Retrotransposon BARE displays strong tissue-specific differences in expression

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Summary

• The BARE retrotransposon comprises c. 10% of the barley (Hordeum vulgare) genome. It is actively transcribed, translated and forms virus-like particles (VLPs). For retrotransposons, the inheritance of new copies depends critically on where in the plant replication occurs.
• In order to shed light on the replication strategy of BARE in the plant, we have used immunolocalization and in situ hybridization to examine expression of the BARE capsid protein, Gag, at a tissue-specific level.
• Gag is expressed in provascular tissues and highly localized in companion cells surrounding the phloem sieve tubes in mature vascular tissues. BARE Gag and RNA was not seen in the shoot apical meristem of young seedlings, but appeared, following transition to flowering, in the developing floral spike. Moreover, Gag has a highly specific localization in pre-fertilization ovaries.
• The strong presence of Gag in the floral meristems suggests that newly replicated copies there will be passed to the next generation. BARE expression patterns are consistent with transcriptional regulation by predicted response elements in the BARE promoter, and in the ovary with release from epigenetic transcriptional silencing. To our knowledge, this is the first analysis of the expression of native retrotransposon proteins within a plant to be reported.

Introduction

The long terminal repeat (LTR) retrotransposons (Class I transposable elements) are ubiquitous in the eukaryotes and account for upwards of 80% of large plant genomes such as maize, wheat and barley (Hordeum vulgare; Liu et al., 2007; Wicker et al., 2009), where they are responsible for many of the changes in genome size and organization over evolutionary time (Hawkins et al., 2009; Choulet et al., 2010). They move by a ‘copy and paste’ mechanism in a lifecycle virtually identical to that of retroviruses, excepting the lack of an extracellular infective phase (Frankel & Young, 1998; Sabot & Schulman, 2006). Autonomous retrotransposons encode the proteins necessary to replicate and then insert into the genome (Sabot & Schulman, 2006). These are expressed as a polyprotein generally in one or two open reading frames (ORFs) and include: Gag, the capsid protein that forms the virus-like particles (VLPs); an aspartic proteinase (AP), which cleaves the polyprotein into functional units; reverse transcriptase (RT) and RNaseH (RH), which produce cDNA from the transcripts of the retrotransposon; integrase (INT), which integrates the cDNA back into the genome (Kumar & Bennetzen, 1999; Wicker et al., 2007). The lifecycle comprises transcription of genomic copies, translation, packaging of transcripts into VLPs, reverse transcription, and targeting of the cDNA copy to the nucleus for integration into the genome (Sabot & Schulman, 2006; Wicker et al., 2007). In the Copia superfamily of LTR retrotransposons the coding domains are organized LTR–gag–ap–in–rt–rb–LTR as one ORF.

Retrotransposon copies may be passed to the next generation in one of two ways: replication as part of the chromosome; or propagation by the combined activities of RNA polymerase II and reverse transcriptase. Both strategies present dilemmas. Accumulation of mutations at the neutral rate during genome replication will eventually lead to the extinction of a retrotransposon family through loss of promoter activity and translational capacity. By contrast, propagation through the retrotransposon lifecycle, while 1000-fold more error-prone (Gabriel et al., 1996) than genome replication, offers the possibility of purifying selection. However, the tissue in which the lifecycle is carried out is critical to whether or not newly propagated copies will be inherited. In order for new copies of plant retrotransposons to be inherited, they must be integrated into the chromosomes of cells that contribute clonally to the floral meristem and ultimately pass into the nuclei of gametes.

