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Nitric oxide (NO) regulates the expression of single-domain cystatins in *Glycine max* (soybean)

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Abstract

Plant cystatins inhibit cysteine proteases and are important in regulating plant development and plant responses to biotic and abiotic stress. Furthermore, nitric oxide plays a signaling role in regulating plant responses to developmental processes, biotic and abiotic stress. With the aim of determining if nitric oxide is involved in the regulation of the expression of single-domain cystatins, we have identified single-domain cystatin genes in soybean (*Glycine max* cv. PAN626) on the basis of sequence homology to a nitric oxide-inducible cystatin (*AtCYS1*, At5g12140) from *Arabidopsis thaliana*. Analysis of the expression of the four cystatin genes revealed that transcript levels of these cystatins are altered by exogenously applied nitric oxide and a nitric oxide synthase inhibitor. Altered expression of these cystatins by nitric oxide and the nitric oxide synthase inhibitor implies that changes in cellular nitric oxide content, which have previously been shown to occur during development and/or biotic and abiotic stress, influence soybean physiological processes that are regulated by cysteine proteases. Recombinant protein expression of one of the cystatins (as a glutathione-S-transferase fusion protein) showed that it has inhibitory activity against the model cysteine protease papain but not the model serine protease trypsin and that it inhibits caspase-like activity in soybean nodule extracts. This serves as evidence that these four plant cystatins are functional cysteine protease inhibitors because of their high degree of primary sequence identity. It also indicates that the single-domain cystatins regulate caspase-like activity, which is known to participate in plant responses to biotic and abiotic stress. We thus conclude that nitric oxide and nitric oxide synthase-like activity regulate the expression of these cystatins, thus influencing soybean caspase-like activity. We also propose a role for this nitric oxide-mediated regulation of cystatin gene expression in the mediation of developmental processes and responses to abiotic stress in soybean.

Keywords: Caspase-like activity, cysteine protease, plant cystatin, protein expression, soybean nodules.
Abbreviations: Ac-DEVD-pNA- N-Acetyl-Asp-Glu-Val-Asp-p-Nitroanilide; BAPNA- Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride; BLAST- Basic Local Alignment Search Tool; DETA- diethylenetriamine; DETA/NO- 2,2'-(hydroxynitrosohydrazono)bisethanimine; E64- l-trans-epoxysuccinyl-leucyl-amido-(4-guanidino)butane; GST- glutathione-S-transferase; HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-NNA- N-ω-nitro-L-arginine; PMSF-phenylmethylsulfonyl fluoride.

Introduction

Plant cystatins (phytocystatins) are proteins with inhibitory activity against cysteine proteases, with roles in the regulation of cysteine proteases in plants during various cellular or physiological processes that include organogenesis, seed development and maturation, storage protein turnover and programmed cell death and in plant responses to biotic and abiotic onslaught (Benchabane et al., 2010). Phytocystatins appear to play a role in plant defence against abiotic stress (Van der Vyver et al., 2003; Zhang et al., 2008). Furthermore, the cysteine protease inhibitory activity of phytocystatins is a component of plant defence against insect attacks, executed via inhibition of insect gut proteases (Arai and Abe, 2000) and serves as a protective tool against infection of plants by pathogens (Carrillo et al., 2011; Gleddie and Michaud, 2000; Valdés-Rodríguez et al., 2010).
Classification of phytocystatins is based on sequence homology, molecular weight and domain organization (Martinez et al., 2009). The smallest (between 9 and 12 kDa) phytocystatin family constitutes single-domain cystatins (Benchabane et al., 2010; Megdiche et al., 2009) whereas phytocystatins with a molecular weight of between 14 and 25 kDa belong to the C-tailed cystatin group (Benchabane et al., 2010; Shyu et al., 2004) while those with a molecular weight of approximately 85 kDa constitute the multicystatin group that contain multiple cystatin domains (Benchabane et al., 2010; Girard et al., 2007). The N-terminal region of phytocystatins is unique to plant cystatins and contains two important regions, i.e. a glycine residue and a conserved motif namely [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N (Zhang et al., 2008). Other conserved regions include a QxVxG (where x is any amino acid) motif centrally located in the protein sequence and proline-tryptophan residues located within the C-terminal region (Arai et al., 2002). Phytocystatins inhibit cysteine proteases by forming a tight, reversible equimolar complex by acting as pseudo-substrates that penetrate the active site, thus blocking access to protein substrates (Benchabane et al., 2010; Fabienne et al., 2002). Cystatins utilizes three signature elements to form a tripartite wedge which fit into the cysteine protease active site during inhibition. The first structural

element which makes contact with the cysteine protease active site is a hairpin loop which contains the conserved QxVxG domain (Chu et al 2011; Nagata et al., 2000). The second structural element to enter the active site is a hairpin loop containing the proline-tryptophan residues which is situated in the C-terminal part of the cystatin protein (Irene et al., 2012). The third element which is essential for strong inhibition of cysteine proteases is the conserved glycine residue located in the N-terminal region (Fabienne et al., 2002).

