Phytomorphology 72 (3&4) 2022, 69–82 DOI 10.56024/ispm.72.3&4.2022/69-82

Genomics and Proteomics Evidence for the Presence of Multiple Forms of S-Nitrosoglutathione Reductase (GSNOR) in *Brassica juncea*

Priyanka Babuta and Renu Deswal*

Molecular Physiology and Proteomics Laboratory, Department of Botany, University of Delhi, Delhi-110007, India

Manuscript received on December 17, 2022; accepted on December 28, 2022

ABSTRACT

S-nitrosylation is considered as an important post-translational mechanism involved in the nitric oxide (NO) based signaling, in turn regulating diverse physiological processes like plant development and response to biotic/abiotic stresses. S-nitrosoglutathione (GSNO) is a crucial player in this mechanism as it transfers NO moiety to target proteins. The level of GSNO in the cells is regulated by a denitrosylating enzyme, S-nitrosoglutathione reductase (GSNOR). GSNOR was previously reported as a single copy gene in most green plants; however recent studies suggested the presence of multiple forms as well. The current study attempts to identify and validate multiple GSNOR forms in *Brassica*. Phylogenetic analysis from six *Brassica* species (*B. rapa, B. nigra, B. oleracea, B. carinata, B. juncea* and *B. napus*) showed evolutionary conservation of GSNORs. Out of 4 sequences identified in *B. juncea*, BjuA025075 contained 2 extra stretches (22 and 12 aa) in comparison with BjuA033715, BjuB036048, BjuA046905. PCR amplification resulted in 4 genomic and 2 coding sequences of GSNOR. The amplified genomic sequences match with sequences derived from the Brassicaceae database (BjuA033715, BjuB036048, BjuA046905 and BjuA025075). Interestingly, two immuno-positive bands in western blotting confirmed multiple forms in *B. juncea*. Furthermore, BjuA025075 showed 10.7 % higher expression as analyzed using RNA-seq data in seed and variation in number of alpha-helices and β-turns as suggested by secondary structure predictions in comparison with BjuA033715, BjuB036048 and BjuA046905. Hence the study confirms the presence of multiple GSNORs in *B. juncea*. Though further characterization of functional aspects of multiple isoforms of GSNOR is required.

Key words: ***Brassica*, denitrosylation, GSNOR, phylogeny, secondary structure, S-nitrosylation, transcript analysis, western blotting**

***Author for correspondence:** Renu Deswal, e-mail: rdeswal@botany.du.ac.in

Introduction

Nitric oxide (NO) research in plants revealed the role of NO as a multifunctional signaling molecule regulating diverse physiological functions in plant cells. The involvement of NO is well documented in both plant development and response to abiotic/biotic stresses (Kolbert et al., 2019). NO signaling is predominantly mediated by S-nitrosylation, a redox-based post-translational mechanism (Hess and Stamler, 2011). The mechanism of S-nitrosylation involves a reversible covalent coupling of NO to a reactive cysteine (Cys) of proteins to form S-nitrosothiol (SNO) (Feng et al., 2019). As NO has a very short lifespan, S-nitrosoglutathione (GSNO, a low molecular weight SNO), acts as a stable NO reservoir. Furthermore, GSNO transnitrosylates target proteins modulating their activity and functions (Lindermayr, 2018; Frungillo et al., 2013). The dynamic process of S-nitrosylation is mainly modulated by non-enzymatic and enzymatic denitrosylation that regulates intracellular GSNO level (Benhar et al., 2010; Kneeshaw et al., 2014). In plants, S-nitrosoglutathione reductase (GSNOR) and Thioredoxin/Thioredoxin reductase based NO removal were discovered as enzymatic modes of denitrosylation. Irreversible GSNO degradation is mainly catalyzed by GSNOR, the master regulator of S-nitrosylation in plants (Jahnova et al., 2019). GSNOR is a zinc-dependent dehydrogenase that was earlier known as glutathione-dependent formaldehyde dehydrogenase due to its role in formaldehyde detoxification (Sakamoto et al., 2002). GSNOR (also ADH5) belongs to the Class III alcohol dehydrogenase family (Frungillo et al., 2013). GSNOR-mediated degradation of GSNO involves its conversion to the oxidized form of glutathione i.e., glutathione disulfide (GSSG) and ammonia in the presence of NADH as a cofactor (Rizza and Filomeni, 2017).

Arabidopsis GSNOR cDNA (1140 bp) encodes a protein of 379 amino acids corresponding to the predicted molecular weight of 42.5 kDa. Each subunit contains two Zn^{+2}, one is essential for its structural, and other for catalytic, function of the protein (Lindermayr, 2018). GSNOR was described as a homodimer of two 43 kDa subunits in tomato (Kubienová et al., 2013). GSNOR is shown to be a highly conserved enzyme in a range of organisms from microorganisms to humans (Liu et al., 2001). Crystal structures of GSNOR from human (PDB ID 2ZFE; Sanghani et al., 2003), *Chlamydomonas reinhardtii* (PDB ID: 7AAS; Tagliani et al., 2020), tomato (PDB ID 4DL9; Kubienová et al., 2013) and *Arabidopsis* (PDB ID 3UKO) are available in protein databases. Structural analysis showed that plants and animals GSNOR are highly similar (Kubienová et al., 2013; Xu et al., 2013).

A single copy GSNOR gene was identified in *Arabidopsis* (Lee et al., 2008), tomato (Kubienová et al., 2013), and tobacco (Diaz et al., 2003). However, some reports have suggested the presence of multiple copies of this gene in *Phaseolus vulgaris*, *Glycine max* and *Lotus japonicus* (Xu et al., 2013; Matamoros et al., 2020). Also, a recent study reported polyploidisation of the wheat genome resulted in multiple genes of GSNOR that showed differential regulation against leaf rust pathogen (Hurali et al., 2022). The *Brassica* lineage separated from *Arabidopsis* and underwent whole genome triplication (WGT) to bring about many important amphidiploid *Brassica* crop species (Laha et al., 2020). Transcriptomic analysis showed differential expression patterns for multiple copies of flowering time genes in *B. napus* for the same Arabidopsis homolog, suggesting considerable sub-functionalization of these genes (Schiessl, 2020).

Although a few studies suggested the presence of multiple GSNOR forms, the information about multiple forms of GSNOR in *Brassica* is not analyzed. The current study was aimed to understand the status of GSNOR at both gene and protein level in different species of *Brassica*. The presence of multiple forms was validated through amplification of genomic and cDNA of *BjGSNOR* genes and multiple immuno-positive bands in western. Transcript analysis and structure prediction suggested differential regulation of GSNOR genes.

Material and Methods

Database search and GSNOR Sequence Retrieval

GSNOR sequences from Brassicaceae were identified using *Arabidopsis thaliana GSNOR* sequence *(*At5G43940) as query sequences at the Brassicaceae database (BRAD) (http://brassicadb.org/brad/blastPage.php) (Chen et al., 2022). Complete sequences with more than 90% query coverage were selected, and 29 CDS and amino acid sequences 17 belonging 6 species in genus *Brassica* and 12 belonging to genera *Arabidopsis*, *Cardamine, Thlaspi, Descurainia, Camelina. Capsella, Boechera and Isatis* were downloaded for further analysis (see Table 1).