The question of where and when in a plant retrotransposons are replicated has been hardly studied; the lifecycles of plant retrotransposons have been examined in only a few cases. One, the BARE retrotransposon of superfamily Copia, accounts for over 10% of the barley genome (Vicient et al., 1999; Wicker et al., 2009). The LTR of BARE contains two promoters that drive

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transcription (Suoniemi et al., 1996; Vicient et al., 2005; Chang & Schulman, 2008; Chang et al., 2013). The presence in the \textit{BARE1} promoter of ABA (abscisic acid)-response elements also found in water stress-induced genes (Suoniemi et al., 1996) and the observed copy number variation of \textit{BARE1} between moist and dry habitats together suggest that the \textit{BARE1} is stress induced (Kalendar et al., 2000), although it is transcribed and translated as well under normal growth conditions (Suoniemi et al., 1996; Jääsäkeläinen et al., 1999; Vicient et al., 2001). The LTRs enclose an internal domain encoding Gag, INT, AP, RT and RH in a single ORF matching the \textit{Copia} organization (Manninen & Schulman, 1993; Suoniemi et al., 1998; Tanskanen et al., 2007).

Expression of \textit{BARE} at the protein level has been demonstrated in extracts from leaves, embryos and callus; the Gag and INT are processed from the polyprotein \textit{in vivo} to the predicted size. The \textit{BARE} family appears widely active; other species of the tribe Triteae, as well as oat (\textit{Avena sativa}) and rice (\textit{Oryza sativa}), express proteins of identical size to \textit{BARE} Gag of barley that react to anti-Gag antiserum (Vicient et al., 2001). In barley, formation of VLPs that contain \textit{BARE} DNA as well as Gag, INT and RT, has been demonstrated (Jääsäkeläinen et al., 1999). \textit{BARE} activity had earlier been demonstrated in extracted whole leaves and embryos, which are complex structures that contain many types of tissues. Here, in order to shed light on the replication strategy of \textit{BARE} in the plant, we have used a combination of immunolocalization and \textit{in situ} hybridization to examine \textit{BARE} expression at a tissue-specific level.

**Materials and Methods**

**Plant materials and growth conditions**

Barley (\textit{Hordeum vulgare} L.) cv Bomi and Golden Promise was raised in 1-l pots in a growth chamber with 18 h, 18°C light and 6 h, 14°C dark cycles. Forty days after germination, at the boot stage before flowering when the first awns were visible – defined as Zadoks stage 49 (Zadoks et al., 1974) – the plants were subjected to drought stress. No water was given to the plants until the pots were dry and the first leaves showed symptoms of withering. Thereafter, the plants were maintained on 50 ml water per day. Samples were collected 3–6 d after the first symptoms of drought. For immunoblotting, barley root tips and steles were collected c. 2–3 cm away from the tips of 3-d-old seedlings, and leaves were collected from 1-wk-old seedlings. The internode, node and inflorescence tissues including seed scales and stamens were prepared from mature, c. 40-d-old plants.

**Protein extractions and immunoblotting**

The anti-Gag and -RT antisera were purified essentially as described earlier (Jääsäkeläinen et al., 1999; Vicient et al., 2001). The RT-RH region of \textit{BARE} was amplified from cDNA with forward (CTACCTGAGGCTGCAAGGCTA) and reverse (CAGCGGTTGCTACATTCCAGA) primers. The PCR product was cloned into pGEMTeasy (Promega) and transformed into JM109 competent cells. A clone containing the correct reading frame was selected and amplified further using primers matching the forward and reverse primers, but with the addition, respectively, of BamHI and XhoI restriction sites. The PCR products were cloned into plasmid PET14b and the plasmid RT-RH/PET14b was transformed into strain BL21(DE3)pLysS \textit{E. coli}. The construct was expressed and then purified on a HiTrap™ nickel metal affinity resin (GE Healthcare Biosciences, Piscataway, NJ, USA) column, then further purified by SDS-PAGE electrophoresis, before injection into the rabbit. The RT antiserum was raised as before (Jääsäkeläinen et al., 1999; Vicient et al., 2001). The proteins were extracted with Strong Denaturing Buffer (SDB) consisting of 60 mM Tris pH 7.5, 60 mM DTT, 2% SDS and proteinase inhibitors (10 μM E-64, 4 μM Pepstatin, 2 μM Leupeptin). Electrophoresis was performed on Bis-Tris gels with high-MW running buffer as described elsewhere (http://openwetware.org/wiki/Sauer:bis-Tris_SDS-PAGE,_the_very_best; Updyke & Engelhorn, 2000). The blotting and immunoreactions were made as previously described (Jääsäkeläinen et al., 1999).

