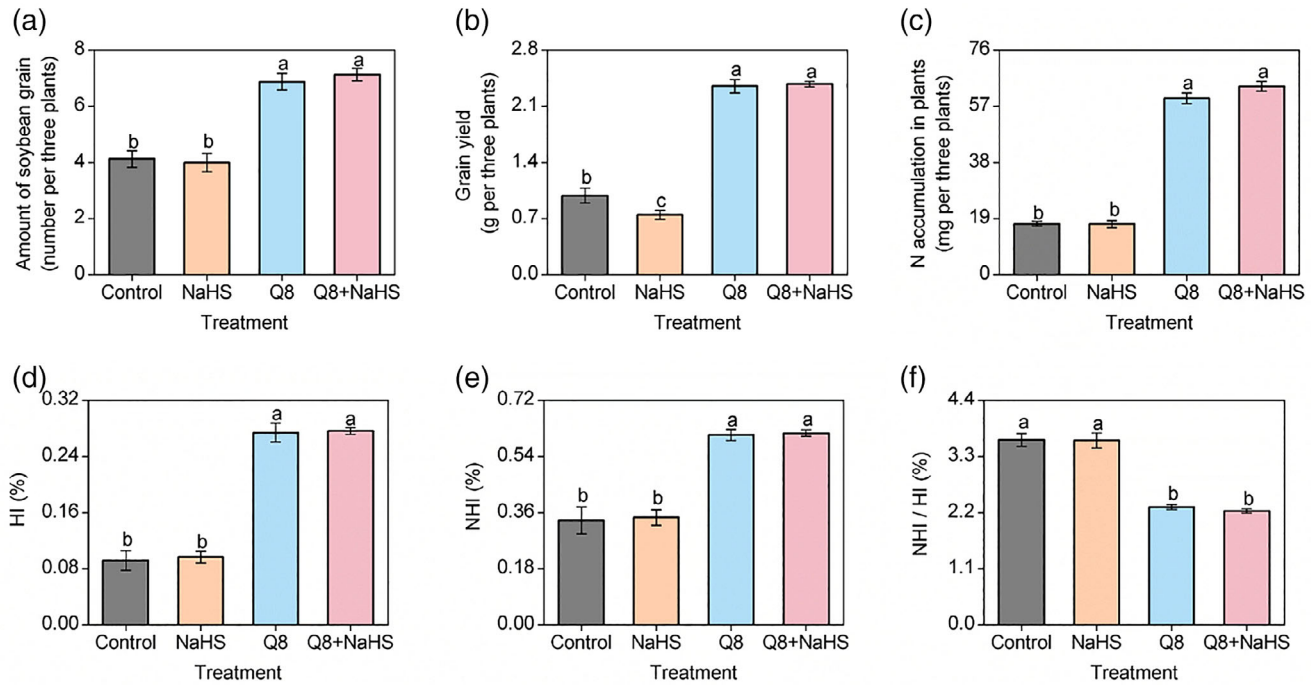

FIGURE 5 Evaluation of soybean grain number (a), grain yield (b), plant N accumulation (c), HI (d), NHI (e), and NHI/HI (f) on the 47th day of reproductive growth under H_2S and rhizobia application. Each value was the mean ± *SE*. Columns marked with different letters indicated significant differences, P < .05. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

content in plant tissues at maturity (on the 47th day) was increased by H_2S and rhizobia (Figure 4). Among them, H_2S and rhizobia significantly enhanced the N content in leaves (Figure 4a). In addition, the inoculation of rhizobia significantly advanced the N content in stems, roots, and soybean seeds (Figure 4b,c,e). Moreover, the synergistic regulation of H_2S and rhizobia generated a more remarkable increase in the N content in stems and roots than rhizobia alone (Figure 4b,c). This may be because H_2S promoted the increase in the N content in nodules in a remarkable manner (Figure 4d). However, only Q8 + NaHS treatment resulted in an increase in the N content in pods (Figure 4f). Therefore, soybean grain numbers and yield, and plant N accumulation at maturity were prominently enhanced by the inoculation of rhizobia (Figure 5a-c). Moreover, rhizobia exhibited significant symbiotic advantages in the improvement of HI and NHI when harvesting yield (Figure 5d,e). Not surprisingly, H_2S also advances this symbiotic function. Moreover, NUE (NHI/HI) in soybeans subjected to N deficiency was evidently reduced by rhizobia (Figure 5f). Because of limited nutrition and growth conditions, these yield parameters at maturity were relatively weak, which differ from the results of the field experiments.

3.4 | The activity of N metabolism enzymes in soybean is affected by H_2S and rhizobia

To explore the effects of H_2S and rhizobia on N assimilation and remobilization, the activity of key enzymes involved in N metabolism was determined. Although H_2S and rhizobia significantly reduced NR activity in roots, only Q8 + NaHS lowered it in leaves (Figure 6a). Compared with control, only NaHS resulted in a striking increase in NiR activity in leaves. However, H_2S and rhizobia distinctly decreased NiR activity in roots (Figure 6b). GS activity in leaves was markedly reduced under Q8 + NaHS compared with the other treatments. Interestingly, H_2S and rhizobia caused an improvement in GS activity in roots (Figure 6c). Additionally, GOGAT activity in leaves decreased