Transcriptional regulation of phytocystatins by biotic and abiotic stress has previously been described (Diop et al., 2004; Hwang et al., 2010; Pernas et al., 2000; Valdés-Rodríguez, 2007; Van der Vyver et al., 2003). Noteworthy is the observation that the expression of some single-domain phytocystatins from different species is transcriptionally modulated by biotic and abiotic stress (Belenghi et al., 2003; Hwang et al., 2010; Pernas et al., 2000) and to date the expression of only one of these phytocystatins has been shown to be responsive to nitric oxide (Belenghi et al., 2003). Moreover, nitric oxide is involved in signalling cascades in response to both biotic and abiotic stress (Belenghi et al., 2003; Courtois et al., 2008; Delledonne, 2005; Delledonne et al., 2003; Diop et al., 2004; Hwang et al., 2010; Pernas et al., 2000; Qiao et al., 2008; Valdés-Rodríguez, 2007) and is required for nodule development (del Giudice et al., 2011; Leach et al., 2010). This raises the question of whether or not nitric oxide modulates the expression of other single-domain cystatins in a similar manner as found in the study by Belenghi et al. (2003). Soybean (*Glycine max*), is a member of the family Leguminosae which has a symbiotic relationship with specific soil bacteria, called rhizobia (Ferguson et al., 2010). This symbiotic interaction results in the establishment of root nodules which become a specialized area in which the rhizobia convert atmospheric nitrogen gas into usable forms of nitrogen for the host plant. The ability of soybeans to develop a symbiotic relationship with nitrogen-fixing rhizobia provides them with an advantage over other plant species and removes the demand for expensive fertilizers for its growth. In addition, soybean production has increased to cover 6% of all arable land in the world which is the highest percentage increase in area under production among crops annually (Hartman et al., 2011). Soybean is used as food for human consumption (Ferguson et al., 2010; Yamada 2012), in industrial products such as cosmetics and hygiene products (Ferguson et al., 2010), in livestock feed (Hartman et al., 2011) and in biofuel production (Hossain et al., 2010). Therefore, highly adapted soybean varieties with vigorous nodule yield stability under all environmental conditions are important to sustain soybean production worldwide. We thus used the amino acid sequence of AtCYS1 (Belenghi et al., 2003) to identify its homologues in soybean and characterize the responses of the homologues to altered nitric oxide content in soybean nodules. This is the first report on the influence of altered nitric oxide content on gene expression of single-domain cystatins in root nodules. Furthermore, the fact that a nitric oxide synthase inhibitor altered the expression of these single-domain cystatins implies that plant-derived nitric oxide synthase activity influences cysteine protease activity, a finding that has not been demonstrated in plants before. Given that caspase-like enzymatic activity is implicated in a wide range of plant physiological processes and caspases are cysteine proteases amicable to inhibition by cystatins (Carrillo et al., 2011;

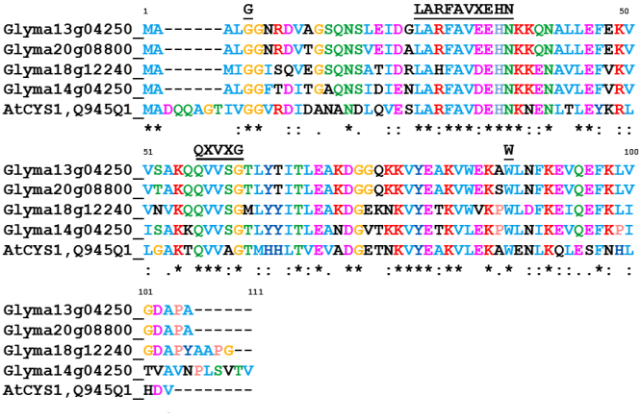

Fig 1. Comparison of the full length amino acid sequences of AtCYS1 and its four phytocystatin homologues in soybean (Glyma13g04250, Glyma20g08800, Glyma18g12240 and Glyma14g04250). Conserved amino acid residues characteristic of phytocystatins are indicated above the aligned sequences. These are the conserved N-terminal glycine (G), the N-terminal motif (LARFAVXEHN), the conserved centrally located motif (QXVXG) and the conserved C-terminus tryptophan residue (W).

Chichkova et al., 2004; Nissen et al., 2009; Rojo et al., 2004; Wang et al., 2010), we also determined if one of the AtCYS1 homologues alters caspase-like activity in soybean nodules.

Results

Identification and sequence analysis of AtCYS1 homologues in soybean

BLAST searches using AtCYS1 amino acid sequence as the query against SoyBase identified a soybean EST (accession number ACBU1760) encoding a protein homologous to AtCYS1. Upon analysis of the sequence in Phytozome, this EST was found to correspond to the soybean gene Glyma13g04250. Three other proteins; namely Glyma20g08800, Glyma18g12240 and Glyma14g04250; were identified in Phytozome as homologues of Glyma13g04250 and AtCYS1, based on the number of amino acid residues of the full-length proteins and the degree of similarity of their amino acid sequences (Fig 1). As depicted in Fig 1, all the four soybean proteins contain the conserved N-terminal G (glycine) residue and N-terminal motif [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N together with the centrally located QxVxG motif and the conserved C-terminal W (tryptophan) residue that are found in typical plant cystatins (Margis et al., 1998). These features suggest that the four proteins are potentially single-domain phytocystatins.

Effect of altered root nodule nitric oxide content on expression of AtCYS1 homologues

We evaluated the effect of altering the level of nitric oxide, in soybean tissue, on the expression (transcript levels measured by semi-qRT-PCR) of the genes encoding the four AtCYS1 homologues identified here; by either increasing the nitric oxide content in the tissues using the nitric oxide donor DETA/NO or reducing the nitric oxide content in the tissues using the nitric oxide synthase inhibitor L-NNA. This was done together with analysis of the expression of the genes in roots, leaves and nodules of soybean to establish the spatial

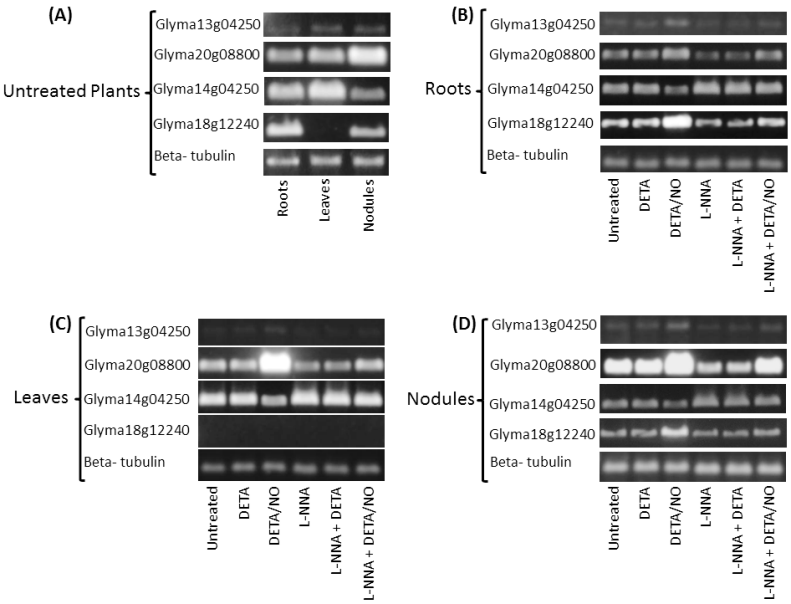

Fig 2. Spatial expression pattern of the four single-domain phytocystatins and the effect of altered root nodule nitric oxide content on their expression. (A) Representative agarose gel showing semi-qRT-PCR analysis on roots, leaves and nodules of soybean plants grown in nitrogen-free nutrient solution without any treatment. β-tubulin was used as a reference gene for normalization of expression levels. (B, C and D; respectively) Effect of exogenously applied nitric oxide (generated from 10 µM DETA/NO) or inhibition of nitric oxide synthase-like activity (imposed by treating plants with 1 mM L-NNA) on the expression of the gene encoding the four soybean single-domain phytocystatins in roots, leaves and nodules of soybean; respectively. DETA/NO at a final concentration of 200 µM was used to reverse the effects of the nitric oxide synthase inhibitor by simultaneously treating the plants with 200 µM DETA/NO and 1 mM L-NNA. Negative controls for the DETA/NO treatments were evaluated after treatment of plants with 10 µM DETA, whereas negative controls for the reversal of inhibition of nitric oxide synthase-like activity were set up by simultaneous treatment with 1 mM L-NNA and 200 µM DETA. β-tubulin was used as a reference gene for normalization of expression levels.

