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

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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

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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 redoxbased 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 glutathionedependent 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). GSNORmediated 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	Lin02508 (1140 bp)

Name	Primer Sequence	Tm (°C)	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

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

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 4°C. 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 (BioRad) 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 antirabbit 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 $p \le 0.05$.

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 ± 2.4 Å (for BjuA033715 and BjuA046905); 1.69, 0.95 ± 0.05 and 3.3 ± 2.3 Å (Bju036048); 0.5, 0.78 ± 0.10 , 5.8 ± 3.6 Å Table 3: Protein Identity Matrix of Brassica GSNORs generated by Clustal MUSCLE. Highlighted cells indicate lower levels of pairwise identity.

1	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 1	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 9 B002800 9	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 9	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	89.86	98.68	99.21	99.21	99.21	98.94	98.15	98.15	98.15	98.15	98.15	98.42
Bni B037807 9	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 9	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 A 06g 044700 9	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 9	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 A 09g020720	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
3caC04g21887 9	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 9	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 9	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

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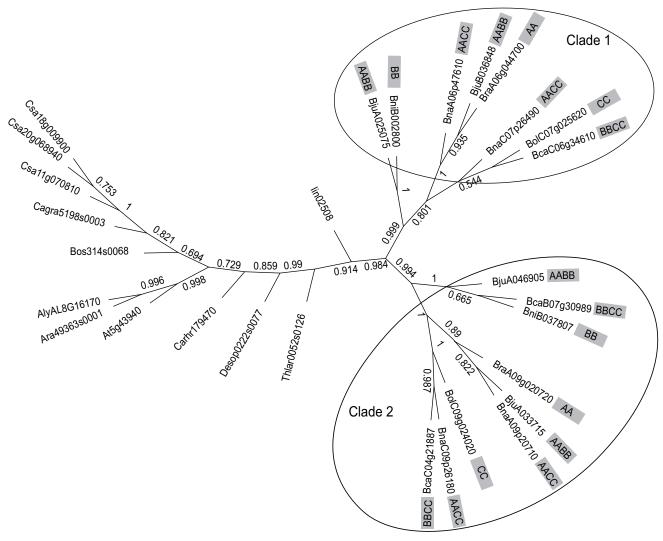


Figure 1: Evolutionary relationships of GSNOR protein sequences belonging to members of the family Brassicaceae. Sequences were aligned using Clustal MUSCLE and the phylogenetic tree (NJ) with 1000 bootstrap replicates (bootstrap values in red) was estimated using Mega 11 version 11.0.11 software.

