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Protein Storage Vacuoles of *Brassica napus* Zygotic Embryos Accumulate a BURP domain Protein Whose Gene is Tightly Controlled Spatially and Temporally and its perturbation distorts the PSV

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ABSTRACT

BNM2 is a prototypical member of the enigmatic BURP domain protein family whose members contain the signature $FX_{6-7}GX_{10-28}PX_{25-31}CX_{11-12}$ $X_2SX_{45-56}CHX_{10}CHX_{25-29}CHX_2$ $TX_{15-16}PX_5CH$ in the *C*-terminus. This protein family occurs only in plants, and the cognate genes vary very widely in their expression contexts in vegetative and reproductive tissues. None of the BURP family members has been assigned any biochemical function. *BNM2* was originally discovered as a gene expressed in microspore-derived embryos (MDE) of *Brassica napus* but we found that MDE do not contain the corresponding protein. We show that BNM2 protein production is confined to the seeds and localized to the protein storage vacuoles (PSV) even though the transcript is found in vegetative parts and floral buds as well. In developing seeds, transcript accumulation precedes protein appearance by more than 18 days. RNA accumulation peaks at ~20 days post anthesis (DPA) whereas protein accumulation reaches its maximum at ~40 DPA. Transgenic expression of *BNM2* does not abrogate this regulation to yield ectopic protein production or to alter the temporal aspect of BNM2 accumulation. Overexpression of *BNM2* led to spatial distortion of storage protein accumulation within PSV and to some morphological alterations of PSVs. However, the overall storage protein content was not altered.

INTRODUCTION

Transcripts corresponding to the so-called BURP domain proteins have been found in representatives of all land plants and there is no evidence of occurrence of these in other organisms. The BURP domain was termed as such by Hattori et al (1998) based on the conservation of amino acid (aa) sequences of the deduced BNM2 protein of rapeseed/canola, USP of bean (Unknown Seed Protein; (Bassuner et al., 1988)), RD22 of Arabidopsis (Response to Dehydration 22; (Yamaguchi-Shinozaki and Shinozaki, 1993)), and the β subunit of Polygalacturonase 1 of tomato (PG1β; (Zheng et al., 1992)). All deduced BURP proteins contain an *N*-terminal hydrophobic domain. Some BURP domain proteins contain in addition a short conserved segment and/or a repeated domain interposed between the *N*-terminus and a universally conserved *C*-terminus. The *N*-terminal domain acts as a signal peptide in USP (Bassuner et al., 1988). The *C*-terminus contains a signature feature that is conserved in all members of the BURP family. The overall identity among the ~225-aa *C*-termini of all BURP proteins is very low at 6%, although pairwise identities can be higher depending on the taxonomic relatedness of the plants where the coding sequences have been found. Against this backdrop of overall low identity some aa are highly conserved in their positions over the length of the domain, resulting in a signature of $FX_{6-7}GX_{10-28}PX_{25-31}CX_{11-12}$ $X_2SX_{45-56}CHX_{10}CHX_{25-29}CHX_2\ TX_{15-16}PX_5CH$.

The spatial and temporal expression aspects of *BURP* gene family members vary substantially. Some are developmentally regulated: *USP*, the very first of the BURP protein genes to be identified, is abundantly expressed in field bean embryos (Bassuner et al., 1988). *BNM2* is from a collection of genes that are highly expressed in microspore-derived embryos

(Hattori et al., 1998). *PG1β* expression occurs in ripening tomato (Zheng et al., 1992). *RAFTIN* is expressed in rice and wheat anthers (Wang et al., 2003). *SCB1* is a soybean seed coat protein gene expressed in early stages of seed development (Batchelor et al., 2002). *SALI3-2* and *SALI5-4a* are aluminum-induced in soybean roots (Ragland and Soliman, 1997). *ASG-1* is expressed in immature pollen, aposporous embryo sac during microsporogenesis and young embryos of both apomictic and sexual plants of guinea grass (Chen et al., 1999; Chen et al., 2005). *GhRDL*, an *RD22*-like gene, is expressed in cotton fibers, which are derived from the trichomes of the outer integument of ovules (Li et al., 2002). Some BURP protein genes are hormone or stress-responsive: *ADR6* is auxin-downregulated (Datta et al., 1993); *Arabidopsis thaliana RD22* and its homolog in *B. napus*, *BnBDC1*, are dehydration/mannitol-, ABA-, and salt-inducible (Yamaguchi-Shinozaki and Shinozaki, 1993; Yu, 2004). Of all these, the impact of downregulating the genes is known only for the *PG1β* (Watson et al., 1994) and *RAFTIN* (Wang et al., 2003). PG1β is a non-catalytic polypeptide found in ripening tomato; its transgenic suppression leads to altered pectin metabolism but it is not required for pectinase activity and its suppression does not affect tomato ripening. RAFTIN is found in the tapetum, Ubisch bodies and microspore exines; suppression of *RAFTIN* in rice abrogates pollen development and causes male sterility. Although numerous BURP domain protein-encoding sequences have been found, PG1β is the only BURP domain protein isolated in protein form from a plant source.