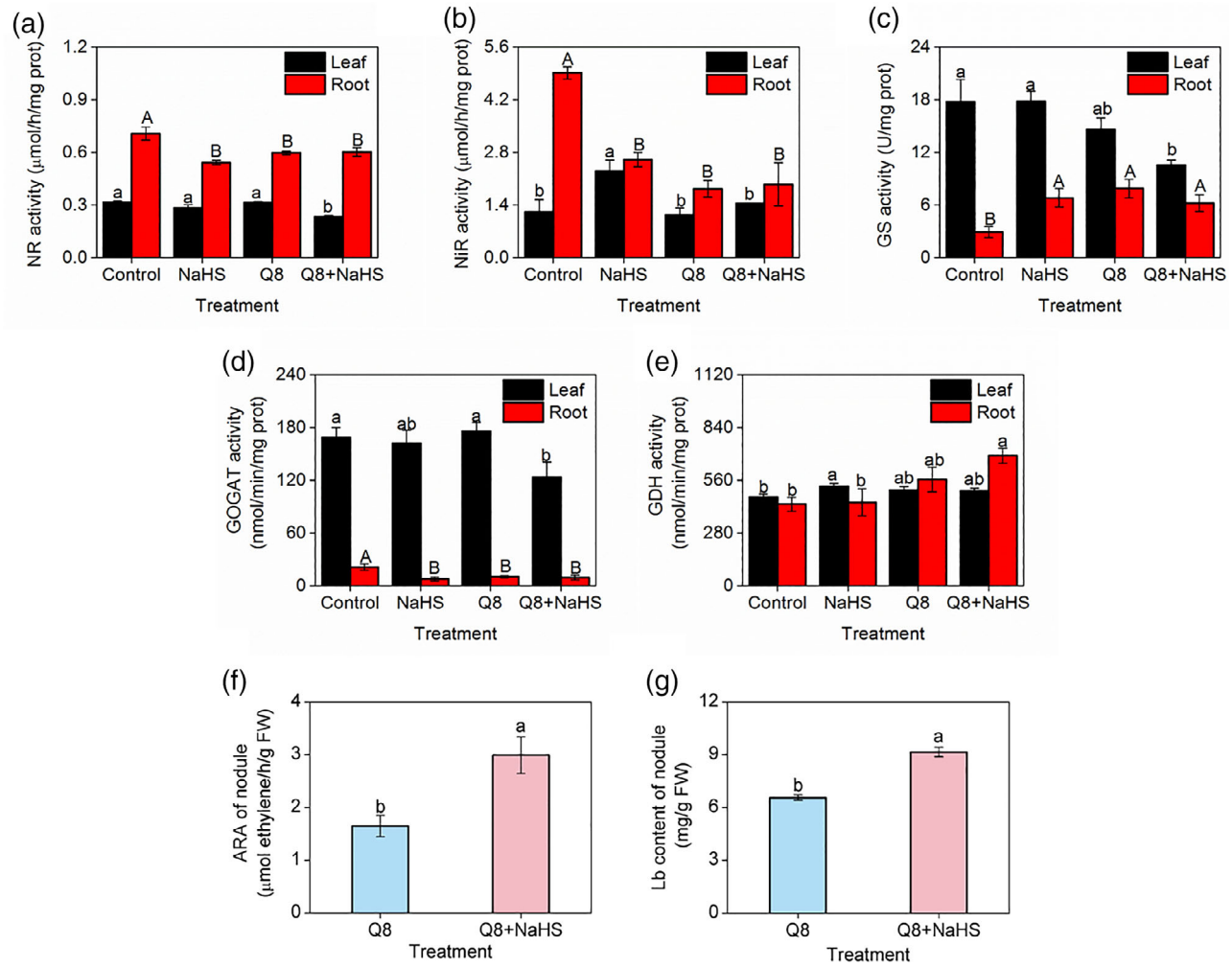

FIGURE 6 H_2S and rhizobia affected the activity of enzymes associated with N metabolism, which include NR (a), NiR (b), GS (c), GOGAT (d), and GDH (e) in L-3 leaves and entire roots from the 26th day. Furthermore, H_2S enhanced ARA (f) and Lb content (g) of nodules in soybeans on the 26th day post inoculation with rhizobia. Each value was the mean ± *SE*. Columns marked with different letters indicated significant differences, $P < .05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

significantly under the treatment of Q8 + NaHS, and in roots, it was also notably reduced by both H_2S and rhizobia (Figure 6d). Surprisingly, both H_2S and rhizobia enhanced GDH activity in leaves and roots, which was the most exceptional under NaHS or Q8 + NaHS treatment, respectively (Figure 6e). In addition, a comparison of the two treatments inoculated with rhizobia indicated that H_2S promoted symbiosis between soybean and rhizobia by prominently enhancing the ARA and Lb content in nodules (Figure 6f,g).

3.5 | H_2S and rhizobia regulate the abundances of important proteins involved in N metabolism

The protein abundance of NR, Rubisco LSU, and GS1/2 in leaves was higher than that in roots of soybean in all of the treatments (Figure 7a,b). The NR antibody signal detected in leaves was observably higher under NaHS and Q8 + NaHS treatments than that under control and Q8 treatments (Figure 7a,c). Compared with control, H_2S behaved synergistically with rhizobia to significantly decrease the level of NR protein in roots, which was increased under treatment with H_2S or rhizobia alone (Figure 7b,c). H_2S and rhizobia enhanced the abundance of Rubisco LSU protein in leaves, particularly under Q8 and Q8 + NaHS treatments (Figure 7a,d). Additionally, the synergistic effect of H_2S and rhizobia significantly increased the abundance of GS1/2 protein in soybean leaves, which controlled the uptake and assimilation of N in plants (Figure 7a,e). Furthermore, H_2S and rhizobia substantially reduced the level of GS1/2 protein in roots (Figure 7b,e).

3.6 | H_2S and rhizobia coregulate the expression of genes involved in soybean N metabolism

In addition to assessing the enzymes and proteins involved in N metabolism, related gene expression was also detected, which regulates the protein modification and enzyme function in the plant. The transcription level of *GmNR* in leaves was heightened by H_2S and rhizobia, and the *GmNR* expression under Q8 and Q8 + NaHS treatments was approximately twofold that of control (Figure 8a). However, H_2S and rhizobia significantly downregulated the expression level of *GmNR* in roots (Figure 8a). Moreover, the application of H_2S and rhizobia distinctly reduced the expression abundance of *GmNiR* in leaves, but the synergistic effect of H_2S and rhizobia prominently upregulated the expression level of *GmNiR* in roots (Figure 8b). The expression abundance of *GmGS1* in leaves was reinforced by NaHS