(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

	10 • • • • • • • • • • • •	20 · · · · · · · ·	30 40		60 70 · · · · · · · · · · · ·	80 90
BraA06g044700 BraA09g020720		MATQGQV	GMYACLFHLKPY	LLLSVNLISTPISSI.	AVAYEPNKPLVIEDVQVAP	PQAGEVRIKILFTA
BniB037807	• • • • • • • • • • • • • • • •					
Bn/8002800 Bca807g30989						
BcaC04g21887 BcaC06g34610						
BcaC06g34610 BolC07g025620						
BolC09g024020 BnaA06p47610						
BnaA09p20710						
BnaC07p26490 BnaC09p26180						
BjuA025075	MORYANDDLSTFHL	LFVFV.VDSSD				
BjuA033715 BjuB036848						
B/uA046905						
	100	110	120 130	140	150	170 180
BroA06g044700	LHTDAYTWSGKDP	1		PGDHVIPCYQAECREC]] [
BraA09g020720	L	EGLFPCILGHE	V			V G V MMN D R K S R F S V
Bni8037807 Bni8002800	· · · · · · · · · · · · · · · · · · ·		· · · · V · · · · · · · · · · · · · · ·	• • • • • • • • • <mark>• •</mark> • • • • • •		S
BcaB07g30989			• • • • • • • • • • • • • • • • • • •	· · · · · · · · · · · · · · · · · · ·		S
BcaC04g21887 BcaC06g34610		• • • • • • • • • •	A	• • • • • • • • • <mark>• •</mark> • • • • • •	• • • • • • • • • • • • • • • • • • • •	
BolC07g025620				<mark></mark>		
BolC09g024020 BnaA06p47610			· · · · V · · · · · · · · · · · · · · ·			
BnaA09p20710			· · · V · · · · · · · · · · · · · · · ·			
BnaC07p26490 BnaC09p26180			A	• • • • • • • • • • • • • • • • • • •		
Bj@A025075						\$
BjuA033715 BjuB036848			· · · · V · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·		
BjuA046905			· · · · V · · · · · · · · · · · · · · ·	••••••		
	190	200	210 220	230	240 250	250 270
BraA06g044700	NGKPIYHEMGTSTE		AKIDPQAPLEKVCLLG		- GLGAVWN TAK V EPGSN VA	
BraA09g020720						
BniB037807 BniB002800						
BcaB07g30989						••••••
BcaC04g21887 BcaC06g34610	D		· · · · · · K · · · · · · · · · · · · ·			
BolC07g025620 BolC09g024020	D			· · · · ·		· · · · · · · · · · · · · · · · · · ·
BnaA06p47610			· · · · · · · · · · · · · · · · · · ·			· · · · · · · · · · · · · · · · · · ·
BnaA09p20710						
Boa(07o26490	D					
BnaC07p26490 BnaC09p26180						
BnaC09p26180 BjuA025075					11.	
BnaC09p26180 BjuA025075 BjuA033715 BjuB036848					۹L	
BnaC09p26180 BjuA025075 BjuA033715					1L	
BnaC09p26180 BjuA025075 BjuA033715 BjuB036848	280	290		J20	330 340	350 360
BraCO9p26180 BjuA025075 BjuA033715 BjuB036488 BjuA046905 BraA06g044700	280				1L	350 360
ВпаСО9́р26180 ВјшА025075 ВјшА033715 ВјшВ036848 ВјшА046905	280	290 1 · · · 1 · · • 1		320	1L	350 360
Brac09p26180 BjuA025075 BjuA025075 BjuA035715 BjuA046905 BruA06g044700 BruA06g044700 BruB0220720 BruB037807	280	290 1 · · · 1 · · • 1	300 330 	320 EVIVDLTDGGVDYSFE	1L	350 360 1 4 4 5 4 5 4 5 4 5 4 5 4 5 4 5 4 5 4 5
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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.

А

65NOR CONA , kb ladde В 2.6 kb 2.3 kb 1.3 kb 1.1 kb 1.9 kb

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 subfunctionalization 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

	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	$0.93{\pm}0.06$	3.5±2.4Å
BjuA025075	0.5	0.78 ± 0.10	5.8±3.6Å