USP, the archetypal member of the BURP family, is highly expressed in bean embryos. However, the predicted storage protein has remained undetectable in bean or in transgenic plant seeds or elsewhere, indicating some constraints in USP synthesis or

accumulation. Seed development can be divided into three successive phases: early morphogenesis, storage reserve accumulation and maturation phase (West and Harada, 1993; Hills, 2004; Vicente-Carbajosa and Carbonero, 2005; Gutierrez et al., 2007). In the first phase, cell division and differentiation occur. The second phase includes cell expansion and accumulation of nutrient reserves. The third phase involves desiccation and acquisition of quiescence. *B. napus* accumulates two major storage proteins, mostly in the second phase: cruciferin (12S globulin; 60% of total seed proteins) and napin (2S albumin; 20% of total seed proteins)(Crouch and Sussex, 1981). These storage proteins are initially produced as pro-proteins and subsequently processed by vacuolar processing enzymes and/or aspartic proteinases to yield heterooligomeric cruciferin and heterodimeric napin (Schwenke et al., 1981; Ericson et al., 1986; Yamada et al., 2005; Otegui et al., 2006). Protein storage vacuoles (PSV) biogenesis is a very complex process. The sorting of storage protein from endoplasmic reticulum (ER) to PSV as well as PSV biogenesis are very elaborate processes that are not completely understood (Okita and Rogers, 1996; Neuhaus and Rogers, 1998; Herman and Schmidt, 2004; Vitale and Hinz, 2005).

In a recent large-scale collection of *B. napus* zygotic embryo (ZE) expressed sequence tags (EST), we found many clones corresponding to *BNM2*. In agreement with Hattori et al (1998), *BNM2* has been found in other MDE cDNA libraries as well (Boutilier et al., 1994; Joosen et al., 2007; Malik et al., 2007; Tsuwamoto et al., 2007). We report that BNM2 protein is not present in MDE but is produced in seeds and accumulated in PSV. Accumulation of BNM2 is under tight spatio-temporal control. There is a substantial lag between mRNA synthesis and protein accumulation. The attempts to uncouple this control by over-expression

of *BNM2* caused distortion of PSV without any significant alteration to the BNM2 content,
68 suggesting a tight control by the cell over production of this enigmatic protein component.

RESULTS

70 ### *BNM2* Transcription is Active in Zygotic and Microspore-Derived Embryos

Analysis of our *B. napus* EST collections from ZE showed that there are two groups of
72 *BNM2* genes expressed during embryogenesis; one corresponds to the cDNA characterized by Hattori et al (1998) and the other is 94% identical to the above in the open reading frame
74 (ORF). As *B. napus* is an amphidiploid (AA CC genome), resulting from natural hybridization between *Brassica rapa* (AA) and *Brassica oleracea* (CC) (Kimber and McGregor, 1995), we
76 tentatively considered these two types of *BNM2* as homeologous representatives because our ESTs have been generated from a doubled-haploid line of *B.napus* (DH12075; all ESTs
78 deposited in GenBank). We then isolated the *BNM2* sequences of the diploid species by polymerase chain reaction. Based on sequence identity between the PCR products and the
80 cDNA ORFs above, we assigned the two *BNM2* cDNA representatives to *B. rapa* (*BNM2A*) and *B. oleracea* (*BNM2C*). *BNM2A* and *BNM2C* occur in ESTs from ZE, MDE, and whole
82 seeds of *Brassica* spp reported in Genbank (Supplemental Table S1). *BNM2A* occurs at more than 3-fold abundance in ZE relative to MDE. *BNM2A* encodes a deduced polypeptide of 282
84 aa with a molecular mass of 32.1 and predicted pI of 7.23, and *BNM2C* encodes a deduced polypeptide of 281 aa with molecular mass 31.9 and a predicted pI of 6.33

86 ### *BNM2A* and *BNM2C* Are Transcribed in Developing Seeds and Also in Vegetative Tissues

The expression patterns of *BNM2A* and *BNM2C* were determined by reverse transcription-PCR (RT-PCR). A region that shows 65% difference in the 3′ untranslated regions (UTR) of *BNM2A* and *BNM2C* was used for designing gene-specific primers (Supplemental Fig. S1). As shown in Fig. 1A, *BNM2A* transcripts were detected in roots, stems, flower buds, radicle, and young cotyledons but not in 30-day old cotyledons, leaves, and open flowers. *BNM2C* expression was generally weaker than *BNM2A*, and was evident in radicle and roots. The expression of both *BNM2A* and *BNM2C* was, however, more pronounced in the embryo and seed coat tissues of developing seeds (Fig. 1B). *BNM2A* and *BNM2C* transcripts were detectable as early as 3 DPA. They were abundant in 17-DPA and 28-DPA samples but declined thereafter; *BNM2C* transcripts diminished sooner than *BNM2A* transcripts. The steady-state level of *BNM2C* transcripts was generally lower than that of *BNM2A* transcripts in both vegetative tissues and ZE, with the exception of 17- and 28-DPA embryos where the difference was not so obvious. In MDE, both *BNM2A* and *BNM2C* were comparably expressed.

The Arabidopsis genome contains a gene whose deduced protein (AT1G49320) is ~75% identical to the deduced proteins of *BNM2A* and *BNM2C* (Supplemental Fig. S2). Similar to *BNM2A*, *AT1G49320* was found to be expressed in cotyledons, radicle, floral buds, open flowers, roots, and developing seeds, but not in leaves (Fig. 1C). Notably, *AT1G49320* expression in seeds was relatively poor unlike *BNM2* expression in *B. napus*.