pattern of expression of these genes under steady state conditions. All the soybean cystatin genes and β-tubulin are expressed in the roots and nodules whereas Glyma18g12240 was not expressed in the leaves (Fig 2A). Furthermore, none of the treatments altered the expression of β-tubulin in any of the tissues when compared to the untreated controls whereas DETA/NO increased the expression of Glyma13g04250, Glyma20g08800 and Glyma18g12240 in roots, nodules and leaves (Fig 2B-D). However, DETA/NO resulted in reduced levels of expression of Glyma14g04250 in all the tissues evaluated in this study (Fig 2B-D). DETA did not alter the expression of the cystatin genes when compared to the untreated controls. L-NNA decreased the expression of Glyma13g04250, Glyma20g08800 and Glyma18g12240 in all the tissues (Fig 2B-D). On the other hand, L-NNA increased the expression of Glyma14g04250 in all tested tissues (Fig 2B-D). The reduced expression of Glyma13g04250, Glyma20g08800 and Glyma18g12240 (L-NNA treatments) was reversed by DETA/NO to a level similar to the untreated controls in all the tissues whereas DETA could not reverse the effect of L-NNA on the expression of any of the genes (Fig 2B-D). Furthermore, DETA/NO reversed the effect of L-NNA for Glyma14g04250 to the level of the untreated controls (Fig 2B-D). Glyma13g04250 expression in leaves and nodules was approximately 2-fold higher than its expression in roots (Fig 3). On the other hand, the expression of Glyma20g08800 was increased by approximately 0.3-fold in leaves compared to roots and increased by approximately 0.75-fold in nodules compared to roots (Fig 3). There was no difference in the expression levels of Glyma14g04250 in roots and leaves but its expression levels were decreased by 0.5-fold in nodules compared to the expression levels of Glyma14g04250 in roots and leaves (Fig 3). Expression of Glyma18g12240 decreased by approximately 0.4-fold in nodules compared to its expression in roots and expression of this cystatin in leaves was undetectable (Fig 3). Exogenously applied nitric oxide (generated from the nitric oxide donor DETA/NO) increased the level of expression of Glyma13g04250 in roots (by approximately 0.3-fold) and leaves (by approximately 0.45-fold) compared to the expression of this gene in untreated roots and untreated leaves respectively, whereas DETA (which is chemically analogous to DETA but lacks the nitric oxide moiety found in DETA/NO and thus does not release nitric oxide) did not alter the expression of the gene in roots and leaves (Fig 4A and Fig 5A). On the other hand, inhibition of nitric oxide synthase activity by treatment of soybean with L-NNA resulted in approximately a 0.3-fold decrease in the expression of Glyma13g04250 in roots and approximately a 0.17-fold decrease in the expression of this gene in leaves compared to its expression in roots and leaves of untreated plants, respectively (Fig 4A and Fig 5A). However, application of DETA to the L-NNA-treated plants did not reverse the inhibitory effect of the nitric oxide synthase inhibitor on the expression of Glyma13g04250 in roots and leaves (Fig 4A and Fig 5A). On the other hand, supplementation of nitric oxide to the L-NNA treatment reversed the inhibitory effects of L-NNA on the expression of Glyma13g04250 in roots and leaves to levels similar to its expression in roots and leaves from untreated plants. Expression of Glyma20g08800 was induced to levels approximately 0.4-fold higher in roots and to levels approximately 0.75-fold higher in leaves, respectively in response to the nitric oxide donor, whereas DETA did not

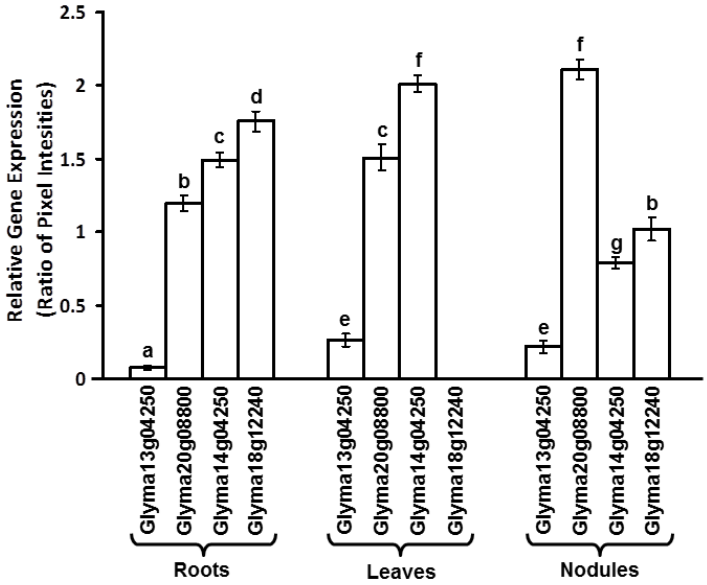

Fig 3. Estimation of the levels of gene expression in different organs of soybean. Densitometry was used to estimate the levels of expression of the four soybean phytocystatins in roots, leaves and nodules. Values were calculated as gene expression relative to the values for β-tubulin in each organ. Three gels resulting from three independent experiments were generated and analysed. Values are thus means ± standard deviations from these three independent experiments ($P < 0.05$).