**Paraffin embedding and immunolabeling**

For immunolocalizations, plant tissues were prepared under 4% paraformaldehyde fixative (PFA) buffered either with phosphate-buffered saline (PBS) or 10 mM Hepes pH 7.2, for 10–20 min. The fixative was then changed to one containing 167 mM EDTA and incubated for 1–3 h at 32°C, followed by fixation without EDTA for 1–3 d at + 4°C. Samples were dehydrated in an ethanol series and xylene and then embedded in paraffin. Sections of 3–9 μm thickness were cut, the paraffin removed by xylene, and the sections rehydrated with an ethanol series. Some sections were further cleaned by incubation in 0.05% Tween-20 and the antigens were unmasked by heating the samples in a microwave oven in 10 mM sodium citrate buffer to 100°C. The buffer temperature was increased at a rate of 6.5°C min$^{-1}$ to avoid boiling and excessive bubble formation. The final temperature of + 86°C was reached within 12 min. The buffer was cooled down below + 40°C and sections washed with PBS. The sections were blocked with 5% BSA/PBS for 35 min before 1 h 30 min incubation with primary antibody in all cases. Sections were washed with PBS and further incubated for 40 min with anti-rabbit IgG conjugated with alkaline phosphatase. This secondary antibody was detected with a commercial Fuschin staining kit (DAKO Fuschin Substrate Chromogen System, K0625; DAKO Denmark A/S, Glostrup, Denmark) according to the manufacturer’s directions. Color development was allowed to proceed for 10 min for samples from normally watered plants and 5 min from drought-stressed plants, the times being chosen to optimize the balance between specific staining and background. Sections were mounted in Faramount (DAKO S3025) and photographs were taken with an Olympus (Tokyo, Japan) Provis microscope equipped with digital camera.

\textit{In situ} hybridization

A previously sequenced clone (accession AJ295226) containing the full-length \textit{BARE1} \textit{gag} was used as template for PCR
amplification with the forward primer SacI-1 (ATACGAGCT CATGGCTCGCGGA) and the reverse primer EcoRI-2 (GGAATTCTTTTTCTTTTGCC), which match the ends of gag and contain the restriction sites SacI and EcoRI, respectively. The PCR product was digested with SacI and EcoRI and then cloned into plasmid pSPT18 (DIG RNA Labeling Kit; Roche). The DIG-labeled antisense RNA probe was transcribed with T7 RNA polymerase and the sense-strand control probe was synthesized by SP6 RNA polymerase, following the supplier’s protocol. The probes were treated with DNase I (Ambion) and gel-purified. The tissue sections were first deparaffinized in xylene and then hydrated through an ethanol series. After hydrolyzing the protein, the sections were post-fixed in 4% paraformaldehyde and then hydrated through an ethanol series. The hybridization was done in 800 μl hybridization solution containing 100 μl 10× in situ salts (3 M NaCl, 100 mM Tris pH 8, 100 mM NaH2PO4 pH 6.8, 50 mM EDTA), 400 μl deionized formamide, 200 μl 50% dextran sulfate, 10 μl 100× Denhardt’s solution, 100 μl tRNA (10 mg ml−1), and 2 μl RNase inhibitor. The probe was first denatured for 2–5 min at 80°C in 10 μl hybridization buffer before the remaining buffer was added. The hybridization was made overnight at 55°C. After hybridization, the slides were washed and blocked with blocking solution (1% blocking reagent in Maleic acid buffer, pH 7.5), anti-Dig antibody 1 : 100 added to each slide, and the slides incubated for 1 h. Hybridizations were detected by adding 150 μl of NBT/BCIP (Promega) premix to each slide, incubating in darkness for 12 h, and then visualized.

Transcription factor binding sites

Searches for binding sites in the BARE1 LTR that recognize transcription factors were carried out using online programs such as PlantPan (Plant Promoter Analysis Navigator; Chang et al., 2008) as well as with SIGNAL SCAN (Prestridge, 1991). The LTR of accession Z17327 served as the query sequence.

