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ORIGINAL ARTICLE

Cloning and molecular characterization of high-affinity nitrate transporter gene *BjNRT2.1* from Indian mustard

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Abstract Inducible high-affinity nitrate transporter gene (*BjNRT2.1*) was isolated and structurally characterized from Indian mustard (*Brassica juncea* L.) seedlings grown in 10 μM nitrate solution. By performing 5′RACE-PCR, the full length cDNA of 1593 bp was obtained. Alignment and comparative analyses with other NRT2 proteins showed that BjNRT2.1 encodes a member of HATS family. Highly conserved motifs between AtNRT2.1 and BjNRT2.1 suggested that it is an orthologue of AtNRT2.1. Hydropathy plot of BjNRT2.1 predicted 12 membrane spanning α-helices with both C- and N-terminal inside. Reliability of this model was supported by the presence of major facilitator superfamily and nitrate-nitrite porter signatures associated with NRT2 transporter and conserved arginine residues. Induction of *BjNRT2.1* transcripts at 10 μM nitrate concentration only in roots confirmed that it is root-specific and a member of inducible HATS gene family.

Keywords *Brassica juncea* · Full length cDNA · High affinity nitrate transporter · Nitrogen · Root-specific expression

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Introduction

Indian mustard (*Brassica juncea* (L.) Czern and Coss) is an important source of vegetable oil after soybean and palm oil. Brassica requires 40–50 kg more N as compared to cereals (30 % more than wheat) (Colton and Sykes 1992). However, the NUE in *Brassica* species varies between 12 and 36 % depending upon N source and water availability (Gan et al. 2008). Poor NUE is of great concern in *Brassica species*, therefore, improving NUE is an important alternative for sustainable agriculture.

Nitrate uptake into plants from soil via roots involves two basic transport system identified by kinetic studies: high-affinity (HATS), operating under low external concentration ($K_m < 200$ μM), and low-affinity (LATS) operating under higher external concentrations (>50 mM) without saturation (Crawford and Glass 1998). In *Arabidopsis*, entire family of *NRT2* genes comprising of seven members that controls the flux of nitrate from soil into root tissues and throughout the plant system has been characterized. *AtNRT2.1* belongs to inducible HATS and is strongly expressed in root plasma membrane under nitrate starvation and down-regulated under increasing nitrate concentration (Filleur and Daniel-Vedele 1999). More than 40 full length cDNA sequences encoding *NRT2* transport components have been identified sharing 25 % similarity to *crnA* gene of *Aspergillus nidulans* (Trueman et al. 1996) and 43 % with *CrNRT2* of *Chlamydomonas reinhardtii* (Zhou et al. 2000). The *NRT2* genes cloned and characterised from crop plants include *BnNRT2* (*B. napus*) (Faure-Rabasse et al. 2002), *GmNRT2* (*Glycine max*) (Amarasinghe et al. 1998), *NpNRT2.1* (*N. plumbaginifolia*) (Fraisier et al. 2000), *HvNRT2.1* (BCH1) and *HvNRT2.2* (BCH2) from barley (Vidmar et al. 2000) and *TaNRT2.1* (Yin et al. 2007) from *Triticum aestivum*. In

the present study, an attempt was made to isolate a full-length cDNA of high-affinity nitrate transporter from Indian mustard.

Materials and methods

Plant growth conditions

Seeds of Indian mustard (*B. juncea,* $2n = 36$, AABB) var. 'Pusa Bold' were surface sterilized with 70 % ethanol for 2 min, followed by sodium hypochlorite (1.5 % v/v) and Tween-20 (0.1 % v/v) solution for 20 min and finally washed with distilled water. Sterilized seeds were spread on germination paper in a petridish and kept in dark at room temperature. On third day, seedlings were transferred to nylon mesh placed on a plastic container filled with 3 l of deionized water and allowed to grow for 3 days. On 7th day after germination, full strength Hoagland solution was supplied to the seedlings in 1 l containers. The whole set-up was raised under controlled environment at National Phytotron Facility, New Delhi. Growth conditions were maintained at 25/15 °C day/night temperature with 14/10 h light/dark cycle under a photon flux density of 360 μmol m^{-2} s^{-1} and 90 % relative humidity. Composition of nutrient solution used was: 0.5 mM K_2SO_4, 0.05 mM $CaHPO_4{\cdot}2H_2O$, 0.5 mM H_3PO_4, 2 mM $MgSO_4$, 3 μM $FeCl_3$, 3 μM EDTA, 1 μM H_3BO_3,1 μM $ZnCl_2$, 0.2 μM $CuCl_2{\cdot}2H_2O$, 0.5 μM $MnCl_2$, 0.075 μM H_2MoO_4. Nitrate treatment was provided as KNO_3 at three concentrations, viz, 10 μM (very low-N), 50 μM (low-N) and 5,000 μM (control), determined on the basis of physiological parameters such as biomass, leaf area, root traits and nitrate reductase activity. The pH of solution was adjusted to 5.6 throughout the experiment. The solution was changed at 2 days interval and aerated continuously using aquarium pumps and tubing. About 20 seedlings per container were raised for 20 days. Sampling was done on 28 days after germination and tissues were immediately frozen in liquid nitrogen for RNA isolation.