Table 4: Confidence Score for accuracy of predicted

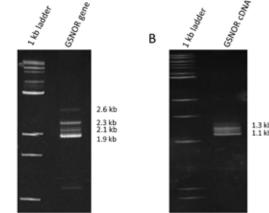
structures

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	11
BjGSNOR.cDNA1 BjGSNOR-1 BjGSNOR-2 BjGSNOR-3 BjGSNOR-4	A T G G C G A C T C A A G G T C A G G T T A T C A C T A T G G C G A C T C A A G G T C A G G T T A T C A C A	100 40 50 60 70 TGCAAAGGTATGTTCTTT - GA A - A - CCCTAT - TGCAAAGGTATGTATGAAA - GCCTATCTACGGTAGTCACATATA TGCAAAGGTACGCAAATGGCTACTTTCTCA - ACCT - TGCAAAGGTACGCAAACGATGA - TCTCTCA - ACCTTTCATCTA
BjöSNOR.¢DNA1 BjöSNOR-1 BjöSNOR-2 BjöSNOR-3 BjöSNOR-4	CACTCTGAGTTTATATGAAACCCTATC	100 110 120 130 140 1 1 1 1 1 1 1 TACGTIGTIGTCGGA TCAATCTAATTTCGA CTCCCATCTCCT TG TAAGGTIGTTGTTGATTT TTTGACTCC GATCTCC GATCTCC ACGGTTGTTGATTTCATTCGATCTGGTTTCTCTCTCA EAC
BJGSNOR.¢DNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	C A A A G C T G C G G T G G C C T A C G A G C C C A A A G C T G C G G G G G G C C T A C G A G C C C A A A C A T A G C T G C G G T G G C C T A C G A G C C G A A C A T A G C T G C G C T G C C T A C G A G C C G A A C A T A G C T G C G C T A C G A G C C G A A C A T A G C T G C G C T A C G A G C C G A A C A C A G C C G A A C A C	170 180 180 200 210 CAAACCTCTCATCATCGAAGATGTTCAAGTCGCTCCCCCTCAG CAAACCTCTCATCATCGAAGATGTTCAAGTCGCTCCCCCTCAG 100
BjGSNOR.cDNA1 BjGSNOR-1 BjGSNOR-2 BjGSNOR-3 BjGSNOR-4	6 C C G G C G A G G T T C G G A T C A A G A T C C T A G C C G G C G A G G T T C G G A T C A A G A T C C T A G C C G G T G A G G T T C G G A T C A A G A T C C T C G C C G G T G A G G T T C G C A T C A A G A T C C T C T	240 250 260 270 280 TTCACGGCTCTCTGTCACACGCGACGCCTACACTTGGAGCGGCA TTCACGGCTCTCTGTCACACCGACGCCTACACTTGGAGCGGCA TTCACGGCTCTCTGTCACACGGACGCCTACACTTGGAGCGGCA TTCACGGCTCTCTGCCACACGCCGACGCCTACACTTGGAGCGGCA TTCACCGCTCTCTGTCACACCGACGCCTACACCTGGAGCGGCA TTCACCGCTCTCTGTCACACCGACGCCTACACTTGGAGCGGCA TTCACCGCTCTCTGTCACACCGACGCCTACACTTGGAGCGGCA
BjGSNOR.cDNA1 BjGSNOR-1 BjGSNOR-2 BjGSNOR-3 BjGSNOR-4	AGG AGGTTCACTCTT - TTCTCTCCAC TC AGGTTCACACTCCTTCTCCCCCCATCT AGGTCCGTCTGA - ATCTCCTTCTTACT	310 320 350 340 350 GCTACGGCT TAGGG ATTCAAATCTCT TCATTCATCGCTATCGAT TTAGGGATCCAAATTTCT ACTACTGATTCAATCGATCGAGTCCATTTATTAGGGATCTAACATCT TCGATTGAT TCGAGTCGATTTATTAGGGATCTAACATCT TCGATTGAT TCGAGTCGATTTATTAGGGATCAACATCT
BJGSNOR.¢DNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	G A T C C - T C T T T C C A T T T G C A G G A T C C G A T T C T C C A T T C T C T C G A T G C A G G A T C T T T G A T T G T G G A T T T A G G A T C	380 390 400 410 410 CTGAAGGTCTCTTCCCATGTATCCTCGGTCATGAAGCTGCTGG CTGAAGGTCTCTTCCCATGTATCCTCGGTCATGAAGCTGCTGG CCGAAGGTCTCTTCCCATGTATCCTCGGTCATGAAGCTGCTGG CGAAGGTCTCTTTCCCTGTATCCTCGGTCATGAAGCTGCTGG CCGAAGGTCTCTTCCCTGTATCCTCGGTCACGAGGCTGCTGG CCGAAGGTCTCTTCCCTTGTATCCTCGGTCACGAGGCTGCTGG
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	GTAAGTTCCCCAATCATCATGTAATAT	450 460 470 480 490 TTACTCCCTCCCTTCCGAAAAGATTTATGTTTTAGAAAAAATAA TAG CTCCCACTTTTTAAGTTAGGTTTCTT - ACTGTTATATAA
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4		520 530 540 550 540 GTAATGGCACTCCAGCTGGAGTGGCCTATCCTGATTCGAGTAGG GTAGTGGAGTGGAGTAGC CTAGCTGGATTCGAGTAGC GTAGCTGGACTCGAACTGGAGAGA C C TACTGCT CTTAGCTTTTAACTAAAAAA ATCAAATCAT
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4		560 600 610 620 610 T T G C C C G G A T G G T G A T C C C A G G G G A T A T A A A A A A A A A T A A T I <
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	AATAATAATTTAGTTACAAAAAATACA	TTTTTAATGTAAACTTCAAACAATTATTAAAATTTTATTTGTT TÅ AGAAACGTCTCTTAG TGTTTTTAGTT
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	AAAAGTTGTGTACAATAGTTAATTATT	730 740 750 760 770 1 1 1 1 1 1 1 T T T A T A A A T G T G A A C A T T T G C T G A C A A A A A A A A A A A A A T T A A A A G A G A - C T G T A T C T T A T A T A A A G A G A - C T G T A T C T T A T A T T A
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	CGCTAAAAACCCCAACCTAAGAGAC	800 810 810 850 840 TTGAGATTCTCCCCCAATGCTAAAGATCTTTTTTTTGTTTG
BjöSNOR.cDNA1 BjöSNOR-1 BjöSNOR-2 BjöSNOR-3 BjöSNOR-4	850 T G G G A C A G G G T G T G A G A G T G T G G T G G G A C A G G G T T G T T G A G A G T G T T G G T G G G A C A G G G T T G T T G A G A G T G T T G G T G G A G C A G G A T T G T G A G A G T G T T G G	BTO B

