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Arabidopsis thaliana locus At5g62890, a nucleobase-ascorbate transporter family member, is preferentially expressed in carpel transmitting tract and tapetal cells

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Abstract

The expression pattern for Arabidopsis locus At5g62890 (MQB2.190) is investigated at the molecular and whole plant level. MQB2.190 belongs to the nucleobase-ascorbate transporter (NAT) family which includes transporters of purines, pyrimidines and ascorbate. A full-length cDNA clone was sequenced verifying that MQB2.190 is actively expressed and encodes a NAT protein. Northern blot and RT-PCR analysis reveal that MQB2.190 is expressed at a low level in a variety of tissues, but is preferentially expressed in flowers. Whole plant gene expression was investigated by generating transgneic Arabidopsis with the MQB2.190 promoter driving *GUS* gene. Visible GUS staining was detected in the central tissue of carpels and siliques and faintly in anthers, but was absent in young seedlings, roots, leaves, stems or other floral tissues. MQB2.190 transcripts were precisely localized to the central transmitting tract of the carpel and in the tapetal cell layer in anthers using RNA in situ hybridization.

Keywords: Arabidopsis; Nucleobase-ascorbate transporters; Gene expression; Carpel; Transmitting tract; Tapetum

1. Introduction

The transport of purines and pyrimidines in plants is governed by at least two distinct families of integral membrane transporters. Purine permeases (PUP) have been identified only in plants. One member of this multigenic family in Arabidopsis has been shown to transport adenine and cytosine with high affinity [1]. In contrast, nucleobase-ascorbate transporters (NAT) are ubiquitous and are present in bacterial, fungal, protozoan, plant and mammalian genomes [2]. Microbial NAT proteins include uracil permeases, high affinity uric acid-xanthine transporters and general purine transporters, while mammalian NAT proteins transport ascorbate. In plants the maize NAT Leaf permease 1 (LPE1) functions as a high capacity, high affinity transporter of uric acid-xanthine that binds, but does not transport ascorbate [3].

Purines and pyrimidines play key roles in plant metabolism, both at the cellular and whole plant level. Although the biochemistry of nucleobase synthesis and catabolism is known in detail [4], the transport of nucleobase compounds within and between cells is less well characterized. Intracellular transport of nucleobases is involved in RNA synthesis and catabolism, as plastids are the primary site for nucleobase synthesis. Intercellular transport of nucleobases occurs in between endosperm and cotyledons in germinating seed [5]. Extensive intracellular and intercellular transport of purines uric acid and xanthine mark the biosynthesis of ureides in nitrogen fixing tropical legumes such as soybean [6]. Ascorbate is involved in several roles in plant biochemistry, including the detoxification of oxygen radicals in chloroplasts formed during photosynthesis, and may be involved in the extra-cellular

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expansion of cell walls [7]. Although no plant ascorbate transporter genes have been identified, ascorbate transport across plastid and plasma membranes has been documented [8].

Plant genomes contain numerous NAT genes (e.g. 12 independent NATs are present in the Arabidopsis genome). Plant NAT proteins may have overlapping but distinct solute transport profiles, including highly specific purine transporters (such as LPE1), general purine transporters, pyrimidine transporters and ascorbate transporters. It is also likely that plant NAT genes display unique expression patterns to serve local requirements for nucleobase or ascorbate transport. Members of other multigenic families in plants display a variety of tissue and/or development expression patterns including sugar transporter genes [9] and amino acid transpoter genes [10]. In this report we characterize Arabidopsis gene At5g62890 (MQB2.190) a member of the NAT family. A variety of independent techniques are used to investigate the restricted expression pattern of this locus.

2. Material and methods

2.1. Plant material

Arabidopsis thaliana L. ecotype C24 was used for RNA isolation, reverse transcriptase-polymerase chain (RT-PCR) analysis, transgenic plant production and in situ analysis. Plants were grown at 21–24 °C under 75–125 $\mu Em^{-2}\ s^{-1}$ illumination 12 h light/12 h darkness.

2.2. DNA sequence analysis

DNA sequencing was performed at the W. M. Keck, Biotechnology Resource Laboratory, Yale University, USA. DNA and protein sequences were analyzed using Lasargene software (DNASTAR, Madison, WI), ClustalW [11] and TMHMM v.2.0 [12].