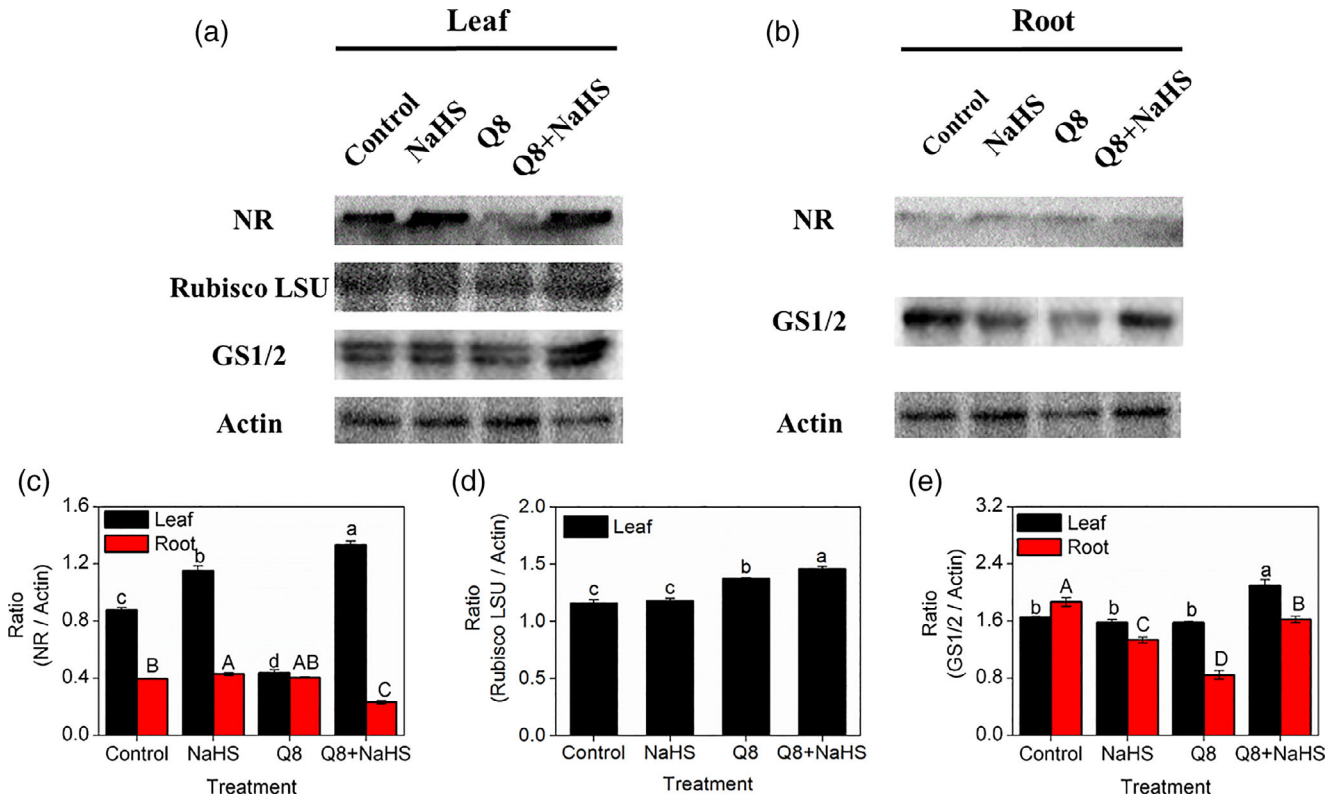

FIGURE 7 Western blot analysis of NR, Rubisco LSU, and GS1/2 protein involved in N metabolism in soybean L-3 leaves and entire roots from the 26th day (a). The relative expression levels were shown as the ratio of NR:β-actin (b), Rubisco LSU:β-actin (c), and GS1/2:β-actin (d) using the ImageJ software. Data were expressed as the mean ± *SE*. Columns marked with different letters indicated significant differences at $P < .05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