Sequence Alignment and Phylogenetic Analysis

The 29 GSNOR sequences, including 17 from *Brassica*, were aligned using Clustal MUSCLE using default

Table 1: Gene IDs (CDS length) of GSNOR sequences retrieved from the Brassicaceae database (BRAD)

Species	Gene IDs
B. rapa (AA)	BraA06g044700 (1140 bp), BraA09g020720 (1221 bp)
B. juncea (AABB)	BjuA033715 (1140 bp), BjuA046905 (1140 bp), BjuB036048 (1140 bp), BjuA025075 (1242 bp)
B. nigra (BB)	BniB037807 (1140 bp), BniB002800 (1140 bp)
B. carinata (BBCC)	BcaB07g30989 (1140 bp), BcaC06g34610 (1140 bp), BcaC04g21887 (1140 bp)
B. oleracea (CC)	BolC07g025620 (1140 bp), BolC09g024020 (1140 bp)
B. napus (AACC)	BnaA06p47610 (1140 bp), BnaA09p20710 (1140 bp), BnaC07p26490 (1140 bp), BnaC09p26180 (1140 bp)
Arabidopsis lyrata	AlyAL8G16170 (1140 bp)
Arabidopsis halleri	Ara49363s0001 (1140 bp)
Cardamine hirsuta	Carhr179470 (1140 bp)
Thlaspi arvense	Thlar0052s0126 (1140 bp)
Descurainia sophia	Desop0222s0077(1140 bp)
Camelina sativa	Csa11g070810 (1140 bp), Csa18g009900 (1140 bp), Csa20g068940 (1140 bp)
Capsella grandiflora	Cagra5198s0003 (1140 bp)
Boechera stricta	Bos3148s0068 (1140 bp)
Isatis indigotica	Iin02508 (1140 bp)

Table 2: Primers used to amplify GSNORs from *B. juncea*

Name	Primer Sequence	Tm (ºC)	GC (%)
GSNOR-F1	5' ATG GCG ACT CAA GGT CAG GGT 3'	66.9	52.3
GSNOR-R4	5' TCATTTGCTGGTATCGAGGACAC 3'	60.6	47.8

parameters (https://www.ebi.ac.uk/Tools/msa/muscle/). This alignment of 29 sequences was used to construct a phylogenetic tree based on the neighbor-joining (NJ) method (Saitou and Nei, 1987) with 1000 bootstrap replicates and distances computed using the Poisson model, pairwise deletion, and uniform rates in MEGA 11 version 11.0.11 (Tamura et al., 2021).

Amplification of cDNA and genomic GSNORs

DNA and total RNA was isolated from 6-day old *B. juncea* seedlings (200 mg) using CTAB and Trizol method, respectively. The first strand cDNA was synthesized from 2 µg of total RNA using MMLV-RT (New England Biolabs) following manufacturer's instructions. DNA and cDNA were used as templates to amplify BjGSNORs using primers (manually designed) listed in Table 2. The thermocycler program included 3 min/ 94°C, 32 cycles of 30 s/ 94°C, 45 s/ 55°C, 1.30 min/ 72°C (for cDNA) and 2.30 min/ 72°C (for DNA), and a final extension step of 72°C for 10 min. The amplified PCR products were resolved on 6-8% TBE-Polyacrylamide Gel (TBE-PAGE). The amplicons were cloned in pGEMT-Easy vector (Promega) as per manufacturer's instructions and sequenced using automated sequencer (Applied Biosystems).

The exon-intron structures of the GSNOR genes in multiple sequence alignments of 4 genomic and 1 cDNA sequences were analysed using the Clustal MUSCLE tool (https://www.ebi.ac.uk/Tools/msa/muscle/).

Extraction of Proteins

Extraction of seed proteins was performed following Sakamoto et al. (2002) with some modifications. The seeds were homogenized in an extraction buffer [25 mM Tris-Cl buffer (pH 7.4) containing 30% glycerol and 2.5 mM DTT] with the ratio of sample to extraction buffer at 1:10. The homogenate was spun at 12,000 g for 25 min at 4ºC. The supernatant was used for SDS-PAGE and western blotting.

SDS-PAGE and Western Blotting

Western blotting was performed following Towbin et al. (1984). Briefly, the seed protein extracts were resolved on the SDS-PAGE (15%) and transferred onto a nitrocellulose membrane using a wet transfer (Bio-Rad) at 400 mA for 1 h at 4 °C. The membrane was probed using rabbit anti-GSNOR antibody (1:5000, 2 h) and alkaline phosphatase conjugated goat anti-rabbit antibody (1:10,000 for 30 min) as primary and secondary antibodies, respectively (Agrisera). Nitroblue Tetrazolium (NBT) and 5-bromo-4-chloro-3- indolyl phosphate (BCIP) were used as substrates. Reaction was stopped with double distilled water.

Secondary Structure Prediction

The secondary and three-dimensional (3-D) structures were predicted using the iterative threading assembly refinement (I-TASSER) server (Yang et al., 2015; Zheng et al., 2021). The secondary structures were predicted using the PDBsum tool (http://www.ebi.ac.uk/thornton-srv/databases/cgibin/pdbsum/GetPage.pl?pdbcode=index.html). The stereochemical quality of the predicted structures was inspected using PROCHECK analysis (Laskowski et al., 1993).

Transcript Analysis

Data on transcript expression (Illumina) were downloaded from NCBI SRA databases from a previous study (Mathur et al., 2022). High-quality reads were mapped on the *B. juncea* var. Varuna genome sequence using STAR aligner (Dobin et al., 2013). Differentially expressed genes were identified using DESeq2 v1.30.1 (Love et al., 2014). The raw feature counts obtained were normalized using variance stabilizing transformation (vst). One-way ANOVA and post-hoc Tukey's HSD test was used to statistically determine the significant differences at $p \leq 0.05$.

Results

Identification and Sequence Retrieval of GSNORs in Brassica

GSNORs were identified using *A. thaliana* GSNOR (At5G43940) as a query using the BLASTN tool available at the BRAD against the CDS database of Brassicaceae species. Complete sequences with more than 90% query coverage were selected for further analysis. A total of 17 sequences were identified in *B. rapa* (2), *B. nigra* (2), *B. oleracea* (2), *B. carinata, B. juncea* (4) and *B. napus* (4). The length of all

GSNORs was 1140 bp except BraA09g020720and BjuA025075 that were longer with 1221 bp and 1242 bp respectively. The variation in length of genes may lead to sub-functionalization. For phylogenetic analysis, GSNOR were also retrieved for other members of Brassicaceae family available at BRAD; *Arabidopsis lyrata, Arabidopsis halleri, Cardamine hirsuta, Thlaspi arvense, Descurainia sophia, Camelina sativa, Capsella grandiflora, Boechera stricta* and *Isatis indigotica*. The length of all these sequences was 1140 bp.

Sequence Alignment and Phylogenetic Analysis

To estimate phylogenetic relationships among GSNORs belonging to the Brassicaceae family, a phylogenetic tree was constructed using MEGA 11 software. The phylogeny of 29 GSNORs from 16 Brassicaceae species strongly supported the distinct monophyly of *Brassica* (Fig. 1). *Brassica* GSNORs consisted of 2 distinct clades (Clades II and III). Both the clades consisted of diploid (AA/BB/CC) and their tetraploid (AABB/BBCC/AACC) species. Interestingly, GSNORs from tetraploids were grouped along with GSNOR from one of their diploid parent species. Other Brassicaceae members, *C. hirsuta*, *T. arvense*, *D. sophia*, *Arabidopsis*, *C. sativa*, *C. grandiflora* and *B. stricta*, were separated (Clade I) from *Brassica*.

An alignment of 17 GSNOR amino acid sequences belonging to *Brassica* species was further analysed, showing 97-99 % identity among all sequences except BraA09g020720 and BjuA025075 (Fig. 2). These two sequences contain extra stretches of sequences and share only 91% identity with each other (highlighted in Table 3). Despite some dissimilarity, NAD binding, substrate binding and Zn^{+2} binding sites were conserved in all sequences. Among allotetraploids, *B. juncea* showed variation among identified GSNORs, therefore, *B. juncea* was selected for further validation of different forms of GSNOR. To know the number of genes present in *B. juncea,* PCR amplification was attempted.

