Running title: *Tissue-specific protein deposition*

Corresponding author: Eva Stoger, Institute for Molecular Biotechnology, Biology VII, RWTH Aachen, Worringerweg 1, 52074 Aachen, Germany, Telephone +492418026525, Fax +492418020145, e-mail: eva.stoger@molbiotech.rwth-aachen.de
The intracellular fate of a recombinant protein is tissue-dependent.¹

Georgia Drakakaki²,³, Sylvain Marcel², Elsa Arcalis, Friedrich Altmann, Pablo Gonzalez-Melendi, Rainer Fischer, Paul Christou, and Eva Stoger*

Institute for Molecular Biotechnology, Biology VII, Aachen University, Worringerweg 1, 52074 Aachen, Germany (G.D., S.M., E.A., R.F., E.S.); Department for Chemistry, Glycobiology Division, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna (F.A.); Centro de Investigaciones Biologicas, CSIC, Ramiro de Maeztu 9, 28040-Madrid, Spain (P. G.-M.); ICREA, Universitat de Lleida, Department de Produccio Vegetal i Ciencia Forestal, Av. Alcalde Rovira Roure, 191, E-25198 LLEIDA, Spain (P.C.).
Footnotes:

1 **Financial Source**: The work was supported by the Sofia Kovalevskaja Prize awarded by the Alexander von Humboldt Foundation.

2 G. Drakakaki and S. Marcel have contributed equally to the work.

3 **Present address**: G.D. Department of Botany and Plant Sciences, University of California, Riverside CA 92521.

* **Corresponding author**: Eva Stoger, Institute for Molecular Biotechnology, Biology VII, RWTH Aachen, Worringerweg 1, 52074 Aachen, Germany, Telephone +492418026525, Fax +492418020145, e-mail: eva.stoger@molbiotech.rwth-aachen.de
Abstract

Recombinant proteins directed to the secretory pathway in plants require a signal peptide for entry into the endoplasmic reticulum. In the absence of further targeting information, such proteins are generally secreted via the default pathway to the apoplast. This has been well-documented in protoplasts and leaf tissue, but the trafficking of recombinant proteins in seeds and other storage tissues has rarely been investigated. We used *Aspergillus niger* phytase as a model glycoprotein to compare the intracellular fate of a recombinant protein in the leaves and seeds of rice. Using fluorescence and electron microscopy, we showed that the recombinant protein was efficiently secreted from leaf cells as expected. In contrast, within endosperm cells it was retained in ER-derived prolamin bodies and protein storage vacuoles. Consistent with our immunolocalization data, the phytase produced in endosperm cells possessed oligomannose and vacuolar-type N-glycans (Man$_3$(Xyl)(Fuc)GlcNAc$_2$), whereas the phytase produced in leaves contained predominantly secretion-type N-glycans (GlcNAc$_2$Man$_3$(Xyl)(Fuc)GlcNAc$_2$). The latter could not be detected in preparations of the endosperm-derived phytase. Our results show that the intracellular deposition and modification of a recombinant protein is tissue dependent.
INTRODUCTION

In eukaryotic cells, polypeptides destined for the secretory pathway contain a short, N-terminal transit signal peptide, which facilitates co-translational import into the endoplasmic reticulum (ER). Such proteins may then be secreted to the cell surface or directed to intracellular domains of the endomembrane system. In the latter case, further positive sorting information is deemed necessary. For example, three different categories of signals are known to direct proteins to vacuolar compartments and these may comprise short peptide tags or structural information rather than a peptide sequence (Neuhaus and Rogers, 1998). In the absence of any sorting information, it is generally accepted that proteins in the plant endomembrane system follow the default pathway and are secreted to the apoplast (Denecke et al., 1990).