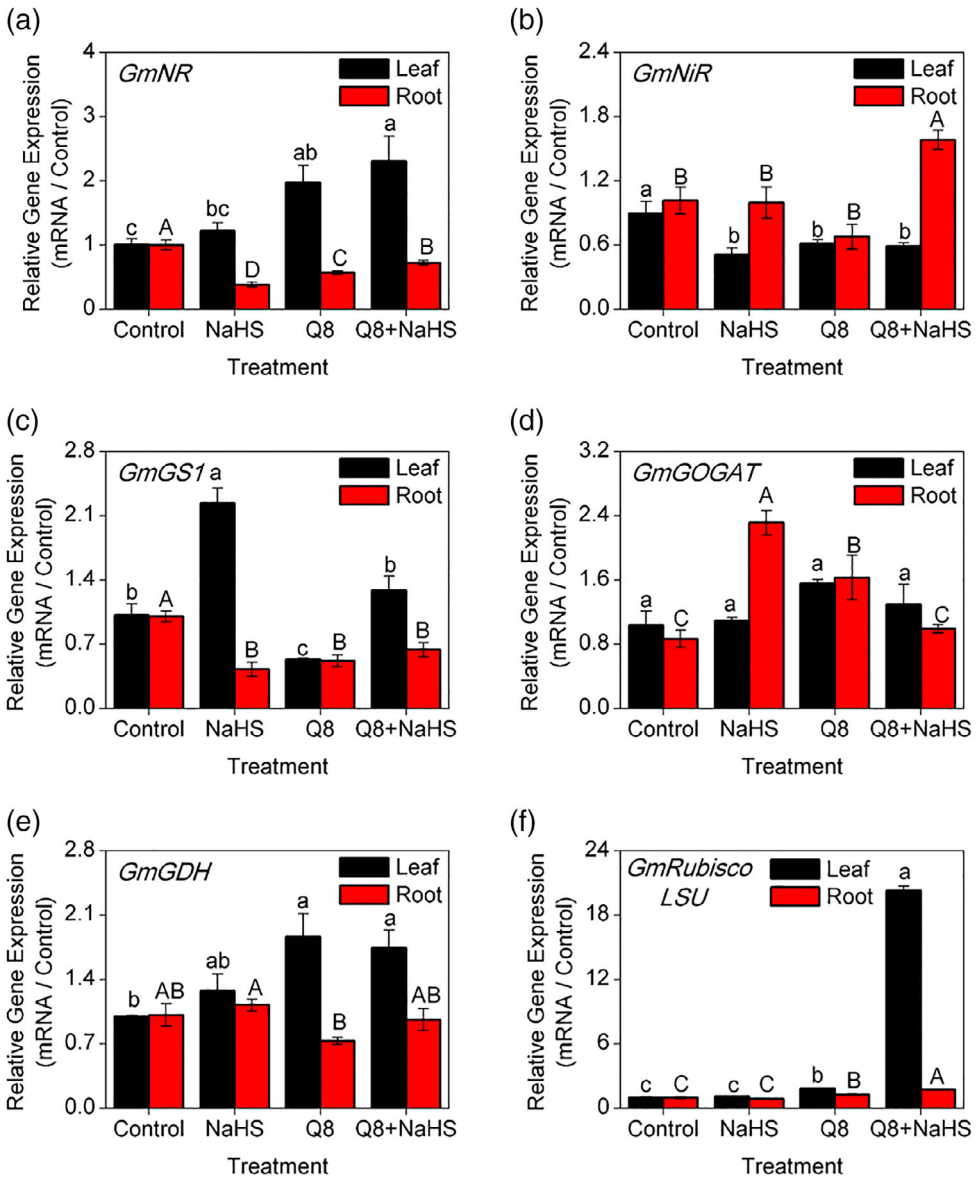

FIGURE 8 Gene expression of *GmNR* (a), *GmNiR* (b), *GmGS1* (c), *GmGOGAT* (d), *GmGDH* (e), and *GmRubisco LSU* (f) encoding N metabolism enzymes in soybean L-3 leaves and entire roots from the 26th day under different treatments. The relative mRNA levels of each gene were normalized to Zmactin2 mRNA expression. Data were expressed as the mean ± *SE* of three replicates. Columns marked with different letters indicated significant differences at $P < .05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

and Q8 + NaHS treatments, but both H_2S and rhizobia apparently decreased the expression of *GmGS1* in roots, similar to the expression of *GmNR* (Figure 8c). There was no apparent difference in the gene transcription of *GmGOGAT* in leaves under different treatments, although *GmGOGAT* expression in roots was notably enhanced under NaHS or Q8 treatment alone, particularly under NaHS treatment (Figure 8d). H_2S and rhizobia upregulated the relative expression of *GmGDH* in leaves, and higher expression was observed under Q8 and Q8 + NaHS treatments. However, the expression of *GmGDH* in roots did not distinctly change following treatment with H_2S and rhizobia (Figure 8e). Although the separate addition of H_2S had no significant effect, treatment with rhizobia inoculum resulted in a significant increase in the expression of *GmRubisco LSU* in roots and leaves. In particular, the synergistic regulation of H_2S and rhizobia was the most effective, particularly in leaves (Figure 8f).

3.7 | The expression level of *SAG*s is regulated by H_2S and rhizobia

H_2S and rhizobia significantly reduced the expression level of *GmCysP1* in leaves compared with control, whereas its expression in roots was markedly downregulated under Q8 and Q8 + NaHS treatments (Figure 9a). However, the expression of *GmUBE2* and *GmUBC4* genes in leaves was significantly enhanced under Q8 and Q8 + NaHS treatments that contained rhizobia (Figure 9c,d). Moreover, the synergistic effect of H_2S and rhizobia distinctly upregulated the expression of *GmUBE1* in roots (Figure 9b). The expression level of *GmUBC4* in roots was significantly enhanced by the addition of H_2S and rhizobia, which was highest under treatment with NaHS alone (Figure 9d). However, both H_2S and rhizobia significantly decreased the expression level of *GmUBE2* in roots (Figure 9c). Additionally, H_2S and

