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SHORT COMMUNICATION

Targeting and processing of membrane-anchored YFP fusion proteins to protein storage vacuoles in transgenic tobacco seeds

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Abstract

Seeds that store proteins in protein storage vacuoles are attractive bioreactors for producing and storing large amounts of pharmaceutical proteins. However, foreign proteins expressed in transgenic plants are subjected to the delivery and modification processes present within plant cells. Here, it is demonstrated that unique membrane sequences deliver a yellow fluorescent protein (YFP) to the seed protein storage vacuoles in transgenic tobacco (*Nicotiana tabacum* L.) plants, where the YFP is then separated from its membrane anchors. This precise targeting and separation is required for the successful delivery of useful proteins to seed protein storage vacuoles for their stable accumulation in transgenic crops.

Keywords: membrane anchors, *Nicotiana tabacum*, protein storage vacuole, seed bioreactor, vacuolar sorting receptor, yellow fluorescent protein

Introduction

Plant cell cultures have been tested and used over several decades as bioreactors to produce recombinant proteins (Hellwig *et al.*, 2004). However, the use of transgenic plants to produce recombinant proteins (i.e. human lysosomal enzymes and antibodies that have pharmaceutical applications) has several advantages over mammalian or bacterial expression systems, including less cost and freedom from viral contamination.

Plant cells contain lytic vacuoles and protein storage vacuoles (PSVs) (Okita and Rogers, 1996; Vitale and Raikhel, 1999), and seeds accumulate abundant proteins in the latter during their development (Bewley and Black, 1994). PSVs in most seeds, including tobacco, are compound organelles that contain three morphologically and functionally distinct subcompartments: matrix, globoid and crystalloid (Jiang *et al.*, 2000, 2001, 2002). Proteins reach the vacuoles via distinct vesicular transport pathways that are mediated by vacuolar sorting receptor (VSR) proteins (Neuhaus and Rogers, 1998; Shimada *et al.*, 2003; Vitale and Hinz, 2005). Because seeds are rich in proteins sequestered in PSVs, this makes them attractive as potential bioreactors for producing and storing large amounts of recombinant proteins. However, when these proteins are expressed in transgenic seeds (and other plant tissues), they are subject to both targeting and modification, and therefore, a strategy is required to ensure that they are delivered to their correct compartments (PSVs) for stable accumulation. This can occur either directly via the endoplasmic reticulum (ER) or from the ER to the Golgi apparatus (Jiang and Rogers, 1998). In addition, recombinant proteins may undergo seed-specific N-linked glycosylation modifications before they reach the PSVs. Such changes could affect the function or enzymatic activity of a seed-derived recombinant protein (Séveno *et al.*, 2004). These potential problems are addressed in this study of the expression and targeting of membrane-anchored fusion proteins to seed PSVs in transgenic tobacco plants.

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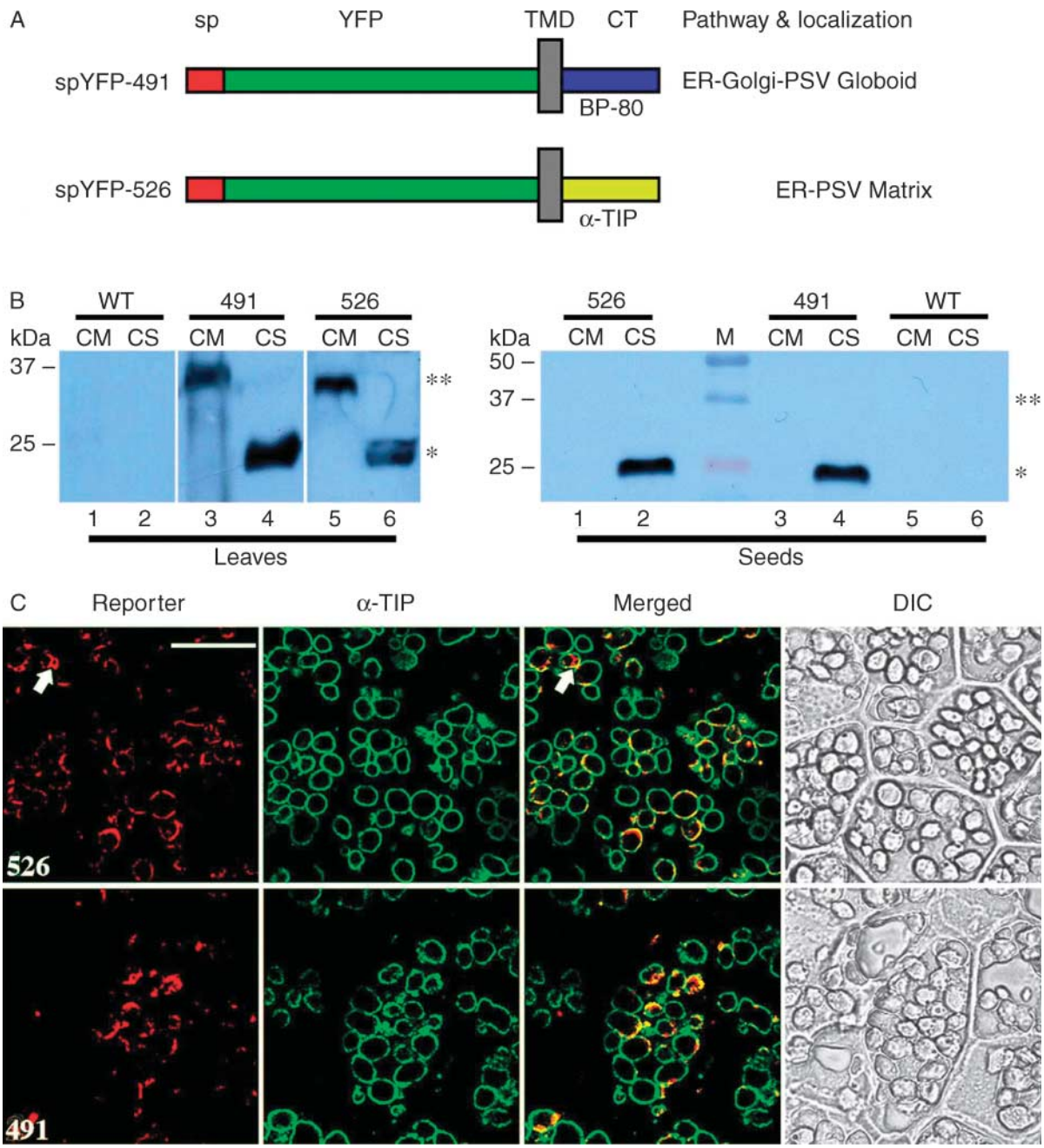