	990 1000 1010 1020 1050	1010 1010
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	66T C C G C T A C T G G T G T G G G G T C A T G A T G A A G A C G A C G T A G A A G C G G T T C T C G G T T 1010 1000 </td <td>A A T G G G A A A C C C A T A A T G G G A A A C C C A T A A T G G G A A A C C C A T</td>	A A T G G G A A A C C C A T A A T G G G A A A C C C A T A A T G G G A A A C C C A T
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	1060 1070 1080 1000 1000 TTATCACTTCATGGGTACCTCCACGTTAGTCAGTACACTGTTGTCATGACGTCA TTATCACTTCATGGGTACCTCCACGTTAGTCAGTACACTGTTGTCATGACGTCA TTATCACTTCATGGGTACCTCCACGTTAGTCAGTACACTGTTGTCATGACGTCA TTATCACTGTCATGGGTACACTCCACGTTAGTCAGTACACTGTTGTCATGATGTCA TTATCACTTCATGGGTACCTCCACGTTAGTCAGTACACTGTTGTCATGATGTCA TTATCACTTCATGGGTACCTCCACGTTAGTCAGTACACTGTTGTCATGATGTCA TTATCACTTCATGGGTACCTCCACGTTAGTCAGTACACTGTTGTCATGATGTTA TTATCACTTCATGGGTACCTCCACGTTAGTCAGTACACTGTTGTCATGATGTTA	G T G T C G C C A A G A T C G T G T C G C C A A G A T C G T G T C G C C A A G A T C G T G T T G C C A A G A T C G T G T G G C T A A A A T C
BJGSNOR ¢DNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	III IIII III IIII III IIII III III IIII	IIII IIII IIII IIIIIIIIIIIIIIIIIIIIIII
	1200 1210 1220 1230 1240	1250 1260
BJGSNOR.¢DNA1 BJGSNOR+1 BJGSNOR+2 BJGSNOR+3 BJGSNOR+4	TCAAACTCAGATTGATTATGTGCACTCCCTGTTACACGTTTTACTGCTACATATTA TTGGTTGATTATGTGCAGTCCTTGTAACACGTTTTACTGCTACATATTA AGTG TCACTGTTTCGTAGTCT TCACTTT GATTAAATGCAGTC	TGTTTATTGAAGCA
NGSNOR.cDNA1 NGSNOR-1 NGSNOR-2	1270 1280 1280 1300 1300 1310 	TTTGCTATCTAATA
BJGSNOR-3 BJGSNOR-4	TTCA- TTTGTTGT TACCCA-	
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2	1340 1350 1360 1370 1370 1380 TAATGTTATGAACCTATTAGGTCTTGGGAGTTTTTTTGGGGGATGTGAAGTCTATGTT ACAAGGTC	1390 1400 TCTTGATCCTATTA
BJGSNOR-3 BJGSNOR-4	G	
	1410 1420 1430 1440 1450	1460 1470
JGSNOR.cDNA1 JGSNOR-1 JGSNOR-2 JGSNOR-3	GAATACTGCAAACTTGATTGATTATGTGCAGTCTTTGTTACACATTTTATGTGCTC	
SIGSNOR-4		
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3	1480 1490 1500 1510 1510 1510 T C T C T T T G G A G T G T G T T A C T A C C C A T T A T G T T G T C A A A G C G A C A T C A C T A A T A A - T G - T G T G A A G T G A T T C C T A G C T A T T A A T T T T A T G C A A C A T C A C T G A T A T T C T G T T T A	GATTGATCTACATG
SJGSNOR-4	TCTCTTTTTGAAA	
GSNOR cDNA1	1550 1560 1570 1580 1580 1590	1600 1630
NGSNOR-1 NGSNOR-2 NGSNOR-3 NGSNOR-3	TTTTCTTTGCCATCCTGTTTATTCTATCTGATAGGGAATCTAAACTAGTAGCCCAC GTTTCTTCACCATTCTATTTTTTTGCAT ATCT T TCTTC TTG CGATGACTACCTCTGTTTACAG T	TTTCTCTGTGAATT
	1630 1650 1660 1660	1670 1680
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	T T T T G G T G AT AT T G A AG T A G C T A A C AG T AT T AT AT C AT G T T T C T G AT AC T A T T T T T C T G AT AT T A A A C T C T A T G G T C C C T A A C AG T AG AT C AT G T T T G T G AT C T A C C T C T AG C AG T AT C T A T G T AT C T G AT C T G AT C T G AT C AT G A A C AG T A A A T C AT G T AT C T T G AT C AT G	C T T G G A G C T C A G G T C T T G G A G C T T A G G C C T T G G A G C T T A G G C C T T G G A G C
	1690 1700 1710 1720 1730	1740 1750
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	1600 1700 1710 1720 1720 1720 TGTTTGGAATACTGCAAAAGTAGAACCAGGGTCTAATGTTGCCATCTTTGGTCTG TGTTTGGAATACTGCAAAAGTAGAACCAGGGTCTAATGTTGCCATCTTTGGTCTG AGTTTGGAACCTGCAAAAGTAGAACCAGGGTCAAATGTTGCCATTTTGGGTCTG AGTTTGGAACCTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTCGGTCTG AGTTTGGAACACTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTTGGGTCTG AGTTTGGAACACTGCAAAAGTAGAACTTGGCCTGGGTCAAATGTTGCCATTTTTGGTCTTG	GGACTGTTGGACTT GGACTGTTGGACTT GCACTGTTGGGCTT GGACCGTGGGGGCTT