Amplification of BjGSNORs

Amplification of GSNOR was performed by PCR using DNA and cDNA as template. The amplification using DNA as template resulted in 4 products at 1.9, 2.1, 2.3 and 2.6 kb (Fig. 3A). On the other hand, PCR of GSNOR using cDNA as template amplified two products at 1.1 and 1.3 kb (Fig. 3B). The amplified products were cloned in pGEMT vector and were sequenced for further validations.

The sequence of 1.1 kb matched with cDNA sequence of BjuA033715, whereas 2.6 kb (BjGSNOR-1), 2.3 kb (BjGSNOR-2), 2.1 kb (BjGSNOR-3) and 1.9 kb (BjGSNOR-4) matched with genomic sequences of BjuA025075, BjuB036048, BjuA046905 and BjuA033715 respectively. The cloning of one of the GSNOR cDNA amplicons (1.3 kb) was unsuccessful. The exon-intron structure of GSNOR sequences was determined using multiple sequence alignment of genomic sequences with cDNA sequence. The result showed 9 exons and 8 introns in all the GSNORs (Fig. 4). However, variation in the length and nucleotide sequences was observed in all 4 genomic GSNOR sequences. The major variations in the length and sequence similarity among introns of different BjGSNORs genes were observed in intron 3 and intron 4. The length of intron 3 ranged from 97–428 whereas intron 4 ranged from 103–491 bp. This variation in the intron length and sequence composition among BjGSNOR genes may affect expression pattern of these genes (Jo and Choi, 2015).

Transcript Analysis of BjGSNORs

To further investigate if these genes have a differential role, the expression profile of these genes were analyzed in *B. juncea* seeds using Illumina RNA-seq data. The transcript abundance of *BjGSNOR* genes in seeds was graphically represented (Fig. 5A). A comparative expression analysis showed that the relative expression of BjuA025075 was 10.7, 6.2 and 10.7% higher than BjuA033715, BjuB036048 and BjuA046905 respectively, indicating differential expression.

GSNOR Western Blotting

To analyze multiple forms of GSNOR at protein level, western blotting of GSNOR was performed in seeds of *Arabidopsis* and all 6 species of the *Brassica* Triangle of U (Fig. 5B). The results showed the presence of 1 immuno-positive band in *Arabidopsis* and 2 immuno-positive bands in *B. juncea* (Fig. 5C).

Predicted Three-Dimensional Structure

To further discern any differences in structure that might support the presence of variants, 3D structure models for BjGSNORs were predicted using online server I-TASSER software and confidence score was used to measure accuracy of prediction. The C-score, estimated TM-score and estimated RMSD for the most accurate structures were 1.57, 0.93±0.06 and 3.5±2.4Å (for BjuA033715 and BjuA046905); 1.69, 0.95±0.05 and 3.3±2.3Å (Bju036048); 0.5, 0.78±0.10, 5.8±3.6Å

Table 3: Protein Identity Matrix of *Brassica* GSNORs generated by Clustal MUSCLE. Highlighted cells indicate lower levels of pairwise identity.

	Bju A025075	Bni B002800	Bca C06g34610	Bol C07g025620	Bna C07p26490	Bni B037807	Bca B07g30989	Bra A06g044700	Bna A06P47610	Bju B036848	Bju A046905	Bra A09g020720	Bca C04g21887	Bna C09p26180	Bju A033715	Bol C09g024020	Bna A09p20710
Bju A025075	100	98.93	98.12	98.12	97.86	98.12	98.12	98.12	98.12	98.12	97.86	91.75	97.05	97.05	97.05	97.05	97.32
Bni B002800	98.93	100	99.21	99.21	98.94	99.21	99.21	99.21	99.21	99.21	98.94	98.15	98.15	98.15	98.15	98.15	98.42
Bca C06g34610	98.12	99.21	100	100	99.74	98.94	98.94	99.47	99.47	99.47	99.21	98.42	98.42	98.42	98.42	98.42	98.68
Bol C07g025620	98.12	99.21	100	100	99.74	98.94	98.94	99.47	99.47	99.47	99.21	98.42	98.42	98.42	98.42	98.42	98.68
Bna C07p26490	97.86	98.94	99.74	99.74	100	98.68	98.68	99.21	99.21	99.21	98.94	98.15	98.15	98.15	98.15	98.15	98.42
Bni B037807	98.12	99.21	98.94	98.94	98.68	100	100	99.47	99.47	99.47	99.74	98.94	98.68	98.68	98.94	98.94	99.21
Bca B07g30989	98.12	99.21	98.94	98.94	98.68	100	100	99.47	99.47	99.47	99.74	98.94	98.68	98.68	98.94	98.94	99.21
Bra A06g044700	98.12	99.21	99.47	99.47	99.21	99.47	99.47	100	100	100	99.74	98.94	98.94	98.94	98.94	98.94	99.21
Bna A06P47610	98.12	99.21	99.47	99.47	99.21	99.47	99.47	100	100	100	99.74	98.94	98.94	98.94	98.94	98.94	99.21
Bju B036848	98.12	99.21	99.47	99.47	99.21	99.47	99.47	100	100	100	99.74	98.94	98.94	98.94	98.94	98.94	99.21
Bju A046905	97.86	98.94	99.21	99.21	98.94	99.74	99.74	99.74	99.74	99.74	100	99.21	98.94	98.94	99.21	99.21	99.47
Bra A09g020720	91.75	98.15	98.42	98.42	98.15	98.94	98.94	98.94	98.94	98.94	99.21	100	99.21	99.21	99.47	99.47	99.74
BcaC04g21887	97.05	98.15	98.42	98.42	98.15	98.68	98.68	98.94	98.94	98.94	98.94	99.21	100	100	99.21	99.21	99.47
Bna C09p26180	97.05	98.15	98.42	98.42	98.15	98.68	98.68	98.94	98.94	98.94	98.94	99.21	100	100	99.21	99.21	99.47
Bju A033715	97.05	98.15	98.42	98.42	98.15	98.94	98.94	98.94	98.94	98.94	99.21	99.47	99.21	99.21	100	99.47	99.74
Bol C09g024020	97.05	98.15	98.42	98.42	98.15	98.94	98.94	98.94	98.94	98.94	99.21	99.47	99.21	99.21	99.47	100	99.74
Bna A09p20710	97.32	98.42	98.68	98.68	98.42	99.21	99.21	99.21	99.21	99.21	99.47	99.74	99.47	99.47	99.74	99.74	100