Gene isolation, cloning and sequencing

RNA was extracted from roots as per the manufacturer's protocol (PureLink RNA Mini kit, Ambion) and cDNA made using two-step cDNA synthesis kit (SSIII First Strand synthesis, Invitrogen). The gene-specific primers for *BjNRT2.1* were designed using cDNA sequence of *BnNRT2* (AJ293028):

Forward: 5′-GGC TCT AGA TGG GTG ATC CTA GTG GCG AG-3′

Reverse: 5′-TGA GCT CTC AAA CAT TGT TTG GTG TGT TCT C-3′.

Gene amplification was done using high-fidelity Phusion^{TM} DNA polymerase. Amplicon of 1.34 kb was extracted and purified from gel using QIAquick Gel Extraction kit (Qiagen). The PCR product was cloned in pGEM-T Easy vector (Promega) and transformed chemically competent cells of *E. coli* (DH5-α). Blue-white screening of recombinants was done on Luria agar containing 1 μg ml^{-1} ampicillin. Transformed colonies were confirmed by colony PCR and restriction digestion with *Eco RI*. Positive clones were sequenced using T7 and SP6 universal primers along with an internal primer, 5′-GCA ATA TCA GAT GCA TAG CCT CCT-3′. The obtained sequences were analyzed using software Vector NTI ver. 10 (Invitrogen). A partial sequence was obtained lacking about 260 bp at 5′, while it was complete at 3′.

5′-Rapid amplification of cDNA ends

To obtain full length coding sequence of the gene, 5′ RACE-PCR was performed, using three gene specific primers (GSP) and Abridged Universal Amplification Primer (AUAP). The following GSP primers were designed according to the sequence data obtained from *B. juncea* cDNA fragment.

GSP1: 5′-AGCGTTTCCTATGTCT-3′ (16 bp), at position 311
GSP2: 5′-TCACGGATGATGGGGACGAGTGG-3′ (22 bp), between position 252 and 274
GSP3: 5′-GCAGCTGCGAATGTGGAGACAA-3′ (21 bp) between position 229 and 252
AUAP: 5′-GGCCACGCGTCGACTAGTAC-3′

Reverse transcription (RT) was performed using total root RNA for the first strand cDNA synthesis according to the manufacturer's protocol (Invitrogen). RT product obtained from this reaction was used as a template for 5′RACE-PCR. First strand cDNA synthesis was primed using GSP1. Tailed cDNA was then amplified by PCR using a mixture of three primers: a nested GSP2, which anneals 3′ to GSP1; and a combination of a complementary homopolymer-containing anchor primer and corresponding adapter primer, which permits amplification from the homopolymeric tail. This allows amplification of unknown sequences between GSP2 and 5′-end of the mRNA. A 260 bp product was amplified by RACE-PCR. To obtain the full length cDNA, RT-PCR was performed on cDNA isolated from roots using gene specific primers (forward primer with XbaI: 5′-GCTCTAGA ATG GGT GAT CCT AGT GGC GAG-3′, and reverse primer with SacI:

5′-TGAGCTC TCA AAC ATT GTT TGG TGT GTT CTC-3′), which produced 1.59 kb fragment.

Tissue specific expression analysis

Total RNA was extracted from roots and leaves from seedling grown at 10, 50 and 5,000 μM nitrate concentrations as above. RT-PCR was performed using gene specific primers and the PCR product was run on 1 % agarose gel.

Quantitative real-time (qRT) PCR analysis

For gene induction analysis, *Brassica* seedlings were grown as mentioned above in complete nutrient solution for 15 days followed by N starvation for another 5 days by removing nitrate from the basal solution. For inducing high-affinity transporter, the seedlings were transferred to solutions containing a range of nitrate concentrations (0, 5, 10, 20, 30, 40, 50, 5,000 μM) and roots were sampled after 30 and 120 min. Total RNA from root was extracted and treated with RNase-free DNase I (Invitrogen) for complete removal of genomic DNA. First strand cDNA was synthesized as described above. For normalization of real-time data, *BnACT* (actin) was used as a reference gene (F 5′-AGC AAC TGG GAT GAC ATG GAG-3′; R 5′- CTC CTC AGG AGC AAT ACG GAG CTC GT-3′). Gene specific primers were designed from *BjNRT2.1* sequence (F 5′-CTT TGT CCC TGG TTG GTT GC-3′; R 5′- GCT CAA CTC AAC TCC CAT GGA-3′). qRT-PCR was carried out in a 25 μl reaction volume containing reverse transcribed template, primers and SsoFast^{TM} EvaGreen$^{®}$ Supermix (Bio-Rad) on MiniOpticon real time PCR system (CFB-3120EDU, Bio-Rad). Reaction conditions were: 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 62 °C for 20 s. The gene expression analysis had three biological replicates. The change in threshold cycle ($\Delta\Delta C_T$) was calculated for each sample and gene expression was expressed as normalised fold change.