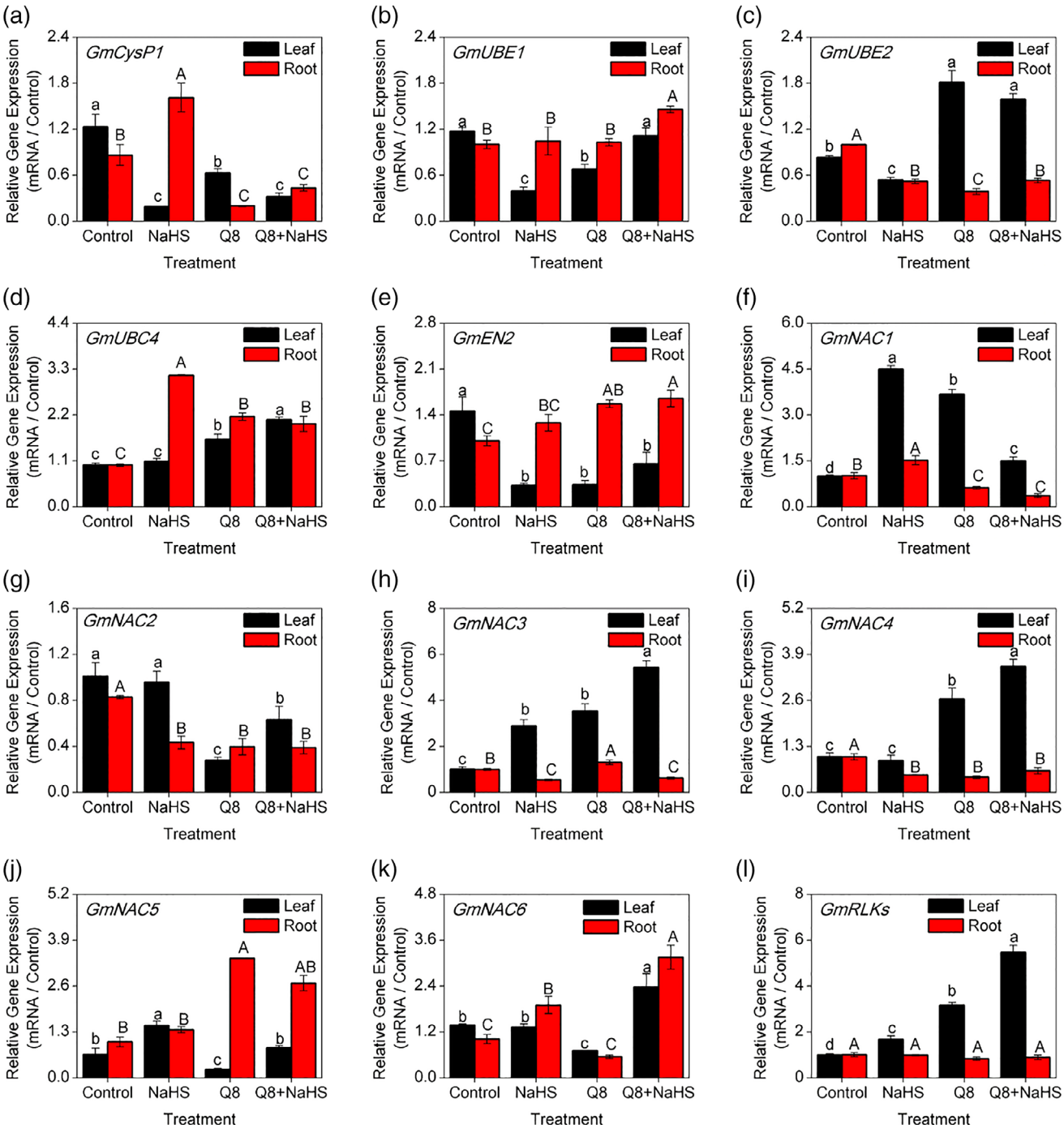

FIGURE 9 Expressions of *SAG*s in soybean L-3 leaves and entire roots from the 26th day under different treatments were detected, including *GmCysP1*(a), *GmUBE1* (b), *GmUBE2* (c), *GmUBC4* (d), *GmEN2* (e), *GmNAC1* (f), *GmNAC2* (g), *GmNAC3* (h), *GmNAC4* (i), *GmNAC5* (j), *GmNAC6* (k), and *GmRLKs* (l). The relative mRNA levels of each gene were normalized to Zmactin2 mRNA expression. Data were expressed as the mean ± *SE* of three replicates. Columns marked with different letters indicated the difference at $P < .05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS

rhizobia prominently reduced the transcriptional level of *GmEN2* in leaves compared with control but upregulated its expression in roots, which was prominently reinforced under Q8 + NaHS treatment (Figure 9e).

H_2S and rhizobia treatment considerably strengthened the transcriptional levels of *GmNAC1* and *GmNAC3* in leaves (Figure 9f,h). The difference was that the expression level of *GmNAC1* in leaves treated with NaHS or Q8 alone was twice as high as that of Q8 + NaHS treatment, whereas *GmNAC3* expression in leaves was the most prominent under the combined action of H_2S and rhizobia (Figure 9f,h). Additionally, Q8 and Q8 + NaHS treatments significantly weakened the expression level of *GmNAC2* in leaves, but the transcription of

GmNAC4 in leaves was distinctly increased under these two treatments (Figure 9g,i). The gene expression of *GmNAC5* in leaves was improved by treatment with NaHS alone, whereas the expression abundance of *GmNAC6* in leaves was only notably improved by the synergy of H_2S and rhizobia (Figure 9j,k). In the soybean roots, the expression levels of *GmNAC2* and *GmNAC4* were severely downregulated by H_2S and rhizobia, but the gene expression of *GmNAC5* was reinforced under the treatment inoculated with rhizobia (Figure 9g,i,j). The transcriptional level of *GmNAC6* in roots was strengthened by the treatment containing H_2S, particularly under Q8 + NaHS (Figure 9k). The application of H_2S and rhizobia boosted the transcriptional level of *GmRLKs* in leaves, which was the highest due to the synergy of H_2S and rhizobia (Figure 9l).

4 | DISCUSSION

This experiment focused on the effects of H_2S and rhizobia on the biomass, nodulation, N content, leaf senescence and NRE in soybeans during vegetative growth, and the N content in different tissues, grain yield, and NUE at the mature period. Moreover, we investigated whether H_2S and rhizobia regulate the N metabolism-related enzyme activities, protein and gene expression, and expression of *SAG*s in leaves and roots when N metabolism and remobilization are vigorous in soybeans. The process by which organic N is recycled and exported to young leaves and seeds is well known to be an important determinant of plant yield (Guiboileau et al., 2012). This process is particularly true in cereal crops such as wheat and rice, because a necessary percentage of the grain N is derived from the remobilization of N stored in the vegetative tissues before flowering (Kong et al., 2016). Furthermore, understanding N accumulation in leaves during flowering and N regeneration in leaves after flowering may have a specific value for breeding programmes, aiming to optimize senescence duration and intensify N fertilizer utilization and NUE (Gaju et al., 2014). Our results demonstrated that in indoor planting experiments, H_2S and rhizobia could synergistically facilitate the N assimilation and remobilization through regulating N metabolism and the transcriptional levels of *SAG*s during N deficiency-induced senescence, ultimately enhancing the biomass, plant N accumulation, and grain yield in soybeans (Figure S3). Moreover, our experiments may provide a theoretical and scientific basis for future field experiments.

4.1 | H_2S and rhizobia jointly regulate the growth, N content in different tissues, and yield parameters in soybeans

In this study, H_2S and rhizobia observably improved the growth phenotype of shoots and roots in soybean under N deficiency (Figure 2a, b). Abundant amounts of research have also recorded the crucial effects of H_2S on plant growth under abiotic stress. For example, H_2S significantly promoted growth conditions, including shoot height, root length and biomass in plants under Al toxicity (Qian et al., 2013), high temperature (Zhou, Wang, Ye, & Li, 2018), and Pb stress (Chen et al., 2018), whereas rhizobia can establish symbiosis with legumes by forming nodules, which are used to absorb gaseous N and direct it as a nutrient for legumes. Therefore, treatment with rhizobia not only increased the number and dry weight of nodules but also the biomass and N content in plants (Khaitov, 2018; Samudin & Kuswantoro, 2018). Consistently, rhizobia clearly enhanced nodule formation (Figures 2 and 3). Furthermore, convincing data demonstrated that H_2S and rhizobia markedly increased the shoot biomass in soybean under N deficiency (Figure 3a). Additionally, as a signalling molecule, NO prominently alleviated the senescence induced by phytohormone and salt stress (Hung & Kao, 2003, 2004; Kong, Xie, Hu, Feng, & Li, 2016). In this study, from the leaf phenotype, H_2S and rhizobia effectively relieved the senescence of higher leaves in the corresponding development stage induced by N deficiency (Figure 2c). Additionally, senescence-induced leaf yellowing was obviously suppressed by H_2S via the activation of key energy metabolic enzymes and the inhibition of chlorophyll degradation (Li et al., 2017; Wei et al., 2017). This explains why the amount of fallen leaves recorded was decreased by H_2S and rhizobia (Figure 3d). Simultaneously, the interaction of H_2S and rhizobia more effectively enhanced nodule numbers and shoot biomass and alleviated N deficiency-induced leaf senescence (Figures 2 and 3), suggesting that H_2S resulted in an improvement in a rhizobia-soybean symbiont. This mechanism appears to be similar to the manner in which presoaking wheat seeds in a solution of H_2O_2 strengthened the acceleration of N-fixing snails on plants, generating a greater fresh and dry weight and higher seed number and grain yield (Jafariyan & Zarea, 2016).