For glycoproteins, the subcellular destination and the path taken by the protein through the endomembrane system determine the final structure of N-glycan moieties. In the first stage of N-glycosylation (which takes place in the ER), the precursor oligosaccharide Glc₃Man₉GlcNAc₂ is cotranslationally added to the protein. As the protein moves through the endomembrane system, this core is modified by various glycosidases and glycosyltransferases to form the final glycan structure (Lis and Sharon, 1993; Lerouge et al., 1998). ER-resident glycoproteins contain high-mannose-type N-glycans whereas proteins passing through the plant Golgi apparatus contain complex-type N-glycans that are mainly characterized by the presence of α(1-3) fucose and/or β(1-2) xylose. These residues are linked to the proximal N-acetyl glucosamine and the β-mannose residues of the core (Lerouge et al., 1998). While secreted glycoproteins contain terminal GlcNAc residues in addition to the core fucose and xylose, these terminal residues are trimmed off by enzymes either en route to the vacuole or within the vacuole. (Lerouge et al., 1998).
The endomembrane system is well structured not only in cells that secrete proteins, but also in those specialized for protein storage. The major seed storage proteins of plants pass through the endomembrane system en route to the protein bodies in which they accumulate, and these bodies may either be ER-derived structures or vacuolar compartments (Müntz, 1998). In rice endosperm cells, approximately 60-80% of the storage proteins are glutelins and approximately 5-18% are prolams depending on the genotype and quantification method (Huebner et al., 1990; Li and Okita, 1993). These two classes of proteins accumulate in distinct storage compartments with different origins (Okita and Rogers, 1996). Glutelins accumulate in large protein storage vacuoles (PSVs) that are electron-dense and irregularly-shaped (Krishnan et al., 1986). In contrast, spherical prolam body (PB-I) form through the direct deposition and aggregation of prolams in the ER lumen and remain enclosed by ER membranes (Li et al., 1993). The mechanism by which rice prolams are retained in the ER is not completely understood, and no defined ER-retrieval or retention signal has been identified. It has been suggested that retention is achieved by binding to the ER-resident molecular chaperone BiP, which facilitates the folding and assembly of prolams into insoluble aggregates (Li et al., 1993). The assembly process itself may also contribute to ER retention (Shewry and Halford, 2002) and may be promoted by the increased local concentration of prolams brought about by spatially-regulated translation. Recent evidence suggests that prolam mRNA is preferably directed to the cortical subdomain of the ER through a mechanism which involves two cis-acting elements (Hamada et al., 2003a and 2003b; Crofts et al. 2004).

The subcellular localization and sorting of recombinant proteins in storage organs such as cereal seeds has rarely been investigated. Indeed, in many cases, the subcellular localization has been inferred by extrapolating data obtained from protoplasts. However, recent reports have indicated that recombinant proteins expressed in seeds can be deposited in unexpected
places. For example, Chikwamba et al. (2003) found that recombinant *Escherichia coli* heat-labile enterotoxin subunit B (LT-B) was deposited in the starch granules of maize seeds, instead of being secreted by the default pathway as expected. Similarly, an antigenic glycoprotein from human cytomegalovirus was expressed in tobacco seeds with the aim of developing an oral vaccine (Tackaberry et al., 1999). The protein was expected to be secreted but was instead found in protein storage vacuoles, although no targeting studies were carried out to establish its route (Wright et al., 2001). Recent observations of recombinant phytase, a normally secreted protein, revealed unexpected deposition in prolamin aggregates within the vacuole of transgenic wheat endosperm cells (Arcalis et al., 2004). Such observations show that further research into the trafficking of recombinant proteins in storage tissues is necessary, and suggest that the specialized architecture and function of endosperm cells could influence the intracellular route of a given recombinant protein.

We therefore carried out experiments in which *Aspergillus niger* phytase was used as a model glycoprotein to compare intracellular trafficking in de-differentiated tissue (callus), leaves and seeds of transgenic rice. This molecule was particularly suitable for our investigation as it has been shown to be secreted in other heterologous systems in which it has been expressed, it is very stable within plant cells, and it is highly glycosylated (Vervoerd et al., 1995; Li et al., 1997; Lucca et al., 1999; Brinch-Pedersen et al., 2000; Ullah et al., 2002; 2003). Therefore, glycosylation analysis could be carried out to complement data from fluorescence and electron microscopy studies, providing further evidence for tissue-specific intracellular localization. Together, the results from these experiments show that the same protein can be directed to different compartments or locations in the cell as a function of the tissue in which it is expressed. Since the destination of a protein can influence both its stability and modification, these results are particularly significant where the aim is to produce active recombinant proteins in plants.
RESULTS