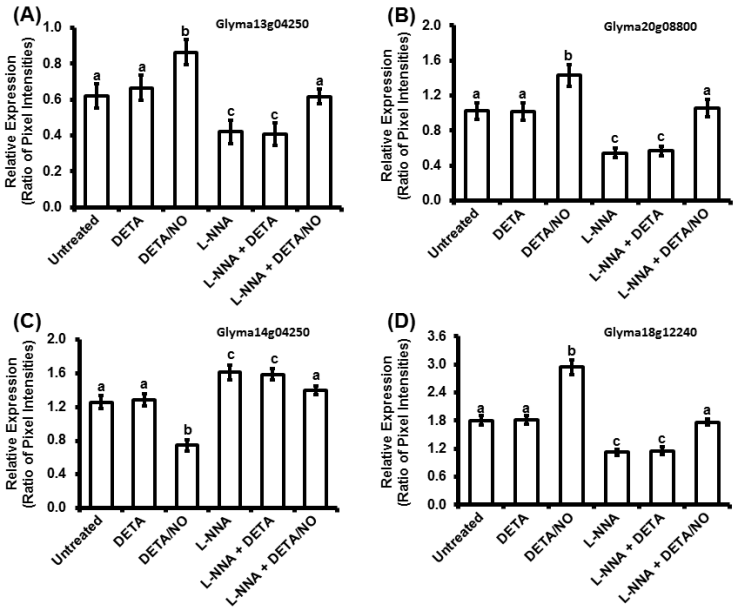

Fig 4. Nitric oxide-mediated changes in gene expression in roots of soybean. Estimation of expression levels of (A) Glyma13g04250, (B) Glyma20g08800, (C) Glyma14g04250 and (D) Glyma18g12240 in response to altered nitric oxide content in roots. Densitometry was used to calculate gene expression levels relative to the values for β-tubulin in roots for each treatment. Three gels resulting from three independent experiments were generated and analysed. Values are means ± standard deviations ($P < 0.05$, $n = 3$).

change the expression levels of this gene in roots and leaves (Fig 4B and Fig 5B). The nitric oxide synthase inhibitor L-NNA decreased the expression of Glyma20g08800 in roots and leaves to levels that are approximately 0.4-fold lower and approximately 0.3-fold lower than roots and leaves of untreated plants, respectively, whereas DETA did not reverse the inhibitory effect of L-NNA on the expression of Glyma20g08800 in roots and leaves (Fig 4B and Fig 5B). On the other hand, exogenous application of the nitric oxide donor simultaneously with the nitric oxide synthase inhibitor resulted in expression levels of Glyma20g08800 in roots and leaves similar to those of roots and leaves from untreated plants (Fig 4B and Fig 5B). Exogenously applied nitric oxide reduced the expression of Glyma14g04250 in soybean roots (by approximately 0.4-fold) and leaves (by approximately 0.25-fold) compared to its expression in roots and leaves of untreated soybean plants, respectively, whereas the nitric oxide synthase inhibitor (L-NNA) induced the expression of this gene in roots and leaves (to levels that are approximately 0.3-fold higher and approximately 0.25-fold higher, respectively) than its expression in roots and leaves of untreated soybean (Fig 4C and Fig 5C). Treatments with DETA did not alter the expression of Gyma14g04250 in soybean roots and leaves but simultaneous treatment with L-NNA and the nitric oxide donor returned the expression levels of this gene in roots back to the levels of the untreated controls, whereas simultaneous treatment of soybean with L-NNA and DETA resulted in Glyma14g04250 expression

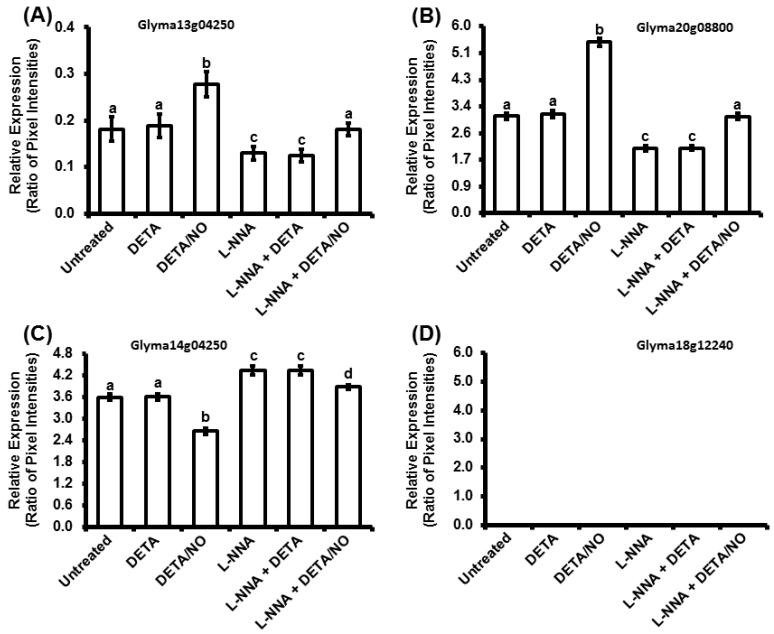

Fig 5. Effects of altered nitric oxide content on gene expression in leaves of soybean. Measurement of expression levels of (A) Glyma13g04250, (B) Glyma20g08800, (C) Glyma14g04250 and (D) Glyma18g12240 in response to altered nitric oxide content in leaves. Densitometry was used to calculate gene expression levels relative to the values for β-tubulin in leaves for each treatment. Three gels resulting from three independent experiments were generated and analysed. Values are means ± standard deviations ($P < 0.05$, $n = 3$).