Under N deficiency, nodules will form that will provide N to plants through BNF (Stougaard, 2000). Ammonia, as the primary metabolic product of BNF, is converted to urea for long-distance transport, which will then provide organic N resources for the construction of N-containing biomolecules in legumes (Shelp & Ireland, 1985). In this study, the N contents in leaves, stems, roots, fallen leaves, and nodules decreased overall due to N deficiency, but they were considerably strengthened by treatment with rhizobia (Figure S2A-E). Similarly, rhizobia alleviated the effects of drought stress on the biomass and N content of legume seedlings (Pereyra, Hartmann, Michalzik, Ziegler, & Trumbore, 2015). The higher N content in leaves treated by Q8 and Q8 + NaHS might well be primarily due to the high N content in L-4 and L-5 leaves (Figure S1D,E). Interestingly, the N contents in lower and fallen leaves were all decreased by H_2S and rhizobia (Figures 4d and S1A,B). Chlorophyll content in different leaf blades also displayed a similar tendency for N is required for chlorophyll biosynthesis, just as H_2S controlled chloroplast biosynthesis and N metabolism by acting on photosynthetic mechanisms and N metabolism-related gene transcription (Rizwan et al., 2019; Zhang et al., 2010). One possible reason for these unusual findings was that the chlorophyll and N contents in senescent leaves were gradually reduced for the development of new leaves or organs during senescence by N remobilization caused by proteolysis, which was consistent with previous studies (Masclauxdaubresse, Reisdorfcren, & Orsel, 2010; Poret et al., 2017; Zhang et al., 2015). This also corresponded

to the distinct augmentation of NRE in leaves (Figure S2F). The improvement in N recycling during oilseed rape leaf senescence caused by N deficiency was dependent on a high NRE, which was associated with an increase or induction of senescence-associated protease activity (Poret et al., 2019). Moreover, the synergistic effect of H_2S and rhizobia on the increase of N content in higher leaves was more conspicuous (Figure S1D,E), which could be explained as H_2S contributed to the continuous transport and accumulation of N in a soybean-rhizobia symbiont. Additionally, H_2S reduced the N content in nodules (Figure S2E), suggesting that H_2S might encourage N transfer from the nodules to other tissues during N deficiency-induced senescence. Indeed, N remobilization will occur during senescence for almost all species (Maillard et al., 2015). Therefore, H_2S and rhizobia might aid in the absorption and remobilization of N by promoting the maximum utilization of N in aging leaves and nodules during soybean senescence caused by N deficiency, ultimately facilitating plant growth and alleviating leaf senescence.

Precisely, because H_2S and rhizobia alleviated the inhibition of N deficiency on vegetative growth in soybean, the N contents in leaves, stems, roots, seeds, and pods were strengthened at maturity (Figure 4), which eventually caused the improvement in grain yield, plant N accumulation, HI, and NHI (Figure 6). Consistent with our findings, H_2S enhanced the N content in plants exposed to high Zn by regulating an antioxidant defence, resulting in an increase in yield (Kaya, Ashraf, & Akram, 2018). *Rhizobium* inoculation significantly increased N fixation efficiency, pod number, and grain yield in legumes (Gresta, Trostle, Sortino, Santonoceto, & Avola, 2019; Pereira, Mucha, Goncalves, Bacelar, & Marques, 2019). Therefore, the synergistic effects of H_2S and rhizobia produced optimal results, which might be drawn from the elevated N content in nodules (Figure 4d). However, the NUE (NHI/HI) was evidently reduced by rhizobia (Figure 5f). This could take place because without rhizobia, the plants were faced with severe stress induced by N deficiency, which produces the highest recycling of N in soybeans for high NUE. However, rhizobia inoculation could mitigate the effects of N deficiency on soybean via forming nodules and fixing gaseous N, accounting for the reduction in NUE. Therefore, H_2S played a pivotal role in the vegetative and reproductive growth of soybean.

4.2 | H_2S and rhizobia synergistically regulate N metabolism by enhancing N contents and related enzyme activity, protein, and gene expression in soybean

N metabolism includes N absorption, assimilation, and remobilization, which plays an essential role in plants under low N conditions (Kant, Bi, & Rothstein, 2011). In this study, H_2S and rhizobia affected N assimilation and remobilization by regulating the enzyme activities, protein abundances, and gene expressions associated with N metabolism during N deficiency-induced soybean senescence. For example, NR is essential for N metabolism in plants (Di Martino, Palumbo, Vitullo, Di Santo, & Fuggi, 2018). H_2O_2 promotes N assimilation and photosynthetic NUE by increasing the NR activity in mustard under Ni stress (Khan, Khan, Masood, Per, & Asgher, 2016). Moreover, NO increased the N absorption capacity by intensifying NR activity and then mediating inorganic N absorption, such as ammonium and nitrate, at the transcriptional level in rice (Sun et al., 2015). In contrast, H_2S and rhizobia synergistically reduced NR activity in soybean leaves and roots during senescence (Figure 6a). Primary N assimilation enzymes, including NR, NiR, GS, and GOGAT, were downregulated as with leaf aging, whereas N regenerating enzymes, such as GDH, were upregulated (Masclaux, Valadier, Brugiere, Morot-Gaudry, & Hirel, 2000). These results were consistent with those of Arabidopsis leaves, as NR and GS activity decreased and GDH activity strengthened with aging (Diaz et al., 2008). However, the expression of NR proteins and genes in leaves was precisely opposite to the change in NR activity. H_2S and rhizobia interactively reinforced the protein and gene expression of NR in leaves, whereas they were reduced in roots (Figures 7c and 8a). Compared with NH^{4+} treatment, partial nitrate nutrition treatment significantly enhanced the NR activity in rice cultivars, while downregulating the gene expression of *NIA1* but increasing the expression level of *NIA2* (Sun et al., 2015). Therefore, there may be a complex regulatory mechanism between enzymatic functional activity and gene expression that involves the transcriptional regulation of many factors, suggesting that protein function may not only be regulated by a single factor and that there may be feedback regulation between the protein and enzyme (Stitt & Gibon, 2014). Thus, H_2S and rhizobia might directly decrease NR activity in leaves during senescence, which, in turn, causes the upregulation of transcriptional level and protein abundance of NR. In contrast, the gene expression and protein level of NR in roots were decreased by the interaction of H_2S and rhizobia, ultimately causing a reduction in NR functional activity. Similarly, N fertilizer application increased the transcriptional level and enzyme activity of NR in roots by 220.0% and 5.0%, respectively, and that in leaves by 51.5% and 13.8%, respectively (Liao et al., 2019). In addition, it is possible that the activity or molecular regulation of different N metabolic enzymes differed in shoots and roots under salt stress (Teh, Shaharuddin, Ho, & Mahmood, 2016). Because NiR is also a primary N assimilation enzyme, its activity in leaves during senescence was also at a low level, which may be due to a decrease in the transcriptional level of *GmNiR*. However, H_2S and rhizobia acted directly on the NiR enzyme in roots, distinctly reducing its activity, whereas positive feedback regulated *GmNiR* expression in roots (Figures 6b and 8b). The expression of NiR and GS2 in plant leaves was inhibited under almost all stress conditions during natural aging (Pageau, Reisdorf-Cren, Morot-Gaudry, & Masclaux-Daubresse, 2006).