Bioinformatics

The full length mRNA and amino acid sequences for *NRT2.1* gene from *B. juncea* was queried against the nucleotide and protein database of NCBI (http://www.ncbi.nlm.nih.gov) using BLAST program. Multiple sequence alignment of selected homologous sequences was performed through ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) for identifying conserved regions. The amino acid sequence of NRT2 family members from *A. thaliana* were downloaded from "The *Arabidopsis* Information Resource (TAIR)" (www.arabidopsis.org) and multiple sequence alignment was carried out with *BjNRT2.1*. Theoretical pI and molecular weight of amino acid sequence was calculated using ExPASy server and presence of motifs in the sequence was identified by MotifScan. The location of protein was predicted by PSORT (http://psort.hgc.jp/form.html). The transmembrane helices (TMH) were predicted by MemBrain (Shen and Chou 2008; http://chou.med.harvard.edu/bioinf/MemBrain/) and the 3-D structure was generated by Phyre^{2} server (Kelley and Sternberg 2009; http://www.sbg.bio.ic.ac.uk/phyre2/).

Results and discussion

Isolation and cloning of full length coding sequence

A 1.34 kb cDNA fragment was cloned and sequenced. Homology search of nucleotide sequence revealed similar sequences of high-affinity nitrate transporters from various plant species. At DNA level, the cloned fragment showed maximum identity with *B. rapa* (NRT2, subsp. Pekinensis: 99 %, chinensis: 91 %), *B. napus* (BnNRT2: 88 %), *Arabidopsis* (AtNRT2.1: 88 %). The cloned cDNA fragment was incomplete at 5′ end, therefore, a 260 bp nucleotide sequence was obtained by 5′-RACE PCR. The full length cDNA was obtained by cloning and re-sequencing (Fig. 1). The open reading frame (ORF) of cloned gene consisted of 1593 bp encoding 530 amino acids (Fig. 2). The nucleotide sequence was submitted to NCBI (Acc. No. JQ305139) and designated as *BjNRT2.1*.

Comparison of BjNRT2.1 with other members of plant NRT2 family

The sequence comparison between cloned *B. juncea* (JQ305139) and *B. napus* (AJ293028) showed a difference

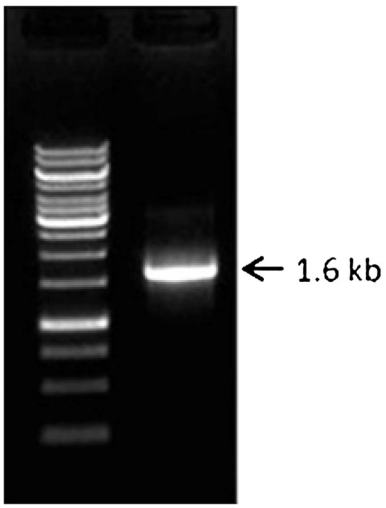

Fig. 1 Amplified product of *NRT2.1* obtained after 5′-RACE PCR from the roots of *Brassica juncea* cv. Pusa Bold. Seedlings were grown hydroponically with 10 μM nitrate for 28 days