BJGSNOR-1 BJGSNOR-2 BJGSNOR-8	TGTTT GGAATACT GCAAAAGTAGAAC CAGGGT CTAATGTT GCCAT CTTT GGTCT TG GTTT GGAATACT GCAAAAGTAGAAC CAGGGT CTAATGTT GCCAT CTTT GGTCT TG AGTTT GGAACACT GCAAAAGTAGAAC CT GGGT CAAATGTT GCCAT TTT CGGT CT TG AGTTT GGAACACT GCAAAAGTAGAGC CT GGGT CAAATGTT GCCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT AGGAC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT AGGAC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TTT TGGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT GGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT GGT CT TG AGTTT GGAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT GGT CT TG AGTT TG GAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT GGT CT TG AGTT TG GAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT GGT CT TG AGTT TG GAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT GGT CT TG AGTT TG GAACACT GCAAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT GGT CT TG AGTT TG GAACACT GCAAAGT TG AGC CT GGGT CAAATGT TG CCAT TT TT TG GT CT TG AGTT TG	1810 1820
BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4 BJGSNOR-1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3	TGTTT GGAATACT GCAAAAGTAGAAC CAGGGT CTAATGTT GCCAT CTTT GGT CTTG GTTT GGAATACT GCAAAAGTAGAAC CAGGGT CTAATGTT GCCAT CTTT GGT CTTG AGTTT GGAACACT GCAAAAGTAGAAC CT GGGT CAAATGTT GCCAT TTT CGGT CTTG AGTTT GGAACACT GCAAAAGTAGAAC CT GGGT CAAATGTT GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT AGGAC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT AGGAC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT TTT T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT T T T T GGT CTTG AGTTT GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT T T T T GGT CTTG AGTT T GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT T T T T GGT CTTG AGTT T GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT T T T T GGT CTTG AGTT T GGAACACT GCAAAAGT T GAGC CT GGGT CAAATGT T GCCAT T T T T GGT CTTG	66 A C T G T T G G A C T T 66 A C T G T T G G A C T T 66 A C C G T G G G G G C T T 66 A C C G T G G G G G C T T 1810
jasnor-1 jasnor-2 jasnor-3 jasnor-4 jasnor-4 jasnor-1 jasnor-1 jasnor-2 jasnor-3	16111166AATACT6CAAAAGTA6AACCA66GTCTAATGT16CCATCTT16GTCT16 16111166AATACT6CAAAAGTA6AACCA66GTCTAATGT16CCATCTT16GTCT16 AGTAT6GAACACT6CAAAAGTA6AACCA66GTCTAATGT16CCATTT1C6GTCT16 AGTAT6GAACACT6CAAAAGTA6AACCA66GTCAAATGT16CCATTTT1C6GTCT16 AGTAT6GAACACT6CAAAAGTA6A6CCT66GTCAAATGT16CCATTTT16GTCT16 AGTAT6GAACACT6CAAAAGTA6A6CCT66GTCAAATGT16CCATTTT16GTCT16 AGTT76GAACACT6CAAAAGTA6A6CCT66GTCAAATGT16CCATTTT16GTCT16 AGTT76GAACACT6CAAAAGTA6A6CCT66GTCAAATGT16CCATTTT16GTCT16 6CT6 6CT6 6CT61AA6CTACTACCTC T1CTAAAACTTCT 6CT61AA6CTACT6CCT T1CTAAAAATTTCT 6CT61AA6CTACT6CCT T1CTAAAACTTCT 6CT61AA6CTACT6CCT T1CTAAAACTTCT 6CT61AA6CTACT6CCT T1CTAAAACTTCT 6CT61AA6CTACT6CCT T1CTAAAACTTCT 6CT61AA6CTACT6CCT T1CTAAAACTTCT 6CT61AA6CTACT6CCT T1CTAAACTTCT 6CT61AA6CTACT6CCCT T1CTAAACTTTTTTTTTTTCCCCAT6G 6CT61AA6CTACTACCCCCTTTCTAAGACTTCATCTTTTTTTTTT	66 A C T G T T G G A C T T 6 G A C T G T T G G A C T T 6 G A C G T T G G G C T T 6 G A C C G T G G G G C T T 1810 1820 A A G T G A A C G T T T T G A A G T G A A C G T T T T G T A A G A G A A C G T T T C T G A A G A G A A C T T T C T G A A G A G A A G T T T C T G 1850 1980