BNM2 Protein Accumulates Only in Seeds and Only Long After Transcript Accumulation Becomes Evident

A synthetic polypeptide for the aa residues 27-41 (YTSRKLISNNEQEGQ) of BNM2A was used for raising antiserum against BNM2. This peptide fragment is identical to its counterpart from BNM2C in all but the first aa, which is a cysteine in the latter. The specificity of the BNM2 antiserum is shown in Fig. 2. *E. coli* cells expressing a 59-kD *GST-BNM2* fusion but not those expressing *GST* alone showed an immuno-detectable product (Fig. 2A). In *B. napus* mature seeds, two positive bands (~36 ka and ~29 kD, Fig. 2B) were found in 1D PAGE and these were resolved into 4 immuno-positive spots in 2D PAGE (Fig. 2B). These results would be consistent with the presence of isoforms of BNM2 or processed versions. Note that BNM2A and BNM2C have an *N*-terminal hydrophobic region that is predicted to be a signal peptide for secretory targeting (www.expasy.com; targetP and WoLF PSORT (Horton and Park, 2006; Emanuelsson et al., 2007).

Despite the presence of *BNM2* transcript in the stem, root and floral bud tissues (Fig. 1A), BNM2 protein was not detectable in these organs (Fig. 2C). Similarly, MDE contained an abundant level of the transcript but had no detectable BNM2 protein (Fig. 1B and Fig. 2C). In contrast, the seeds approaching maturity as well as mature seeds contained BNM2 protein (Fig. 2D). Notably, whereas much younger seeds (less than 17 DPA) contained a relatively abundant quantity of *BNM2* mRNA (Fig. 1B), the polypeptide was undetectable in them as shown for the 10- and 18-DPA seeds. At 30 DPA, the protein became detectable and progressively more prominent as the seeds matured. In contrast, cruciferin and napin were abundant in 30-DPA seeds (Fig. 2E). As the seeds proceeded to germinate (between 24 h and 48 h post imbibition), BNM2 became undetectable just as in the case of the seed storage protein cruciferin (Fig. 2F).

BNM2 Localizes to Protein Storage Vacuoles

To determine the cellular location, we fused a GFP (Teerawanichpan et al., 2007) to the *C*-terminus of full-length BNM2A and screened particle-bombarded tobacco epidermal cells for GFP fluorescence. As shown in Fig. 3A, the unfused GFP control showed fluorescence almost throughout the cells, whereas BNM2A-GFP highlighted a network, suggesting ER localization of BNM2A. We surmise that this is mediated by the *N*-terminal signal peptide region of the BURP protein. We extended this investigation by electron microscopy and sub-cellular fractionation of the PSV. As shown in Fig. 3B-C, BNM2 was found in the PSV. The PSV fraction contained two forms of BNM2, and the persistence of unprocessed polypeptides might be responsible for the second form.

Constitutive Expression of *BNM2* Does Not Cause Accumulation of the Protein Product in Transgenic Leaves

Given the observed lag between accumulation of the transcripts and production of BNM2 protein, we attempted to perturb this apparent temporal regulation by constitutive production of the mRNA under the control of a *CaMV 35S* promoter. In addition, we wanted to determine if BNM2 could be produced in vegetative parts. Sixteen independent primary transgenics (T0) that were selected for kanamycin resistance were screened by PCR and by GUS staining (see Materials and Methods). The *BNM2* transcript level in transgenic plants was further evaluated by RT-PCR (Fig. 4A). *BNM2A* transcripts were found in the leaves of 7 transgenic plants (Line #1, 6, 8, 12, 14, 16, and 19) but not in the empty-vector control. Five transgenic lines with relatively high transcript levels (Line #1, 6, 8, 12, and 14) were further analyzed for protein production (Fig. 4B). The leaves, however, did not contain any BNM2

proteins. The mature seeds on the other hand showed up to 1.5 fold greater level of BNM2 protein in one of the transgenic lines (Line 1). Thus, despite the production of *BNM2* transcripts, the transgenic leaves were unable to accumulate BNM2 proteins. We also investigated protein accumulation in developing seeds of two transgenic lines (Line 1 and Line 6). BNM2 protein was not detectable in 15 DPA seeds but became evident in 30 DPA seeds (Fig. 4C). This result suggested that post-transcriptional control of *BNM2* expression had been maintained in the transgenic seeds such that BNM2 was not produced at an earlier time relative to its native temporal control in control cells. Alternatively, transgenically produced protein might have been rapidly degraded by the native control processes.

Perturbation of *BNM2A* Expression Causes Abnormal PSV in Seeds

The gross morphology of the vegetative parts, flowers and seeds of *CaMV 35S-BNM2A* plants was comparable in untransformed and vector control plants. However, cytological analysis of seeds showed notable differences. As illustrated for *CaMV 35S-BNM2A* Line 1, the embryos contained fewer PSVs and the size of the PSVs was larger in comparison with untransformed plants as seen by light microscopy (Fig. 5A-B). Electron microscopy revealed that untransformed embryos contained almost uniformly electron dense and smooth-contoured PSVs (Fig. 6A-B). The occasional lacunae that are seen are due to phytate globoids being lost from the sections. In contrast, the transgenic seeds produced a number of deformed and rough-edged PSVs (Fig. 6C-F). The electron density in these PSVs was relatively low in the center but more intense at the periphery (Fig. 6C-D). In addition, *CaMV 35S-BNM2A* seeds had more electron dense particles in the cytosolic region when compared with the control seeds (Fig. 6B and 6E), which is likely due to more free ribosomes.