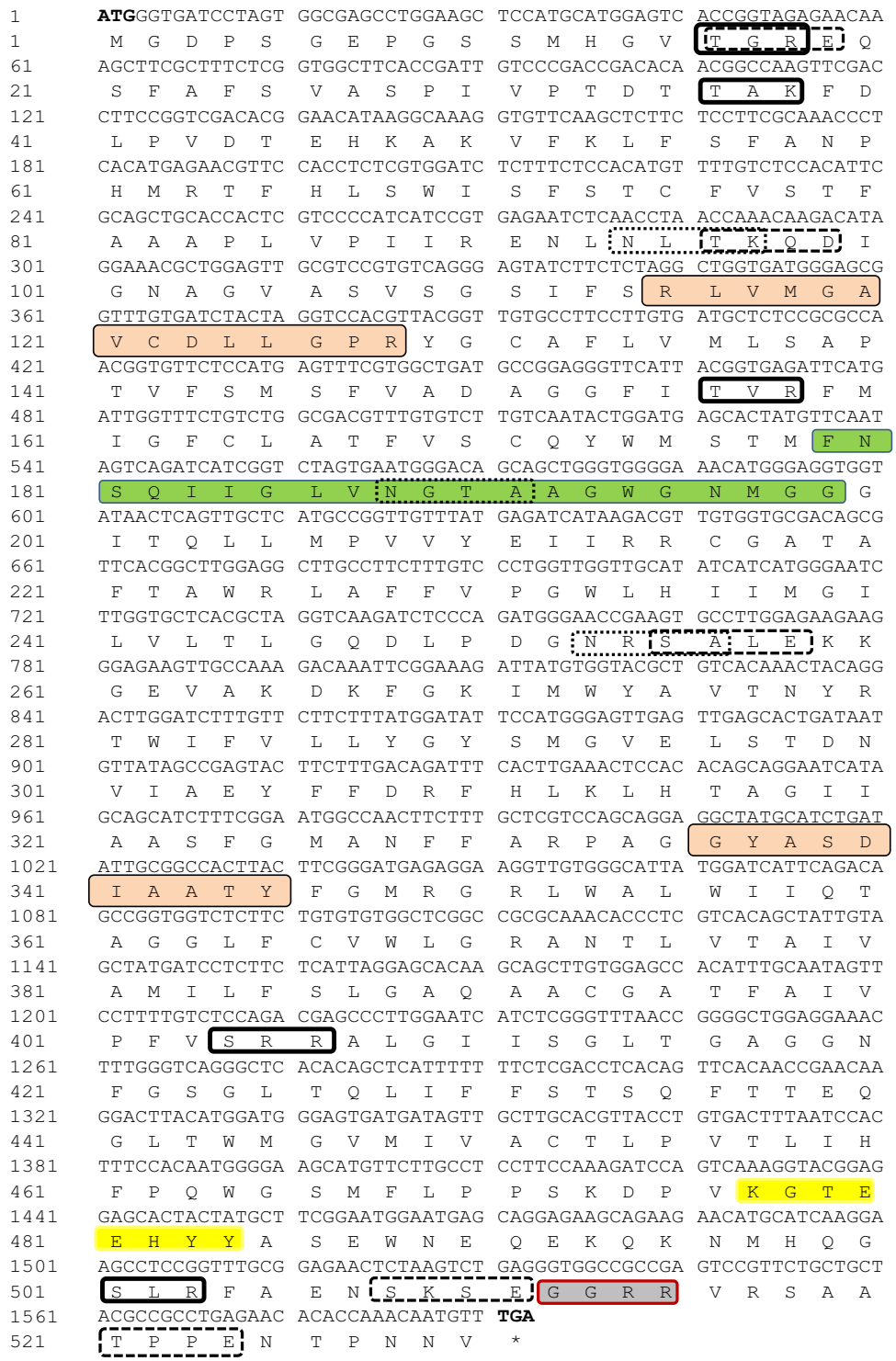

Fig. 2 Nucleotide and deduced amino acid sequences of *BjNRT2.1*. The initiator ATG and terminator TGA are in *bold*. Conserved motifs are marked: five putative protein kinase C (*solid black box*), five casein kinase II (*dashed black box*), three asparagine glycosylation sites (*dotted black box*), amidation site (*highlighted in grey*), nitrate/nitrite porter signature motif (*highlighted in green*) and two major facilitator superfamily (MFS) (*highlighted in pink*). (Color figure online)

of 19 nucleotides and 15 amino acids. The similarity between two species was 90.1 and 97.2 % at DNA and protein level, respectively. The NRT2 of *B. rapa* (96 %) and *B. napus* (95 %) were closely related to *B. juncea* as it shares the 'AA' genome from both species. A comparative sequence analysis of *BjNRT2.1* with NRT2 of *Arabidopsis*

Table 1 Homology between BjNRT2.1 and typical high affinity nitrate transporter (NRT2 proteins) from various plant species