*Generation and selection of transgenic rice plants expressing phytase*

The *A. niger phyA* gene was expressed constitutively in transgenic rice plants under the control of the enhanced CaMV 35S promoter (present in construct pTRA-PhyA-AH). It was also expressed specifically in the endosperm of transgenic rice seeds using the glutelin-1 (Gt1) promoter (present in construct pLPL-phyA). The Gt1 promoter not only facilitates high-level expression but also prevents contamination of the recombinant enzyme with phytase expressed from tissues surrounding the endosperm, since the promoter’s activity is strictly endosperm specific. In both constructs, the *phyA* gene was preceded by a murine N-terminal signal peptide, which mediates entry into the plant ER (Torres et al., 1999). No further targeting information was included, so the expectation was that the enzyme would be secreted to the apoplast via the default pathway.

Several independent transgenic lines were tested by immunoblot analysis of leaf and seed extracts (Fig. 1A). The accumulation levels achieved with the Gt1 promoter in seeds and the 35S promoter in leaves were comparable and exceeded 0.5% of total extracted protein (Fig. 1A and B). We selected two representative transgenic rice plants expressing phytase constitutively, and one representative plant expressing phytase specifically in the endosperm for in depth analysis.

*The relative molecular mass of phytase depends on the tissue where it is expressed*

Recombinant phytase extracted from rice leaves and seeds was analyzed by immunoblot (Fig. 1). In the leaves of rice plants expressing phytase constitutively, two variants of the recombinant protein were detected with different apparent molecular masses (65 and 75 kDa; Fig. 1C, lane 2). Both variants had greater molecular masses than that calculated for the
unglycosylated polypeptide (49kDa; Van Hartingsveldt et al., 1993), but lower molecular masses compared to the hyperglycosylated phytase isolated from *Aspergillus* (Figure 1A and B). Seed phytase migrated as a single band with an apparent molecular mass between 60 kDa and 65 kDa (Fig.1C, lane 1). As expected, there was only a small amount of seed phytase in the constitutively-expressing plants reflecting the lower activity of the CaMV 35S promoter in rice endosperm (Figure 1C, lane 6). No significant differences were observed in the molecular mass of seed-derived phytase irrespective of the promoter used (Figure 1, compare lanes 1 and 6). No phytase was detected in the leaves and seeds of wild type plants (Fig. 1, lanes 3 and 4).

**The intracellular localization of recombinant phytase differs in leaves and endosperm**

The subcellular localization of recombinant phytase was analyzed by fluorescence and electron microscopy. As expected for a protein with a signal peptide but no further targeting information, the recombinant phytase was secreted in both callus and leaves. This was confirmed in regenerating callus tissue by electron microscopy: the phytase clearly appeared in the apoplast (Fig. 2A). Secretion of the recombinant protein was also confirmed in transgenic rice leaves (Fig. 2B and C).

In rice endosperm tissue, an overview of recombinant phytase localization was produced by fluorescence microscopy (Fig. 3A). This showed that, in contrast to the data for leaf and callus, fluorescence was confined to inner spherical structures of the endosperm tissue and no signal was detected in the intercellular space. In order to define precisely the structures involved, ultrathin sections were processed and analyzed by electron microscopy. Figure 3B shows a general view of a rice endosperm cell, where the main compartments of an endosperm cell can be observed. The two different types of protein bodies can be distinguished (Figs 3B, D, E and F): the glutelin-containing storage vacuoles are electron-
dense and have an irregular shape, while the spherical ER-derived prolamin bodies are surrounded by a ribosome-studded ER membrane.

Gold labeling was equally distributed between the protein storage vacuoles and the prolamin bodies, and no additional labeling was observed in the apoplast or any other cell compartment (Figs. 3 C-F). No qualitative difference in endosperm labeling was observed between plants expressing phytase constitutively (Figs. 3 B-E) or only in seeds (Fig. 3F). Sections of non transgenic seeds were not labeled.

**Phytase mRNA is translated on the cisternal ER**

In rice endosperm cells, clear evidence has been presented for active prolamin mRNA targeting to the cortical ER, where the prolamin bodies are formed (Choi et al., 2000). We carried out in situ hybridization analysis of ultratin sections of rice endosperm to determine whether the transgene mRNA, encoding phytase, was targeted in the same manner. No labeling was detected on the cortical ER surrounding the prolamin bodies (Fig. 4) and only the cisternal ER was labeled, indicating that the protein is not initially targeted to the cortical ER.