In some cells, there were milder aberrations (Fig. 6F). Immuno-gold electron microscopy localized BNM2 to PSV, where cruciferin and napin were also found. All these were evenly distributed in control samples (Fig. 6G-I). On the other hand, in the PSVs of the *BNM2* over-expressing transgenic lines both BNM2 and the major storage proteins were clustered and restricted to the electron dense regions (Fig. 6J-L).

Constitutive *BNM2* Expression Does Not Advance Production of BNM2 Proteins in Seeds

Assuming from the above that PSV are likely required to house transgenically produced BNM2, we studied the vacuolar morphology of developing seeds in vector control and *CaMV 35S-BNM2A* transgenic plants; for the latter, seeds from T1 plants (i.e second generation transgenics) were used. In 20 DPA control seeds, most cells showed only 2 or 3 vacuoles and these that did not stain positively for proteins; the staining was positive only in a few cells (Fig. 7A). As the seeds developed further to 30 DPA stage, almost all of the large vacuoles had been converted to PSV with proteins (Fig. 7B). The PSV stained heavily in 40 DPA seeds (Fig. 7C). In transgenics, there were no differences at 20 DPA (Fig. 7D). At 30 DPA, there were cells with both large unstained vacuoles and stained PSVs; this contrasted with corresponding control seeds that did not contain as many such cells (Fig. 7E). The protein content in PSV had also dramatically increased during the 30-40 DPA period as in the case of the control (Fig. 7F). Thus, there was no evidence of protein accumulation or vacuolar distortion at 20 DPA despite the use of a constitutive promoter.

DISCUSSION

BURP domain proteins are plant-specific and are enigmatic. There are 162 deduced proteins annotated as BURP in Uniprot (www.pir.uniprot.org ; June 2008). The EST databases in GenBank have >10,000 sequences that have the BURP domain. The diversity of their gene expression contexts – ranging from tapetum to stem to seeds and inducibility by aluminum or drought stress – is perplexing. There is no evidence of catalytic activity for BNM2 or for any of the previously characterized BURP proteins. The deduced structure does not offer a hint of function for any BURP protein, except to suggest membrane targeting. Although the ~225-aa BURP domains in the large family share only 6% aa identity, there are 21 aa occurring through the length of BURP domain that are highly conserved. It has been suggested that the presence of six cysteines in BURP domain might be involved in intramolecular disulfide bond formation in protein folding (Treacy et al., 1997; Hattori et al., 1998). The repeated units, which is present in some BURP proteins such as the β subunit of PG1 (Zheng et al., 1992) and SCB1 (Batchelor et al., 2002), might be involved in cell wall matrix binding. The absence of any repeated units in BNM2 suggested a potentially different intracellular location for this protein, and we found the protein in ER and PSV.

Only a very few BURP proteins/genes have been studied so far. The consequences of suppressing *PG1β* in tomato (Watson et al., 1994) and *RAFTIN* in rice (Wang et al., 2003) suggest relevance of the former to pectin metabolism but not to ripening and the latter to pollen development. The PG1β polypeptide has been isolated from plant tissues (Zheng et al., 1992) and others such as RAFTIN (Wang et al., 2003) and SCB1(Batchelor et al., 2002) have only been found immuno-diagnostically. For a vast majority of the *BURP protein* genes, there

is no evidence of protein production. Notably, the protein product of *USP*, the first of the BURP family protein gene to be identified has not been found in its native expression domain (cotyledons of field bean), despite the copious production of USP transcripts (Bassuner et al., 1988; Baumlein et al., 1991). Our results and those of Van Son et al. (2009; the accompanying paper) provide the first report that shows the presence of a BURP protein in PSV. BNM2 is targeted to ER by its *N*-terminal signal peptide and the protein is transported to PSVs during late embryogenesis. The unusual seed phenotypes, including PSV deformation, improper deposition of seed storage proteins, and an increase in free ribosome in cytosolic compartment when *BNM2* gene is overexpressed might indicate a role for BNM2 in PSV biogenesis in *B. napus*. This cytological defect is also observed when the BNM2 homolog in Arabidopsis, *AtUSPL1* or *AT1G49320* is over-expressed in Arabiodopsis as shown by Van Son et al., 2009.

BNM2 has been undetectable in MDE although *BNM2* was originally identified as an abundantly expressed gene in MDE (Hattori et al., 1998) and its expression is recognized as a marker for embryogenic microspore cultures (Boutilier et al., 2002; Joosen et al., 2007; Malik et al., 2007; Tsuwamoto et al., 2007). Although MDE contain the transcripts of several embryogenesis-related genes (Taylor et al., 1990; Malik et al., 2007) and some proteins/enzymes involved in production of storage lipid (Taylor et al., 1990; Holbrook et al., 1991), at the sub-cellular level the morphology and cytology of MDE vary from that of ZE (Yeung et al., 1996). Unlike the ZE that, at mid-point of maturation, show vacuoles being transformed into PSV, MDE do not contain appreciable PSV. PSVs are highly specialized organelles and the milieu of PSV (Okita and Rogers, 1996) may be crucial for BNM2 protein assembly and stability. This likely explains the absence of BNM2 in MDE.