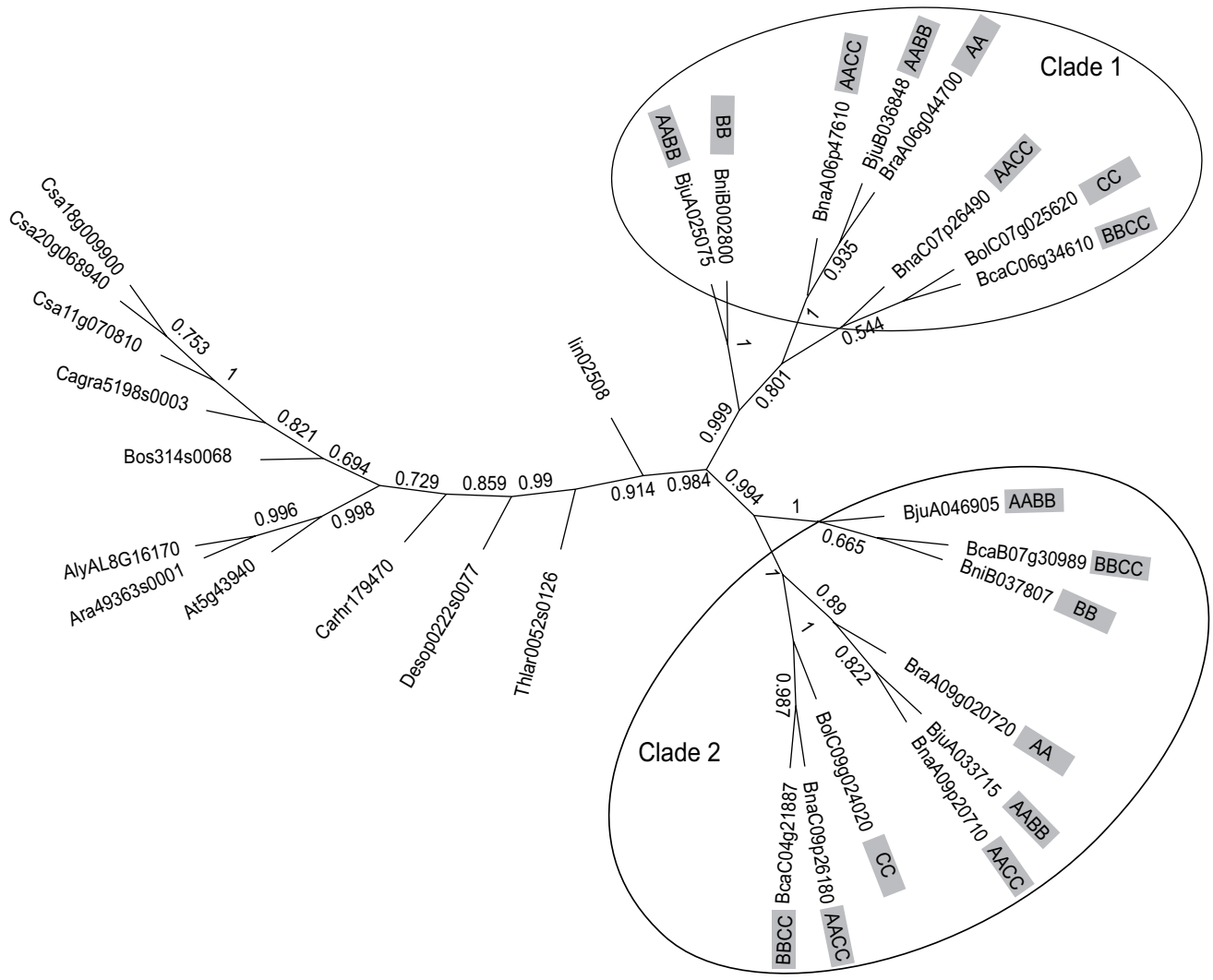


Figure 1: Evolutionary relationships of GSNOR protein sequences belonging to members of the family Brassicaceae. Sequences were aligned using Clustal MUSCLE and the phylogenetic tree (NJ) with 1000 bootstrap replicates (bootstrap values in red) was estimated using Mega 11 version 11.0.11 software.

(Bju025075) respectively (Table 4). The BjuA033715, BjuB036048 and BjuA046905structure showed highest homology with *Arabidopsis thaliana* alcohol dehydrogenase (PDB ID 4RQT), whereas BjuA025075 showed homology with *Solanum lycopersicum* GSNOR (PDB ID 4DL9). The stereochemical properties of the predicted 3D structures were validated by subjecting the PDB files to the PDBsum server and analyzed by the PROCHECK server. The structures revealed that each monomer of BjuA033715, BjuB036048 and BjuA046905 was characterized by 19 alpha helices, 3 sheets and 30 β-turns. On the contrary, BjuA025075 showed 18 helices, 4 sheets and 53 β-turns (Fig. 6). Also, one of the residues (204) in the extra stretch of BjuA025075 was observed to be involved in NADH binding. This suggests multiple forms may have differential roles. The difference in BjuA025075 structure suggested functional diversities of BjGSNORs.

Discussion

Genome duplication and polyploidy is identified as the driving force behind the evolution and diversification of *Brassica* species (Laha et al., 2020). Additionally, in spite of availability of genomes of several species, impact of genome duplication and polyploidisation has not been analyzed for GSNOR genes in *Brassica*. GSNOR is a denitrosylating enzyme that maintains the level of S-nitrosylation/ denitrosylation, thereby protecting cells from inimical effects of an oxygen metabolism disorder excess of NO (Jahnová et al., 2019). GSNOR was initially identified as single copy gene in plants, whereas multiple gene copies were

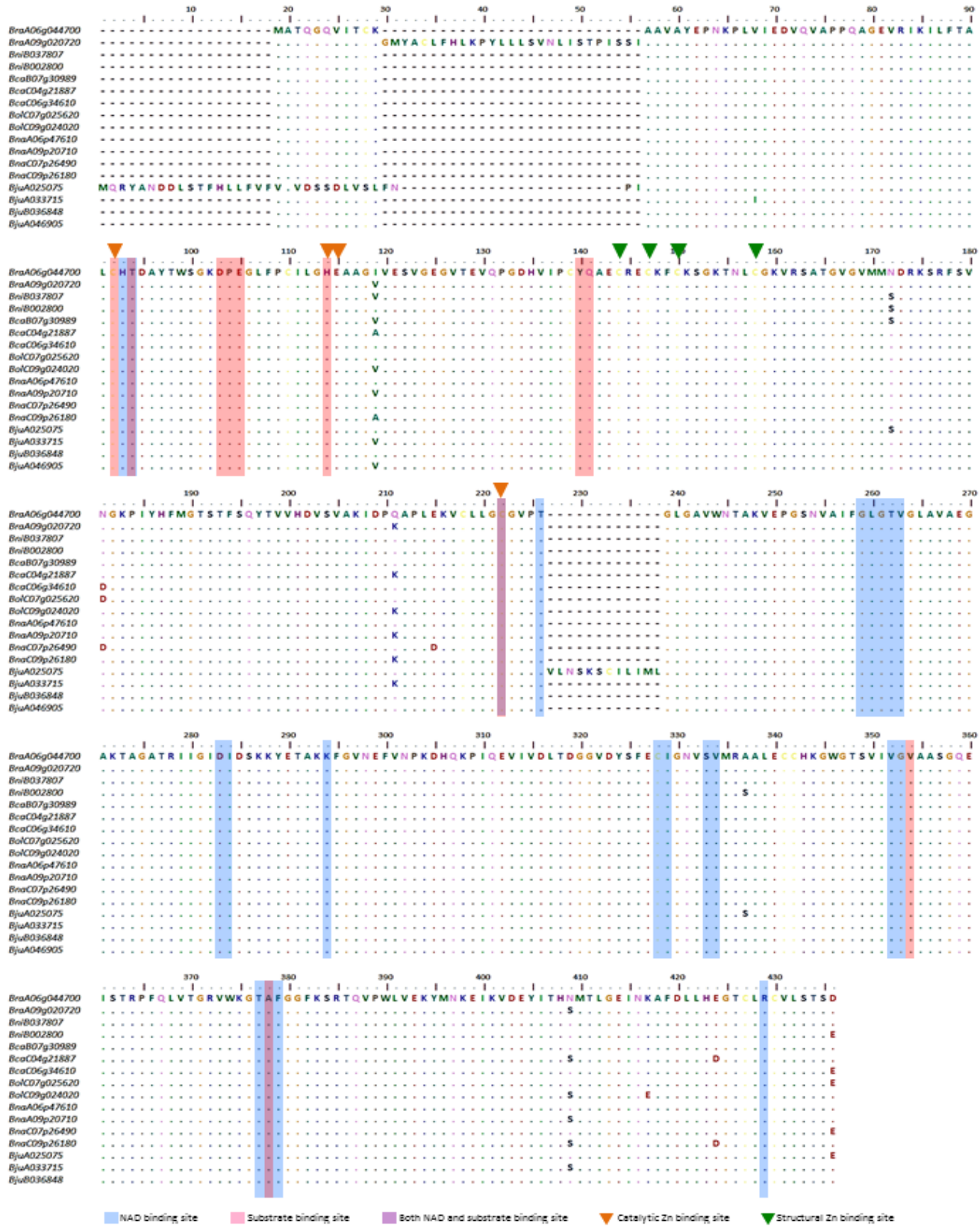


Figure 2: Multiple Sequence Alignment of GSNOR amino acid sequences across Brassicas showing substrate and cofactor binding sites. *Brassica* GSNORs were aligned using Clustal MUSCLE.