**The modification of recombinant phytase differs between leaves and seeds**

To determine whether the tissue-specific localization of recombinant phytase was also reflected by its glycosylation pattern, we analyzed the N-glycan structures of phytase isolated from leaves and endosperm. Endosperm-specific phytase expression prevented contamination with phytase expressed in other parts of the seed, such as the embryo. The protein samples were concentrated and separated by SDS-PAGE. The phytase bands were excised separately
from the gel, digested with trypsin, and their identity was confirmed by peptide mass fingerprinting.

In the case of leaf-derived phytase, the major protein band had a molecular mass of 75 kDa (Figure 5, insert, left lane), and corresponded to phytase carrying the complex N-glycan GlcNAc₂Man₃XylFucGlcNAc₂ with terminal GlcNAc residues (Fig. 5, RL5). This glycan, structure, also known as GnGnXF (Wilson et al., 2001), is typically found on secreted glycoproteins (Lerouge et al., 1998). Its presence is consistent with the intercellular localization of phytase in leaves. A minor protein band with a molecular mass of 65 kDa (Figure 5, insert, left lane) carried smaller glycans (Fig. 5, RL6). The main peak corresponded to the vacuolar-type N-glycan Man₃XylFucGlcNAc₂ (MMXF) indicating that glycan trimming had taken place in the vacuole. A second peak represented MMX which is the above glycan without fucose (Fig. 5).

In the case of endosperm-derived phytase, (which migrated as single band between 60 kDa and 65 kDa; Figure 5 insert, right lane), we identified two distinct major glycan families (Fig. 5, RS1): The predominant glycan structure was MMXF (or Man₃XylFucGlcNAc₂). Paucimannosidic-type N-glycans such as MMXF, MUXF (Man₂XylFucGlcNAc₂) and MMX (Man₃XylGlcNAc₂) are considered typical for vacuolar glycoproteins (Lerouge et al., 1998). Approximately 17% of the N-glycans of the endosperm-derived phytase were of the oligomannose type and had thus not been modified in the Golgi. This minor component could reflect the presence of phytase which had not passed through the trans-Golgi. Importantly, in endosperm-derived phytase we did not detect significant amounts of complex-type glycans with terminal GlcNAc residues, as typically found on apoplastic proteins. It is likely that the different glycan structures also account for the differences in molecular mass that were observed with phytase isolated from leaves and seeds.
DISCUSSION

Although many recombinant proteins with various targeting signals have been expressed in different plant species and organs, little attention has been paid to their precise intracellular localization. This is particularly the case for proteins expressed in specialized tissues such as the endosperm (Hood, 2004). Evidence for the tissue-specific deposition of proteins in plants has been provided in only a handful of previous studies (Dixon et al., 1991; Carzaniga et al., 1994; Kjemtrup et al., 1995). For example, there is evidence that a small amount of phytohemagglutinin, a PSV-resident seed protein, is secreted to the apoplast of bean root tissues (Kjemtrup et al., 1995). Furthermore, the pathogenesis-related protein PR-1, which is normally secreted, was found to accumulate in the vacuoles of tobacco leaf crystal idioblasts (Dixon et al., 1991). These examples of endogenous proteins indicate that protein sorting may be modulated in a tissue or cell-specific manner, but a methodical investigation has been lacking thus far.

We therefore embarked on a systematic comparative study using a model glycoprotein – Aspergillus niger phytase – and showed that the subcellular fate of this recombinant protein differed according to the cell type in which it was expressed. A. niger phytase has been expressed previously in yeast, plants and animal cells, and is generally secreted as an extracellular enzyme when a signal peptide for entry into the ER is present (Vervoerd et al., 1995; Li et al., 1997; Han et al., 1999; Richardson et al., 2001; Wang et al., 2003). In line with these observations, our data show that phytase is secreted to the apoplast in rice leaves and callus, following the default pathway as expected. Surprisingly however, phytase is not secreted from endosperm tissue, irrespective of the promoter used. Rather, recombinant phytase was found to accumulate in both ER-derived prolamin bodies (PB-I) and in protein storage vacuoles (PSVs).
PB-Is are protein deposits that are formed within the lumen of the rough ER and remain surrounded by the ER membrane. A major factor contributing to prolamin body formation in rice seeds is the sorting of prolamin mRNA to specific, PB-associated ER subdomains, a process that involves interaction with specific RNA-binding receptors (Choi et al., 2000; Hamada et al., 2003; Crofts et al., 2004). In order to find out how and why phytase is deposited in such an unexpected manner, it was important to determine whether a similar mechanism is involved in the sorting of phytase mRNA. Our findings strongly suggest that phytase deposition in PBs does not rely on RNA targeting, since only the cisternal ER was labeled by phytase antisense RNA. It is more likely that diffusion and passive trapping (rather than transcriptional control) leads to the incorporation of phytase within the prolamin aggregates.