While *BNM2* is active in zygotic embryos and floral buds (Hattori et al., 1998; Joosen et al., 2007; Malik et al., 2007) and vegetative parts (this study), BNM2 protein has not been detectable in locations other than the seed. Thus, BNM2 is a seed-specific protein even though its gene is also expressed elsewhere. In this respect it resembles other seed protein genes such as *phaseolin*, *legumin B4*, *Late Embryogenesis Abundant proteins* (*LEA*) and *napin* that have broader expression domains (Boutilier et al., 1994; Vicient et al., 2000; Vicient et al., 2001; Zakharov et al., 2004)

A notable difference in comparison with other seed proteins, cruciferin and napin, the transcription of *BNM2* occurs sooner but the accumulation of BNM2 protein is not evident until 20 days thereafter; the lag between transcript peak and protein peak is at least 18 days. This is notably longer than that found with the production of other storage proteins as illustrated in Supplemental Fig. S3 (Crouch and Sussex, 1981; Delisle and Crouch, 1989; Sjodahl et al., 1993). This data indicates that BNM2 is tightly regulated at post-transcriptional level and it cannot be advanced by *CaMV 35S* promoter. However, the cytology of developing seeds indicates that this anomalous appearance of seeds occurs around 30 DPA in *CaMV 35S-BNM2A* when BNM2 protein accumulates. At least three potential mechanisms can account for this: (a) inhibition of translation (by miRNA, for example); (b) requirement for accessory factors for translation; (c) rapid degradation of BNM2 at early stages until the proteolytic processes are hindered, for example, by the production of proteinase inhibitors at later stages. Post-transcriptional gene regulation has been previously reported to control the expression of other seed protein genes (ie. *late embryogenesis abundant*; *LEA*, USP and cruciferin; (Bassuner et al., 1988; Taylor et al., 1990; Bies et al., 1998)) as well as a stress-induced gene,

BN28 (Boothe et al., 1995). Treatment of MDEs with abscisic acid (ABA) substantially induces *cruciferin* mRNA but has a negligible effect on the protein level (Taylor et al., 1990). Arabidopsis *LEA* protein gene, *EM6* also shows a delay in protein translation (Bies et al., 1998). Moreover, either treatment of immature siliques with ABA or over-expression of *ABI3* in vegetative tissues induce *EM1* and *EM6* transcript levels without any increase in the protein levels (Parcy et al., 1994; Bies et al., 1998). However, BNM2 does not contain repeated units, presumably allowing the protein to be transferred to PSV through the ER network.

CONCLUSION

BNM2 is a plant-specific seed protein that is targeted to the ER and destined to PSV during the mid-stage to late embryogenesis. This gene is tightly regulated at the post-transcriptional level. Over-expression of *BNM2* in *B. napus* alone is unable to advance the accumulation of BNM2 in the seed or to afford protein production elsewhere. However, BNM2 protein starts to appear in 30 DPA transgenic seeds and to accumulate at 1.5-fold greater level compared to the untransformed plants. Overproduction of BNM2 in these transgenic lines causes an anomalous morphology of PSV. Taken together with the work reported in Van Son et al. (2009), the zygotic embryo-associated BURP proteins are involved in PSV biogenesis.

MATERIALS AND METHODS

Plant Materials and Transformation

Arabidopsis thaliana cv. Columbia (Arabidopsis) and *Brassica napus* (cv. DH12075) seeds were surface-sterilized in 20% (v/v) (for Arabidopsis) and 100% (v/v) sodium hypochlorite (for *B. napus*) for 10 min, thoroughly rinsed with sterile distilled water, and

germinated on MS medium (Murashige and Skoog, 1962). For expression analysis, Arabidopsis and *B. napus* seeds were germinated on MS medium and subsequently transferred to soil in a growth cabinet at 22°C with a 16-h photoperiod (120 μE m^{-2} s^{-1}). For the germination assay, *B. napus* seeds were germinated on paper soaked with water at 22°C under 16 h (120 μE m^{-2} s^{-1})/8 h (light/dark) regime and samples were collected at 2, 6, 12, 18, 24, 48 h time points. *B. napus* (cv. Topas) MDE were from the studies of Ferrie and Keller (2007) and a kind gift from A. Ferrie. Six-week old Arabidopsis plants were transformed with *Agrobacterium tumefaciens* GV3101 [pMP90] (Koncz and Schell, 1986) by a floral dipping method (Clough and Bent, 1998). Agrobacterium-mediated transformation of *B. napus* cotyledonary petiole explants was performed according to Moloney et al (1989).

RNA Isolation and Reverse transcription–PCR (RT–PCR)

Total RNA was extracted from 100-mg leaf tissues using the RNeasy plant mini kit under conditions detailed by the supplier (Qiagen) and treated with DNaseI (Invitrogen, 1 unit μl^{-1}). First-strand cDNA was synthesized at 42°C for 2 h, using 0.5 µg of oligo$(dT)_{12\text{-}18}$ primers, 1-µg total RNA isolated from appropriate *B. napus* tissues, and 200 units of SUPERSCRIPT II Reverse Transcriptase (Invitrogen). An appropriate amount (0.5-2µl) of the first-strand reaction was subsequently used as a template for 25-µl PCR reaction in the presence of 2.5 units of *Taq* DNA polymerase (Invitrogen). Primers OL5002 (5′-ATTACTTCTCTTCAAAGAAAAATT-3′) and OL5505 (5′-TAGCCAGCAACACTTTTTTATTT-3′) were used to generate a 955-bp *BNM2A* amplicon, primers OL5504 (5′-ATTACTCTCGTCAAAGAAAAATA-3′) and