**Results**

**BARE proteins are expressed and processed throughout the plant during development**

The BARE ORF encodes a polyprotein predicted to be 153 kD (Fig. 1). According to the lifecycle model for retrotransposons and retroviruses, as well as previous results (Jääskeläinen et al., 1999), this polyprotein is cleaved by the integral AP proteinase to release the RT-RH complex, Gag and INT. Immunoblotting with an anti-Gag antibody (Fig. 1) reveals successive processing of the polyprotein to generate the expected intermediate forms of the polyprotein containing Gag with one or more of the other domains cleaved off. The full-length polyprotein, as well as forms with 89 and 55 kD intermediates and the 31 kD mature Gag forms, are detected in varying relative proportions in all organs analyzed (Fig. 1b). The amount of mature Gag detected varied from tissue to tissue, being most abundant in nodes, in developing embryos and in germinating embryos and scutellum, whereas the unprocessed polyprotein was weakly detected in root steles and internodes.

**BARE is expressed as RNA and protein in apical meristematic regions**

The variability in BARE protein expression seen in immunoblotting of barley organs raised the question of whether BARE might be differentially expressed in a tissue-specific manner within the organs. To address this, immunolocalizations with anti-Gag antiserum were carried out. The Gag showed intense localization in root tips in the region of the root initial cells, as visualized in longitudinal sections (Fig. 2a), particularly in the root cap and in cell files of the cortex corresponding to the expected position of the vasculature. Cross-sections from the root apex (Fig. 2b–d) show strong staining for Gag in cells across the stele at the level of the meristem. A combination of immunolocalization and DAPI staining of nuclei in meristem cells at various stages of division (Fig. 2f–h) showed that the Gag is associated with the cytoplasm, but is not restricted to the perinuclear region. The nuclei themselves appear devoid of Gag. The nondividing cells in the root cap (Fig. 2e) showed localization also in cytoplasmic granules, which are likely to be amyloplasts and associated statoliths. In an extended view (Supporting Information Fig. S1a), Gag is found throughout the root but appears most abundant in the vascular initials, meristem and root cap. The BARE RT (Fig. S1b) is detectable above background concentrations throughout the root
in a pattern resembling Gag, but at a lower abundance as expected from the stoichiometry of VLPs (Yap et al., 2000).

The axillary shoot apical meristems of young plantlets (Fig. 3a) displayed strong Gag expression. The meristematic tissues giving rise to leaf primordia and the inflorescence, already formed as a rudimentary spike by 18 d after germination (Fig. 3a), stained intensely for the presence of Gag, while the pre-immune controls (Fig. 3b) showed no response. Gag was abundant also in the pro-vascular tissue between the shoot and radicle, but not in the parenchyma. BARE RNA showed a localization pattern similar to Gag (Fig. 3c).

Earlier in germination, before differentiation of the floral meristem and leaf primordia, Gag protein and BARE transcripts are present only at low concentrations in the embryonic shoot (Figs 4b,e, S2a,b). However, at the same stage, the scutellum and aleurone displayed intense staining for Gag, with high concentrations in vascular tissues of the embryonic axis (Fig. S2c,d), although the starchy endosperm displayed only minor and spotty staining (not shown, but similar to Fig. S4a). This tissue-specific expression pattern cannot be resolved in the whole-embryo immunoblots (Fig. 1). Closer examination revealed that the scutellar epithelium and four sub-epithelial cell layers strongly express the Gag protein, but an adjacent cell layer that separates the scutellum from the embryo had very little Gag (Fig. S2c).

Given the sharp polarization of Gag localization in the scutellum, we examined the correlation between the presence of the protein and BARE RNA in this tissue by in situ hybridization (Fig. 4). Across the scutellum and embryo as a whole, the amounts of RNA and Gag corresponded (Fig. 4b,e), the scutellum at 4 d after germination showing intense Gag protein expression. In sharp contrast, the cell layer of the scutellum adjacent to the embryo shows little Gag (Fig. 4, Fig. S2c) but the BARE RNA is present more strongly there than in the surrounding cells (Fig. 4c). Hence, it appears that the RNA produced in this cell layer is not used to synthesize Gag protein in the same cells.