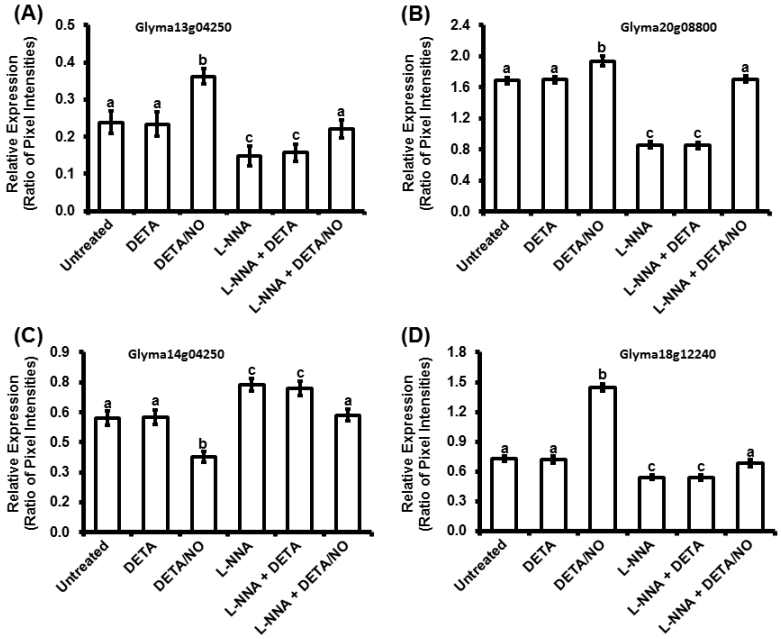

Fig 6. Nitric oxide-mediated changes in gene expression in root nodules of soybean. Estimation of expression levels of (A) Glyma13g04250, (B) Glyma20g08800, (C) Glyma14g04250 and (D) Glyma18g12240 in response to altered nitric oxide content in nodules. Densitometry was used to calculate gene expression levels relative to the values for β-tubulin in roots for each treatment. Three gels from three independent experiments were generated and analysed. Values are means ± standard deviations ($P < 0.05$, $n = 3$).

levels in roots and leaves similar to those observed in roots and leaves of plants treated with L-NNA, respectively (Fig 4C and Fig 5C). However, simultaneous treatment with L-NNA and the nitric oxide donor, even though it reversed the effects of L-NNA on the expression of Glyma14g04250 in leaves, the expression levels did not return to the levels of expression in the leaves from untreated soybean since these levels were still 0.14-fold higher than the levels in leaves of untreated plants (L-NNA + DETA/NO in comparison to untreated in relation to L-NNA, Fig 5C). Application of the nitric oxide donor augmented the expression of Glyma18g12240 in roots by approximately 0.7-fold compared to the expression of this gene in untreated roots, whereas DETA did not alter the expression of the gene in roots (Fig 4D). However, the nitric oxide synthase inhibitor (L-NNA) resulted in approximately a 0.35-fold decrease in the expression of Glyma18g12240 in roots compared to its expression in roots of untreated plants, whereas application of DETA to the L-NNA-treated plants did not reverse the inhibitory effect of the nitric oxide synthase inhibitor on the

expression of Glyma18g12240 in roots (Fig 4D). On the other hand, supplementation of nitric oxide in the L-NNA treatment reversed the inhibitory effects of L-NNA on the expression of Glyma18g12240 in roots to levels similar to its expression in roots from untreated plants. Irrespective of whether or not the plants were treated with the NO donor or nitric oxide inhibitor, expression of this gene was not detectable in leaves (Fig 5D). Increased gene expression in root nodules was observed for Glyma13g04250 (increased by 0.45-fold), Glyma20g08800 (increased by 0.2-fold) and Glyma18g12240 (increased by 1-fold) in response to treatment with the nitric oxide donor when the expression of these genes was compared to their corresponding expression levels in nodules from untreated soybean, whereas DETA did not alter the expression of these genes in comparison to nodules from untreated plants (Fig 6A, B and D). On the other hand, the expression of Glyma14g04250 in nodules was attenuated to levels approximately 0.3-fold lower in response to exogenously applied nitric oxide than in nodules from untreated plants (Fig 6C). The nitric oxide synthase inhibitor (L-NNA) lead to decreased gene expression in root nodules for Glyma13g04250 (decreased by approximately 0.4-fold), Glyma20g08800 (decreased by approximately 0.5-fold) and Glyma18g12240 (decreased by approximately 0.25-fold) in comparison to the expression of these genes in nodules from untreated soybean (Fig 6A, B and D). On the other hand, the expression of Glyma14g04250 in nodules was elevated to levels approximately 0.35-fold higher in response to the nitric oxide synthase inhibitor than in nodules from untreated plants (Fig 6C). However, simultaneous treatment of soybean with L-NNA and DETA/NO reversed the effects of L-NNA of the expression of all four genes in the nodules back to the levels seen for the expression levels of these genes in nodules from untreated plants, whereas simultaneous treatment of the plants with L-NNA and DETA did not reverse the effects of L-NNA of the expression of these genes in nodules (Fig 6).

Recombinant protein expression and purification

Expression of Glyma13g04250 as a GST fusion recombinant protein [expressed from pET-41a (+) with Glyma13g04250 in the multiple cloning site] yielded an approximately 35 kDa protein whereas the control expression in which the target protein was only GST [expressed from pET-41a (+) without Glyma13g04250] yielded an approximately 25 kDa protein (Fig 7A), indicating that Glyma13g04250 has a molecular weight of approximately 10 kDa, which is the expected molecular weight for a protein composed of 97 amino acids. Purification of the crude protein extract from cells expressing the recombinant protein produced fractions of relatively pure GST-Glyma13g04250 protein of approximately 35 kDa (Fig 7B).

Assays for inhibitory activity of Glyma13g04250 against protease activity

To evaluate if Glyma13g04250 is a cystatin with specificity against cysteine protease activity rather than a general protease inhibitor, the protease inhibitory activity of the recombinant protein was tested against a reference plant cysteine protease (purified papain) and the serine protease trypsin. This was done in parallel with the commercially established cysteine protease inhibitor E-64 as a positive control. Recombinant Glyma13g04250 reduced the protease activity of papain by approximately 0.5-fold whereas both boiled recombinant Glyma13g04250 and GST had no significant effect of the protease activity of papain, whilst E-64 decreased the protease activity of papain by approximately 0.93-fold (Fig 7C). On the other hand, recombinant Glyma13g04250 had no effect on the protease activity of trypsin (Fig 7C). The purified recombinant Glyma13g04250 (as a GST fusion protein) was tested for inhibitory activity against caspase-like protease activity in extracts obtained from soybean root nodules. Caspase-like protease activity was chosen because caspases are cysteine proteases and they regulate programmed cell death in plants (Carrillo et al., 2011, Chichkova et al., 2004; Nissen et al., 2009; Rojo et al., 2004; Wang et al., 2010) and Glyma13g04250 is a homologue of a cystatin (AtCYS1) involved in the regulation of programmed cell death during the hypersensitive response (Belenghi et al., 2003). Recombinant Glyma13g04250 reduced caspase-like protease activity in the nodule extracts by 0.4-fold compared to extracts without the recombinant Glyma13g04250 whereas no significant effect on nodule extract caspase-like activity was observed when boiled recombinant Glyma13g04250 or native GST were used instead of recombinant Glyma13g04250 (Fig 7D).