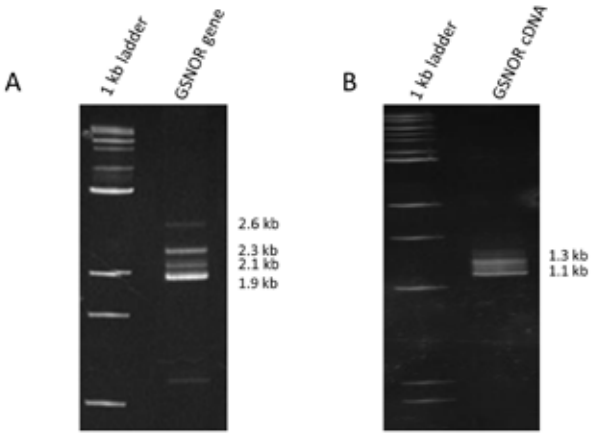


Figure 3: Validation of multiple copies of BjGSNOR genes (A) Amplification of genomic and (B) cDNA sequences of GSNOR. The amplicons were resolved on 6-8% TBE-PAGE.

also identified in *Phaseolus vulgaris*, *Glycine max* and *Lotus japonicus* due to genome duplication (Xu et al., 2013; Matamoros et al., 2020). Therefore, in the present study, evidence for the existence of multiple forms of GSNOR were presented using genome wide identification, transcript and western analysis. The results could provide a scientific basis for future understanding in the area of NO homeostasis.

Genome-wide identification showed multiple copies of GSNOR genes in six different species of *Brassica* (*B. rapa, B. nigra, B. oleracea, B. carinata, B. juncea* and *B. napus*). Hybridized species (*B. juncea* and *B. napus)* have doubled the number of genes as compared to parent species (*B. rapa, B. nigra* and *B. oleracea)* except *B. carinata.* This may be because of gene loss during duplication events. Presence of multiple gene copies of GSNOR in *Brassica* was supported by a wheat genome that contains three copies of GSNOR due to polyploidisation. However, the three copies identified were 381 aa in length and shared high sequence similarity (Hurali et al., 2022). Phylogenetic relationship based on Neighbor-Joining method and tree-building revealed that the clustering of homologs in a lineage-specific manner suggesting the multiplication of GSNOR genes in *Brassica* is genome specific (Fig. 1) and outcome of large-scale duplication, including WGT. It is already reported that multiple copies of flowering locus C genes due to WGT led to sub-functionalization in the genus *Brassica* (Akter et al., 2021). Sequence alignment analysis showed at least 97% homology indicating evolutionary conservation except BraA09g020720 and BjuA025075. To confirm the *in silico* results, the presence of multiple genes

Table 4: Confidence Score for accuracy of predicted structures

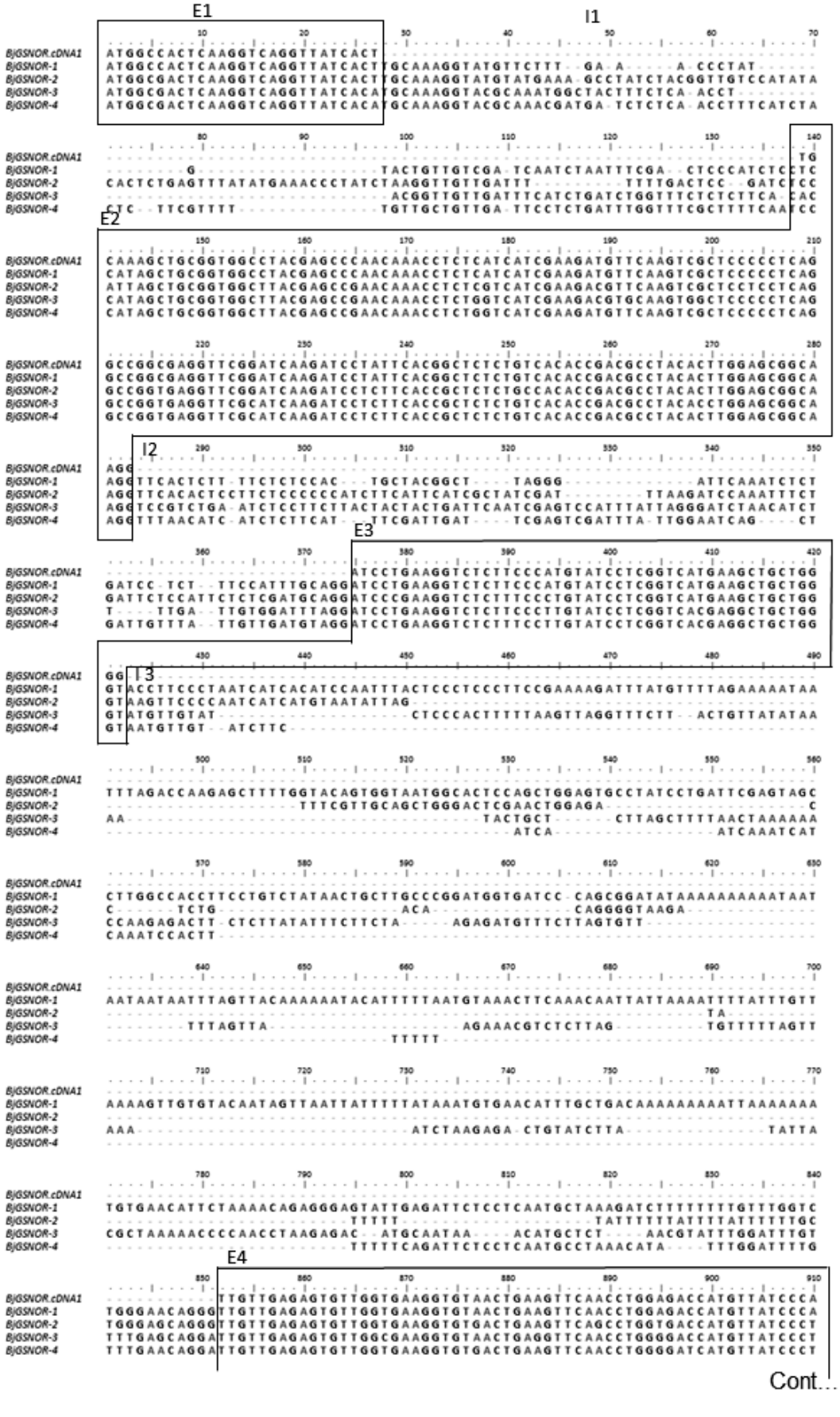
	C-Score	Estimated TM-score	Estimated RMSD
BjuA033715	1.57	0.93±0.06	3.5±2.4Å
BjuB036048	1.69	0.95±0.05	3.3±2.3Å
BjuA046905	1.57	0.93±0.06	3.5±2.4Å
BjuA025075	0.5	0.78±0.10	5.8±3.6Å