Fig. 2 Immunolocalization of BARE capsid protein Gag in barley (Hordeum vulgare) root tips. (a) Longitudinal section of root tip from germinating embryo (bar, 200 μm). (b–d) Cross-section of the root tip approximately corresponding to the position shown by the line on (a). (b) Immunolocalization, (c) combined DAPI staining and immunolocalization, (d) DAPI staining. (e) Higher magnification of cells from the root cap with Gag immunolocalization (top row), combined DAPI staining and immunolocalization (middle row), and DAPI staining (bottom row). (f–h) Higher magnification of cells from cross-sections corresponding to (b–d) with nuclei at various stages of division. Rows are as for (e).

Fig. 3 Expression of Gag and BARE RNA in barley (Hordeum vulgare) shoots. Longitudinal sections of barley shoot tips 18 d after germination. (a) Reaction to anti-Gag IgG. (b) Reaction to pre-immune serum as the control. (c) In situ hybridization with antisense probe corresponding to gag region. (d) Control in situ hybridization with sense probe for gag. The primordial spike containing floral and (at base) leaf meristems (F), parenchymatous ground tissue (P) and pro-vascular tissue (V), and axillary meristems (X) are labelled. Bar, 500 μm.
**BARE** Gag and RT are present in barley ovaries and developing grains

The genomes of cells in the ovule of Arabidopsis are demethylated and consequently transcribe TEs (Slotkin *et al.*, 2009); with this in mind, we examined the corresponding tissues in barley for **BARE** protein expression. As shown in Fig. 5 (and in greater detail for Gag in Fig. S3), Gag and RT are strongly localized to specific cell types in the pre-fertilization ovule of barley. The cells of the ovary wall show only a weak Gag signal, except for those of the provascular tissue connecting the ovary to the spike. However, large concentrations of Gag are present in the chlorenchymatous tissue adjacent to the ovule, as well as in the outer and inner integuments and chalaza of the ovule. Within the ovule, the antipodal cells are strongly stained. RT displays the same localization pattern as Gag, albeit at the expected lower concentrations. During grain development, Gag accumulated in the aleurone (Fig. S4) and embryo; by maturity (Fig. 1), the embryo is rich in Gag.

**BARE** Gag is strongly expressed and drought responsive in vascular tissues

The presence of **BARE** Gag in the floral and embryo provascular tissues led us to examine the vasculature of adult plants. Immunolocalization on internode cross-sections (Fig. 6a,b) showed that Gag accumulates in the vascular bundles but not in the surrounding parenchyma. The amount of Gag depends on the position of the bundle within the plant; more is seen in axillary stems and leaf sheaths than in the main stem (Fig. 6a,b). Vascular bundles of the outer leaf sheath display a small amount of Gag. Within the vasculature, Gag shows specific localization. It is not seen in the collenchymatic tissue surrounding the vascular bundles (Fig. 6a,b). Within the...
vascular bundles, Gag is not seen in the xylem vessels or in the peripheral cells, but is highly concentrated in companion cells surrounding the phloem sieve tubes (Fig. 6d,e). Gag localization to the companion cells is clearer in longitudinal sections (Fig. 6g,h), where the dense staining of nuclei is apparent (Fig. S5).

Gag expression in the vascular tissue was strengthened by drought treatment, in a variety-dependent manner. Golden Promise shows more wilting throughout the plant in response to drought treatment than does cv Bomi. Even for Bomi, immunoreactions on sections require shorter incubations (5 vs 10 min) in drought-treated (Fig. 6b,e,h) than in control stems for similar staining levels in the vasculature. Immunoblots, which both resolve the precursor forms from the processed Gag that is assembled into VLPs and allow samples to be processed together, show that cv Golden Promise responded to drought more strongly in terms of Gag than did cv Bomi (Fig. 7). The drought-treated samples, particularly in the 5th internode and the 6th node, contain more mature Gag than samples from normally watered plants. Little uncleaved polyprotein was detected in the drought-treated samples, whereas it was still detectable in normally watered plants, particularly in the nodes. The amount of the 90 kD processed form of the polyprotein remained unchanged throughout.