2.3. RNA isolation, Northern and RT-PCR analysis

Total RNA was isolated from plant tissue using TRIzol (Gibco BRL, Gaithersburg, MD) for Northern analysis or using RNAqueous-4PCR (Ambion, Austin, TX) for RT-PCR analysis. Ten micrograms of RNA was fractionated on agarose gels [13] and transferred to ZetaProbe GT membranes (Bio-Rad, Richmond, CA) according to the manufacturers recommendations. Northern blots were probed with [^{32}P]dCTP labeled [14] 2.0 kb fragment from clone AA05E06 and hybridized as previously described [15]. RT-PCR reaction was performed using RETROscript (Ambion, Austin, TX). Primers PQ14-1 5′ GTTTCTCTGTCTTCCTGGGCTGT 3′ and PQ14-2 5′ GACAGATGGGAAGTACTTGTTGAG 3′ were used to amplify MQB2.190 sequences spanning genomic intron sequences and primers 18SA 5′ TTCGGGATCGGAGTAATGATTAACAGG 3′ and 18SB 5′ TGCACCACCACCCATAGAATCAAGAA 3′ to the 18S rRNA gene as controls. PCR amplification conditions were 95 °C 3 min; 94 °C 20 s; 55 °C 30 s; 72 °C 1 min, with amplification for 18S rRNA control 18 cycles and for amplification of MQB2.190 sequences 30 cycles.

2.4. Plasmid construction

Promoter-*GUS* construct was generated by amplifying sequences from P1 clone MQB2 (GB# AB009053) using primers MQB2-1 5′ AAGCTTACACACTTGATTGACTAATC 3′ and MQB2-2 5′ GGATCCGATTCCTTCCTTAAATCTCTCTCT 3′. The resulting 2440 bp fragment, containing the MQB2.190 Promoter region, was cloned into the HindIII and BamHI restriction endonuclease sites of pBI101 (Clontech, Palo Alto, CA) to create plasmid pNS374. For in situ sense and anti-sense probes, the 311 bp product of the above mentioned RT-PCR reactions was cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) to generate plasmid pQI-14. *Escherichia coli* strains DH5α and Top10 F′ (Invitrogen, Carlsbad, CA) were used to propagate plasmid DNA.

2.5. Transgenic plant production

Plasmid pNS374 was transferred into *Agrobacterium tumefaciens* strain C58C1 (GV2260) by electroporation. Transformation into Arabidopsis followed previously detailed procedure [16]. Transgenic seedlings were selected on 0.5 MS media supplemented with 30 μg ml^{-1} kanamycin. Plants were grown to T2 or T3 generation.

2.6. Histochemical analysis

For histochemical staining plants were grown on synthetic media or in soil. GUS expression analysis was performed on plants or plant organs grown to different developmental stages. Staining occurred at 37 °C overnight incubation with 2 mM 5-bromo-4-chloro-3-indoly-β-D-glucuronid acid (Gold Biotechnology, St. Louis, MO) in Buffer 0.1 M Sodium phosphate, pH 7.0 with K_3FeCn_6 and K_4FeCN_6 as previously described [17]. Plant tissue was cleared after staining in 70% ethanol.

2.7. In situ analysis

Arabidopsis flowers were fixed in formalin-acetic acid (10% formaldehyde, 5% acetic acid, 50% ethanol) and imbedded in Paraplast X-tra (Fischer Scientific, Pitts-

burgh, PA). In situ procedure followed manufactures recommendations using DIG labeling Kit Sp6/T7 (Boehringher Mannheim, Indianapolis, USA). Sense and anti-sense DIG labeled transcripts were generated by cleaving pQI-14 with HindIII and XhoI restriction endonuclease and polymerizing with T7 DNA polymerase or Sp6 polymerase respectively.