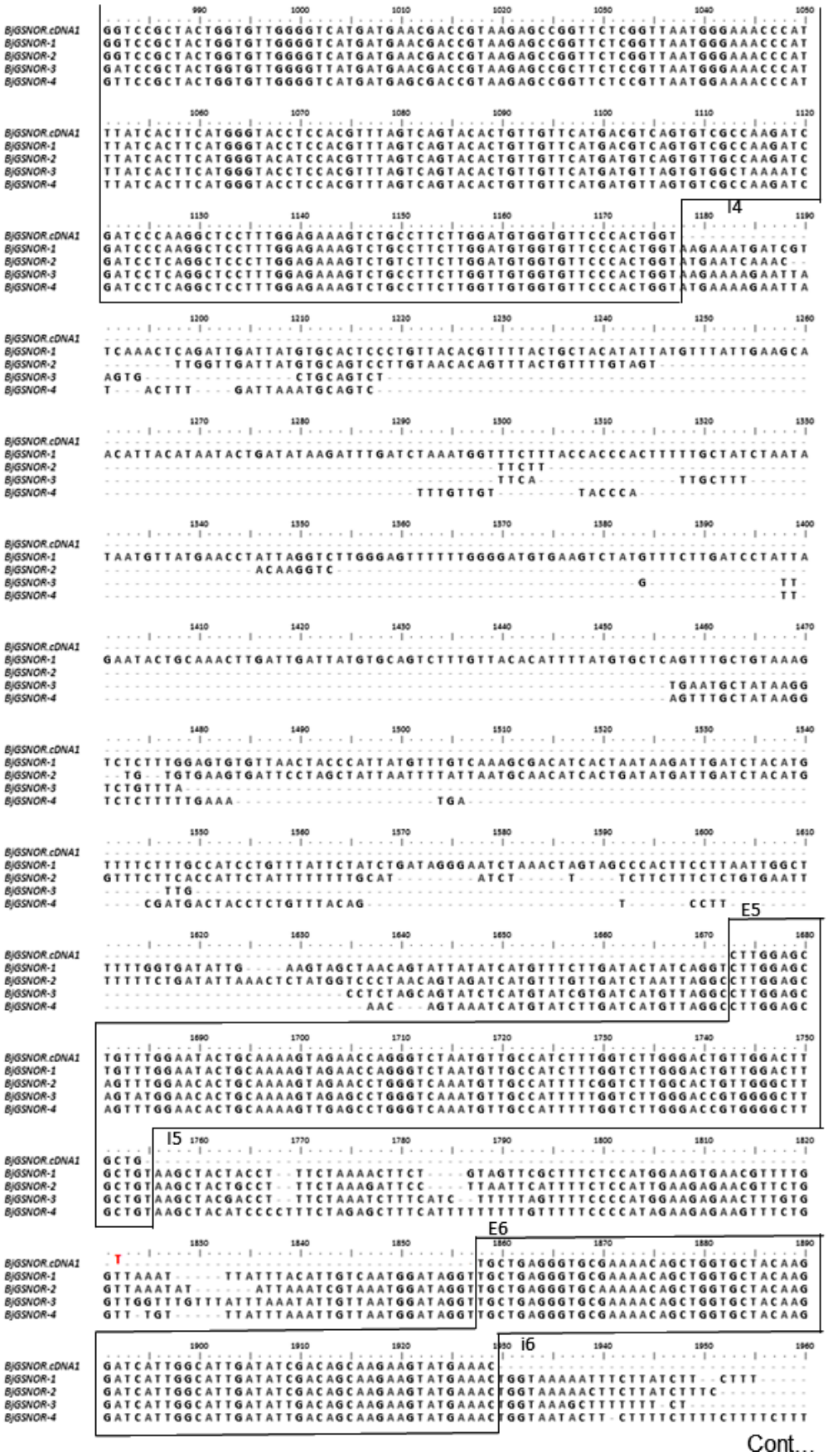
were experimentally validated. The confirmation of multiple GSNORs at protein level was proved by 2 immuno-positive bands of GSNOR on western blot. To the best of our knowledge, this is the first study to report multiple immuno-positive bands of GSNOR in plants. Earlier, *Arabidopsis* (Lee et al., 2008), tomato (Gong et al., 2019), pepper (Rodríguez-Ruiz et al., 2017) and poplar (Cheng et al., 2015) showed single immuno-reactive band on GSNOR western blot.

The results were validated at genomic and transcript level in *B. juncea* using PCR. As expected, the results showed amplification of 4 full length genes ranging from 1.9 kb to 2.4 kb. It is widely accepted that coding transcripts are better determinants of physiological roles of genes. Earlier reports suggested that multiple gene copies may produce the same transcripts. Therefore, GSNOR transcripts were analyzed using PCR amplification that resulted in 2 amplicons (1.1 and 1.3 kb). Further, expression analysis using Illumina RNA-seq data also showed differential expression of 4 genes in *B. juncea*. Variation in coding sequence length of 1 out of 4 GSNOR genes explained the amplification of only 2 amplicons at cDNA level.

Three-dimensional structures are major determinants of functional properties of a protein. These properties are mainly analyzed on the basis of protein secondary structures. Structural analysis showed high homology of all BjGSNORs with *A. thaliana* alcohol dehydrogenase (PDB ID 4RQT except BjuA025075 that was similar to *S. lycopersicum* GSNOR (PDB ID 4DL9). In addition, the secondary structure showed double the number of β-turn in BjuA025075 in comparison with other BjGSNOR proteins. The β-turns are considered the third important secondary structure (after helices and β-strands) that plays a crucial role in stability of protein's structure. Therefore, differential properties of GSNOR at expression and protein structure level may hint towards subfunctionalisation of GSNOR in *B. juncea.*

In conclusion, the present study provides insights into the identification and validation of multiple GSNOR


E1
I1
E2
I2
E3
I3
E4
BjGSNOR.cDNA1
BjGSNOR-1
BjGSNOR-2
BjGSNOR-3
BjGSNOR-4
Cont…


I4
E5
I5
E6
i6
Cont...