Immunolocalization experiments revealed that a large proportion of the recombinant phytase was sequestered into glutelin-containing protein storage vacuoles. There are several different routes by which proteins can reach these organelles (Brandizzi and Hawes, 2004; Wenzel et al., 2005; Vitale and Hinz, 2005; Robinson et al., 2005; Herman and Schmidt, 2004). For example, vicilin, legumin and $\alpha$-TIP are transported through dense vesicles, while other seed proteins such as ricin appear to be transported in clathrin-coated, Golgi-derived vesicles (reviewed by Vitale and Hinz, 2005). In cotyledon cells, vesicles are released from the ER and become internalized into vacuoles (Hara-Nishimura et al., 1998) or fuse directly with the storage vacuoles (Toyooka et al., 2000), thus bypassing the Golgi. In wheat endosperm cells, glutenins reach the PSV via large ER-derived protein bodies that are incorporated into the PSV by a phagocytotic process (Levanony et al., 1992).

In rice endosperm cells, PSVs consist mainly of glutelins that have passed through the Golgi (Okita and Rogers, 1996), although there is also recent evidence for ER-derived vesicles (Takahashi et al., 2005). Thus we cannot rule out the possibility that recombinant phytase
could have reached the PSVs without passing through the Golgi. To investigate this possibility, we characterized the glycan profile of phytase, focusing specifically on core α(1,3)-fucose and β(1,2)-xylose residues which represent late Golgi modifications. We observed typical vacuolar-type glycan structures such as Man$_3$XylFucGlcNAc$_2$ and Man$_3$XylGlcNAc$_2$ suggesting that the protein had traveled via the Golgi complex to the protein storage vacuole, where terminal GlcNAc residues are removed (Lerouge et al., 1998). Approximately 17% of the N-glycans were of the oligomannose type (Man$_{4\text{ to }6}$GlcNAc$_2$), perhaps corresponding to the phytase detected in ER-derived prolamin bodies. In agreement with our immunolocalization data, we did not detect significant levels of complex glycans with terminal GlcNAc residues or Lewis$^a$ structures, as typically found on secreted glycoproteins.

This is in sharp contrast to the glycan profile of phytase isolated from rice leaves. Here, the most prevalent structures were complex glycans GlcNAc$_2$Man$_3$XylFucGlcNAc$_2$ and Lewis$^a$ structures as routinely found on secreted proteins. Only a small proportion of leaf-derived phytase had a lower molecular mass and contained trimmed glycans typical of vacuolar proteins. This vacuolar fraction of the phytase population in leaves could not be detected by immunolocalization, perhaps indicating that the protein was diluted below the detection threshold by the contents of the vacuolar compartment. However, based on the combined evidence from electron microscopy and glycan analysis, it is clear that most of the recombinant phytase accumulated in the leaf apoplast.