3. Results

3.1. Characterization of full-length MQB2.190 cDNA

Two expressed sequence tag (EST) clones displaying high levels of nucleic acid similarity to chromosome locus At5g62890 (genomic MQB2.190 sequences) were obtained from stock centers or individual researchers. EST ATTS1009 (Gb# Z25495) was isolated from a floral cDNA library while EST AA05E06 (Gb#BE037863) was isolated from a library of salt stressed plant tissue. The complete sequence of each EST clone was determined and can be found under Genbank # AF466824 or AF466198 respectively. EST ATTS1009 clone contains a partial cDNA of 965 bp corresponding to the latter half of the gene. EST AA05E06 clone contains a full-length cDNA of 2059 bp, including a 150 bp 5′ UTR, a 310 bp 3′ UTR and an open reading frame of 1599 bp (Fig. 1). The DNA sequence of the full-length clone AA05E06 contains identical DNA sequence to the theoretical open reading frame predicted from analysis of genomic sequence data of P1 clone MQB2 (Gb# AB009053) and chromosome 5 (Gb# NC003076.1) [18,19]. Two previously sequenced cDNA clones (Gb# AY063011 and AY035003) share sequence identity with the complete sequence of AA05E06, but are well short of full-length.

3.2. Comparison of the deduced amino acid sequence of MQB2.190 with other nucleobase-ascorbate transporters

Clone AA05E06 corresponds to the expressed version of Arabidopsis gene MQB2.190 and clearly encodes a member of the NAT family. The predicted protein encoded by the AA05E06 open reading frame contains 532 amino acids. Secondary structure analysis reveals that the MQB2.190 protein is an integral membrane protein with predicted 10–12 trans-membrane spanning domains depending upon the algorithm used [12] (Fig. 1). The MQB2.190 protein shares a high degree of secondary structural similarity (Fig. 1) and amino acid sequence similarity (Fig. 2 and Table 1) with other plant and mammalian NAT proteins. Overall amino acid sequence similarities are highest between MQB2.190 and other plant NAT proteins, lower with mammalian vitamin C transporters and least compared to fungal and bacterial NATs. NAT proteins, including MQB2.190 protein, contain a conserved nucleobase-ascorbate transporter signature motif, (Q/E/P)ENXGX$_4$TX$_2$SR [20]. This motif is present in a domain critical for nucleobase recognition and transport function in Aspergillus UapA and UapC purine transporters [21]. High levels of amino acid sequence similarity are shared among NAT proteins in this region (Fig. 2).

3.3. MQB2.190 gene expression

To investigate the expression pattern of MQB2.190 Northern blot analysis was performed using RNA isolated from different tissues. Ten micrograms of total RNA was fractionated, blotted and probed with full-length MQB2.190 cDNA sequences (Fig. 3A). It is clear that MQB2.190 is expressed at a high level in floral tissue, but is not abundant in stem, rosette leaf or root tissues (Fig. 3A). To determine if MQB2.190 is expressed at low levels undetected by Northern blot analysis, total RNA was subjected to RT-PCR analysis (Fig. 3B). The amount of RNA from stem, leaf, root and flower was standardized using 18S RNA specific primers to amplify a 445 bp product, and then used to determine the linear range for product formation over a series of amplification cycles using gel electrophoresis. Volume adjustments were made to ensure a standard amount of rRNA (and presumably mRNA) in each sample. MQB2.190 specific primers that span intron sequences were then used to amplify cDNA through thirty cycles of PCR. The resulting product size of 311 bp reflects detection of MQB2.190 mRNA and not genomic sequence in the sample (Fig. 3B). It is clear that MQB2.190 is expressed in root, stem, and leaf tissue at low levels.

3.4. Promoter-reporter transgenic analysis

Transgenic Arabidopsis containing the MQB2.190 promoter-*GUS* construct were generated to determine the plant wide expression patterns. A genomic DNA fragment containing 2440 bp directly upstream of the start of translation of MQB2.190 was fused to *E. coli GUS* sequences to generate a transcriptional fusion plasmid pNS374. Three independent transgenic Arabidopsis lines exhibiting GUS activity were generated. Analysis was performed on T2 and T3 progeny. Staining was performed on 10-day-old seedlings, adult root, rosette leaf, inflorescence stem, flowers and siliques. As shown in Fig. 4A staining is most evident in carpel tissue. No staining was observed in young seedlings, adult root, leaf or stem tissue (data not shown). GUS activity first appears in young flowers at the top of the carpel below the stigma and progresses down the carpel with increased age (data not shown). In developing transgenic siliques GUS activity is restricted to the entire