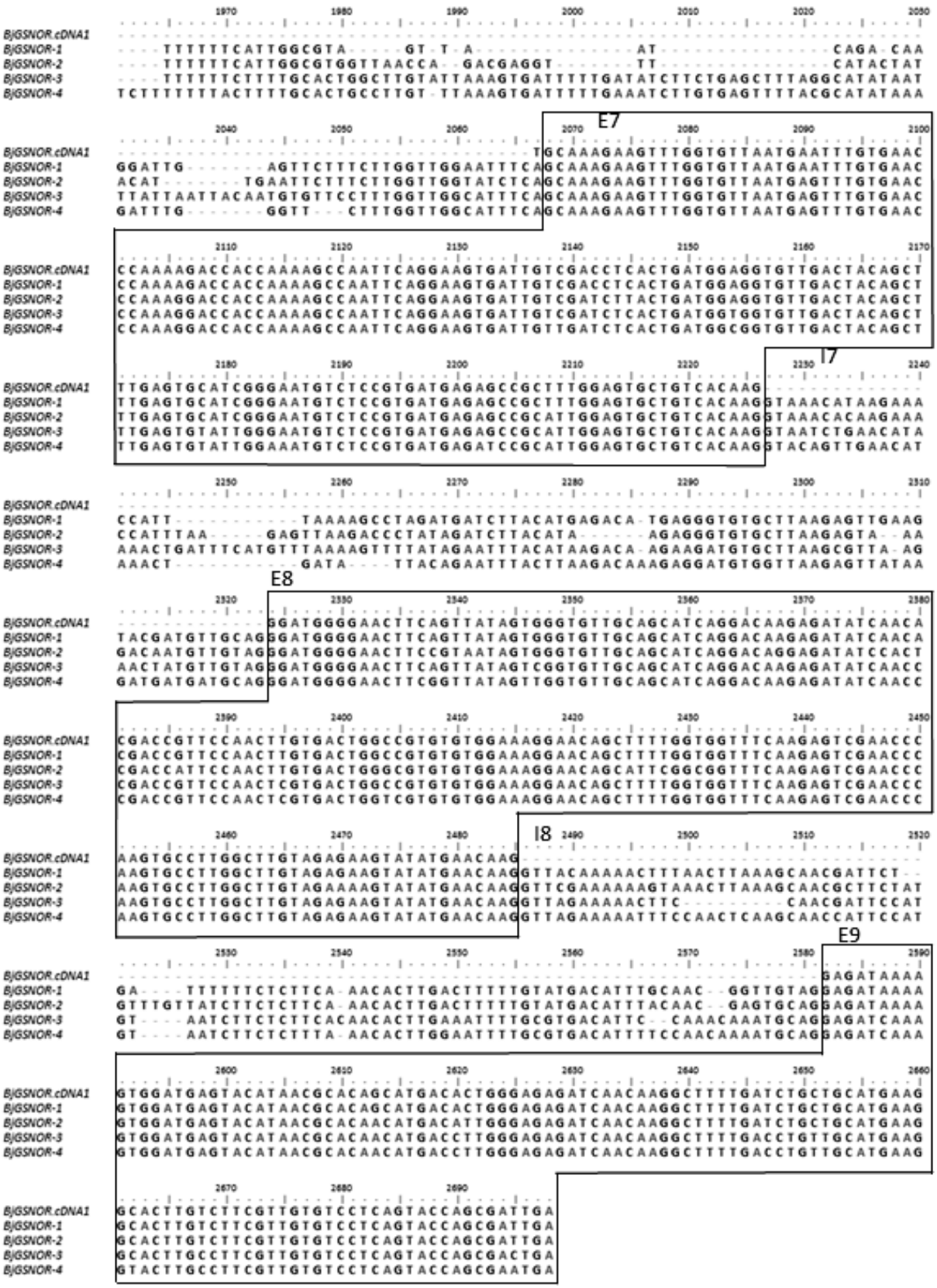


Figure 4: Multiple Sequence Alignment of BjGSNOR DNA and cDNA sequences showing exon (E) and Intron (I) structure of GSNOR genes in *Brassica juncea*. Exons are represented in boxes.

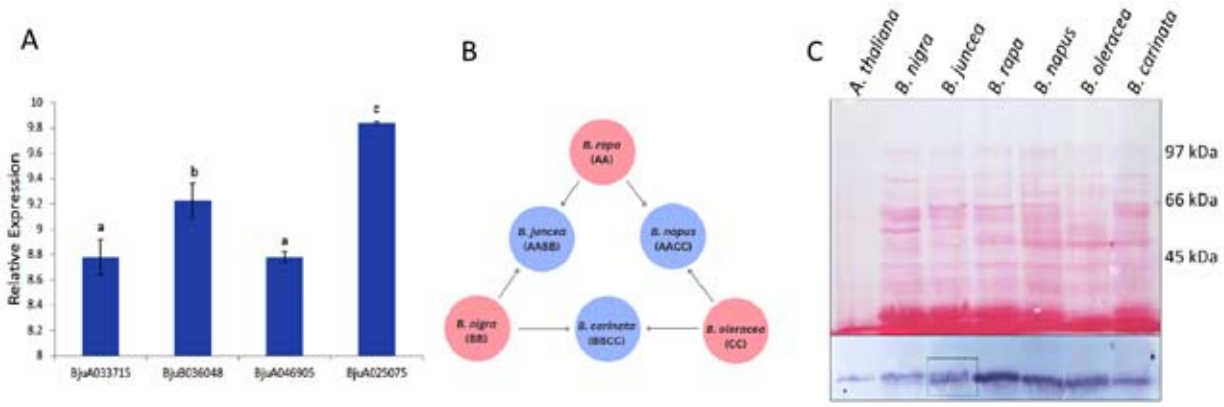


Figure 5: (A) Bar graph showing differential expression of `BjGSNOR in *B. juncea* seeds (B) Triangle of U for *Brassica* (C) Detection of multiple immuno-positive bands of GSNOR.

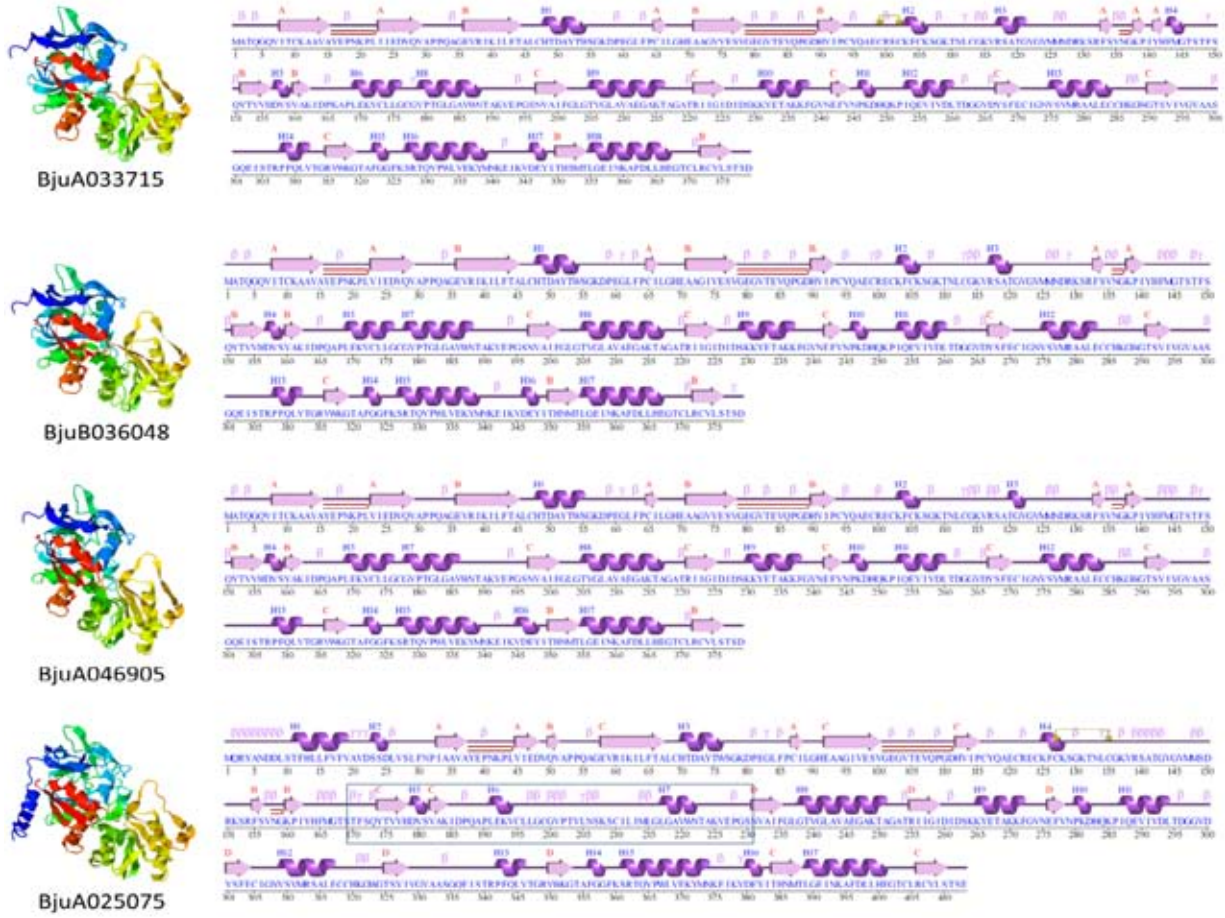


Figure 6: Three Dimensional structure and schematic diagram showing secondary structural elements of BjGSNORs predicted using I-TASSER (left) and PDBsum (right) tools respectively.

genes in *Brassica*. Phylogenetic analysis revealed evolutionary conversation among genera. The presence of multiple forms were confirmed by PCR and western blot analysis in *B. juncea*. Structural prediction and transcriptional analysis suggested different forms may have differential roles. Though further characterization of functional aspects of multiple isoforms of GSNOR is required, the present study provides preliminary evidence of the presence of GSNOR isoforms in *Brassica* species.

Acknowledgements

This work was partially supported by a FRP grant provided by Institute of Eminence, University of

Delhi [IOE/2021/12/FRP]. PB availed senior research fellowship from UGC, India.