OL5507 (5′-TTTTCAAACATTACAAATACAAAGAGAATAC-3′) were used to generate a 961-bp *BNM2C* amplicon. Primers OL5407 (5′-ATTGAGATCTAAGATCACTTGAACACTTATAAA-3′) and OL5409 (5′-TTACTTTGTTACCCACACAATGTTATCAAG-3′) were used to generate a 976-bp *AT1G49320* amplicon. Primers OL5538 (5′-ACTACGAGCAGGAGATG-3′) and OL5539 (5′-GAGCACAATGTTACCGT-3′) were used to generate a 232-bp internal control for a housekeeping gene, *actin2* (GenBank accession no. AF111812). The PCR conditions for *BNM2A*, *BNM2C*, and *AT1G49320* were 30 cycles of 94°C, 1 min, 56°C, 30 sec, and 72°C, 1 min; for *actin2*: 29 cycles of 94°C, 30 sec, 56°C, 30 sec, and 72°C, 30 sec. A 25-µl aliquot of *BNM2A*, *BNM2C* and *AT1G49320* reactions and a 10-µl aliquot of *actin2* reactions were further used for agarose gel analysis.

Construction and Expression of *GST-BNM2* Fusion in *E. coli*

Amplification of *BNM2A* ORF was performed using BD Advantage 2 Polymerase Mix (Clontech) with primers OL5739 (5′-TGCACCGCGGCTTCTTTGCGATTCTCTGTC-3′ and OL5740 (5′-TGCAGCTCGAGTTACTACTTTGATACCCACACAATATTATC-3′). The PCR reaction conditions were as follows: 35 cycles of 94°C for 45 sec, 55°C for 30 sec, and 72°C for 1 min, and final incubation at 72°C for 10 min. The *BNM2A* amplicon was cloned in-frame into *Sac*II-*Xho*I sites of pET41a vector (Novagen), yielding pPT120. Plasmids pET41a and pPT120 were transformed into *E. coli* (BL21 (DE3) pLysS, Novagen). A single colony of *E. coli* was inoculated in 2 ml LB supplemented with 50 mg L^{-1} kanamycin and 34 mg L^{-1} chloramphenicol and grown at 37°C to Abs_{600} of 0.6 to1.0. Gene expression was induced by 1 mM IPTG for 6 h. Cell pellet was resuspened in 200 µl Tris-HCl (pH 8.0). An aliquot of 20

328 µl of samples were mixed with 2X SDS sample buffer, heated to 95°C for 5 min, and then fractionated on 12.5% SDS-PAGE.

330 **Protein Extraction, 1D and 2D PAGE**

One hundred mg of samples of various stages of developing seeds were homogenized 332 with 400 µl buffer M [50 mM MOPS/NaOH buffer, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol (DTT), 60 µg ml^{-1} phenylmethanesulphonyl fluoride]. The supernatant was 334 collected by centrifugation at 16,000*g* at 4°C for 10 min and the protein concentration was determined using a Bradford Protein Assay Kit (Bio-Rad). For 1 D-PAGE, 30 µg of protein 336 extracts were mixed with 2X SDS sample buffer, heated to 95°C for 5 min, and then fractionated on 12.5%/15% SDS-PAGE. The gel was either subjected to Coomassie Blue 338 staining or western blotting. For 2 D-PAGE, 500 µg of protein were precipitated with 4 volumes of 10% (w/v) trichloroacetic acid/acetone containing 0.07% β-mercaptoethanol, 340 incubated at -20°C for 2 h, and centrifuged at 16,000*g* at 4°C for 15 min. The pellet was washed with cold acetone containing 0.07% β-mercaptoethanol and resuspended in 300 µl of 342 isoelectic focusing (IEF) sample buffer (8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte (Bio-Rad), 0.001% (w/v) Bromophenol Blue). ReadyStrip™ IPG strip 344 (pH 3-10 non-linear; 17 cm; Bio-Rad) was passively rehydrated with protein sample at room temperature for overnight and subjected to isoelectric focusing (IEF) using a Protein IEF Cell 346 (Bio-Rad) at a maximum current of 50 μA/strip and a final voltage of 10,000 V for a total of 60,000 V· h (rapid ramp). Prior to SDS-PAGE, the protein strip was equilibrated sequentially 348 with SDS equilibration buffer (375 mM Tris-HCl; pH 8.8, 20% glycerol, 2% SDS, 6 M Urea) supplemented with 2% (w/v) DTT and SDS equilibration buffer supplemented with 2.5%

(w/v) Iodoacetamide for 15 min each. The protein strip was subsequently fractionated on 15% SDS-PAGE.

Production of BNM2 Antiserum and Immunodetection

A synthetic polypeptide for the aa residues 27-41 (YTSRKLISNNEQEGQ) of BNM2A was used for raising BNM2 antiserum. This region of BNM2A was chosen due to its high identity with the corresponding region of BNM2C (CTSRKLISNNEQEGQ) and its high hydrophilicity and antigenicity (Protean program, Lasergene 7; DNASTAR). The BNM2A antiserum was purified by HYDRA® peptide immuno-affinity purification column (Charles River Laboratories).