Species	Gene name	Accession number	Score bits	Amino acid identities	% identities	Gaps	No. of (predicted) transmembrane helices
Brassica juncea	*BjNRT2.1*	AEZ68614	1,088	530/530	100	0	12
Brassica rapa subsp. chinensis	*BrNRT2*	AFK65609	1,055	521/530	96	0	12
Brassica napus	*BnNRT*	CAC05338	1,045	504/530	95	0	12
Arabidopsis thaliana	*AtNRT2.1*	NP_172288	1,038	498/530	94	0	12
Arabidopsis thaliana	*AtNRT2.2*	NP_172289	920	449/523	86	7/523	12
Arabidopsis thaliana	*AtNRT2.4*	NP_200885	936	444/523	85	0	12
Ricinus communis	*RcNRT*	XP_002523687	930	440/530	83	0/530	12
Glycine max	*GmNRT2*	NP_001236444	915	432/530	82	0	12
Cucumis sativus	*CsNRT2*	AAS93686.3	904	427/530	81	0	12
Medicago truncatula	*MtNRT*	XP_003596775	900	425/529	80	3/529	12
Nicotiana tabacum	*NpNRT2*	CAD89798	894	420/530	79	0	12
Solanum lycopersicum	*SlNRT2.1*	NP_001234134	883	416/530	78	0	12
Solanum lycopersicum	*SlNRT2.3*	NP_001234127	871	409/531	77	1/531	12
Triticium aestivum	*Ta NRT2*	AAG01172	778	366/501	73	7/501	12
Hordeum vulgare	*HvNRT2.6*	ABG20829	776	363/497	73	6/497	12
Oryza sativa	*OsNRT2*	NP_001045658	812	383/529	72	8/529	12
Zea mays	*ZmNRT2*	NP_001105195	777	372/523	71	8/523	12
Daucus carota	*DcNRT*	AAL99362	747	373/538	69	11/538	11
Arabidopsis thaliana	*AtNRT2.6*	NP190092	764	372/536	69	8/536	12
Arabidopsis thaliana	*AtNRT2.3*	NP_200886	757	370/533	69	5/533	12
Arabidopsis thaliana	*AtNRT2.5*	NP_172754	613	290/477	61	2/477	12
Populus trichocarpa	*Pt NRT*	662054	605	290/493	59	1/493	12
Gossypium hirsutum	*GhNRT*	AFI74368	581	286/497	58	3/497	12

showed that *BjNRT2.1* was closely related to *AtNRT2.1* (94 % similarity). This suggests the possibility that the cloned gene might be an orthologue of *AtNRT2.1*, which is specifically expressed in roots, hence it was named *BjNRT2.1*. In *Arabidopsis*, seven NRT2 genes have been identified (Orsel et al. 2002; Okamoto et al. 2003). Of these, NRT2.1 is most critical for nitrate uptake under low-N based on its expression pattern and HATS activity. Filleur et al. (2001) showed the first functional evidence that AtNRT2.1 proteins belong to inducible HATS by complementation of *atnrt2* mutants.

Sequence analysis and characterization of deduced NRT2 protein

Protein–protein BLAST and multiple sequence alignment of BjNRT2.1 (AEZ68614) exhibited a high degree of sequence similarity (ranging from 58 to 96 %) with the members of HATS NRT2 family of plant nitrate transport proteins (Table 1). Sequences with more than 80 % average similarity included *B. rapa* (AFK65609), *B. napus* (CAC05338), *Arabidopsis* (NTR2.1, NP_172288; NRT2.2, NP_172289; NRT2.4, NP_200885), castor (XP_002523687), soybean (NP_001236444) and cucumber (AAS93686.3). The analysis of deduced amino acid sequence of BjNRT2.1 predicted a protein with molecular weight of 57.6 kD and pI 8.79, suggestive of a basic protein as expected for a member of NRT2 family. Figure 2 represents various motifs present in the amino acid sequence of BjNRT2.1. Five putative protein kinase C sites were identified at positions 16–18 (TGR), 36–38 (TAK), 156–158 (TVR), 404–406 (SRR) and 501–503 (SLR), five putative casein kinase II phosphorylation sites at positions 16–19 (TGRE), 96–99 (TKQD), 255–258 (SALE), 508–511 (SKSE) and 521–524 (TPPE) and one putative tyrosine phosphorylation site at position 477–484 (KGTEEHYY). Presence of putative protein kinase C and casein kinase II domains indicates the regulatory phosphorylation of this protein. Three putative asparagine glycosylation sites were also identified at amino acid positions 94–97 (NLTK), 188–191 (NGTA) and 253–256 (NRSA) suggestive of this protein to be a membrane bound.

The predicted localization of the polypeptide was plasma membrane, with certainty threshold value of 0.60. The transmembrane propensity analysis revealed twelve putative transmembrane domains in clusters of 6 + 6 with both N- and C- terminal inside (Fig. 3a). The loops between TMHs 3 and 4 and between 9 and 10 were shortest containing two residues each, while those between 6 and 7 were the longest