Discussion

We have engaged in a study of the influence of nitric oxide on the expression of four (Glyma13g04250, Glyma20g08800, Glyma18g12240 and Glyma14g04250) soybean cystatins belonging to the single-domain cystatin family of plant cystatins. Judging from the significant similarity of the four cystatins to AtCYS1 both from primary sequence data and conserved sequence motifs found in the cystatins, together with the length (number of amino acids) and approximate molecular weight of the four cystatins (± 10 kDa) and the inhibitory activity of recombinant Glyma13g04250 on both the cysteine protease enzymatic activity of papain and the caspase-like enzymatic activity of soybean nodule protein extracts, we conclude that Glyma13g04250 and the three other cystatins studied here are cystatins belonging to the single-domain cystatin family. This is corroborated by the fact that recombinant Glyma13g04250 lacks inhibitory activity against the serine protease trypsin and its fusion partner GST also lacks any protease inhibitory activity.

With the exception of Glyma18g12240 which is not expressed in leaves but only expressed in roots and nodules, the other single-domain cystatins studied are expressed in roots, nodules and leaves. Given that cystatins play a role in regulating protein turn-over towards the regulation of developmental processes in plants (Hwang et al., 2010; Martinez et al., 2009; Valdés-Rodríguez, 2007), the differential expression seen amongst the cystatins in the different organs studied here may be a reflection of the various roles these cystatins play in regulating developmentally-driven control of protein turn-over in the various organs investigated here. These organ-specific differences may also be indicative of differences in the extent to which each of the cystatins regulate such developmental processes. However, further study is required to establish the role of each of these cystatins in regulating development in the different plant organs. The induction of expression of Glyma13g04250, Glyma20g08800 and Glyma18g12240 upon treatment with a nitric oxide donor in all organs studied here (except for the fact that no expression of Glyma18g12240 was detected in leaves), together with the attenuation of expression of these genes by a nitric oxide synthase inhibitor, indicates a role of nitric oxide in regulating cystatin expression and suggests that nitric oxide synthase activity may play a role in the regulation of cystatin gene expression. The fact that the effects of the nitric oxide

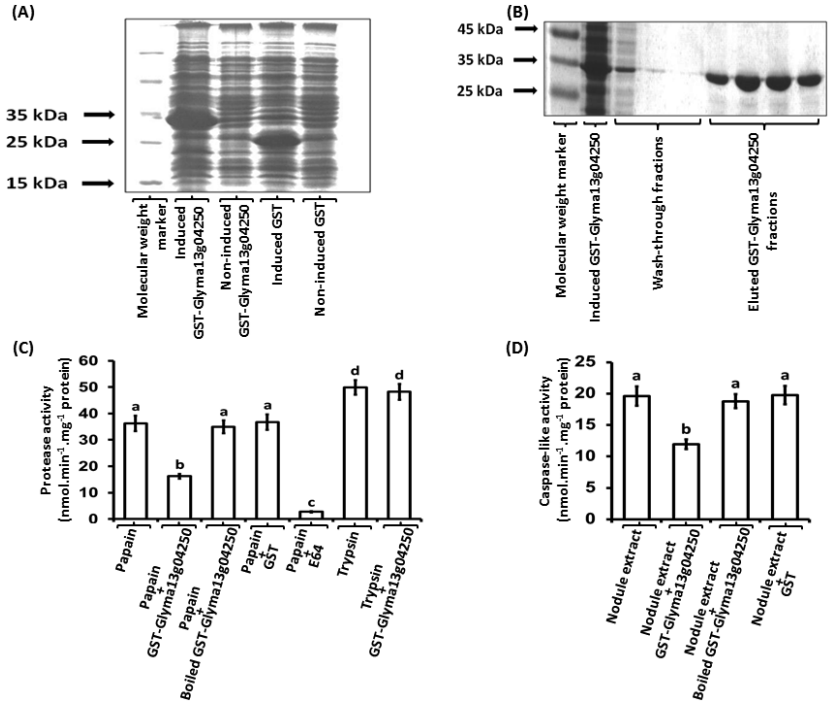

Fig 7. Recombinant protein expression, purification and cystatin activity of Glyma13g04250. (A) SDS- PAGE gel of *E. coli* crude protein extracts showing induced and non-induced recombinant Glyma13g04250 (GST fused to Glyma13g04250); and induced and non-induced GST. Protein molecular weight marker was loaded as a size standard to estimate protein molecular weight. (B) SDS-PAGE gel of crude *E. coli* extracts and purified recombinant Glyma13g04250 (GST fused to Glyma13g04250). (C) Protease activity of papain or trypsin in the absence or presence of recombinant Glyma13g04250 or boiled recombinant Glyma13g04250 or purified GST or in the presence of the cysteine protease inhibitor E-64. Values are means from three independent experiments with readings done in duplicates and error bars represent standard errors ($P < 0.05$). (D) Caspase-like activity in soybean root nodule crude protein extracts in the absence or presence of recombinant Glyma13g04250 or boiled recombinant Glyma13g04250 or purified GST. Values are means ± standard deviations ($P < 0.05$, $n = 3$).

synthase inhibitor are reversed by the nitric oxide donor, together with the observation that the effects of the nitric oxide donor are not imitated by an analogous chemical that does not release nitric oxide confirms that the effects observed on gene expression for the four cystatins studied here are a result of changes in nitric oxide content in the plant tissue. The mechanism by which exogenously applied nitric oxide (represented here by DETA/NO) and endogenously synthesized nitric oxide (from nitric oxide synthase-like activity in the case of this study in which a nitric oxide synthase inhibitor was used) influences cystatin gene expression is unknown but may involve *cis*-acting elements occurring in the promoters of these genes. In light of the fact that several potential *cis*-acting elements have been proposed to regulate gene expression in response to nitric oxide (Palmieri et al., 2008), identification and characterization of the promoters in the genes encoding the four cystatins reported here will be useful in understanding the molecular basis for the nitric oxide-mediated regulation of the expression of these cystatins. Such characterization becomes more pertinent and relevant when consideration is given to the fact that one of the cystatins, namely Glyma14g4250, oppositely regulated by nitric oxide in comparison to the other three cystatins reported here because exogenously applied nitric oxide attenuates its expression whereas the nitric oxide synthase inhibitor induces its expression. Identification of differences in potential *cis*-acting elements amongst the promoters of the cystatins studied here, which would determine which transcription factors interact with the promoters of the cystatins, will form part of our near future investigations together with characterization of these promoters and their potential transcription factor partners to improve our understanding on how nitric oxide regulates the expression of these genes. Such understanding may assist in elucidating the role played by these cystatins in controlling soybean development and soybean responses to abiotic stresses, which is the main focus of our future efforts, given the fact that roles for cysteine protease activity in regulating nodule development via a nitric oxide-mediated mechanism (Leach et al., 2010) and regulating plant abiotic stress tolerance by cystatins (Zhang et al., 2008) have been demonstrated.