BjGSVOR-1 BjGSVOR-2 BjGSVOR-2 BjGSVOR-4 BjGSVOR-4 BjGSVOR-1 BjGSVOR-2 BjGSVOR-4 BjGSVOR-4 BjGSVOR-1 BjGSVOR-2 BjGSVOR-2 BjGSVOR-3	TGTTTGGAATACTGCAAAAGTAGAACCAGGGTCTAATGTTGCCATCTTTGGTCTTG GTTTGGAATACTGCAAAAGTAGAACCAGGGTCTAATGTTGCCATCTTTGGTCTTG AGTTTGGAACACTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTCGGTCTG AGTTTGGAACACTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTCGGTCTTG AGTTTGGAACACTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTTGGTCTTG AGTTTGGAACACTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTTGGTCTTG GCTG GCTGTAAGCTACTGCCT. TTCTAAAACTTCT. GTAGTTCCATTGCTTCCCATGG GCTGTAAGCTACTGCCT. TTCTAAAACTTCT. GTAGTTCCATTTTCCCCATGG GCTGTAAGCTACTGCCT. TTCTAAAACTTCT. TTCTAATTCCATTGCTTTCCCCATGG GCTGTAAGCTACGCCT. TTCTAAAACTTCT. TTTTTGTTTTCCCCATGG GCTGTAAGCTACGCCT. TTCTAAAGATTCC. TTTTTTGTTTTCCCCATGG GCTGTAAGCTACGCCC. TTCTAAAGCTTCC. TTTTTTGTTTTCCCCATGG GCTGTAAGCTACATCCCCTTTCTAGGACCTTCCATTTTTTTT	66 A C T G T T G G A C T T 66 A C T G T T G G G C T T 66 A C C G T G G G G C T T 66 A C C G T G G G G C T T 1110 11
BJGSNOR-1 BJGSNOR-2 BJGSNOR-3	TGTTTGGAATACTGCAAAAGTAGAAACCAGGGTCTAATGTTGCCATCTTTGGTCTTG GTTTGGAACACTGCAAAAGTAGAAACCAGGGTCTAATGTTGCCATCTTTGGTCTTG AGTTTGGAACACTGCAAAAGTAGAAACCAGGGTCAAATGTTGCCATTTTCGGTCTTG AGTTTGGAACACTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTTGGTCTTG AGTTTGGAACACTGCAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTTGGTCTTG AGTTTGGAACACTGCAAAAGTTGAACCTGGGTCAAATGTTGCCATTTTTGGTCTTG GCTG GCTG GCTG GCTG AGCTAGCACCTCCT ICTAAAGCTACCCCT ICTAAAGCTACCCCT ICTAAAGCTACCCCCTTTCTAAAGATTCC ICTAAGCTACCGCCCT ICTAAGCTACCGCCCT ICTAAGCTACCGCCCT ICTAAAGCTACCCCCCCTTTCTAAGGATCCC ISSO	66 A C T G T T G G A C T T 66 A C T G T T G G G C T T 66 A C C G T G G G G C T T 66 A C C G T G G G G C T T 1110 11
BjGSVOR-1 BjGSVOR-2 BjGSVOR-2 BjGSVOR-4 BjGSVOR-4 BjGSVOR-1 BjGSVOR-2 BjGSVOR-4 BjGSVOR-4 BjGSVOR-1 BjGSVOR-2 BjGSVOR-2 BjGSVOR-3	16111166AATACT6CAAAAGTAGAACCAGGGTCTAATGTTGCCATCTTTGGTCTTG 16111166AATACT6CAAAAGTAGAACCAGGGTCTAATGTTGCCATCTTTGGTCTTG AGTTTGGAACACT6CAAAAGTAGAACCAGGGTCTAATGTTGCCATCTTTGGTCTTG AGTTTGGAACACT6CAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTGGTCTTG AGTTTGGAACACT6CAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTGGTCTTG AGTTTGGAACACT6CAAAAGTAGAAGCCTGGGTCAAATGTTGCCATTTTTGGTCTTG AGTTTGGAACACT6CAAAAGTAGAACCTGGGTCAAATGTTGCCATTTTTGGTCTTG AGTTTGGAACACT6CAAAAGTAGAAGCTGAGCTGAGGTCAAATGTTGCCATTTTTGGTCTTG AGTTTGGAACACT6CACTACTACT 1700 1780 1700 1800 6CTG 6CTG 6CTGTAAGCTACTACTACTTTTCTCCATGG 6CTGTAAGCTACTACCTC TTAATTCGTAATTTTCCCCATGG 6CTG AAGCTACCACCT TTCTAAAGCATTCC TTAATTCGTTTTCCCCATGG 6CTG AAGCTACCACCT TTCTAAATCTTTCATCT TAAGTCGCTACTACCCT TTAATTCGTAAGGTTCCCCT 6CTG AAGCTACCACCCT TTCTAAATCTTTCCCCCTAGG TTCTTTTTTTTTCCCCCATGG 1850 1850 1850 1870 1850 1850 1860 1870 1850 1850 1870 1870 1850 1850 1870 1870 1850 1850 1870 1870 1850 <t< td=""><td>GG A C TG T TG G A C TT GG A C TG T TG GG A C TT GG A C TG T TG GG C TT GG A C CG TG GG GG C TT IBIO IBIO A A G TG A A CG T TT TG A A G A A G A A CG T TT TG A A G A A G A A CG T T T TG A A G A A G A A CG T T T TG A A G A G A A CG T T T TG A A G A G A G A C T T TG TG A A G A G A G C T A C A A G G C TG G TG C T A C A A G G C TG G TG C T A C A A A A A A A A A A A A A A A A</td></t<>	GG A C TG T TG G A C TT GG A C TG T TG GG A C TT GG A C TG T TG GG C TT GG A C CG TG GG GG C TT IBIO IBIO A A G TG A A CG T TT TG A A G A A G A A CG T TT TG A A G A A G A A CG T T T TG A A G A A G A A CG T T T TG A A G A G A A CG T T T TG A A G A G A G A C T T TG TG A A G A G A G C T A C A A G G C TG G TG C T A C A A G G C TG G TG C T A C A A A A A A A A A A A A A A A A