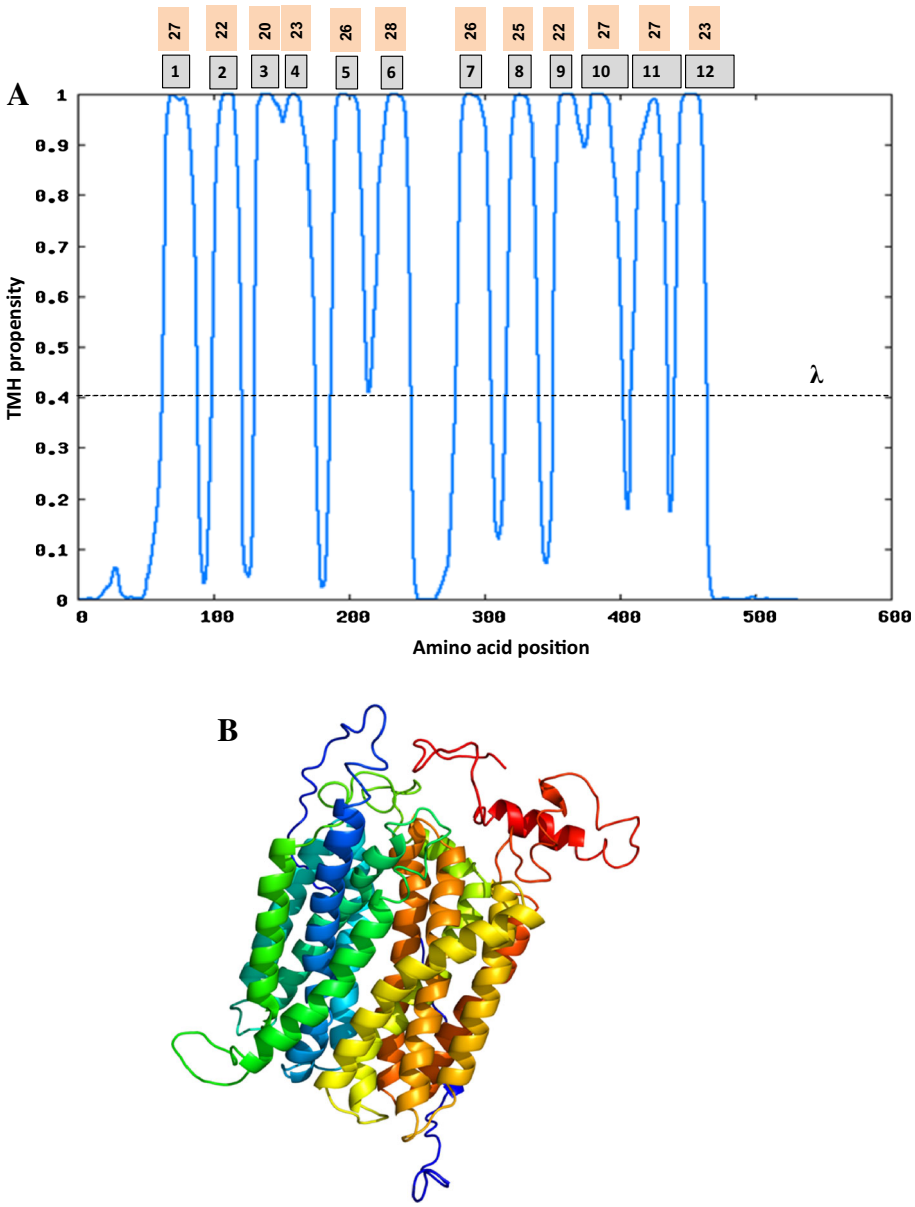

Fig. 3 **A** Transmembrane helices propensity of BjNRT2.1 protein from *B. juncea* as predicted by MemBrain analysis tool (http://chou.med.harvard.edu/bioinf/MemBrain/). λ denotes the base threshold of residue propensity which is greater than or equal to 0.4. Values in *grey boxes* denote the TM helices and those in *pink boxes* represent the number of amino acid residues in each TMH. **B** 3-D structure of transmembrane helices of BjNRT2.1 predicted by Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/). 81 % residues were modeled at >90 % confidence. (Color figure online)

containing 33 residues. The 6th TMH possessed 28 amino acid residues, while TMHs 1, 10 and 11 contained 27 residues each. The 3rd TMH was smallest possessing 20 residues. The characteristic major facilitator super-family (MFS) sequence at positions 114–128 (MFS I) and 335–345 (MFS II) were also observed in the studied protein sequence. The signature motifs of nitrate transporters, NNP (nitrate/nitrite porter) sequences were also present at position 178–198 (Fig. 2) which has an important role in substrate binding (Unkles et al. 2012). The presence of two MFS sites and a signature motif of NPP in BjNRT2.1, reported in a topologically equivalent position in AtNRT2.1, are the additional diagnostic features. The 3-D structure of BjNRT2 was generated from the top 20 hits of protein database (PDB), with 81 % residues modeled at more than 90 % confidence (Fig. 3b). The structure showed presence of 16 α-helices, out of which four were small with residues ranging from three to nine and were not enough to span the plasma membrane. However, twelve α-helices were large enough to traverse the membrane consisting of amino acid residues ranging from 20 to 28. Pairwise sequence alignment of AtNRT2.1 and BjNRT2.1 showed that the length and position of 12 TMHs were very similar in the two proteins (Fig. 4). The first putative high-affinity nitrate transporter gene, *AtNRT2.1*, was isolated from *Arabidopsis*, which showed similarity with high-affinity nitrate transporters from yeast, fungi, algae and other higher plants (Filleur and Daniel-Vedele 1999).