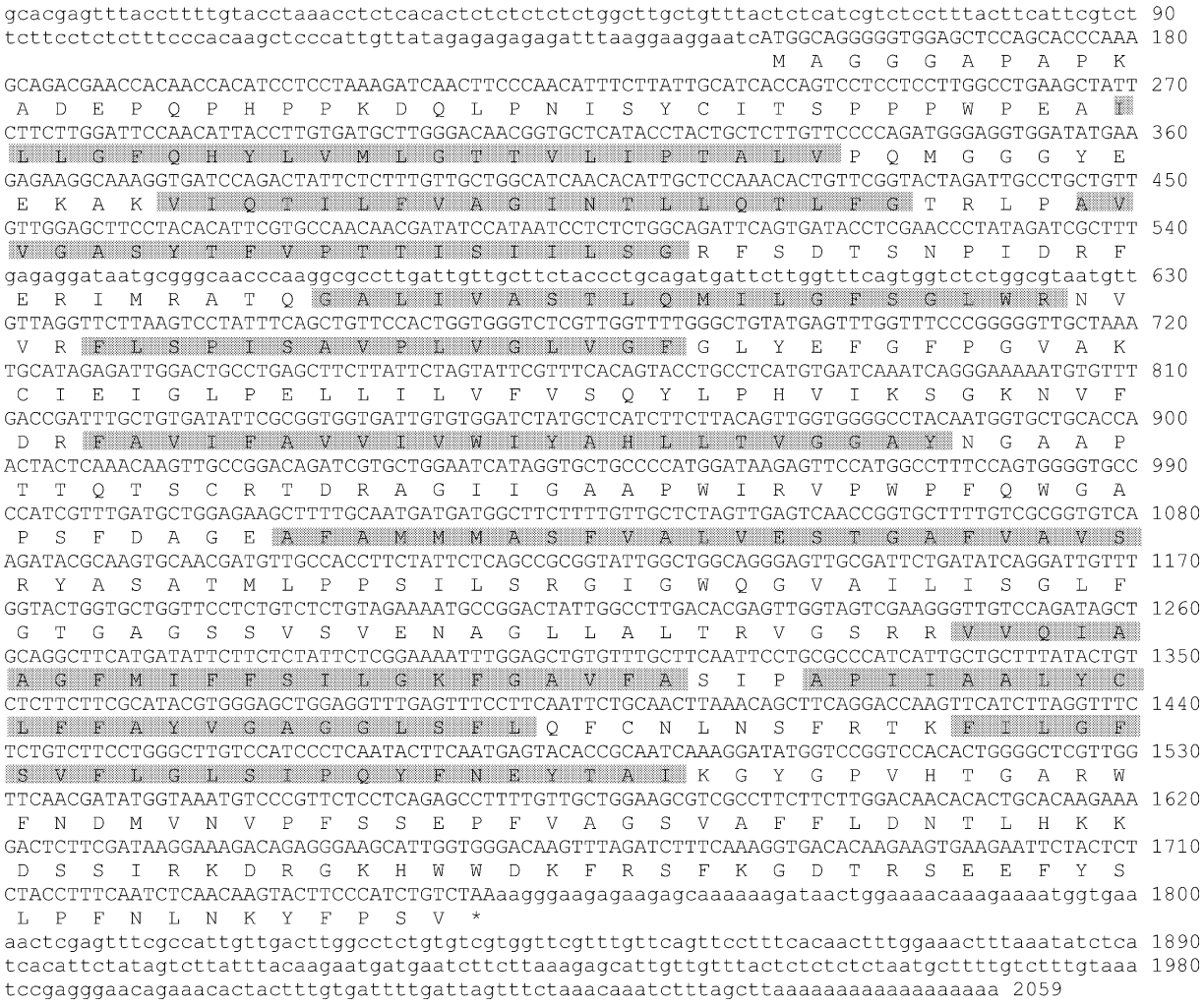

Fig. 1. Nucleotide sequence of a full-length MQB2.190 cDNA AA05E06. Nucleotides in lower case represent 5′ UTR and 3′ UTR sequences while upper case nucleotides correspond to the longest ORF. Proposed protein sequence is indicated below the coding region as single letter amino acid abbreviations. Gray shaded regions in the predicted amino acid sequence represent putative membrane spanning domains as predicted by TMHMM v 2.0 [12].

length of central carpel tissue including the style, but GUS activity is not evident in the stigmatic region, developing seeds or in carpel walls (Fig. 4B). Mature flowers display very faint and diffuse GUS staining in anthers (Fig. 4C).