BJGSNOR.¢DNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	1870 1880 1990 2000 2010
BJGSNOR.¢DNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2040 2050 2060 207 2070 E7 2080 2090 2000 2000 2000 2000 2000 2000
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2110 2110 2120 2130 2140 2150 2150 2160 2160 2170 2170 2160 2170 2170 2160 2170 2170 2160 2170 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2160 2160 2170 2160 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2160 2160 2170 2160 2160 2170 2160 2160 2160 2160 2170 2160 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2160 2170 2170 2160 2170
BJGSNOR.¢DNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2180 2100 2210 2220 2220 2220 2220 2220 2220 2220 2220 2220 2220 2220 2220 2220 2220 2220 2200 2220 2200 2000 2200 2200
BJGSNOR.¢DNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2250 2260 2270 2280 2280 2350 2350 2350 2350 2350 2350 2350 235
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2330 2330 2340 23 2340 23
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2390 2400 2410 2410 2410 2410 2410 2410 241
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2440 2470
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2530 2540 2540 2550 2540 2550 2540 2570 2580 2570 2580 25 25 25 25 25 25 25 25 25 25
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2600 2610 2610 2610 2610 2600 2640 2640 2650 2660 G T G G A T G A G T A C A T A A C G C A C G G A T G A C T G G G A G A G A T C A A C A A G G C T T T G A T C T G C T G C A G A A G G T G G A T G A G T A C A T A A C G C A C A G C A T G A C A T G G G A G A G A T C A A C A A G G C T T T T G A T C T G C T G C A G A A G G T G G A T G A G T A C A T A A C G C A C A G C A T G A C A T G G G A G A G A T C A A C A A G G C T T T T G A T C T G C T G C A T G A A G G T G G A T G A G T A C A T A A C G C A C A T G A C A T G G G A G A G A T C A A C A A G G C T T T T G A T C T G C T G C A T G A A G G T G G A T G A G T A C A T A A C G C A C A A C A T G A C C T T G G G A G A G A T C A A C A A G G C T T T T G A C C T G T T G C A T G A A G G T G G A T G A G T A C A T A A C G C A C A A C A T G A C C T T G G G A G A G A T C A A C A A G G C T T T T G A C C T G T T G C A T G A A G G T G G A T G A G T A C A T A A C G C A C A A C A T G A C C T T G G G A G A G A T C A A C A A G G C T T T T G A C C T G T T G C A T G A A G G T G G A T G A G T A C A T A A C G C A C A A C A T G A C C T T G G G A G A G A T C A A C A A G G C T T T T G A C C T G T T G C A T G A A G
BJGSNOR.cDNA1 BJGSNOR-1 BJGSNOR-2 BJGSNOR-3 BJGSNOR-4	2670 2680 2690 G C A C T T G T C T T C G T I G T G T C C T C A G T A C C A G C G A T T G A G C A C T T G T C T T C G T I G T G T C C T C A G T A C C A G C G A T T G A G C A C T T G T C T T C G T I G T G T C C T C A G T A C C A G C G A T T G A G C A C T T G C C T T C G T I G T G T C C T C A G T A C C A G C G A T T G A G C A C T T G C C T T C G T T G T G T C C T C A G T A C C A G C G A A T G A G T A C T T G C C T T C G T T G T G T C C T C A G T A C C A G C G A A T G A