The evidence above demonstrates that recombinant phytase is localized and modified differently in endosperm and leaf tissues. Since the same molecule can behave differently in alternative tissues of the same plant, it is likely that the cell type has an effect on the default pathway, i.e. protein sorting may be regulated in a cell-specific manner. This hypothesis has been put forward previously but there has been no systematic attempt to verify it (Dixon et al.,
Indeed there have been several anecdotal accounts of unusual protein targeting in storage tissues, but until now no comprehensive analysis in different tissues of the same plant. For example, we found in a previous study that recombinant phytase accumulates in protein bodies within the storage vacuole of wheat endosperm (Arcalis et al., 2004). Similarly, Yang et al. (2003) reported the unexpected accumulation of recombinant human lysozyme in endosperm cells, in a pattern that matches precisely our data for recombinant phytase. The deposition of lysozyme in leaves was not investigated, and since lysozyme is not glycosylated, no precise tracking could be performed. To interpret the unexpected localization of recombinant lysozyme in endosperm cells, the authors suggested that human lysozyme might contain cryptic intrinsic sorting determinants. However, there is no significant sequence (or structural) similarity between \textit{A. niger} phytase and human lysozyme, so unless these proteins happen to have distinct PSV sorting determinants, it is likely that the sorting process is not based on intrinsic features of the protein. We have also previously studied the localization of a recombinant immunoglobulin in rice endosperm, and neither the assembled antibody nor its free components were detected in the apoplast (Nicholson et al., 2005). These data support our hypothesis that the storage function of cereal endosperm cells is able to override the default pathway that directs proteins to the apoplast in leaf cells.

Interestingly, recombinant human lactoferrin isolated from maize and rice seeds contained almost exclusively (98%) paucimannose-type N-glycans with $\beta$(1,2)-xylose and $\alpha$(1,3)-linked core fucose (Samyn-Petit et al., 2001 and 2003), indicating localization within a vacuolar compartment. In contrast, recombinant lactoferrin from tobacco leaves contained a significant amount of processed glycans with terminal GlcNAc residues (Samyn-Petit et al., 2003).

The above reports support the existence of tissue-specific protein sorting, and provide cumulative evidence that the unique features of specialized storage tissues such as the cereal endosperm may significantly affect the localization and consequent modification of
recombinant proteins. Given the specialized architecture of endosperm cells, it is likely that this will influence the intracellular route of recombinant proteins, and it is tempting to speculate that the default secretion machinery is modified in a moribund storage tissue such as endosperm.

The fact that rice endosperm expresses predominantly storage proteins that from their intrinsic characteristics tend to aggregate may also alter the fate of recombinant proteins within the default secretory pathway. Aggregation-based sorting has been suggested for a number of seed storage proteins (Shewry et al., 2002; Wenzel et al., 2005), and it is conceivable that proteins destined for secretion become passively trapped in a storage protein network. Thus, due to their abundance, storage proteins may exert a dominant effect on the subcellular localization of recombinant proteins resulting in tissue-specific sorting and modification of recombinant proteins in rice and other cereals.

While further research is needed to elucidate the underlying mechanisms of tissue-specific protein sorting, it is important to realize the far-reaching practical implications arising from our findings, particularly for the production of recombinant proteins. In this context, the subcellular destination of the recombinant protein and its glycan structure may influence protein recovery, as well as its activity, longevity, stability and allergenicity. By learning more about the mechanisms of protein sorting in storage tissues, we will have better control over the yield, structure and functionality of recombinant proteins produced in plants, and will be able to choose particular plant tissues as production platforms to generate proteins tailored to our specific needs.
MATERIALS AND METHODS

Vectors and plant transformation

For seed-specific phytase expression, we used the plasmid construct pLPL-phyA, containing the Aspergillus niger phyA gene preceded by the N-terminal signal peptide of the murine immunoglobulin kappa chain and under the control of the rice glutelin-1 promoter (Gt-1, a kind gift from Dr. T. Okita; Washington State University, Washington). This construct has been described previously (Arealis et al. 2004). For phytase expression in callus and leaf tissue, the coding region was amplified from the pLPL-phyA vector by PCR using forward primer phyEco (5’-ACG CGA ATT CGT ACA CAA TCA GA-3’) and reverse primer phyNot (5’-CAT GCG GCC GCA GCA AAA CAC TCC-3’) to introduce new restriction sites. This product was then inserted into a pTRA-based vector (AY027531) containing the enhanced 35S promoter and the nopaline synthase terminator, using the EcoRI and NotI restriction sites. The resulting vector was designated pTRA-PhyA-AH. The plasmids were introduced into rice callus derived from mature embryos (Oryza sativa L., cv. Bengal) by microprojectile co-bombardment with a plasmid carrying the hpt marker gene encoding hygromycin phosphotransferase (Sudakhar et al., 1998). Plants were grown in a 90% humidity phytochamber with a 12-h photoperiod (28°C day, 24°C night).