Fig. 4 Alignment of deduced amino acid sequence of *B. juncea* (Bj) and *A. thaliana* (At) NRT2.1. *Bars* and *colon* correspond to identities and similarities, respectively. The *bold* highlighted amino acids represent the 12 transmembrane helices (TMH) predicted by MemBrain program. Pairwise sequence alignment was done by EMBOSS Needle (EMBL-EBI)

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                                                  TMH I
Bj    1 MGDPSGEPGSSMHGVTGREQSFAFSVASPIVPTDTTAKFDLPVDTEHKAK     50
        |||.:|||||||||||||||||||||.||||.||.|||||||||||||||.
At    1 MGDSTGEPGSSMHGVTGREQSFAFSVQSPIVHTDKTAKFDLPVDTEHKAT     50
                                                  TMH I
Bj   51 VFKLFSFANPHMRTFHLSWISFSTCFVSTFAAAPLVPIIRENLNLTKQDI    100
        ||||||||.|||||||||||||||||||||||||||||||||||||||||||
At   51 VFKLFSFAKPHMRTFHLSWISFSTCFVSTFAAAPLVPIIRENLNLTKQDI    100
                TMH II                                TMH III
Bj  101 GNAGVASVSGSIFSRLVMGAVCDLLGPRYGCAFLVMLSAPTVFSMSFVAD    150
        |||||||||||||||||||||||||||||||||||||||||||||||||:|
At  101 GNAGVASVSGSIFSRLVMGAVCDLLGPRYGCAFLVMLSAPTVFSMSFVSD    150
                TMH IV                                TMH V
Bj  151 AGGFITVRFMIGFCLATFVSCQYWMSTMFNSQIIGLVNGTAAGWGNMGGG    200
        |.|||||||||||||||||||||||||||||||||||||||||||||||||||
At  151 AAGFITVRFMIGFCLATFVSCQYWMSTMFNSQIIGLVNGTAAGWGNMGGG    200
                                              TMH VI
Bj  201 ITQLLMPVVYEIIRRCGATAFTAWRLAFFVPGWLHIIMGILVLTLGQDLP    250
        |||||||:|||||||:|||||||:||||||||||||||.||||||
At  201 ITQLLMPIVYEIIRRCGSTAFTAWRIAFFVPGWLHIIMGILVLNLGQDLP    250
                                                  TMH VII
Bj  251 DGNRSALEKKGEVAKDKFGKIMWYAVTNYRTWIFVLLYGYSMGVELSTDN    300
        ||||:.|||.||||||||||:||||||||||||||||||||||||||||||
At  251 DGNRATLEKAGEVAKDKFGKILWYAVTNYRTWIFVLLYGYSMGVELSTDN    300
                                          TMH VIII
Bj  301 VIAEYFFDRFHLKLHTAGIIAASFGMANFFARPAGGYASDIAATYFGMRG    350
        |||||||||||||||||:|||.||||||||||||||||.||.||||||
At  301 VIAEYFFDRFHLKLHTAGLIAACFGMANFFARPAGGYASDFAAKYFGMRG    350
                TMH IX                        TMH X
Bj  351 RLWALWIIQTAGGLFCVWLGRANTLVTAIVAMILFSLGAQAACGATFAIV    400
        |||.|||||||||||||||||||||||:|||:|||:||||||||||||||
At  351 RLWTLWIIQTAGGLFCVWLGRANTLVTAVVAMVLFSMGAQAACGATFAIV    400
                                  TMH XI                TMH XII
Bj  401 PFVSRRALGIISGLTGAGGNFGSGLTQLIFFSTSQFTTEQGLTWMGVMIV    450
        |||||||||||||||||||||||||||||:||||||.|||||||||||||||
At  401 PFVSRRALGIISGLTGAGGNFGSGLTQLLFFSTSHFTTEQGLTWMGVMIV    450

Bj  451 ACTLPVTLIHFPQWGSMFLPPSKDPVKGTEEHYYASEWNEQEKQKNMHQG    500
        ||||||||:|||||||||||||.|||||||.|||.||||||||||||||||
At  451 ACTLPVTLVHFPQWGSMFLPPSTDPVKGTEAHYYGSEWNEQEKQKNMHQG    500