Figure 1. Expression, processing and subcellular localization of membrane-anchored yellow fluorescent protein (YFP) fusion proteins in transgenic tobacco plants. (A) Structures of membrane-anchored YFP fusions. These two fusion proteins contain a signal peptide (sp) from the barley cysteine protease aleurain, the yellow fluorescent protein (YFP), the transmembrane domain (TMD) of BP-80, and the cytoplasmic tail (CT) of BP-80 (for construct spYFP-491) or α-TIP (tonoplast intrinsic protein; for construct spYFP-526). Their predicted transport pathways and subcellular localization within the subcompartments of protein storage vacuoles (PSV) in seeds are also indicated. ER, endoplasmic reticulum. (B) Western blot analysis of expressed proteins in vegetative tissues and mature seeds of transgenic tobacco. Cell membrane (CM) and cell soluble (CS) proteins were isolated from leaves (left panel) and mature seeds (right panel) of transgenic (spYFP-491 or spYFP-526) or wild-type (WT) tobacco plants, followed by SDS-PAGE and Western blot analysis with green fluorescent protein (GFP) antibodies. Double and single asterisks indicate the positions of the full-length fusion protein and the processed/free YFP. Molecular mass in kDa is also indicated. M, molecular mass markers. (C) Subcellular localization of the YFP fusion proteins 491 and 526 in transgenic mature tobacco seeds. Paraffin thin sections were prepared from mature transgenic tobacco seeds, followed by double-labelling with GFP antibodies

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Materials and methods

Cloning of the spYFP-491 and spYFP-526 constructs with a proaleurain signal peptide was carried out as described previously (Tse *et al.*, 2004). Transgenic tobacco (*Nicotiana tabacum* L.) plants expressing these two constructs were obtained via *Agrobacterium*-mediated transformation and regeneration of kanamycin-resistant plantlets (Jiang *et al.*, 2000). Transgenic plants were either maintained in magenta boxes by regular transfer of shoots to fresh Murashige and Skoog (MS) medium, or transferred to a greenhouse and grown to maturity for seed collection.

For Western blot analysis, tobacco leaves or seeds were first ground to a fine powder in liquid N_2 before suspension in buffer A, containing 50 mM Tris–HCl, pH 7.4, 125 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 μgml^{-1} leupeptin (Sigma, St Louis, Missouri, USA). After centrifugation at 10,000 *g* for 15 min, the supernatant was separated from the pellet and designated the cell soluble fraction (CS); the membrane pellet was then re-suspended in buffer A and designed the cell membrane fraction (CM). Proteins were then separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis using affinity-purified anti-green fluorescent protein (anti-GFP) antibodies (Molecular Probes Inc., Eugene, Oregon, USA).