GS is not only a key enzyme in N assimilation and remobilization but also a core multifunctional enzyme because it plays a crucial role in ammonium fixation (Veliz, Roberts, Criado, & Caputo, 2017). Similar to the change in NR, the activity and protein abundance of GS were differentially expressed in leaves and roots. For instance, H_2S acted synergistically with rhizobia to significantly decrease GS activity in leaves, which could cause an increase in GS protein abundance and *GmGS1* expression. In contrast, GS activity in roots during senescence may be markedly upregulated by the direct regulation of H_2S and rhizobia, which stimulated a decrease in GS protein accumulation and

GmGS1 expression in response (Figures 6c, 7e, and 8c). However, GS primarily includes cytosolic GS1, which is mainly involved in the transport of stored N sources during seed germination and the transfer and reuse of N sources during leaf senescence, and chloroplast type GS2, which is mainly involved in the assimilation of ammonia produced by photorespiration and nitrate reduction. Therefore, the enzymes that play a major role in leaves or roots during senescence may be different. This could be comparable with the situation in *Helianthus annuus* leaves in which elevated CO_2 reduced the activity of the N assimilation enzymes (NR and GS), increased the deamination activity of GDH, greatly improved the transcriptional level of GS1, and decreased the expression of GS2, which promoted the mobilization of N in leaves during senescence (De la Mata, De la Haba, Alamillo, Pineda, & Agueera, 2013). Moreover, during the reproductive phase, elevated CO_2 promoted nitrate assimilation in leaves by enhancing NR activity in wheat, whereas in the late reproductive stage, N assimilation was inhibited due to lower *GS2* gene expression and lower GS activity (Sailo, Verma, Pandey, & Jain, 2013).

NO reinforced the enzyme activity and gene expression of Fd-GOGAT in wheat under low N conditions (Balotf et al., 2018). However, enzyme activity, protein abundance, and the transcriptional level of Fd-GOGAT in leaves were declining significantly when the leaves became senescent (Masclaux et al., 2000; Zeng et al., 2017). Because Fd-GOGAT accounts for up to 96% of the total GOGAT in leaves (Coschigano, Melo-Oliveira, Lim, & Coruzzi, 1998), the total GOGAT activity was committed to assess Fd-GOGAT activity. Therefore, downregulation of the total GOGAT activity in leaves and roots during soybean senescence was also promoted by H_2S, which might simultaneously stimulate an increase in *GmGOGAT* (NADH-dependent) expression to assimilate ammonium (Figures 6d and 8d). Additionally, as described above, the N remobilization enzyme GDH is also induced during the soybean aging process. H_2S and rhizobia were likely to enhance GDH activity in leaves by promoting the accumulation of *GmGDH* mRNA during senescence and directly inducing the augmentation of GDH activity in roots (Figures 6e and 8e), which aids in the remobilization and transport of N. Similarly, to the manner in which the accumulation of *GS1* and *GDH* mRNA increased during aging (Pageau et al., 2006), elevated CO_2 greatly strengthened the deamination activity of GDH, which in turn promoted the remobilization of N in leaves during senescence (De la Mata et al., 2013). Gibberellin (GA) slowed the senescence of *Paris polyphylla* while delaying the decline in the activity of N remobilization enzymes, including GS1 and GDH, as well as the reduction of N, chlorophyll, and soluble protein contents (Yu, Fan, Wei, Yu, & Li, 2012). Additionally, H_2S significantly enhanced the content of ARA and Lb in nodules in rhizobia-inoculated plants (Figure 6f,g), indicating that H_2S promoted N metabolism and remobilization of symbiotic systems by enhancing the N fixation capacity of nodules. This was consistent with our previous findings that H_2S effectively facilitated the nodulation and N fixation of the soybean-rhizobia symbiotic system (Zou et al., 2019). As is well known, Rubisco is crucial for photosynthesis (Bloom & Lancaster, 2018). Additionally, with concentrations up to 50% of the total soluble protein, Rubisco is the most abundant protein in plants and is thought to be an important N storage protein, thus playing a central role in N metabolism and regulating N dynamics in leaves (Ishida, Shimizu, Makino, & Mae, 1998; Ullmann-Zeunert et al., 2012). Interestingly, we detected that H_2S-rhizobia synergism markedly enhanced Rubisco LSU blots in leaves along with its gene expression in soybean leaves and roots (Figures 7d and 8f). In addition, a deficiency of N not only limited the formation and stability of chloroplast proteins in the matrix and thylakoids but also affected the activities of enzymes such as Rubisco, NR, and GS, eventually increasing proteolytic activity and N mobilization to young developmental tissues during aging (Busheva et al., 1991). Thus, H_2S might have effectively promoted N storage in the Rubisco protein for better remobilization. Therefore, H_2S and rhizobia promoted the absorption, assimilation, and remobilization of N by regulating the enzymatic activity, transcriptional level and protein abundance of key N metabolic enzyme in leaves and roots, and the content of ARA and Lb in nodules during N deficiency-induced soybean senescence.