Materials and methods

Identification and sequence analysis of AtCYS1 homologues in soybean

The Basic Local Alignment Search Tool (BLAST) was used to search for Expressed Sequence Tag (EST) sequences in the SoyBase (http://soybase.org) database, using AtCYS1 amino acid sequence as the query sequence. The amino acid sequence of the closest match in SoyBase was used as a BLAST query in Phytozome (www.phytozome.net) to identify, based on sequence identity, soybean homologues of AtCYS1. The NCBI protein and DNA accession numbers for AtCYS1, Glyma13g04250, Glyma20g08800, Glyma18g-12240 and Glyma14g04250 is provided in Supplementary Table 2.

Effect of altered nitric oxide content on expression of four AtCYS1 homologues

Surface sterilization of seeds of soybean (*Glycine max* L. merr. cv. PAN 626) was done by incubating the seeds for 10

min in 0.35% sodium hypochlorite. The sodium hypochlorite was discarded and seeds were washed five times with sterile distilled water. The seeds were imbibed in sterile distilled water for 1 hour, and then inoculated with *Bradyrhizobium japonicum* in the form of peat-based HiStick2 Soybean Inoculant (Becker Underwood Ltd., West Sussex, United Kingdom). The *G. max* seeds were sown in 1 litre of filtered silica sand (98% SiO_2, Rolfes® Silica, Brits, South Africa) that had been pre-soaked in distilled water, in 15 cm diameter plastic pots (one plant per pot). Germinated seedlings were grown on a 25/19°C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m^{-2}.s^{-1} during the day phase. Soybean plants were irrigated, at intervals of three days, with 200 ml of nitrogen-free nutrient solution consisting of 1 mM K_2SO_4, 2 mM $MgSO_4$, 3 mM $CaCl_2$, 1 mM K_2HPO_4 buffer at pH 7.3, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM ZnSO4, 2 µM $CuSO_4$, 2 µM Na_2MoO_4, 0.1 µM $CoSO_4$, 50 µM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3 at the VC stage (when the unifoliolate leaves are fully expanded) until they reached the V4 stage (fourth trifoliolate leaves and four nodes present). At this stage, plants were treated by applying 200 ml of nitrogen-free nutrient solution supplemented with either a nitric oxide donor or a nitric oxide synthase inhibitor or appropriate controls, followed by harvesting of plant material 24 hours after the treatments. For these treatments, plants were supplied with either nitrogen-free nutrient solution (named as untreated), or nitrogen-free nutrient solution containing the nitric oxide donor 2,2'-(hydroxynitrosohydrazono) bisethanimine (DETA/NO) at a final concentration of 10 µM (referred to as DETA/NO), or nitrogen-free nutrient solution containing DETA (diethylenetriamine, which serves as a control for DETA/NO since it is chemically analogous to DETA/NO except that it lacks the nitric oxide moiety found in DETA/NO and thus does not release nitric oxide, designated as DETA) at a final concentration of 10 µM, or nitrogen-free nutrient solution containing the nitric oxide synthase (EC 1.14.13.39) inhibitor N-ω-nitro-L-arginine (L-NNA) at a final concentration of 1 mM (which we named as L-NNA), or nitrogen-free nutrient solution containing L-NNA at a final concentration of 1 mM in combination with DETA/NO at a final concentration of 200 µM (designated as L-NNA + DETA/NO), or nitrogen-free nutrient solution containing L-NNA at a final concentration of 1 mM in combination with DETA at a final concentration of 200 µM (referred to as L-NNA + DETA). Previous work has shown that DETA/NO at a final concentration of 10 µM elevates NO content in plant tissue whereas the same concentration of DETA does not alter plant tissue NO content (Keyster et al., 2011). Furthermore, concentrations of L-NNA (1 mM) significantly reduce NO content in plant tissue and this reduction is reversed close to the levels found in untreated plant tissue when L-NNA (final concentration of 1mM)-treated plants are supplemented with a final concentration of 200 µM of DETA/NO (Keyster et al., 2010). Leaves, roots and nodules from each set of treatments were harvested, snap-frozen in liquid nitrogen and stored at -80°C until used for RNA extraction. RNA extraction was done from each treatment using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. To eliminate any contaminating DNA, the total RNA from each treatment sample was treated with RNase-free DNase (Fermentas) according to the manufacturer's instructions. This was followed by reverse transcription for first strand cDNA synthesis on 0.2 µg of total RNA from each treatment sample, using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions, with the oligo(dT)18 primer (Fermentas) used for the first strand cDNA synthesis. For semi-quantitative reverse transcription polymerase chain reaction (semi-qRT-PCR), 2 µl from each first strand cDNA synthesis reaction were used as template for PCR, using gene-specific primers (Suppl. Table 1). PCR conditions were as follows: 1 min at 95°C, followed by 20 cycles of PCR cycling (30 sec at 95°C, 30 sec at 60°C and 1 min at 72°C), then 10 min at 72°C; catalyzed by 1.25 Units of TrueStart™ Hot Start Taq DNA Polymerase (Fermentas). This was followed by electrophoresis of 10 µl of each PCR product on a 1% agarose gel stained with GelRed™ (Biotium Inc). Gel image acquisition was done on the AlphaImage 2200 system (Alpha Innotech Corporation). Densitometry analysis was done using the Spot Denso tool (AlphaEase FC Imaging Software, Alpha Innotech Corporation). Individual gels from three independent treatments, as described above, were used for expression analysis. Expression levels were expressed as ratios relative to the values of the untreated samples, with beta-tubulin as the reference gene. The PCR products were sequenced on an ABI Prism 377 DNA sequencer using the Big Dye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems) to confirm the identity of the amplified products.