Bj  501 SLRFAENSKSEGGRRVRSAATPPENTPNNV    530
        |||||||:|||||||||||||||||||||||
At  501 SLRFAENAKSEGGRRVRSAATPPENTPNNV    530
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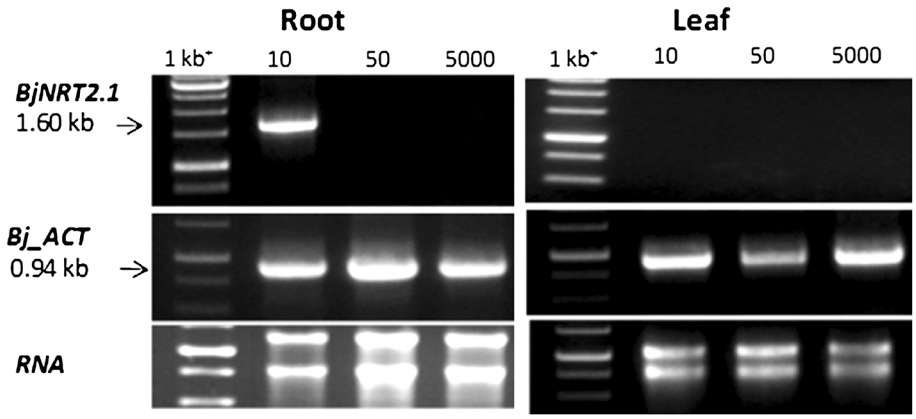
Fig. 5 Tissue specific expression of high-affinity nitrate transporter gene *BjNRT2.1* extracted from root and leaf of *B. juncea* grown at very low (10 μM), low (50 μM) and sufficient (5,000 μM) nitrate concentration



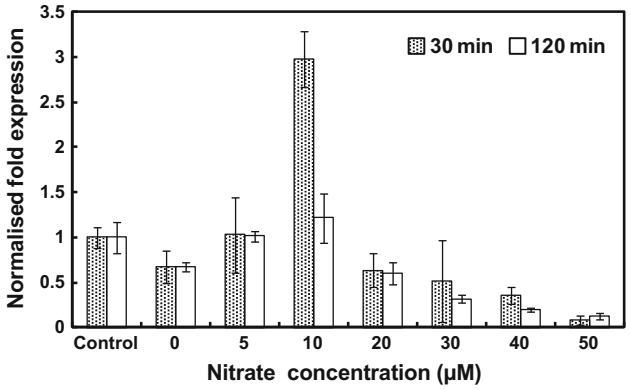


Fig. 6 qRT-PCR analysis of high affinity nitrate transporter, *BjNRT2.1*, in the roots of *B. juncea* seedlings. The plants were starved for nitrate for 5 days and *BjNRT2.1* was induced by subjecting the plants to a range of nitrate concentrations ranging between 0 and 50 μM. Expression level at control (5,000 μM) nitrate concentration was taken as 1. Roots were sampled at 30 and 120 min after induction. Values are the means of three biological replications ± standard error

Tissue-specificity and expression analysis of *BjNRT2.1*

To check the tissue-specific expression of *BjNRT2.1*, RT-PCR of RNA extracted from roots and leaves of plants grown at very low-, low- and control nitrogen were performed (Fig. 5). No expression was found in leaf tissue, while there was significant expression in root tissues at very low-N (10 μM). To examine the potential induction of *BjNRT2.1* by nitrate in the lower concentration range (from 0 to 50 μM), transcript abundance was assessed by qRT-PCR in roots of *Brassica* seedlings (Fig. 6). After thirty-minutes of induction at 10 μM nitrate concentration, the expression of *BjNRT2.1* increased by three folds with respect to control. No significant increase in expression was noted beyond 10 μM and after 2 h of induction. The real-time-PCR data suggested that the high-affinity transporter system was activated within a short period once the N depleted medium was provided. *BjNRT2.1* was, thus, highly induced under very-low N in roots and nitrate concentrations beyond 10 μM suppressed its expression, suggesting that it belongs to root-specific inducible HATS family. In *Arabidopsis* different reports have cited different nitrate concentrations for induction of *AtNRT2.1* in roots. Zhou et al. (1999) observed highest expression of *AtNRT2.1* in 100 μM nitrate and lowest in 15 mM nitrate grown plants, while Filleur and Daniel-Vedele (1999) reported its induction at 50 μM. Similar root specific expression of *NRT2.1* have been reported for *NpNrt2* at 10 μM nitrate concentration (Krapp et al. 1998). Tomato seedlings N-starved for 36 h and induced by 0.2 mM nitrate for 4 h showed dominant expression of *LeNRT2.1* in root hairs (Ono et al. 2000). In wheat roots, *TaNRT2.1* was highly expressed at 0.05 and 1.5 mM nitrate (Yin et al. 2007). These differences in nitrate concentration in lower range required for induction of high-affinity nitrate transport varying with plant species also justifies the differences in rate/dose of N required by various crops for optimum productivity. From the qPCR data and bio-informatics evidence it can be concluded that the isolated gene belongs to the family of NRT2 genes isolated from Indian mustard, which is an orthologue of *AtNRT2.1,* and belongs to inducible HATS gene family.

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