4.3 | H_2S interacts with rhizobia to promote the remobilization of N nutrition in soybeans by regulating the gene expression of *SAG*s

Plant senescence is a process in which cells are controlled by internal and external factors to degrade and eventually cause the death of tissues. Physiological and biochemical changes that occur during aging are not identical to those of normal growth stages. Moreover, senescence is a highly regulated and tightly controlled process involving the upregulation and downregulation of many *SAG*s (Yang et al., 2018; Zheng et al., 2016). For example, reactive oxygen species and NO played an important role in the induction of primary leaf senescence by regulating the expression of *SAG*s in *Litchi chinensis* (Yang et al., 2018). H_2S alleviated postharvest senescence in plants by regulating antioxidant defences and the expression of *SAG*s, including chlorophyll degradation and cysteine proteases (Li, Hu, et al., 2014; Zheng et al., 2016). Moreover, H_2S delayed leaf senescence in *Arabidopsis* under drought stress by regulating the expression of *SAG12* (Jin et al., 2018). Despite this, it is unclear whether H_2S is involved in the regulation of the senescence process in the soybean-rhizobia symbiont. Therefore, the expression abundance of *SAG*s was measured, including the genes involved in protein degradation, nucleic acid degradation, transcription factors, and receptor-like protein kinases.

The auxin response factor (*GmARF10*) positively regulated soybean leaf senescence by affecting the transcription of the senescence-associated marker *GmCysP1* (Li, Zeng, Zhang, & Zhao, 2014). We also observed that the synergy of H_2S with rhizobia obviously reduced the expression of *GmCysP1* in leaves and roots during senescence (Figure 9a). During plant senescence, various pathways may lead to the degradation of proteins and other macromolecules (Graaff & Kunze, 2006). Ubiquitination, as an effective means of protein degradation, refers to the process of specifically modifying a target protein through the action of a series of specific enzymes, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating

enzyme (E2), and ubiquitin-protein ligase (E3; Hellmann & Estelle, 2002). Numerous genes involved in the ubiquitination pathway were upregulated during leaf senescence in *Arabidopsis* (Breeze et al., 2011). The expression of *GmUBE1*, *GmUBE2*, and *GmUBC4* that encode ubiquitination in leaves and roots were upregulated or downregulated by H_2S and rhizobia (Figure 9b-d). Moreover, during N remobilization, plants could also induce significant protein degradation, such as those engaged in chloroplast degradation, autophagy, and the ubiquitin-26S proteasome pathway (Liu, Wu, Yang, Liu, & Shen, 2008). The amino acids released from protein degradation were then remobilized and served as the primary form of N, which was required for maintaining normal plant development under low N conditions (Sanders et al., 2010). Therefore, H_2S and rhizobia might promote the effective utilization and remobilization of N in aging tissues by regulating protein degradation during senescence. The senescence and wilting of pea leaves were accompanied by the DNA degradation, which was closely related to the increase in endonuclease activity (Aleksandrushkina, Kof, Seredina, Borzov, & Vanyushin, 2008). In this study, H_2S and rhizobia regulated the degradation of nucleic acids in plants by reducing the expression of *GmEN2* in leaves and upregulating the transcription of *GmEN2* in roots (Figure 9e). During protein and nucleic acid degradation, nutrients that accumulated in aging tissues, particularly N, are remobilized to the growing vegetative or reproductive organs (Lim et al., 2007). Therefore, macromolecules degradation might be controlled by H_2S and rhizobia during N deficiency-induced senescence, which triggered the mobilization and recycling of N in soybean.

TFs associated with aging are also indispensable in the coordinated control of shoot senescence and effective N remobilization. As a TF family, the NAC gene family plays a conserved role in plant leaf senescence (Hollmann, Gregersen, & Krupinska, 2014; Yang et al., 2015). For example, the expression of the transcription factor *HvNAC026* and serine-type protease *SCPL51* was upregulated during senescence in barley flag leaves under standard N supply (Hollmann et al., 2014). The regulatory function of NAC in soybean leaf senescence has been confirmed. During soybean senescence, H_2S and rhizobia enhanced the transcription of the *GmNAC1*, *GmNAC3*, *GmNAC4*, *GmNAC5*, and *GmNAC6* genes and downregulated the expression of *GmNAC2* in leaves (Figure 9f-k). As previously reported, *PvNAC1* and *PvNAC2* were expected to improve nutrient use efficiency of switchgrass by genetic manipulation (Yang et al., 2015). Simultaneously, the functional grouping of *SAG*s in switchgrass indicated that transcription and protein degradation play a crucial role in regulating plant senescence. More importantly, the coexpression networks predicted that the NAC TFs and other TF family members played an essential role in coordinating carbohydrate, N, and lipid metabolism; protein modification/degradation; and transport processes during senescence (Yang et al., 2016). In this study, H_2S and rhizobia upregulated or downregulated NAC TFs expression in roots while regulating their expression in leaves. In addition, the synergetic effects of H_2S and rhizobia were highlighted more, suggesting that H_2S could coordinate with rhizobia to regulate senescence in soybean caused by N deficiency by affecting the expression of NAC TFs in leaves and roots. Receptor-like protein kinases are deemed to act as important cell surface receptors and are involved in a variety of biological processes, such as aging and stress responses (Zheng et al., 2018). Recent research revealed that the expression of *RLKs* was upregulated during leaf senescence in *Arabidopsis* (Li et al., 2019). Consistent with our findings, H_2S and rhizobia prominently enhanced the gene expression of *GmRLKs* in soybean leaves during senescence (Figure 9l). *Arabidopsis* experienced a decrease in total N, free amino acid, and soluble protein contents due to leaf aging and the increased transcription of N remobilization markers such as cytosolic GS, GDH, and CND41-like protease (Diaz et al., 2008). Therefore, we hypothesized that H_2S and rhizobia could synergistically mediate N assimilation and remobilization in soybeans by regulating the activity of N-metabolizing enzymes, their corresponding protein, and gene expression and the expression of *SAG*s during senescence.

5 | CONCLUSION

Can hydrogen sulfide (H_2S), as an important signalling molecule, play a pivotal role in soybean-rhizobia symbionts? Here, we demonstrated that H_2S could act synergistically with rhizobia to accelerate N assimilation and remobilization by regulation of the expression of *SAG*s during N deficiency-induced senescence. Ultimately, H_2S enhanced the biomass, N contents, and yield in soybean during vegetative and reproductive growth, presumably through interactions with rhizobia under conditions of N deficiency.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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