Immunoblot analysis

Total soluble protein was extracted from plants by homogenization in PBS containing 500 mM NaCl, 10 mM ascorbic acid and 5 mM β-mercaptoethanol (pH 8.0). After centrifugation at 17500 x g and 4°C, the supernatants were boiled for 10 minutes and separated on a 12% (w/v) SDS-PAGE gel under reducing conditions. Immunoblot analysis was carried out.
according to standard protocols using a 1:10000 dilution of rabbit antiserum, which was kindly provided by Dr. A. Ohmann (Novozyme, Kalundborg, Denmark).

**Protein extraction and glycan analysis**

Phytase was isolated from protein extracts essentially as described by Arcalis et al. (2004), with the slight modification that leaf samples were heated to 58°C after extraction. The phytase fraction was enriched by ammonium sulfate fractionation, dialyzed in PBS and concentrated by ultrafiltration before loading on a 12% (w/v) SDS-PAGE gel. The bands corresponding to phytase were excised from the Coomassie-stained gel after electrophoresis under reducing conditions and subjected to tryptic digestion as described elsewhere (Katayama et al., 2001). The extracted and dried peptides were dissolved in 95:5:0.1 v/v/v water/acetonitrile/trifluoroacetic acid (TFA) and analyzed by MALDI-TOF mass spectrometry (MS). Further preparation and MS analysis of N-glycans was carried out as described by Kolarich and Altmann (2000).

**Microscopy**

Developing rice grains (14 days after fertilization) were bisected transversely and the embryo removed. The half grain formerly containing the embryo was processed for recombinant protein analysis by western blot. The remaining half was fixed and processed for microscopy as described below.

Portions of the immature endosperm from wild type and transgenic rice plants were cut into small pieces with a razor blade under PBS (pH 7.4). The tissue pieces were fixed in 4% (w/v) paraformaldehyde and 0.5 % (v/v) glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) overnight at 4°C. Samples were dehydrated through an ethanol series and then infiltrated and
polymerized in LRWhite resin. The weak fixation and low temperature (-20°C) helps to increase the sensitivity of detection, but compromises the quality of the tissue sections.

For light microscopy, 1-µm sections were stained in methanol blue. For electron microscopy, sections showing silver interference colors were stained in 2% (w/v) aqueous uranyl acetate. The sections were observed using a Philips EM-400 transmission electron microscope.

Sections mounted either on glass slides for fluorescence microscopy or on gold grids for electron microscopy were preincubated in 5% (w/v) bovine serum albumin (BSA Fraction V) in phosphate buffer (0.1 M, pH 7.4) and then incubated with the appropriate dilution (1:100) of polyclonal rabbit anti-phytase. Sections were then treated with the secondary antibody diluted in phosphate buffer (0.1 M, pH 7.4). This was a goat anti-rabbit IgG Alexa Fluor® 594 for fluorescence microscopy and a goat anti-rabbit IgG labeled with 10-nm gold particles for electron microscopy.

**In situ hybridization to ultrathin sections**

Digoxigenin-labeled antisense and sense phytase RNA probes were synthesized by *in vitro* transcription as previously described (Highett et al., 1993). Probe size was reduced to approximately 100 bases by a mild carbonate hydrolysis (Cox et al., 1984). Gold grids carrying the sections were incubated for 3 h at 37°C in a humid chamber with the hybridization mixture containing 200 ng µl⁻¹ digoxigenin-labeled probe, 1 µg µl⁻¹ unlabeled RNA transcribed from a plasmid containing an unrelated sequence, 50% deionized formamide, 10% dextran sulfate, 300 mM NaCl, 10 mM PIPES (pH 8.0), 1 mM EDTA. After washing in PBS, the hybridization signal was detected by a primary mouse anti-digoxigenin antibody (Sigma) and a secondary anti-mouse IgG labeled with 10-nm gold particles (BioCell). The antibodies were applied at 1:5000 in 3% BSA in PBS and at 1:25 in PBS,
respectively, for 1 h at room temperature. Finally, the sections were stained with 2% (w/v) aqueous uranyl acetate for 40 min.
ACKNOWLEDGEMENTS

The authors would like to thank Duncan Keen for excellent technical assistance, Richard Twyman for critical assessment and help with manuscript preparation, and Thomas Rademacher for providing the pTRA vectors. The work was financially supported by a Sofia-Kovalevskaja grant from the Alexander von Humboldt Foundation.
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**FIGURE LEGENDS**