3.5. *In situ analysis*

A more detailed view of wild type floral expression for MQB2.190 was investigated via in situ analysis. MQB2.190-specific sense and anti-sense probes were

Fig. 2. Comparison of MQB2.190 protein and representative NAT proteins in the conserved motif region. Amino acids are represented as single amino acid abbreviations. MQB2 = MQB2.190 protein; T9J22 = *Arabidopsis thaliana* NAT T9J22.18 (GB# AF370518); LPE1 = maize Leaf Permease 1 [34]; SVCT1 & SVCT2 = Rat acsorbate transporters [35]; BsPbuX = *Bacillus subtilus* xanthine transporter [36]; BsPyrP = *Bacillus subtilus* pyrimidine transporter [37]; UapA = *Aspergillus nidulans* uric acid-xanthine transporter [38]; UapC = *Aspergillus nidulans* purine transporter [39]. Consensus refers to nucleobase-ascorbate transporter signature motif [20].

Table 1
Comparison of deduced MQB2.190 protein to representative NAT protein amino acid sequences

NAT	Species	Solute transport	Percent amino acid*		Accession
			Identity	Similarity	
MQB2.190	*Arabidopsis thaliana*	Unknown	–	–	This work
LPE1	*Zea mays*	Uric acid/xanthine	58.1	86.1	U43034
T9J22.18	*Arabidopsis thaliana*	Unknown	48.2	82.1	AC002505
SVCT1	*Rattus norvegicus*	Ascorbate	28.4	62.4	AF080452
SVCT2	*Rattus norvegicus*	Ascorbate	30.0	63.2	AB038145
PBUX	*Bacillus subtlus*	Xanthine	18.9	54.6	X83878
PYRP	*Bacillus subtilus*	Uracil	17.4	51.3	M59757
UAPA	*Aspergillus nidulans*	Uric acid/xanthine	15.7	48.2	X71807
UAPC	*Aspergillus nidulans*	Oxidized purines	18.6	53.0	X79796

* Amino acid comparisons performed with ClustalW [11].

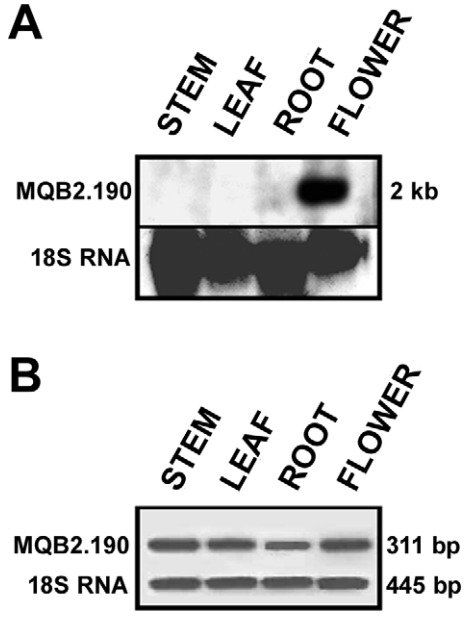

Fig. 3. (A) RNA blot analysis of MQB2.190 expression. Total RNAs from stem, leaf, root and flowers fractionated on a 1% agarose gel. The top membrane was probed with a radiolabeled MQB2.190 cDNA probe, above and the bottom duplicate membrane was probed with a radiolabeled 18S rDNA probe. (B) RT-PCR analysis of RNA isolated from stem, leaf, root and flowers. MQB2.190-specific bands at 311 bp and rRNA-specific controls at 445 bp.

hybridized with floral cross sections. A transverse section through a developed flower reveals that MQB2.190 expression is restricted to the septum central transmitting tissue, but not the surrounding septal epithelial layer in the central carpel (Fig. 5A). Expression is also observed in tapetal cells in anthers (Fig. 5A). No expression in other carpel cells (placental, repulum, ovary or vascular bundles) is observed (Fig. 5A), nor is there evident expression in the sense control (Fig. 5B). This data shows that the transgenic promoter analysis faithfully reflected the wild type tissue expression pattern for MQB2.190 and that a majority of the transcriptional control resides in the upstream region of the gene.