Fixation and preparation of seeds for paraffin-embedded sections, and their labelling and analysis by epifluorescence and confocal immunofluorescence, have been described previously (Jiang *et al.*, 2000; Li *et al.*, 2002; Tse *et al.*, 2004). For double-labelling, using two polyclonal antibodies, GFP antibodies were used to label yellow fluorescent protein (YFP) fusion proteins, followed by detection using a rhodamine-conjugated Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA), while antibodies raised against a synthetic peptide corresponding to the cytoplasmic tail of α-tonoplast intrinsic protein (α-TIP) (Jauh *et al.*, 1999) were used to label the PSV tonoplasts and were detected by fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). All confocal images were collected using a Bio-Rad Radiance 2100 system (UK). Images were processed using Adobe Photoshop software as described previously (Jiang and Rogers, 1998). All antibodies were used at 4 μgml^{-1} in both Western blot analysis and confocal immunofluorescent studies.

Results

Previously, it was proposed that unique membrane sequences could be used as anchors to deliver recombinant proteins to seed PSVs via distinct vesicular pathways, resulting in their stable accumulation and correct modifications (Jiang and Sun, 2002). As a first step to test this hypothesis, transgenic tobacco plants were generated expressing two YFP fusions under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter (Fig. 1A, constructs spYFP-491 and spYFP-526), in which the transmembrane domain (TMD) and cytoplasmic tail (CT) sequences of BP-80, a member of the vacuolar sorting receptor (VSR) protein family from pea, target the YFP to the PSV via the Golgi apparatus, while the attachment of the CT of α-TIP directs the YFP fusion to the PSV matrix directly from the ER, bypassing the Golgi apparatus (Jiang and Rogers, 1998) (Fig. 1A). Western blot analysis with GFP antibodies to detect YFP was then performed, using proteins extracted from both leaves and mature seeds of transgenic tobacco plants expressing both fusions. As shown in Fig. 1B (left panel, lanes 3–6), when proteins extracted from leaves were used for analysis, various amounts of both full-length YFP membrane fusion proteins and soluble free YFP proteins were detected in the cell membrane (CM) and cell soluble (CS) fractions, respectively. In contrast, when proteins isolated from mature seeds were used for Western blot analysis, only free YFP proteins were detected in the CS fraction (Fig. 1B, right panel, lanes 1–4), indicating that the YFP was completely separated from the membrane anchors within the transgenic tobacco seeds. The detection of YFP proteins was specific to transgenic plants because no band was detected in wild-type (WT) control plants (Fig. 1B, lanes 1–2 in left panel and lanes 5–6 in right panel). The differences in protein processing between leaves and seeds might be due to the absence of PSVs from vegetative tissues, where these fusion proteins might remain in intermediate prevacuolar compartments (Bethke and Jones, 2000; Tse *et al.*, 2004). Confocal immunofluorescence studies using double-labelling and detection in paraffin sections of transgenic tobacco seeds demonstrated that both YFP fusion proteins (red) are located inside the PSVs (green, the bounding membrane of which is marked by anti-α-TIP antibodies) of transgenic seeds (Fig. 1C). Therefore, both YFP fusions were targeted correctly to seed PSVs when they were expressed in transgenic tobacco

(detecting YFP fusion proteins inside of the PSVs, red) and α–TIP antibodies (detecting the PSV tonoplast bounding membrane, green) with detection by confocal immunofluorescence. Merged images showed YFP reporters (red) in relation to PSV tonoplasts (green). The morphology of the sampled cells is shown as DIC (differential interference contrast) images. Arrows indicate examples of PSV localization of the GFP reporter. Scale bar = 50 μm.

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plants. The distinct patterns of YFP fusion proteins (red, Fig. 1C) may indicate that they are localized to distinct PSV subcompartments in the transgenic seeds. Using commercial GFP proteins as standards in Western blot analysis, it was estimated that the soluble GFP can account for as much as 0.5–5% of the total soluble (water extractable) seed proteins in transgenic tobacco plants, and it is likely that much larger amounts of recombinant proteins can be obtained if a seed-specific promoter is used (Jiang and Sun, 2002).

Discussion

These results demonstrate that the membrane-anchored strategy for delivering YFP proteins to seed PSVs works well in transgenic tobacco plants. Seeds provide several advantages over other plant tissues, including cell cultures. First, seed PSVs are natural and ideal compartments for storing recombinant proteins; second, protocols are available for the easy purification of seed PSVs or other organelles (Jiang *et al.*, 2000; Mo *et al.*, 2003), so that the recombinant proteins within them can be enriched before their extraction and purification; third, and most attractively, it appears that YFP are separated from the membrane anchors naturally by the cell enzymatic system upon reaching the seed PSVs, which would allow direct purification of free recombinant proteins without the further need to separate the target protein from the membrane anchors; fourth, the direct ER to PSV transport pathways avoid unwanted plant-specific Golgi modifications (such as complex N-linked glycosylation) to recombinant proteins. A goal is to apply this delivery system for the production of active pharmaceutical proteins, such as human lysosomal enzymes in seeds of transgenic crops.

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