References

Akter, A., Itabashi, E., Kakizaki, T., Okazaki, K., Dennis, E. S., & Fujimoto, R. 2021. Genome triplication leads to transcriptional divergence of FLOWERING LOCUS C genes during vernalization in the genus *Brassica*. Frontiers in Plant Science, 11: 619417.

Benhar, M., Thompson, J.W., Moseley, M.A. & Stamler, J.S. 2010. Identification of S-nitrosylated targets of thioredoxin using a quantitative proteomic approach. Biochemistry, 49: 6963–6969.

Chen, H., Wang, T., He, X., Cai, X., Lin, R., Liang, J., Wu, J., King, G. & Wang, X. 2022. BRAD V3. 0: an upgraded Brassicaceae database. Nucleic Acids Research, 50 (D1): D1432-D1441.

Cheng, T., Chen, J., Ef, A., Wang, P., Wang, G., Hu, X. & Shi, J. 2015. Quantitative proteomics analysis reveals that S-nitrosoglutathione reductase (GSNOR) and nitric oxide signaling enhance poplar defense against chilling stress. Planta, 242: 1361-1390.

Díaz, M., Achkor, H., Titarenko, E. & Martínez, M. C. 2003. The gene encoding glutathione-dependent formaldehyde dehydrogenase/ GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. FEBS Letters, 543: 136-139.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M. & Gingeras, T. R. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics, 29: 15-21.

Feng, J., Chen, L. & Zuo, J. 2019. Protein S-nitrosylation in plants: current progresses and challenges. Journal of Integrative Plant Biology, 61: 1206-1223.

Frungillo, L., De Oliveira, J.F.P., Saviani, E.E., Oliveira, H.C., Martínez, M.C. & Salgado, I. 2013. Modulation of mitochondrial activity by S-nitrosoglutathione reductase in *Arabidopsis thaliana* transgenic cell lines. Biochimica et Biophysica Acta (BBA) – Bioenergetics, 1827: 239-247.

Gong, B., Yan, Y., Zhang, L., Cheng, F., Liu, Z. & Shi, Q., 2019. Unraveling GSNOR-mediated S-nitrosylation and multiple developmental programs in tomato plants. Plant and Cell Physiology, 60: 2523-2537.

Hess, D.T. & Stamler, J.S. 2011. Regulation by S-nitrosylation of Protein Posttranslational Modification. Journal of Biological Chemistry, 287: 4411-4418.

Hurali, D. T., Bhurta, R., Tyagi, S., Sathee, L., Sandeep, A. B., Singh, D., Mallick, N., Vinod & Jha, S. K. 2022. Analysis of NIA and GSNOR family genes and nitric oxide homeostasis in response to wheat-leaf rust interaction. Scientific Reports, 12: 803.

Jahnová, J., Luhová, L. & Petřivalský, M. 2019. S-nitrosoglutathione reductase—the master regulator of protein S-nitrosation in plant NO signaling. Plants, 8: 48.

Jo, B. S. &, Choi, S. S. 2015. Introns: The functional benefits of introns in genomes. Genomics & Informatics, 13: 112-118.

Kneeshaw, S., Gelineau, S., Tada, Y., Loake, G. J. & Spoel, S.H. 2014. Selective protein denitrosylation activity of Thioredoxin-h5 modulates plant Immunity. Molecular Cell, 56: 153-62.

Kolbert, Z.S., Barroso, J.B., Brouquisse, R., Corpas, F.J., Gupta, K.J., Lindermayr, C. & Hancock, J.T. 2019. A forty year journey: The generation and roles of NO in plants. Nitric Oxide, 93: 53-70.

Kubienová, L., Kopečný, D., Tylichová, M., Briozzo, P., Skopalová, J., Šebela, M. & Petřivalský, M. 2013. Structural and functional characterization of a plant S-nitrosoglutathione reductase from *Solanum lycopersicum*. Biochimie, 95: 889-902.

Laha, S.D., Dutta, S., Schäffner, A.R. & Das, M. 2020. Gene duplication and stress genomics in Brassicas: Current understanding and future prospects. Journal of Plant Physiology, 255: 153293.

Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. Journal of Applied Crystallography, 26: 283-291.

Lee, U., Wie, C., Fernandez, B. O., Feelisch, M. & Vierling, E. 2008. Modulation of nitrosative stress by S-nitrosoglutathione reductase is critical for thermotolerance and plant growth in *Arabidopsis*. Plant Cell, 20: 786-802.

Lindermayr, C. 2018. Crosstalk between reactive oxygen species and nitric oxide in plants: key role of S-nitrosoglutathione reductase. Free Radical Biology & Medicine, 122: 110-115.

Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J. & Stamler, J. S. 2001. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature, 410: 490-494.

Love, M.I., Huber, W. & Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology, 15: 1-21.

Matamoros, M. A., Cutrona, M. C., Wienkoop, S., Begara-Morales, J. C., Sandal, N., Orera, I. & Becana, M. 2020. Altered plant and nodule development and protein S-nitrosylation in *Lotus japonicus* mutants deficient in S-nitrosoglutathione reductases. Plant and Cell Physiology, 61: 105-117.

Mathur, S., Paritosh, K., Tandon, R., Pental, D. & Pradhan, A. K. 2022. Comparative Analysis of Seed Transcriptome and Coexpression Analysis Reveal Candidate Genes for Enhancing Seed Size/Weight in *Brassica juncea*. Frontiers in Genetics, 13: 814486.

Rizza, S. & Filomeni, G. 2017. Chronicles of a reductase: biochemistry, genetics and physio-pathological role of GSNOR. Free Radical Biology & Medicine, 1, 10: 19-30.

Rodríguez-Ruiz, M., Mioto, P., Palma, J.M. & Corpas, F.J. 2017. S-nitrosoglutathione reductase (GSNOR) activity is down-regulated during pepper (*Capsicum annuum* L.) fruit ripening. Nitric Oxide, 68:.51-55.

Sakamoto, A., Ueda, M., & Morikawa, H. 2002. *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase is an S-nitrosoglutathione reductase. FEBS Letters, 515: 20-24.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-442.

Sanghani, P. C., Robinson H., Bennett-Lovsey R., Hurley T. D. & Bosron W. F. 2003. Structure-function relationships in human Class III alcohol dehydrogenase (formaldehyde dehydrogenase). Chemico-Biological Interactions, 144: 195-200.

Schiessl, S. 2020. Regulation and subfunctionalization of flowering time genes in the allotetraploid oil crop *Brassica napus*. Frontiers in Plant Science, 11: 1824.

Tagliani, A., Rossi, J., Marchand, C.H., De Mia, M., Tedesco, D., Gurrieri, L., Meloni, M., Falini, G., Trost, P., Lemaire, S.D., Fermani, S. & Zaffagnini, M. 2020. Structural and functional insights into nitrosoglutathione reductase from *Chlamydomonas reinhardtii*. Redox Biology, 38: 101806.

Tamura, K., Stecher, G. & Kumar, S. 2021. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution, 38: 3022-3027.

Towbin, H. & Gordon, J. 1984. Immunoblotting and dot immunobinding—current status and outlook. Journal of immunological methods, 72: 313-340.

Xu, S., Guerra, D., Lee, U. & Vierling, E. 2013. S-nitrosoglutathione reductases are low-copy number, cysteine-rich proteins in plants that control multiple developmental and defense responses in *Arabidopsis*. Frontiers in Plant Science, 4: 430.

Yang, J. Yan, R., Roy, A., Xu, D., Poisson, J. & Zhang, Y. 2015. The I-TASSER Suite: Protein structure and function prediction. Nature Methods, 12: 7-8.

Zheng, W., Zhang, C., Li, Y., Pearce, R., Bell, E.W. & Zhang, Y. 2021. Folding non-homology proteins by coupling deep-learning contact maps with I-TASSER assembly simulations. Cell Reports Methods, 1: 100014.