4. Discussion

In this study we have investigated the expression patterns of one member of the multigenic nucleobase-ascorbate transporter family in Arabidopsis. Two cDNAs corresponding to the expressed version of locus At5g62890 or MQB2.190 were sequenced. Analysis of the full-length cDNA clone AA05E06 reveals that the encoded protein shares many features common to NAT proteins. The MQB2.190 protein is predicted to be an integral membrane protein with at least ten membrane spanning domains (Fig. 1). MQB2.190 protein shares high levels of amino acid sequence similarity with other NAT members, particularly in a domain important for nucleobase specificity and transport function (Table 1 and Fig. 2). Although MQB2.190 protein is similar to other NAT members with know transport functions, it is not feasible to assign function based on amino acid sequence comparisons.

The expression of MQB2.190 is novel, displaying a preference for transmitting tract and tapetal cells. MQB2.190 message is highly expressed in flowers as determined by Northern blot analysis (Fig. 3A). It is likely that the gene is expressed elsewhere at low levels as matching EST clones have been isolated from floral, mixed tissues and equilibrated root cDNA libraries. Results show that RT-PCR analysis, but not Northern blot analysis detects limited MQB2.190 gene expression in roots, leaves and seedlings (Fig. 3B). Since the RT-PCR was not quantitative or semi-quantitative, the apparent discrepancy in floral expression levels between Northern blot and RT-PCR analysis most likely represents a plateau of product amplification. Both promoter-*GUS* analysis and in situ analysis reveal that MQB2.190 is preferentially expressed in transmitting tract tissue and in the tapetum. The identical localization of GUS activity in the transgenic plants and the in situ results in wild type plants shows that the promoter region contains sufficient information to direct cell-specific expression in carpel and anthers. This expres-

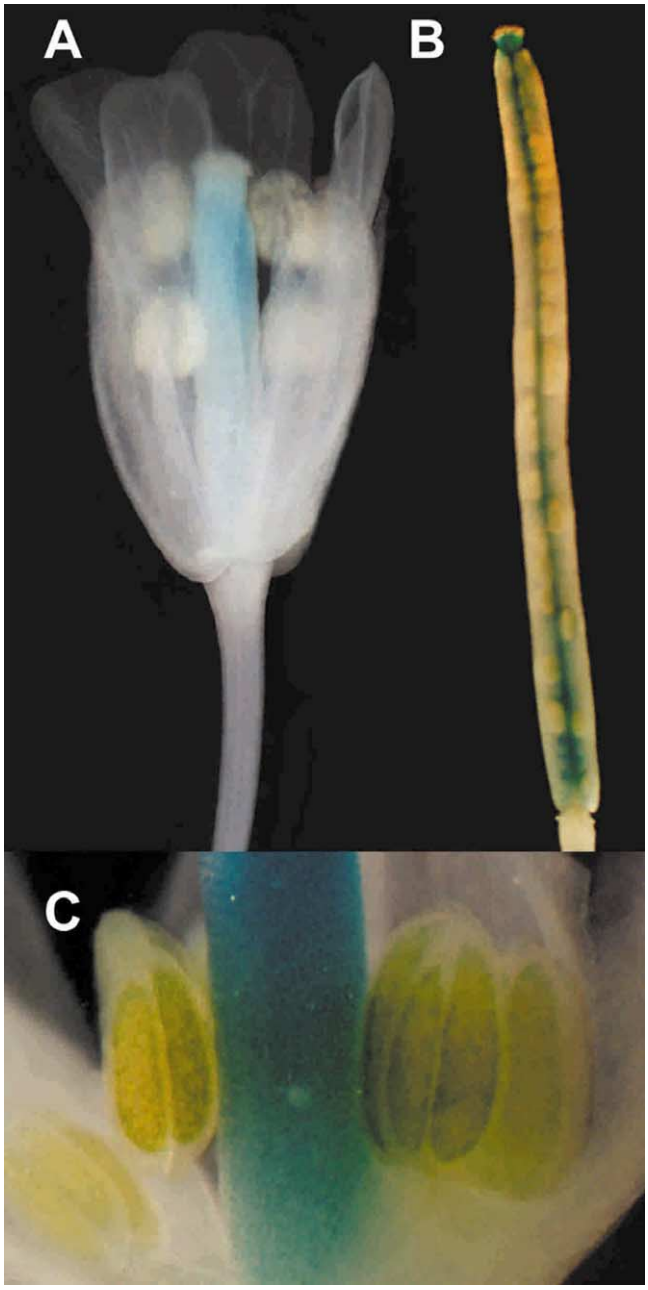

Fig. 4. Expression pattern of MQB2.190 Promoter-GUS fusion in transgenic flowers. (A) GUS activity in immature flowers; (B) GUS activity in developing silique; (C) GUS activity in mature carpel and anthers.