Discussion

For retrotransposons, their replicative lifecycle both enables their copy number to grow into the many thousands in the host genome and provides a feedback loop for maintaining activity while deleterious mutations of genomic copies can occur at the neutral rate, in the absence of selective advantage for the plant. However, for newly replicated copies of BARE or other retrotransposons to be inherited, integration events inevitably must occur in cells giving rise clonally to the next generation. The lifecycle requires the presence of retrotransposon-encoded RNA and proteins, something that has not been examined before in plants within organs or tissues. Using antibodies raised to the capsid protein Gag, the most abundant of the retrotransposon-encoded proteins, we were able to examine the question. We observed large differences in expression level from tissue to tissue. The strong Gag and RNA localization in the floral meristems and mature embryos, in particular, appear consistent with a strategy of replication within cells giving rise ultimately to gametes so that newly integrated copies can be inherited; it also corresponds to expression of BARE RNA from the TATA2 of the LTR (Chang & Schulman, 2008; Chang et al., 2013).
Nevertheless, strong protein expression was seen also in tissues that do not give rise to gametophytes, such as vascular tissues, aleurone and scutellum. This may be a byproduct of either BARE promoter response elements needed for replication in target tissues or generalized activation associated with epigenetic state. In root tips, the Gag localization resembles that of auxin transport pathways in Arabidopsis (Marchant et al., 1999).

Localization of BARE Gag in the vascular tissue or vascular initials but not in the surrounding parenchyma was seen elsewhere in the plant as well. There was little mature Gag compared to the unprocessed polyprotein seen in root tips, and comparatively little nuclear staining with anti-Gag antibodies there. This suggests that, in root tips, the BARE lifecycle is hindered by lack of polyprotein processing to form Gag and thence VLPs, which then can be localized to the nucleus (Kim et al., 2004). The polyprotein is processed by the AP, a step that could be a replication control point; aspartic proteinase inhibitors, which would be required for such control, are known for plants (Headey et al., 2010) and present in phloem exudates (Walz et al., 2004). Callus cells also contain comparatively little mature Gag for the amount of polyprotein detected.

Gag expression is increased by drought treatment, and the 153-kD polyprotein disappears in favor of the mature 32-kD Gag under drought stress. This indicates that either the polyprotein is processed by the AP or it is degraded in the stressed plants. The appearance of the mature size Gag and the presence of 95 kD partially processed polyprotein in the stressed plants supports the processing scenario. The increase in Gag seen in the vascular tissue as a response to drought mirrors the presence of an abscisic acid (ABA) response element (Suoniemä et al., 1996) in the LTR (ABI4, Table S1) and the eco-geographic correlation between high BARE copy number and water scarcity (Kalander, 2008). Moreover, ABA is translocated in the phloem (Hartung et al., 2002), synthesized companion cells and xylem parenchyma cells as well as in root tips and hypocotyls (Koiwai et al., 2004), and produced in response to water stress (Davies & Zhang, 1991). Hence, the BARE Gag and RNA localization pattern in the vasculature is consistent with a role for ABA in BARE activation.

In germinating grains, Gag protein was very abundant and localized in the scutellum in particular, and mirrored the overall level of BARE RNA expression. The scutellum plays an important role in the germination of monocot seeds, by both secreting hydrolytic enzymes from its endothelium, absorbing the glucolytic and proteolytic products, and translocating them to the embryo (Potokina et al., 2002). The scutellum differentially expresses at least tens of genes (Potokina et al., 2002), and shows specific or differential expression of transcription factors (Postma-Haarsma et al., 1999; Isabel-LaMoneda et al., 2003; Kovalchuk et al., 2012). The Gag accumulation pattern matched that of the SAD transcription factor of barley, a DOF-class protein that binds the pyrimidine box and activates transcription of a GA-induced promoter (Isabel-LaMoneda et al., 2003). The BARE LTR contains 15 putative promoter elements (Table S1) matching motifs of GAMYB, DOF, AMYBOX2, TATCCAOSAMY and WBOXHVIS01, all of which are associated with gibberellin response or regulation of amylase activity or sugar metabolism (Huang et al., 1990; Gubler et al., 1999; Yanagisawa & Schmidt, 1999; Sun et al., 2005; Haseneyer et al., 2008; Gaur et al., 2011).