For immunodetection, the polypeptides were electroblotted onto a polyvinylidene difluoride membrane (Hybond-P, Amersham), and the membrane was probed with BNM2A antiserum (BNM2; 1:1,000), C2 cruciferin antiserum (CRU2; 1:10,000), or rAt2S2 napin antiserum (1:10,000), followed by goat anti-rabbit IgG (H+L) Alkaline phosphatase conjugate (Bio-Rad), and colorimetric detection with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Roche), according to the supplier's instructions. Low SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) was used as a standard marker for protein size determination.

DNA Sequencing and Analysis

All synthesis and sequencing work were performed by the DNA Technologies Unit at the Plant Biotechnology Institute, National Research Council of Canada. Nucleotide sequence and aa sequence comparisons were performed using Lasergene 7 (DNASTAR Inc.).

Amino Acid Sequence Alignment

The aa sequences of BNM2A, BNM2C and AT1G49320 were aligned using ClustalW program hosted by the European Bioinformatics Institute (Chenna et al., 2003), with default parameters, including the Blosum scoring matrix and a gap penalty of 10.

Generation of *BNM2A-GFP* Fusion and *CaMV 35S-BNM2A* Construct

The vector backbone for the *BNM2A-GFP* construct is a derivative of pTZ19R (accession number Y14835; (Mead et al., 1986) in which the 5′-*Hin*dIII-through-*Kpn*I-3′ of the original multiple cloning site had been replaced with a *Hin*dIII-*Xba*I-*Pst*I-*Xho*I-*Spe*I-*Kpn*I poly-linker obtained by annealing 5′-AGCTTTCTAGAATCGCTGCAGTATCGTCTCGAGA-3′ and 5′-CTAGTCTCGAGACGATACTGCAGCGATTCTAGAA-3′. This modified pTZ19R also contained an *Emerald GFP* ORF and *nopaline synthase* (*NOS*) terminator at *Spe*I-*Kpn*I sites and *Sac*I-*Eco*RI sites, respectively. This vector was designated as pPT100 (Teerawanichpan et al., 2007). The *2XCaMV 35S* promoter from pHS571 (G. Selvaraj and R. Hirji, unpublished work) was cloned into *Hin*dIII-*Xba*I sites of pPT100, yielding pPT107. The *BNM2A* ORF without stop codon was amplified using OL5519 (5′-TGCACTGCAGATTACTTCTCTTCAAAGAAAAATTATCTAT-3′) and OL5520 (5′-TGCACTCGAGCTTTGATACCCACACAATATTATC-3′) and cloned into *Pst*I and *Xho*I sites of pPT107, yielding *BNM2A-GFP* construct (pPT109). The PCR amplification of *BNM2A* amplicon was performed using BD Advantage 2 Polymerase Mix (Clontech). The PCR reaction parameters were as follows: 35 cycles of 94°C for 45 sec, 55°C for 30 sec, and 72°C for 1 min, and final incubation at 72°C for 10 min. For the GFP alone control, the *Emerald GFP* was subcloned into a derivative of pGKK14 (Datla et al., 1991), yielding

pTH15. In pTH15, the *Emerald GFP* was flanked by *tac* promoter (tacP) and *NOS* terminator. The tacP in pTH15 was subsequently replaced with *2XCaMV 35S* promoter to give a GFP control construct (pPT40).

For *CaMV 35S-BNM2A* construct, the *BNM2A* cDNA including 5′ and 3′ UTR, was excised from the pDNR-LIB vector (Clontech) by digesting the plasmid with *Ava*I, and subsequently end-filled, and ligated into *Sma*I site of a plant binary vector pHS737 (G. Selvaraj and R. Hirji, unpublished work), resulting in pPT90. pPT90 contained *BNM2A* driven by *CaMV 35S* promoter, *neomycin phosphotransferase* (*nptII*) as a selection marker, and a *β-glucuronidase* (*GUS*) as a reporter.

Biolistic Transformation of Tobacco Epidermal Tissues and GFP Detection

Prior to bombardment, tobacco leaves were transferred to half strength MS solid medium. Plasmid DNA (5 μg), which was coated on gold particles (3 mg; 1 μm outer diameter), was subsequently bombarded onto tobacco leaves, using the PDS 1000/He biolistics system (Bio-Rad) with 1,100 psi He pressure under 25 inch Hg vacuum. After bombardment, epidermal tissue was held at 22°C under 16 h/8 h (light/dark) regime for 24 h to allow for transient expression of the introduced genes. The bombard leaf tissues were examined for GFP fluorescence under a confocal laser scanning microscope (CLSM; Zeiss LSM 510 META, Carl Zeiss) equipped with a GFP filter set (excitation: 488 nm; barrier filter: 507-529 nm).

PSV Isolation

PSV was isolated from *B. napus* mature seeds according to the glycerol protocol as described by Gillespie et al (2005), with some modifications. Approximately 30 mg of seeds

were ground with a pestle, 100 ml of 50% (v/v) glycerol were added and pulverized in a blender. The debri were first filtered with cheesecloth and the fitrate was centrifuged at 3000*g* at 4°C for 15 min twice to remove the remaining cell debri. The supernatant was centrifuged at 12000*g* at 18°C for 25 min. The PSV pellet was resuspended and washed with 50% (v/v) glycerol for 3 times. The purified PSV was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and subjected to protein analysis.

Light Microscope, Transmission Electron Microscopy and Immunogold Labeling

Semi-thin sections (1 μm) of *B. napus* seed were stained with 0.5% toluidine O blue for 1.5 min, rinse with distilled water, and examined under Leica DMR fluorescence microscope. Transmission electron microscopy and immunogold labeling experiment of ultrathin sections (50-70 nm) of *B. napus* mature seeds were performed as previously described (Wang et al., 2003). C2 cruciferin (CRU2; 1:1,000) or BNM2 (1:100) antiserum was used for immunogold labeling experiment.