**Figure 1.** Immunoblot detection of recombinant phytase in extracts from leaves and seeds of transgenic plants. The proteins were detected by rabbit anti phytase antiserum followed by AP-labeled secondary antibodies. **A.** S1, S2, S3 and S4: Transgenic seeds from independent transgenic lines expressing phytase (endosperm-specific expression); L1-L6 Leaf samples from independent transgenic lines expressing phytase constitutively. Twenty µg and 10 µg of TSP (total soluble protein) of each sample were loaded. Asp: 100 ng and 50 ng of *Aspergillus niger* phytase; M: MW marker. **B.** Selected transgenic lines. Lanes 1-3: Twenty µg, 10 µg and 5 µg of TSP of a selected leaf sample (constitutive expression); lanes 4-6: Twenty µg, 10 µg and 5 µg of TSP of a selected seed sample (endosperm-specific expression); lane 7: MW marker; lanes 8-10: 200 ng, 100 ng and 50 ng of *Aspergillus niger* phytase. **C. 1.** Transgenic seeds expressing phytase (endosperm-specific expression), **2.** transgenic leaves (constitutive expression), **3.** wild type seeds, **4.** wild type leaves, **5.** size marker, **6.** Transgenic endosperm (constitutive expression). Twenty µg of TSP (total soluble protein) were loaded.

**Figure 2. Immunolocalisation of recombinant phytase in rice callus and leaves.**

**A.** Callus. Abundant gold labeling can be seen in the apoplast. **B- C.** Leaf. Gold labeling can be seen in the apoplast and on the cell wall. Abbreviations: apo- apoplast, cw- cell wall, chl- chloroplast, v- vacuole. Bars 0,5 µm

**Figure 3. Immunolocalization of recombinant phytase in rice endosperm.**

**A-E.** Constitute expression. **A.** Fluorescence microscopy. Phytase accumulates in protein storage organelles within the cells (arrowheads). No signal was detected in the apoplast
(arrows). B-F. Electron microscopy. B. General view of a rice endosperm cell showing the prolamin bodies (pr), the protein storage vacuoles (PSV), the rough endoplasmic reticulum (rER), the starch granules (s), and the apoplast (apo). C. Enlargement of area 1 as outlined in B. No gold particles are found decorating the apoplast. D. Enlargement of area 2 as outlined in B. Labeling is seen in the prolamin bodies and in the protein storage vacuoles. No significant labeling is seen in the starch or in the cytoplasm. E. Detail of a labeled protein body and PSV in rice endosperm. F. Seed specific expression. Labeling is seen in the prolamin bodies and the protein storage vacuole. No labeling is seen in the endoplasmic reticulum or the cytoplasm. Abbreviations: apo - apoplast, pr – prolamin bodies, PSV – protein storage vacuole, ER – endoplasmic reticulum, s – starch granule. Bars: 20 µm (A), 1 µm (B-F).

**Figure 4. In situ hybridization of recombinant phytase mRNA in rice endosperm 14 days after pollination.**

CER: cisternal ER; PSV: protein storage vacuole; Pr: Prolamin protein bodies.

Unlabelled arrows indicate 10 nm gold particles decorating the phytase transcript. Bar = 500 nm.

**Figure 5. Glycoanalysis of recombinant phytase derived from rice endosperm or leaves.**

The bands corresponding to phytase isolated from rice endosperm (RS1) and leaves (RL5 and RL6) were separately excised from the Coomassie-stained gel (insert) after electrophoresis under reducing conditions. RL5 and RL6 designate the major and the minor phytase band from rice leaves, respectively (see insert). N-Glycans were enzymatically released, isolated and analyzed by MALDI-TOF MS. Glycan structures were assigned on the basis of the molecular mass of the sodium adducts. In the structure cartoons, black squares depict GlcNAc, grey circles mannoe, white circles galacotse, triangles fucose and stars xylose. The
vacuolar type glycans MMXF, MMX and MUXF were identified as the major N-glycan structures in endosperm-derived phytase (RS1), whereas complex structures with terminal GlcNAc residues or Lewis\(^a\) epitopes dominated in phytase isolated from leaves (RL5).