Hence, the BARE protein expression pattern and the promoter analyses, taken together, are consistent with transcriptional regulation by gibberellin and expression in the embryo and seed.

Strong Gag localization and BARE RNA expression was seen in floral meristems and axillary shoot apical meristems, but not in the shoot apical meristem of young seedlings before transition to floral spike formation when Gag appeared in the tissue. We haven’t examined RNA and Gag expression in the stages between the transition to flowering and spikelet maturation. However, their presence in the floral spike implies that new BARE insertions there will be carried clonally to the ovary even if BARE expression is downregulated at intervening stages. The expression of BARE in the floral spike appears to be a good strategy to favor passage of new retrotransposon copies into the following generation.

It has been generally held that uncontrolled replication of retrotransposons is selected against, due to the potentially deleterious effects of disruptive integration into genes and of genome size growth. BARE and other retrotransposons of high copy number in large genomes such as barley are found mainly in ‘seas’ of repetitive DNA between ‘gene islands’, mitigating mutagenic effects (Shirasu et al., 2000; Wicker et al., 2005; Baucoum et al., 2009). Moreover, in meristems, a deleterious retrotransposon insertion causing cell death or delayed replication would not be inherited due to consequent replacement of that cell by its neighbors. The repeat regions are also generally heterochromatic, suggesting that most retrotransposon copies in them will be epigenetically silenced (Mirouzé et al., 2009; Szabé & Kakutani, 2011). Some retrotransposons, moreover, have been shown to be post-transcriptionally silenced by small RNAs (Slotkin & Martienssen, 2007; Slotkin, 2010). However, we show here that Gag is present in the pre-fertilization ovary, in the chlorenchyma, integuments, chalaza and antipodal cells. The expression of BARE in these cells consequently may reflect demethylation as seen in the ovule of Arabidopsis for the production of siRNAs targeted at TEs (Slotkin et al., 2009; Calarco & Martienssen, 2011).

The data presented here suggest that BARE employs a replication strategy that could explain its high copy number in the genome, in contrast to retrotransposons such as Tnt1 or Tos17, which are rare in the genome and quiescent during plant development, but mobilized by tissue culture and transcriptionally activated by some stresses (Grandbastien et al., 2005; Cheng et al., 2006). In this regard, the importance of ABA in regulating flower development under water stress in Arabidopsis (Fitzpatrick et al., 2011) and its accumulation in spikes of drought-sensitive wheat (Ji et al., 2011) may help explain both the vascular and floral spike expression and the drought responsiveness of Gag in the vasculature that we report here. However, it remains to be established, within the tissues and cells in which BARE is expressed, what further control mechanisms may be operating to restrict BARE propagation.
The advent of new copies depends on the lifecycle of the retrotransposon being completed. Blocks, in cells ultimately passing nuclei to the next generation, to transcription, translation, packaging into VLPs, reverse transcription, nuclear localization or integration will attenuate retrotransposon propagation. Hence, our work establishes the basis for probing the relationship between the RNA and protein expression patterns and integration events. To answer the question of which RNA and protein expression sites ultimately give rise to newly integrated copies in the following generation, it will be necessary to create marked cDNA copies and retrotransposon proteins under tissue-specific promoters and monitor the fate of these constructs transgenic lines. This task is currently in progress.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Immunolocalization of retrotransposon *BARE*-encoded Gag and RT in roots.

Fig. S2 Immunolocalization of Gag in barley grains.

Fig. S3 Immunolocalization of Gag in the pre-fertilization ovule.

Fig. S4 Immunolocalization of Gag in developing barley grains.

Fig. S5 Gag Immunolocalization in drought-stressed barley vascular tissues of the internode.

Table S1 Putative transcription factor binding sites in the LTR of *BARE1*

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