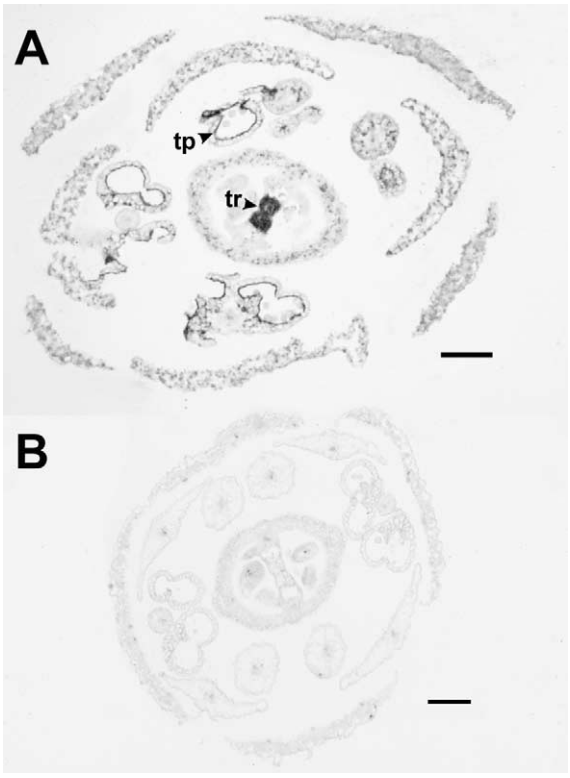

Fig. 5. In situ analysis on floral transverse sections in wild type Arabidopsis hybridized with an (A) anti-sense MQB2.190 probe; or (B) sense MQB2.190 probe. tr = transmitting tract, tp = tapetum. Scale bar = 100 μm.

sion pattern contrasts with other genes displaying either transmitting tract-expression patterns [22] or tapetum-specific expression [23]. Transgenic promoter-reporter gene analysis reveals that MQB2.190 is expressed from the top non-stigmatic region in carpels early in development and at maturity is expressed through out the length of the carpel. Although some genes (e.g. *ATCEL2* [24]; *Spatula* [25], *Kanadi* [26] *or Cup-shaped cotyledon 2* [27]) display transient expression in the central transmitting tract region early in carpel development, expression is often observed in other plant tissues or floral organs or is absent in mature carpels. MQB2.190 is present the entire length of the carpel septum unlike the 9612 gene of tomato that exhibits an expression pattern restricted to the upper 2/3 of the transmitting tract [28].

The restricted expression pattern of MQB2.190 points to a role in influencing pollen development and pollen tube growth. Both the tapetal cells and transmitting tract cells are highly secretory and serve as a conduit for nutrients, enzymes or matix components essential to the developing pollen cells or growing pollen tube respectively [29]. How might a NAT protein serve in the transmitting tract? MQB2.190 protein may transport nucleobase-like compounds to serve the needs of maturing pollen cells in the anther and for germinating pollen in the transmitting tract. Physiological studies have shown that germinating pollen actively imports nucleosides, however pollen tube membranes exhibit little nucleoside transport and nucleobase transport has not been shown [30–32]. Alternatively MQB2.190 may serve as an extra cellular transporter of ascorbate. Ascorbate transport has been detected in plant plasma membranes [8] and is believed to aid in cell wall expansion [33] and

may play a role in pollen tube growth. The restricted expression patterns suggest that a mutation in MQB2.190 would result in male and female sterile plants if the MQB2.190 protein serves an essential role. Future experiments are aimed at determining the solute transport function of MQB2.190 and to generate MQB2.190 mutants to address its role in anthers and carpels.

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