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.

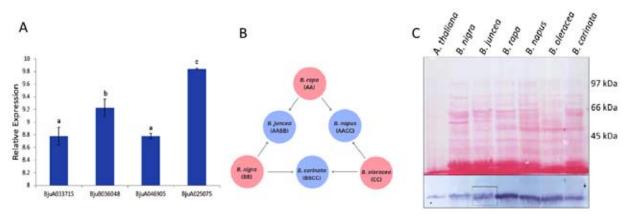


Figure 5: (A) Bar graph showing differential expression of `BjGSNOR in *B. juncea* seeds (B) Triangle of U for *Brassica* (C) Detection of multiple immuno-positive bands of GSNOR.

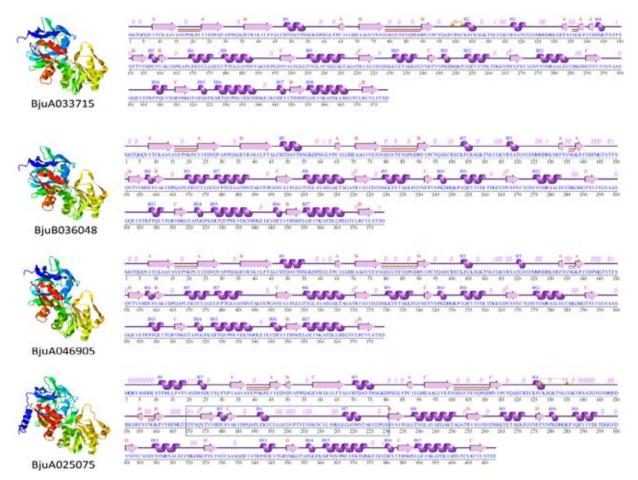


Figure 6: Three Dimensional structure and schematic diagram showing secondary structural elements of BjGSNORs predicted using I-TASSER (left) and PDBsum (right) tools respectively.

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.

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