Recombinant protein expression and purification

First strand cDNA, synthesized as described above, from RNA extracted from nodules of untreated plants for one of the candidate phytocystatin genes (Glyma13g04250) was used as a template in PCR amplification of the gene for cloning towards recombinant expression to determine the inhibitory activity of the protein against proteases. The first strand cDNA (2 µl) was used in a PCR with the following oligonucleotides (10 pmol of each) as primers: Forward Primer, incorporating a *BamH*I restriction enzyme site as underlined, (5′-GTTGAGGGATCCATGGCAGCACTTGG-TGGCAATC-3′) and Reverse Primer, incorporating a *Sal*I restriction enzyme recognition sequence that is underlined, (5'-GTCAATGTCGACCTATGCAGGTGCATCTCCAAC-3'). PCR conditions were as follows: 1 min at 95°C, followed by 30 cycles (30 sec at 95°C, 30 sec at 60°C and 1 min at 72°C), then 10 min at 72°C; catalyzed by 1 U of *Pfu* DNA Polymerase (Fermentas). The PCR product was sequenced on an ABI Prism 377 DNA sequencer using the Big Dye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems) and cloned in frame as a 5′ *BamH*I - 3′ *Sal*I fragment (underlined in primer sequences above) in pET-41a(+) expression vector (Novagen) and introduced into *E. coli* KRX cells (Promega). Unmodified pET-41a(+) was used as a control to produce glutathione-S-transferase (GST) in *E. coli* KRX cells using a method similar to that used for pET-41a(+) harboring the Glyma13g04250 cDNA. Overnight cultures (in Luria Bertani supplemented with 30 µg/ml kanamycin) of the *E. coli* KRX cells harboring the expression construct or unmodified pET-41a(+) were diluted at a ratio of 1:100 in auto-induction media (LB, 0.05% glucose, 0.1% rhamnose and 30 µg/ml kanamycin) and grown for 18 h at 25 °C. After induction, the cells were harvested by centrifugation and lysed by sonication in 10 mM phosphate buffer (pH 8) containing Triton X-100 at a final concentration of 0.5%. Soluble fractions were analyzed by 12% SDS-PAGE (BioRad) and proteins were detected by SimplyBlue™ SafeStain (Invitrogen). Protein purification was done using glutathione agarose (Sigma) chromatography. Binding to the resin was conducted at 4°C for 2 h with constant gentle shaking, followed by a wash of the resin with 10 resin

volumes of the appropriate purification buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT and 0.5 mM EDTA). The target protein was recovered by eluting the bound protein from the glutathione agarose resin with 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0). Protein quantification was performed using the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories Inc.) as instructed by the manufacturer.

Assays for inhibitory activity of Glyma13g04250 against protease and caspase-like activity

The specificity of Glyma13g04250 as a cystatin was evaluated using 50 µg of serine protease (trypsin) or 50 µg of cysteine protease (papain) (both from Sigma) prepared in a buffer containing 100 mM Tris-HCI (pH 7.2) and 50 mM $CaCI_2$ in the absence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Recombinant Glyma13g04250 (50 µg) or GST (50 µg) was added to the trypsin or papain reaction mixture followed by incubation for 10 min at 25°C. The reaction was initiated by addition of 0.5 mM of the protease substrate Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) (Sigma), followed by incubation at 37°C for 60 min. Protease activity was determined, based on the cleavage of BAPNA, by measuring absorbance at 405 nm every 20 min, using the extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$ for p-nitroaniline. A reference reaction, for inhibition of papain, was set up in which 10 µM of the synthetic cysteine protease inhibitor, l-trans-epoxysuccinyl-leucyl-amido-(4-guanidino)butane (E-64), was used instead of Glyma13g04250 or GST. Glyma13g04250 was tested for cystatin activity on soybean nodule extracts by modifying a previously described procedure (Keyster et al., 2012). Protein extraction was done from 200 mg of soybean root nodule tissue, which was homogenised in 2 ml of buffer containing 100 mM Tris-HCI (pH 7.2), 5 mM $MgCI_2$, 2 mM EDTA, 10% (v/v) glycerol and 10 mM β-mercaptoethanol. For this caspase-like (and thus cysteine protease) activity determination, 1 mM PMSF was added to the nodule extract to inhibit other classes of proteases (e.g. serine proteases) in the homogenate (cysteine protease inhibition by PMSF is abrogated in the presence of β-mercaptoethanol) prior to incubation of the mixture at 37°C for 5 min. Inhibition of caspase-like activity by Glyma13g04250 was evaluated by adding 50 µg of recombinant Glyma13g04250 to the nodule extract, followed by incubation for 10 min at 25°C. Subsequently 10 µl of 5 mM N-Acetyl-Asp-Glu-Val-Asp-*p*-Nitroanilide (Ac-DEVD-pNA) (Sigma), which is a caspase substrate, was added to the reaction mixture and incubated at 37°C for 60 min. Caspase-like cysteine protease activity was determined by measuring absorbance at 405 nm every 20 min, using the extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$ for *p*-nitroaniline. As controls for the assay, 50 µg of either boiled recombinant Glyma13g04250 (boiled at 95°C for 20 min) or GST were substituted for recombinant Glyma13g04250.

Statistical Analysis

GraphPad Prism 5.03 software was used for statistical analysis. Data were analyzed by One-way analysis of variance (ANOVA) and means were compared by the Tukey-Kramer test at the 5% level of significance.

Conclusion

Cysteine proteases, specifically caspase-like cysteine proteases, are some of the drivers of abiotic stress-induced root nodule senescence in soybean. Hence, strategies to inhibit cysteine proteases are of utmost importance for insuring optimum growth of soybean in nitrogen-limited soils. Plant cystatins are natural inhibitors of cysteine proteases and play diverse roles in physiological processes in plants. Therefore, in this study we used the amino acid sequence of AtCYS1 to identify its homologues in soybean. Furthermore, we used recombinant DNA technologies to characterize the closest soybean homologue to AtCYS1 to determine if these soybean homologues are cystatins. We also tested the responses of these homologues to altered nitric oxide content. We thus conclude that Glyma13g04250, Glyma20g08800, Glyma18g12240 and Glyma14g04250 are single-domain phytocystatins for which the expression of the encoding genes is responsive to changes in nitric oxide content in soybean. Furthermore, we suggest that the involvement of nitric oxide in the regulation of the expression of these four cystatins may contribute to the regulation of caspase-like enzymatic activity in soybean, which may have implications for the regulation of processes that involve programmed cell death, development and abiotic stress responses in plants since both the activity of cysteine proteases and the signaling roles of nitric oxide are involved in these processes.

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