Examination of *CaMV 35S-BNM2A* transgenic plants

B. napus transgenic plants were selected on MS medium supplemented with 50 mg L^{-1} kanamycin. The presence of the gene cassette was confirmed by PCR of *BNM2A* amplicon with OL5002 and OL5505 and 5-bromo-4-chloro-3-indolyl-β-glucuronic acid-based β-glucuronidase (GUS) assay (Jefferson et al., 1987). To examine the expression level of *BNM2A* in transgenic plants, the RNA was isolated from 1-month old leaf tissues and subjected to RT-PCR analysis, using primers, OL5002 and OL5505.

Accession Numbers

BNM2A cDNA; FJ203999, genomic *BNM2A*; FJ204000, *BNM2C* cDNA; FJ204001, genomic
438 *BNM2C;* FJ204002

Supplemental material

440 **Supplemental Figure S1.** Nucleotide sequence alignment of genomic *BNM2A* and *BNM2C.*

Supplemental Figure S2. Amino acid sequence alignment of BNM2A, BNM2C and
442 AT1G49320.

Supplemental Figure S3. Transcript and protein level during embryogenesis of BNM2,
444 cruciferin and napin.

Supplemental Table S1. The number of *BNM2A* and *BNM2C* clones present in various stages
446 of zygotic and microspore embryo EST libraries.

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454

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Figure legends

Figure. 1. Expression profile of *BNM2A* and *BNM2C* in *B. napus* and *AT1G49320* in Arabidopsis. RT-PCR analysis of *BNM2A* and *BNM2C* expression in various tissues at different developmental stages. *BNM2A* cDNA and *BNM2C* cDNA were used as templates to demonstrate primer gene specificity. The lowest panel is the control RT-PCR of a house keeping gene, *actin2*. Primers and PCR conditions are described in Materials and Methods. DPI; day post imbibition, DPA; days post anthesis, MDE; microspore-derived embryos.

Figure 2. Accumulation of seed proteins in *B. napus*. Panels A and B are controls for the antiserum. Pre-immune serum control is shown in Panel C. A, Immunodetection of BNM2 in *E.coli* expressing *GST-BNM2* fusion but not *GST* alone. Asterisks indicate degraded GST-BNM2 proteins. B, Immunodetection of BNM2 in *B. napus* mature seeds on 1D (left panel) and 2D PAGE (right panel). The right panel shows a portion of the blot from a 2D PAGE. The gel was resolved with a ReadyStrip™ IPG strip pH 3-10 non-linear. Panels C - F are blots of plant proteins. Solid and open arrow heads show 29 kD and 36 kD immune reactive bands,

632 respectively, with antiserum raised against BNM2 peptide. The arrows denote cruciferin or napin bands that react with respective antiserum.

634 **Figure 3.** Localization of BNM2 protein. A, *N. tabacum* leaves bombarded with the indicated constructs that show uniform (GFP) or network of fluorescence (*BNM2A-GFP* fusion). B,

636 Immunodetection of BNM2 among PSV proteins resolved on 1D PAGE; solid and open arrow heads indicate 29 kD and 36 kD bands. C, Electron microscopic localization of BNM2 in

638 mature seeds of *B. napus*. Gold-labeled BNM2 antibodies were used. Arrow heads point to BNM2. PSV; protein storage vacuoles, PG; phytate globoids, LB; lipid bodies, CW; cell wall.

640 **Figure 4.** Expression analysis of *BNM2* in transgenic *B. napus* lines. A, RT-PCR analysis of *BNM2A* expression in leaf tissue of vector control (VC) and independent *CaMV 35S-BNM2A*

642 transgenic plants (1-19); the bottom panel shows RT-PCR of *actin2*. B, Immunodetection of BNM2 proteins in leaf tissue and mature seed of vector control and *CaMV 35S-BNM2A* lines. The

644 relative amount of BNM2 protein was determined by comparing the intensity of BNM2 (both 29 kD + 36 kD) bands to the cruciferin band, with a reference value of 1.0 for the vector control. C,

646 Immunodetection of BNM2 and cruciferin in 15-DPA and 30-DPA seeds of *CaMV 35S-BNM2A* Line 1 and 6. Solid and open arrow heads show 29 kD and 36 kD immune reactive bands,

648 respectively. Arrow indicates immune reactive band of cruciferin.

Figure 5. Toluidine blue O stained sections of mature seeds.

650 **Figure 6.** Electron microscopic investigation of sections of mature seeds from control and transgenic lines of *B. napus* expressing *BNM2* under the control of a *CaMV35S* promoter.

652 Panels A to F show sub-cellular features in an untransformed (A and B) and *CaMV 35S-BNM2A* line (C-F). Panels G to L show immunogold lebelling of proteins (arrow heads) with

654 cruciferin antiserum (CRU) or BNM2 antiserum (BNM2). PSV; protein storage vacuoles, PG; phytate globoids, LB; lipid bodies, CW; cell wall, RB, ribosome.

656 **Figure 7.** Appearance of PSV as observed by toluidine blue O staining of sections of developing seeds from control and transgenic plants. Solid and open arrow heads denote

658 stained PSV and large unstained vacuoles, respectively.